MARKERS OF INFLAMMATION AND IMMUNOSUPPRESSION AS PREDICTORS OF ORGAN FAILURE IN HUMAN ACUTE PANCREATITIS

by

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Academic dissertation

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To my family

Abstract

Acute pancreatitis is an acute inflammation of the pancreas initiated by intracellular activation of pancreatic digestive enzymes. It is characterised by local and systemic activation of inflammatory cells, with overproduction of pro-inflammatory mediators. Systemic inflammation is accompanied by development of an anti-inflammatory counter-reaction that may result in impaired host immunity. It is believed that these inflammatory mediators play a significant role in the progression of local pancreatic inflammation into systemic disease with involvement of other organ systems. Usually acute pancreatitis is a mild, self-limited disease. However, some 20 to 30% of patients develop severe disease, with local pancreatic complications or systemic organ dysfunction or both. Because the development of vital organ failure is the major cause of morbidity and mortality in acute pancreatitis, early identification of patients likely to develop organ failure would be of clinical value.

The aim of the present study was to find markers to predict development of organ failure as well as give insight into the development of immunosuppression in patients with acute pancreatitis.

This clinical study consists of four parts. All patients studied were admitted to Helsinki University Central Hospital with acute pancreatitis. In the first study, serum levels of mast cell tryptase, vascular endothelial growth factor, and basic fibroblast growth factor were studied during the first week of hospitalisation in 70 patients, of whom 20 developed organ failure. In the second study, monocyte human leucocyte antigen (HLA)-DR expression was studied in 314 consecutive patients on admission. In the third study, plasma anti-inflammatory cytokines and monocyte HLA-DR expression were determined during their hospital stay in 27 and 47 patients with mild and severe disease, respectively; 20 patients with severe disease developed organ failure. In the fourth study, 19 prognostic markers were studied at hospital admission in 33 consecutive patients who developed organ failure and 99 matched control patients to find the best single marker and a combination of markers to accurately predict development of organ failure.

Our results show that organ failure usually develops early in the course of acute pancreatitis, suggesting that predictive tests should be carried out immediately on hospital admission. In patients who developed organ failure, circulating tryptase levels were higher than in other patients, indicating that mast cell activation may be involved in the pathophysiology of remote organ dysfunction. Furthermore, a compensatory anti-inflammatory reaction determined by the circulating anti-inflammatory cytokines interleukin-6 or interleukin-10 developed early, reflected disease severity, and correlated with development of immunosuppression. Flow cytometric determination of monocyte HLA-DR expression was a useful method to monitor immunosuppression. Low HLA-DR expression predicted development of organ failure at least as well as did high C-reactive protein or acute physiology and chronic health evaluation (APACHE) II score. Failure to recover initially low HLA-DR expression was related to secondary infections later in the course of acute pancreatitis. High circulating interleukin-6 and interleukin-10 levels within the first 24 hours of disease onset may predict the development of organ failure, whereas at hospital admission, the combination of interleukin-10 more than 50 pg/ml or serum calcium less than 1.65 mmol/l predicted organ failure with better diagnostic accuracy than did any single marker or the APACHE II score.

In conclusion, acute pancreatitis provoked systemic inflammation which began with systemic activation of inflammatory cells possibly including mast cells. Co-occurrence of anti-inflammatory cytokines in the circulation with pro-inflammatory mediators preceded development of systemic manifestations and resulted in early deactivation of monocytes. Measurement of interleukin-10 in combination with calcium provided an accurate method to predict organ failure at hospital admission. Monitoring of monocyte HLA-DR expression in patients with organ failure may provide a means to predict secondary infections occurring later in the course of the disease.

Contents

Original publications

The following original publications are referred to in the text by their Roman numerals I to IV.

I Mentula P, Kylänpää M-L, Kemppainen E, Eklund KK, Orpana A, Puolakkainen P, Haapiainen R, Repo H. Serum levels of mast cell tryptase, vascular endothelial growth factor and basic fibroblast growth factor in patients with acute pancreatitis. Pancreas 2003;27:e29-e33.

II Mentula P, Kylänpää-Bäck M-L, Kemppainen E, Takala A, Jansson S-E, Kautiainen H, Puolakkainen P, Haapiainen R, Repo H. Decreased HLA (human leucocyte antigen)-DR expression on peripheral blood monocytes predicts the development of organ failure in patients with acute pancreatitis. Clinical Science 2003;105(4):409-417.

III Mentula P, Kylänpää M-L, Kemppainen E, Jansson S-E, Sarna S, Puolakkainen P, Haapiainen R, Repo H. Plasma anti-inflammatory cytokines and monocyte human leucocyte antigen-DR expression in patients with acute pancreatitis. Scandinavian Journal of Gastroenterology 2004; 39(2):178-187.

IV Mentula P, Kylänpää M-L, Kemppainen E, Jansson S-E, Sarna S, Puolakkainen P, Haapiainen R, Repo H. Early prediction of organ failure by combined markers in patients with acute pancreatitis. British Journal of Surgery 2005;92:68-75.

Abbreviations

ACD	acid-citrate-dextrose
ALI	acute lung injury
AP	acute pancreatitis
APACHE	acute physiology and chronic health evaluation
ARDS	adult respiratory distress syndrome
AST	aspartate aminotransferase
AUC	area under curve
bFGF	basic fibroblast growth factor
C/B ratio	cost/benefit ratio
CAPAP	carboxypeptidase B activation peptide
CD	clusters of differentation
CI	confidence interval
CRP	C-reactive protein
CT	computed tomography
ELISA	enzyme-linked immunosorbent assay
ENA-78	epithelial neutrophil-activating protein-78
ERCP	endoscopic retrograde cholangiopancreaticography
FiO_2	inspiratory oxygen fraction
FITC	fluorescein isothiocyanate
GM-CSF	granulocyte-macrophage colony-stimulating factor
GRO-α	growth-related oncogene-α
HLA	human leucocyte antigen
ICAM	intercellular adhesion molecule
ICE	interleukin-1 converting enzyme
IFN	interferon
IL	interleukin
IL-1ra	interleukin-1 receptor antagonist
IL-2R	interleukin-2 receptor
IQR	inter-quartile range
LDH	lactate dehydrogenase
mAb	monoclonal antibody

MCP	monocyte chemoattractant peptide
MHC	major histocompatibility complex
MIP	macrophage inflammatory protein
MODS	multiple organ dysfunction score
MODS	multiple organ dysfunction syndrome
MOF	multiple organ failure
NF	nuclear factor
NO	nitric oxide
$PaCO_2$	arterial partial pressure of carbon dioxide
PAF	platelet-activating factor
PAI	plasminogen activator inhibitor
PaO_2	arterial partial pressure of oxygen
PAR	protease-activated receptors
PE	phycoerythrin
PLA_2	phospholipase A_2
RFU	relative fluorescence units
ROC	receiver-operating characteristic
sIL-2R	soluble interleukin-2 receptor
SIRS	systemic inflammatory response syndrome
TAP	trypsinogen activation peptide
TGF	transforming growth factor
TNF	tumour necrosis factor
VCAM	vascular cell adhesion molecule
VEGF	vascular endothelial growth factor

1 Introduction

Acute pancreatitis (AP) is a common cause of acute abdominal pain and is the most frequent pancreatic disease, for which alcohol abuse and gallstone disease are the two most common causes (Lankisch et al. 2001). It is usually a mild, self-limited disease showing only minimal and transient systemic manifestations including fever, tachycardia, hypovolaemia or tachypnea. However, some 20 to 30% of patients develop severe disease with pancreatic complications including necrosis, abscess or pseudocysts, or systemic organ dysfunction (Bradley 1993). Systemic organ dysfunction may affect one or more remote organ systems. Respiratory failure and renal failure are the two types of organ dysfunction most commonly encountered (Heath et al. 1995). Development of vital organ dysfunction is, in AP, the major cause of morbidity and mortality (De Beaux et al. 1995). About half the deaths occur within the first week, typically from organ failure (McKay et al. 1999). Later in the course of the disease, secondary infections lead to a second attack of systemic inflammation, resulting in increase in organ dysfunctions and death (Bone 1996a).

AP is an inflammation of the pancreas with inconsistent involvement of regional tissues and remote organ systems. Inflammation is initiated by intracellular activation of pancreatic proenzymes and autodigestion of the pancreas (Steer and Meldolesi 1988, Grady et al. 1998). Irrespective of the aetiological factor, destruction of the pancreatic parenchyma induces an inflammatory reaction at the site of injury characterised by infiltration of activated leucocytes. Infiltrating inflammatory cells play a central role in determining AP severity (Steer 2002). Local leucocyte-derived production of inflammatory mediators may become amplified, resulting in systemic activation of inflammatory cells and overwhelming production of inflammatory mediators. This process is considered responsible for the systemic manifestations of the disease (Norman 1998). Although a number of inflammatory mediators in this process have been characterised, the exact pathophysiologic mechanisms that determine the course of the disease remain to be established.

At present, no specific treatment exists that would prevent development of systemic complications in patients with AP. Several early supportive treatments or

interventions may, however, be beneficial (Yousaf et al. 2003), making early identification of patients bound to develop organ dysfunction important. Tests available for prediction of the disease severity are in general considered inaccurate during the earliest phase of the disease. A new simple and rapid test is thus needed for identification of these high-risk patients so that their monitoring and intensive care can be initiated without delay.

Increasing understanding of the pathogenesis of systemic inflammation and multiple organ dysfunction syndrome may provide us with drugs to inhibit development of organ dysfunction or ameliorate already developed physiological disturbances (Bernard 2003). In contrast to sepsis-related organ failures, a therapeutic window exists between onset of symptoms and development of organ dysfunction in AP (Norman 1998). Experimental studies suggest that introduction of anti-inflammatory treatment early enough may be able to prevent further development of organ dysfunction (Norman et al. 1995c, Rongione et al. 1997). An early predictive test would be essential for clinical studies of these future treatments.

Immune-mediated immunosuppression may play an important role in the development of secondary infections in the later course of AP. Because treatment of patients with these late complications remains a challenge with high mortality rates, novel methods to diagnose and treat these patients are also needed. Monitoring of immunosuppression and even therapy that would restore impaired host defense mechanisms may provide additional tool for this clinical issue (Döcke et al. 1997).

The main purpose of this clinical study was to find early prognostic tests to identify patients bound to develop organ failure. At first we explored whether the serum levels of mast cell tryptase and vascular endothelial growth factor (VEGF) correlate with the development of organ dysfunction. Secondly, an anti-inflammatory counter-reaction and development of immunosuppression were studied with relationship to clinical outcome. Thirdly, we investigated whether prognostic markers or a combination of these predict organ failure on hospital admission.

