Mononuclear Phagocyte Activation in Acute Pancreatitis
A Clinical and Experimental Study

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Summary of thesis

The pathophysiology of the systemic illness seen in patients with acute pancreatitis is unknown but there are similarities with the systemic complications of sepsis, prompting the suggestion that similar endogenous mediators may be responsible. Central to this hypothesis is the role of activated mononuclear phagocytes. The long acting somatostatin analogue, octreotide (Sandostatin) has been shown to improve hepatic reticuloendothelial cell function, an effect which may be beneficial in acute pancreatitis by enhancing the clearance of endotoxin from portal blood and preventing systemic mononuclear phagocyte activation. This thesis addresses two main questions. Firstly the potential role of octreotide in the treatment of acute pancreatitis and secondly the role of mononuclear phagocyte activation in the pathophysiology of the systemic complications of acute pancreatitis.

Octreotide in acute pancreatitis

58 patients with moderate or severe acute pancreatitis who were admitted to hospitals within the West of Scotland over an 18 month period were randomised to receive octreotide, 40μg/h by continuous intravenous infusion, or placebo in addition to standard supportive therapy. Patients were comparable in age, sex, aetiology and severity of disease on admission. There was no significant difference in the incidence of complications (53.6% octreotide group and 43.3% placebo group) or mortality (octreotide group 18%; placebo group 20%). The results of this study indicate that octreotide is of little or no benefit in the treatment of acute pancreatitis.

Mononuclear phagocyte activation in acute pancreatitis

Monocytes were isolated from the peripheral blood of 28 patients with moderate or severe acute pancreatitis and the in-vitro secretion of these cytokines measured at intervals during the first week of illness. Sixteen patients (57%) developed systemic complications. Peak TNFα secretion was significantly higher in patients who developed systemic complications (median = 18.5ng/ml, IQR 5.5-28.5) than in those with an uncomplicated course (3.7ng/ml, 2.3-6.4, $P<0.01$). Similarly, peak IL-6 and peak IL-8 secretion were significantly higher in the complicated group (IL-6; complicated: median = 48.9ng/ml, IQR 12-71, uncomplicated 16.3ng/ml, 9.9-24.8, $P<0.05$; IL-8; complicated: median = 754ng/ml, IQR 683-857, uncomplicated: median = 602ng/ml, 496-756, $P<0.05$). No significant difference in peak IL-1 secretion was observed between the two groups.

This study demonstrates that the systemic complications of acute pancreatitis are associated with a significant increase in monocyte secretion of TNFα , IL-6 and IL-8 suggesting that, as in sepsis, these cytokines play a central role in the pathophysiology of the disease.

The role of IL-1β and proteolytic degradation of parathyroid hormone (PTH) in the pathophysiology of pancreatitis-associated hypocalcaemia was studied. Serum levels of PTH, calcium and albumin were measured daily for five days in 41 selected patients with moderate to severe acute pancreatitis. PTH was measured by means of a two-site immunoradiometric assay specific for the intact peptide. A rise in PTH levels was observed more commonly in patients with a complicated or fatal outcome than in those with an uncomplicated course (complicated; 87.5% of 16 patients, uncomplicated; 24% of 25 patients, $P<0.001$, Chi-square test). In the presence of hypocalcaemia, although PTH levels were variable, raised levels of PTH were found more frequently in the complicated group (complicated; 7 of 8, uncomplicated; 2 of 7, $P=0.035$, Fisher's exact test).

This study confirms that an appropriate rise in PTH occurs in response to the hypocalcaemic stimulus in patients with acute pancreatitis, with no evidence of significant PTH degradation. There was a significant negative correlation between serum calcium levels and the secretion of IL-1β by monocytes but this was mainly a consequence of the results obtained from one patient with very low serum calcium associated with high monocyte IL-1β secretion.

Increased production of pro-inflammatory cytokines by monocytes and mononuclear phagocytes would be expected to result in neutrophil activation and therefore raised plasma levels of polymorphonuclear elastase (PMNE). Measurement of PMNE in acute pancreatitis may therefore be a simple method of assessing the degree of leucocyte activation which would allow the early identification of patients at risk of developing systemic complications. Thirty seven patients entered into the randomised, controlled trial of octreotide had serial measurement of PMNE carried out. The mean age of these patients was 68 (range 32-88) and gallstones were identified as an aetiological factor in 21 (57%). Plasma PMNE levels were significantly higher in those patients with systemic complications on each of the days on which it was measured (day 1 PMNE complicated median=250ug/l, IQR 143-656, uncomplicated median 139, IQR 74.7-254, $P<0.05$; day 3 complicated median=224.1, IQR 139-382, uncomplicated median=86.1, IQR 51.4-159.7, $P<0.002$; day 5 complicated median=178.5, IQR 117. Highest levels of PMNE were observed on day 1 of the hospital admission in all but nine of the 37 cases and of these, seven subsequently developed systemic complications. There was a strong positive correlation between IL-1β secretion on the first day after admission and the plasma level of PMNE ($r=0.961$, $P<0.001$). There was no significant correlation between the secretion of TNFα ($r=0.061$), IL-6 ($r=0.282$) or IL-8 ($r=0.288$) and PMNE levels on day 1.

Part 1

Chapter 1

Introduction, hypothesis and aims of the thesis

1.1 General introduction

It is over 100 years since Sir Reginald Fitz described the clinical and pathological features of acute pancreatitis[1]and although supportive measures have greatly improved since then, there remains no specific treatment and many aspects of the pathophysiology of the disease are still poorly understood.

Patients with severe acute pancreatitis commonly develop a systemic illness in the first few days after admission characterised by cardiovascular, respiratory and renal impairment. For many years this was thought to be a consequence of the release of "toxic factors" from the peritoneal exudate, produced as a result of protease activation [2]. More recently, however, it has been observed that the systemic organ dysfunction seen in acute pancreatitis is similar to that seen in patients with sepsis, burns or multiple trauma and it has been suggested that similar endogenous mediators may be responsible for the systemic illness seen in these conditions [3].

There is now strong evidence to suggest that the systemic illness in sepsis is mediated by cytokines as a result of mononuclear cell activation [4]. This has led to recent attempts to examine mononuclear phagocyte activation in acute pancreatitis but results have so far been inconclusive [5,6].

In the assessment of any therapeutic agent in acute pancreatitis, the pathophysiology of the illness must be considered. Somatostatin, and more recently its long-acting analogue, octreotide, have been suggested as being of possible benefit in the treatment of acute pancreatitis. Not only does somatostatin suppress pancreatic secretion, generally thought to be beneficial in acute pancreatitis, but it has also been reported to enhance

hepatic reticuloendothelial activity [7]. This effect would be expected to reduce the likelihood of systemic endotoxaemia and subsequent mononuclear phagocyte activation [8].

This thesis examines the effect of octreotide in the treatment of acute pancreatitis and also explores the role of mononuclear phagocyte activation and cytokine production in the pathophysiology of some of the systemic manifestations of the disease.

1.2 Hypothesis

The systemic complications commonly seen in severe acute pancreatitis are the consequence of unregulated or excessive mononuclear phagocyte activation with the subsequent production and release of excessive quantities of pro-inflammatory cytokines.

The function of the hepatic reticuloendothelial system is important in the removal of toxic factors, particularly bacterial endotoxin, from the portal circulation and in patients with severe acute pancreatitis this is impaired or its functional capacity is exceeded. The use of an agent which enhances hepatic reticuloendothelial function may therefore reduce the morbidity and mortality due to the systemic complications of acute pancreatitis by preventing systemic endotoxaemia and excessive extrahepatic mononuclear cell activation.

1.3 Aims of the thesis

1. To examine the therapeutic effect of the long-acting somatostatin analogue, octreotide, in the management of patients with acute pancreatitis.

2. To determine the role of mononuclear phagocyte activation in the pathophysiology of the systemic complications of acute pancreatitis.

3. To determine the effect of octreotide on mononuclear cell activation.

4. To examine the role of cytokines in the pathophysiology of the hypocalcaemia of acute pancreatitis.

1.4 Plan of thesis

This thesis is presented in two parts In part 1, the relevant literature is reviewed and a clinical trial with octreotide in patients with moderate and severe acute pancreatitis is described. In part 2, there is the laboratory work concerning the role of mononuclear phagocyte activation in the pathophysiology of the systemic complications of pancreatitis.

Part 1 begins with a review of the relevant literature with regard to the pathophysiology of acute pancreatitis and its treatment following which the relevant literature on somatostatin and its analogues in acute pancreatitis is reviewed. A clinical trial with octreotide in acute pancreatitis is then described.

In part 2, The development of the methods used in the laboratory study are described following which the results of an experimental study into mononuclear phagocyte activation are presented. The role of polymorphonuclear cell activation in acute pancreatitis is explored in chapter 6 and a study on the pathophysiology of hypocalcaemia and its relationship to mononuclear phagocyte activation in acute pancreatitis is presented in chapter 7.

Chapter 2

Introduction and review of the literature

2.1. Epidemiology

Acute pancreatitis is a common disorder accounting for some 1234 hospital admissions in Scotland in 1985 [9].There has been an apparent increase in the incidence of acute pancreatitis over the last 30 years, rising from around 36 cases per million in 1969 to 246 cases per million in 1985, the largest increase being in young males [9]. A smaller increase in incidence of 26% to 73 cases per million was reported in a similar study from the Bristol area over a similar period of time [10]. In the Scottish study, the case mortality fell from 18% to 5.6% [9] although a similar reduction was not seen in the Bristol area with case mortality remaining unchanged at 22% for the years 1968-1969 and 1978-79 [10]. In as many as 35% [10]-42% [11] of cases, the diagnosis of acute pancreatitis was only made at post-mortem. The reduction in case mortality seems likely to be related to improvements in diagnosis as the total number of deaths from acute pancreatitis has changed little [9].

2.2 Aetiology

The single most common cause of acute pancreatitis remains gallstones which account for approximately 50% of cases in the West of Scotland [12]. The incidence of gallstone pancreatitis varies widely between geographical areas, accounting for as many as 57% of cases in the Leicester area [13] but only 25% in Germany [14] and 17% in New York [15]. Alcohol accounts for the majority of the remainder of cases accounting for 32-69% of cases with miscellaneous causes accounting for around 4-20% [12-14]There remain a 12-15% [12-15] of patients in whom no cause is identified and these are grouped as "idiopathic". There is growing suspicion that many of these cases are in

fact due to very small gallstones or even biliary sludge [16]. Less common causes of acute pancreatitis are listed in **table 1.1**

Obstructive causes	Choledocholithiasis Ampullary carcinoma Ascaris infestation Pancreas divisum
Toxins	Alcohol Scorpion venom Drugs
Infection	Coxsackie B Mumps Epstein-Barr virus CMV Hepatitis A, B, non-A non-B HIV
Metabolic	Hyperlipidaemia Hypercalcaemia
Miscellaneous	Peptic ulcer Crohns Vasculitis Trauma, ERCP Hypothermia

Table 1.1 Causes of acute pancreatitis

2.3 Clinical presentation and diagnosis

The characteristic presentation of acute pancreatitis is that of sudden abdominal pain. The site of the pain is usually the epigastric but pain also commonly occurs in the right hypochondrium. Rarer sites include the left hypochondrium and lower abdomen. The pain commonly radiates to the back and this may be the only site of pain. Vomiting and retching are often present and may be the main complaint. Physical examination reveals abdominal tenderness and guarding, usually restricted to the upper abdomen but occasionally more generalised. Bowel sounds are usually absent and, especially in more advanced cases, abdominal distension may be a prominent feature. Varying degrees of dehydration are present

depending on vomiting and the duration of symptoms prior to presentation. Tachycardia and, occasionally, hypotension may also be present. Specific clinical signs are rare but body wall ecchymosis, seen as flank staining (Grey-Turner's sign, [17]) or periumbilical staining (Cullen's sign, [18]) is present in around 3% of cases [19]. Such signs, since they typically do not develop until after the fourth day of hospital admission [19], seldom contribute to the initial diagnostic assessment.

With these presenting features, it can readily be appreciated that acute pancreatitis can mimic almost any acute intra-abdominal pathology, in particular perforated peptic ulcer and acute cholecystitis. The diagnosis therefore rests upon the measurement of serum levels of pancreatic enzymes.

2.3.1 Pancreatic enzymes

Amylase

Amylase is a starch-splitting enzyme which is specific for the α1,4 glucosidic linkage of the starch molecule. It is secreted mainly by the salivary glands and the exocrine pancreas with most of the amylase normally present in the serum originating from these sources. The use of urinary amylase measurements in the diagnosis of pancreatic disease was suggested by Wohlgemuth in 1910 but it was not until 1929 that Elman et al described a patient with acute pancreatitis in whom raised levels of serum amylase were found [20] and suggested the usefulness of serum amylase determination for the diagnosis of pancreatic diseases. Over the years this diagnostic test has proven its worth and is now the cornerstone of diagnosis of acute pancreatitis.

The level of serum amylase required for a diagnosis of acute pancreatitis has been a matter of some debate. Ranson, in his study of peritoneal lavage for the treatment of acute pancreatitis, accepted any level above the normal

range in association with an appropriate clinical history [15] as being sufficient for a diagnosis of acute pancreatitis. Other authors have accepted only patients with a threefold [13] or fourfold [12] rise. Few conditions which clinically mimic acute pancreatitis are associated with a rise in serum amylase of this magnitude, the exception being acute mesenteric infarction which is associated with amylase levels elevated to the diagnostic range in 8% of patients [21]. Difficulty in diagnosis occasionally occurs in patients with mild elevations in serum amylase levels in whom perforated duodenal ulcer cannot be excluded.

Amylase is excreted in the urine and in such cases of diagnostic doubt, measurement of urinary amylase has been suggested. However, studies have failed to demonstrate any advantage of urinary amylase levels over measurement of serum amylase alone[22]. Since renal clearance of amylase strongly influences the level of urinary amylase, the use of the amylase to creatinine ratio has also been suggested as a means of improving the specificity of urinary amylase measurements. However, studies have failed to confirm the usefulness of this approach[22].

Lipase

Levels of serum lipase rise in association with acute pancreatitis and parallel the rise of serum amylase. Several studies have shown that lipase levels are more sensitive and specific for the diagnosis of acute pancreatitis [23,24]than serum amylase. However, like amylase, other conditions such as perforated duodenal ulcer and mesenteric infarction may be associated with elevated levels[25]. The availability of the assay is limited in UK laboratories, therefore lipase levels are infrequently measured.

Trypsin

The majority of serum immunoreactive trypsin circulates as trypsinogen. Although serum levels of trypsinogen rise in patients with acute pancreatitis[26], at present, trypsin assays are not generally available on a routine basis.

2.3.2 Other Diagnostic Aids

Plain Abdominal Radiographs

Several abnormalities on plain abdominal radiographs have been described in patients with acute pancreatitis. These were studied by Davis et al in 100 patients with acute pancreatitis and 100 patients undergoing routine intravenous urography[27]. The most commonly observed abnormalities in the pancreatitis group were the sentinel loop (26%), thought to represent a localised duodenal ileus, colon cut-off sign- due to dilatation of the ascending colon with oedematous swelling in the transverse colon (18%) and loss of definition of the left psoas shadow (70%). However, while these features may be more common in radiographs of patients with acute pancreatitis compared with healthy controls, many of these features are commonly seen in radiographs of patients with other abdominal emergencies such as acute cholecystitis or perforated duodenal ulcer. For example, in one study comparing acute pancreatitis patients to those with other acute abdominal emergencies[28], duodenal abnormalities were observed in 21% of patients with perforated duodenal ulcer or acute cholecystitis. Plain abdominal radiographs therefore have little to offer in the diagnosis of acute pancreatitis and serve mainly to exclude other pathology such as perforated abdominal viscus or intestinal obstruction.

Ultrasound and CT Scanning

In the early stages of acute pancreatitis, the presence of bowel gas overlying the pancreas limits the usefulness of ultrasound in the diagnosis of acute pancreatitis[29]. In a study from Glasgow[29], even in cases of mild pancreatitis, the pancreas was not visualised in almost 60% of cases. In contrast, CT scanning plays an invaluable role in the assessment of the pancreas in acute pancreatitis in demonstrating characteristic pancreatic swelling and peripancreatic fluid collections. Although CT may be useful in the rare case of diagnostic doubt, its main role lies in the diagnosis of local pancreatic complications.

2.4 Prediction of Severity

Acute pancreatitis ranges in severity from a rapidly fatal fulminating disease to a mild, self-limiting illness which settles within a few days. At either extreme the prediction of severity presents no particular difficulty but in a large group of patients who lie between these two extremes, objective grading of severity is difficult and many scoring systems and assays have been proposed over the years to improve the accuracy of clinical assessment.

2.4.1 Multiple Factor Scoring Systems

In 1976, Ranson described the prospective evaluation of multiple clinical, biochemical and haematological indices in the prediction of severity of acute pancreatitis[15]. Retrospective analysis of 43 factors revealed 11 which were of prognostic value **(table 1.2)**. In patients with less than 3 positive factors, the mortality was 1.2% and the incidence of major complications 3.7%. In those with 3 or more positive factors, the major morbidity and mortality was 62%. A modified version of this system was later utilised by Imrie and colleagues in the analysis of patients taking part in a clinical trial

of intravenous aprotinin **(table 1.3)**[12]. Again, three or more positive criteria within 48h of admission were associated with a worse prognosis. This modified version was later validated by Blamey et al[30], who suggested that the transaminase level be excluded. Using the original system, 79% of 405 episodes of acute pancreatitis were correctly classified. Imrie later excluded age as a prognostic factor as there was a tendency to overestimate the severity of patients with gallstone pancreatitis, who tended to be older[31].

Despite the apparent accuracy of these scoring systems, several problems remain. Firstly, all multiple factor scoring systems require 48 hours of measurement prior to categorisation of patients. Secondly, the accuracy of these systems in correctly predicting severe disease may have been overstated. Using Imrie's modification of the Ranson criteria, Blamey and colleagues found that only 31% of patients with 3 or more positive factors had severe pancreatitis[30]. The system proved more useful in identifying those with mild disease with only 8.4% with less than 3 positive factors developing severe pancreatitis. Consequently, there have been numerous attempts to find an assay which allows accurate prediction of severity on admission to hospital.

On Admission	Within 48h
Age >55	Haematocrit fall >10%
Glucose >200mg/1ml	Calcium < 8mg/1ml
WBC>16000/mm^3	Base deficit >4mEq/l
GOT>250 Sigma Frankel units/1ml	Blood urea nitrogen increase >5mg/1ml
LDH>300IU/l	Fluid sequestration >6l
	PaO_2<60mmHg

Table 1.2 Ranson's prognostic criteria in acute pancreatitis.

Within 48h of admission
PaO_2<60mmHg
Albumin<32g/l
Calcium <2mmol/l
WBC>15x10^9/l
AST/ALT>1u/l
LDH>600u/l
Glucose >10mmol/l (in the absence of diabetes)
Urea>16mmol/l (not responding to therapy)
Age >55years

Table 1.3 Modified prognostic criteria (Imrie et al, 1978)

APACHE II Score

The APACHE II system uses 12 routinely available physiological and biochemical measurements, coupled with a score for age and pre-existing health status to give a score which can be used to grade the severity of illness in an individual patient[32]. Although studied most widely in intensive care units, the system has been successfully applied to the assessment of patients with acute pancreatitis[33,34]. When the APACHE II score on admission is measured, a cut-off score of 5 allows accurate prediction of severity in over 60% of cases[34]. The positive predictive value, however, is only 0.4 although even this is higher than has been reported with multiple factor scoring systems[30]. Increasing the cut-off to 7 slightly improves the specificity of the test without altering the positive predictive value. Peak APACHE II score allows even better discrimination between mild and severe groups. An APACHE II score of greater than 7 has been accepted as being indicative of severe acute pancreatitis by the Atlanta Symposium[35]. The advantages of the APACHE II system are that all the criteria for its measurement are readily available on admission to hospital, thus for clinical trials the cut-off level can be manipulated to improve either the sensitivity

or the specificity of the test as is deemed most appropriate. Furthermore, the system can be used to monitor the course of the illness[33,34].

2.4.2 Single prognostic indicators

C-Reactive Protein

C-reactive protein (CRP) is an acute phase protein synthesised by the liver in a variety of disease states. In acute pancreatitis, several studies have reported that levels of CRP may be used to identify those patients with severe attacks[36-41]. Uhl and colleagues[37] suggested a cut-off for peak CRP of 120mg/l which accurately predicted outcome in excess of 80% of cases. Similar results were reported by Gross et al with a cut-off of 100mg/l[36]. In a study from Glasgow, Wilson and colleagues found a cut-off peak level of 210mg/l to be more accurate[34]. In all studies, peak levels of CRP were reached between the second and fourth days after admission. Therefore, although diagnostic accuracy was similar to that achieved using multiple factor scoring systems, measurement of CRP was unable to give earlier prognostic information.

Polymorphonuclear Elastase

Polymorphonuclear elastase (PMNE) is released from activated neutrophils in a variety of conditions and has recently been assessed in patients with acute pancreatitis[36,37,42]. Dominguez-Munoz and colleagues[42] found very encouraging results in a series of 182 patients of whom 28 had severe acute pancreatitis. Using a cut-off level of 250ug/l on admission to hospital. the authors reported a positive predictive value of 80% with a negative predictive value of 98%. The positive predictive value of a level of greater than 300ug/l at 24h rose to over 97%. In a smaller study, Uhl and colleagues[37] reported similar peak levels of PMNE on day one of the illness, with levels of greater than 120ug/l accurately predicting necrotising

pancreatitis in 84% of cases. However, in all cases, PMNE was measured retrospectively on stored plasma samples and the possibility of observer bias cannot be excluded. The use of PMNE has not been evaluated in a prospective study.

Trypsinogen activation peptides (TAP)

The activation of trypsinogen has long been considered the key event in the development of acute pancreatitis The measurement of trypsinogen activation has recently become possible with the development of an assay for the peptides released from the trypsinogen molecule after its activation to trypsin. In a series of 55 patients, workers in Glasgow and London measured urinary TAP levels in 55 patients[43] with acute pancreatitis of whom 15 were described as having severe attacks. Of these latter patients, 12 were correctly predicted as severe on the basis of a cut-off level of 2nmol/l of urinary TAP on admission to hospital (positive predictive value 0.8). Using the same figure, only 4 of the remaining 40 patients were incorrectly graded as severe (negative predictive value 0.9). However, included in those patients with "major complications" are two with ileus, one with jaundice, two with duodenal obstruction and three with haemorrhage from undefined sources. It is therefore unclear how many of these patients genuinely had severe acute pancreatitis by accepted criteria. The value of urinary TAP and other activation peptides such as PLAP (phospholipase A_2 activation peptide) in the prediction of severity of acute pancreatitis remains to be determined.

2.5 Complications of acute pancreatitis

The clinical course of severe acute pancreatitis is characterised by the development of local complications or systemic organ failure. These have recently been defined by an international working party meeting in Atlanta in 1992[35]. Their recommendations are summarised below.

2.5.1 Local Complications

Acute fluid collection

This was defined as a collection of fluid in or near to the pancreas occurring early in the course of the illness (within 4 weeks of the onset of acute pancreatitis) and lacking a defined wall of fibrous or granulation tissue.

Pancreatic necrosis

This is present if, on intravenous contrast-enhanced CT scan, focal or diffuse areas of non-enhancement of greater than 3cm or more than 30% of the gland are present. This is typically associated with peri-pancreatic fat necrosis. The distinction between sterile and infected pancreatic necrosis depends culture of tissue or fluid from percutaneous needle aspiration although, in many centres the distinction is made on clinical grounds.

Acute pseudocyst

This is defined as a collection of pancreatic juice enclosed by a wall of fibrous or granulation tissue 4 or more weeks after an attack of acute pancreatitis. Therefore an acute fluid collection which persists for more than 4 weeks and aquires a fibrous wall becomes by definition an acute pseudocyst.

Pancreatic abscess

This is defined as a collection of pus, usually in proximity to the pancreas which is associated with little or no pancreatic necrosis. The presence of pancreatic necrosis is associated with a very much worse prognosis and would be included in the definition of "infected pancreatic necrosis".

2.5.2. Systemic complications

Organ failure in acute pancreatitis is defined by the following criteria:

Shock

Systolic blood pressure < 90mmHg

Pulmonary insufficiency

PaO_2 < 60mmHg

Renal failure

Serum creatinine greater than 177umol/l after rehydration

2.5.3 Incidence of complications

The incidence of the above complications in acute pancreatitis is difficult to determine as definitions have varied with time and between authors. In a Glasgow study[12] pseudocyst occurred in 5.5% of cases but this included patients who would now be defined as having acute fluid collections. The number of local complications which are found will also depend on how avidly they are sought. In one series the incidence of fluid collections in acute pancreatitis was 53%[44]. The incidence of pancreatic necrosis depends largely upon how many patients undergo CT scanning. Thus in a study from Leicester, the incidence of pancreatic necrosis was 1.5%[13] whereas in another study from the same area[45] where 45% of patients had early CT

scanning, at least 23% of patients had pancreatic necrosis. Similarly, the incidence of local complications depends upon the definitions used and the frequency of biochemical sampling. The incidence of respiratory complications varies greatly between studies and was found to be as high as 47% when repeated blood gas measurements were made[12]. Severe renal failure is less common at 3-4% [12,13].

2.6 Pathogenesis of Acute Pancreatitis

It is now over 100 years since the first comprehensive account of acute pancreatitis by Sir Reginald Fitz[1]. Seven years later, Chiari proposed that the pancreatic damage was the result of autodigestion of the gland by activated pancreatic enzymes. Since then, our understanding of the pathophysiology of the disease has progressed remarkably little despite many years of intensive research.

2.6.1 Gallstone Pancreatitis

It was Opie who first noted the association between an obstructing ampullary stone and fatal acute pancreatitis[46]. Since then controversy has persisted over the exact sequence of events in gallstone pancreatitis. Subsequent large studies cast doubt on the ampullary obstruction theory. In a post mortem study of 1 cases of fatal pancreatitis Shader and Paxton found obstructing ampullary calculi in only three cases[47]. In patients undergoing operation for gallstone pancreatitis, Kelly found obstructing calculi in only 5%[48].

The finding that if the stools of patients with pancreatitis and proven gallstones are screened for stones, they will be found in 85-95% of cases[48,49] suggests that gallstone migration is the important aetiological factor in these cases. Certain features of the biliary tract in patients with gallstones and a history of pancreatitis are compatible with an increased

tendency towards migration. The cystic duct tends to be wider than in patients with gallstones and no history of pancreatitis and the gallstones are smaller and more numerous[29,50,51].

More recently the concept of transient ampullary obstruction has been introduced. Acosta[52] found ampullary obstruction in 63% of patients undergoing early operation for gallstone pancreatitis. The incidence dropped from 75% at <48 hours to 25% at >4 days suggesting that pancreatitis is associated with transient ductal obstruction. He also found that persisting obstruction was associated with fulminating disease and suggested that the duration of obstruction may determine the severity of the disease.

Like Acosta, Armstrong[50] found the incidence of ampullary obstruction decreased with time, falling from 66% with operation under 48 hours to 3.8% at greater than 22 days. In those without obstruction, gallstones are usually recovered from the stool, confirming gallstone migration.

It would seem likely that gallstone pancreatitis is related to the migration of a stone with transient obstruction of the ampulla of Vater. The mechanism by which such a stone initiates pancreatitis remains controversial but there are three main theories;

- Bile reflux
- Duodenal reflux
- Ductal obstruction.

Bile reflux

Bile reflux was initially championed by Opie as the pathogenesis of gallstone pancreatitis[46]. He postulated that, following the obstruction of the ampulla of Vater by a stone, bile would be allowed to reflux along the pancreatic duct via the common channel. There are two main objections to this theory;

1.Bile itself is unlikely to be pathogenic.

It has been assumed that pancreatitis is initiated by the intrapancreatic activation of protease precursors. Bile does not cause trypsin activation[53] and bile introduced into the pancreatic duct at low pressures will not initiate pancreatitis[54]. The entire biliary output of experimental animals can be routed through the pancreatic duct without causing pancreatitis[55]. Infected bile under normal pressure does, however, cause pancreatitis[54]. In experimental models, it is necessary to inject bile at high pressure for pancreatitis to occur and the degree of damage caused is related to the pressure used. Such pressures are unlikely to be generated under physiological conditions.

2.Reflux is unlikely to occur.

For bile reflux to occur following ampullary obstruction, a functioning common channel must exist. This was demonstrated at cholangiography in only 23% of cases in a study by Acosta[52].

The type of obstruction usually present prevented bile reflux by obstructing the pancreatic duct. Although others have claimed to demonstrate bile reflux more commonly in patients with pancreatitis, this has been in the recovery period where gallstone obstruction was not present and thus a functioning common channel more likely. The methods used for demonstrating bile reflux involve operative cholangiography which utilises

high injection pressures unlikely to be obtained under normal physiological conditions. The normal pressure gradient between the pancreatic duct and common bile duct would also be expected to prevent bile reflux. Pancreatic duct pressures have been found to be 2-3 times greater than those in the common bile duct, both in normal subjects and in a variety of pathological conditions[56]. One study[57] however, has demonstrated slightly greater common bile duct pressures in patients with common bile duct stones. Even in these patients, the pancreatic duct sphincter pressure remained greater than 3 times the common bile duct pressure at all times. It is difficult to explain bile reflux under these conditions.

Duodenal reflux

McCutcheon[58] offered his "fresh approach" to the pathogenesis of pancreatitis: as bile alone is unlikely to activate pancreatic proteases, he suggested that duodenal contents containing enterokinase may gain access to the pancreatic duct, thus initiating pancreatitis. Factors normally preventing duodenal reflux include; the oblique course of the pancreatic duct which tends to become compressed as it passes through the duodenal muscle, the sphincter of Oddi and the mucosal folds at the papilla. It was proposed that the presence of a stone in the ampulla caused fixed dilatation, overcoming these factors and favouring the reflux of duodenal contents. In experimental animals, the formation of a closed duodenal loop, with the resultant rise in intraduodenal pressure, is associated with the onset of haemorrhagic pancreatitis[59]. This is prevented by the ligation of the pancreatic duct[60] suggesting that duodenal contents are responsible. McCutcheon and Race[61] demonstrated reflux of barium into the pancreatic duct, confirming this as the aetiological factor. Stimulation with cholecystokinin has been shown to favour duodenal reflux after the formation of a closed loop[62]. Such conditions are, however, unlikely in

gallstone pancreatitis. Perfusing the pancreatic duct with bile mixed with duodenal content at physiological pressures does not cause pancreatitis[55]. It has been suggested that duodenal reflux may explain the occasional occurrence of pancreatitis after a Bilroth II gastrectomy[58].

Pancreatic duct obstruction

Although pancreatic duct obstruction is rarely found in post mortem studies of acute pancreatitis, being found in only one of three patients in one series[47], in patients undergoing early intervention it is found much more frequently[49]. At least transient obstruction of the ampulla of Vater is a likely step in the pathogenesis of the disease. Pancreatic duct obstruction may occur either as a direct consequence of an impacted stone or as a result of the subsequent oedema.

In experimental animals, pancreatic duct obstruction alone does not result in the development of haemorrhagic pancreatitis[63]. Ligation of the pancreatic duct leads to mild pancreatic oedema and hyperamylasaemia followed, if the obstruction is prolonged, by acinar cell atrophy and fibrosis[64,65]. If ductal ligation is combined with secretagogue stimulation, however, more severe pancreatitis develops, accompanied by areas of fat necrosis[66]. A similar effect is seen if ductal ligation is followed with a meal[67].The development of pancreatic necrosis depends upon the subsequent reduction of pancreatic blood flow[68]. In the clinical situation, the common presentation of pancreatitis is with simple pancreatic oedema and hyperamylasaemia accompanied by abdominal pain, all of which may be caused by ductal obstruction alone. It has been observed that the onset of pain in gallstone pancreatitis often follows a large meal. It has been suggested that the variable course of the disease and the development of pancreatic necrosis may depend upon the subsequent extent of pancreatic

ischaemia. Further support for the ductal obstruction theory comes from case reports of pancreatitis induced by the obstruction of the pancreatic duct by Ascaris Lumbricoides[69].

One experimental study examined the effects of biliary and pancreatic obstruction, both independently and in combination[70]. It was found that pancreatic duct ligation alone resulted in the development of pancreatic oedema and hyperamylasaemia but the addition of bile duct ligation resulted in pancreatic necrosis. The underlying mechanism was not known but biliary obstruction was previously shown to increase pancreatic secretion in the same species[71] which may have contributed to the development of acute pancreatitis.

2.6.2 Alcohol-induced Acute Pancreatitis

Many patients with alcohol-induced acute pancreatitis subsequently develop chronic pancreatitis, with recurring attacks of pain and pancreatic insufficiency but acute pancreatitis does not progress to chronic pancreatitis in all cases[72]. Subclinical pancreatic damage has been demonstrated in heavy drinkers[73] and there is little doubt that in many patients presenting with acute alcoholic pancreatitis, pancreatic damage has already occurred. In any discussion of the pathophysiology of acute alcoholic pancreatitis, there is therefore considerable overlap with chronic pancreatitis and at the present time it is unclear why some patients present with a seemingly isolated attack of acute pancreatitis and others progress to chronic pancreatitis.

The mechanism by which alcohol causes pancreatic damage remains a subject of some debate. Three main theories have been proposed: these are the ductal plug hypothesis, the toxic-metabolic hypothesis and the large duct flow/reflux hypothesis.

Ductal plug hypothesis

The ductal plug hypothesis is based on the observation that the administration of alcohol to experimental animals results in changes in the composition of pancreatic juice which favour the development of proteinaceous plugs which may obstruct small pancreatic ducts leading to the development of inflammation and consequent degeneration of the area drained by that duct.

In dogs, alcohol administration results in an increase in protein concentration in pancreatic juice with associated decreases in secretory volume and bicarbonate concentration[74]. Plugs with a concentric, laminar structure were found within the ducts. Other investigators have failed to find ductal plugs in similar experiments on rats[75,76]. Evidence from human studies suggests that increased protein concentration of pancreatic juice is found in patients with a high alcohol intake[77]. Decreased levels of secretion of lithostathine, a protein which attenuates calcium carbonate crystallisation[78], have also been identified in the pancreatic juice of chronic alcoholics which may be one predisposing factor in the development of chronic calcifying pancreatitis. Increased serum levels of another family of proteins, known as pancreatitis associated protein (PAP) have been demonstrated in acute pancreatitis and have been suggested as a marker of subclinical pancreatic injury[79]. Their role in the pathophysiology of acute pancreatitis is uncertain and it appears that they are secreted as part of the acute phase response following the initial pancreatic damage[80].

As mentioned in the preceding section, experimental models of simple duct obstruction do not lead to the development of acute pancreatitis but to chronic atrophy and this mechanism may therefore be of more relevance to the pathophysiology of chronic rather than acute pancreatitis. It is also of relevance that in experimental pancreatitis, the administration of alcohol

does not worsen the severity of pancreatitis induced by duct obstruction[81], whereas it significantly worsens the severity of secretagogue-induced pancreatitis.

Toxic-metabolic Hypothesis

Ingested ethanol is metabolised in the liver to ethanaldehyde. Both ethanol itself and ethanaldehyde are toxic to cells and the basis of the toxic-metabolic hypothesis is that ethanol and its metabolites are directly injurious to pancreatic acinar cells resulting in the development of pancreatitis[82]. It has been demonstrated that ethanaldehyde combined with a short period of ischaemia can induce acute pancreatitis in an ex-vivo model[83] and there is evidence that the quantity of alcohol ingested in the week prior to a first attack of acute pancreatitis is an important determinant of the severity of the attack[83].

Large duct Flow/Reflux Hypothesis

This theory simply suggests that alcohol facilitates the development of acute pancreatitis by either duodeno-pancreatic or biliary-pancreatic reflux. The bile of alcohol-fed rats is more toxic to the pancreas than bile from control rats when injected into the pancreatic duct[84]. Intragastric alcohol increases the permeability of the pancreatic duct in cats and allows the induction of acute pancreatitis by activated pancreatic enzymes infused into the duct[85]. Such models may explain cases of alcohol-induced pancreatitis in the absence of chronic pancreatic damage but the relevance of the complex experimental models employed to the development of pancreatitis in humans is uncertain.

It can be readily appreciated that no one theory satisfactorily explains the development of alcohol-induced acute pancreatitis in man, and in

particular, it is unknown which, if any, of these mechanisms are responsible for the development of an acute attack of pancreatitis in a chronic alcoholic.

2.6.3 Cellular events in experimental pancreatitis

Under normal circumstances, pancreatic zymogen enzymes are synthesised on the rough endoplasmic reticulum of the acinar cell and transported with zymogen enzymes to the Golgi complex. Here, the two classes of enzyme are separated, the lysosomal enzymes into lysosomes and the zymogens into secretory granules. This occurs by means of the recognition of a 6-phosphorylated mannose residue unique to the lysosomal enzymes. Cathepsin-B, a lysosomal hydrolase, is capable of trypsinogen activation and this separation prevents intracellular activation of proteases. In addition, a potent trypsin inhibitor is synthesised and transported with the zymogen enzymes[86].

In three divergent experimental models of acute pancreatitis, alterations in the normal pattern of enzyme secretion result in the potential for intracellular activation of pancreatic enzymes. In a choline deficient, ethionine supplemented diet model, Steer et al[87] have demonstrated an intracellular accumulation of zymogen granules, the contents of which are subsequently discharged into lysosomes. In a caerulin hyperstimulation model, zymogen accumulation did not occur but large intracellular vacuoles appeared which contained both lysosomal and digestive enzymes. In a ductal obstruction model of acute pancreatitis, Ohshio et al[63] have demonstrated that cathepsin-B, a lisosomal hydrolase capable of the activation of trypsinogen, is found within zymogen granules and it has therefore been suggested that intracellular, rather than extracellular, zymogen activation is the first event in the pathogenesis of acute

pancreatitis. However, the relevance of these findings in experimental acute pancreatitis to acute pancreatitis in man is not known.

2.7 Management of Acute Pancreatitis and its Complications

The mainstay of management of acute pancreatitis remains supportive and although many specific medical therapies have been proposed, none has been shown to be better than standard supportive care when assessed in clinical trials.