2 Review of the literature

2.1 Historical background

The term pancreas comes from the Greek words *pan*, all; *kreas*, flesh, but its origin has never been accurately determined. In 1889, pathologist Reginald Heber Fitz (1843-1913) was the first to provide a systematic description of clinical AP (Fitz 1889, Leach et al. 1990). Only 7 years later did Hans Chiari (1896) postulate that the pathogenetic mechanism of AP was autodigestion (O´Reilly, 2001). Eugene Lindsay Opie (1873-1971) was the first to describe gallstones as an aetiological factor for AP in 1901 (Opie 1901, McClusky et al. 2002). The association between alcohol abuse and pancreatitis was described later by Symmers (1917). In the early twentieth century, the treatment recommended for AP was emergency surgery (Moynihan 1925). Recognition of high mortality rates (between 50% and 78%) and following the invention of a test in 1927 to measure serum amylase levels (Elman 1927), a more conservative approach was adopted in the treatment of AP (Mikkelsen 1934) until the 1970s, when early surgical treatment for severe AP was again adopted (McClusky et al. 2002). The following decades provided several surgical options including total pancreatectomy, open and closed peripancreatic drainage, open packing, blunt necrosectomy, staged reoperation necrosectomy with delayed primary closure over drains, and necrosectomy with continuous closed local lavage – all with variable success.

The earliest surveys of respiratory and renal failure in AP were published in the mid-twentieth century (Paxton and Payne 1944, Stein et al. 1959, Roseman et al. 1960). Adult respiratory distress syndrome (ARDS) in patients with AP was first described in 1967 by Ashbaugh and colleagues. The first description of sequential multiple organ failure was published in the early 1970s concerning surgical patients (Tilney et al. 1973). Edmondson and Berne were the first to suggest that measurement of a single biochemical factor (i.e., serum calcium) might be a useful predictor of outcome in AP (Edmondson and Berne 1944). In 1974, Ranson and co-authors provided several prognostic signs of severe AP, and further improvement in diagnosis and severity assessment of AP followed introduction of computed tomography (CT) (Haaga et al. 1976, Kivisaari et al. 1983, Balthazar et al. 1990). Because of improved

understanding of the mechanisms underlying the pathogenesis of AP and multiple organ dysfunction syndrome, and improved diagnostic methods and favourable results after conservative management during the last two decades, surgical treatment of AP has been limited mainly to those few patients with severe AP with infected pancreatic necrosis and not responding to intensive conservative treatment later in the course of the disease (Yousaf et al. 2003).

2.2 Clinical manifestations and classification

AP usually has an acute onset characterised by upper abdominal pain that may radiate to the back. Abdominal pain may be accompanied by nausea, vomiting, fever, and tachycardia. Abdominal findings in clinical examination vary from mild tenderness to rebound. Ileus and even shock may be present at admission (Ranson 1997). Rare clinical findings include bruising of the flank (Grey Turner's sign) or periumbilical bruising (Cullen's sign) (Dickson and Imrie 1984). In some cases, clinical presentation is atypical, with absence of abdominal pain (Wilson and Imrie 1988, Lankish et al. 1991). Laboratory examinations in most patients show leukocytosis and elevated pancreatic enzyme levels in blood or urine (Smotkin and Tenner 2002).

Reported mortality rates for AP vary considerably in hospitalized series (2-22%), which can be explained by differing patient selection. In addition to in-hospital mortality, a third of patients with fatal AP are not admitted to hospital and are diagnosed post-mortem (Appelros and Borgstrom 1999, Andersson and Andren-Sandberg 2003). Furthermore, 10% of severe AP patients surviving initial hospitalisation die within a few years (Halonen et al. 2003). The mortality rate increases dramatically in the elderly and is not affected by aetiology (McKay et al. 1999, Eland et al.2000). Recurrent episodes seem to have a lower risk of death (Appelros and Borgstrom 1999), although conflicting results are also reported (Gullo et al. 2002). In epidemiological studies, the overall mortality rate of AP ranges from 1 to 3/100 000 person years (Wilson and Imrie 1990, Appelros and Borgstrom 1999, Floyd et al. 2002). Case-mortality in population-based studies ranges from 7 to 15% (McKay et al. 1999, Eland et al. 2000, Floyd et al. 2002). Despite increasing incidence of AP, its mortality rate has been decreasing (Jaakkola and Nordback 1993,

McKay et al. 1999, Eland et al. 2000, Floyd et al. 2002), which may indicate either improved treatment or increased incidence of the mild AP with its minimal mortality.

2.2.1 Mild acute pancreatitis

A clinically based classification system for AP was established in an international symposium in Atlanta, in the USA, in 1992, in which AP was classified as either mild or severe (Bradley 1993). The majority (70 - 80%) of cases present with mild AP. The histological finding is interstitial oedema; peripancreatic fat necrosis may or may not be present. Patients with mild AP respond to appropriate conservative treatment with prompt normalization of physical signs and laboratory values. Mild AP is characterised by uneventful recovery, minimal organ dysfunction, and absence of pancreatic complications (Bradley 1993).

2.2.2 Severe acute pancreatitis

About 20 to 30% of patients develop severe AP (Steinberg and Tenner 1994), which is associated with remote organ failure or at least one local pancreatic complication, or both (Bradley 1993).

2.2.2.1 Local pancreatic complications

Local pancreatic complications include pancreatic necrosis, abscess, and pseudocyst. A diffuse or focal area of nonviable pancreatic parenchyma defines pancreatic necrosis, which is typically associated with peripancreatic fat necrosis; haemorrhage may be present. Microscopically, there is extensive interstitial fat necrosis with vessel damage, and necrosis affecting acinar cells, islet cells, and the pancreatic ductal system (Nevalainen and Aho 1992, Kloppel and Maillet 1993). Pancreatic necrosis usually develops within the first four days in the course of AP (Isenmann et al. 1993). Diagnosis of pancreatic necrosis is based on findings in dynamic contrast-enhanced CT (Kivisaari et al. 1983). Prerequisite for diagnosis are focal or diffuse, well-defined zones of non-enhanced (less than 30 Hounsfield units) pancreatic parenchyma that are larger than 3 cm or involve more than 30% of the pancreas (Balthazar et al. 1990).

A pancreatic abscess is a circumscribed intra-abdominal collection of pus with a positive microbial culture located in the proximity of the pancreas. Purulent material contains little or no pancreatic necrosis. Pancreatic abscesses are considered to arise

as a consequence of limited pancreatic necrosis with subsequent liquefaction and secondary infection usually later in the course of severe AP, often at least four weeks after onset (Bittner et al. 1987).

A pancreatic pseudocyst may occur as a result of AP and disruption of the pancreatic duct. It is a collection of extravasated pancreatic fluid enclosed by a wall of fibrous or granulation tissue, which arises at least four weeks after onset of AP (Bradley et al. 1976). Fluid collections during the first four weeks of AP lack a defined inflammatory wall and are called acute fluid collections, most of which regress spontaneously, while some progress to become pseudocysts or abscesses (Bradley 1993).

2.2.2.2 Organ failure

Depending on the criteria for organ failure, it is associated with severe AP in 20 to 80% of cases and is the major cause of morbidity and mortality (Heath et al. 1995, de Beaux et al. 1995, Tenner et al.1997). Organ failure develops early in the course of AP (Isenmann et al. 2001, Johnson et al. 2001) and is responsible for mortality within the first week, which accounts for about half the AP mortality (McKay et al. 1999). Organ failure in AP shows the same characteristics as organ failure induced by sepsis, major surgery, or trauma (Wilson et al. 1998). Lung injury, i.e., acute lung injury (ALI) or ARDS, is the most prominent organ failure and is present in the majority of patients with organ failure (Atabai and Matthay 2002, Vincent et al. 2002). Other organ systems that may be affected include the renal, hepatic, cardiovascular, haematologic, gastrointestinal, neurological (Deitch 1992), endocrine (Marik and Zaloga 2002), and immune systems (Kox et al. 2000).

The term “multiple organ dysfunction syndrome” (MODS) was recommended by the consensus conference of the American College of Chest Physicians and the Society of Critical Care Medicine to define the presence of altered organ function in an acutely ill patient such that homeostasis cannot be maintained without intervention (Bone et al. 1992). The terms “multiple organ failure” (MOF) (Deitch 1992) or simply “organ failure” (Bradley 1993) has been used for the same clinical entity, which is characterised by sequential development of the dysfunction and eventually failure of at least one organ system. No uniformly accepted consensus criteria for multiple organ failure exist. The use of several different scoring systems including the MOF

score initially published by Goris and co-authors (1985), the multiple organ dysfunction score (MODS) (Marshall et al. 1995), and others (Vincent et al. 1996, Le Gall et al. 1996), or criteria (Bradley 1993) for defining organ failure makes comparison of studies difficult. In the Atlanta classification system, organ failure in AP is defined as shock (systolic blood pressure less than 90 mmHg), pulmonary insufficiency (PaO_2, 60 mmHg or less), renal failure (creatinine level, higher than 177 µmol/l after rehydration), or gastrointestinal bleeding (more than 500 ml/ 24 hours) (Bradley 1993).

2.2.2.3 Secondary infections

Patients with severe AP are susceptible to secondary infections including infected pancreatic necrosis, pancreatic abscess, and generalized infection like sepsis. Pancreatic necrosis becomes infected at an overall frequency of 30 to 70%, and becomes more common with duration of disease (Beger et al. 1986). Infected pancreatic necrosis is associated with prolonged hospital stay and increases mortality (Rau et al. 1997b). Gram-negative bacteria cause most infections, but one-third are polymicrobial, including also anaerobes and fungi. Bacterial species in pancreatic infections suggest that the intestine may be the main source of pathogens, but the biliary system may also serve as a route of bacterial contamination in biliary AP (Räty et al. 1998). Possible mechanisms to promote the passage of bacteria across the intestinal barrier include decreased bowel motility and overgrowth of indigenous microflora, impaired host immunity, and injury to the bowel mucosa due to MODS. Bacteria may then translocate into mesenteric lymph nodes and eventually into the bloodstream (Cicalese et al. 2001).

2.3 Epidemiology

Worldwide, the incidence of AP varies considerably: from 2/100 000 person years to 73/100 000 person years, one of the highest incidences being in Finland (Bourke 1975, Thomson et al. 1987, Jaakkola and Nordback 1993). Several studies have shown an increasing trend in annual incidence during recent decades (Jaakkola and Nordback 1993, Eland et al. 2000, Floyd et al. 2002). The prevalence of two main aetiological factors (gallstones and alcohol abuse) may explain variations and increase in incidence (Jaakkola and Nordback 1993), which may not be attributable only to improved diagnostic methods (Eland et al. 2000). Direct comparison of incidences is

difficult because of differing diagnostic criteria in these epidemiological studies (Dufour and Adamson 2003). Recurrent episodes of AP have a great impact on incidence values, accounting for over a third of all cases (Appelros and Borgstrom 1999). AP is more prevalent in men and is relatively uncommon in children (Benifla and Weizman 2003).

2.4 Aetiology

Numerous aetiological factors have been associated with AP, but about 70 to 80% of patients have either gallstones or a history of alcohol abuse (Sakorafas and Tsiotou 2000). Worldwide, these causes affect a respective 41% and 32% of victims (Lankisch et al. 2001), with considerable variation in the predominance of these two main aetiological factors among countries (Gullo 2002). Alcohol consumption correlates with incidence of alcoholic pancreatitis, the most common aetiological factor in Finland (Jaakkola and Nordback 1993, Räty et al. 2003). Females are more likely to have gallstones as an aetiological factor for AP (Lankisch et al. 2001), although men are more likely to develop AP than women when gallstone disease is present (Lowenfels et al. 2000). Other rare causes of AP are metabolic (hypercalcaemia, hyperlipidemia, toxins, drugs, genetic mutations in rare hereditary AP), mechanical obstruction (endoscopic retrograde cholangiopancreaticography or ERCP, pancreas divisum, post-trauma, pancreatic tumour, worms, foreign bodies, dysfunction or stenosis of the sphincter of Oddi, biliary sludge), vascular (ischaemia, vasculitis), infection, and inflammatory bowel disease (Sakorafas and Tsiotou 2000). In about 10% of cases aetiology is idiopathic. The aetiology of AP is likely multifactorial, including genetic factors, because only a minority of patients with common bile duct stones or suffering from alcohol abuse do develop AP (Singh and Simsek 1990).

2.5 Pathogenesis of acute pancreatitis

Pathogenesis of AP involves three steps: at first a triggering factor is needed to initiate pancreatic acinar cell injury by poorly understood mechanisms. Secondly, after several intracellular events, pancreatic proenzymes (zymogens) become activated intracellularly, resulting in acinar cell injury. Acinar cell injury is followed by local

inflammation of the pancreas, which involves activation of several inflammatory cells and release of inflammatory mediators.

2.5.1 Triggering factors

Mechanisms by which different aetiological factors initiate AP are incompletely understood. Initially, a common-channel theory was proposed as an explanation of how an impacted gallstone in the ampulla of Vater can induce AP by causing bile reflux into the pancreatic duct (Opie 1901). Later, experimental results demonstrated that pancreatic duct obstruction alone is capable of triggering AP (Lerch et al. 1993). Several possible sequels of duct obstruction, including refluxed biliary-pancreatic secretions, pancreatic duct hypertension, and/or aberrant acinar cell secretion, may lead to progression of AP (Lightner and Kirkwood 2001). The pathogenesis of alcoholic pancreatitis may involve several mechanisms not yet fully understood. One mechanism proposed is that alcohol or its oxidative or non-oxidative metabolites (Werner et al. 2002, Wilson and Apte 2003, Criddle et al. 2004) sensitize acinar cells to intracellular zymogen activation (Gorelick 2003).

2.5.2 Intrapancreatic digestive enzyme activation

Pancreatic digestive enzymes are stored in pancreatic acinar cells as inactive proenzymes (zymogens). Zymogens are synthesized in the endoplasmic reticulum and stored in zymogen granules in the apical pole of acinar cells. Under physiological conditions, zymogens are secreted into pancreatic fluid and pass via the pancreatic ductal system into the duodenum, where activation takes place by enteropeptidase. According to the current hypothesis, the crucial step in AP is intracellular activation of zymogens, especially trypsinogen, resulting in active trypsin, which is capable of activating other zymogens (Hofbauer et al. 1998). Zymogen activation is accompanied by secretory blockage of these activated digestive enzymes (Grady et al. 1998). In experimental studies, the triggering event is immediately followed by an increase in cytosolic calcium ions, which is needed for further progression (Raraty et al. 2000, Krüger et al. 2000). Secondly, defective sorting of newly synthesized proteins in the Golgi stack probably results in co-localization of zymogens and lysosomal hydrolases within cytoplasmic vesicles (Steer and Meldolesi 1988, Otani et al. 1998, Singh et al. 2001), where trypsinogen is catalytically activated by these lysosomal hydrolases (Halangk et al. 2000, Van Acker et al. 2002). Ultrastructurally,

zymogen granules on the apical pole of acinar cells are replaced by vacuoles, in which further zymogen activation takes place (Gorelick 2003). Activation of zymogens leads to acinar cell injury, and activated enzymes and their activation peptides escape into the interstitium of the pancreas and into the circulation.

2.5.3 Inflammation

Inflammation is a physiological protective response to tissue injury produced by mechanical (such as trauma, burns, surgery), chemical (such as AP) or microbial stimuli in infection. Inflammation is a complex system controlled by highly amplified humoral and cellular responses. Humoral responses include plasma-derived enzyme cascades like complement, kinin, coagulation or fibrinolytic cascades and numerous cell-derived mediators. Inflammatory cell activation leads to synthesis and release of soluble inflammatory mediators and altered expression of cell membrane-bound receptors and complexes (Delves and Roitt 2000). In the circulation, white blood cells (polymorphonuclear leucocytes, monocytes, and lymphocytes) and platelets are all involved in inflammatory processes. Macrophages and mast cells are present in tissues, and upon inflammation, circulating inflammatory cells also are sequestered in inflamed tissues. Vascular endothelial cells form a barrier between the circulation and tissues. In inflammatory processes, activation of endothelial cells is essential for inflammatory cell sequestration in tissues.

2.5.3.1 Inflammatory cells

Phagocytes

Polymorphonuclear neutrophils, the most numerous leucocytes in the blood, play a pivotal role in acute inflammation. Activation of neutrophils is manifested by increased integrin CD11b/CD18 and decreased L-selectin expression on the cell surface. This altered adhesion-molecule expression facilitates neutrophil extravasation at the site of inflammation (Repo and Harlan 1999). Activated neutrophils secrete a wide variety of inflammatory mediators including the pro-inflammatory cytokines interleukin (IL)-1β, tumour necrosis factor (TNF)-α, and interferon (IFN)-γ and anti-inflammatory cytokines like IL-1 receptor antagonist (IL-1ra), IL-6, and transforming growth factor (TGF)-β (Davies and Hagen 1997, Opal and Depalo 2000).

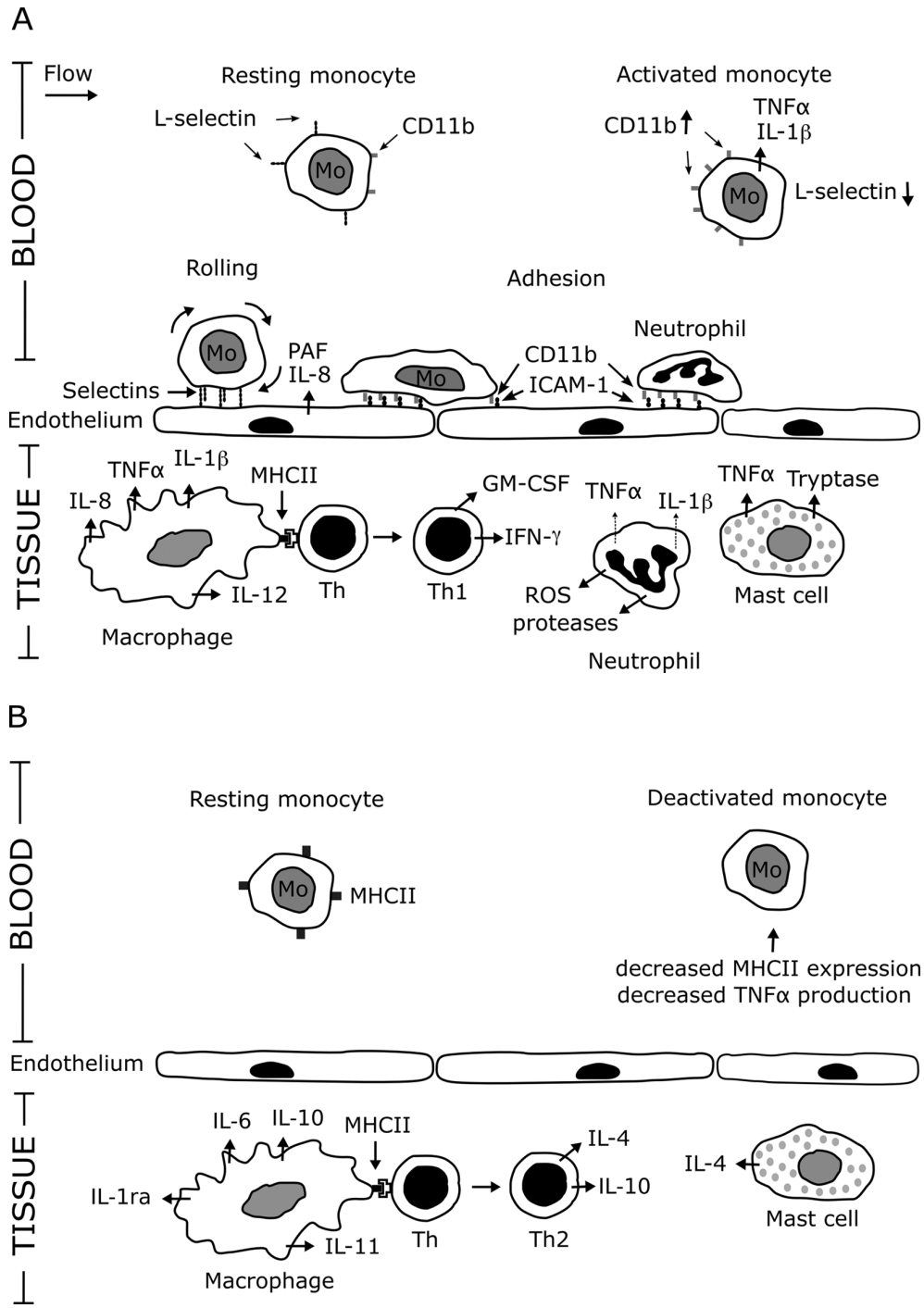

Figure 1. A. Pro-inflammatory reaction: phagocyte-endothelial cell interactions including rolling and adhesion, release of major pro-inflammatory mediators by activated inflammatory cells, T-helper (Th) cell activation and differentiation into Th1 cells, activation of circulating monocytes, and tissue destruction mediated by activated neutrophils releasing reactive oxygen species (ROS) and proteases. **B.** Anti-inflammatory reaction: Release of anti-inflammatory cytokines, T-helper (Th) cell activation and differentiation into Th2 cells, deactivation of circulating monocytes. MHC II – major histocompatibility complex II; ICAM – intercellular adhesion molecule; GM-CSF – granulocyte-macrophage colony-stimulating factor; IFN – interferon; IL – interleukin; IL-1ra – interleukin-1 receptor antagonist; PAF – platelet-activating factor; TNF – tumour necrosis factor

In addition to cytokine production, neutrophils also produce lipid mediators such as platelet-activating factor (PAF) and leukotriene B_4, both of which share pro-inflammatory activities (Bulger and Maier 2000). Neutrophils mediate their destructive effect through generating reactive oxygen species, through phagocytosis, and by releasing proteolytic enzymes (Dallegri and Ottonello 1997) (Figure 1A).

Circulating monocytes, upon migration into tissues, undergo tranformation into tissue macrophages. In inflammatory processes, tissue macrophages play an important role in modulating and chemoattracting other inflammatory cells because of being the main source of pro-inflammatory cytokines (IL-1β, TNF-α, IL-12) (Norman et al. 1995b, Fink and Norman 1996), chemokines (IL-8, monocyte chemoattractant peptide MCP-1) (Strieter et al. 1999), major anti-inflammatory cytokines (IL-1ra, IL-6, IL-10) (Opal and DePalo 2000), and lipid mediators (PAF, leukotrienes, prostaglandins, and thromboxane) (Bulger and Maier 2000). The principal task of monocytes/macrophages like neutrophils is phagocytosis; they also generate reactive oxygen species. Moreover, monocytes/macrophages play a fundamental role in the immune response by presenting antigens to lymphocytes during the development of specific immunity (Delves and Roitt 2000). Monocytes/macrophages express major histocompatibility complex (MHC)-class II antigens on the cell surface (Koppelman et al. 1997). Expression of MHC class II antigen (human leucocyte antigen, HLA-DR) on monocytes/macrophages is a prerequisite in the presentation of phagocytosed-processed antigen to T-helper cells for the elaboration of a specific immune response (Wolk et al. 2000, Tonegawa 1988) (Figure 1A).