2.7.1 Supportive measures

On presentation, pain, vomiting and dehydration are the main problems. Pain is usually controlled with intramuscular morphine derivatives, pethidine being commonly used as it is thought to have less potential to cause sphincter of Oddi spasm than morphine itself. Vomiting often resolves quickly after admission to hospital but if persistent can be alleviated by nasogastric suction. In patients with more severe attacks, nasogastric suction is often used routinely, as in these patients prolonged ileus is common. Clinical trials, however, have shown no benefit in terms of clinical outcome, duration of pain, or duration of hyperamylasaemia with the routine use of nasogastric suction in patients with acute pancreatitis, although the number of patients studied was small and patients with severe pancreatitis were not studied[88]. Intravenous fluids are given to correct dehydration and a urinary catheter is passed in order to assess the adequacy of resuscitation. In more severe cases and in the elderly, central venous pressure monitoring is commonly employed. The choice of fluid used for resuscitation varies but in acute pancreatitis, in addition to fluid lost through prolonged vomiting, there is loss of protein-rich fluid into the peritoneum and interstitial space therefore a combination of colloid and crystalloid fluid replacement is often recommended. The majority of

patients will settle within a few days on this management but a proportion will go on to develop either local or systemic complications.

2.7.2 Management of Local Complications

Acute Fluid collection

Acute fluid collections are common in acute pancreatitis may occur in more than 50% of severe cases[44]. They are more common in patients with alcohol-related pancreatitis and are often asymptomatic[89]. Many of these will settle spontaneously and require no intervention but if large and causing pressure effects or if infection supervenes then intervention is required. If early intervention is necessary, the choice lies between formal laparotomy and external drainage or percutaneous drainage under CT or ultrasound guidance. There is little doubt that percutaneous drainage can be carried out safely[90,91] but recurrence is common and formal laparotomy with external drainage may be more appropriate in some cases, particularly when there is much debris present or the collection is locculated.

Acute pseudocyst

When there is a well-defined wall around the fluid collection, internal drainage may be employed if the cyst fails to resolve or if complications supersede. A period of observation by repeated ultrasound or CT is warranted if the pseudocyst is small and asymptomatic[89]. Internal drainage may be carried out by cystogastrostomy if the pseudocyst lies in close approximation to the posterior wall of the stomach or by cystojejunostomy when the cyst lies in the body or tail of pancreas[89]. Success has been reported with endoscopic cystogastrostomy,[92-94] although at present this technique is restricted to specialist centres.

Infected pancreatic necrosis

Patients with infected pancreatic necrosis have a high mortality if surgery is not undertaken[89]. In contrast, specialist centres have reported mortality rates as low as 10-11% [89,95] for patients with infected pancreatic necrosis managed surgically. The details of the type of surgery undertaken vary between centres but the essential principles are laparotomy with wide debridement of devitalised tissue (necrosectomy) with either prolonged postoperative lesser sac lavage and drainage[95] or open packing[89]. In both cases, re-operation is often required for the removal of further necrotic tissue. It can be readily appreciated that pre-operative CT scanning is essential in order to differentiate between pancreatic abscess (which can be successfully managed with simple external or internal drainage) and infected pancreatic necrosis.

2.7.3 Management of systemic complications

Respiratory impairment

Arterial hypoxaemia is common in acute pancreatitis, with 50% of patients developing pO_2 levels of 8kPa or less during the first three days following admission[96]. Detailed study of gas exchange patterns in these patients has demonstrated right to left shunting as the main cause of hypoxaemia[97], which is similar to the findings in patients with adult respiratory distress syndrome (ARDS) occurring as a result of sepsis. In one study, 50% of patients with hypoxaemia had radiological abnormalities, the majority being pleural effusions, particularly left sided[97]. Initial management is by the administration of oxygen by facemask but close monitoring is required as pulmonary function can deteriorate rapidly[97]. Early deteriorating respiratory function necessitates management within an intensive care unit and early assisted ventilation. When respiratory function begins to deteriorate later in the course of the illness, a septic complication (i.e.

pancreatic abscess or infected pancreatic necrosis) should be suspected and appropriate investigations instituted.

Renal impairment

As with pulmonary insufficiency, renal failure may occur early in the course of the illness when it is associated with inadequate fluid resuscitation or fulminant acute pancreatitis, or later when it is often the result of the development of pancreatic sepsis[98]. The important factor early in the illness is prevention of this complication by adequate fluid resuscitation but once established, treatment is supportive with haemofiltration. Dopamine is often used to augment renal perfusion in cases of incipient renal failure[98] but is of unproven value.

Cardiovascular impairment

Adequate fluid resuscitation is important, particularly in the early stages of acute pancreatitis and in the majority of patients the circulation can be sustained with crystalloid, colloid and blood [98]. The onset of refractory shock is associated with a grave prognosis in acute pancreatitis, whether occurring early or late in the illness. Treatment is supportive with inotropes and fluids and in addition, these patients are usually in need of artificial ventilation and renal support. Myocardial infarction may complicate acute pancreatitis, particularly in the elderly, and was thought to be a contributory factor in 43% of deaths in one study[12]

Biochemical complications

Mild hyperglycaemia is commonly seen in the early stages of acute pancreatitis and may be a consequence of the stress response and relative pancreatic endocrine insufficiency[99]. It rarely requires specific treatment. Hypocalcaemia is also commonly seen and is associated with the more

severe cases. The majority of cases of observed hypocalcaemia are the result of hypoalbuminaemia[100] but true hypocalcaemia does occur. This subject is discussed in detail in chapter 5.

2.7.4 Role of early ERCP

Two studies have now been carried out which suggest a role for early ERCP and sphincterotomy in patients with gallstone pancreatitis. Neoptolemos and colleagues in Leicester[101] found a significant reduction in morbidity in patients with predicted severe, gallstone pancreatitis if ERCP and sphincterotomy was undertaken within 72h of admission. Although a study from Hong Kong[102] failed to confirm this reduction in morbidity when ERCP was applied to all patients with gallstone pancreatitis, there was a significant improvement in outcome in the subgroup with predicted severe disease. In neither study was there any morbidity directly related to ERCP. Rather than concluding that ERCP is safe under such circumstances, it would be prudent to assume that such reductions in morbidity can only be achieved if a suitably skilled and experienced endoscopist is available.

2.7.5 Medical management of acute pancreatitis

No specific therapy has been found to be successful in the treatment of acute pancreatitis but proposed treatments have fallen into two main categories; inhibition of pancreatic secretion and counteraction of pancreatic enzymes.

Inhibition of pancreatic enzymes

Systemic Antiprotease Therapy

Activation of pancreatic proteases has been considered to play a central role in the development of pancreatic autodigestion and in the development of the early systemic illness in patients with severe pancreatitis[103]. Aprotinin, a polypeptide trypsin-kallikrein inhibitor obtained from the bovine parotid, was introduced by Frey in the 1950's[12]. Many encouraging reports appeared in the European literature during the late 1950's and early 1960's but these were mainly uncontrolled studies with small numbers of patients in whom subjective impressions of clinical improvement were observed[104]. Subsequent controlled clinical trials were conducted but in many cases the number of patients studied was too small to enable conclusions to be drawn on the efficacy of aprotinin[105-107], Experimental work suggesting that the original doses used in these early studies was too small led to subsequent trials with higher doses of aprotinin. Trapnell et al[104] reported a randomised, controlled trial of high dose aprotinin which demonstrated a significant reduction in mortality in the treatment group. However, this trial was subsequently criticised as the mortality in the control group was 25% which was higher than that commonly reported in other UK centres. Subsequent trials with high dose aprotinin failed to demonstrate any effect on mortality or the development of complications[12,108,109] and it is now generally accepted that intravenous aprotinin has no role to play in the management of acute pancreatitis.

Gabexate mesilate (Foy) is a synthetic antiproteases which has the theoretical advantage of being able to inhibit intracellular proteases[14]. A multi-centre controlled trial, recruiting 223 patients with moderate and severe acute pancreatitis, has reported no evidence of improvement in outcome with 4g/day of gabexate mesilate intravenously[14].

Fresh frozen plasma is a rich source of naturally occurring antiproteases, particularly alpha-2-macroglobulin but neither low dose[13] (2 units daily for three days) or high dose[45] (8 units daily for three days) made any impact on clinical outcome.

Peritoneal Lavage

Toxic factors have been identified in the peritoneal exudate present in experimental pancreatitis which cause hypotension when injected into healthy dogs[2]. Peritoneal lavage, aimed at removal of these factors, improved survival in a number of experimental models of acute pancreatitis[110,111]. Many uncontrolled series of patients were reported in whom therapeutic peritoneal lavage appeared to improve the clinical course in acute pancreatitis[112-114]. The first randomised trial was carried out by Stone and Fabian and reported in 1980[115]. They observed clinical response in the majority of patients treated with peritoneal lavage and also in patients in the control group who failed to improve and were subsequently given peritoneal lavage. The criteria for clinical improvement were rather subjective, however, and overall there was no significant difference in mortality between the two groups of patients. This did not prevent the authors from claiming that their observations offered "vigorous support" for the use of peritoneal lavage in acute pancreatitis. to Enthusiasm was such that many centres were utilising peritoneal lavage as a primary treatment in patients with severe pancreatitis until, in 1985, Mayer and colleagues reported the results of a multi-centre trial conducted between Leeds, Bristol and Glasgow[116] in which 91 patients with prognostically severe acute pancreatitis were studied. There was similar mortality and morbidity in the treatment and placebo groups and the power of the study was sufficiently high to exclude a therapeutic effect of peritoneal lavage.

Intraperitoneal antiprotease therapy

Following the failure of peritoneal lavage, a controlled trial of therapeutic lavage with the addition of the protease inhibitor, aprotinin was carried out between centres in Leeds and Glasgow. Patients with mild pancreatitis were excluded and 203 patients were eventually recruited[117]. Once again, mortality and morbidity were similar in the two groups of patients, allowing confident exclusion of a beneficial effect with this treatment.

Inhibition of pancreatic secretion.

In 1948, Paxton and Payne reported the high mortality associated with surgical management of patients with acute pancreatitis and suggested a conservative management regime consisting of fasting, intravenous fluids, nasogastric suction, analgesia and atropine to "rest the pancreas"[118]. Forty five years later, our management has changed little and, although atropine is no longer used, drugs which may suppress pancreatic secretion continue to be the subject of therapeutic trials.

Glucagon

Glucagon reduces pancreatic secretion and was first suggested for use in the management of acute pancreatitis by Knight and colleagues in 1972[119]. Since then there have been five randomised controlled trials conducted of which four[119-123] studied too few patients with severe pancreatitis for valid conclusions to be drawn. The one large trial[108,109] recruiting 257 patients, was conducted under the auspices of the MRC and compared glucagon with aprotinin as well as with placebo. No difference in mortality or morbidity was observed between either of the treatment groups and the placebo group.

Cimetidine

Secretin is responsible for the stimulation of bicarbonate secretion during the intestinal phase of pancreatic secretion. It was suggested that cimetidine, by reducing gastric acid output, may cause a reduction in pancreatic stimulation by secretin[124] and might therefore improve outcome in acute pancreatitis. No effect of cimetidine was observed in two small randomised trials[124,125] and the availability of more potent suppressers of pancreatic secretion precluded the further study of cimetidine in this context.

Somatostatin and somatostatin analogues

Somatostatin is a potent inhibitor of pancreatic secretion and its use in the treatment and prophylaxis of acute pancreatitis is reviewed in chapter 2.

Antibiotics

Septic complications are the single most common cause of death in patients with severe acute pancreatitis and the use of prophylactic antibiotics has been a point of controversy in the management of patients since the mid 1970's. Early trials with ampicillin[126-128] failed to demonstrate any reduction in the incidence of complications or mortality, although all involved small numbers of patients with mainly alcoholic pancreatitis. Subsequent studies showed that the penetration of ampicillin and many other antibiotics into pancreatic tissue is inadequate to reach therapeutic levels[129]. A recent study in 120 patients undergoing pancreatic surgery compared blood levels and pancreatic tissue levels obtained with 10 different antibiotics[130]. Although all achieved therapeutic plasma levels, in only a proportion were high levels seen in pancreatic tissue. Of these, ciprofloxacillin, ofloxacin and imipenem were active against the majority of organisms present. Following this, a multicentre trial was carried out to assess the effect of imipenem in patients with pancreatic necrosis[131]. There was a statistically significant reduction in

the incidence of pancreatic sepsis in those patients treated with imipenem 0.5g tid, although there was no significant reduction in overall mortality. The diagnosis of pancreatic sepsis in this study depended upon positive bacterial cultures which may have been influenced by the administration of Imipenem. The number of patients undergoing surgery for pancreatic necrosis associated with a septic clinical picture was not different in the two groups of patients and the routine administration of prophylactic antibiotics cannot be supported on the evidence of this study alone.

2.8 Mediators of the Systemic Illness in Acute Pancreatitis

In consideration of the potential role of any therapeutic agent in acute pancreatitis, it is necessary to examine the pathophysiology, not only of the underlying pancreatic inflammation, but also of the systemic manifestations of the illness. It is well-recognised that the late, septic complications of acute pancreatitis result in organ failure and it is assumed that this is a consequence of the same mechanisms which are responsible for organ failure due to other forms of sepsis. What is less clearly understood is the mechanism of the early systemic illness which still results in considerable morbidity and a high proportion of deaths from acute pancreatitis[116].

2.8.1 Activated proteases

For many years the systemic manifestations of acute pancreatitis have been attributed to the actions of activated proteases released from the damaged pancreas. Proteases, complexed with the antiprotease molecules alpha-1-antitrypsin and alpha-2-macroglobulin, have been demonstrated in the peritoneal fluid, lymph and plasma of animals with experimental acute pancreatitis[132]. Overwhelming of these antiproteases was associated with hypotension and death. In man, however, free trypsin activity has never been demonstrated[133,134] and it would appear that in man, unlike in

experimental pancreatitis, overwhelming of the natural antiproteases is a rare event[134]. This coupled with the universal failure of antiproteases in clinical trials has led to the original hypothesis being questioned.

2.8.2 Acute pancreatitis and sepsis

More recently, it has been observed that the multiple organ failure which often precedes death in patients with fulminating pancreatitis is similar to that seen in patients with sepsis, burns and trauma[3]. When the haemodynamic parameters of patients with severe acute pancreatitis were measured they were found to closely resemble those seen in patients with sepsis, a high output, low-resistance state being the typical findings[135,136]. Pancreatic infection is rarely present in the early stages of acute pancreatitis [89] but the similarity between acute pancreatitis and sepsis has led to the suggestion that similar endogenous mediators may be responsible for the systemic manifestations of both conditions[3]. In sepsis there is now strong evidence to suggest that the development of systemic complications is a result of the activation of mononuclear phagocytes and the subsequent release of pro-inflammatory cytokines which acting through interactions with other cells result in end organ damage [4].

2.8.3 Cytokines in Sepsis

1. Tumour Necrosis Factor Alpha (TNFα)

TNFα was initially known also as cachectin as a result of its independent discovery as both a serum factor causing tumour necrosis[137] and as an agent causing hypertriglyceridaemia and cachexia in experimental infection[138,139]. TNFα is a 17kDa 157 amino acid polypeptide cytokine[140] which is produced predominately by cells of the monocyte/macrophage lineage although its production by NK-cells, antigen-stimulated T cells and

mast cells has been documented[141]. It is synthesised as a pro-peptide which undergoes proteolytic cleavage to produce the mature polypeptide[140]. A membrane-associated form has also been identified in which the leader protein persists[142]. The main stimulus to its synthesis and release is endotoxin[143] (bacterial lipopolysaccharide) although other in-vivo stimuli are thought to include viruses and fungi[144,145].

A wide variety of physiological effects have now been attributed to TNFα. TNFα acts on endothelial cells to enhance procoagulant activity, increase permeability and induce the production of other inflammatory mediators including Interleukin-1 and platelet activating factor[4]. TNFα induces neutrophil margination and activation and is therefore an important stimulator of the non-specific immune response[146-149]. TNFα also induces the maturation of myeloid cells to monocytes and macrophages and is capable of macrophage activation thus stimulating its own production[150,151]. It can readily be appreciated that all of these effects may be of benefit to the host in the event of a microbial challenge. However, if production is excessive or unregulated the balance may swing towards host injury.

The first evidence of a role for TNFα in the mediation of endotoxin-induced injury followed the discovery by Beutler et al that passive immunisation against endogenous TNFα conferred a survival advantage in mice presented with an endotoxin challenge. Similarly, endotoxin resistant C3H/HeJ mice did not produce TNFα in response to a bacterial challenge[143]. Administration of high doses of TNFα to experimental animals was subsequently reported to result in haemodynamic collapse and death[152]. Metabolic changes similar to those seen in septic shock were observed, namely hyperglycaemia and hyperkalaemia. The histological changes in the lungs, kidneys and gastrointestinal tract of animals dying following a lethal dose of TNFα were strikingly similar to those seen in patients dying from septic shock. Prior administration of anti-TNFα

antibody to primates given experimental bacteraemia prevented the characteristic haemodynamic and metabolic changes and dramatically improved survival[153]. A similar protective effect of anti-TNFα antibody was reported in rabbits presented with a lethal dose of bacterial endotoxin[154]. Following experimental endotoxaemia in humans, a transient rise in plasma TNFα levels was observed[155,156] correlating with signs of systemic toxicity.

The experimental evidence suggesting a key role for TNFα in the pathophysiology of septic shock was subsequently supported by clinical studies. Raised levels of circulating TNFα were detected in patients with meningococcal septicaemia which was associated with a fatal outcome[157,158]. Studies in patients with septic shock admitted to intensive care units have generally confirmed the association between high circulating TNFα levels with the severity of illness and mortality. Damas[159] studied 27 patients and found significantly higher levels of TNFα but not IL-1β in non-survivors than survivors. Although de Groote[160] found no relationship between the severity of sepsis and plasma TNF levels, the patients studied were a heterogeneous group of whom only one died suggesting that the patients were less severely unwell than in the study by Damas in which mortality was 63%. More recently, Calandra[161] have reported persistently raised TNFα levels in patients dying from septic shock compared with transiently raised levels in survivors and both Marks[162] and Offner[163] reported a correlation between the severity of sepsis and plasma TNFα levels. High levels of monocyte TNFα production have also been reported in patients with sepsis following burns[164] suggesting the activated monocyte is the major source of TNFα in sepsis.

2. Interleukin-1 beta (IL-1β)

Interleukin-1 (IL-1) was first identified as a polypeptide co-stimulator of T-cell responses and named "lymphocyte activating factor" but is now recognised as a major mediator of the non-specific inflammatory response[165]. The range of activities attributed to IL-1 are reflected in the variety of names by which this cytokine has been known **(table 1.4)**.

Purification of IL-1 identified two distinct polypeptide species, now known as IL-1α and IL-1β which are actually the products of two distinct genes[166].

The main source of IL-1β is the mononuclear phagocyte although production by endothelial cells, neutrophils and B-lymphocytes has been reported[167]. As with TNFα, LPS is the main stimulus to its production although IL-1β production is also induced by other stimuli, including TNFα[4].

Many of the effects of IL-1β overlap with those of TNFα and both are capable of stimulating the release of the other, thus acting in a synergistic fashion[4]. It is therefore possible that much of the overlap in function observed could be explained by the subsequent induction of secretion of the other. Infusion of IL-1β and TNFα together results in systemic effects at concentrations well below those required when the cytokines are infused separately[168,169]. In addition to its effects in the inflammatory response such as induction of acute phase protein production[167] and neutrophil adherence and activation[170]

IL-1β acts centrally to induce fever[171] secondary to the release of prostaglandins in the hypothalamus and may be involved in the development of anorexia[172]. It also causes the release of adrenal corticosteroids, both by a direct action and by increasing the release of ACTH[173-175]. IL-1β also stimulates the release of pancreatic insulin and

glucagon[176]. A stimulatory effect of IL-1β on myelopoeisis has also been described, an effect which is partly due to induction of release of myelopoetic growth factors[177].

Unlike TNFα, an increase in IL-1β levels has not been demonstrated following endotoxin infusion in experimental animals or human volunteers[155]. During lethal E Coli septicaemia, however, high circulating levels of IL-1 have been reported[178-180] and infusion of IL-1β receptor antagonist significantly improved survival in a primate sepsis model[179]. In clinical studies raised levels of IL-1β have been demonstrated but the prognostic significance has been less than that observed with TNFα. In children with meningococcal septicaemia, Girarden et al demonstrated higher IL-1β and TNFα levels in non-survivors than survivors[158]. Similarly, Waage[157] found that detectable levels of plasma IL-1β were associated with a poor prognosis. Cannon et al[181] detected raised levels of IL-1β in patients with sepsis compared with healthy controls. In patients with septic shock, Calandra[161] reported higher levels of IL-1β in non-survivors than survivors although this difference did not persist beyond the first day of study.

Lymphocyte activating factor
Mitogenic protein
Endogenous pyrogen
B-cell activating factor
Catabolin
Leucocyte endogenous mediator
Haemopoetin-1
Proteolysis inducing factor
Helper peak-1
T-cell replacing factor III
B-cell differentiation factor

Table 1.4: Previous names for interleukin-1

3. Interleukin-6 (IL-6)

Interleukin-6 (IL-6), like IL-1β was previously known by a number of names. **(table 1.5)** All of these molecules have been shown to be an identical 22-29kDa protein now known as IL-6. IL-6 is produced by mononuclear phagocytes, endothelial cells fibroblasts and T-cells in response to cytokines including TNFα and IL-1β[182]. The best described actions of IL-6 are those on hepatocytes and B cells. IL-6 plays an important role in the change of priority in hepatocyte protein synthesis in response to various injurious stimuli resulting in the increased synthesis of acute phase proteins such as C-reactive protein, fibrinogen and α-1-antitrypsin and the decreased synthesis of albumin[182,183]. These effects are generally beneficial to the host[182].

B2 interferon
B-cell stimulatory factor 2
26kDa protein
Hybridoma/plasmacytoma growth factor
Hepatocyte stimulating factor
Monocyte granulocyte inducer type 2

Table 1.5: Previous names for interleukin 6

4. Interleukin-8 (IL-8)

IL-8 is one of a family of more recently described inflammatory mediators which is secreted by mononuclear phagocytes and other cells stimulated by endotoxin. TNFα and IL-1β also induce the secretion of IL-8[184]. IL-8 causes neutrophil aggregation and activation and it is believed that the activation of neutrophils induced by TNFα is, at least in part, a consequence of its effect on IL-8 secretion[184]. IL-8 levels have been shown to be elevated in patients with sepsis and high levels appear to correlate with a poorer prognosis[185] In experimental septic shock in primates, levels of IL-8 peak later than those of IL-1β and TNFα and correlated with the

severity of the septic insult. Levels of IL-8 also rise after an IL-1β infusion[186].

2.8.4 Cytokines in acute pancreatitis

TNFα

In 1988, Rinderknecht first proposed the hypothesis that cytokines may play an important role in the pathophysiology of acute pancreatitis[3]. He cast doubt on the conventional view that activated pancreatic proteases were responsible for the systemic manifestations of acute pancreatitis and suggested that excessive stimulation of neutrophils by phagocytosed cell debris may lead to production of harmful quantities of free oxygen radicals, leukotrienes and the newly described cachectin (tumour necrosis factor) in a situation analogous to septic shock. The first attempt to investigate this hypothesis was by Banks and colleagues. who measured serial plasma levels of TNFα in 27 patients with acute pancreatitis[5]. They found raised levels of TNFα in some patients, including two patients who died of their illness, but found no statistically significant difference in TNFα levels between patients with mild and those with severe disease. In those with raised levels, TNFα levels peaked within the first three days after admission but many patients had TNFα levels which were similar to those observed in healthy controls. The authors concluded that it was unlikely that TNFα played an important pathophysiological role in acute pancreatitis, although they did find evidence of neutrophil activation. Subsequently, Exley and co-workers[6], in 38 patients with prognostically severe acute pancreatitis taking part in a therapeutic trial of fresh frozen plasma, measured serum levels of TNFα during the first week of admission. They found detectable levels of TNFα on admission in 45% of non-survivors compared with 23% of survivors and overall, the levels of TNFα were higher than those reported by Banks et al. In addition, endotoxin was detected in 91% of non-

survivors on admission. These studies demonstrate the problems associated with plasma TNFα measurement. TNFα is thought to act primarily at a paracrine level and a cell-bound form has even been described[142,187]. Circulating levels therefore represent "spillover" into the circulation of excess TNFα and the absence of TNFα in the circulation does not imply that TNFα is not involved in a disease process. Circulating TNFα inhibitors rapidly bind TNFα[188,189] and can interfere with its detection in assays which further complicates the interpretation of these studies[190]. In addition, TNFα has a relatively short circulation half-life[191] so that transiently raised levels may be missed. It is therefore not surprising that these studies have inconclusive results. The presence of high TNFα levels in certain patients with some evidence of an association with the more severe manifestations of the illness provides encouraging evidence to support further study.

Interleukin-6

Several authors have documented raised IL-6 levels in patients with severe pancreatitis. Leser and colleagues[39] described elevated levels of IL-6, measured by bioassay, in patients with complicated acute pancreatitis, with normal or slightly elevated levels of IL-6 in patients with mild attacks. Similar findings were reported by Viedma et al[40] who measured IL-6 by ELISA and by Heath and colleagues[38] who used a bioassay. What is unclear from these papers is the time course of the rise in plasma IL-6 and the variation which occurs between individual patients. Leser[39] described the pattern of IL-6 activity in two individual patients with severe disease, showing peak levels on day 1, falling thereafter in one patient and on day 3 in the second patient. It is not clear how accurately this reflects the findings in the remaining patients or how this relates to the time from onset of symptoms. In the study by Viedma et al[40], persistently raised levels of IL-6 were found but there was no obvious peak in IL-6 levels during the 7 days

of study. Heath et al[38] corrected their results for the time from onset of symptoms and found that IL-6 levels peaked between 24 and 48 hours after symptom onset. It is, however, unclear what degree of variation there was between individual patients.

The relationship between C-reactive protein and IL-6 levels has been studied since the rise in plasma IL-6 has been proposed as the main stimulus to hepatic acute phase protein production. Peak IL-6 levels correlated with peak CRP levels in the studies by Heath et al[38] and Leser et a[39] with the peak in IL-6 preceding the peak in CRP by one day. There was no correlation between IL-6 and CRP levels in the study be Viedma et al[40].

Interleukin-8

Early studies suggest that plasma IL-8 levels are increased in patients with complicated attacks of acute pancreatitis[192] but, compared with IL-6 and TNF, little is known of the role of this cytokine an acute pancreatitis.

2.8.5 Role of Endotoxin in acute pancreatitis

For activated leukocytes to be responsible for the systemic manifestations of acute pancreatitis, then a factor capable of inducing leucocyte activation must be present in these patients. As discussed in the foregoing section, bacterial endotoxin is a potent activator of mononuclear phagocytes and it induces the secretion of cytokines including TNF, IL-6, IL-1 and IL-8. The presence of endotoxaemia in acute pancreatitis was first reported in 1974 in three patients[193], with disappearance of endotoxin from the circulation when the illness resolved. In 1982 Foulis et al reported 24 patients with acute pancreatitis in whom serial assays of serum endotoxin were carried out[194]. Endotoxin was detected on two consecutive days in half of the attacks of acute pancreatitis and in 6 of the 7 patients who developed systemic complications. The limulus amoebocyte lysate assay for endotoxin

has certain limitations therefore assays have been developed which measure the immunological response to endotoxin, therefore indirectly measuring endotoxin exposure. Kivilaakso et al[195] measured titres of antibodies to the enterobacterial common antigen in a series of patients with acute pancreatitis. The enterobacterial common antigen is spatially related to the lipopolysaccharide component of the bacterial cell wall and it was argued that measurement of antibodies to this antigen were a measurement of exposure to enteric bacteria and thence to endotoxin. The authors reported decreased titres on admission in those patients with complicated or fatal pancreatitis, with a rise in levels during the course of the illness in survivors, suggesting endotoxin exposure. However, in 5 of 6 patients with fatal pancreatitis no rise in anti-enterobacterial titres was observed, despite proven gram negative septicaemia in two cases. This exemplifies the difficulty in interpretation of such indirect methods of endotoxin measurement. More recently, an assay has been developed to detect titres of an antibody to the core glycolipid portion of the endotoxin molecule. Depletion of the IgM anti-endotoxin antibody has been demonstrated in patients with both mild and severe pancreatitis and falling IgG levels have been linked with a poor prognosis[196]. This contradicts the argument that raised titres are an indicator of endotoxin exposure but it has been suggested that antibody depletion results in increased exposure to free endotoxin At the present time the complex relationship between endotoxin, its circulating inhibitors and leucocyte activation has not been clearly defined.

2.8.6 Role of the reticuloendothelial system in acute pancreatitis

In health, portal endotoxin is rapidly removed from the circulation by the hepatic Kupffer cells, thus preventing systemic endotoxaemia[8]. The failure of hepatic reticuloendothelial function results in what has been termed

"spillover" of endotoxin into the systemic circulation[8]. In this way, suppression of reticuloendothelial function, increased endotoxin load or a combination of both may be associated with the development of systemic endotoxaemia and the subsequent activation of systemic mononuclear phagocytes. In experimental sepsis, systemic endotoxaemia was associated with decreased hepatic RES phagocytic function[197]. Similarly, depressed RES function associated with systemic endotoxaemia has been demonstrated in patients with obstructive jaundice[198]. As discussed above, acute pancreatitis is also associated with systemic endotoxaemia. In experimental acute pancreatitis, the suppression of reticuloendothelial function with oleic acid was reported to increase mortality[199]. In another study, stimulation of the RES with glucan reduced mortality[200]. There is indirect evidence from clinical studies that the RES is either overwhelmed or its function is suppressed as raised levels of complexed α-2-macroglobulin, normally removed by the RES, are present in the circulation in patients with severe attacks[201].

2.9 Effect of somatostatin and octreotide on reticuloendothelial function

Somatostatin was demonstrated to enhance hepatic RES activity in rats by Szabo in 1983[202]. The clearance of colloidal carbon from the circulation was increased in a dose-dependent fashion by somatostatin which was more effective than the RES stimulant, zymosan. Baxter and colleagues later demonstrated similar effects on colloid clearance with both somatostatin and octreotide[7].

There is therefore evidence that somatostatin and somatostatin analogues may have potential for the treatment of acute pancreatitis by their stimulatory action on reticuloendothelial cell function. The use of these agents in acute pancreatitis is reviewed in the following chapter.

Chapter 3

Somatostatin and somatostatin analogues in acute pancreatitis

Somatostatin, a 14 amino acid peptide was discovered more than 20 years ago in the rat hypothalamus[203]. Since then it has been identified in species from protozoa to man and in a wide range of mammalian tissues[204] As our knowledge of its physiological role expands, so the list of conditions for which it has been suggested to be of potential therapeutic benefit grows longer.

3.1 Physiological role of somatostatin

Somatostatin was discovered more or less simultaneously by two separate groups of researchers. Krulich and colleagues found a substance which inhibited growth hormone release in the rat hypothalamus during studies on growth hormone releasing factor[203] and one year later, Hellman and Lernmark reported the discovery of a factor which inhibited the release of insulin from pancreatic islet cells in-vitro[205]. Unsurprisingly, no connection was made between these two discoveries at that time and it was not until 1973, when workers in the Salk Institute made similar observations to those of Krulich and colleagues that serious attempts were made to isolate and sequence the peptide now known as somatostatin[206,207]. A 14-amino acid peptide was described with a cyclic conformation determined by a disulphide bond linking two cysteine residues **(figure 3.1)**.

Subsequent work has identified an amino-terminal expanded form[208] (somatostatin-28), larger pro-hormones and a number of species specific variants (such as a 22-amino acid somatostatin found in catfish). Although the first descriptions of somatostatin were restricted to its hypothalamic functions, since the description of the first radio-immunoassay for

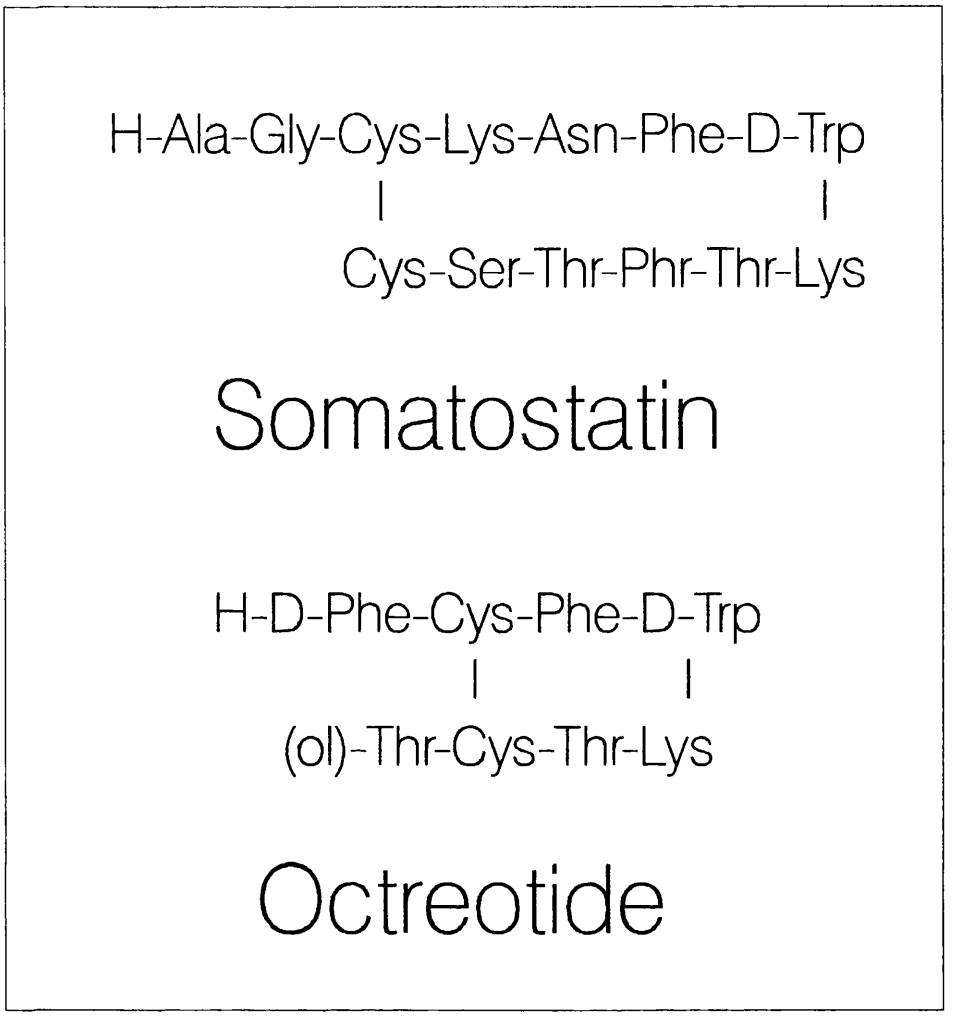


Figure 3.1 : Amino acid sequence of native somatostatin and octreotide

somatostatin[209], somatostatin-containing cells have been identified in most mammalian organs.

Within the nervous system, somatostatin functions as a neurotransmitter and somatostatin-secreting neurones have been identified in the anterior and posterior pituitary, the limbic system, brain stem and spinal cord[204]. Elsewhere, cells containing somatostatin are mainly distributed within the secretory cells of the gastrointestinal tract, the pancreatic islets and the salivary glands[204].

Outside of the nervous system, there is evidence of both endocrine and paracrine functions of somatostatin. Somatostatin inhibits the release of insulin and glucagon within the pancreatic islets[204]. The secretion of a variety of gut hormones is also inhibited as is exocrine pancreatic secretion[204]. Intestinal motility is also inhibited as is gallbladder contraction. Somatostatin also reduces mesenteric blood flow[204].

This extensive range of inhibitory effects on gastrointestinal function suggests a potential variety of therapeutic uses of somatostatin but these have been limited by its short half-life necessitating administration by continuous intravenous infusion, thus making treatment, in anything but the short term, impractical.

3.2 Somatostatin Analogues

The discovery of the wide range of physiological actions of somatostatin inevitably led to the search for analogues with similar or more potent effects and longer duration of action. The first and most widely studied of these analogues is octreotide (SMS 201-995, Sandostatin) manufactured by Sandoz Pharma, Basle, Switzerland. Octreotide is an eight-amino acid peptide[210] which retains the Phe-D-Trp-Lys-Thr sequence in a cyclic conformation **(figure 3.1)** which has been shown to be necessary for the endocrine activity of somatostatins[211]. Octreotide has a longer circulation

half-life than somatostatin-14, of 41-58 minutes after intravenous administration and approximately 113 minutes if administered subcutaneously compared with 2-3 minutes for somatostatin-14. The effects of octreotide were found to be more specific than somatostatin-14 in that growth hormone secretion is inhibited preferentially over insulin secretion[212]. This combined with its increased potency makes it more suitable than somatostatin-14 for treatment of acromegaly.

Octreotide completely inhibits the secretion of pancreatic enzymes, gallbladder contraction and CCK response after a Lundh meal[213]. Pancreatic polypeptide, gastric inhibitory polypeptide and to a lesser extent insulin secretion are also inhibited by even low doses of octreotide[213].

Octreotide has now been investigated in the management of a wide range of gastrointestinal diseases and more recently, interest has focused on its anti-proliferative effects in a variety of gastrointestinal malignancies.

3.3 Use of somatostatin and its analogues in gastrointestinal disease.

In addition to its use in the management of acromegaly, somatostatin or octreotide has been used in the treatment of acute pancreatitis, pancreatic and enterocutaneous fistulae[214,215], pancreatic pseudocysts[216] variceal haemorrhage[217] secretory diarrhoea[218] and the short bowel syndrome[219]. It has also been used successfully in the palliation of some endocrine tumours, most notably carcinoid[220] and is currently under investigation in a variety of other solid tumours.

3.3.1 Gastrointestinal Fistulae

Many small, anecdotal series have been reported on the experience of the use of somatostatin and octreotide in the management of enterocutaneous and pancreatic fistulae. In the largest randomised controlled trial[221], 40 patients were randomised to receive total parenteral nutrition alone or TPN

with somatostatin 250ug/h. The overall fistula closure rate was similar at 81% with TPN alone and 85% with TPN and somatostatin but fistula closure occurred about 7 days earlier in the somatostatin group. This effect was associated with a reduction in morbidity as may be expected and a reduction in the duration of TPN with its attendant problems. It was emphasised, however, that somatostatin simply speeded closure of fistulae which would ultimately close on standard conservative management and would not allow closure of fistulae associated with distal obstruction or other unfavourable factors.

3.3.2 Somatostatin and octreotide in the prevention of complications after pancreatic surgery

In a large, multicentre trial which has provoked much discussion, Buchler and colleagues from one Austrian and various German hospitals compared the effect of placebo and octreotide 300μg/day in patients undergoing resection for periampullary or pancreatic tumours or chronic pancreatitis[222]. There were an impressive 246 evaluable patients, 125 randomised to receive octreotide and 121 to placebo. The incidence of post-operative complications was significantly reduced from 55% to 32% with octreotide treatment. However, this difference is entirely accounted for by the difference in the incidence of pancreatic fistulae.

The overall incidence of pancreatic fistulae was 27% but was 38% in the placebo group and 17% in the octreotide group. The number of patients who developed complications unrelated to pancreatic fistulae was similar (17% octreotide group, 20% placebo group). An important factor to be considered is the definition of pancreatic fistula employed by the authors. This was defined as a leakage of 10ml of fluid more than 3 days postoperatively containing more than 3 times the serum concentration of amylase. Therefore, the expected effect of octreotide on reducing

pancreatic enzyme secretion would naturally reduce the incidence of fistulae using this definition and might be expected to bias the result in favour of the octreotide group regardless of the clinical relevance of such an effect.

This is underscored by the similar length of hospital stay in the two groups of patients. It is not clear how many patients had clinically significant post-operative fistulae which failed to meet the trial criteria on the basis of amylase levels which therefore do not appear in the results.

3.3.3 Somatostatin and octreotide in the management of secretory diarrhoea

Somatostatin inhibits the secretion of intestinal water and electrolytes and the secretion of vasoactive intestinal polypeptide(VIP) and other experimental secretagogues[218]. Octreotide has been successfully used in the treatment of secretory diarrhoea due to VIP secreting tumours, carcinoid syndrome and the diarrhoea often associated with AIDS[218]. However, the evidence of efficacy of octreotide in this context is restricted to case reports and no randomised, controlled trials have been carried out.

3.3.4 Somatostatin and octreotide in the management of pancreatic pseudocysts

The majority of pancreatic fluid collections will settle on conservative management and although there have been sporadic case reports of the use of octreotide in patients with pancreatic pseudocysts[216], no randomised trials have been conducted and there is no conclusive evidence that either somatostatin or octreotide alters the natural history of pseudocysts in any way.

3.3.5 Antitumour effects of octreotide

Recent interest has focused on the potential antitumour effects of octreotide and other somatostatin analogues. Many tumours display somatostatin receptors, a finding which has led to the development of radiolabelled somatostatin analogues to enable imaging of tumours, particularly those of neuroendocrine origin[223]. In-vitro studies have shown that octreotide has an antiproliferative effect on somatostatin receptor positive tumour cell lines and similar effects have been demonstrated in animal models[224]. So far, however, there are no reports of similar antitumour effects in primary human malignancies.

3.4 Somatostatin and octreotide in the treatment of acute pancreatitis

The use of somatostatin in acute pancreatitis was first suggested in 1975[225,226] but despite much experimental and clinical study since that time, its role in the management of this condition remains unclear.

There are four main properties of somatostatin or octreotide which may influence the course of acute pancreatitis.

3.4.1 Mechanism of action

Inhibition of pancreatic secretion.

It is conventional wisdom that "to rest the gland" is beneficial in the management of acute pancreatitis[118]. Although the validity of this assumption has not been established it has led to clinical trials with nasogastric suction[88] cimetidine[124,125] and glucagon[119-123] in the treatment of acute pancreatitis, none of which has been shown to be effective.

Somatostatin causes a dose dependent reduction in exocrine pancreatic secretion, the effect on enzyme secretion being greater than the effect on secretion of bicarbonate and water[227]. Similar effects have been

demonstrated with the somatostatin analogue, octreotide[213,228] In health, the doses of somatostatin used in clinical trials would be expected to cause a 70% reduction in exocrine pancreatic secretion[227]. This action may explain the successful use of somatostatin analogues in the treatment of pancreatic fistulae[221]. However, the secretory status of the pancreas in acute pancreatitis is unknown, as is the effect of somatostatin on the diseased gland. In experimental models at least, pancreatic secretion appears to remain at basal levels[229] and there is evidence that pancreatic exocrine secretion is impaired in the majority of patients following recovery from necrotising pancreatitis[230].

Haemodynamic effects.

Somatostatin reduces pancreatic blood flow in experimental animals[231]. The development of pancreatic necrosis has been linked to hypoperfusion of the gland[232] and vasoconstrictors have been shown to worsen the histological severity of experimental pancreatitis[233]. Conversely, dopamine, which improves pancreatic blood flow, has been found to improve the outcome from experimental pancreatitis[234].

Schroder and colleagues found that, following the induction of pancreatitis, pancreatic blood flow decreased to a greater extent in somatostatin treated piglets than in untreated controls[235]. This would be expected to be associated with a deleterious effect on outcome although no difference between treated and untreated animals was observed in this study.

Cytoprotection/organoprotection.

Cytoprotective properties have been claimed for somatostatin[211,236] on the basis of its ability to reduce the effect of various toxins on gastric mucosal cells and hepatocytes. However, cytoprotection towards the pancreas has

not been demonstrated and experiments have shown that for somatostatin to be effective in this context, pre-treatment is necessary. Obviously in acute pancreatitis, where organ damage will have occurred prior to admission, such pre-treatment would not be possible.

Stimulation of the reticuloendothelial system.

The fourth way in which somatostatin may influence the course of acute pancreatitis is by its stimulatory effect on the phagocytic cells of the reticuloendothelial system. In experimental acute pancreatitis, survival can be improved by stimulation of the RES with either zymosan or glucan[199,237]. Conversely, depression of the RES results in worsened survival[199]. There is also some evidence of RES depression in patients with acute pancreatitis[201].

Both somatostatin and the long acting somatostatin analogue, SMS 201-995, have been shown to increase the clearance of colloidal carbon[7,236]. Somatostatin was found to be more effective in this regard than Zymosan[236]. Somatostatin has also been reported to enhance monocyte phagocytic activity in-vitro[238]. Survival of rats after intraperitoneal injection of endotoxin was also improved with somatostatin therapy[7] which may have been a consequence of RES stimulation.

3.4.2 Somatostatin in experimental pancreatitis

Somatostatin has been examined in most animal models of acute pancreatitis and has met with varying degrees of success **(Table 3.1)**. In the first study to be reported[239], Lankisch and colleagues administered somatostatin at a dose of 100μg/100 g body weight for three hours immediately after inducing pancreatitis in rats by the retrograde injection of sodium taurocholate into the pancreatic duct. Although the serum amylase and lipase were lower in the treatment group, the overall mortality was unchanged. The histological changes in the pancreas were also similar in the two groups.

Two years later, Schwedes et al[240] reported a significant histological benefit with cyclic somatostatin, 62.5μg/h in a canine model of haemorrhagic pancreatitis. This effect was seen with both somatostatin pre-treatment and with somatostatin administered two hours after the induction of pancreatitis. The general condition of the animals in the treated group was also noted to be improved.

In addition to the different species used in these experiments, there are differences in experimental design which may help to explain these contradictory results. In inducing acute pancreatitis, Lankisch et al used sodium taurocholate whereas Schwedes et al utilised the animals own bile. Neither group of workers standardised the injection pressures used, a factor which may influence the severity of pancreatitis produced. There was also a difference in the duration of somatostatin treatment between the two studies. Whereas Lankisch et al discontinued treatment after three hours, Schwedes et al gave somatostatin by continuous infusion for up to 24 hours. Native somatostatin has a half life in the circulation of only two to three minutes and it is possible that prolonged treatment in the second study was responsible for the difference in result.

Author	Model	Survival effect	Histology effect
Lankisch et al 1977	Rat, RIB	No	No
Schwedes et al 1979	Dog, RIB	N/A	Yes
Adler et al 1980	Rat, CH	N/A	Yes
Baxter et al 1985	Rat, PDL	Yes	Yes
Degertekin et al 1985	Mouse,CDED	No	No
Schlarman et al 1987	Dog, RIB	N/A	No
De Rai et al 1988	Rat, CDL	No	Yes

***Table 3.1**: Somatostatin in experimental pancreatitis*

RIB: retrograde injection of bile or taurocholate

CH: ceruelin hypersecretion

PDL: pancreatic duct ligation

CDED: choline deficient, ethionine supplemented diet

CDL: closed duodenal loop

Subsequently, Schlarman et al[241] reported no improvement in pancreatic histology following the infusion of native somatostatin 20μg/kg/h for five hours immediately after the induction of pancreatitis by retrograde bile injection in dogs, although portal and thoracic duct lymph amylase levels were reduced in the treatment group. Similarly, Degertekin et al[242] using a diet-induced model of acute pancreatitis in mice, failed to demonstrate a significant effect on survival or pancreatic histology following the subcutaneous administration of native somatostatin, 30μg every eight hours. However, in view of the short circulation half-life of somatostatin it is unlikely that such a dosage schedule would achieve therapeutic levels in plasma. It is therefore difficult to draw firm conclusions from this study.

A beneficial effect of cyclic somatostatin, 5μg/kg/h, on the histological severity of ceruelin-induced pancreatitis was reported by Adler et al[243], treatment being commenced immediately after the induction of pancreatitis. Similarly, De Rai et al[244] demonstrated reduced histological damage with somatostatin given intravenously for 9 hours. No information on the timing or dose of treatment was given. Baxter et al[245], in addition to demonstrating a histological benefit with somatostatin treatment, found a dramatic improvement in survival in the treatment group. They continued somatostatin, 4μg/kg body weight, for up to 24 hours using a duct-ligation model in the rat. Similar results were seen even if treatment was commenced immediately or delayed for 24 hours after duct ligation. These results suggest that differences in duration of treatment, but not in timing of treatment or size of dose, may explain some of the differences in results obtained.

Differences between the experimental models used may also play a part in the contradictory results observed. In the only study to demonstrate a survival deference, Baxter et al induced acute pancreatitis in rats by ligation of the common bile/pancreatic duct. This model is normally associated with pancreatic oedema[246] although pancreatic necrosis and a high mortality were seen in the control group in this study.

3.4.3 Octreotide in experimental pancreatitis

The development of the long acting somatostatin analogue, octreotide, has led to its use in experimental pancreatitis. Its longer half life allows subcutaneous administration avoiding the need for continuous intravenous infusion. The three experimental studies using octreotide all show some benefit from octreotide administration **(Table 3.2)**.

Author	Model	Survival effect	Histology effect
Baxter et al 1985	Rat, PDL	Yes	Yes
Augelli et al 1989	Dog, RIB	N/A	Yes
Davliakos et al 1990	Dog, ex-vivo	N/A	Yes

Table 3.2: *Octreotide in experimental pancreatitis*

PDL: pancreatic duct ligation

RIB: retrograde injection of bile or taurocholate

ex-vivo: ex-vivo, isolated, perfused pancreas

In the one study to assess survival[245], a dramatic advantage was demonstrated after twice daily subcutaneous octreotide at a dose of 2μg/kg commencing 12 hours after the induction of pancreatitis by pancreatic duct ligation. This survival advantage was of a similar magnitude to that reported by the same authors using native somatostatin. Augelli et al[238] were able to demonstrate a reduction in histological severity in animals given octreotide, 5μg/h, prior to the induction of pancreatitis although no effect was seen if treatment was delayed until pancreatitis was established, despite continuing treatment for 24 hours. Similarly, Zhu et al[247], giving octreotide 2μg/kg/h demonstrated a reduction in histological severity in a rat bile injection model. Once again, this effect was only seen if treatment was commenced prior to the induction of pancreatitis.

In the study by Davliakos et al[248] an ex-vivo model was used and histological benefit was seen after only one injection of octreotide, even if treatment was delayed until one hour after the induction of pancreatitis. However, ex-vivo models have been criticised as being unphysiological[246]

and their usefulness in evaluating treatment effects remains to be established.

In summary, the evidence from experimental studies is inconclusive although in those studies which continue treatment over a prolonged period there is evidence of some improvement in the treatment group. Differences between studies are not explained by the size of dose or the timing of administration in relation to the induction of pancreatitis. Only one study to date has reported an overall reduction in mortality with somatostatin.

3.4.4. Clinical trials with somatostatin and octreotide in acute pancreatitis

The main difficulty in therapeutic trials in acute pancreatitis is that only a very small proportion of those with the disease are likely to benefit significantly from specific treatment and it is therefore necessary to recruit large numbers of patients to be confident of demonstrating a reduction in mortality. As with other proposed treatments in acute pancreatitis, clinical trials with somatostatin have often failed to recruit sufficient patients for meaningful conclusions to be drawn.

Following small, uncontrolled reports in the late 1970's suggesting clinical improvement with somatostatin in acute pancreatitis a multicentre study was carried out[249]. The APTS study (Acute Pancreatitis Treatment with Somatostatin)[250] was conducted between centres in Germany, Switzerland and Greece and was intended to include only patients with severe disease. Severe acute pancreatitis was defined as hyperamylasaemia and abdominal pain with three or more of five severity criteria present; shock, ileus, abdominal distension, white cell count greater than 12000cells/dl and glucose greater than 150mg/dl. Somatostatin 250μg/h or placebo was

infused for up to 7 days. A total of 77 patients were recruited over a two year period, 36 in the treatment group and 41 in the control group.
There were 7 deaths in the control group (17%) and 4 in the treatment group (11%) a difference which fails to reach statistical significance. This led the authors to conclude that somatostatin was of no benefit in acute pancreatitis. However, it is important to realise that this study has insufficient numbers of patients to exclude a survival advantage as large as 50%. It can be readily calculated from sample size nomograms that more than 200 patients would be required in each group to exclude a type II error where the mortality in the control group is only 17%[251].

The authors found that serum pancreatic enzyme levels returned to normal more quickly in the treatment group, but unfortunately no detailed analysis was made of the incidence of complications in the two groups, perhaps reflecting difficulties in collating this information in a multicentre study.

Since 1985 there have been a further three controlled clinical trials reported in the English-language literature, all of which recruited patients with both mild and severe acute pancreatitis **(Table 3.3)**.

	No of Patients	Mortality (Placebo)	Mortality (Treatment)	Complications (placebo)	Complications (Treatment)
Usadel et al, 1985	77	17%	11%	N/A	
Choi et al, 1989	71	5.5%	2.9%	36%	14%
D'Amico et al, 1990	164	8.5%	2.4%	NS	
Gjorup et al, 1992	63	3%	3%	70%	58%

***Table 3.3**: Somatostatin in the treatment of acute pancreatitis*

N/A: Not assessed

NS: Not statistically significant but exact figures not given

Choi and co-workers in Hong Kong[252] conducted a randomised controlled trial comparing somatostatin against standard treatment in patients with acute pancreatitis. Only 71 patients were recruited of whom 15 had prognostically severe disease based on the presence of three or more positive Glasgow criteria. Patients were randomised on admission to standard treatment and somatostatin 100μg/h for two days by continuous intravenous infusion or to standard treatment alone. With such small numbers of patients, it is impossible to assess any effect on mortality alone and indeed there were only three deaths overall, two in the control group and one in the treatment group. There was a statistically significant difference in the complication rate between the two groups which was mainly accounted for by local complications, in particular "pancreatic phlegmon" which the authors defined as inflammatory pancreatic swelling on CT scanning with associated fluid collection.

A multi-centre study was reported by D'Amico et al[253] in which a total of 164 patients were recruited over a two year period. Patients with acute

pancreatitis of every grade of severity were included resulting in an overall mortality of 5.5%. No information was given about the randomisation procedure. Somewhat unusually, patients were divided into those requiring immediate surgery and those needing medical treatment alone based on a clinical and radiological assessment of disease severity. Patients not undergoing immediate surgery were then given either total parenteral nutrition alone or total parenteral nutrition with somatostatin, 250μg/h for five days. Patients who subsequently deteriorated and required surgery were excluded from the study. There was a further sub-division of patients into "haemorrhagic" and "oedematous" pancreatitis based upon the operative findings and clinical course. Overall, there was a trend towards a reduction in mortality in the somatostatin treated group which failed to reach statistical significance (2 vs. 7). Again, there were less local complications in the treatment group compared with the control group (1 vs. 5) although observer bias cannot be excluded and the numbers are too small to allow meaningful statistical analysis. The hospital stay for the group of patients undergoing surgery was reduced in the somatostatin treatment group. Overall, however, the complex design of this study makes interpretation of the results difficult.

Recently, Gjorup et al[254] have reported a small, randomised controlled trial in which 63 patients with acute pancreatitis received somatostatin 250μg/h or placebo for three days. Once again, patients with mild pancreatitis were included and as a result the overall mortality is only 3%. Although no significant differences in mortality or complications were observed between the treatment and placebo groups similar criticisms regarding patient numbers can be applied to this study. It is worth noting, however, that in contrast to the studies by Choi and D'Amico the number of local complications in this study were similar in the two groups.

Several small, controlled studies have been reported in the foreign-language literature[255-257]. However, in all cases, the studies include patients with pancreatitis of every grade of severity and therefore the mortality rates are low. The complication rates are not reported in sufficient detail to allow any meaningful analysis of clinical outcome.

In summary, no sufficiently large trial has yet been performed for firm conclusions to be drawn on the efficacy of somatostatin in acute pancreatitis. There is, however, some evidence of a trend towards a reduction in mortality and complication rate with somatostatin and a meta-analysis of the studies carried out to date suggests that overall, there is an improvement in outcome with somatostatin treatment[258].

3.4.5 Somatostatin and octreotide in the prophylaxis of ERCP-induced pancreatitis

Acute pancreatitis is the most common complication following endoscopic retrograde cholangiopancreatography occurring in up to 11% of cases[259] although asymptomatic hyperamylasaemia occurs in up to 70%[260]. Both somatostatin and octreotide have been evaluated in the prophylaxis of acute pancreatitis following ERCP[261-268] but the results of controlled clinical trials are conflicting. The incidence of acute pancreatitis in these studies is low and most studies do not have the statistical power necessary to exclude a therapeutic effect.

One exception to this is the study by Guelrud et al[264] who studied a particularly high risk group of patients undergoing pancreatic duct sphincter dilatation and found an overall incidence of acute pancreatitis of 43%. Infusion of somatostatin for 12 hours peroperatively resulted in an incidence of acute pancreatitis of 25% compared to 75% in the placebo group. However, another study has reported a significant increase in the

incidence of acute pancreatitis from 11% to 34% following the prophylactic use of octreotide[269], suggesting that the use of octreotide may even be harmful.

Unlike somatostatin, octreotide has not been the subject of a large, controlled clinical trial in the treatment of acute pancreatitis. The following study was conceived as a single-centre pilot study designed to assess the therapeutic potential of octreotide in acute pancreatitis. As discussed above, of the mechanisms of action by which octreotide may influence the outcome from acute pancreatitis it was felt that its action on the hepatic reticuloendothelial system was of most relevance in the prevention of the early systemic complications.

Chapter 4.

A Randomised, controlled trial of octreotide in the treatment of patients with acute pancreatitis

4.1 Patients and Methods

4.1.1 Ethical approval

Consultant surgeons in 9 hospitals within the Glasgow area were approached by the author. The details of the proposed study were explained and approval sought for the inclusion of their patients. Ethical approval was sought by the author from each individual hospital ethical committee and was granted in all cases. Samples of consent forms and patient information sheets were forwarded and approved. The relevant exemption certificate for doctors carrying out clinical trials was obtained (DDX) as was demanded prior to the granting of ethical approval

4.1.2 Study Design.

A double-blind, placebo controlled was conducted. There were two study arms;

1. Patients with acute pancreatitis receiving full supportive therapy and placebo.
2. Patients with acute pancreatitis receiving full supportive therapy and octreotide.

Details of the supportive therapy were necessarily left to the discretion of the medical teams responsible for the individual patients. The use of early ERCP in gallstone pancreatitis was not precluded and there were no restrictions on the timing of surgical intervention. The use of antibiotics was, again, left to the discretion of local medical staff but in all hospitals the use of antibiotics was not routine, but restricted to specific indications.

Patients were assessed on a daily basis by the author and thereafter at regular intervals until discharge from hospital.

4.1.3 Entry criteria

Acute pancreatitis was defined as the acute onset of abdominal pain along with a rise in serum amylase to at least four times the upper reference limit. Patients were eligible for inclusion in the study if they were assessed within 72 hours of the onset of abdominal pain but all efforts were directed at assessing patients within 48 hours of symptom onset.

Patients with mild acute pancreatitis rapidly recover with standard supportive therapy and it was considered desirable to concentrate the study on those patients most at risk of developing complications. As discussed in chapter 2, no single method of assessing severity of the attack is able to adequately differentiate between mild and severe pancreatitis on admission to hospital but the APACHE II scoring system[32] was considered the best available method. It utilises measurements which are readily available in all hospitals and can be used on admission to hospital. In order to include as many of the potentially severe cases as possible, it was decided to use a cut-off admission APACHE II score of 5. This, it was anticipated from the work of Wilson and colleagues[34] in a similar patient population, would give a sensitivity for severe pancreatitis of 95%with a specificity of 54%. It was estimated that at least 40% of the patients so included would develop significant complications.

Patients under the age of 18 were excluded as were females in whom pregnancy could not be excluded.

Written informed consent was a pre-requisite for inclusion in the study. It was anticipated at the outset of the trial that there would be a proportion of patients who would be too ill to be able to give informed consent and a small number of patients in intensive care units for whom this would

obviously be impossible. In these cases, consent from a close relative was obtained. All patients were informed of their right to withdraw from the study at any time.

4.1.4 Statistical Analysis

This trial was conducted as a single-centre pilot study in order to assess the likely magnitude of any effect of octreotide on outcome. It was anticipated that this information would, if the results were encouraging, lead to a large multi-centre study. The information would also allow more accurate estimation of the necessary number of patients required for such a study. As it was unlikely that sufficient patients would be recruited to enable hypothesis testing to be carried out with sufficient statistical power, it was decided to restrict the analysis to the calculation of confidence limits for any difference observed between the two arms of the study. The true effect of octreotide would therefore be expected to lie between these extremes.

4.1.5 Randomisation procedure

Octreotide and placebo were provided in identical glass vials by Sandoz pharmaceuticals. The octreotide vials contained 1mg of octreotide in 5ml while the placebo vials contained 5ml of saline. Labels were removed and replaced with labels identifying the vial as containing "trial drug", with the name of the study and the name of the principal investigator. The vials were assembled in individually numbered treatment packs, each containing either 5 vials of octreotide or 5 of placebo. The packs were numbered sequentially and the allocation of octreotide or placebo to a particular number was determined by a computer-generated random code. The preparation of these packs was carried out by a third party (Dr W Angerson, non-clinical Senior Lecturer, Department of Surgery, Glasgow Royal Infirmary). The randomisation code was kept in a sealed envelope

until termination of the study and all investigators, patients and attending medical staff were not aware of the nature of the trial infusion.

On entry to the study, patients were simply allocated the appropriate, sequentially numbered pack containing 5 days supply of either octreotide or placebo.

4.1.6 Drug administration

If the entry criteria were met and informed consent given, patients were randomised to receive either placebo or octreotide, at a dose of 1mg/day (40mg/h) by continuous intravenous infusion, continuing for 5 days. Intravenous infusion was chosen for administration because of the local discomfort often reported following subcutaneous injection of octreotide which it was thought might introduce observer bias. It also removed the potential problems associated with reliance on ward nursing staff to administer the trial drug. The 5ml vial was diluted with saline in a 50ml syringe to give a final volume of 48ml. This was then infused intravenously via a syringe pump at 2ml per hour. In this way, one vial was infused over 24h. Subsequent infusions were prepared either by the author if present or by the junior medical staff who were left detailed verbal and written instructions. Each day's infusion was prescribed on an intravenous fluid prescription sheet and was signed for by the nurse commencing the infusion. The infusion was continued for 5 days or until full biochemical and clinical resolution if this was earlier.

4.1.7 Patient recruitment

Junior medical staff in each participating hospital were contacted and the details of the study explained. Contact numbers by which the author could be reached was provided. The author was available by radiopage, cellular telephone and failing these by answering machine. A sheet with the

appropriate contact numbers was left in the relevant wards of each participating hospital.

In addition, medical and nursing staff were regularly contacted by the author and reminded of the study, particularly when referral numbers dropped.

On being contacted about a patient, the author visited the hospital involved as soon as other commitments allowed and assessed the patient for inclusion in the study. If entry criteria were met and informed consent granted, the trial infusion was commenced. Patients not meeting the entry criteria were excluded from further study.

4.1.8 Study end-points

Clinical endpoints were death or complications, either local or systemic. These were defined as follows:

Local complications;

- pancreatic necrosis (sterile or infected) as defined on contrast-enhanced CT scan or at laparotomy
- acute fluid collection defined on ultrasound or CT scan.

Systemic complications;

- respiratory insufficiency (pO2<8kPa),
- radiologically confirmed pleural effusion,
- renal impairment (serum creatinine >180umol/l despite fluid resuscitation in the absence of pre-existing renal impairment),
- shock (systolic BP<100).

These definitions were arbitrarily defined, as the study was designed prior to the Atlanta convention[35] where more precise definitions were agreed. The definitions chosen, however, are very similar to those arrived at by the Atlanta convention, the one exception being pleural effusion. This, it was

reasoned, simply represented a fluid collection within the lung and as such should be included as a systemic complication.

The degree of systemic illness was assessed by the acute physiology score (APS), which is a component of the APACHE II score.

In order to simplify the study for participating hospitals, no additional tests were required of the local hospitals but junior medical staff were encouraged to carry out daily measurement of the Glasgow criteria (or until normal). The daily measurement of CRP was also encouraged. All patients were requested to have an abdominal ultrasound carried out prior to discharge in order to identify gallstones if present.

4.2 Results

Between November 1991 and April 1993, 58 patients were recruited to the study. Eight of the nine hospitals who were approached contributed patients to the study **(Table 4.1)**.

Hospital	No of Patients
Glasgow Royal Infirmary	**20**
Stobhill Hospital	**7**
Southern General Hospital	**6**
Hairmyres Hospital	**5**
Monklands Hospital	**5**
Vale of Leven Hospital	**5**
Law Hospital	**5**
Victoria Infirmary	**4**

Table 4.1: Patients recruited from individual hospitals

4.2.1 Aetiology

In 31 patients (17 octreotide group, 14 placebo group), gallstones were present on ultrasound examination or had been previously diagnosed. Of the remainder, 11 gave a history of excessive alcohol consumption. One patient had hypercalcaemia due to primary hyperparathyroidism as a probable cause and one patient developed acute pancreatitis following ERCP. In 14 patients (24%) no definite aetiological factor was identified. Ultrasound is known to be less sensitive when carried out during the acute attack and it is probable that some of those with no known aetiological factor had gallstone-related pancreatitis. Patients seldom had repeat ultrasound carried out during the same hospital admission.

4.2.2 Comparability of groups

The patient groups are compared in **table 4.2**. Twenty-eight patients were randomised to receive octreotide (13 female, 15 male, median age 64, range 25-86) and 30 to receive placebo (19 female, 21 male, median age 73, range 32-92). Patients in the placebo group had a slightly higher proportion of women than the treatment group (63% compared with 46%) and tended to be older with a median age of 73 compared with 64 in the treatment group. Aetiology was similar in the two groups with gallstones identified in 54% of the treatment group and 53% of the placebo group.

	Octreotide	**Placebo**
No	**28**	**30**
Age	**64 (25-86)**	**73 (32-92)**
Male	**15**	**11**
Female	**13**	**19**
Gallstones	**17**	**15**

Table 4.2: Patient characteristics, placebo and octreotide groups

All patients were selected on admission on the basis of the APACHE II score but other measurements of prognostic severity were similar in treatment and placebo groups. Glasgow criteria (median 2 positive factors in both groups) and peak CRP within 48 hours of admission (placebo group median=150mg/ml (IQR 110-276), treatment group=157 (77-232)) were similar in the two groups of patients.

4.2.3 Effect on Mortality

The trial was of insufficient size to exclude a treatment effect on mortality but no difference in mortality was seen between the two groups of patients (octreotide group 5/28 (18%); placebo group 6/30 (20%). There were five early deaths (within the first week), three in the placebo group and two in the treatment group. All had fulminant pancreatitis and failed to respond to maximal supportive therapy. Two patients died within 24h of admission to hospital. Both were elderly and with evidence of multiple organ failure on admission and were not considered for ITU. One of these patients was found at post-mortem to have evidence of coexistent ascending cholangitis with intra-hepatic abscesses. Two of the remaining early deaths were admitted to intensive care and had full ventilatory and circulatory support. One died after 4 days in ITU, the other after only 48h. The remaining early death occurred in a 53 year old lady with severe chronic obstructive airways disease who deteriorated rapidly after admission but was not felt to be a suitable candidate for ventilation in ITU.

Of the late deaths, there were two patients in each group who died from causes not directly related to acute pancreatitis. One had a cerebro-vascular accident and died on day 10, one had a myocardial infarction which contributed to his death on day 9, one elderly lady died of bronchopneumonia on day 20 after making an initial recovery from her pancreatitis and one patient died from iatrogenic causes following the

insertion of a central venous catheter for the purpose of intravenous feeding. However, all four patients had severe acute pancreatitis associated with significant systemic complications. The remaining two deaths were in two patients who died in the post-operative period. One, a 69 year old female died of uncontrolled haemorrhage following a necrosectomy for pancreatic necrosis with fungal infection. The other, a 75 year old man died of multiple organ failure following operative exploration of the common bile duct for ascending cholangitis.

4.2.4 Effect on incidence of complications

The complications which were observed are summarised in **table 4.3**. Complications were recorded in 26 patients, 14 of whom were in the treatment group and 12 in the placebo group. The overall complication rate was therefore 45% which was in keeping with the rate expected with an admission APACHE II cut-off of greater than 5.

Multiple organ failure occurred in 4 patients in the placebo group and three in the treatment group. All patients in this study who developed multiple organ failure died as a consequence. Isolated respiratory insufficiency was the single most common systemic complication and was present in 6 patients in the placebo group compared with 7 in the treatment group. Local complications were recorded in three in the placebo group compared with 4 in the treatment group. Two patients, one in each group, had acute fluid collections on ultrasound. Both settled without surgical or radiological intervention. One patient had an infected fluid collection, by definition a pancreatic abscess, which settled following operative intervention with prolonged external drainage. One patient had pancreatic necrosis on contrast-enhanced CT scan and a second had pancreatic necrosis discovered at post-mortem only. Two patients had infected

pancreatic necrosis Both underwent necrosectomy, with one patient surviving and the other dying following uncontrolled haemorrhage.

	Placebo			**Treatment**	
Patient No	Complication	Death	Patient No	Complication	Death
7	PE, RF		11	MSOF	Day 14
10	RF, IPN		13	RF	Day 10
18	MSOF	Day 4	23	RF	
19	RF, Abscess		24	ARF	
22	MSOF	Day 1	31	RF	
29	RF	Day 14	33	MSOF	Day 2
36	RF, AFC		35	RF	
37	IPN, MSOF	Day 27	39	ARF	
40	RF	Day 20	42	RF	Day 4
44	MSOF	Day 1	43	AFC, PE	
51	RF		48	RF	
58	PE		52	RF, PN	Day 9
			55	RF	
			56	PE, PN	

***Table 4.3**: Complications and deaths in patient groups*

PN: Pancreatic necrosis

IPN: Infected pancreatic necrosis

PE: Pleural effusion

RF: Respiratory failure

ARF: Acute renal failure

MSOF: Multiple systems organ failure

AFC: Acute fluid collection

CVA: Cerebrovascular accident

The overall combined morbidity and mortality rate in the octreotide group was 50% and in the placebo group 40%. There was therefore an observed adverse effect of octreotide on outcome of 10% compared with placebo

(95% Confidence Interval -35 to +15). These results are summarised in **table 4.4**

	Treatment (n=28)	**Placebo (n=30)**	**Difference (95% confidence interval)**
Complicated	9 (32%)	6 (20%)	-12% (-22% to +10%)
Fatal	5 (18%)	6 (20%)	+2% (+22% to -18%)
Total	50%	40%	-10% (-35% to +15%)

***Table 4.4**: Summary of outcome in octreotide and placebo groups*

4.2.5 Influence of Aetiology on Outcome

Thirteen of the 31 patients (42%) with gallstone pancreatitis developed complications of whom 5 died. Of those with pancreatitis of other aetiologies, the complication rate was 48% with 6 deaths. Seven of the 11 (64%) patients with alcohol-related acute pancreatitis developed complications two of whom died. This difference, however, was not statistically significant (P>0.2, Chisquare test).

In the sub-group of patients with gallstone pancreatitis, there was no evidence of a beneficial effect from octreotide. Sixteen patients with gallstone pancreatitis received placebo of whom 7 had complications (44%). In those receiving octreotide there were 6 complications in 15 patients (40%).

4.2.6 Effect on the systemic illness

The degree of systemic illness on admission to the trial (as assessed by the acute physiology score, APS) was similar in the two groups of patients (treatment group; median APS=4, range 1-12, placebo group; median APS=4, range 1-14). There was no difference between groups in peak APS during the infusion period. The time taken for the systemic illness to settle, as measured by the return of the APS to zero was identical in both groups

of patients, occurring at a mean of 3.4 days in the treatment group and 3.8 days in the placebo group.

Patients receiving octreotide had similar serum levels of C-reactive protein to those receiving placebo. No significant difference in CRP levels was observed on any of the 5 days of trial infusion (**Figure 4.1**).

No beneficial influence of octreotide was observed on the incidence of systemic complications. As all patients who developed local complications also had systemic complications, no separate analysis of the effect on systemic complications was necessary.

4.2.7 Effect on plasma enzyme levels

Previous authors have suggested that the administration of somatostatin to patients with acute pancreatitis may be associated with a more rapid return of serum amylase levels to normal. No evidence of this was observed in the present study with similar levels of amylase being observed on each of the days of study in the two groups of patients (**Figure 4.2**).

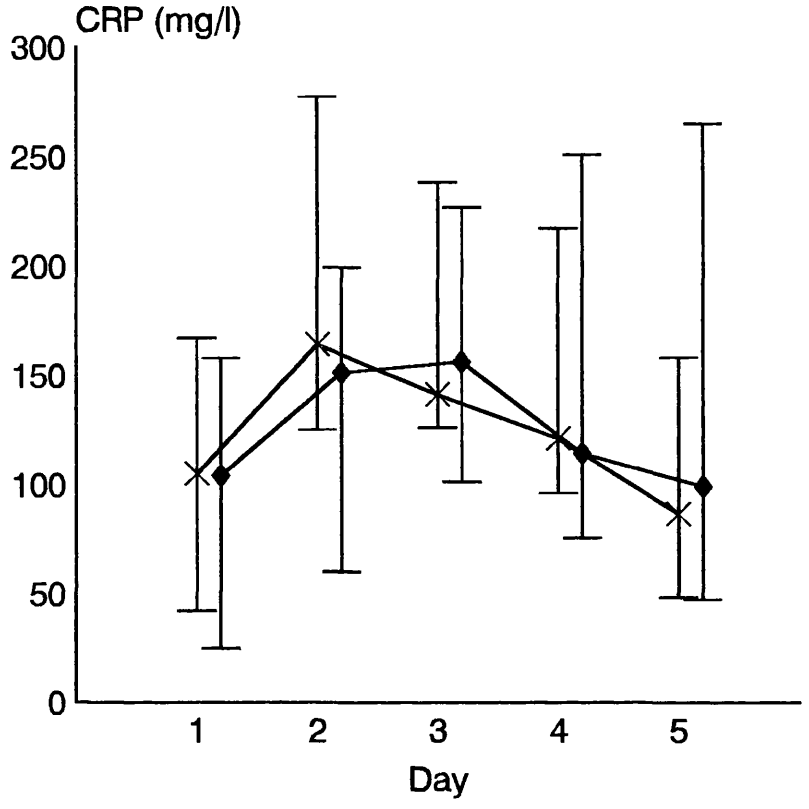
4.2.8 Effect on hospital stay

In survivors, hospital stay was not influenced by the administration of octreotide. Both groups of patients had a median hospital stay of 10 days.

4.3 Discussion

Previous studies with somatostatin or octreotide in acute pancreatitis have been discussed in section **3.4**.