T-lymphocytes

Lymphocytes represent 20% of circulating leucocytes, most of which are T-cells, of which two-thirds are CD4+ (helper) T-cells and the rest CD8+ (cytotoxic) T-cells. Helper T-cells play a pivotal role in development of the specific immune response and mediate their effect either by activating macrophages or by activating B-lymphocytes. Helper T-cells are activated by antigen presented on MHC class II molecules on antigen-presenting cells (Tonegawa 1988). Activated helper T-cells differentiate into either Th1 or Th2 cells, depending on cytokine stimulus (Kelso 1995) (Figure 1). Initially, macrophage-derived pro-inflammatory cytokine IL-12 drives differentiation into Th1 cells, which produce macrophage-activating molecules including IFN-γ,

granulocyte-macrophage colony-stimulating factor (GM-CSF), and TNF-α. Later, anti-inflammatory cytokines (IL-4 and IL-10) favour differentiation into Th2 cells, which inhibit macrophage functions and Th1 responses (through IL-4, IL-10, and IL-13), resulting in the down-regulation of the inflammatory response (Mosmann and Sad 1996). Th2 cells produce B-lymphocyte-activating molecules promoting B-cell-derived antibody production. Cytotoxic T-cells are activated by antigen presented on MHC class I molecules and kill their targets by inducing apoptosis. Activated T-cells produce IL-2, which is the primary stimulator of T-cell proliferation and differentiation (Spellberg and Edwards 2001).

Mast cells

Mast cells are found closely apposed to the vasculature in essentially all tissues. A vast number of stimuli are able to activate mast cells including substance P, complement components, bradykinin, and chemokines (MCP-1 and macrophage inflammatory protein MIP-1α). Upon activation, mast cells release from their intracellular granules vasoactive agents such as histamine, pro-inflammatory cytokines such as TNF-α and proteolytic enzymes such as tryptase (Schwartz 1994) (Figure 1A). Mast cell activation can promote vascular endothelium P- and E-selectin and the intercellular adhesion molecule ICAM-1 expression (Thorlacius et al. 1994, Kubes and Granger 1996) and, specifically, mast cell-derived tryptase can initiate endothelial cell activation by cleaving endothelial PAR-2 (Molino et al. 1997, Coughlin 2000). Early P-selectin-mediated leucocyte-endothelial-cell rolling after tissue trauma is dependent on mast cell degranulation and activation of endothelial cell PAR-2 (Lindner et al. 2000). Indeed, mast cells have been shown to play a critical role in host defense against bacterial infections by modulating neutrophil influx at sites of infection (Echtenacher et al. 1996, Malaviya et al. 1996). In addition, mast cells are an important source of anti-inflammatory cytokine IL-4 and interact with T-helper cell differentiation (Metcalfe et al. 1997).

Platelets

Platelets are activated parallel with endothelial cell and coagulation cascade activation during inflammation. Thrombin can mediate activation of platelets through PAR-1 receptors, which results in increased adhesion molecule expression such as P-selectin on platelet plasma membrane (Coughlin 2000). PAF and interactions with adhesion

molecules on activated endothelial cells may also trigger platelet activation (Repo and Harlan 1999). Activated platelets become adherent, which leads to platelet aggregation and subsequent degranulation of intracellular granules. During degranulation, platelets release a number of cytokines (Wagner and Burger 2003) and growth factors like VEGF (Weltermann et al. 1999). Activated platelets may adhere to circulating phagocytes, and promote leucocyte-endothelial cell adhesion (Repo and Harlan 1999). In conjunction with fibrin, platelets may form a haemostatic plug (Wagner and Burger 2003) and contribute to microcirculatory dysfunction in multiple organ dysfunction syndrome (Gawaz et al. 1997).

Endothelial cells

Endothelium is the lining between blood and tissues. Endothelial cells mediate and modulate vascular permeability, microcirculation, migration of leucocytes into tissues, and blood coagulation. In inflammation, the endothelial cell lining becomes permeable, allowing fluid and plasma protein extravasation. A number of mediators can increase vascular permeability, e.g., bradykinin, substance-P, leukotrienes, and VEGF, some of which are implicated in the pathogenesis of AP (Figini et al. 1997, Grady et al. 2000). In addition, a number of substances (IL-1β, TNF-α, PAF, thrombin, trypsin, tryptase) can activate endothelial cells, which leads to altered expression of cell-surface proteins, increased secretion of pro-inflammatory cytokines and chemokines, and increased vascular permeability (Zimmerman et al. 1999).

Leucocyte recruitment to the site of inflammation involves interactions with endothelial cells. This multi-step process mediated by adhesion molecules involves leucocyte rolling, adhesion, and migration (Figure 1A). Within minutes, upon an inflammatory stimulus, endothelial cells activate and express P-selectin, PAF, and von Willebrand factor (Zimmerman et al. 1999, Aird 2003). This is initiated by activation of the endothelial cell-bound protease-activated receptors PAR-1 and PAR-2 (Coughlin 2000). Expression of E-selectin and IL-8 follows within 2 to 3 hours after cytokine IL-1β and TNF-α activation (Zimmerman et al. 1999). Immunoglobulin superfamily protein (i.e., ICAM-1 and vascular cell adhesion molecule VCAM-1) expression is also induced upon activation. Leucocytes express constitutively L-selectin, and interaction of these endothelial cell selectins and leucocyte selectins initiates rolling of leucocytes in the microcirculation. Rolling promotes activation of

leucocytes by endothelial surface-associated chemokines and chemoattractants such as PAF and IL-8. Upon activation, L-selectin is shed from the cell surface and replaced by the integrin CD11b/CD18 which binds to the ICAM-1 and thus results in firm adhesion of leucocytes to endothelial cells. Leucocyte adherence to endothelium causes increased microvascular permeability (Edens and Parkos 2003), and finally these firmly attached leucocytes transmigrate across the endothelium in postcapillary venules (Repo and Harlan 1999).

Under normal physiologic conditions, endothelium prevents activation of blood coagulation by several mechanisms (Hack and Zeerleder 2001). Inflammatory endothelium, however, turns into a procoagulant surface within hours by losing its anticoagulant properties and by introducing expression of tissue factor that can initiate the coagulation cascade. Activated endothelium also produces and releases plasminogen activator inhibitor (PAI)-1, which inhibits fibrinolysis (Aird 2003). Altered endothelial cell adhesion molecule expression may promote platelet adhesion and activation and consequently formation of intravascular platelet aggregates.

Microvascular blood flow is regulated by endothelium by production of both vasodilators (nitric oxide, NO, and prostacyclin) and vasoconstrictors (endothelins). A number of inflammatory mediators can increase production of NO which results in vasodilation (Hack and Zeerleder 2001). On the other hand, stimulated endothelial cells also produce vasoconstricting endothelins, elevated levels of which have been detected in critically ill patients (Wanecek et al. 2000). Although some of the classical signs of inflammation (erythema and heat) result from vasodilatation, vasoconstriction may become more important in the development of organ dysfunction and pancreatic necrosis (Foitzik et al. 1998).

2.5.3.2 Humoral mediators

Pro-inflammatory cytokines

Cytokines, soluble low molecular-weight proteins secreted not only by inflammatory cells but also by many different cell types, play an important role in regulating the immune response with a wide range of biological effects. Cytokine signalling can be autocrine, paracrine or endocrine, and cytokines can be assigned according to their

effects to either a pro-inflammatory or an anti-inflammatory group, although some of them share both properties. IL-1β and TNF-α are the principal cytokines promoting inflammatory responses (Dinarello 2000) but IL-2, IL-12, IL-18, IFN-γ, and GM-CSF also share pro-inflammatory activities (Figure 1A).

Cells that are typically involved in the immune response can produce IL-1β, most importantly the monocyte-macrophage cell line. IL-1β is synthesized as the inactive precursor pro-IL-1β and is activated by the caspase-1/interleukin-1-converting enzyme (ICE), which also activates pro-IL-18 (Fantuzzi and Dinarello 1999). The principal feature of IL-1β is stimulation of arachidonic acid metabolism. Activation of the IL-1 receptor by IL-1β promotes nuclear translocation of nuclear factor (NF)-κB, leading to increased gene expression of a number of pro-inflammatory mediators including cytokines, chemokines, type II phospholipase A_2 (PLA_2), and adhesion molecules (Dinarello 2000, Abraham 2000). One of the most important properties of IL-1β involves its ability to activate the vascular endothelium and thus facilitate the mobilization, activation, and accumulation of leucocytes for specific localized immune responses (Zimmerman et al. 1999).

Like IL-1β, TNF-α is a multifunctional cytokine acting as a first-line mediator in the pro-inflammatory cytokine cascade (Dinarello 2000). The main source for TNF-α is activated macrophages, but many other cell types can produce it. IL-1β and TNF-α share many biological properties including activation of endothelium (Pober et al. 1996) and promotion of the synthesis of other pro-inflammatory mediators such as IL-8 and PAF (Dinarello 2000). TNF-α is also a potent inducer of anti-inflammatory mediators such as IL-6 and IL-10, resulting a negative feedback loop (Oberholzer et al. 2002). TNF-α signalling occurs through two different receptors, and in patients with AP, soluble forms of the two also occur in high concentrations in the circulation (deBeaux et al. 1996a).

IL-2, produced by activated T-lymphocytes, acts in an autocrine or paracrine fashion through the IL-2 receptor (IL-2R) to stimulate growth and activation of T-lymphocytes (Spellberg and Edwards 2001). Like other interleukin receptors, it exists in a circulating form. Soluble IL-2R (sIL-2R) is released after T-cell stimulation, can

be measured in the circulation, and reflects T-cell activation (Rubin et al. 1985, Rubin and Nelson 1990).

Chemokines

Cytokines with chemotactic properties are called chemokines. These make up a family of small (8–10 kDa), inducible, secreted cytokines with chemotactic and activating effects on various leucocyte subsets. They provide the key stimulus for directing leucocytes to areas of injury (Adams and Lloyd 1997). A number of chemokines have been identified, including IL-8, epithelial neutrophil-activating protein-78 (ENA-78), the growth-related oncogene-α (GRO-α), MCP-1, and MIP-1α and -1β (Strieter et al. 1999). IL-8 is one of the most potent mediators of neutrophil chemotaxis in this family of molecules. The early-response cytokines, TNF-α and IL-1β, are key molecules for inducing IL-8, which is produced by an array of both inflammatory and other cells types including endothelial cells (Striter and Kunkel 1994).

Anti-inflammatory cytokines

Anti-inflammatory cytokines control the pro-inflammatory cytokine response in concert with soluble cytokine receptors. Under physiological conditions, these cytokine inhibitors limit the potentially deleterious effects of sustained or excess inflammatory reactions. Principal anti-inflammatory cytokines include the IL-1ra, IL-4, IL-6, IL-10, IL-11, IL-13, and TGF-β (Opal and DePalo 2000) (Figure 1B).