The main difficulty in conducting clinical trials in acute pancreatitis is the large number of patients which must be recruited to give sufficient statistical power to confidently exclude a beneficial effect on outcome. It has been estimated that in only 3-5% of patients the outcome may be influenced by trial therapy[270]. It is therefore desirable to focus therapeutic



I Median/IQR
✳ Octreotide
◆ Placebo

Figure 4.1 : CRP levels in patient groups

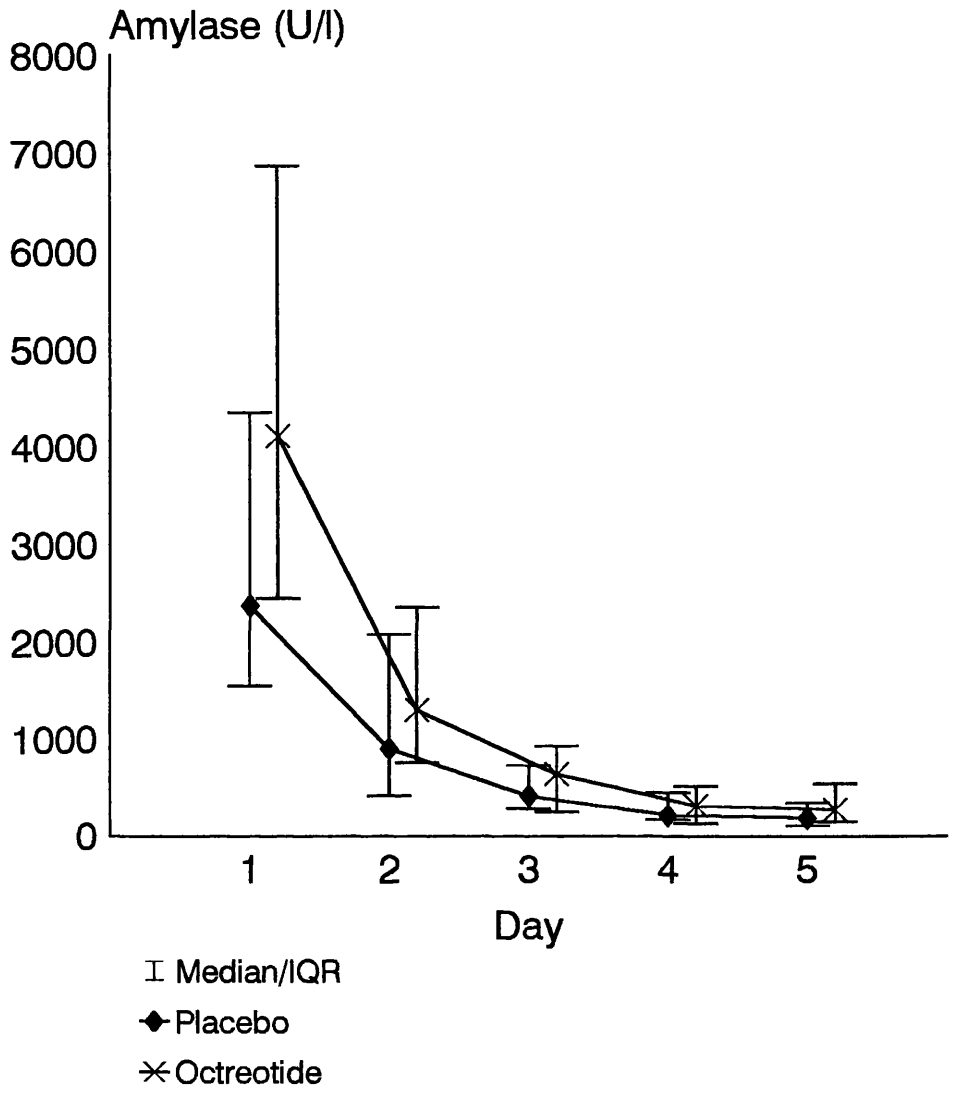


Figure 4.2 : Amylase levels in patient groups

trials on those patients most likely to benefit from treatment, namely those with severe pancreatitis. In the present study, although only 58 patients were studied, those with mild disease were excluded resulting in a mortality and complication rate of 45% which is higher than that of previous studies. No statistically significant difference was observed between groups and, unlike some previous studies[252,253], there was no evidence of a trend towards improved outcome in the octreotide group. The confidence intervals for the difference in complication rate between the two groups indicate that our results are consistent with an influence on outcome ranging from a 35% increase to a 15% reduction in complication rate with octreotide therapy. This indicates that even if octreotide has a beneficial effect in acute pancreatitis, such an effect likely to be small and of little relevance to routine clinical practice. It is equally likely, from the results of this study, that octreotide has a deleterious effect on outcome.

It can be readily calculated from sample size nomograms[251] that in order to demonstrate a beneficial effect of octreotide of 15%, the largest effect consistent with the results of the present study, a study would need to recruit 175 patients in each limb to give a power of 0.85. In the event that the true effect of octreotide was a 10% improvement in outcome, then a trial recruiting 800 patients with prognostically severe pancreatitis would be required. The power of the present study to demonstrate a difference of 15% was 0.15 and it is obvious that such an effect cannot be excluded by this trial. The power of the present study to exclude a beneficial effect of octreotide on outcome of 30% (reducing the complication rate to 10%) was 0.75. From these considerations, the present study indicates that a large, beneficial effect of octreotide on outcome is unlikely. It is also unlikely that a trial of sufficient magnitude to exclude a small beneficial effect of octreotide will ever be conducted.

The use of the APACHE II score may be criticised as a means of grading patients for prognostic purposes as it is less specific than other methods such as multiple factor scoring systems or C-reactive protein levels[34]. However, it is the only readily available method by which patients can be graded for severity on admission to hospital and therefore allows earlier selection of patients for study. The cut-off level of five was chosen to give a high sensitivity and reasonable specificity for severe attacks[34]. The overall mortality rate of 19% in this study confirms the relative severity of disease in the group of patients studied and compares favourably with other trials in which patients with mild pancreatitis have been excluded.

One problem encountered with the use of the APACHE II system was the relatively heavy weighting placed on age. Any patient over the age of 75 automatically qualified for inclusion in the study with an APACHE II score of 6. This may explain the relatively high median age of patients recruited to the present study. Younger patients needed to score more APS points to qualify for inclusion in the study and were therefore more unwell on admission than many of their elderly counterparts.

An admission APACHE II score of greater than 7 has been recommended in order to indicate a prognosis of severe pancreatitis but, while this would reduce the number of mild attacks included in the study, it would also be associated with a reduction in sensitivity resulting in some patients with severe pancreatitis being missed. The APACHE II score of 5 was considered a reasonable compromise, allowing inclusion of almost all of those with severe attacks and excluding at least half of those with mild pancreatitis. It is emphasised, however, that no prognostic scoring system meets all the needs of a clinical trial and the APACHE II system was chosen as the most appropriate of the methods available.

4.4 Possible factors responsible for the failure of octreotide to improve outcome

Notwithstanding the statistical considerations discussed above, the possible reasons why the present study has failed to suggest a benefit from the use of octreotide in acute pancreatitis were examined.

4.4.1. Trial Factors

Drug administration

No studies were carried out to determine the bioavailability of octreotide during the trial infusions. However, the drug was stored and used according to the manufacturer's recommendations. It was kept refrigerated at 4^{o}C until the trial infusion was prepared following which it was used immediately. The drug was diluted 5:48 which is only very slightly more than recommended by the manufacturer (1:9 or 5:45). The continuity of the trial infusion was outwith the direct control of the author but infusions were checked on a daily basis. Brief interruptions to the infusion would not be expected to influence the results greatly in view of the prolonged circulation half-life of octreotide. Interactions with syringes or giving sets used in the study have not been previously reported.

The dose of octreotide used in the present study was 1mg/day (40μg/h) which is greatly in excess of the dose required for maximal suppression of pancreatic secretion[271]. It is higher than that used in previous studies in acute pancreatitis[272] and higher than the dose used in studies in pancreatic surgery[222] both of which demonstrated a therapeutic benefit with octreotide treatment. It is therefore unlikely that inadequate dosage was responsible for the negative findings of this study.

Delay in treatment

There is some evidence from previous experimental studies with somatostatin that pre-treatment is required for therapeutic benefit to be seen in acute pancreatitis[238,247,248]. This would obviously not be possible in the context of a clinical trial but every effort was made to ensure that the trial infusion was commenced as soon as possible after admission. The median delay from symptom onset to trial infusion was 24h (mean 29h, range 6-72h) and it is unlikely that this could be significantly improved.

4.4.2. Octreotide-related factors

The many actions of somatostatin and somatostatin analogues have led to several proposed mechanisms by which they may influence the outcome from acute pancreatitis. Although octreotide has been to be associated with inhibition of pancreatic secretion, reduction in pancreatic blood flow, cytoprotection and reticuloendothelial stimulation, there is no conclusive evidence that these properties are likely to be useful in the treatment of acute pancreatitis. This is discussed in detail in section **3.4.1**.

In conclusion this trial has failed to demonstrate any evidence of a beneficial effect of octreotide in 58 patients with moderate to severe acute pancreatitis. The results of this study indicate that the therapeutic effect of octreotide, if present at all, is small and unlikely to make any impact on the management of patients with acute pancreatitis.

Chapter 5
Development of methods for the study of mononuclear phagocyte function.

5.1 Introduction

Severe acute pancreatitis is commonly associated with a systemic illness characterised by a hyperdynamic circulation[135,136] with varying degrees of impairment of respiratory and renal function. At the extreme end of the spectrum, patients develop multiple organ failure with its associated high mortality. This systemic illness has many similarities to that seen in patients with sepsis and it has been postulated that similar pathophysiological mechanisms may underlie the systemic manifestations of both conditions[3]. In sepsis, there is now considerable evidence that the systemic illness is the result of uncontrolled activation of mononuclear phagocytes and the consequent release of increased quantities of inflammatory mediators. Of these, tumour necrosis factor alpha (TNFα), interleukin-1 beta (IL-1β), interleukin-6 (IL-6) and interleukin-8 (IL-8) are thought to play an important role[4]. In contrast, the pathophysiology of the systemic illness seen in acute pancreatitis is unknown. For many years it was considered to be the result of activated proteases released from the damaged pancreas and the consequent local and systemic release of vasoactive peptides[2,132,273] However, the failure of antiprotease therapy to influence the outcome from acute pancreatitis[12,13,45,108] has led to reappraisal of this theory and the search for alternative mechanisms.

Little is known of the role of mononuclear phagocyte activation in acute pancreatitis. Previous studies have been restricted to the measurement of plasma levels of cytokines, as an indirect measurement of phagocyte function. Previous authors have demonstrated that plasma levels of IL-6 are increased in patients with complicated or fatal acute pancreatitis[38,40] and IL-6 levels have been reported to have prognostic significance early in the

course of the disease[38]. Plasma levels of TNFα have been measured in two studies with inconclusive results[5,6]. Gross and colleagues measured plasma levels of interleukin-8 in 10 patients with acute pancreatitis and found higher levels in those with systemic complications[192]. There have been no studies examining IL-1β in acute pancreatitis. The interpretation of studies on plasma cytokine levels is made difficult by the presence of circulating inhibitors[189] the short circulation half-life of many cytokines[191] and the fact that their main physiological action is at a paracrine level[274]. In order to overcome these problems it was decided to study directly the function of mononuclear phagocytes in patients with acute pancreatitis.

In man, the only mononuclear phagocyte readily available for study is the circulating monocyte. Monocytes are produced in the bone marrow from precursor stem cells and enter the bloodstream as 12-20μm cells with typically bean-shaped nuclei. Monocytes migrate to various tissues where they differentiate according to the tissue site[275]. In the liver they become Kupffer cells, in the lung, alveolar macrophages. Monocytes, like tissue macrophages, are activated by endotoxin and can be induced to release cytokines including TNFα, IL-1β, IL-6 and IL-8. By studying the in-vitro function of monocytes taken from patients with acute pancreatitis, it was reasoned that an indirect measurement could be made of the activation status and secretory function of mononuclear phagocytes in-vivo.

The first stage in this study was to develop a method of separation of monocytes from peripheral blood which would allow the study of monocyte function in patients with acute pancreatitis.

5.2 Monocyte separation - principles

Monocytes comprise less than 5% of peripheral blood leucocytes as determined by esterase activity[276] and several methods have been described for their enrichment. The ideal method would fulfil three main criteria:

Firstly, a high yield of cells is required to enable sufficient numbers of cells to be extracted from a small quantity of blood. Only limited amounts of blood can be taken from patients, particularly when repeated measurements are required and when further blood samples are required for routine biochemistry and haematology.

Secondly, consistent, high levels of purity are required. The levels of cytokine secretion observed in the resulting cell culture would be a function not only of the activation status of the monocytes, but also on the proportion of monocytes present. Therefore, if mixed peripheral blood mononuclear cells were studied, it would be necessary to interpret the results in the light of varying monocyte numbers in the cell populations studied, a factor which would make the final interpretation of the results difficult.

Thirdly, any method for monocyte separation should have a minimal effect on cell function in order that the function of the monocytes in-vitro should be as close as possible to their function in-vivo.

In practice , any method chosen will be a compromise between these three ideals as all the available methods have associated drawbacks.

5.2.1 Density Gradient Separation

Monocytes and lymphocytes are less dense than polymorphonuclear leucocytes and erythrocytes and can be separated from them by centrifugation at low speed over a medium of appropriate density. Metrizoic acid, an iodinated non-toxic compound, is commonly used for this purpose. Lymphoprep (Nycomed UK) is a solution of 9.6%(w/v)

Sodium Metrizoate combined with the erythrocyte aggregating polysaccharide, Ficol. Centrifugation of whole blood on this medium produces a band of cells at the interface layer containing mixed mononuclear cells from which monocytes can be further purified using a variety of methods. Monocytes generally represent 20-30% of the mixed cell population. A variation on this method is the one step separation of monocytes using Nycoprep 1.068. (Nycomed UK). This is a discontinuous density gradient which utilises the fact that monocytes and lymphocytes expel water at different rates in hyperosmotic media.

5.2.2. Separation by adherence

Monocytes rapidly adhere to various surfaces and this property can be used to separate them from other leucocytes. Leucocyte-rich plasma is normally incubated in a tissue culture flask, allowing monocytes to adhere to the plastic surface. The non-adherent cells are then washed off and adherent cells recovered by incubation with a cation chelating agent such as EDTA. Certain substrates, such as gelatin or autologous serum, can be used to coat the plates in order to achieve better monocyte yields. This method, although relatively simple and requiring no specialised equipment, may result in contamination with granulocytes and B-lymphocytes, both of which can adhere under these conditions.

5.2.3. Separation by cell size

Counterflow centrifugal elutriation (CCE) utilises the fact that monocytes are generally larger than lymphocytes to separate monocytes from mixed mononuclear cells. Although this method allows separation with the minimum of interference to the cells it requires relatively large quantities of blood and depends on the availability of specialised equipment. Furthermore, different populations of monocytes with varying size are now

recognised[277] and CCE may differentially select larger monocytes with different functional characteristics from the whole monocyte population.

5.2.4 Separation by immunomagnetic methods.

Monocytes may be selected by methods utilising magnetic microbeads (Dynal) covalently bound to monoclonal antibodies raised against various cell-surface determinants. Removal of T and B-lymphocytes as well as NK-cells results in a relatively pure population of monocytes with minimal interference with cell function. High bead:cell ratios are needed for effective removal of lymphocytes and enrichment of cells by this method is not possible[278]. Recently, a new immunomagnetic method has become available. MACS (magnetic cell separation system) was developed at University of Cologne and utilises small, superparamagnetic particles in a high gradient magnetic field[278]. The advantage of MACS over traditional magnetic cell sorting is that the small microbeads used (50-100nm) do not interfere with cell function or viability[279]. Furthermore, the small magnetic moment of the microbeads allows positively selected cells to be easily removed from a ferromagnetic matrix outside of the magnetic field and therefore enrichment as well as depletion of cells is possible[278].

5.2.5 Fluorescence activated sorting (FACS)

Similar selection of cells by their cell-surface markers can be achieved by labelling with fluorochrome-conjugated antibodies and separation in a flow cytometer with cell sorting capabilities. Although this method allows separation of rare cell populations, including monocytes, it requires expensive equipment and is time consuming if large numbers of cells are required[278].

5.3 Comparison of two methods of monocyte separation

Of the monocyte separation methods available, the MACS method appeared most promising as it could potentially allow high yields of cells from small quantities of blood. There were no reports in the literature on the use of the MACS system for monocyte separation therefore MACS separation was compared with adherence which is a long-established method of monocyte separation.

5.3.1 Methods

120 ml of venous blood was withdrawn from three healthy donors using 2.4% EDTA as anticoagulant. Immediately after collection, blood was mixed 1:1 with phosphate buffered saline (PBS) and layered on Ficol-Hypaque (Lymphoprep, Nycomed UK). 10ml of blood/PBS was pipetted onto 7.5ml of Lymphoprep in sterile 25ml universal containers. After centrifugation at 400g for 30 min at room temperature, the mononuclear cell-rich interface was collected using a sterile pasteur pipette. PBS was added and the cells washed by centrifugation at 600g for 20 min at 4^{o}C. Two further washes with PBS were performed, with centrifugation at 200g to remove platelets. The cell pellet was then resuspended in 1ml of PBS and counted. The cells were then divided into three aliquots and monocyte enrichment was carried out by adherence or magnetic separation. Two monoclonal antibodies recognising monocyte cell-surface antigens were compared, namely anti CD33 and anti-CD14.

MACS SEPARATION (Figure 5.1)

Microbead-conjugated antibody (60ml) was added to each of two aliquots of PBMC's, each containing $3x10^7$ cells. To one aliquot was added anti-CD33 antibody and to the other anti-CD14 antibody. Both were obtained from Bekton Dickinson. The cell suspensions were then incubated for 20

MACS System

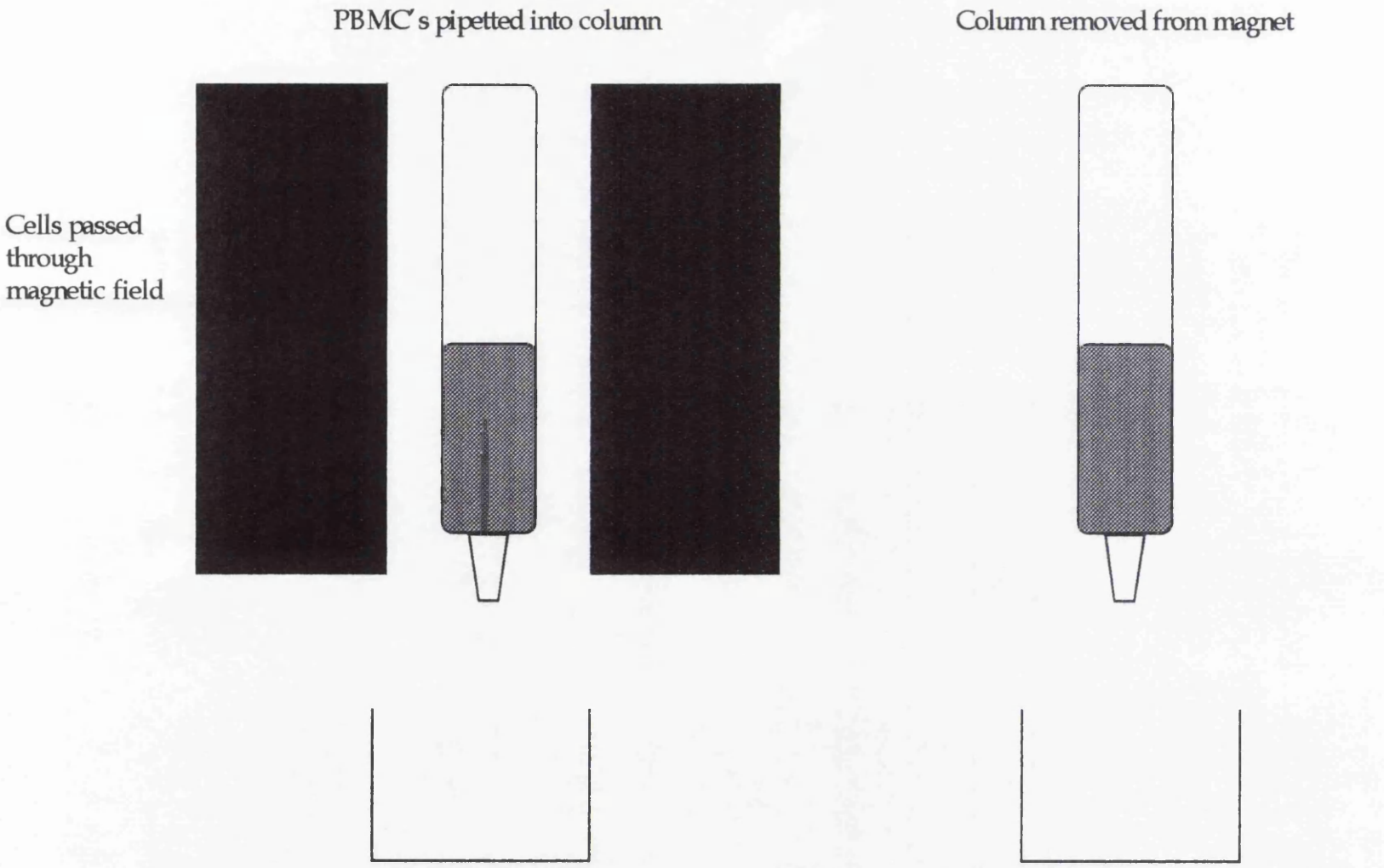


Figure 5.1

min at 4°C following which cell separation was carried out. Following incubation with the microbeads, each aliquot was separated in the MACS, also obtained from Bekton Dickinson. A column ($3x10^7$ cell capacity) was sterilised by flushing from below with 70% alcohol. Following this the column was washed with PBS/5mMol EDTA/0.2% bovine serum albumin (BSA) to remove all traces of alcohol. Finally the column was cooled by the passage of several column volumes of ice-cold PBS/EDTA/BSA. The PBMC's were pipetted from the top of the column and flow rate was controlled using a 22G needle. The column was washed with 3 column volumes of PBS/EDTA/BSA and then was flushed from below to loosen weakly adherent cells. The washing and flushing was then repeated. Finally, the column was washed at a higher flow rate using a 21G needle. The column was then removed from the magnetic field and the retained cells were eluted using cold PBS/EDTA/BSA flushed from the top of the column with short, forceful movements. The positively selected cells were then collected by centrifugation at 600g for 6min and the cell pellet resuspended in AIMV. A viable cell count was then performed by trypan-blue exclusion.

ADHERENCE

Of the remaining PBMC, $4x10^7$ were added to 20ml of RPMI medium supplemented with 10% n

normal human AB serum in a medium sized culture flask. The flask was then incubated for 90min at 37°C in a 5% CO_2 enriched atmosphere. The flask was then carefully washed with warm phosphate buffered saline to remove the non-adherent cells. 5mMol EDTA in PBS was then added to cover the bottom of the plate and the cells were incubated for 15min following which adherent cells were removed using gentle agitation. The resultant cells were then collected by centrifugation at 600g for 6min and

the pellet was resuspended in AIMV medium. A viable cell count was again performed by trypan blue exclusion.

Analysis of purity

The purity of the resultant monocyte-enriched cell suspension was assessed by flow cytometry. 5µl of fluorochrome (FITC) -conjugated anti-CD14 monoclonal antibody was added to 50ml of each cell suspension and incubated at 4°C for 20min. The cells were then washed by centrifugation and the cell pellet resuspended in 1ml of PBS. To one aliquot of the original PBMC suspension was added 5µl of FITC-conjugated murine immunoglobulin to act as a negative control. The cells were then analysed in a flow cytometer (EPICS II, Coulter) and the percentage of CD14 positive cells in the enriched cell populations and in the original PBMC populations was measured.

5.3.2 Results

Cell viability in all experiments was assessed by trypan blue exclusion and was always greater than 95%. The number of cells obtained following separation on Lymphoprep is shown in **table 5.1**. Blood from subject 1 yielded the highest absolute number of cells but the proportion of monocytes as determined by immunofluorescence was the lowest of the three volunteers at 18%.

Subject	No PBMC	% monocytes
1	$1.3x10^8$	18
2	$1.1x10^8$	26
3	$1.15x10^8$	31

Table 5.1: Yield of monocytes from 30ml peripheral blood

Cell separation by immunomagnetic positive selection consistently yielded the highest number of monocytes, the yields being two to three times higher than achieved by adherence. Furthermore, selection using the CD14 cell-surface antigen yielded consistently higher numbers of monocytes than the CD33 method **(table 5.2)** Using selection by anti-CD14 conjugated microbeads, over 30% of the available monocytes in the original PBMC population could be retrieved. .

Subject	**ADHERENCE**	**MACS CD33**	**MACS CD14**
1	13%	27%	33%
2	19%	23%	36%
3	7%	23%	33%
MEAN	13%	24%	34%

Table 5.2: *Final yield of cells after enrichment for monocytes as percentage of the original number of CD14 positive cells.*

The proportion of monocytes in the final cell population was consistently higher with CD14 immunomagnetic separation than with separation by adherence **(table 5.3)**. In two of the three subjects, however, the proportion of monocytes was higher after separation using the CD33 antigen compared with the CD14 antigen.

Subject	ADHERENCE	MACS CD33	MACS CD14
1	84%	94%	89%
2	74%	86%	89%
3	82%	94%	92%
MEAN	80%	91.3%	90.%

Table 5.3: Proportion of cells in final population identified as monocytes by CD14 immunofluorescence

It was concluded that the monocyte yield achieved by magnetic cell separation was higher than that achieved by adherence. In addition, monocyte purity was higher and the time taken for separation was considerably shorter. The average time taken for monocyte separation using adherence was 150min and MACS 25min. MACS separation was therefore quicker and more efficient than adherence under these conditions.

5.3.3 Discussion

Immunomagnetic selection of monocytes proved rapid, easy to perform and was associated with consistently high yields of cells. The purity of the final cell population was around 90% or greater which was higher than was achieved by separation using adhesion. Methods of improving the yield and purity of adherent cells have been described. Using fibroblast exudate-coated culture plates, monocyte yields of 2-7x10^6 cells from 20ml of blood have been achieved, with monocyte purity in excess of 90%[280]. This technique was not assessed in the present study but the results obtained using uncoated plates for adherence were not encouraging.

The rapidity with which monocytes could be separated from PBMC with the immunomagnetic method minimised the time between venepuncture and the availability of cells for culture and *in-vitro* assessment of function. It was felt that this would help to minimise the unwanted effects on cell function. The method was also simple to learn and to use. Repeated separations could be carried out sequentially, allowing the study of several blood samples simultaneously.

Neither CD14 or CD33 is specific to monocytes. CD33 is found on bone marrow progenitor cells but in the peripheral blood, it is only expressed by monocytes. In contrast, CD14 is expressed on peripheral blood monocytes but also, if to a lesser extent, on granulocytes. CD14 is expressed on a higher proportion of monocytes than CD33, however, which may explain the higher yield of cells which was obtained using the CD14 as the target antigen. The other reason for improved cell yields with the CD14 method may have been the selection of granulocytes in addition to monocytes as both display the CD14 antigen. However, the vast majority of granulocytes should have been removed during the initial Lymphoprep separation, as granulocytes are sedimented out at this stage along with erythrocytes. Cellular smears were made of the monocyte suspensions in order to check for the presence of granulocytes. After staining, the smears were examined by light microscopy and only very occasional granulocytes were observed.

It was therefore concluded that optimal monocyte yields could be achieved using the CD14 immunomagnetic method with acceptable levels of purity.

Experiments were then conducted in order to assess the effect of monocyte separation on cell function.

5.4 Analysis of effect of monocyte separation on monocyte function

As outlined above the optimal method of monocyte separation would be associated with minimal effects on cell viability and function. It was reasoned that all methods of monocyte separation would be associated with functional changes which would influence the results obtained from in-vitro studies. However, as all patient samples would be exposed to similar influences, the effect of these influences would be similar for each patient. This would therefore allow comparison of results between individual patients and between patients on separate days of study. It was considered necessary to document as far as possible the effect of the method chosen on monocyte function, if only to exclude uniform cellular activation.

5.4.1 Methods

Four volunteers were chosen from the laboratory staff. 30ml of venous blood was withdrawn into 2.4%EDTA and immediately separated on Lymphoprep as described previously. PBMC were then resuspended in AIM V and counted. The proportion of monocytes in the PBMC suspension was then assessed by flow cytometry. An aliquot of PBMC containing approximately 10^7 cells was taken from each of the four cell suspensions and placed on ice. The remainder of the cells were incubated with anti-CD14-microbeads (20µl per 10^6 cells) for 20 min at 4^oC. The PBMC suspensions were then split into two aliquots. One aliquot was kept on ice and the on the other, magnetic cell separation was carried out as previously described. Monocytes were then separated using the MACS and CD14-conjugated microbeads. Monocyte purity was assessed by flow cytometry. The monocytes and PBMC were then suspended in AIM V medium, adjusted to give a final cell density of $5x10^5$ **monocytes** per ml. The following culture wells were then set up;

1. Monocytes alone

2. Monocytes with LPS 200ng/ml (lipopolysaccharide E Coli E5 B55, Sigma)

3. PBMC alone

4. PBMC with LPS 200ng/ml

5. PBMC with anti-CD14-microbeads

6. PBMC with anti-CD14-microbeads with LPS 200ng/ml

The cells were then cultured for 24h at 37°C following which the supernatants were removed, clarified by centrifugation and frozen at -30°C. TNF was subsequently measured in the supernatants by ELISA.

5.4.2 Results

The results of this experiment are summarised in **table 5.4**. In all cases, visual inspection of the culture wells after 24h confirmed the typical phenotypic changes associated with "flattening" of the monocytes onto the surface of the culture flasks. There was a consistent increase in secretion of TNFα with the addition of LPS to the culture medium. Mean TNFα secretion in the monocyte alone wells was 1.4ng/ml. After LPS stimulation, TNFα levels rose to a mean of 3.6ng/ml (range 1.4-7ng/ml). Mean TNFα secretion in the PBMC alone wells was similar at 1.2ng/ml, and rose to a mean of 2.8ng/ml after LPS secretion (range 1.1-4.1ng/ml). The basal secretion when PBMC to which CD14-microbeads were added was unchanged at 1.2ng/ml but the mean secretion after stimulation with LPS was lower at 1.9ng/ml, although this difference was not statistically significant (T=1.8, P=0.17, two sample t-test).

Culture well	Control 1	Control 2	Control 3	Control 4	Mean	Mean Difference
1	1.2	1.0	0.9	2.6	1.4	
2	2.8	1.4	2.7	7.0	3.6	2.2
3	1.0	0.9	0.8	1.9	1.2	
4	3.5	2.5	1.1	4.1	2.8	1.6
5	1.0	0.9	0.9	1.8	1.2	
6	2.25	1.5	1.3	2.4	1.9	0.7

Table 5.4 *; TNFα secretion (ng/ml)*

Culture well1: monocytes alone (MACS separated with CD14-microbeads)

Culture well 2: monocytes + LPS

Culture well 3: PBMC alone

Culture well 4: PBMC+LPS

Culture well 5: PBMC+CD14

Culture well 6: PBMC+CD14+LPS

5.4.3 Discussion

Basal secretion of TNFα by monocytes, PBMC and PBMC with CD14-microbeads was similar in all four cases. In three of the four subjects, the effect of LPS on TNFα secretion by PBMC was slightly attenuated by the addition of anti-CD14-microbeads to the culture medium. Similar, small responses were found in the case of control 3. The secretion of TNFα by the separated monocytes was similar to that observed in the wells containing PBMC. From these results, the following were concluded;

1. The use of CD14-microbeads does not remove the ability of monocytes to respond to appropriate stimuli in-vitro.

2. CD14 microbeads are associated with no reduction in basal levels of cytokine secretion.

3. There was no evidence of significant cellular activation, as measured by TNF secretion, by CD14-microbeads.

Until recently, the CD14 cell-surface antigen had no known function, although anti-CD14 mAb had previously been shown to induce an oxidative burst in human monocytes[281]. However, in 1990, Wright and colleagues[282] demonstrated that CD14 is in fact a cell-surface receptor for LPS and LPS complexed with a serum factor, lipopolysaccharide binding protein (LBP). Binding of monocytes with anti-CD14 mAb has been shown to inhibit serum-dependent LPS stimulation by up to100%[281] a result which would make the use of the CD14 positive selection method unsatisfactory for the present study. However, different epitopes on the CD14 molecule are recognised by different anti-CD14 mAb. In the study by Couturier et al[281], mAb IOM2 (Immunotech) and My4 (Coultronics) were each associated with complete inhibition of serum-dependent LPS binding. The Becton Dickinson monoclonal antibody, LeuM3 which was used in the present series of experiments and which is conjugated with microbeads for use in the MACS system, recognises an epitope on the CD14 molecule which is not located in the LPS receptor and thus did not inhibit LPS binding[281].

A further factor to be considered in the present study is the use of serum-free medium (AIM V). This was chosen in order to exclude the possible effect of serum on the detection of cytokines in the culture medium but it is now appreciated that under serum-free conditions, LPS binding occurs via CD14-independent mechanism[281] and therefore, binding by CD14 mAb may be irrelevant under these circumstances.

It was therefore considered that the use of positive selection of cells by the anti-CD14-microbeads was an appropriate method for the prospective study of monocyte function in patient groups.

5.5 LPS Titration

In order to find the optimal concentration of LPS for future experiments, a titration of LPS concentration against TNF production was carried out in two of the above control subjects.

5.5.1 Methods

From healthy laboratory volunteers (controls 3 and 4, above), 30ml of venous blood was withdrawn and monocyte separation carried out as described above by Lymphoprep separation followed by CD-14 immunomagnetic monocyte separation.

LPS (E Coli 055 B5, Sigma) was diluted to 50ug/ml in AIM V (Gibco). A 100ul aliquot of this solution was then diluted serially 1:5, by taking 20ul into 80ul of AIM V, followed by 20ul of the resulting solution into a further 80ul of AIM V and so on. This gave 4 concentrations of LPS in AIM V; 400, 2000, 10 000 and 50 000ng/ml. 20ul of these solutions was added to each of 4 wells containing 1ml of AIMV with monocytes at a density of $5x10^5$/ml. This resulted in a final concentration in the wells of 8, 40, 200 and 1000ng/ml of LPS.

The cells were cultured for 24h at 37^oC following which the supernatants were removed and the concentration of TNF measured by ELISA.

5.5.2 Results

The results of this experiment are summarised in **table 5.5**.

Concentration of LPS	8ng/ml	40ng/ml	200ng/ml	1000ng/ml
Control 3	2.3	2.1	2.7	3.2
Control 4	3.1	2.6	7.0	7.9

***Table 5.5**; LPS titration. TNFα concentration in wells in ng/ml*

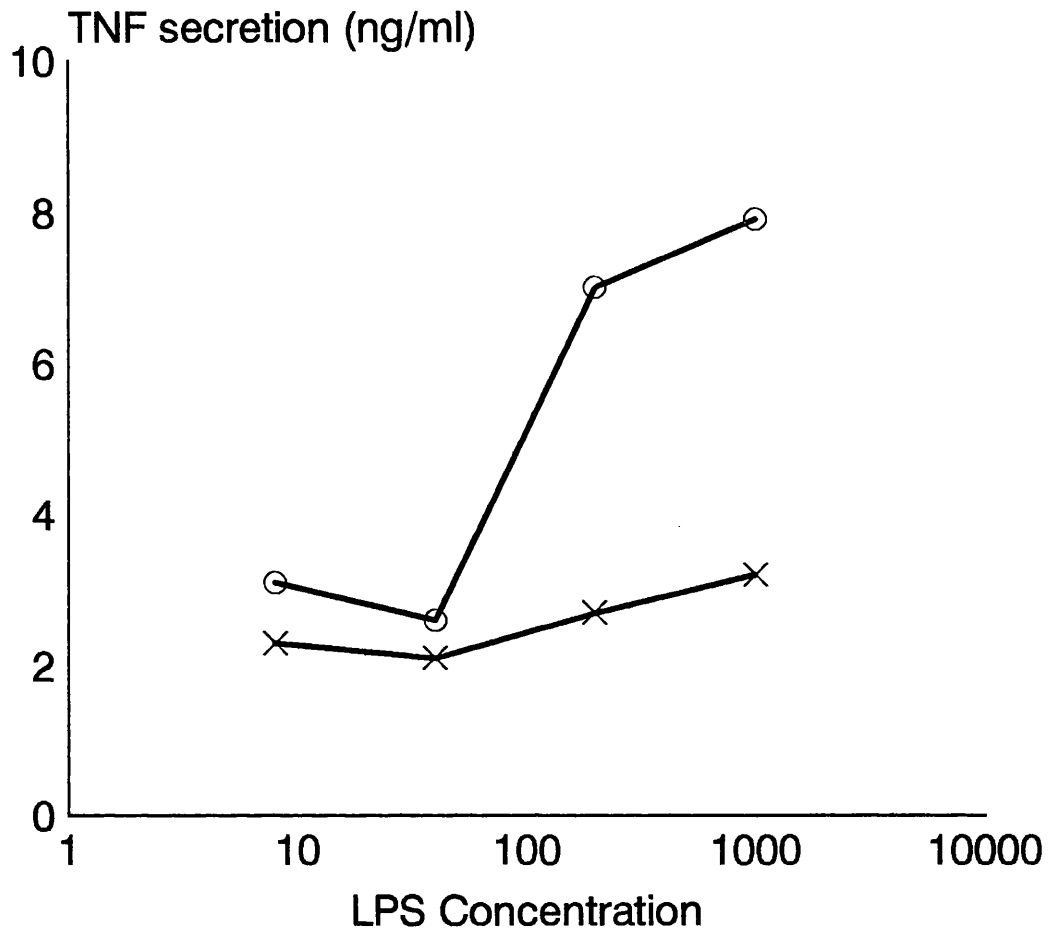
A concentration of LPS which would induce significant cytokine production but which would not cause maximal cellular stimulation was considered ideal. In this way, it was thought more likely that small differences between cell populations would be identified. A final culture well concentration of 200ng/ml of LPS was therefore chosen for all future experiments (**Figure 5.2**).

5.6 Development of technique for patient group

Following these preliminary experiments, these methods were assessed in patients who presented with acute pancreatitis. In particular, it was important to ensure that the proportion of monocytes in peripheral blood from patients with acute pancreatitis was sufficient to give a satisfactory yield of cells. Also, it was not known how the behaviour of monocytes following in-vivo activation might differ from those obtained from healthy donors.

5.6.1 Methods

In three successive patients entered into the octreotide study, blood was withdrawn within 48h of admission for monocyte separation. The blood was transported to the laboratory at room temperature and separation commenced in each case within 1h of venepuncture. The manufacturer's



Control 3

Control 4

Basal secretion was undetectable

Figure 5.2 : LPS titration

recommended methods were used as described above for separation of PBMC and subsequent positive selection of monocytes using the MACS system. The proportion of monocytes in PBMC and in the final positively selected cell population were assessed using flow cytometry as described before.

5.6.2 Results

The results of this experiment are shown in **table 5.6**

Patient No	APACHE II	PBMC No ($x10^6$)	PBMC %CD14	Monocyte No ($x10^6$)	Monocyte % CD14	%extraction
17	6	21	25	<1	80	<11
18	11	8	20	<1	50	<31
19	6	25	51	<1	78	<6

***Table 5.6** MACS separation of monocytes in patients 17, 18 and 19.*

5.6.3 Discussion

These results were considerably worse than was achieved in the control subjects and the numbers of monocytes obtained would have been insufficient for the purposes of the present study. Possible reasons for the poor results in the patient group were considered as follows:

1. Low proportion of CD14+ve cells in peripheral blood.

This reason was excluded by the flow cytometric analysis of the PBMC obtained from these patients. In one patient, 51% of peripheral blood mononuclear cells were CD14 positive, but despite this, less than $1x10^6$ monocytes were eventually obtained.

2. Deterioration in antibody efficacy.

The microbead-conjugated anti-CD14 antibody had been kept refrigerated at 4°C as suggested by the manufacturer. In view of these results, however, a sample of antibody was sent to the manufacturer in Germany for assessment. It was subsequently returned having performed satisfactorily in the laboratory.

3. The disease process could have altered the binding characteristics of the CD-14 antigen resulting in poor extraction rates

4. Alteration in monocyte behaviour resulting in cell loss during separation procedure.

This was considered the most likely explanation for the results obtained in the patient group. As a result, the following conditions were changed;

5.7 Adjustment to methodology

5.7.1 Methods

1. The initial separation of peripheral blood on Ficol was carried out at room temperature to avoid subjecting the cells to unnecessary changes in temperature. It was necessary to incubate the PBMC with the anti-CD14 antibody at 4°C for optimal antibody binding. Following this the MACS separation was carried out using washing buffer cooled on ice and a column which was cooled by the passage of this cooled washing buffer. Following MACS separation the cells were allowed to return to room temperature gradually prior to final resuspension in culture medium and culture at 37°C.

2. Bovine serum albumin (0.7%) and EDTA (5mmol/l) were added to the PBS used for all washes and for the MACS separation. This was in order

to reduce the "stickiness" of the monocytes to the various plastic containers used in the procedures and so minimise cell loss.

These methods were assessed with blood from a healthy control and Two patients with acute pancreatitis.

5.7.2 Results

The results of this experiment are shown in **table 5.7**

Patient No	APACHE II	PBMC No ($x10^6$)	PBMC %CD14	Monocyte No ($x10^6$)	Monocyte % CD14	%extraction
Control	0	50	38	7.6	97	39
26	6	40	28	3	97	26
30	7	12	35	2.2	96	50

***Table 5.7** : Monocyte extraction using modified methodology*

It was therefore decided to use these methods for all subsequent separations and to use only these results for analysis. The detailed methodology finally used is given below.

5.8 Methods used in patient study

5.8.1 Blood Collection

Anterior cubital fossa venepuncture was performed using a 19G butterfly needle with 1% propanol as skin disinfectant. Patients in an intensive care unit with an indwelling arterial cannula had blood withdrawn from it. 35ml of blood was taken into previously prepared 50ml centrifuge tubes containing 3.5ml of sterile 2.4% ethanoldiaminotetraacetic acid (EDTA) in

PBS. Remaining blood was collected into blood bottles, 10ml for serum and 10ml for plasma/EDTA.

5.8.2 Separation of Peripheral Blood Mononuclear Cells

Blood samples were returned to the laboratory at room temperature following which separation of peripheral blood mononuclear cells was carried out using a density gradient method.

Blood was diluted 1:1 with PBS containing EDTA 5mmol/l and bovine serum albumin 0.5% (PBS/EDTA/BSA) and layered 2:1 on ficol-hypaque density gradient medium (Lymphoprep, Nycomed UK).
Blood layered on Lymphoprep was placed in a centrifuge and spun at 600g for 30min at 20°C. Following this, the interface layers were removed using sterile pasteur pipettes and collected in universal containers. PBS/EDTA/BSA was added and the cells washed by centrifugation at 800g for 20min. The cell pellets were then resuspended and collected in one container . Cells were then washed twice at 400g for 12min at 20°C in order to remove platelets. Finally the cell pellet was resuspended in 1ml of PBS/EDTA/BSA and a viable cell count carried out by trypan-blue exclusion.

Trypan-blue is taken up only by dead cells, viable cells excluding it. 50ul of cell suspension was diluted 1:100 in trypan blue and counted in a haemocytometer.

An aliquot of cells was kept aside for subsequent analysis by flow cytometry. To the remainder of the separated PBMC was added anti-CD14 microbeads (Bekton Dickinson), 20ul per 10^6 cells. These were incubated at 4°C for 20 min during which time the separation column was prepared

for use. A column with a cell capacity of $3x10^7$ cells was used. This was sterilised using 70% ethanol and then washed with a total of 20ml of ice-cold PBS. The cell suspension was then passed through the column which was suspended within the MACS magnet and washed with 3 column volumes of PBS/EDTA/BSA. A 22G needle was used to control the flow speed. Following this, the column was flushed from below and a further 3 column volumes of buffer washed through using a 20G needle. The cells washed through were collected in a universal container. The retained cells were then collected by removing the column from the magnetic field and gently flushing the column with 20ml of cold PBS/EDTA/BSA. Both cell fractions were spun at 600G for 5 min at room temperature. The cell pellets were then resuspended in 1ml of AIM V medium at room temperature and a viable cell count carried out using trypan-blue.

The monocyte fraction was diluted to a final cell density of $5x10^5$ cells/ml and 1ml aliquots added to the wells of a 24-well plate. To half of the wells was added bacterial lipopolysaccharride (LPS 055 B5, Sigma) 20ul of a 10ug/ml suspension resulting in a final well concentration of approximately 200ng/ml. The plates were then cultured at 37°C in a 5% CO_2-enriched atmosphere for 24h. Following this the supernatants were removed and spun at 600G for 5 min. The clarified supernatants were then removed and frozen in Eppendorf containers at -30°C.

5.9 Discussion

Traditional magnetic separation systems use large magnetic particles which allow bound cells to be separated using a simple permanent magnet. Large particles, however, interfere with cell viability and may cause activation of cells by cross-linking of cell-surface receptors[278]. Such techniques are usually only used for depletion of cell populations. The use of magnetic

microparticles (50-100nm) has a number of theoretical advantages; the binding reaction is faster, cross-linking of cell-surface receptors is avoided, optical parameters are not affected allowing further analysis by flow cytometry and cell viability should be unaffected[278]. The major disadvantage is the lesser magnetic moment associated with such particles necessitating longer separation times in conventional magnetic fields. The cell separation method used in the present study was based on the use of 50-100nm superparamagnetic particles in a high gradient magnetic field as developed by Miltenyi and co-workers at University of Cologne[278]. Monoclonal antibodies conjugated to these microbeads are available for a wide range of cell-surface markers and the methods have been applied to positive selection of NK-cells with no loss of viability or alteration of function [279].

Dead cells will take up antibody in a non-specific fashion and it was considered possible that the positive selection method might result in a large population of dead cells. However, the viability of the final cell population was always in excess of 95%. Every effort was made to minimise the trauma to cells during the separation process, including the avoidance of sudden temperature changes, in order to minimise the proportion of dead cells present.

The monocytes which were obtained using these methods retained their function with regard to cytokine secretion, and their ability to respond to an LPS stimulus was not impaired.

There were obvious differences between subjects in the proportion of the available monocytes which were successfully extracted using these methods. Monocytes are not a functionally homogenous group of cells and it is now known that smaller, immature monocytes secrete less cytokines in response to an appropriate stimulus than larger, mature cells[283]. Any tendency of this methodology to select one type of monocyte over another

may therefore directly influence the results obtained. However, both functionally immature cells and mature cells are normally CD14 positive[283] and there is no apparent reason why the methodology used in the present study should select one type in favour to another. In contrast, selection of monocytes by cell size, as in countercurrent centrifugal elutriation and one-step density separation, may tend to select a functionally distinct group of cells. The results of the above experiment suggested that higher extraction rates occurred when a higher proportion of monocytes was present in the initial PBMC population. It was therefore assumed that the monocyte separation process removed a fraction of the initial monocyte population which was representative of the monocyte population as a whole and which was similar in nature between individual subjects.

5.10 Measurement of Cytokines

Two methods are available for the measurement of cytokines; bioassay and immunoassay.

5.10.1 Bioassays

Bioassays utilise a biological effect of the substance under test to measure the quantity of the substance present in the test medium. In the case of TNF, cytotoxicity towards the murine cell line L-929 has been widely used as the basis of a bioassay[284]. Although bioassays have the theoretical advantage that only bioactive cytokines are measured, there is considerable overlap in function between many cytokines and synergistic effects are also possible, such as is present between TNFα and IL-1[284]. Therefore, such an assay often lacks specificity for the cytokine under consideration.

5.10.2 Immunoassays

Immunoassays utilise highly specific monoclonal and polyclonal antibodies to cytokines in order to accurately quantify the amount of cytokine present in a test medium. These have the advantage over bioassays of rapidity and specificity for the cytokine under test. Biological activity of the test cytokine is, however, ignored[284]. Two types of immunoassay are available depending on whether quantification is achieved with radiolabelled antibody (radioimmunoassay, RIA) or by enzyme-linked antibody causing a quantifiable colour change in the presence of an appropriate substrate (enzyme-linked immunosorbent assay, ELISA).

All cytokines in the present study were measured by ELISA. IL-6, IL-1β and IL-8 were measured using commercially available kits. In the case of TNFα, an in-house ELISA was developed.

5.11 Development of TNFα ELISA

The principle of the ELISA technique is outlined below.

5.11.1 Principles

The substance to be tested, in this case a cytokine, is "captured" from the test medium by antibody which is coated on the surface of a plastic plate. The "capture antibody" is coated in sufficient density to allow binding of all of the antigen present. A second antibody is then added which is linked to an enzyme, usually phosphatase or peroxidase. It is essential that this antibody binds to a different epitope on the appropriate antigen. After the excess antibody is washed off, the remaining "bound" antibody can be quantified by adding a substrate solution which changes colour at a rate determined by the quantity of enzyme, and thus the quantity of the second antibody, present in the test well.

A TNFα ELISA was developed by modification of a technique described by McIntyre et al[190].

5.11.2 Antibodies used

A critical factor in the development of an ELISA system is the availability of two antibodies recognising distinct epitopes on the antigen molecule. For this reason, one monoclonal and one polyclonal antibody is often used, the polyclonal antibody hopefully recognising several epitopes on the antigen molecule. Paired monoclonal antibodies are also occasionally used. It was not possible to purchase ant-TNFα antibodies which had been tested in an ELISA system and several antibodies were tested before an optimal pairing was achieved.

As "capture" antibody, polyclonal rabbit anti-hTNFα (Genzyme Corporation, Cambridge, MA, USA, product code IP 300) was used.

As second antibody, monoclonal anti-hTNFα was used (Boeringher-Mannheim GmbH, Germany, Cole 195, Cat No. 1141325)

This was based on a double antibody sandwich technique[284] utilising polyclonal capture antibody and monoclonal anti-TNFα as secondary antibody. Bound secondary antibody was then quantified in two stages using firstly, biotinylated anti-mouse monoclonal antibody followed by streptavidin-conjugated alkaline phosphatase (both Boeringher Mannheim). Plates were read at 415nm and results calculated against a standard curve which was generated with serial dilutions of recombinant human TNFα (Genzyme).

5.11.3 Buffers

Carbonate Buffer

Na_2CO_3 1.59g

$NaHCO_3$ 2.93g

Dissolved in distilled, deionised water and made to 1litre. pH corrected to 9.6 with 4M HCl.

Wash Buffer

Phosphate buffered saline (Sigma) with 0.1%Tween

Alkaline Buffer

Diethanolamine 100g/l , pH10

Alkaline Substrate

Phosphatase substrate 2mg/ml

5.11.4 Methods

1. Immulon II assay plates were used. Preliminary experiments were carried out to determine the optimal concentrations of polyclonal and monoclonal antibodies. Dilutions of 1/1000 of each were found to be optimal.
Rabbit polyclonal anti-TNF (Genzyme) was used as capture antibody.
This was diluted in carbonate buffer at a dilution of 1/1000 with 50ul being added to each well.
2. The plates were incubated for 2 hours at 37^{o}C in a humidified box.
3. The plate contents were discarded and 100ul of PBS with 2.5%BSA added to each well following which the plates were incubated at 4^{o}C overnight. This step ensures blocking of non-specific binding sites in the microtitre wells and improves the specificity of the assay.
4. The plates were washed twice with PBS/Tween.

5. Serial dilutions of TNF standard (Genzyme) were added to each well with concentrations from 20 to 0.15ng/ml. The plates were incubated for 1h at 37°C.

7. The plates were washed twice in PBS/Tween.

8. Anti-hTNFα monoclonal antibody (Boeringher Mannheim) was then diluted , 1/1000, in PBS and 50ul aliquots added to the wells.

9. The plates were incubated for 1h at 37°C.

10. The plates were washed twice in PBS/Tween.

11. Biotinylated anti-mouse (Boeringher-Mannheim) was diluted 1/4000 and 50ul added to each well.

12. The plates were incubated for 1h at 37°C.

13. The plates were washed twice in PBS/Tween.

14. Streptavidin alkaline phosphatase (Boeringher-Mannheim) was diluted 1/4000 and 50ul added to each well.

15. The plates were incubated for 30min at 37°C.

16. The plates were washed twice using PBS/Tween.

17. 100ul of alkaline substrate (Sigma, 2mg/ml) in alkaline buffer was added to each well.

18. The absorbence was read at 415nm after 20min and thereafter until satisfactory readings were obtained.

5.11.5 Calculation of results

Standard curves were prepared with the data from the standard wells. The mean absorbence between the three triplicate wells was calculated and plotted against the concentration in ng/ml. The graph was linearised by logarithmic transformation of both X and Y axes and an equation calculated for this line (using Microsoft Cricket Graph). Unknown samples were then calculated by solution of this equation for X (concentration).

5.11.6 Reproducibility of results

Methods

Two plates were prepared with three concentrations of TNFα (0.15, 1.5 and 15ng/ml) each plated 15 times per plate. The plates were treated as above, standard curves calculated and results obtained to calculate within-plate variability.

Eight plates were prepared with single wells containing 0.15, 1.5 and 15ng/ml of TNFα. Plates were treated as above, standard curves calculated and results for these wells calculated against the standard curves in order to calculate between-plate variability.

Results

The results of these experiments are summarised in **tables 5.8, 5.9 and 5.10.**

Plate 1	Mean TNF	Standard deviation	% CV
0.15ng/ml	0.2	0.02	10
1.5ng/ml	1.34	0.13	9.7
15ng/ml	18.1	3.5	19.4

***Table 5.8** Within-plate variability*

Plate 2	Mean TNF	Standard deviation	% CV
0.15ng/ml	0.16	0.015	9.4
1.5ng/ml	1.4	0.19	13.6
15ng/ml	13.2	1.9	14.4

***Figure 5.9** Between-plates variability*

	Mean TNF	Standard deviation	% CV
0.15ng/ml	0.16	0.029	18
1.5ng/ml	1.27	0.212	16.5
15ng/ml	17.2	3.48	19.7

Table 5.10 Linearity of standard curve

Two typical standard curves are shown in figure 5.3. The curves were consistently straight between 0.5 and 10ng of TNF/ml.

5.12 IL-6 , IL-8 and IL-1β ELISA

Initial attempts were made to develop in-house ELISA assays for both IL-6 and IL-1β but no suitable antibody combination was found and these attempts were eventually abandoned. All assays of IL-6, IL-1β and IL-8 were carried out using commercially available ELISA kits (Amersham UK).

Methods

1. Samples and standards were added to each well of a pre-coated 96 well plate. The ELISA kit was designed for plasma samples and a preliminary test run indicated that the appropriate dilution for the monocyte culture supernatant samples was 1/100 in the case of IL-6 and IL-1 and 1/1000 in the case of IL-8.

2. The plates were then incubated for 2 hours at room temperature.

3. The plates were then washed three times following which peroxidase-conjugated polyclonal anti-IL-6 or IL-1β as appropriate was added to each well and plates incubated for 2h at room temperature..

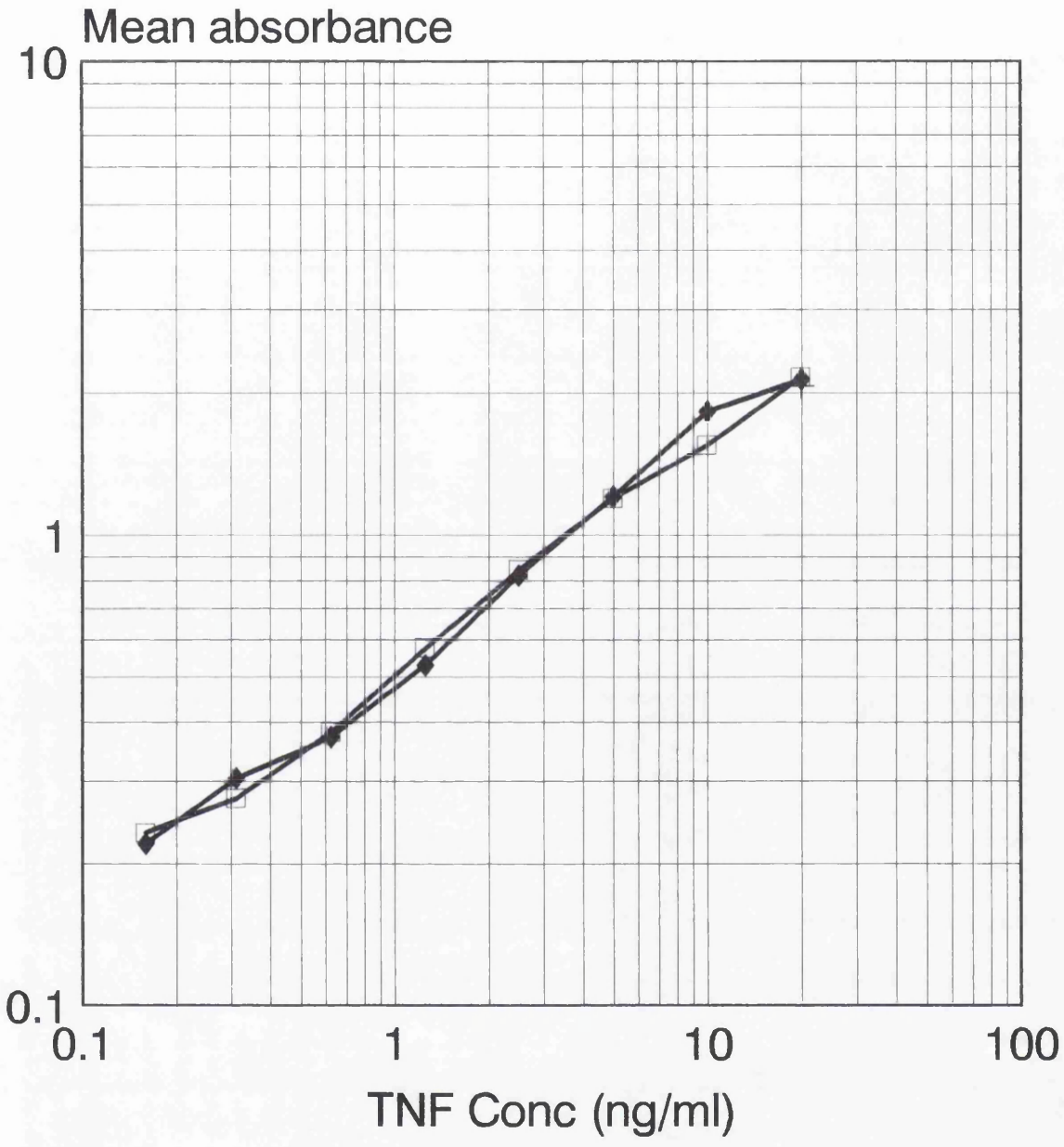


Figure 5.3 ; Typical standard curves

4. The plates were then washed three times and substrate solution (stabilised hydrogen peroxide with tetramethylbenzidine) added to each well and the plates incubated for 20min at room temperature. Stop solution (1M sulphuric acid) was then added to each well and the plates read on a spectrophotometer at a wavelength of 450nm.

5. Standard curves were then obtained after logarithmic conversion of the optical density readings and standard concentrations. Regression equations were then calculated for the linear standard curve and this was used to calculate the concentration of IL-6, IL-8 and IL-1β in the samples.

Reproducibility

IL-6.

The IL-6 assay had a sensitivity of 0.35pg/ml. The within assay variation was between 2.7%CV to 4.2%CV. Between plates variation was between 2.4%CV and 7%CV depending on sample concentration.

IL-1β

The IL-1β assay had a sensitivity of 0.3pg/ml. The within assay variability was between 2.3 and 3.1%CV. The between plates variability was 3.4%CV to 7.2%CV depending on sample concentration.

IL-8

The IL-8 assay had a sensitivity of 3.3pg/ml for cell culture samples. The within assay variability was <10% and the between assays variability was also <10%.

Chapter 6

Study of peripheral blood monocyte activation in patients with acute pancreatitis.

6.1 Introduction

Using the methods developed above, a study was undertaken to examine the role of mononuclear phagocyte activation in the pathophysiology of the systemic illness in acute pancreatitis. The aims of this study were twofold.

6.2 Aims

1. To assess the degree of activation of peripheral blood mononuclear cells in patients with acute pancreatitis in relation to the development of systemic complications.

2. To determine if the administration of octreotide to patients with acute pancreatitis was associated with a reduction in monocyte activation, as a results of its putative effect on reticuloendothelial function.

6.3 Patients

Twenty-eight sequential patients entered into the octreotide trial described in chapter 3 were studied (patient no.30-58). Patient 44 was not studied as she died before blood was obtained.

6.4 Methods

It was intended that blood should be taken on the first, third and fifth days after admission for the study of monocyte function. Where possible this was carried out but in patients who died early in the course of the illness this was obviously not possible. In other instances, insufficient monocytes were obtained to enable monocyte cultures to be established and complete

data for all three proposed days of study were therefore not obtained for every patient.

The first blood sample was usually taken on the morning after the trial drug infusion was commenced. 30ml of venous blood was withdrawn and placed in a 50ml centrifuge tube containing 3.5ml of 2.4%EDTA in PBS. These tubes were prepared in batches under sterile conditions by myself. The blood was then immediately taken to the laboratory at room temperature and monocyte separation carried out using the methods described above.

Monocytes were suspended in AIM V medium at a final cell density of $5x10^5$ cells/ml and cultured for 24h at 37°C in a CO_2-enriched, humidified incubator. The culture supernatants were then removed using a pasteur pipette and clarified by centrifugation. The supernatants were then transferred to sterile Eppendorf containers using a pasteur pipette and labelled appropriately. The Eppendorf containers were then stored in racks at -30°C.

TNFα, IL-6, IL-1β and IL-8 were measured by ELISA using the methods described above. All results were expressed in ng/ml.

Results were compared on each day of study and, in addition, peak levels were compared between patients. Statistical analysis was by Mann-Whitney U test for non-parametric data.

The systemic complications which were recorded were as follows.

Respiratory insufficiency; arterial PO_2 < 8kPa requiring either prolonged facemask oxygen or assisted ventilation.

Renal insufficiency; serum creatinine > 180uml/l despite fluid resuscitation.

Shock; systolic blood pressure < 100mmHg.

Pleural effusion; confirmed radiologically.

6.5 Results

Of the 28 patients studied 14 were male and 14 female with a mean age of 58 (range 25-92). Gallstones were identified as a cause of acute pancreatitis in 16. Of the remainder, nine gave a history of chronic alcohol excess and in three patients the aetiology of acute pancreatitis was not identified. The delay from onset of symptoms to first blood sample varied from 12 to 102 hours (median 36h).

Sixteen patients had evidence of systemic complications during the first 5 days of illness **(table 6.1)**. A further two patients had evidence of coexisting cholangitis, as defined by the presence of jaundice and fever, (patients 46 and 50) and, in view of the known effects of sepsis on cytokine production, these patients were excluded from further analysis. Both had an otherwise uncomplicated clinical course. The remaining ten patients had no evidence of systemic complications during the course of their stay.

Patient No	Complication	Death
31	RF	
33	MSOF	Day 2
34	ARF	
35	RF	
36	RF	
37	MSOF	Day 27
39	ARF	
40	RF	Day 20
42	RF	Day 4
43	RF	
48	RF	
51	RF	
52	RF	
55	RF	
56	RF	
58	PE	

Table 6.1: *Complications*

Those patients with systemic complications are described below and are identical to those with systemic complications described in chapter 2 with the exception of patient 34. For the purposes of the clinical trial, patient 34 was considered to have an uncomplicated course as all biochemical and clinical signs of systemic illness had settled within 24h of starting the trial infusion. However, at the time when the blood sample was taken for the study of monocyte function the patient had biochemical evidence of renal impairment and clinical evidence of a significant systemic illness.

Patient 31 was admitted to ITU within 24h of hospital admission due to deteriorating respiratory function. He was ventilated for 9 days during which time there was no deterioration in renal function and despite a persistent tachycardia, remained haemodynamically stable. He developed a pancreatic fluid collection which was defined on CT at day 16 but this settled with conservative management. There was no evidence of pancreatic necrosis.

Patient 33, like patient 31, was admitted to ITU soon after admission and required full ventilatory and circulatory support. He became anuric overnight and died on the following day of multiple organ failure.

Patient 34 was admitted almost 60h after the onset of abdominal pain and had evidence of renal impairment at admission. He was grossly acidotic with H^+ levels of 78mmol/l. He was treated with fluid resuscitation and his renal function improved rapidly. He took his own discharge on day 2 while suffering alcohol withdrawal symptoms.

Patient 35 had early respiratory impairment treated by facemask oxygen but was admitted to ITU with ARDS on day 7. He required assisted

ventilation for 5 days and subsequently made an uneventful recovery, being discharged on day 22.

Patient 36 had early renal and respiratory impairment. Her serum creatinine initially rose but returned to normal after 4 days. She had hypoxia requiring facemask oxygen for 5 days and subsequently developed an acute fluid collection which settled on conservative management.

Patient 37 had prognostically severe acute pancreatitis and initial hypoxia which required facemask oxygen for the duration of her hospital stay (27 days). She had clinical evidence of a right basal consolidation on day 12 and remained hypoxic. On day 23 she developed acute renal failure and underwent laparotomy and necrosectomy for infected pancreatic necrosis on day 27. She died several hours postoperatively from uncontrolled bleeding.

Patient 39 had a rising urea and creatinine from admission which was associated with high volumes of dilute urine. He also had hypoxia for 4 days after admission along with a sizeable pleural effusion and required facemask oxygen for 5 days. His renal impairment eventually settled without the need for haemodialysis.

Patient 40 was an elderly lady who required facemask oxygen for 3 days after admission for hypoxia. She initially made a good recovery but developed bronchopneumonia and died on day 21. Her family refused permission for a post-mortem.

Patient 42 had long-standing chronic obstructive airways disease and was severely hypoxic on admission. She was initially managed with facemask

oxygen but developed a cerebrovascular accident on day 3, dying on day 4. Once again, post-mortem was refused.

Patient 43 was hypoxic for 7 days after admission but was managed with facemask oxygen. She also developed an acute fluid collection and a large pleural effusion.

Patient 48 had a laparotomy because of diagnostic doubt and was found to have acute pancreatitis at operation. He developed respiratory insufficiency postoperatively but this had resolved by day 4. He subsequently made an uneventful recovery.

Patient 51 was hypoxic for 5 days and treated by prolonged facemask oxygen. His recovery, however, was otherwise rapid and he was discharged from hospital on day 7.

Patient 52 had a complicated course with early hypoxia and renal impairment. He developed Gray-Turner's sign on day 5 along with radiological evidence of a basal consolidation. He had a staphylococcus aureus septicaemia on day 4 and an acute fluid collection on ultrasound. He died on day 8 and at post-mortem was found to have necrotising pancreatitis with evidence of a recent myocardial infarction

Patient 55 had respiratory impairment from admission which necessitated care in the intensive care unit. She was managed on facemask oxygen initially but was ventilated on day 5 for a further 5 days. Her subsequent recovery was uneventful.

Patient 56 had respiratory impairment on days 1 and 2 and had facemask oxygen continued for 5 days. He also developed a pleural effusion but was discharged on day 14 with no further complications.

Patient 58 had respiratory insufficiency requiring 3 days of facemask oxygen along with a large pleural effusion and tachycardia. Subsequent recovery was uneventful and she was discharged on day 14.

TNFα

Complications

Peak cytokine secretion is shown in **figure 6.1**. Peak lipopolysaccharide-stimulated TNFα secretion by monocytes isolated from patients with systemic complications was higher (median=18.5ng/ml, IQR=5.5-28.5) than by those isolated from patients with an uncomplicated course (3.7ng/ml, 2.3-6.4 $P<0.01$). Highest levels of monocyte TNFα secretion were seen on days one and three after admission and were significantly higher in the complicated group (**figure 6.2**). There was a significant positive correlation between peak monocyte TNFα secretion and the degree of systemic illness as assessed by the maximum Acute Physiology Score (APS, component of the APACHE II score) during the five days of study ($r=0.658$, $P<0.001$) (**figure 6.3**).

IL-6

Peak IL-6 secretion by monocytes (**figure 6.1**) was significantly higher in patients who developed systemic complications (48.9ng/ml, 12.1-71) than in those with an uncomplicated course (16.3ng/ml, 14.2-37.9 $P<0.05$). Highest levels of monocyte IL-6 secretion were also seen on days one and three after admission and on these days were significantly higher in the complicated group (**figure 6.4**). There was a significant correlation

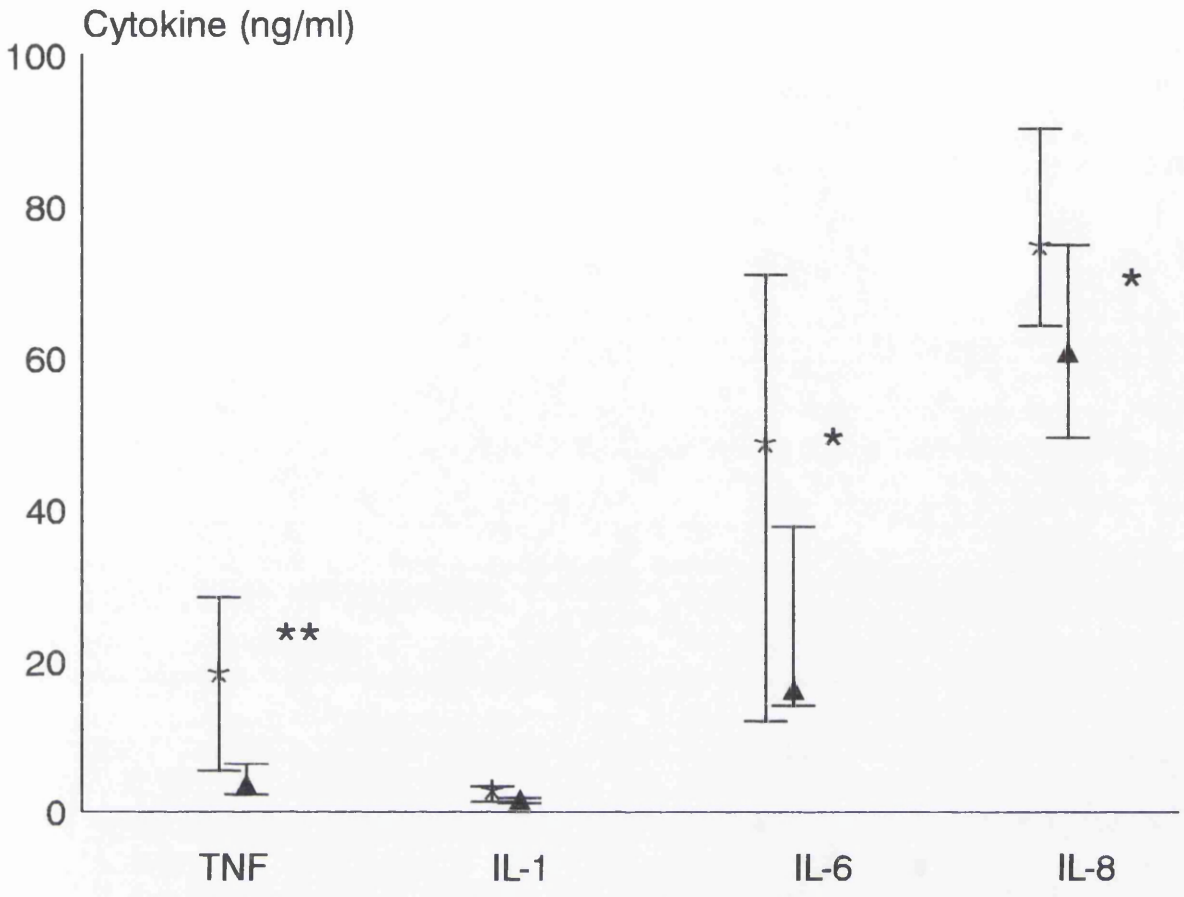


Figure 6.1;Peak cytokine secretion in complicated and uncomplicated groups.(IL-8 result 1/10 of actual result)

I Median/IQR

⋆ Complicated

▲ Uncomplicated

**P<0.01

*P<0.05

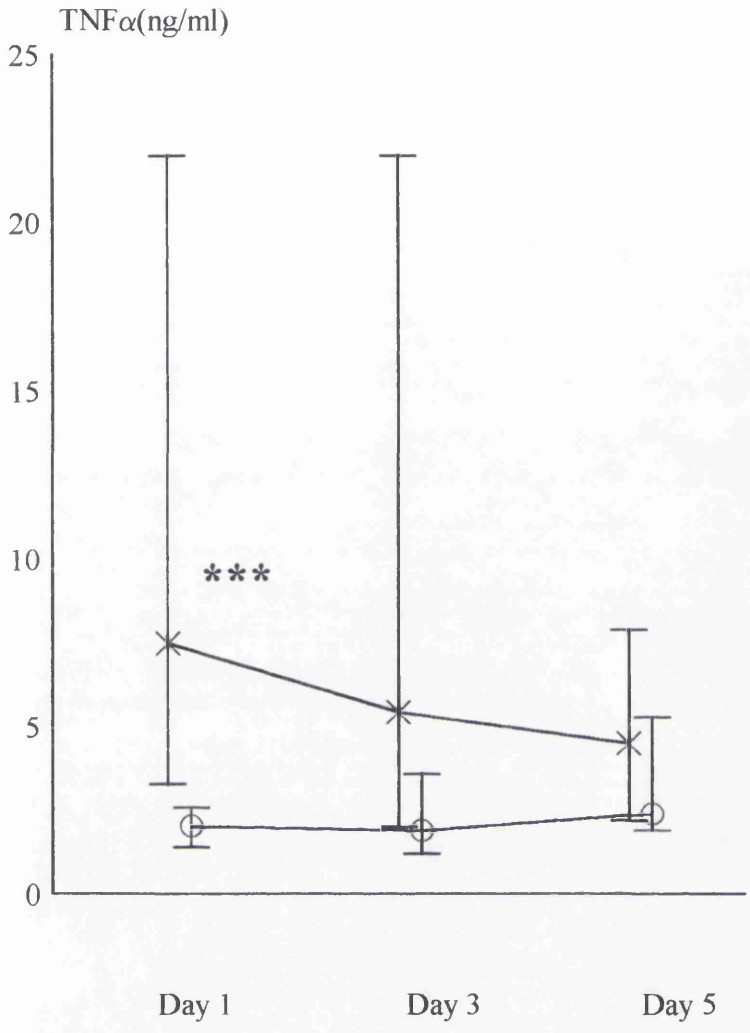


Figure 6.2 : TNF secretion by monocytes
****P<0.01*

⌶ *Median/IQR*
✳ *complicated*
⊖ *uncomplicated*

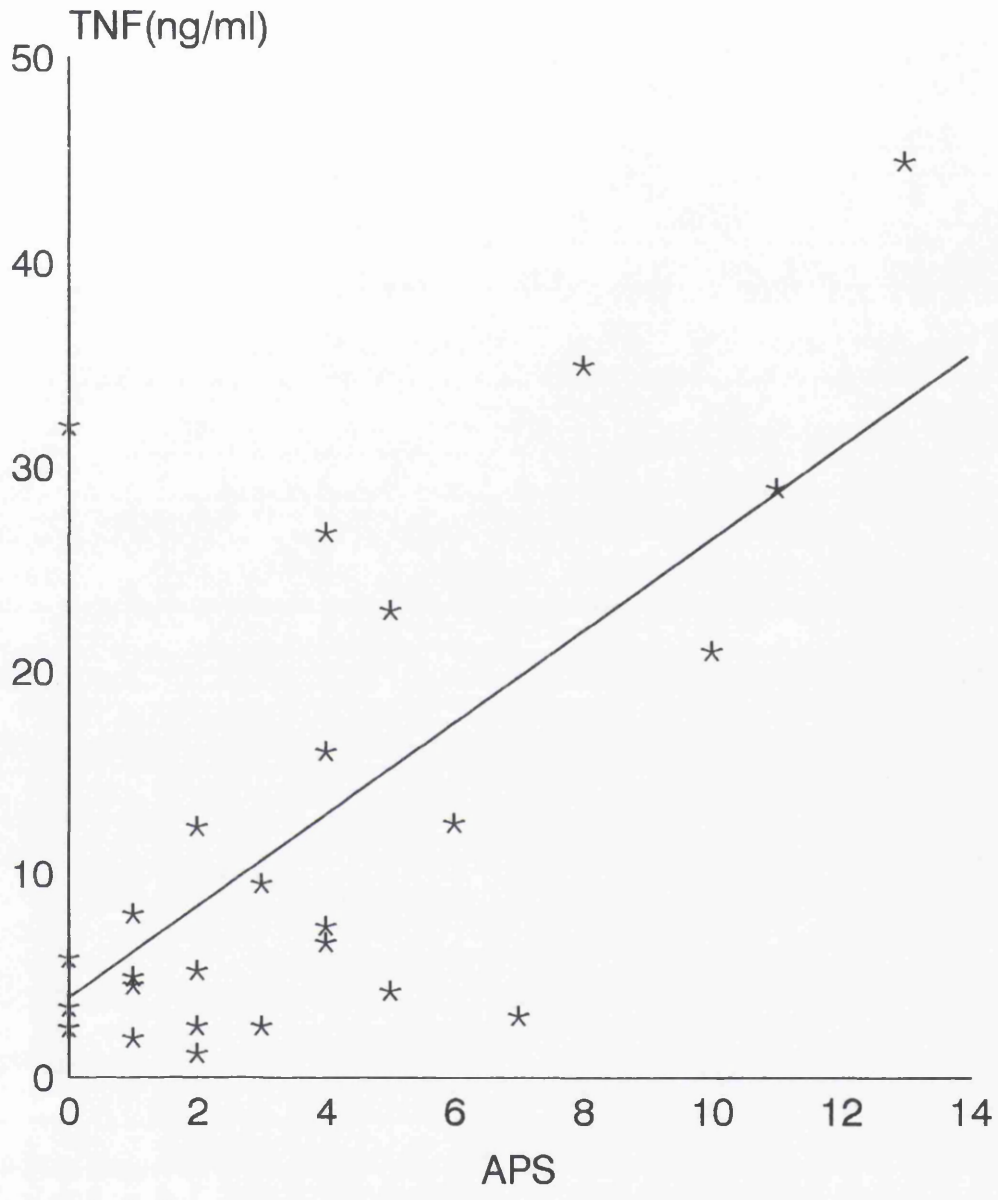


Figure 6.3: Peak TNF levels and peak APS. r=0.658, P<0.001

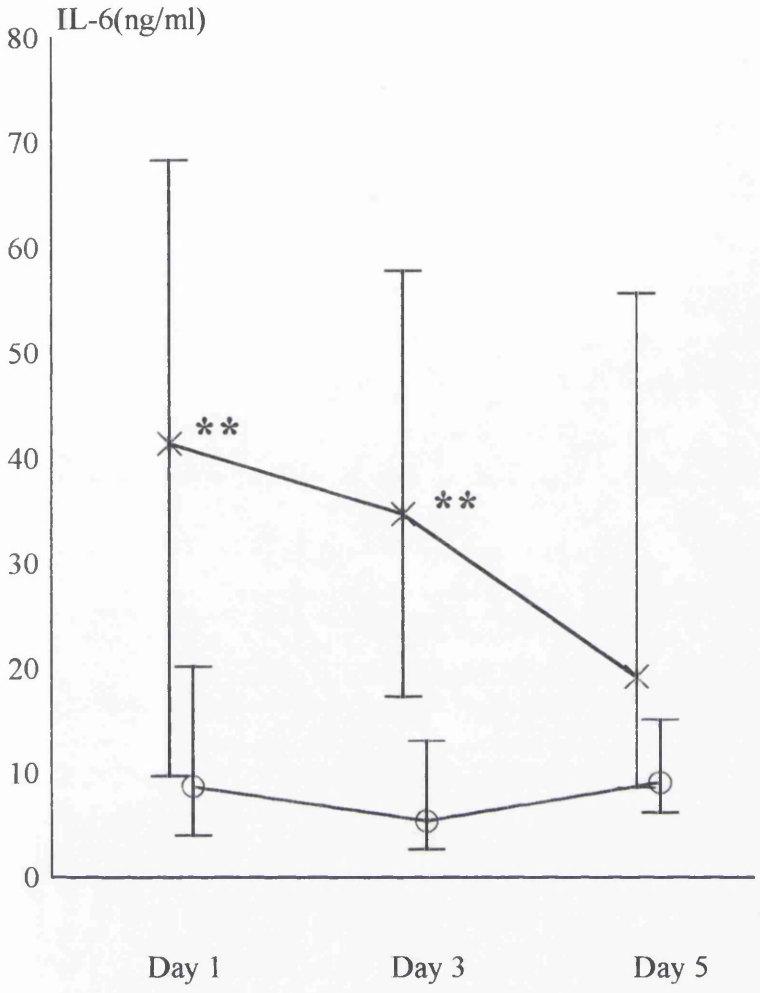


Figure 6.4: IL-6 secretion by monocytes
***P<0.02*

I *Median/IQR*
⋇ *complicated*
↔ *uncomplicated*

between peak monocyte IL-6 secretion and the peak APS ($r=0.587$, $P<0.01$) and between peak IL-6 and TNFα secretion ($r=0.678$, $P<0.001$).(**figures 6.5, 6.6**)

IL-8

Peak monocyte IL-8 secretion (**figure 6.1**) was significantly higher in the group of patients with systemic complications (748ng/ml, IQR 643-901), than in those with an uncomplicated course (608ng/ml, 496-749, $P<0.05$). Once again, highest levels of monocyte IL-8 secretion were observed on days one and three (**figure 6.7**) and there was a positive correlation between peak monocyte IL-8 secretion and the peak APS ($r=0.492$, $P<0.02$) (**figure 6.8**)

IL-1β

In contrast to the findings with TNFα, IL-8 and IL-6, systemic complications were not associated with significantly higher peak monocyte IL-1β secretion (complicated group 2.9ng/ml, 1.4-3.4; uncomplicated group 1.65ng/ml, 1.2-1.9 P=NS) although significantly higher levels of monocyte IL-1β secretion were observed on day five in the complicated group (**figure 6.9**). There was no significant correlation between peak monocyte IL-1β secretion and peak APS or between peak monocyte IL-1β secretion and peak monocyte TNFα or IL-6 secretion.