IL-10 is the most potent anti-inflammatory cytokine (Opal et al. 1998). It inhibits Th1 cytokine production, and down-regulates pro-inflammatory cytokine and chemokine synthesis in monocytes and macrophages (Opal and DePalo 2000). It also promotes the shedding of TNF receptors into the circulation (Joyce et al. 1994). IL-10 down-regulates MHC class II cell-surface expression on monocytes (Koppelman et al. 1997), which results in monocyte anergy and immunosuppression. IL-6 has been regarded as a pro-inflammatory cytokine, but recently it has been shown to act predominantly as an anti-inflammatory cytokine (Opal and DePalo 2000). IL-6 is a potent inducer of acute-phase protein responses in the liver. It also attenuates synthesis of IL-1β and TNF-α (Xing et al. 1998) and promotes synthesis of IL-1ra and release of soluble TNF receptor (Tilg et al. 1994).

IL-1ra, a specific antagonist to IL-1β, binds competitively to the IL-1 receptor and thus blocks IL-1-mediated responses at receptor level; concentrations over 100-fold those of IL-1β can be measured in the circulation (Dinarello 1998). IL-4 is a pleiotropic cytokine which inhibits Th1 responses and suppresses pro-inflammatory cytokine and chemokine production of monocytes/macrophages; it also stimulates synthesis of IL-1ra (Brown and Hural 1997). IL-11 attenuates pro-inflammatory cytokine synthesis in macrophages (Trepicchio et al. 1997). IL-13 down-regulates the production of IL-1β, TNF-α, IL-8, and MIP-1α. In contrast to IL-10, it upregulates MHC class II antigens on monocytes (de Waal Malefyt et al. 1993).

Others

Other humoral mediators of inflammation include complement components; these mediate inflammation by increasing blood vessel permeability, vasodilatation, neutrophil adhesion and activation, and chemotaxis (Hartwig et al. 2001). Kinins such as bradykinin are small vasoactive peptides generated by enzyme cascades and are closely linked to the clotting and complement cascades. Their vasoactive properties include vasodilatation and increased vascular permeability (Griesbacher et al. 2003). A number of lipid mediators are also produced during inflammation, including prostanoids (e.g., prostaglandins), leukotrienes, and PAF. They are not stored in tissues, but are synthesized within seconds in response to stimuli (Bulger and Maier 2000). One, PAF, has a number of pro-inflammatory effects including phagocyte and platelet activation, activation of endothelial cells, and enhancement of vascular permeability (Zhao et al. 2003). PLA_2, the key and rate-limiting enzyme of arachidonic acid metabolism, generates substrates for a number of lipid mediators (Bulger and Maier 2000). Increased circulating concentrations of the extrapancreatic isoform PLA_2-IIA have been detected in many inflammatory diseases, including AP (Grönroos and Nevalainen 1992, Nevalainen et al. 1993, 2000), and it plays an important role in development of systemic inflammatory response syndrome (SIRS) (Bone et al. 1992, Hietaranta et al. 1999) and of distant organ dysfunction in AP (Grönroos and Nevalainen 1992, Tsukahara et al. 1999).

Evidence is also increasing that VEGF may participate in inflammatory processes. VEGF expression is induced by hypoxia (Tuder et al. 1995) and cytokines (Cohen et al. 1996, Thickett et al. 2002), and it is released from activated neutrophils (Gaudry et

al. 1997) and from platelets during clotting (Weltermann et al. 1999). It directly increases vascular permeability and induces endothelial cell-adhesion molecule expression (Senger et al. 1983, Hippenstiel et al. 1998, Kaner et al. 2000), thus supporting the rolling and adhesion of leucocytes; it is also chemotactic for mast cells (Gruber et al. 1995). In addition to vascular permeability, VEGF induces angiogenesis (Senger et al. 1993). Another angiogenic factor in vivo is basic fibroblast growth factor (bFGF). Expression of the bFGF gene is increased in the pancreas of patients with AP and may be involved in pancreas regeneration (Ebert et al. 1999).

2.5.4 Local inflammation and acinar cell death

Acinar cell injury triggers local inflammation of the pancreas, which is characterised by sequestration of neutrophils within the pancreas and by oedema (Nevalainen and Aho 1992). Acinar cell apoptosis or necrosis or both are present. Mild oedematous pancreatitis is associated with acinar cell apoptosis, whereas the predominant finding in severe necrotizing AP is necrosis (Bhatia 2004). The mechanisms of acinar cell apoptosis and necrosis are poorly understood, and several mechanisms may be involved. Following acinar cell injury, activation of transcription factor NF-κB within acinar cells leads to expression of many pro-inflammatory mediators including TNF-α (Steinle et al. 1999). In addition, PAF is very likely generated by phospholipase A_2, which hydrolyzes membrane phospholipids that eventually lead to the release of PAF from acinar cells (Zhou et al 1993). Both PAF and TNF-α have been implicated also in acinar cell apoptosis (Gukovskaya et al. 1997, Sandoval et al. 1996). Intra-acinar cell caspase activation may also play a role in mediating apoptosis in AP (Gukovskaya et al. 2002). Infiltrating neutrophils may be responsible for acinar cell necrosis, through several mechanisms including release of proteolytic enzymes and production of reactive oxygen species (Dallegri and Ottonello 1997, Bhatia 2004). Neutrophil-derived reactive oxygen species have been shown to exacerbate pancreatic injury by facilitating trypsinogen activation (Gukovskaya et al. 2002, Steer 2002). Probably the most likely mechanism in pancreatic necrosis is the inflammation-induced microvascular dysfunction that leads to tissue ischaemia and eventually to necrosis (Sanamura et al. 1998, Foitzik et al. 2002).

The pivotal role of neutrophils in AP is well documented (Sandoval et al.1996), although other cell types are also involved in the activation, chemoattraction, and sequestration of neutrophils. These include macrophages, acinar cells, endothelial cells, T-lymphocytes (Demols et al. 2000), nerve endings, and possibly mast cells (Braganza 2000). In addition to TNF-α and PAF production, injured acinar cells release active protelytic and lipolytic enzymes such as trypsin, elastase, carboxypeptidase A, and lipase, which may also play a role in inflammatory system activation (Jaffray et al. 2000a). Macrophages and infiltrating neutrophils are the main source of pro-inflammatory cytokines (e.g., TNF-α and IL-1β) in the pancreas (Norman et al. 1995b, Fink and Norman 1996). Pancreatic elastase can induce macrophage-derived cytokine and chemokine production (Zhang et al. 2003); trypsin may activate endothelial cells via PAR-2 receptors (Couglin 2000) and is able to activate complement (Acioli et al. 1997).

Substance P and neurokinin are released from nerve endings during the early course of experimental AP, and these may contribute to increased vascular permeability either directly through endothelial cells or via mast-cell activation (Grady et al. 2000, Bhatia et al. 2003). Activation of the kallikrein-kinin system also plays an important role in the early increase in vascular permeability in AP. Acinar cells probably release kallikrein, which catalyses kininogens into kinins such as bradykinin that affect vascular tone and permeability (Griesbacher et al. 2003).

2.6 Pathogenesis of multiple organ dysfunction syndrome

2.6.1 Systemic inflammatory response syndrome

Systemic inflammation in AP is considered to contribute to the development of distant organ dysfunction. Local inflammation is generally tightly controlled at the pancreas. Loss of local control or an overly activated inflammatory response results in an exaggerated systemic response characterised by systemic release of both pro-inflammatory and anti-inflammatory cytokines and activation of circulating leucocytes. It is accompanied by endothelial cell activation in distant organs. Clinically, this response is defined as SIRS (Bone et al. 1992) and is characterised by two or more of the criteria presented in Table 1.

Table 1. Criteria for systemic inflammatory response syndrome (Bone et al. 1992)

Body temperature	>38 ºC or <36 ºC
Heart rate	>90 beats per minute
Respiratory rate	>20 breaths per minute or $PaCO_2$ <4.3 kPa
Leucocyte count	>12 x 10^9/l or <4 x 10^9/l, or 10% immature forms

$PaCO_2$ – arterial partial pressure of carbon dioxide

2.6.1.1 Primary event

The severity of local pancreatic inflammation correlates with development of systemic inflammation. Local inflammation triggers the systemic inflammatory response by a mechanism that is poorly understood, but it may involve systemic release of the pro-inflammatory cytokines IL-1β and TNF-α (Fink and Norman 1995, Norman et al. 1995b, Norman 1998) or other soluble pro-inflammatory mediators (Denham et al. 1997). IL-1β and TNF-α levels correlate with severity in both experimental (Gloor et al. 1998b) and clinical AP (Exley et al. 1992, Chen et al. 1999a, Mayer et al. 2000), and specific antagonisms of both of these, as well as indirect inhibition by anti-inflammatory cytokine IL-10, attenuate systemic inflammatory response and subsequent organ dysfunction (Norman et al. 1995c, Hughes et al. 1996, Kusske et al. 1996). Although a number of studies support the theory that these pro-inflammatory cytokines serve as a link between local and systemic inflammation, other factors may also be involved. Recently, pancreatic elastase has been shown in AP to stimulate pro-inflammatory cytokine synthesis of tissue macrophages, and thus provide an alternative mechanism of systemic activation of inflammation (Jaffray et al. 2000b, 2000c, Murr et al. 2002). Trypsin and activation of circulating trypsinogen in AP may also contribute to development of lung injury (Hartwig et al. 1999).

2.6.1.2 Amplification

Experimental studies have shown that during AP, pro-inflammatory cytokines are synthesized also in distant organs and that this begins several hours later than synthesis of these mediators in the pancreas (Norman et al. 1997). This extrapancreatic cytokine production contributes considerably to systemic cytokine levels (Gloor et al. 2000). From the pancreas, pro-inflammatory mediators are released into portal venous blood, and to a lesser extent into the lymphatic and

systemic circulation via the thoracic duct (Montravers et al. 1995). Portal venous blood enters the liver, which has been shown to play an essential role in development of the subsequent multiple organ failure (Closa et al. 1999, Dhainaut et al. 2001). In AP, the liver may be the source of elevated systemic levels of PLA_2-IIA (Nevalainen et al. 2000, Talvinen et al. 2001). In the liver, tissue macrophages (i.e., Kupffer cells) up-regulate pro-inflammatory cytokine production (Gloor et al. 2000). Blockage or inhibition of Kupffer cell cytokine production in experimental AP reduces systemic cytokine levels, ameliorates lung injury (Folch et al. 2000, Gloor et al. 2000), and improves survival (Gloor et al. 1998). Blockade of the action of early-acting cytokines (TNF-α and IL-1β) has beneficial effects on distant organ injury in experimental settings (Norman et al. 1995a, Hughes et al. 1996). Tissue-derived amplification results in high tissue concentrations of pro-inflammatory cytokines, although plasma concentrations are substantially lower due to their short half-life (Beutler et al. 1985).

2.6.1.3 Distant organ injury

The crucial step in development of distant organ injury is activation of endothelial cells by pro-inflammatory mediators (Strieter and Kunkel 1994). Activated endothelium facilitates neutrophil extravasation and activation (Repo and Harlan 1999) in conjunction with chemokines (Strieter et al. 1999). In tissues, neutrophils promote pro-inflammatory cytokine production (Abraham 2003) and mediate tissue injury through generation of reactive oxygen species and proteolytic enzymes (Dallegri and Ottonello 1997). In severe experimental AP, increased expression of endothelial cell adhesion molecules occurs in the lungs (Lundberg 2000), and blockade of leucocyte-endothelial cell interaction prevents sequestration of neutrophils and ameliorates lung injury (Frossard et al. 1999, Lundberg et al. 2001) and also improves survival (Inoue et al. 1995). Neutrophil-endothelial cell adhesion induces increased endothelial cell permeability (Edens and Parkos 2003), leading to accumulation of protein-rich extracellular fluid that disturbs gas exchange in the lungs (Sznajder and Wood 1991), impairs oxygen delivery to tissues (Leach and Treacher 2002), and results in circulatory hypovolaemia with adverse haemodynamic effects (Isenmann et al. 2003). Procoagulant endothelium and activation of platelets may dispose to formation of a haemostatic plug (Aird 2003) that, in conjunction with microcirculatory dysfunction (Menger et al. 2001, Foitzik et al. 2002), ultimately can lead to tissue ischaemia and result in irreversible organ damage.

2.6.2 Compensatory anti-inflammatory response syndrome

2.6.2.1 Anti-inflammatory cytokines

With synthesis and release of pro-inflammatory mediators, anti-inflammatory cytokines and specific cytokine inhibitors are concomitantly produced. During systemic inflammation, they may either provide insufficient control over pro-inflammatory activities that ultimately lead to distant organ dysfunction or may overcompensate and inhibit the immune response, rendering the host at risk for systemic infection (Bone 1996a). High circulating levels of the anti-inflammatory cytokines IL-6, IL-10, IL-11, and IL-1ra have been documented in experimental (van Laethem et al. 1998, Gloor et al.2000) and clinical AP (Mayer et al. 2000, de Beaux et al. 1996, Simovic et al. 1999, Chen et al. 1999). Gene knockout models of IL-10 and IL-6 have shown that both are important in down-regulating pro-inflammatory cytokine production in AP; the absence of either anti-inflammatory cytokine results in worse survival rates (Gloor et al. 1998, Cuzzocrea et al. 2002). Although during AP a pro-inflammatory reaction is likely to predominate in tissues, in the circulation, anti-inflammatory cytokines prevail over pro-inflammatory ones (Mayer et al. 2000, Cavaillon et al. 2001, Dugernier et al. 2003). Because the synthesis and systemic release of anti-inflammatory cytokines is regulated partly by pro-inflammatory cytokines (Opal and DePalo 2000), high circulating anti-inflammatory cytokine levels may reveal an overwhelming pro-inflammatory reaction in tissues.

Genetic factors may play an important role in regulating the anti-inflammatory response: IL-1ra polymorphism contributes to susceptibility to severe sepsis (Fang et al. 1999), and a polymorphic allele in the IL-10 gene is associated with lower stimulated interleukin-10 release and increased mortality in the critically ill (Lowe et al. 2003).

2.6.2.2 Monocyte deactivation and immunosuppression

A compensatory anti-inflammatory response contributes to development of immunosuppression, which renders the host susceptible to secondary infections. Patients with severe AP show in skin testing an impaired response to recall antigens, denoting a state of cellular immunosuppression (Garcia-Sabrido et al. 1989). Defective host defense mechanisms include functional disturbances in monocytes and

macrophages that include persistent decrease in HLA-DR expression and diminished synthesis of pro-inflammatory cytokines (Döcke et al. 1997). IL-10 may be responsible to some extent for a decreased monocyte HLA-DR expression (Koppelman et al. 1997, Klava et al. 1997, Sachse et al. 1999, Fumeaux and Pugin 2002) leading to impaired antigen presentation capacity which suppresses helper T-cell activation (Tonegawa 1988, Wolk et al. 2000). Decreased HLA-DR expression is associated with adverse outcome including septic complications and increased mortality in trauma and sepsis and in surgical patients (Livingston et al. 1988, Hershman et al. 1990, Cheadle et al. 1991, van den Berk et al. 1997, Tschaikowsky et al. 2002). Moreover, in patients with AP, decreased monocyte HLA-DR expression is related to disease severity (Richter et al. 1999, Gotzinger et al. 2000, Kylänpää-Bäck et al. 2001b) and to septic complications later in the course of the disease (Satoh et al. 2002). According to the two-hit hypothesis of MODS, septic complications in patients with initial overactive SIRS may lead to an exaggerated secondary inflammatory response and possibly to death (Bone 1996a).

2.7 Diagnostic methods

No definitive single method exists to diagnose AP. Its diagnosis is based on clinical presentation in conjunction with laboratory tests or radiology or both. The serum or plasma amylase level is increased within 2 to 12 hours of onset of AP and returns to normal within 3 to 5 days, whereas lipase rises within 4 to 8 hours but remains elevated for 8 to 14 days (Tietz et al. 1993). Elevated amylase is not specific for AP (Clavien et al. 1989), but the specificity for serum amylase in determining AP can be increased by using a cut-off of more than 2- to 3-fold the normal upper limit (Smotkin and Tenner 2002). Urine amylase level or the pancreas-specific isoenzyme of amylase level in plasma can also be measured (Clave et al. 1995). Levels of serum or urinary trypsinogen-2 are elevated in AP (Hedström et al. 1996a, Hedström et al. 1996b), and a rapid urine test is available for diagnosis (Kemppainen et al. 1997, Kylänpää-Bäck et al. 2000). Contrast-enhanced CT is a highly specific method to diagnose AP (Clavien et al. 1988), but in mild forms of the disease findings may be normal (Balthazar et al. 1994), and in the most severe forms of the disease, impaired renal function may limit use of the contrast medium due to its nephrotoxicity.

In these circumstances, magnetic resonance imaging may provide an alternative method with similar results (Hirota et al. 2002, Arvanitakis et al. 2004). Contrast-enhanced CT is the gold standard for diagnosis of pancreatic necrosis (Balthazar 2002); it is useful in localizing abscesses (Mithöfer et al. 1997) and may show the presence of gas in necrotic tissue or in fluid collections, indicating infection. However, diagnosis of infectious complications is based on positive microbiological culture or Gram staining obtained either by fine-needle aspiration or from a surgical sample (Rau et al. 1997).

2.8 Prediction of disease severity

2.8.1 Background

Although no specific therapy for AP is available today, early identification of patients who will develop a severe form of the disease has clinical importance. Some interventions such as emergency ERCP and endoscopic sphincterotomy may be beneficial in severe biliary AP (Neoptolemos et al. 1988, Fan et al. 1993b), and for patients with severe AP, early intensive fluid resuscitation and monitoring may be of benefit (Brown et al. 2002), as is the case in patients with sepsis (Rivers et al. 2001). Delayed admission to an intensive care unit after hospital admission (Brivet et al. 1999) and delayed transferred admission to a specialist centre (de Beaux et al. 1995) have been shown to increase the risk of death. During the first 48 hours, clinical assessment alone of severe AP has shown low sensitivity (34% to 47%) but high specificity (Wilson et al. 1990); therefore, several methods have been developed to improve prediction of the clinical course of AP.

2.8.2 Scoring systems

Ranson and co-workers (1974) provided the first prognostic criteria (Table 2) for predicting severe AP. Since then, a modification for biliary aetiology (Ranson 1982) plus other criteria described by Imrie and colleagues (Imrie or Glasgow score) have been available (Imrie et al. 1978, Osborne et al. 1981, Blamey et al. 1984). The drawback of these scoring systems is a delay of 48 hours in characterising disease severity. Other scoring systems including APACHE II (Knaus et al. 1985, Wilson et al. 1990) and APACHE III (Knaus et al. 1991, Williams and Simms 1999) have been used in prediction of outcome in AP with results similar to those of the Ranson score

(Chatzicostas et al. 2002, Eachempati et al. 2002). APACHE II can be used on admission to hospital, although its complexity limits its routine use (Toh et al. 2000). Multiple organ dysfunction scores have also served for prediction of outcome of severe AP (Halonen et al. 2002), but because they were developed to describe the severity of MODS, they are not useful in early prediction of severe AP.

Table 2. Early objective prognostic signs in AP determined by Ranson in 1974.

At admission or diagnosis
Age > 55 years
White blood cell count > 16 x 10^9/l
Blood glucose level > 200 mg/dl (> 11.1 mmol/l)
Serum lactic dehydrogenase concentration > 350 IU/l
Serum glutamic oxaloacetic transaminase > 250 Sigma-Frankel units/dl
During initial 48 hours
Haematocrit decrease > 10%
Blood urea nitrogen increase > 5 mg/dl (> 1.8 mmol/l)
Serum calcium level < 8 mg/dl (< 2 mmol/l)
Arterial partial pressure of oxygen < 60 mmHg (< 8 kPa)
Base deficit > 4 mmol/l
Estimated fluid sequestration > 6000 ml

2.8.3 Laboratory tests

A great number of laboratory tests have been subjected to examination in predicting the course of AP. These include tests for markers of inflammation and for acute phase proteins, pancreatic enzymes and derivatives, and for other markers; some of these tests are widely available in routine laboratory diagnostics.

2.8.3.1 Acute phase proteins

Among acute phase proteins, CRP has been extensively studied in AP (Puolakkainen et al. 1987, Wilson et al. 1989). The peak CRP value is usually reached within 48 to 72 hours of disease onset and predicts severe AP at least as well as do complex scoring systems (Wilson et al. 1989, Chen et al. 1999). However, the increase in CRP

relatively late in the course of AP makes it inefficient for early assessment of severity (Sandström and Borgström 2002). Although CRP values of >150 mg/l 48 h after onset of symptoms indicate severe AP (Dervenis et al. 1999), the optimal CRP cut-off level in predicting severe AP depends on the timing of measurement (Müller et al. 2002, Sandström and Borgström 2002), and for prediction of organ failure cut-off level may differ. Serum amyloid-A is another acute phase protein shown to predict severe AP, performing at least as well as CRP (Pezzilli et al. 2000, Mayer et al. 2002).

2.8.3.2 Inflammatory mediators

The prognostic roles of a variety of inflammatory mediators in AP have been studied extensively. Among pro-inflammatory cytokines, high IL-1β and IL-18 levels may have an impact on prediction of severe disease (Mayer et al. 2000, Rau et al. 2001, Wereszczynska-Siemiatkowska 2002), whereas levels of TNF-α are usually undetectable in clinical samples (Exley et al. 1992, Paajanen et al. 1995). Although elevated levels of IL-12 are documented in patients with AP, little is known about its role in predicting severity (Pezzilli et al. 1999). Circulating levels of soluble cytokine receptors such as TNF-receptors and sIL-2R rise early, are related to systemic complications in AP, and may be more accurate predictors than is CRP (de Beaux et al. 1996a, Mayer et al. 2000). IL-8 and other chemochines such as GRO-α, ENA-78, and MCP-1 have also been tested in prediction of a complicated course of AP, and show good predictive power (Rau et al. 1997a, Shokuhi et al. 2002, Rau et al. 2003).