Influence of aetiology and therapy

Of the 16 patients who developed systemic complications, 8 had evidence of gallstones. On each day of study, there was no significant difference between levels of secretion of the 4 cytokines in patients with proven gallstones and patients with acute pancreatitis of other aetiologies (**figures 610-6.13**). There was no significant difference in peak cytokine secretion

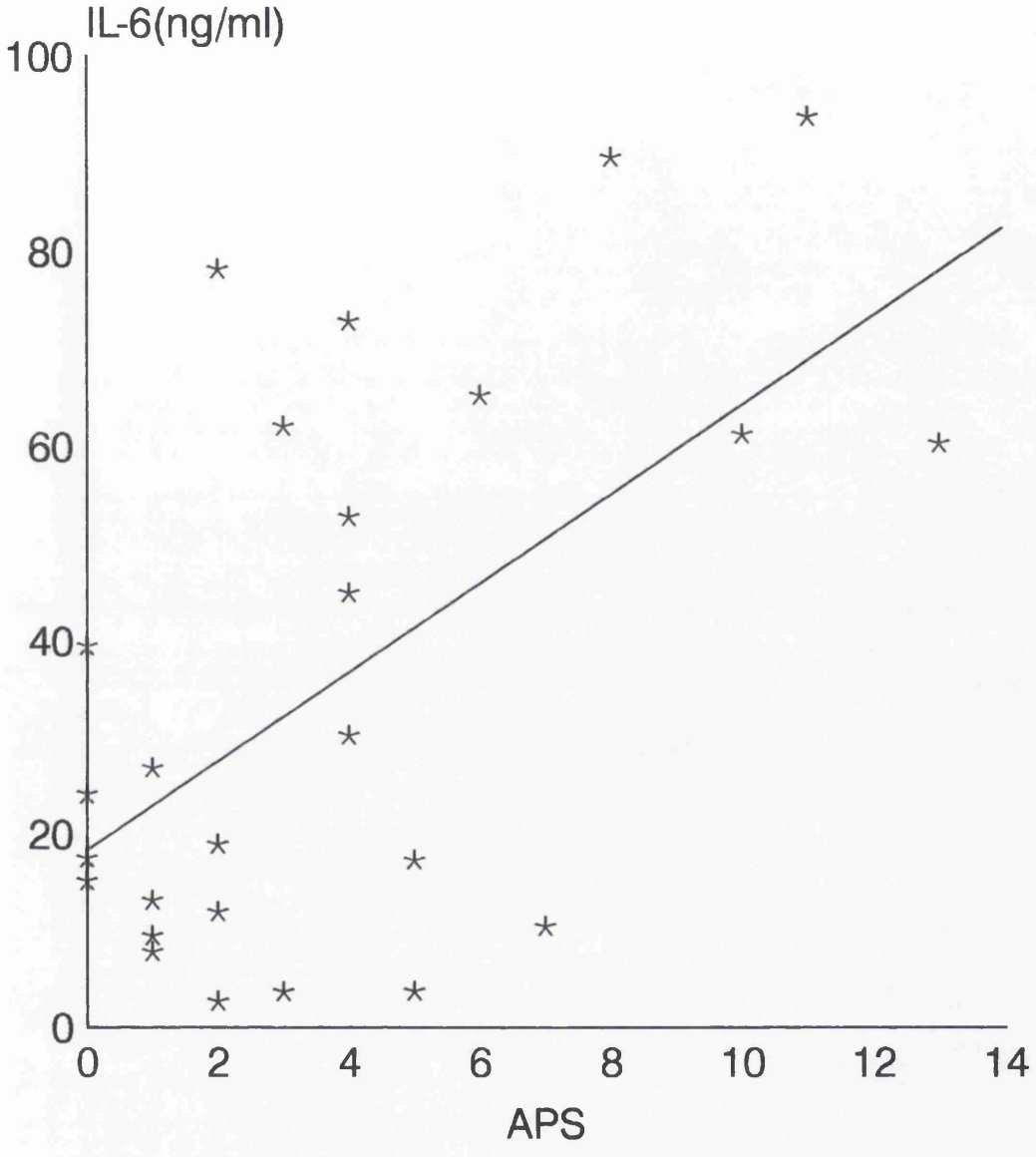


Figure 6.5: Peak IL-6 secretion and APS. r=0.587, P<0.01

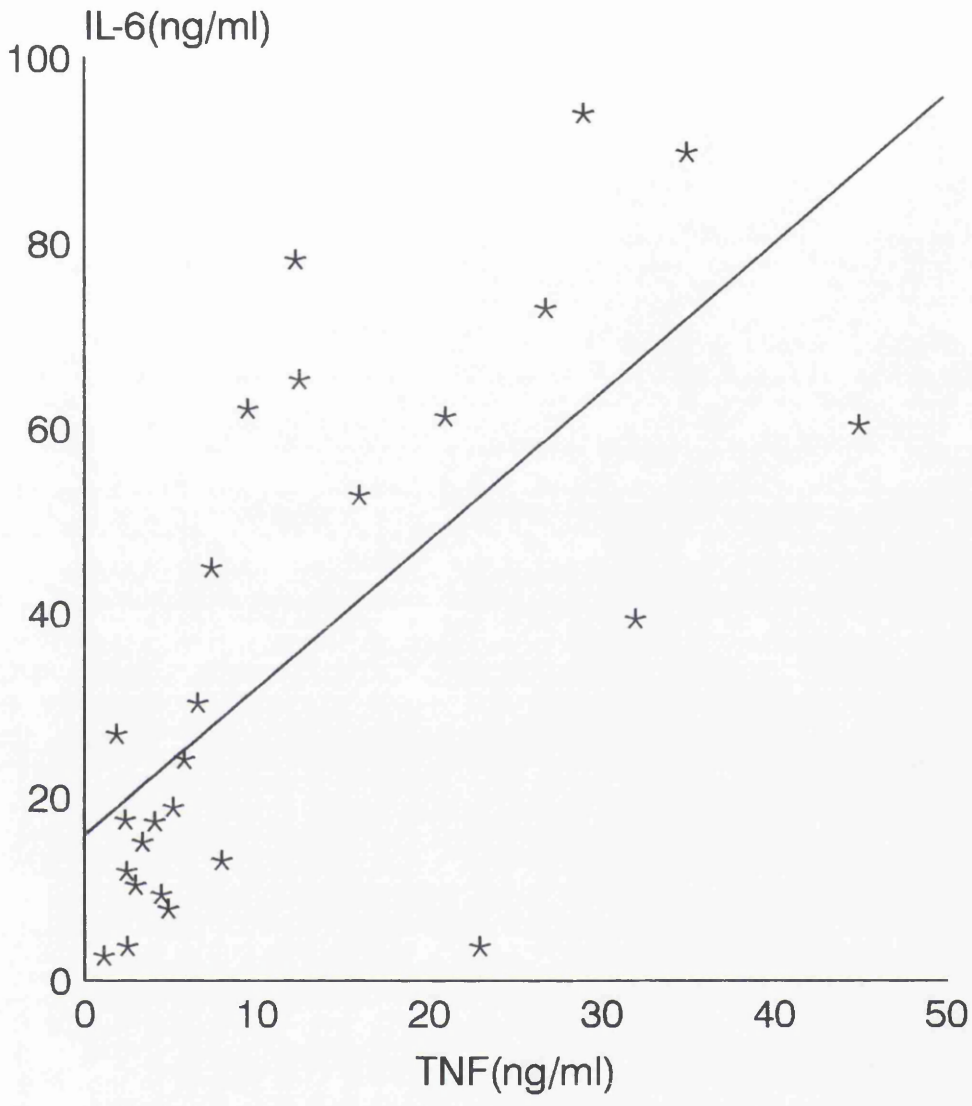


Figure 6.6: Peak IL-6 and peak TNF secretion. r=0.678, P<0.001.

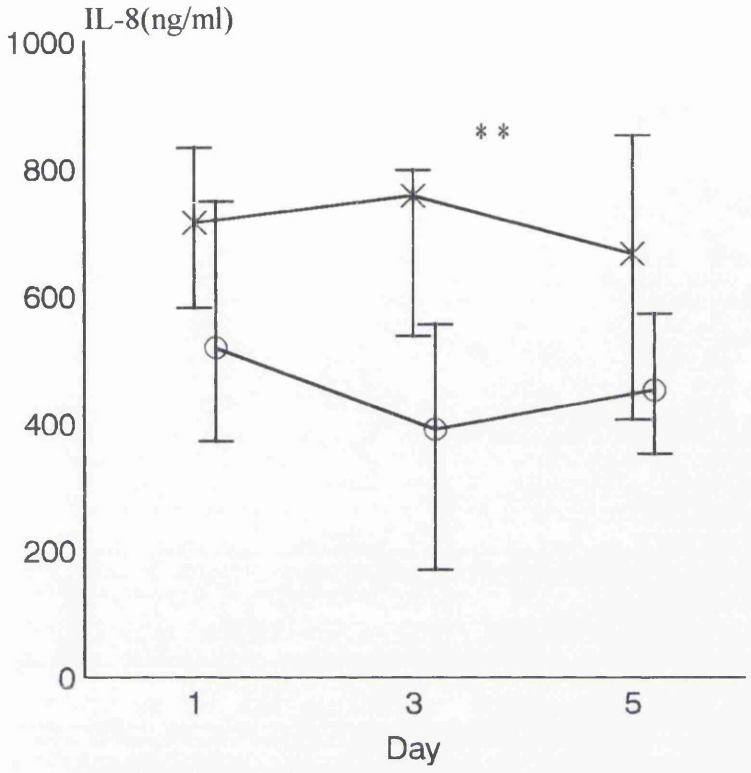


Figure 6.7: IL-8 secretion by monocytes
***P<0.05*

Median/IQR

complicated

uncomplicated

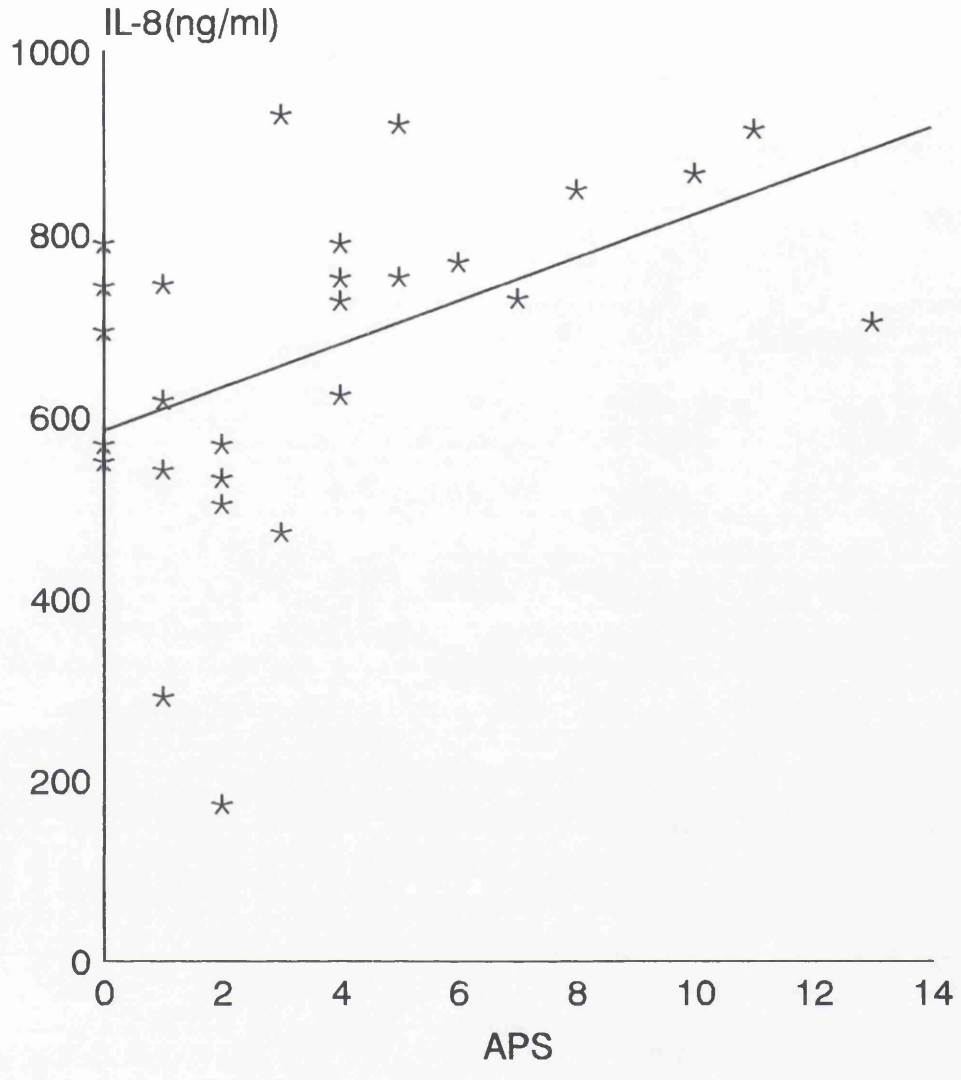


Figure 6.8: Peak IL8 levels and peak APS. r=0.492, P<0.02

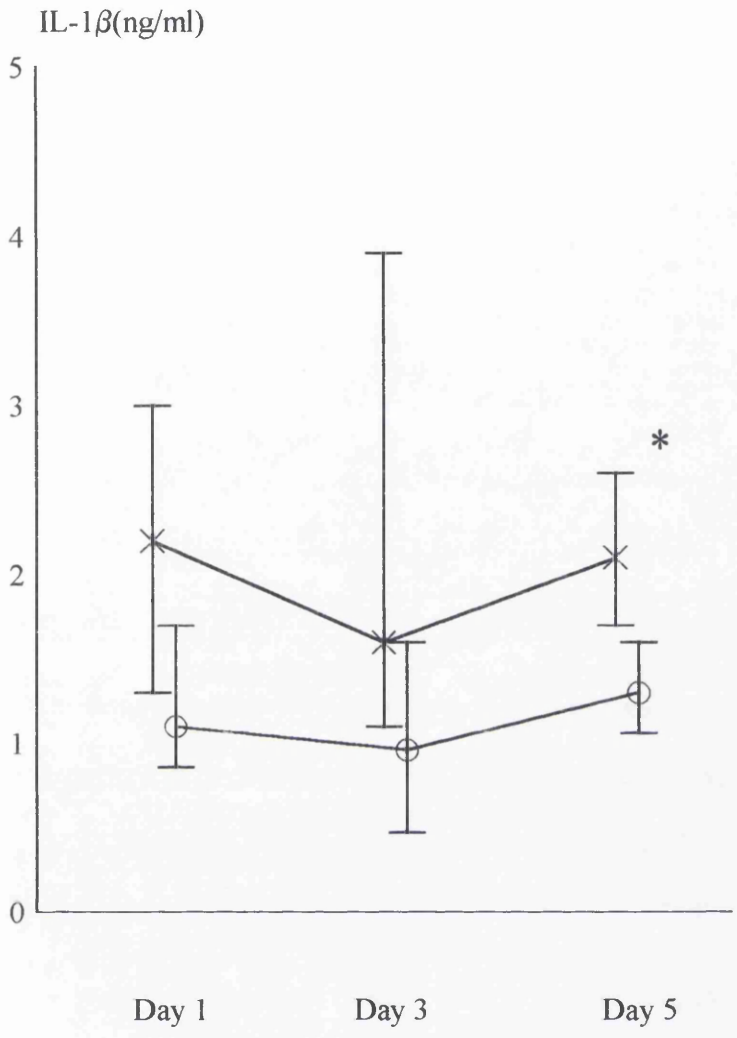


Figure 6.9: IL-1 secretion by monocytes
**P<0.05*

I *Median/IQR*

⊗ *complicated*

⊖ *uncomplicated*

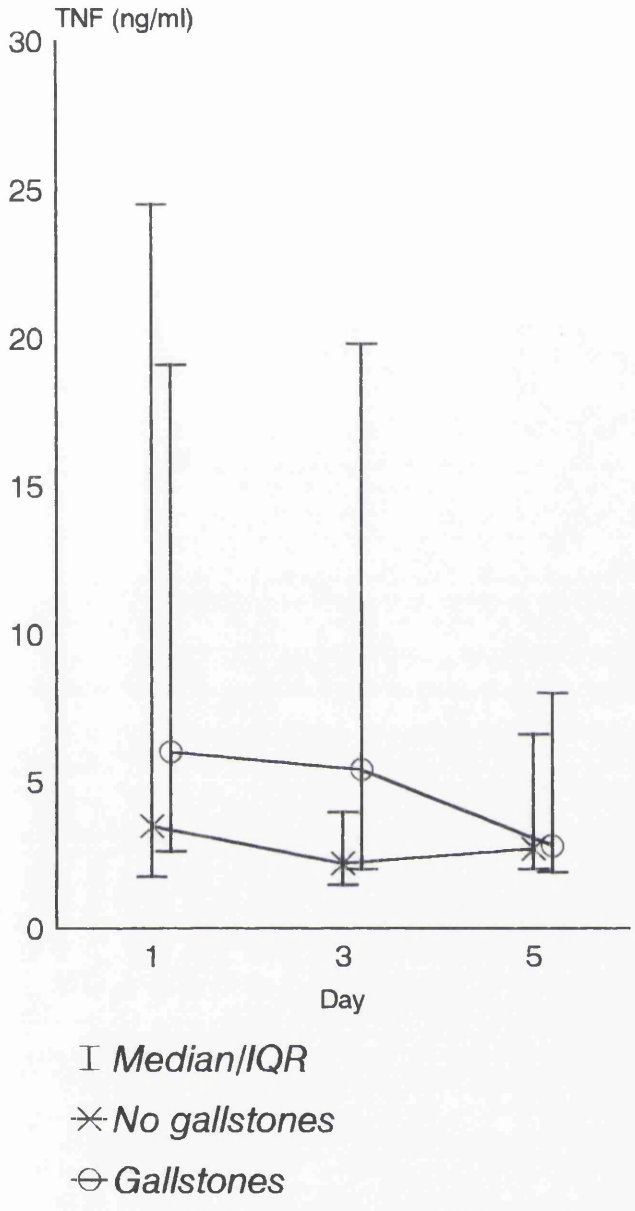
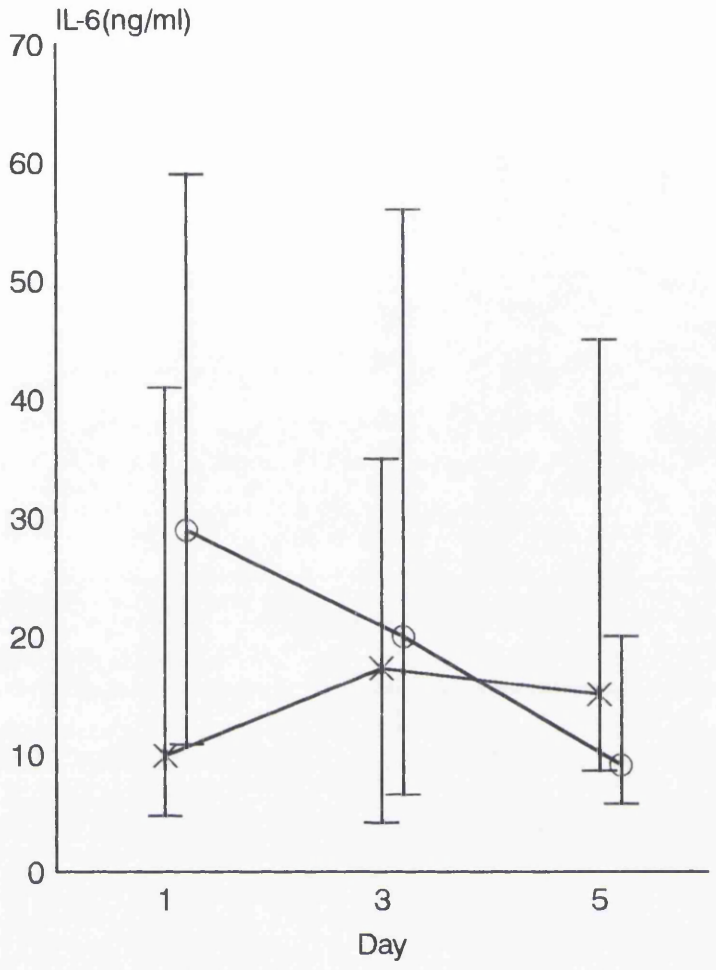


Figure 6.10; TNF secretion: effect of aetiology



Median/IQR

No gallstones

Gallstones

Figure 6.11; IL-6 secretion: effect of aetiology

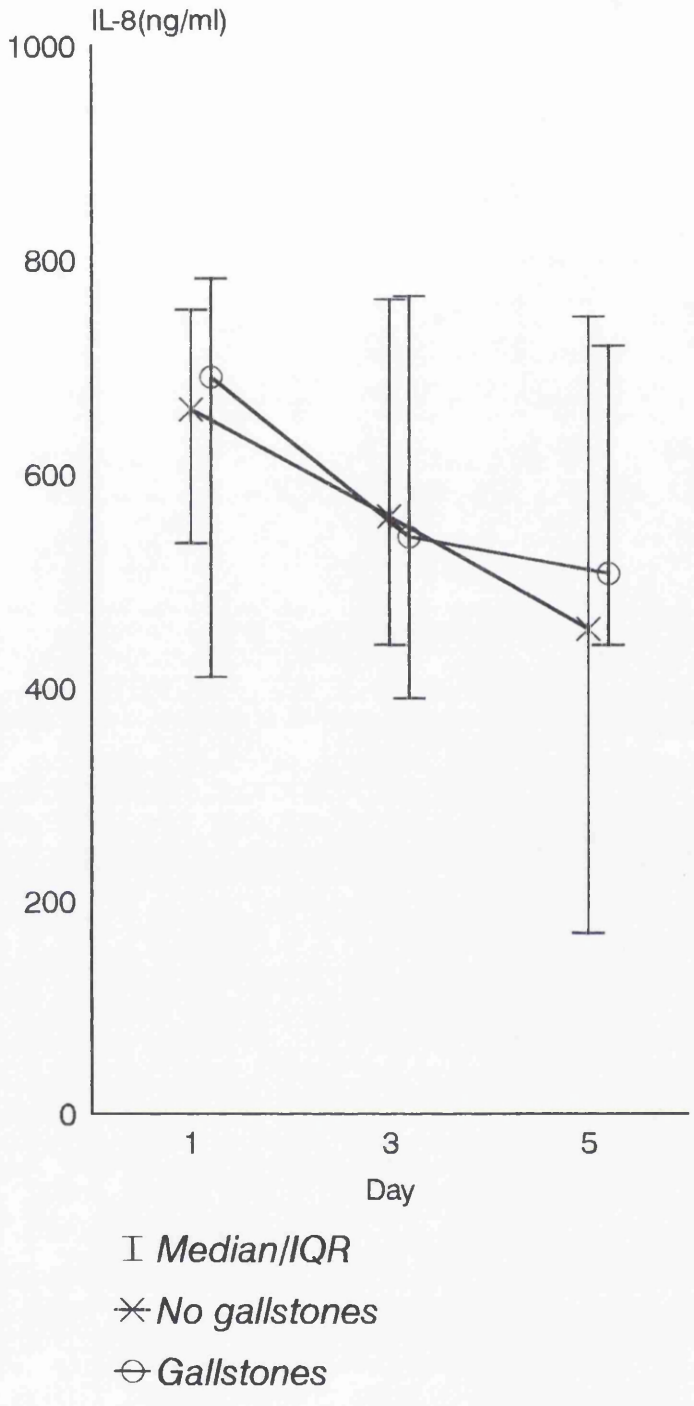
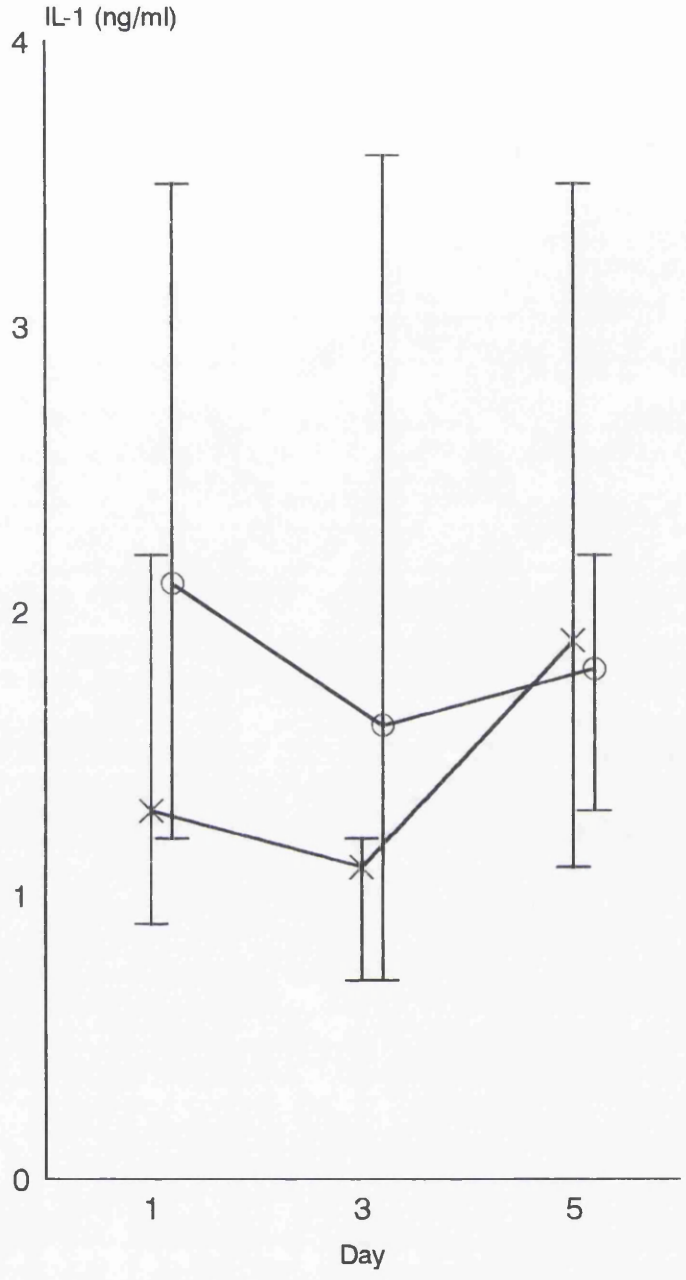


Figure 6.12; IL-8 secretion: effect of aetiology



I *Median/IQR*

⊹ *No gallstones*

⊖ *Gallstones*

Figure 6.13; IL-1 secretion: effect of aetiology

between those with gallstone related acute pancreatitis and those with acute pancreatitis of other aetiologies (**figure 6.14**): (peak TNFα, no gallstones median 5.95ng/ml, IQR 3.1-20.4, gallstones 8.75ng/ml, 5-26.4; peak IL-1β, no gallstones median 1.45ng/ml, IQR 1.1-1.3, gallstones 2.25ng/ml, 1.6-4.0; peak IL-6 no gallstones median 17.4ng/ml, IQR 9.7-60, gallstones 32.4ng/ml, 14.6-61; peak IL-8 no gallstones median 741ng/ml, IQR 582-766, gallstones 735ng/ml, 535-790)

Effect of Octreotide on Monocyte Activation

The possible effect of octreotide on cytokine secretion was examined. Ten of 14 patients in the octreotide group developed complications (71%) compared with six of 12 in the placebo group (50%). On each day of study, secretion of TNFα, IL-1β, IL6 and IL-8 was not significantly different in patients receiving placebo or octreotide (**figures 6.15-6.18**). There were no significant differences between peak levels of cytokine secretion between patients in the placebo and octreotide groups (**figure 6.19**): (peak TNFα secretion placebo group median 12.8ng/ml, IQR 2.5-28.1, octreotide group 6.6ng/ml, 3.94-14.6; IL-6 placebo group median 28.5ng/ml, IQR 14.5-55.1, octreotide group 20.7ng/ml, 11.6-62.3; IL-8 placebo group median 744ng/ml, IQR 594-804, octreotide group 716ng/ml, 549-774; IL-1β placebo group median 2.7ng/ml, IQR 1.4-4.2, octreotide group 1.8ng/ml, 1.3-2.9).