Several studies of AP have shown IL-6 to be an early marker of severity (Viedma et al. 1992, de Beaux et al. 1996, Brivet et al. 1999, Simovic et al. 1999). Elevation of circulating IL-6 precedes the acute phase response (Leser et al. 1991, Heath et al. 1993) and may predict systemic complications and death (Mayer et al. 2000). Compared to pro-inflammatory cytokines, anti-inflammatory cytokines such as IL-6 might serve as more useful factors for early prediction of prognosis (Chen et al. 1999a). High circulating IL-1ra levels occur in patients with severe AP (Brivet et al. 1999), and serum IL-1ra may serve as an early marker of severe AP (Mayer et al. 2000). Several studies have shown that high circulating IL-10 is related to severe AP and fatal outcome (Mayer et al. 2000, Simovic et al. 1999, Brivet et al. 1999, Chen et al. 1999), although some conflicting data also exist (Pezzilli et al. 1997). IL-10 levels peak within the first 24 hours of disease onset and thus may provide a better signal

than CRP in early assessment of severity (Chen et al. 1999). Although elevated serum IL-11 levels appear in patients with severe AP, in its prediction, IL-11 appeared inferior to IL-10 (Chen et al. 1999).

Procalcitonin, a marker of systemic inflammation and sepsis (Vincent 2000), has also been studied in patients with AP. Several studies (Rau et al. 1997a, Kylänpää-Bäck et al. 2001a, 2001c), but not all (Frasquet et al. 2003), indicate its possible role in predicting severe AP. Elevated catalytically active PLA_2 levels reflect severity of AP (Puolakkainen et al. 1987, Viedma et al. 1992). Although pancreatic parenchyma also secretes PLA_2 in AP, now it is known that elevated levels of catalytically active PLA_2 in AP are mainly of non-pancreatic origin, representing mainly an inflammation-inducible type II PLA_2 (Nevalainen et al. 1993, Hietaranta et al. 1999).

Several markers of endothelial cell activation such as soluble forms of adhesion molecules (E-selectin, P-selectin, ICAM-1) and soluble trombomodulin are elevated in AP and may serve as predictors of the complicated course of the disease (Kaufmann et al. 1999, Powell et al. 2001, Mantke et al. 2002, Wereszczynska-Siemiatkowska et al. 2003), although some inconsistency exists (Kingsnorth et al. 1995, Kylänpää-Bäck et al. 2001c). An early event in AP is phagocyte activation, confirmed by elevated polymorphonuclear elastase concentration (Uhl et al. 1991) or by increased monocyte or neutrophil surface antigen CD11b expression (Kylänpää-Bäck et al. 2001b). Phagocyte activation has been shown to reflect disease severity and may serve as an early predictor of severe AP (Wereszczynska-Siemiatkowska et al. 2003). A decrease in monocyte HLA-DR expression occurs early in the course of AP, reflects disease severity (Kylänpää-Bäck et al. 2001b, Richter et al. 1999), and may be a predictive marker of sepsis (Satoh et al. 2002), but it has not been evaluated in early prediction of systemic complications.

2.8.3.3 Pancreatic proenzymes and derivatives

Although circulating amylase levels are not helpful in assessment of AP severity (Ranson 1982), many other pancreas-derived substances have been demonstrated to predict severe disease. These include trypsinogen-2 (Hedström et al. 1996b, Lempinen et al. 2001), trypsinogen activation peptide (TAP) (Neoptolemos et al. 2000, Kemppainen et al. 2001), carboxypeptidase B activation peptide

(CAPAP)(Müller et al. 2002), and trypsin-2-alpha-1-antitrypsin complex (Hedström et al. 2001). The advantage of these markers is that they can be detected early in the course of the disease; in addition, they are specific for AP. Compared to CRP, both urinary TAP and trypsinogen-2 seem to be slightly better in predicting severe AP (Lempinen et al. 2003, Neoptolemos et al. 2000), providing, however, only a marginal benefit over CRP (Windsor 2000).

2.8.3.4 Miscellaneous

Laboratory markers included among the Ranson criteria may also be used independently or in different combinations for predicting severe AP (Fan et al. 1993a). Low platelet count (Ranson et al. 1977) and high serum creatinine and aspartate aminotransferase (AST) may also indicate severe AP (Fan et al. 1993a). All these markers are signs of AP's systemic manifestations. High levels of creatinine or urea reflect renal dysfunction. Hyperglycaemia may be a consequence of increased gluconeogenesis in hepatocytes (Dhainaut et al. 2001) and of impaired endocrine function of the pancreas. Lactate dehydrogenase (LDH) is an intracellular enzyme detectable in virtually all tissues. A study of LDH isoenzymes in AP suggests that elevated levels are mainly of extrapancreatic origin (Chen et al. 1992) and thus may reflect cell damage in distant organs such as the lungs. Hypocalcaemia in sepsis and AP may be a consequence of sequestration of circulating calcium and albumin into extracellular space due to increased microvascular permeability (Bhattacharya et al. 1985, Carlstedt et al. 2000), although other mechanisms may be involved, as well (Zaloga 2000). Low serum calcium alone has been shown to predict organ failure within 48 hours after ERCP (Kawa et al. 2000). However, markers that reflect systemic alterations in homeostasis may not be predictive of organ dysfunction, but instead suggest its dynamic nature (Cryer et al. 1999, Buter et al. 2002).

2.8.4 Radiology

Contrast-enhanced CT is not only diagnostic, but can serve as a prognostic tool. Determination of pancreatic necrosis (Kivisaari et al. 1983), its extent (Isenmann et al. 1999) or its localization to the head of the pancreas (Kemppainen et al. 1996) may indicate a more severe course. The Balthazar CT grading and severity index correlates with clinical outcome in AP (Balthazar et al. 1985, Balthazar et al. 1990), and in predicting severe AP, CT severity index may be even better than other severity

indexes (Chatzicostas et al. 2003). However, CT is inaccurate in predicting organ failure (Chatzicostas et al. 2003), because organ failure occurs in only half the patients with pancreatic necrosis (Tenner et al. 1997). MRI could also serve in severity assessment with an accuracy similar to that of CT and may prove useful in clinical situations where CT is contraindicated (Arvanitakis et al. 2004).

2.9 Treatment

No specific medical treatment for AP exists. Treatment is mainly conservative, consisting of fluid resuscitation in the early phase of the disease, monitoring of organ function, and supportive treatment for organ failures (Yousaf et al. 2003). Results from three trials concerning ERCP in biliary AP (Neoptolemos et al. 1988, Fan et al. 1993b, Folsch et al. 1997) suggest that early ERCP and endoscopic spincterotomy are mandatory for patients with cholangitis or biliary obstruction. Several studies (Sainio et al. 1995, Nordback et al. 2001) including meta-analyses (Villatoro et al. 2004) have shown that prophylactic antibiotic treatment in severe AP reduces mortality and risk for infected necrosis. Recently, the first double-blind placebo-controlled trial involving patients with severe AP (Isenmann et al. 2004) showed no benefit from prophylactic ciprofloxacin-metronidazole combination in severe AP; that there were only five patients with infected necrosis in the control group suggests, however, that the series were overcrowded with mild cases. Although enteral nutrition seems to be well tolerated (McClave et al.1997) and may reduce infectious complications and severity of AP (Kalfarentzos et al. 1997, Windsor et al. 1998, Gupta et al. 2003), data are insufficient to allow firm conclusions as to its effectiveness and safety (Al-Omran et al. 2003).

Pancreatic necrosis should be managed non-operatively (Bradley and Allen 1991, Foitzik et al. 1995). Surgical necrosectomy should be considered in cases of infected pancreatic necrosis (Rau et al. 1997b, Beger and Isenmann 1999), although non-operative management in some of those patients may be successful (Nordback et al. 2001, Adler et al. 2003). Patients with abdominal compartment syndrome (Gecelter et al. 2002) or with uncontrollable intra-abdominal haemorrhage or gut necrosis benefit from early surgical intervention, but for other indications the consensus is that it is

best to delay surgery; no generally accepted consensus exists, however, on the exact indications for and timing of any intervention.

Several experimental studies and clinical trials have tried to discover medical treatment to prevent development of multiple organ dysfunction or ameliorate already existing multiple organ failure. In experimental models of AP, anti-inflammatory therapy has shown promising results in preventing severe AP (Norman et al. 1995a, Rongione et al. 1997) and subsequent organ failure and death (Hughes et al. 1996), but clinical trials have shown inconsistent results or have failed (Kingsnorth et al. 1995, McKay et al. 1997, Johnson et al. 2001, Deviere et al. 2001, Dumot et al. 2001) – resembling the results of anti-inflammatory trials in patients with sepsis (Fisher et al. 1994, Freeman and Natanson 1995).

Among possible ways to inhibit systemic inflammation and the development of organ dysfunction in the complex network of pro-inflammatory and anti-inflammatory mediators and intercellular interactions, are anti-inflammatory therapy with IL-10 (Kusske et al. 1996, Rongione et al. 1997), IL-1ra (Norman et al. 1995a, Tenaka et al. 1995), anti-TNF antibody (Hughes et al. 1996), or PAF antagonist (Kingsnorth et al. 1995, McKay et al. 1997, Johnson et al. 2001), or anti-adhesion therapy with anti-ICAM-1 antibody (Lundberg et al. 2001). Activated protein C has both antithrombotic and anti-inflammatory properties and has shown promising results in treatment of severe sepsis (Bernard 2003) and might also prove beneficial in severe AP. Endothelin receptor blockage can reduce capillary leakage in AP and improve microcirculation (Foitzik et al. 1998, Eibl et al. 2002).

It is possible that in the complex network of inflammation, no single method is sufficient to prevent organ failure, and an early administration of combination of drugs, each with a distinct mechanism of action, may produce better results (Norman 1998). However, in clinical AP, patients usually present at a fairly late stage of the disease, when SIRS and organ dysfunction are already present (Johnson et al. 2001). Organ dysfunction may thus not be preventable, although some therapies can inhibit organ deterioration. Later in the course of AP, a compensatory anti-inflammatory reaction results in immunosuppression, and anti-inflammatory therapies may become harmful (Opal et al. 1998, Oberholzer et al. 2002). In order to recover from

immunosuppression, patients may instead benefit from pro-inflammatory treatment (Volk et al. 1996, Döcke et al. 1997, Kox et al. 1997), a fact which makes it important to monitor patients' immunoinflammatory state (Volk et al. 1999).

2.10 Summary

The inflammatory basis of AP and subsequent organ failure is indisputable, although exact mechanisms remain to be established. In addition to the inflammatory mediators already known, in the pathogenesis of AP, other factors may also be involved. The compensatory anti-inflammatory response syndrome and immunosuppression may play important roles in the development of infectious complications in patients with AP, and their clinical relevance needs to be evaluated. In predicting severity of AP, inflammatory mediators seem promising. However, most studies involving predictive markers have ignored the timing of development of organ failure and are predicting outcome when organ failure may already have occurred (Cryer et al. 1999). This was the case in a recent multi-center trial of the PAF-antagonist Lexipafant, which predicted severe AP as an APACHE II score $\geq$7, which led to inclusion of patients most of whom already had organ dysfunction; this ruined the primary end-point of the study (Johnson et al. 2001). Most studies with predictive markers have predicted severe AP according to the Atlanta classification, although the clinically more relevant issue would have been prediction of organ failure. Moreover, some studies have been mainly descriptive, have lacked any comparison to the gold standard, and have drawn conclusions from a relatively small number of patients without a significant difference between markers. Additional studies are therefore needed to address these issues.