6.6 Discussion

Pro-inflammatory cytokines have been demonstrated to be elevated in plasma from patients with acute pancreatitis[38-40,192,285] but the source of these cytokines and their relevance to the pathophysiology of the disease process is unclear. This study has demonstrated for the first time that monocyte secretion of the pro-inflammatory cytokines, TNFα, IL-6 and

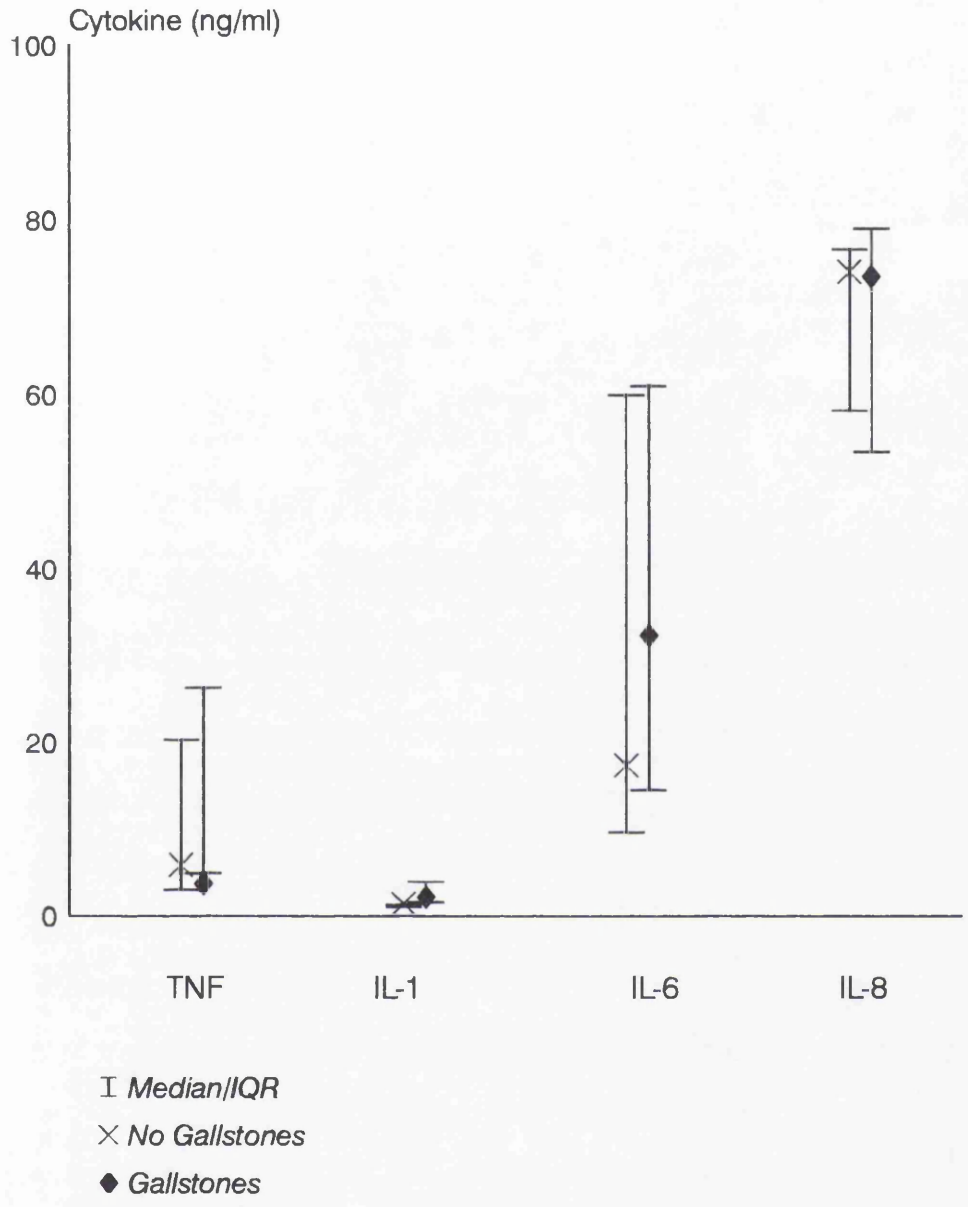


Figure 6.14; Effect of aetiology on cytokine secretion (result for IL-8 1/10 actual result)

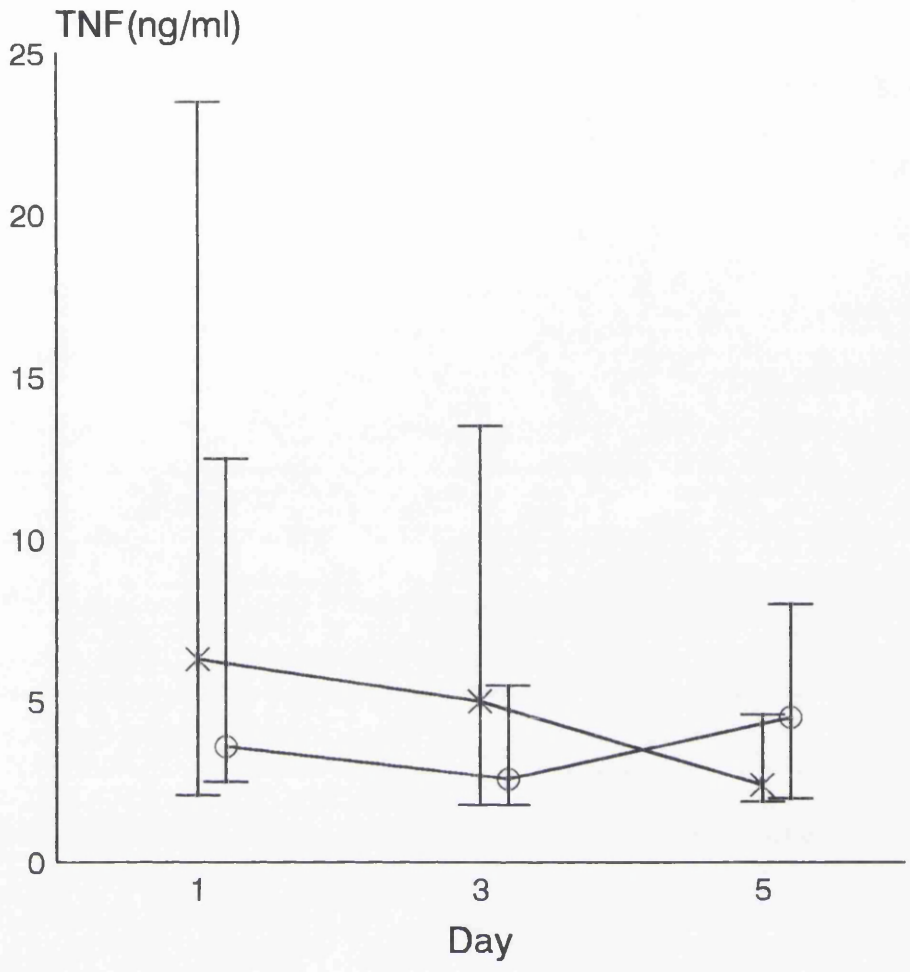


Figure 6.15; TNF secretion: effect of octreotide

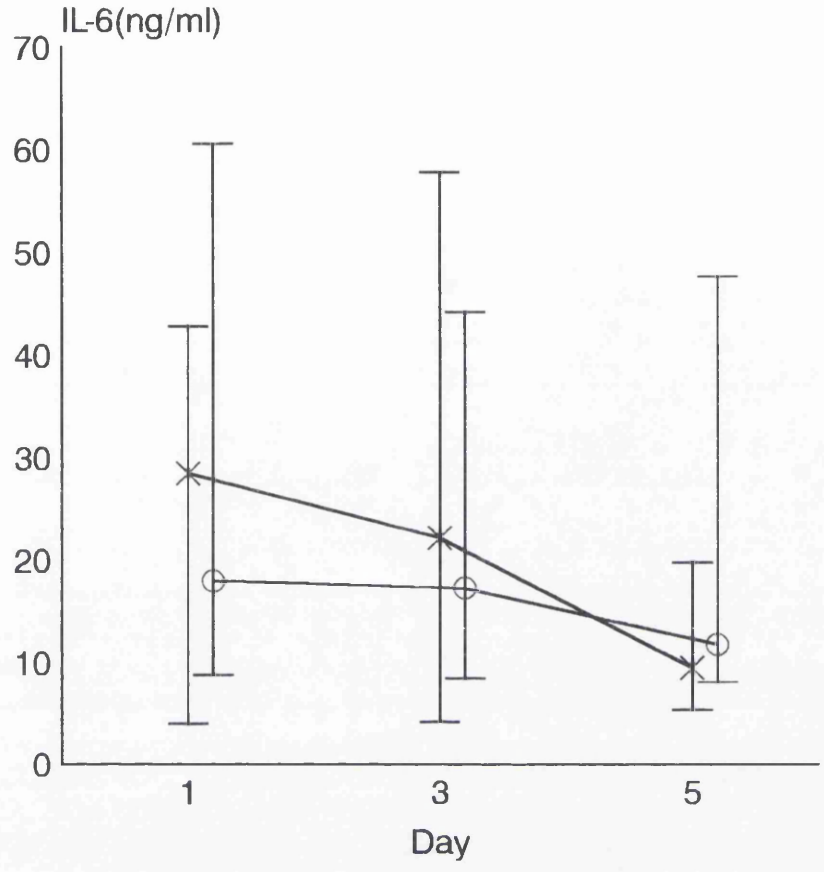


Figure 6.16; IL-6 secretion: effect of octreotide

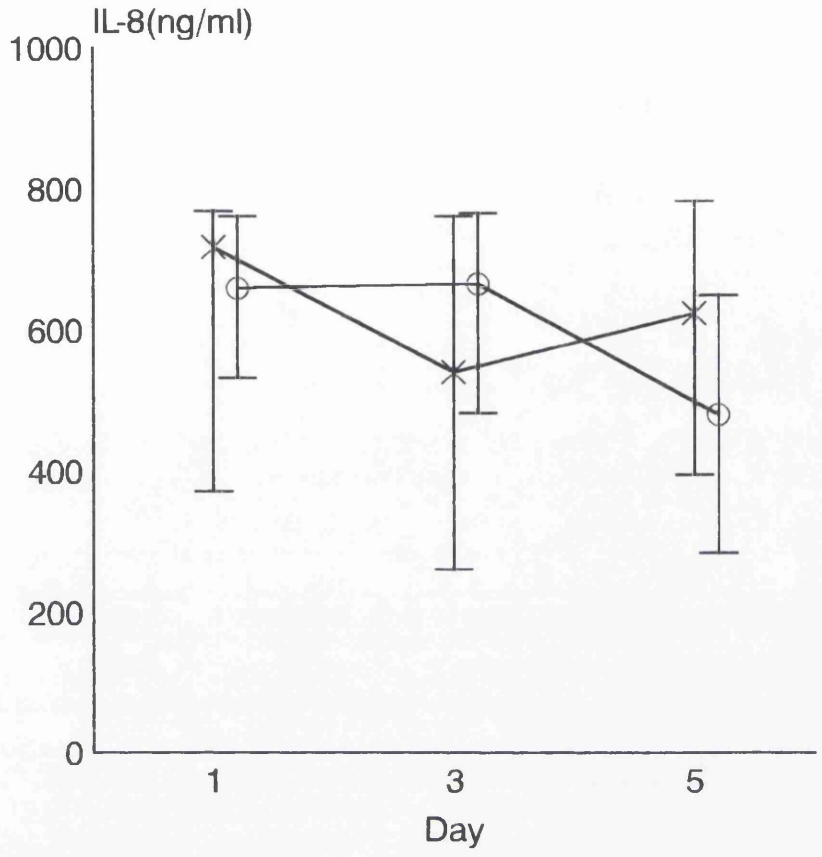
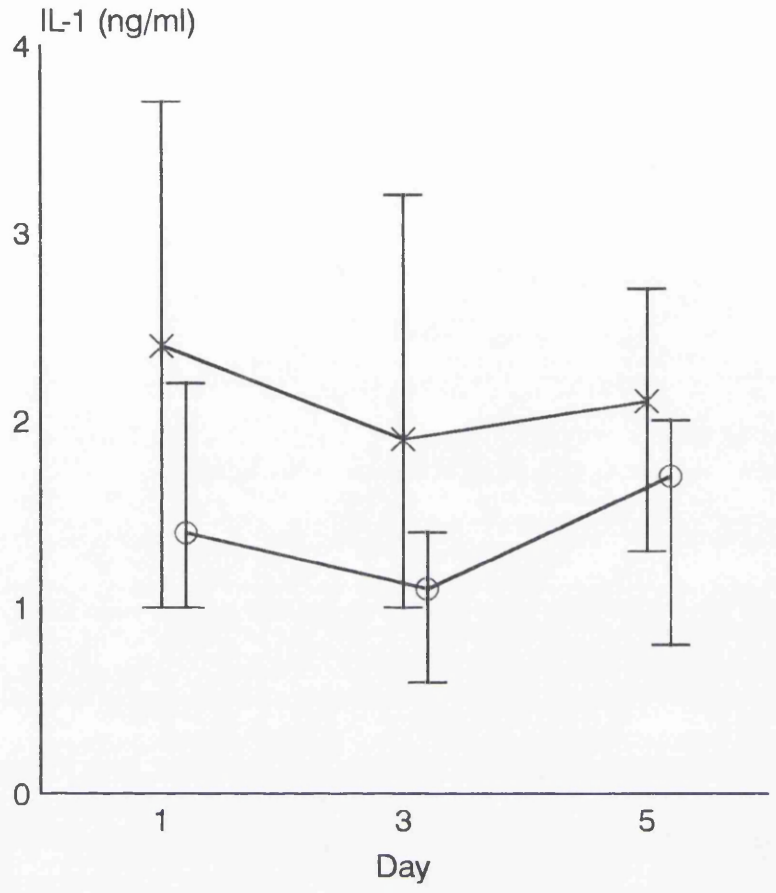


Figure 6.17; IL-8 secretion: effect of octreotide



I *Median/IQR*

⋇ *Placebo*

⊖ *Octreotide*

Figure 6.18; IL-1 secretion: effect of octreotide

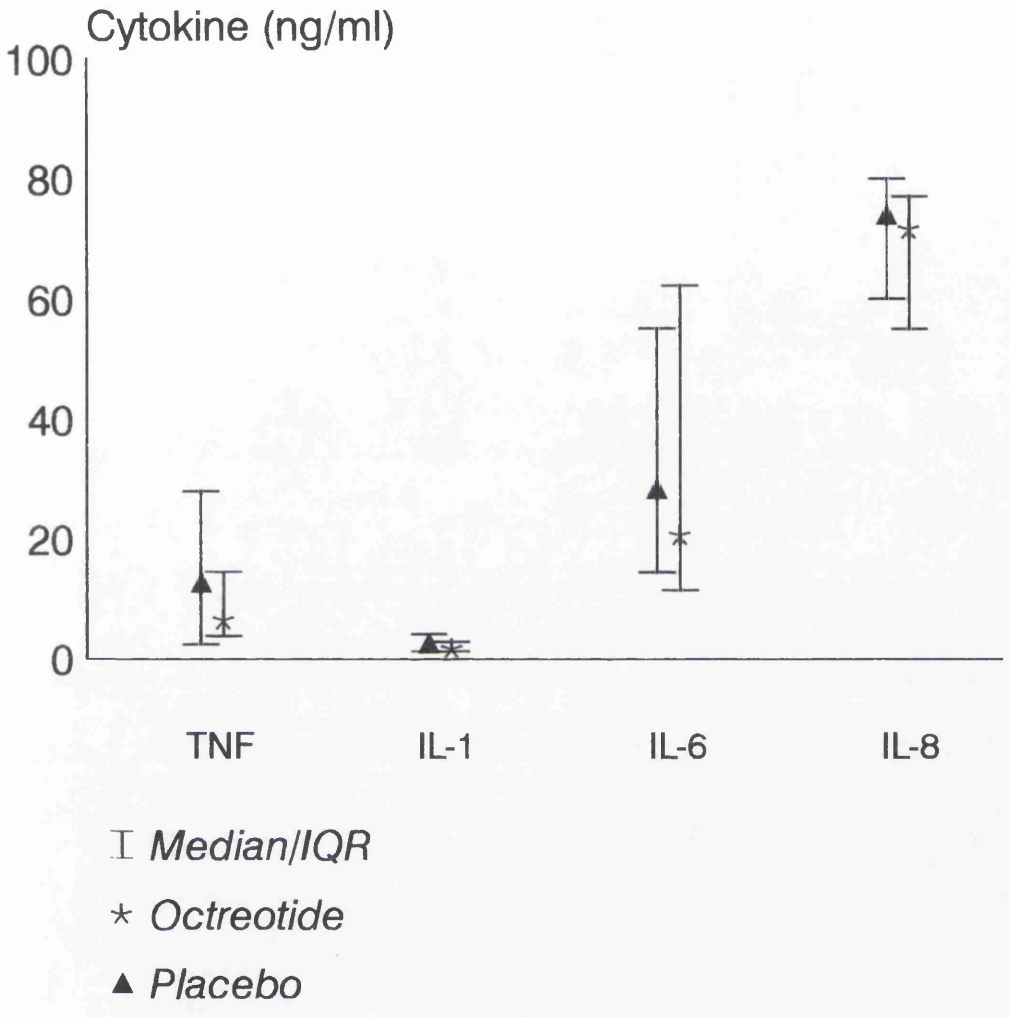


Figure 6.19; Effect of octreotide on peak cytokine secretion.(IL-8 result 1/10 of actual result)

IL-8 is increased in patients with systemic complications in acute pancreatitis. There was also a strong correlation between peak IL-6, IL-8 and TNFα secretion and the peak acute physiology score. These findings, coupled with the known physiological effects of TNFα, IL-8 and IL-6, strongly suggests that these cytokines are early mediators of the systemic illness in acute pancreatitis.

It is recognised from previous reports that raised plasma levels of IL-6 are present in patients with severe acute pancreatitis, with peak levels occurring within the first three days after admission[38-40]. In the present study, the highest levels of monocyte IL-6 secretion were observed during the first three days following admission, being similar to the time course of the peak in plasma levels observed by previous authors. This provides further evidence that IL-6 is involved in the mediation of the systemic illness associated with acute pancreatitis and furthermore, suggests that mononuclear phagocytes are a principal source of IL-6 in acute pancreatitis. The main target organ of IL-6 is the liver[182] and within the liver, it is exclusively taken up by parenchymal cells. After binding to the hepatocyte IL-6 receptor, IL-6 induces a shift in hepatocyte protein synthesis away from albumin and transferrin towards acute phase reactants such as C-reactive protein, fibrinogen and alpha-1-antitrypsin[182]. This response is generally thought to be protective to the host since IL-6 is not associated with the vascular damage seen with TNFα and IL-1β.

In acute pancreatitis, many of the circulating factors which have been shown to have prognostic significance may be indirectly linked to the actions of IL-6. Low serum albumin levels are commonly seen in acute pancreatitis with levels less than 32g/l being included in the multiple factor prognostic scoring systems of both Ranson et al and Imrie et al. Low serum albumin levels may be a consequence of loss of albumin into the peritoneal

cavity or extravascular space as a result of capillary leak but may also reflect the actions of IL-6 on hepatocyte function[182]. Low albumin levels are also seen in patients with sepsis where intraperitoneal albumin loss is less prominent.

C-reactive protein levels have been shown by several groups to have prognostic significance in patients with acute pancreatitis[37,39,41,286,287]. Peak levels of greater than 210mg/ml[41] or 150mg/ml[39] have been reported to indicate severe pancreatitis and this has been attributed to the associated rise in plasma IL-6 which has been shown to correlate with and precede the rise in serum CRP[39]. In the present study, there was only a weak correlation between peak CRP levels and peak IL-6 production (r=0.33). This finding may be a consequence of the fact that cells other than circulating monocytes are capable of secreting IL-6 in-vivo. Fibroblasts, endothelial cells and fixed tissue macrophages all produce IL-6[182] and may contribute more to the circulating pool of IL-6 than monocytes. In addition, Kupffer cells have been shown to be capable of the production of large quantities of IL-6 and these may be a main source of circulating IL-6 in acute pancreatitis[288]. Polymorphonuclear neutrophils have also been shown to express large quantities of IL-6 following experimental endotoxaemia[289]. However, the functional status of circulating monocytes would be expected to be indicative of the secretion of IL-6 by other cytokine-producing cells, although quantitatively other cells may play a more important role. A further factor influencing the IL-6 results may be that IL-6 production by monocytes is induced by TNFα rather than directly by endotoxin. In the in-vitro conditions of this study, IL-6 production may be simply a reflection of TNFα production in response to endotoxin and not of the functional status of monocytes in relation to IL-6 production. However, against this is the weak positive correlation between peak IL-6 secretion and TNFα secretion (r=0.672).

Previous studies on TNFα in the peripheral blood of patients with acute pancreatitis have met with inconclusive results[5,6]. Furthermore, by measuring plasma levels of TNFα, these studies take no account of possible transient rises in TNFα or of increased TNFα production at a cellular level. The presence of TNFα inhibitors and binding proteins further complicates the measurement of plasma TNFα and makes the interpretation of negative results difficult[189]. By studying monocyte TNFα secretion directly, the present study avoids these difficulties and provides a measure of the activation status of mononuclear phagocytes at each time point which it is assumed reflects the likely behaviour of these cells in-vivo. The present study provides the first conclusive evidence of a role for TNFα in the mediation of the systemic complications of acute pancreatitis. TNFα is produced by monocytes, fixed tissue macrophages and other cell types, the main stimulus to its production being endotoxin[141,210,290]. It is capable of reproducing the clinical and histological effects of sepsis-induced multiple organ failure and there is now good evidence that excessive or unregulated production of TNFα is pivotal to the development of systemic complications in sepsis[4]. There are many clinical and experimental similarities between the pattern of illness in septic patients and those with severe acute pancreatitis but whereas the role of TNFα and other cytokines is now generally accepted in sepsis, a similar role for TNFα in acute pancreatitis has not been established. The main reasons for this are the failure of studies in pancreatitis to reproduce the findings of similar studies in sepsis in demonstrating raised plasma levels of TNFα in association with the development of complications and the absence of studies examining TNFα in experimental pancreatitis. It is worth examining both of these facts in light of the results of work presented since the present study was commenced.

In the first study to examine TNFα in acute pancreatitis, Banks and colleagues from Leeds[5] studied 27 patients with a clinical and biochemical diagnosis of acute pancreatitis, taking daily venous blood samples during the first week of hospitalisation. Receptor-bound elastase was measured by ELISA and TNFα by radioimmunoassay. Eight of their patients were graded as severe of whom 4 died. The exact nature of the complications which occurred is not reported and in two of the severe group only local complications occurred.

Plasma levels of elastase were significantly higher in the patients graded as severe compared with the mild group although there was considerable overlap between groups and the highest level was in one patient who had an uncomplicated course. Levels of TNFα were variable and in many patients remained normal. The median TNFα levels in the severe group were higher than in the mild group but not significantly so. The authors concluded that neutrophil activation occurred in severe acute pancreatitis but that there was no evidence to suggest macrophage activation.

In a second study, Exley and co-workers from London and Leicester[6] studied 38 patients with prognostically severe acute pancreatitis (as defined by the presence of three or more positive Glasgow criteria). Venous blood was withdrawn on the first, third and seventh days after admission and serum TNFα measured by ELISA. In this selected group of patients there were 11 deaths and a further 3 patients with significant complications who survived. Day 1 serum TNFα was more frequently measurable in the non-survivors (45% v 23%) but of the patients who had no detectable TNFα at presentation, 4 developed multiple organ failure and one developed adult respiratory distress syndrome (ARDS) indicating a poor association between TNFα levels and the development of systemic complications. There was a stronger association between TNFα levels and complications in those with gallstone pancreatitis.

In both of these studies the authors have examined the association between TNFα levels and all complications whereas it would be more relevant to restrict analysis to those with systemic complications. Many patients have evidence of minor degrees of systemic organ involvement, particularly respiratory impairment, who have an otherwise uncomplicated outcome and may have been classified as such in both of these studies.

In another study, De Beaux and colleagues[291] measured TNFα and plasma levels of soluble TNFα receptors in patients with acute pancreatitis. TNFα itself was rarely present in plasma but increased levels of circulating receptors were seen in those patients with complicated attacks., suggesting that the levels of TNFα receptor may be a better indicator of TNFα release than measurement of TNFα itself.

The main criticism of these studies, however, is the use of plasma or serum TNFα levels as indicators of macrophage TNFα production. As has already been discussed, the short circulation half-life of TNFα and the unpredictable effect of TNFα-binding proteins on ELISA detection make conclusions based on such data difficult and it is certainly not possible to exclude a pathophysiological role for TNFα on the basis of such studies.

The second problem in assessing the role of TNFα in acute pancreatitis is the absence, until recently, of studies in experimental acute pancreatitis. The vast majority of the research which has led to our current understanding of the pathophysiology of sepsis has come from experimental models where TNFα infusions have been used to reproduce the effects of septic shock and where anti-TNFα antibodies have been shown to protect against the deleterious effects of endotoxin infusion. There is no model of acute pancreatitis which is universally accepted as being representative of the human disease with many causing death by rapid and overwhelming protease activation, a process which does not appear important in man. At a more practical level, most models involve

the use of rats or dogs where measurement of cytokines in these species is very much more difficult than in mice, for which antibodies and commercially available cytokine ELISA kits are available. Recently, however, plasma TNFα has been measured in a model of acute pancreatitis with some success. In a bile injection model in the rat, workers from Belfast have identified two peaks in TNFα plasma levels following the induction of acute pancreatitis[292]. The same group have recently demonstrated raised intraperitoneal TNFα in association with peritoneal endotoxin in the bile injection model (S Dolan, personal communication). Formela and colleagues in Liverpool have demonstrated apparent TNFα production by pancreatic acinar cells in a microvascular ischaemia model of acute pancreatitis[293]. It has been suggested, but is as yet unproven, that the early TNFα peak in experimental pancreatitis mat be the result of local, pancreatic TNFα production with a late peak corresponding to the activation of systemic monocytes and macrophages. This work is, however at an early stage and the results of ongoing studies are awaited.

No evidence was found of a rise in monocyte IL-1β secretion in association with systemic complications. It is interesting to note that reports on serum levels of IL-1β in human sepsis are variable and, unlike TNFα and IL-6, levels do not appear to correlate with mortality[161,181]. There are no published reports on plasma IL-1β levels in acute pancreatitis and the role, if any, of this cytokine in acute pancreatitis remains unclear.

IL-8 is a potent activator of neutrophils and may be the key cytokine involved in the neutrophil activation associated with acute lung injury[294]. In the present study, increased IL-8 secretion by monocytes was increased in association with the development of systemic complications and reports are now beginning to appear suggesting that serum IL-8 levels are increased in

patients with acute pancreatitis[192,285,295]. IL-8 is secreted not only by LPS-stimulated mononuclear phagocytes but also by other cells, particularly endothelial cells, after activation by TNFα[184]. In this way IL-8 is thought to be the principal secondary mediator of TNFα-induced neutrophil activation. Although monocyte IL-8 production is increased in association with the development of systemic complications, this may not accurately represent the in-vivo role of IL-8, the prime source of which may be endothelial cells and other cell groups activated by TNFα.

Four patients who developed severe systemic complications had persistently low levels of monocyte TNFα secretion. Three of these patients had alcohol-related acute pancreatitis and this raises the possibility that cytokine secretion is partly dependent on aetiology. Alcohol has a variety of effects on the immune system and has been shown to downregulate TNFα production by human monocytes[296]. However, raised levels of cytokine secretion were found in other patients with alcoholic pancreatitis and overall there was no difference in peak cytokine secretion in those patients with pancreatitis due to gallstones and those with pancreatitis of other aetiologies.

Another explanation for the findings in these three patients may have been delay in presentation, allowing the peak in TNFα secretion to be missed. However, the time from onset of symptoms to first blood sample in this group was similar to that in the remainder of the patients. In three of these patients, however, IL-8 secretion was high by the time of first blood sample. As IL-8 has been found to peak later than TNFα, this is suggestive that an earlier rise in TNFα may have been missed. In the remaining patient, secretion of all three cytokines was persistently low the reason for this being unclear.

Octreotide has been reported to stimulate reticuloendothelial function in-vivo and monocyte function in-vitro[7,238] therefore the possible influence of its administration to the patients in this study was examined. However, peak levels of cytokine secretion were similar in patients receiving octreotide with those receiving placebo and a similar systemic complication rate being observed in the two groups of patients. There was therefore no evidence in the present study that octreotide administration reduced the degree of monocyte activation in these patients.

The main stimulus to mononuclear phagocyte activation is bacterial endotoxin. For many years it has been known that patients with severe acute pancreatitis frequently have endotoxaemia[6,193-196] and that this is more commonly observed in patients with complicated disease. In acute pancreatitis, the combination of a reduction in splanchnic blood flow and local peritonitis may result in an increase in transluminal absorption of endotoxin with subsequent passage through the portal circulation. Impaired hepatic reticuloendothelial function may facilitate the passage of endotoxin into the systemic circulation. This sequence of events may explain the activation of circulating mononuclear phagocytes observed in this study.

A further consideration is the recent finding that monocyte cytokine production is not simply determined by the degree of cellular activation but that there are differences between individuals. Molvig et al[297] demonstrated that, within individuals, the secretion of TNFα and IL-1β were closely correlated and suggested that in HLA-DR2 positive individuals, monocyte responses to endotoxin are lower than in HLA-DR2 negative individuals. Further evidence of a role for HLA haplotype in the determination of monocyte responses to endotoxin came from Santamaria and colleagues[298] who confirmed the influence of HLA-DR2 haplotype on cytokine secretion and found that certain haplotypes in individuals heterozygous for the HLA-

DR2 haplotype could also be classified as low or high secretors of TNFα and IL-1β. The explanation for these findings probably lies in the location of the TNFα gene which is situated in the major histocombatibility complex on chromosome 6. Allelic polymorphisms within the TNFα gene have been identified[299] and the association between HLA class II haplotype and TNFα secretion may reflect linkage disequilibrium between the TNFα allele and the nearby class II locus. In this way TNFα secretion in response to endotoxin may be genetically determined and certain individuals may therefore be more at risk of developing the associated systemic complications.

In summary, increased monocyte release of the inflammatory cytokines, TNFα, IL-8 and IL-6 but not IL-1β, has been demonstrated in association with the development of systemic complications of acute pancreatitis. This provides evidence that the systemic effects of acute pancreatitis may be mediated by activation of mononuclear phagocytes and increased cytokine release which, if confirmed by further study, may lead to trials of new therapeutic agents designed to modulate this aberrant host response.

Chapter 7

Polymorphonuclear elastase in acute pancreatitis as a marker of neutrophil activation

7.1 Introduction

Many of the effects of the cytokines released from mononuclear phagocytes are mediated by neutrophils[300], which release free oxygen species and proteolytic enzymes causing endothelial damage[301,302]. Neutrophil aggregation and superoxide production have been demonstrated in experimental models of pancreatitis and antibodies to neutrophils attenuate the severity of lung injury in experimental pancreatitis[303,304]. Increased neutrophil superoxide production has also been demonstrated in patients with acute pancreatitis[305]. Polymorphonuclear elastase (PMNE) is an enzyme released by neutrophils and increased plasma levels of PMNE are though to reflect neutrophil activation. Raised levels of PMNE have been demonstrated in the plasma and bronchoalveolar lavage fluid of patients with septic shock[306] and also in the plasma of patients with acute pancreatitis[36,37,42].

In the preceding chapter, monocyte activation w as demonstrated in patients with acute pancreatitis. Increased production of pro-inflammatory cytokines by monocytes and mononuclear phagocytes would be expected to result in neutrophil activation and therefore raised plasma levels of PMNE. Measurement of PMNE in acute pancreatitis may therefore be a simple method of assessing the degree of leucocyte activation which would allow the early identification of patients at risk of developing systemic complications.

7.2 Aims

Levels of PMNE were compared with the degree of monocyte cytokine production in individual patients to assess the relationship between PMNE and the cytokine response. The potential of plasma PMNE as a prognostic marker in acute pancreatitis was also assessed.

7.3 Patients

Thirty seven patients entered into the randomised, controlled trial of octreotide described in chapter 4 had serial measurement of PMNE carried out. All patients were therefore pre-selected on the basis of an admission APACHE II score of greater than 5. The mean age of these patients was 68 (range 32-88) and gallstones were identified as an aetiological factor in 21 (57%).

7.4 Methods

Patients had venous blood withdrawn on days 1,3 and 5 after admission. Blood was transported to the laboratory on ice where it was spun in a refrigerated centrifuge and plasma immediately withdrawn with a pasteur pipette. Care was taken not to include cells at the interface layer which may contain neutrophils. The plasma was then frozen at -70^{o}C prior to measurement of PMNE by ELISA. PMNE was measured in one batch after slow thawing of the plasma samples.

Systemic complications which occurred in these patients were defined as in chapter 1:

Respiratory insufficiency; arterial PO_2<8kPa

Pleural effusion

Renal insufficiency; serum creatinine>180ug/l after fluid resuscitation

Shock; systolic BP<100mmHg.

MSOF; combination of any two of the above.

Polymorphonuclear Elastase Measurement

PMNE was measured using a commercially available ELISA (Merck).

The principle of this assay is as follows;

Antibody fragments (Fab') against human granulocyte elastase are covalently bound to horseradish peroxidase to form antibody-peroxidase conjugates. In these conjugates, the peroxidase activity is inhibited by the high local H_2O_2 concentrations in the substrate solution. In the presence of PMNE, immune aggregates form which allows peroxidase to catalyse the reaction;

H_2O_2 + phenol + 4-aminophenazone $\rightarrow$ quinonimine dye.

The quantity of quinonimine dye formed is proportional to the degree of peroxidase activity and thus to the quantity of PMNE present in the sample.

The assay was adapted for use in an auto analyser in the Department of Biochemistry, Glasgow Royal Infirmary.

Results were expressed in μg/l and statistical analysis was by the Mann Whitney U test.

7.5 Results

7.5.1 Relationship between PMNE levels and cytokine secretion

Highest levels of PMNE were observed on day 1 after admission. Day 1 monocyte cytokine production was therefore compared with PMNE levels in 11 patients with acute pancreatitis (patients 30-40).

There was a strong positive correlation between IL-1β secretion on the first day after admission and the plasma level of PMNE (**Figure 7.1**) ($r=0.961$, $P<0.001$). However, when the very high result for patient 37 was omitted, this was no longer statistically significant. There was no significant

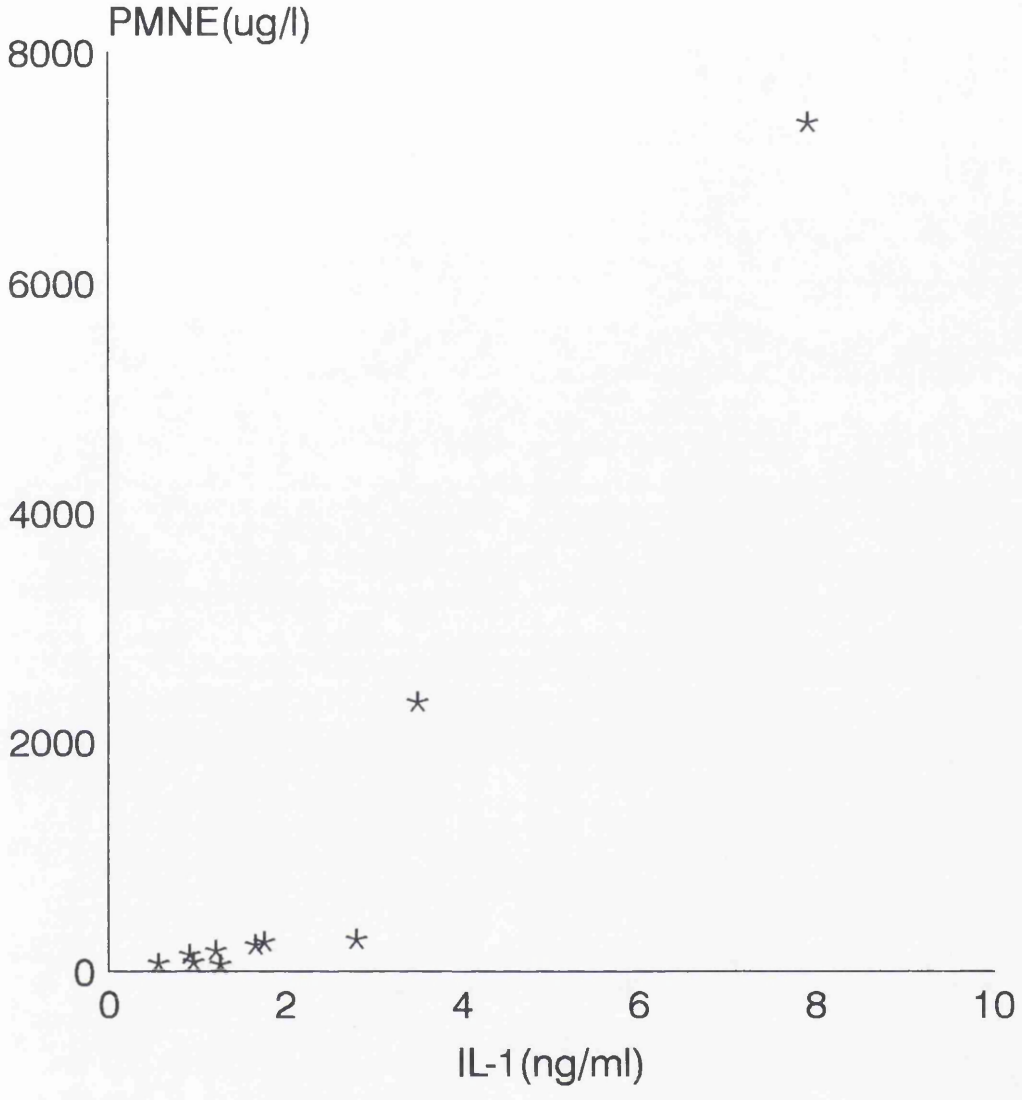


Figure 7.1: Relationship between IL-1 secretion and PMNE levels on day 1. r=0.961, P<0.001)

correlation between the secretion of TNFα (r=0.061), IL-6 (r=0.282) or IL-8 (r=0.288) and PMNE levels on day 1.

7.5.2 Neutrophil activation and systemic complications

Of the 37 patients studied, 15 developed systemic complications of whom 8 died as a result of their illness.

Plasma PMNE levels were significantly higher in those patients with systemic complications on each of the days on which it was measured (**Figure 7.2**)(day 1 PMNE complicated median=250μg/l, IQR 143-656, uncomplicated median 139, IQR 74.7-254, P<0.05; day 3 complicated median=224.1, IQR 139-382, uncomplicated median=86.1, IQR 51.4-159.7, P<0.002; day 5 complicated median=178.5, IQR 117. Highest levels of PMNE were observed on day 1 of the hospital admission in all but nine of the 37 cases and of these, seven subsequently developed systemic complications.

7.5.3 Use of PMNE as a prognostic indicator

For prognostic purposes, a cut-off level of PMNE on admission to hospital of 250μg/l has been suggested[42]. Using this cut-off, 6 of the 15 patients with complications were correctly predicted to have severe disease and only 3 of the patients with an uncomplicated outcome incorrectly assigned to the severe group. Five of the seven patients who died had PMNE levels of more than 300ug/l on admission. The sensitivity, specificity, positive and negative predictive values are given in **table 7.1** Lowering the cut-off was associated with an improvement in sensitivity but an associated reduction in specificity with an overall reduction in accuracy.

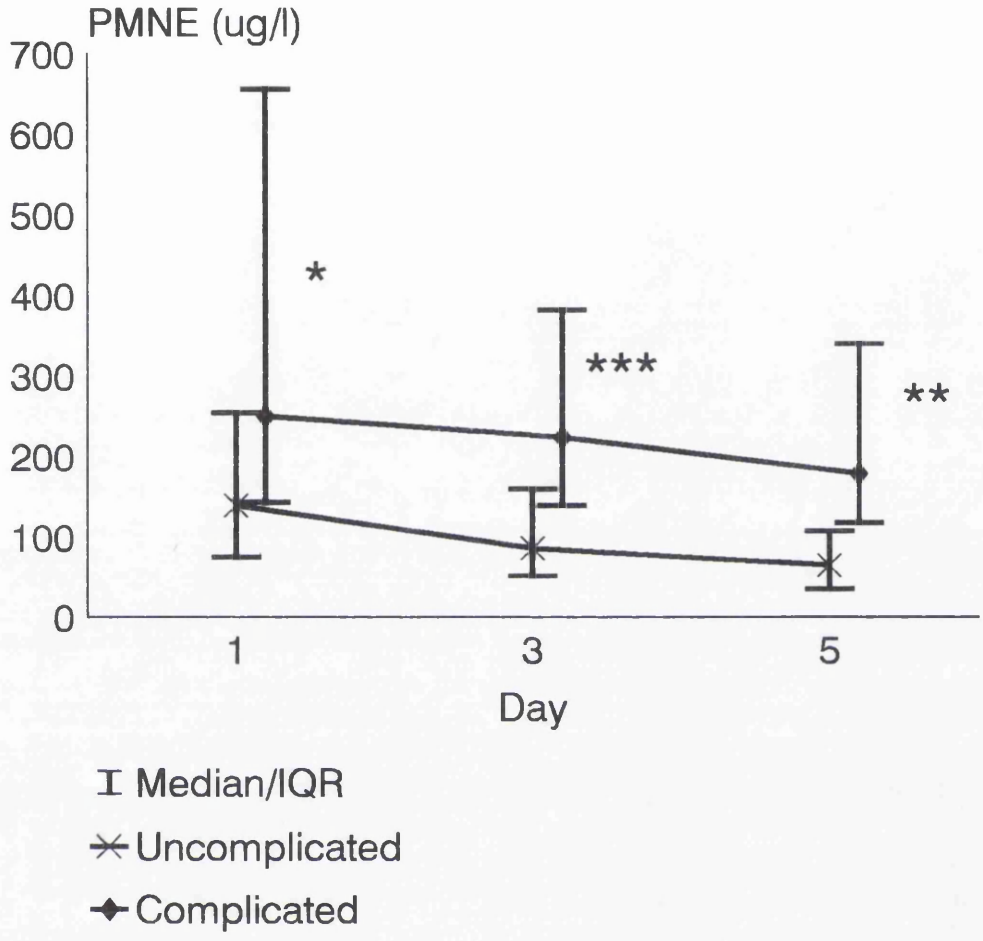


Figure 7.2 Granulocyte elastase in 37 patients with acute pancreatitis
** P<0.05, **P<0.02, ***P<0.002*

	300µg/l	200µg/l	100µg/l
Sensitivity%	**37.5**	**56.3**	**81.3**
Specificity%	**85.7**	**61.9**	**33.3**
Positive predictive value%	**66.7**	**52.9**	**48.1**
Negative predictive value%	**64.3**	**65**	**70**
Correctly predicted%	**64.9**	**59.5**	**54**

Table 7.1: PMNE as prognostic indicator in acute pancreatitis.

These results were compared with peak C-reactive protein levels within 48h of admission and prognostic scoring by the Glasgow criteria. CRP levels were less accurate than day 1 PMNE levels and had the added disadvantage of requiring 48h before prognostic scoring could be carried out. Glasgow criteria, while still requiring 48h to elapse, were associated with similar overall accuracy as day 1 PMNE levels, using a 300ug/l cut-off **(Table 7.2)**.

	Peak CRP>150mg/l	Glasgow Score≥3
Sensitivity%	**68.8**	**43.8**
Specificity%	**38.1**	**76.2**
Positive predictive value%	**45.8**	**58.3**
Negative predictive value%	**61.5**	**64**
Correctly predicted%	**51.4**	**62.2**

Table 7.2: CRP and Glasgow prognostic score in acute pancreatitis

7.6 Discussion

The early, high levels of PMNE in patients with systemic complications suggest that granulocyte activation is an early event in these patients. The very strong correlation between IL-1β secretion by monocytes and day 1 PMNE levels was an unexpected finding given the poor association which was found between IL-1β levels and the development of systemic complications reported in chapter 6. In addition there was a poor association between PMNE levels and the secretion of TNFα, IL-6 and IL-

8. IL-1β has not been shown to activate neutrophils and, with the small number of patients studied, it may be a chance finding. Indeed, much of the association between IL-1β secretion and PMNE levels is due to one very high result for patient 37.

The absence of an association between monocyte production of granulocyte-stimulating cytokines such as TNFα and IL-8 and plasma PMNE levels early in the course of the illness suggests that, if neutrophil activation is the source of PMNE, then systemic mononuclear cell activation is not responsible for this. One explanation may be that early PMNE is the result of local, pancreatic neutrophil activation as a result of local inflammation at a stage in the disease when systemic mononuclear cell activation has not occurred.

PMNE, measured on the first day of hospital admission, has been reported to accurately discriminate between patients with complicated acute pancreatitis and those with mild attacks[36,42]. Gross and colleagues[36] studied 75 patients with pancreatitis of all grades of severity. Of these, 41 were graded as "severe" by the authors on the basis of the development of two or more complications. Patients with one complication or an uncomplicated outcome were graded as "mild". A level of PMNE of greater than 400μg/l was associated with a positive and negative predictive value for severe or lethal pancreatitis of 82% and 81% respectively. This was more accurate than multiple factor scoring systems or C-reactive protein (CRP) levels. The proportion of patients defined as having severe pancreatitis was unusually high at nearly 55% and the reason for this may lie in the rather arbitrary definitions for complicated disease employed in this study.

In a study by Dominguez-Munoz and colleagues[42] 182 patients had serial measurement of PMNE levels. Of these, 28 were considered to have severe pancreatitis on the basis of the development of either local or systemic

complications. Once again the complications which were recorded are poorly defined and, for example, severe hypoxia was only recorded as a complication if associated with radiological changes. "Pancreatic phlegmon" is included in the list of local complications but is not defined and this term has been abandoned[35]. Remarkable results were reported in this study with admission PMNE levels of more than 300ug/l associated with a positive predictive value of 79.4% and negative predictive value of 98.1%. In a third study, Uhl and colleagues[37] reported 84% overall accuracy for PMNE in predicting necrotising pancreatitis with a cut-off level of 120ug/l within 5 days of admission which was similar to that achieved with CRP. No information on the accuracy in predicting complicated pancreatitis was given.

These results were not matched by the present, smaller study which had a PPV and NPV of 66.7% and 64.3% respectively. However, as was found in the study by Gross, day 1 PMNE levels were marginally more accurate than multiple factor scoring or peak CRP levels. The reason for the lesser accuracy achieved in the present study may lie in the fact that patients had already been selected on the basis of an admission APACHE II score of greater than 5 and the study population was therefore biased towards a more severe attack of pancreatitis. The definitions of complications were also different from those employed in previous studies although the definitions in the present study are closest to those suggested by the Atlanta symposium. Even allowing for these factors, day 1 PMNE levels provided better discrimination than the 48h Glasgow score or CRP level. There was, however, considerable overlap between PMNE levels in those with complicated and uncomplicated attacks.

In conclusion, the results of this study provide evidence of early neutrophil activation in acute pancreatitis, particularly in those patients who subsequently develop systemic complications. No evidence was found of an association between monocyte cytokine production and neutrophil activation as measured by plasma PMNE levels.

Chapter 8

Cytokine secretion, parathyroid hormone and the pathophysiology of hypocalcaemia in acute pancreatitis

8.1 Introduction

In chapter 6, the similarities between acute pancreatitis and sepsis have been discussed in terms of the alterations in mononuclear phagocyte function which are present in both groups of patients. Hypocalcaemia was thought to be a systemic complication unique to acute pancreatitis as a result of proteolytic cleavage of parathyroid hormone or saponification in areas of fat necrosis[307]. However, it has been demonstrated that similar biochemical disturbances are also a feature of severe sepsis[308,309]. The nature of the hypocalcaemic stimulus in both sepsis and acute pancreatitis remains unknown but of interest is the finding that bolus injections of IL-1β are capable of causing reductions in serum ionised calcium in an experimental model[310]. In this way, mononuclear phagocyte activation may play a role in the development of hypocalcaemia just as it is proposed to play a role in the pathophysiology of other systemic manifestations of acute pancreatitis.

In patients with sepsis, high levels of PTH are seen in association with hypocalcaemia in non-survivors[309] but for more than 15 years, there has been debate about the nature of the parathyroid response to the hypocalcaemia seen in acute pancreatitis. Although variable or low levels of parathyroid hormone (PTH) in the presence of hypocalcaemia have been reported by some authors, [307,311-314] others have found high levels of circulating PTH in the presence of low serum calcium. [315,316]

Biologically active PTH is an 84 amino acid polypeptide which, both within the parathyroid gland and at peripheral sites, undergoes proteolytic

modification resulting in the formation of biologically inactive peptide fragments[317]. All of the published studies to date on PTH levels in acute pancreatitis have utilised radio immunoassays based on antisera recognising either unspecified or carboxy-terminal amino acid sequences which detect variable proportions of PTH fragments in addition to the intact peptide[318]. Since accelerated proteolytic degradation of intact PTH has been demonstrated in sera from patients with severe acute pancreatitis[319,320] it is possible that the conflicting results of previous studies are the result of the molecular heterogeneity of circulating PTH in this condition. The development of two-site immunoradiometric assays for PTH allows measurement of intact PTH without interference from PTH fragments[318] In this study circulating levels of intact PTH were measured in order to assess the true nature of the parathyroid response in acute pancreatitis. In a number of patients, PTH fragments were also measured in order to assess whether proteolytic cleavage of PTH was involved in the pathophysiology of pancreatitis-associated hypocalcaemia.

Calcium homeostasis was therefore examined in patients with acute pancreatitis in order to determine the role of IL-1β secretion and proteolytic cleavage of parathyroid hormone (PTH) in the pathophysiology of hypocalcaemia.

8.2 Patients and methods

Patients

Forty three patients entered into the randomised, controlled trial of octreotide described in chapter 4 had serial measurement of PTH, calcium and albumin carried out. All patients were therefore pre-selected on the basis of an admission APACHE II score of greater than 5.

Assays

Venous blood was withdrawn into glass tubes and allowed to clot on ice prior to centrifugation at 600g (4°C) for 10min. Serum was removed and frozen immediately at -30°C prior to assay. Serum calcium, albumin and intact PTH were measured on the same sample. Calcium and albumin were measured on a routine auto analyser. Calcium was adjusted for albumin using the formula Ca(adj)=(47-alb)x0.02+Ca (where Ca(adj) = adjusted calcium, alb = albumin and Ca = total calcium) [321]. Intact PTH was measured by immunoradiometric assay (Nichols Institute Diagnostics, San Juan Capistrano, USA). This utilised two affinity purified polyclonal antibodies to PTH, one specific for the mid-molecule region and C-terminal end and the other for the N-terminal portion. The mid-molecule/C-terminal antibody was bound to plastic beads and the N-terminal antibody was supplied radio labelled with ^{125}I. Samples and standards were incubated for 24h with antibody-coated beads and ^{125}I-labelled antibody following which the beads were washed to remove unbound antibody then bound ^{125}I-labelled antibody quantified by counting for one minute in a gamma counter. Results were expressed in pmol/l. The normal range of intact PTH in our laboratory is less than 5pmol/l. After determining calcium and intact PTH levels, hypocalcaemic samples were assayed for the presence of PTH mid-molecule/C-terminal fragments using a specific radio immunoassay (Nichols Institute Diagnostics, San Juan Capistrano, USA). This is a

competitive protein binding assay in which samples and standards were first incubated with goat anti-hPTH(44-68) followed by ^{125}I-PTH. Antibody bound PTH(44-68) was then precipitated using anti-goat precipitant and ^{125}I-PTH quantified in the resulting pellet by counting in a gamma counter. The quantity of PTH(44-68) in the original sample, being inversely proportional to the amount of bound ^{125}I-PTH(44-68) was then calculated by reading against a standard curve.

8.3 Results

Patients

Of 43 patients studied, two were excluded, one with primary hyperparathyroidism and one with chronic renal failure, both conditions which are normally associated with high levels of circulating intact PTH. The 41 remaining patients consisted of 19 males and 22 females, mean age 70 (range 35-92). The aetiology of acute pancreatitis was gallstones in 23, alcohol in 7 and undetermined in 11. There were 16 patients who developed complications of whom 6 died **(Table 8.1)**.

Hypocalcaemia

Calcium levels < 2.2mmol/l were detected in 62% of all samples. After correction for serum albumin, only 19% of all samples had calcium levels < 2.2mmol/l, consisting of 29 samples taken from 15 patients. 50% of patients who developed complications and 28% of the uncomplicated group developed hypocalcaemia during the 5 days of the study ($P>0.1$, chi square test). Two patients with fatal acute pancreatitis received intravenous calcium gluconate as treatment for hypocalcaemia although neither exhibited tetany. No significant difference in serum adjusted calcium levels

Patient No	Complication	Death
11	MSOF	Day 14
13	CVA, RF	Day 10
18	MSOF	Day 4
19	RF, Infected fluid collection	
22	MSOF	Day 1
24	ARF	
27	ARF, MI	
31	RF	
33	MSOF	
35	RF	
36	ARF, RF, Fluid collection	
37	ARF. Infected pancreatic necrosis	
39	ARF, RF	
40	RF	Day 21
42	CVA, RF	Day 4
43	RF, Fluid collection	

MSOF = multiple systems organ failure, RF = respiratory failure (defined as pO2<8kPa for >24h requiring high flow oxygen therapy or assisted ventilation), ARF = acute renal failure (defined as serum creatinine >200mmol/l for >24h), MI = myocardial infarction, CVA = cerebrovascular accident.

***Table 8.1:** Complications in patients entered into study*

was seen between the uncomplicated and complicated groups on any of the first five days after admission (**Figure 8.1**).

Parathyroid hormone

Intact PTH levels were significantly higher in patients with a complicated outcome on four of the five days studied (**Figure 8.2**). There was, however, no significant correlation between adjusted calcium levels and intact PTH on individual days. Peak PTH levels of more than 6pmol/l were observed more frequently in the complicated than the uncomplicated group (14 of 16 patients, complicated group; 6 of 25 patients, uncomplicated group ($P<0.001$, Chi-square test). In the complicated group, individual and mean PTH levels peaked around the second day of admission. Intact PTH levels in the patients who developed hypocalcaemia were variable but a rise in PTH (>6pmol/l) was observed more frequently in the complicated group (7 of 8, complicated group; 2 of 7, uncomplicated group; P=0.035, Fisher's exact test).

PTH fragments

Serum samples from hypocalcaemic patients were analysed for PTH mid-molecule/C-terminal fragments. Low levels of PTH fragments were found associated with low intact PTH levels. Intact PTH levels correlated with the levels of PTH fragments (**Figure 8.3**, $r_S = 0.743$, $P<0.002$, Spearman rank correlation).

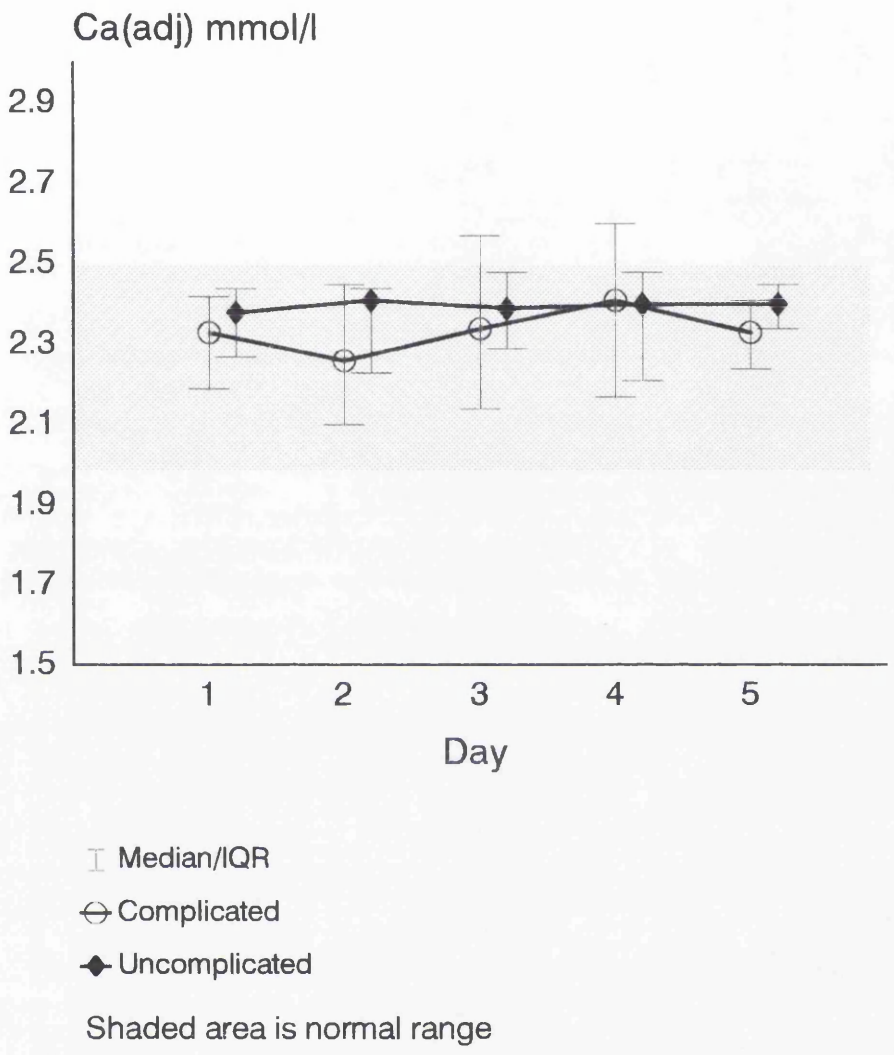


Figure 8.1: Calcium levels in patients with acute pancreatitis

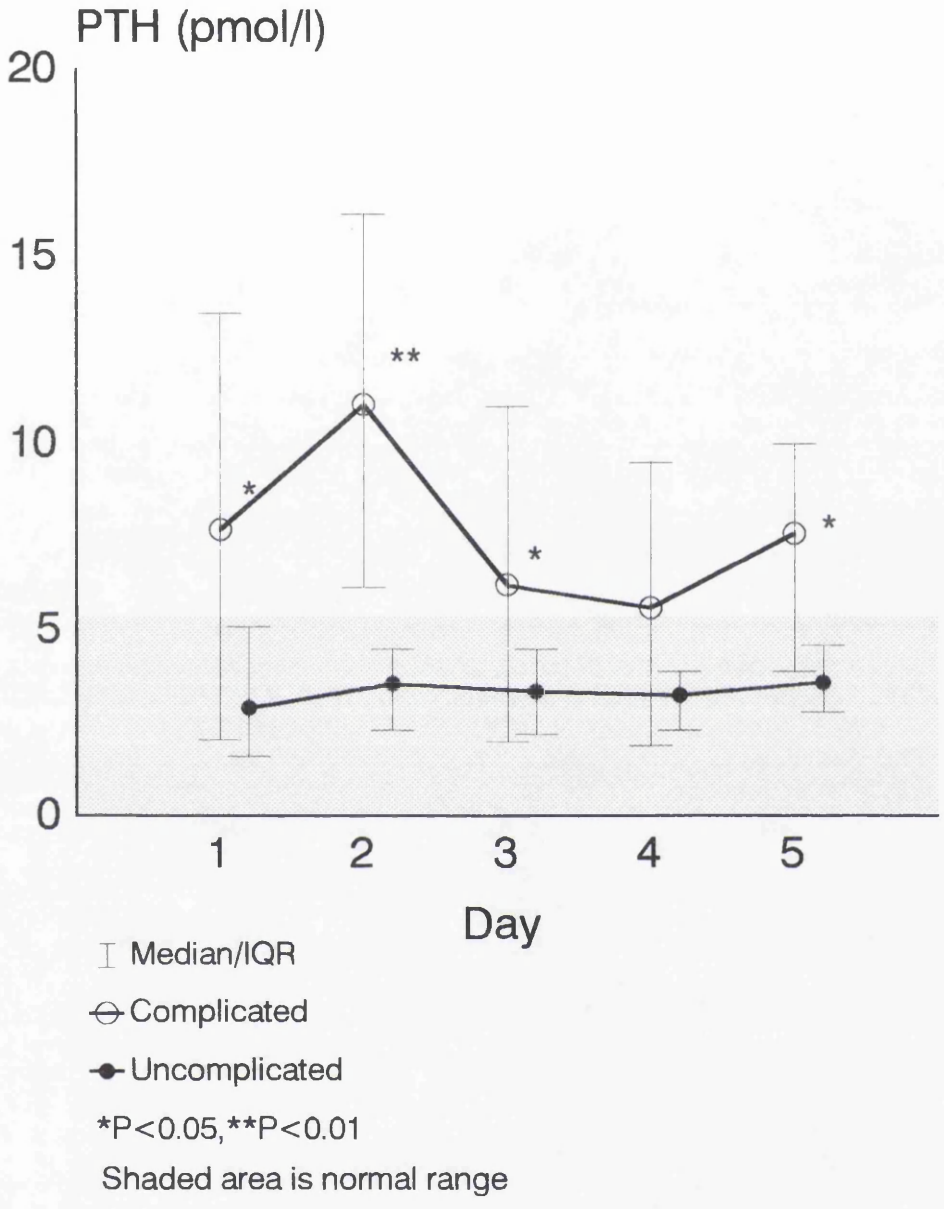


Figure 8.2: PTH levels in patients with acute pancreatitis

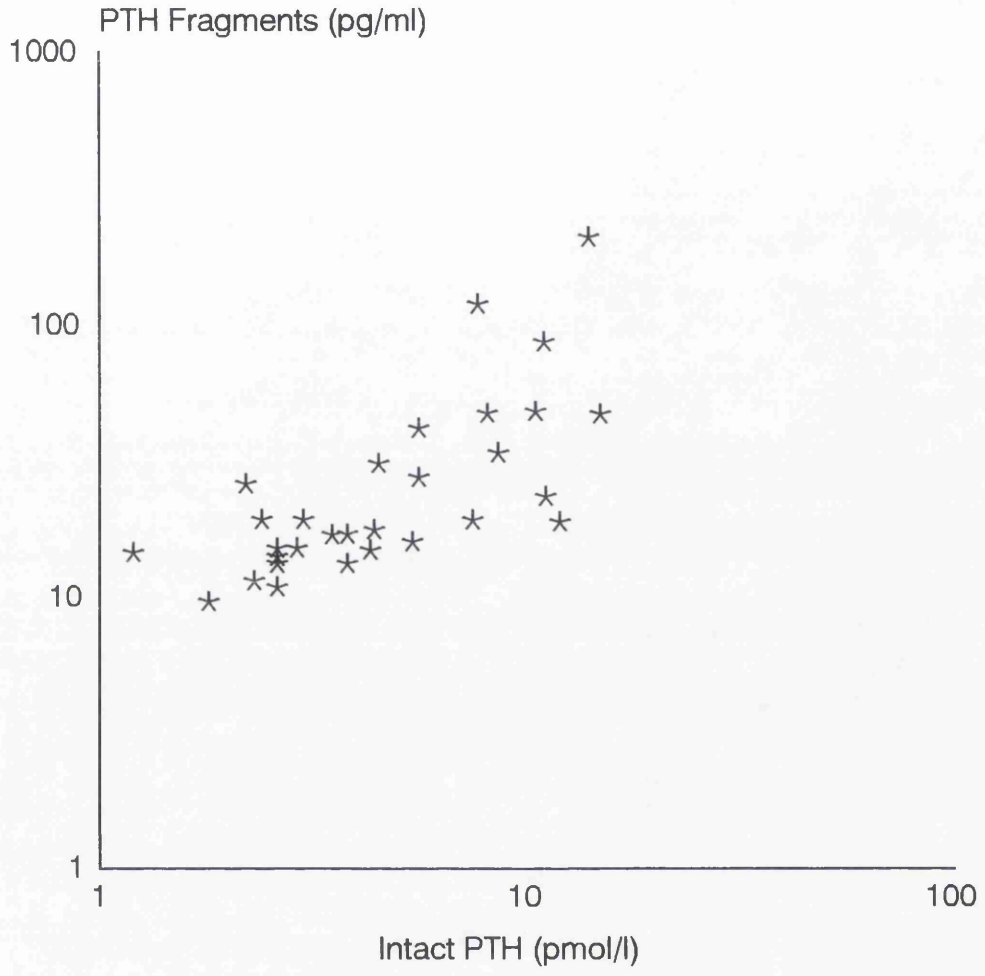


Figure 8.3 : PTH and PTH Fragments in hypocalcaemic patients

IL-1β secretion and hypocalcaemia

In 14 patients (patients 30-43), monocyte IL-1β production was measured as described in chapter 6. Peak levels of monocyte IL-1β production were compared with plasma levels of PTH and calcium. There was no correlation between peak PTH levels and IL-1β secretion by monocytes ($r=0.007$). There was, however a significant correlation between the lowest serum adjusted calcium level and peak IL-1β secretion ($r=0.698$, $P<0.01$) although this result is a consequence of the high IL-1β result for patient 37. If this result is omitted, no significant correlation was demonstrated.

8.4 Discussion

The majority of cases of hypocalcaemia observed in acute pancreatitis are the result of hypoalbuminaemia, with a reduction in total but not ionised calcium[322]. However, despite correction for albumin, hypocalcaemia may still occur[314,315,322] a finding which has been confirmed by direct measurement of ionised calcium[316,323,324]. The failure of homeostatic mechanisms to maintain normal levels of serum ionised calcium has led to studies investigating the nature of the parathyroid response in acute pancreatitis but reports in the literature are conflicting. Condon et al[307] studied PTH levels in 12 patients with hypocalcaemia and found undetectable levels of PTH in plasma from 8 of these although no correction for serum albumin was made and therefore the true incidence of hypocalcaemia may have been lower. Similarly, in 9 patients with hypocalcaemia, Robertson et al[311] found normal levels of PTH with calcium levels returning to normal following administration of bovine PTH. Weir et al[312] reported low ionised calcium in 8 of 11 patients with acute pancreatitis. PTH levels in these patients were variable, with high levels in 5 of the 8 patients and undetectable levels in the remaining three. McMahon et al[313] found lower levels of PTH in patients with severe

pancreatitis when compared to those with mild disease, despite lower levels of serum calcium in the severe group. In contrast, Imrie et al[325] studied more than 90 patients with serial blood sampling and reported high levels of PTH in both normocalcaemic and hypocalcaemic subjects. The highest levels of PTH were observed on the day of admission which fell over the next two days in association with a rise in serum calcium. Similar findings were reported in a series of 6 patients with acute pancreatitis admitted to an intensive care unit[316] although in this study PTH levels peaked later, around the second day. McMahon et al[314], in a second study of 18 patients of whom 6 developed hypocalcaemia, reported low levels of plasma PTH despite urinary calcium and phosphate levels suggestive of a renal response to PTH.

In all of these studies, assays for PTH were based on polyclonal antisera to PTH which, in addition to the intact molecule, would detect varying quantities of PTH fragments depending on the concentration and nature of fragments present and the affinity of the antisera. In the present study, an assay was used which was specific for the intact PTH molecule and did not detect PTH fragments even in high concentration. The present results indicate that high levels of circulating intact PTH are present in patients with acute pancreatitis, in the presence of both normal and low serum calcium. However, the correlation observed between PTH fragments and intact PTH in the present study suggests that the use of PTH fragment assays is not sufficient to explain the discrepancies between the present findings and those of some previous reports.

A rise in PTH was observed more frequently in those patients who developed complications, with peak levels occurring towards the second day after admission. This is in contrast to the findings of Imrie et al[315] where the highest levels were seen in the first sample taken after admission. The reason for this discrepancy may be the result of differences in timing of

blood samples between the two studies or in handling of samples prior to assay.

In uncomplicated acute pancreatitis, intact PTH levels seldom rose beyond the normal range, even in the presence of hypocalcaemia. There are several possible explanations which may explain this finding. Firstly, the calculated ionised calcium levels may not reflect true ionised calcium levels in this group of patients. All 6 patients with hypocalcaemia and low PTH levels had a serum albumin of less than 32g/dl and the results may reflect inaccuracies in correcting total calcium for such low albumin levels. Although close correlation between corrected and ionised calcium in acute pancreatitis has been reported, [324] Croton et al found no correlation during the first 36h of illness[323]. Nomograms for the correction of calcium for albumin levels have been shown to be unreliable in patients with sepsis[310] and it is possible that a similar situation may exist in acute pancreatitis, perhaps as a result of a varying affinity of calcium for albumin in pathological states. Secondly, it may be the result of proteolytic degradation of intact PTH *in-vivo* or *in-vitro*, although increased levels of PTH fragments were not observed in these samples as might be expected if this were the case.

Hypocalcaemia is not unique to acute pancreatitis but occurs in other critically ill surgical patients. [309] Such patients have been reported to exhibit changes in serum PTH similar to those observed in this study, with a rise in PTH accompanied by low ionised calcium occurring in non-survivors[309]. Similarly, patients who survived had normal levels of PTH despite hypocalcaemia suggesting that both in critical surgical illness and acute pancreatitis, the magnitude of the parathyroid response, rather than the level of serum calcium, correlates with outcome. Whether a rise in PTH may be detrimental under such circumstances by allowing further intracellular accumulation of calcium, remains a matter of speculation.

One possible explanation for the hypocalcaemia which occurs in both sepsis and in patients with acute pancreatitis is the action of IL-1β which is capable of inducing experimental hypocalcaemia. In the present study, one patient with a serum calcium level of less than 2mmol/l had greatly increased monocyte IL-1β secretion but in the remainder of the patients, all of whom had serum calcium levels of greater than 2mmol/l, there was no correlation between IL-1β secretion and serum calcium. It remains possible that IL-1β or other cytokines play a key role in the development of hypocalcaemia but further study is obviously required.

Recent work by Ward and colleagues[326] has suggested a role for calcium in the initial acinar cell insult during the development of acute pancreatitis but it is unlikely that this mechanism explains the development of hypocalcaemia during the course of the illness.

In conclusion, this study has shown that raised levels of intact PTH occur in patients with complicated or fatal acute pancreatitis. Normal levels of PTH are present in the majority of patients with uncomplicated acute pancreatitis, even in the presence of hypocalcaemia. No evidence of proteolytic degradation of PTH was found and, in fact, higher levels of PTH were more common in patients who developed complications of acute pancreatitis. No convincing evidence was demonstrated of a relationship between monocyte IL-1β secretion and hypocalcaemia. The nature of the hypocalcaemic stimulus in acute pancreatitis remains unclear.

Chapter 9.

General discussion, conclusions and suggestions for further work

9.1 Conclusions

The principal aims of this thesis were to investigate the role of mononuclear phagocyte activation in acute pancreatitis and the possible effect of octreotide on outcome, by virtue of its suggested effect on reticuloendothelial function. The main findings were as follows;

1. Monocyte activation occurs in acute pancreatitis, and there was evidence to support the hypothesis that they are involved in the pathophysiology of the development of systemic complications..

2. Octreotide has little or no effect on outcome in acute pancreatitis and did not demonstrably reduce the degree of mononuclear phagocyte activation.

9.2 Discussion

In acute pancreatitis, improvements in supportive care and resuscitation have led to a reduction in the mortality associated with the early systemic illness. Patients continue to suffer significant morbidity and early deaths do still occur. In the clinical trial described in chapter 4, there were 5 early deaths due to systemic organ complications which was, in fact, the biggest single cause of death. Much attention has been focused on the potential role of surgery in the later stages of pancreatitis, particularly with regard to the treatment of infected pancreatic necrosis. In the present study, however, there was only one death directly attributable to this complication. These results would suggest that much could still be achieved by focusing any new treatment on the early systemic illness.

Previous attempts at treatment based on antiprotease therapy or peritoneal lavage have proved unsuccessful and the results presented in chapter 6 of this thesis provide evidence that any future treatment should be directed towards the prevention of mononuclear phagocyte activation or the antagonism of mononuclear phagocyte products.

The main stimulus to mononuclear phagocyte activation is bacterial endotoxin and as discussed in chapter 2, there is some evidence that endotoxaemia occurs in patients with acute pancreatitis.

The effects of TNFα and IL-8 are mainly mediated by neutrophils and in chapter 7, raised levels of neutrophil elastase confirmed the presence of neutrophil activation early in the course of acute pancreatitis. Previous authors have demonstrated neutrophil activation by similar methods. The present study was, however, unable to demonstrate a link between monocyte cytokine secretion and neutrophil activation, as measured by PMNE levels.

There is therefore evidence in support of the individual components the proposed hypothesis, with endotoxaemia, mononuclear phagocyte activation and neutrophil activation each having been demonstrated in patients with acute pancreatitis.

9.3 Further study required

There is now evidence that endotoxaemia, monocyte/macrophage activation, cytokines and neutrophil activation are involved in the development of the systemic effects of acute pancreatitis. However, all studies have been restricted to the study of isolated components of the complex cascade of events. In this thesis, evidence has been presented that

monocyte activation occurs in acute pancreatitis but no relationship between this and neutrophil activation was demonstrated. Endotoxaemia was not assessed and it is not known whether this would have shown a correlation with monocyte activation. Before the hypothesis as outlined above can be generally accepted, there is a need for a study which seeks to determine whether the separate components of this proposed mechanism can be demonstrated simultaneously. Measurement of anti-endotoxin core antibody together with mononuclear phagocyte activation measured by both in-vitro cytokine secretion and cell surface activation markers, could be combined with direct and indirect measurement of neutrophil function. In addition, further research is needed in order to explain the reasons for differing reactions to endotoxin between individuals. As outlined above, this may have a genetic basis or it may be a consequence of the degree of endotoxaemia present. Assessment of TNFα allelic polymorphism associated with measurement of monocyte TNFα secretion in patients with acute pancreatitis may help to explain this further.

Indirect measurement of gut perfusion can be easily carried out using gastric tonometry and such a study would help to explain the mechanism behind the appearance of endotoxin in acute pancreatitis. Other studies of intestinal barrier function could be combined with this.

Little is known of the role of T-cells in the immune disturbances seen in acute pancreatitis. Curley et al provided some evidence of diminished T-helper cell numbers[327] but further study is required. In particular, the role of monocyte regulatory cytokines such as IL-10 and IL-4 awaits investigation. IL-4 has been shown to suppress monocyte TNFα, IL-1β and IL-8 secretion[328-331] and more recently, IL-10 has been identified as a factor which inhibits monocyte IL-1β and TNFα secretion[332]. IL-10 is also produced by LPS-stimulated monocytes and may therefore have a role in autoregulation of monocyte function[332]. IL-4 is produced by T-helper cells

and the reduction in T-helper cells seen in some patients with acute pancreatitis may therefore be important in the determination of monocyte responses to endotoxin.

9.4 Future treatment options

Confirmation of the proposed hypothesis by such studies could lead to new therapeutic strategies in acute pancreatitis.

Prevention of endotoxaemia

Initial resuscitation of patients with acute pancreatitis could be improved in order to prevent the splanchnic hypoperfusion associated with the development of endotoxaemia. Additional agents such as dopamine may selectively improve splanchnic perfusion and there are reports that dopamine may improve the outcome from experimental pancreatitis. Improvement of gut barrier function by enteral nutrition may also be beneficial. Selective gut decontamination was suggested by Foulis and colleagues as long ago as 1982 [194] and there has recently been a study of selective gut decontamination in patients with severe pancreatitis carried out in the Netherlands[333], the results of which are promising. Prevention of endotoxaemia by enhancement of hepatic RES activity is another option but, as was reported in chapter 4 of the present thesis, it is unlikely that octreotide will prove useful in this regard.

Anti-endotoxin antibody

Human polyclonal antibodies to the polysaccharide side chain and core regions of the endotoxin molecule was used in patients with gram negative sepsis by Ziegler et al[334]with a reported reduction in mortality from 39 to 22%. However, the use of pooled antisera carries with it the risk of transmission of blood-borne viral infection and more recently, monoclonal

antibody to the lipid-A moiety on the endotoxin molecule has become available. In a study of over 500 patients, Ziegler et al[335] reported no overall effect of anti-endotoxin although there was a reduction in mortality in the sub-set of patients with culture-positive gram-negative septicaemia. Two subsequent studies with a different monoclonal antibody has conflicting results in patients with gram negative sepsis[336,337]. Despite these results, anti-endotoxin antibody may be worth considering as a therapeutic trial in acute pancreatitis.

Cytokine Inhibitors

Antibodies to TNFα are under investigation in patients with sepsis. The association between high monocyte TNFα production and the systemic manifestations of acute pancreatitis provides a rationale for similar clinical trials in acute pancreatitis. Antibody to IL-8 prevents experimental lung injury[294] and such antibodies may in the future be developed for clinical trials.

Prevention of neutrophil activation

Clinical trials are currently underway with a platelet activating factor antagonist inhibitor. PAF is a cytokine released from endothelial cells, activated leukocytes and platelets which has potent effects on neutrophil activation and which has been implicated in the pathophysiology of acute pancreatitis[338,339]. Its place in the complex cascade of events has yet to be elucidated but with the development of a non-toxic peptide inhibitor, studies in experimental pancreatitis have given encouraging results.

A large, multi-centre clinical trial with a PAF antagonist in patients with acute pancreatitis is currently underway.

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Appendix 1
Abbreviations used in thesis

ACTH: adrenocorticotrophic hormone
AIMV: adoptive immunotherapy medium five
AFC: acute fluid collection
APACHE: acute physiology and chronic health evaluation
APS: acute physiology score
ARDS: adult respiratory distress syndrome
ARF: acute renal failure
BSA: bovine serum albumin
CCE: countercurrent centrifugal elutriation
CDED: choline deficient ethionine supplemented diet
CDL: closed duodenal loop
CH: ceruelin hyperstimulation
CRP: C-reactive protein
CT: computerised tomography
CVA: cerebrovascular accident
DDX: doctors and dentists exemption (certificate)
EDTA: ethyl-diamino tetra-acetic acid
ELISA: enzyme-linked immunosorbent assay
ERCP: endoscopic retrograde cholangiopancreatography
FACS: fluorescence-activated cell sorting
IL-1α: interleukin 1 alpha
IL-1β: interleukin 1 beta
IL-6: interleukin 6
IL-8: interleukin 8
IPN: infected pancreatic necrosis
IQR: interquartile range
ITU: intensive therapy unit
LPS: lipopolysacharride
MACS: magnetic cell separation system
MSOF: multiple systems organ failure
N/A: not assessed
NPV: negative predictive value
NS: not significant
PAF: platelet activating factor
PBMC: peripheral blood mononuclear cells
PBS: phosphate buffered saline
PDL: pancreatic duct ligation
PE: pleural effusion
PLAP: phospholipase A_2activation peptide
PMNE: polymorphonuclear elastase
PN: pancreatic necrosis
PPV: positive predictive value
PTH: parathyroid hormone
RES: reticuloendothelial system
RF: respiratory failure
RIA: radioimmunoassay

RIB: retrograde injection of bile
TAP: trypsinogen activation peptide
TNFα: tumour necrosis factor alpha

Appendix 2
Publications and presentations

At the present time, two papers have been published from work presented in this thesis.

McKay CJ, Imrie CW, Baxter JN; Somatostatin and somatostatin analogues - are they indicated in the management of acute pancreatitis? Gut 1993;34;1622-1626.

McKay C, Beastall GH, Imrie CW, Baxter JN. Circulating intact parathyroid hormone levels in acute pancreatitis. Br J Surg 1994;81:357-60.

Two further papers have been accepted for publication

McKay C, Gallagher G, Brookes B, Imrie CW, Baxter JN. Monocyte activation is associated with the systemic complications of acute pancreatitis. Br J Surg, 1996 (in press).

McKay C, Baxter JN, Imrie CW. Mononuclear phagocyte function in acute pancreatitis. Scand J Gastroenterol 1996 (in press).

The work described in this thesis has been widely presented at meetings in the UK, Europe and the USA, including the Surgical Research Society, Pancreatic society of Great Britain and Ireland, European Pancreatic Club, International Hepatopancreaticobiliary Association and the American Gastroenterology Association.

Abstracts from these presentations have been published as follows;

McKay C, Brooks B, Gallagher G, Baxter JN. Imrie CW. Monocyte activation in acute pancreatitis is related to the degree of systemic illness. Digestion 1992;52:104-105.

McKay C, Gallagher G, Brooks B, Baxter JN, Imrie C. Monocyte production of tumour necrosis factor in acute pancreatitis is related to outcome . Gut 1993;34:A1294

McKay C, Gallagher G, Imrie CW, Baxter JN. Monocyte production of tumour necrosis factor in acute pancreatitis is related to outcome. Br J Surg 1993;80:649

McKay C, Gallagher G, Imrie CW, Baxter JN. Monocyte production of tumour necrosis factor in acute pancreatitis is related to outcome. Gut 1993;S3;PDII/3:553.

McKay CJ, Beastall G, Baxter JN, Imrie CW. Circulating intact parathyroid hormone in acute pancreatitis. Br J Surg 1993;80:1480.

McKay C, Baxter JN, Imrie CW. A randomised, controlled trial of octreotide in moderate to severe acute pancreatitis. Digestion 1994;55:316

McKay C, Baxter JN, Imrie CW. Systemic complications in acute pancreatitis are associated with increased cytokine release by monocytes. Digestion 1994;55:316-7

McKay C, Imrie CW, Baxter JN. Systemic complications in acute pancreatitis are associated with increased monokine production. Br J Surg 1994;81:1816

McKay C, Baxter JN, Imrie CW. Octreotide in acute pancreatitis - a randomised, controlled trial. Br J Surg 1994;81:1814