3 The present investigation

3.1 Aims of the study

The main purpose of the present study was to find markers or a marker profile to predict the development of organ failure in patients with AP. Specific aims were:

1. To explore whether serum levels of mast cell-derived tryptase and VEGF correlate with development of organ dysfunction in AP.
2. To study the time-course of humoral and cellular markers of immunosuppression in relationship to outcome of AP.
3. To investigate whether prognostic markers or their combination predicts organ failure in AP and when these markers should be measured.

3.2 Materials and Methods

3.2.1 Patients

The local ethics committee approved the study protocols, and informed consent was obtained from each patient. All patients studied had AP and were admitted to Helsinki University Central Hospital within 72 hours of symptom onset. The first study included 70 non-consecutive patients with AP admitted between August 1997 and May 2000; 31 had mild disease; of the 39 with severe AP, 21 developed organ failure. The second study included 314 consecutive AP patients admitted between September 1998 and July 2001. In the third study, we collected prospectively 238 consecutive patients admitted between August 1998 and October 2000; 147 had mild and 91 had severe disease; 20 of the latter developed organ failure. The study included the first consecutive 27 of the 147 patients with mild AP, the first consecutive 27 of the 71 patients with severe AP with local complications only, and all 20 patients who developed organ failure. The fourth case-control study was based on 351 consecutive AP patients admitted between August 1998 and January 2002. Of these, 33 developed organ failure and were included in the study as cases. Three age- and sex-matched controls for each case, with admission dates closest to that of the case, were selected from among the remaining 318 AP patients, resulting in 99 control patients.

3.2.2 Diagnosis and classification

Diagnosis of AP was based on typical clinical findings including acute onset of epigastric pain, nausea and vomiting, and elevated serum amylase concentration at least 3-fold the upper reference limit or was based on typical appearance of AP on computed tomography, or was based on a combination of these criteria. Patients were retrospectively categorised into those with mild AP or severe AP according to the Atlanta classification (Bradley 1993). Patients with severe disease were further subcategorised into those with only local complications, recovering without organ failure, versus those developing organ failure. Organ failure was defined as respiratory failure necessitating mechanical ventilation or renal failure necessitating haemodialysis, or both. Criteria for initiating mechanical ventilation were tachypnoea (respiratory rate > 35/minute) or need for inspiratory oxygen fraction (FiO2) > 0.6 in order to maintain arterial partial pressure of oxygen (PaO_2) > 8 kPa, or both. Haemodialysis was started in patients with significant reduction in renal function indicated by increased concentrations of serum creatinine (> 300 mmol/l) and serum urea (> 40 mmol/l) and progressive metabolic acidosis (pH < 7.28) in serial measurements with or without anuria or oliguria (urine output < 500 ml /24 hours).

3.2.3 Scoring systems

Three scoring systems were used to describe patients' clinical condition. Appropriate physiological and laboratory data were collected to calculate Ranson score (Table 2) (Ranson et al. 1974) (I, II, III), Multiple Organ Dysfunction Score (MODS) (Table 3) (Marshall et al. 1995) (I, II, III), and APACHE II score (Table 4) (Knaus et al. 1985) (I-IV).

Table 3. Multiple Organ Dysfunction Score (MODS) (Marshall et al. 1995)

	SCORE				
Organ system	0	1	2	3	4
Respiratory: PaO_2/FiO_2, mmHg (kPa)	>300 (>40)	226-300 (30.1-40)	151-225 (20.1-30)	76-150 (10.1-20)	≤75 (≤10)
Renal: Serum creatinine, µmol/l	≤100	101-200	201-350	351-500	>500
Hepatic: Serum bilirubin, µmol/l	≤20	21-60	61-120	121-240	>240
Cardiovascular: PAR, l/min	≤10	10.1-15	15.1-20	20.1-30	>30
Haematological: platelet count (E9/l)	>120	81-120	51-80	21-50	≤20
Neurological: Glasgow Coma Score	15	13-14	10-12	7-9	≤6

FiO_2, fraction of inspired oxygen; PaO_2, arterial partial pressure of oxygen; PAR, pressure adjusted heart rate = heart rate multiplied by the ratio of central venous pressure to the mean arterial blood pressure

Table 4. APACHE II score (Knaus et al. 1985)

	High abnormal range					Low abnormal range			
Physiological variable	4	3	2	1	0	1	2	3	4
Temperature, rectal (°C)	≥41	39-40.9		38.5-38.9	36-38.4	34-35.9	32-33.9	30-31.9	≤29.9
Mean arterial pressure (mmHg)	≥160	130-159	110-129		70-109		50-69		≤49
Heart rate	≥180	140-179	110-139		70-109		55-69	40-54	≤39
Respiratory rate	≥50	35-49		25-34	12-24	10-11	6-9		≤5
Oxygenation									
a)FiO_2>0.5 A-aDO_2 (kPa) A-aDO_2=FiO_2x95-$PaCO_2$-PaO_2	≥66.7	46.7-66.6	26.7-46.6		<26.6				
b)FiO_2<0.5 PaO_2					>9.3	8.1-9.3		7.3-8.0	<7.3
Arterial pH	≥7.7	7.6-7.69		7.5-7.59	7.33-7.49		7.25-7.32	7.15-7.24	<7.15
Serum sodium (mmol/l)	≥180	160-179	155-159	150-154	130-149		120-129	111-119	≤110
Serum potassium (mmol/l)	≥7	6.0-6.9		5.5-5.9	3.5-5.4	3.0-3.4	2.5-2.9		<2.5
Serum creatinine (μmol/l)	≥300	169-299	124-168		53-123		≤52		
Haematocrit (%)	≥60		50-59.9	46-49.9	30-45.9		20-29.9	<20	
Leucocyte count (E9/l)	≥40		20-39.9	15-19.9	3-14.9		1.0-2.9	<1	
Glasgow Coma Score (GSC) Score=15 – actual GCS									
Age points: age ≤44, 0 points; 45-54, 2 points; 55-64, 3 points; 65-74, 5 points; and ≥75, 6 points									

FiO_2, fraction of inspired oxygen; A-aDO_2, alveolar-arterial oxygen difference; PaO_2, arterial partial pressure of oxygen; $PaCO_2$, arterial partial pressure of carbon dioxide

3.2.4 Analytical methods

3.2.4.1 Blood samples

Blood samples were taken by venipuncture at admission (I-IV), and on days 1, 2, and 7 after admission (I), or on days 1, 2, 3, 7, 14, and 21 after admission (III) unless the patient was discharged earlier from hospital. Blood samples for flow cytometry and for plasma measurements were anticoagulated with pyrogen-free acid-citrate-dextrose (ACD) (Terumo Europe N.V., Leuven, Belgium) (II, III, IV). Immediately after withdrawal, these samples were cooled in an ice-cold water bath and then kept at 0 °C until processed for flow cytometry within 24 hours. From the rest of the sample, plasma was separated by centrifugation at +4 °C and stored in aliquots at –70 °C. Blood samples for serum measurements were taken concurrently. After coagulation and centrifugation of these samples, they were either tested directly as a part of the hospital laboratory routines (II, IV), or the aliquots of serum were frozen and stored at –20°C (I).

3.2.4.2 Flow cytometry

Measurement of phagocyte surface marker expression was done with a FACSort flow cytometer and CellQuest software (Becton Dickinson, San Jose, CA, USA). CD11b expression was measured in neutrophils and monocytes (IV) and HLA-DR expression in monocytes (II, III, IV).

Reagents

Monoclonal antibodies (mAb) were as follows: anti-CD14 fluorescein isothiocyanate (FITC) conjugated mAb ($IgG_{2b,}$ clone MFP9), anti-HLA-DR phycoerythrin (PE) conjugated mAb ($IgG_{2a,}$ clone L243), anti-CD11b PE-conjugated mAb (IgG_{2a}, clone D12), and mouse IgG_{2a} PE-conjugated mAb. FACS lysing solution was used for cell washing and red cell lysing. All reagents were from Becton Dickinson.

Cell labelling

Paired 25-µl aliquots of each blood sample were stained with saturating concentrations of mAbs. To measure monocyte surface marker expression, the first sample was double-labelled with FITC-conjugated anti-CD14 mAb and PE-conjugated anti-HLA-DR mAb or anti-CD11b PE-conjugated mAb, and the other sample with FITC-conjugated anti-CD-14 mAb and PE-conjugated irrelevant mouse IgG_{2a} mAb. For neutrophil CD11b expression measurements, the first sample was labelled with anti-CD11b PE-conjugated mAb and the other with PE-conjugated irrelevant mouse IgG_{2a} mAb. After staining, the non-bound mAb molecules were removed by washing, then red cells were lysed with FACS lysing solution, and the cell pellet was resuspended in ice-cold 0.5% formaldehyde in saline.

Measurement of expression

Monocytes were identified on the basis of their CD14-positive fluorescence and light-scatter properties, and neutrophils based on their light-scatter properties. CD11b antigen expression was determined from 5 000 neutrophils and 2 000 monocytes, and results were presented in relative fluorescence units (RFU), which was the geometric mean channel of the CD11b PE fluorescence histogram.

HLA-DR antigen expression of monocytes was evaluated by determining the HLA-DR fluorescence intensity of monocytes (II) and the proportion of positively fluorescing monocytes (II, III, IV). An HLA-DR histogram and a mouse IgG_{2a} (control) histogram of 2000 monocytes were developed. Monocyte HLA-DR fluorescence intensity, expressed as RFU, was obtained by subtracting the median channel number of the IgG_{2a} histogram from the median channel number of the respective HLA-DR histogram. The proportion of HLA-DR-positive monocytes was measured in two ways: Firstly, by a threshold method (II) an electronic gate was set

manually so that it included the brightest 3 to 5% of the cells stained with mouse IgG_{2a} mAb. Then the same gate served to determine the proportion of positively fluorescing cells in the respective sample stained with anti-HLA-DR mAb. Laboratory technicians unaware of each patients' clinical status performed this step. Secondly, by a modified histogram subtraction method (Overton 1988) (II, III, IV) the control histogram (mouse IgG_{2a}) was smoothed and then subtracted from the respective HLA-DR histogram (Figure 2). The histogram differential represents HLA-DR-positive monocytes, the proportion of which was calculated by CellQuest software.

3.2.4.3 Immunoassays and other laboratory tests

Serum tryptase measurements were done with fluoroimmunoassay UniCAP Tryptase (Pharmacia & Upjohn, Uppsala, Sweden) (I). Plasma levels of IL-1ra, IL-4, IL-10, and IL-11, and serum levels of VEGF and bFGF were determined by enzyme-linked immunosorbent assay (ELISA) kits (Quantikine, R&D Systems, Minneapolis, MN, USA) (I, III, IV). Plasma levels of IL-13 were determined by ELISA kit (CytElisa, Alpco Diagnostics, Windham, NH, USA) (III). Plasma levels of IL-1β, IL-6, and sIL-2R were determined by a chemiluminescent immunoassay (Immulite®, DPC, Los Angeles, CA, USA) (III, IV). Plasma procalcitonin levels were determined by immunoluminometric assay (LUMItest®, Brahms Aktiengesellschaft, Berlin, Germany) (IV). All measurements were done according to the manufacturers' protocols. Serum levels of AST, calcium, creatinine, CRP, glucose, LDH, urea, and blood haematocrit, leucocyte, and platelet counts were determined as part of hospital laboratory routines (II, IV).

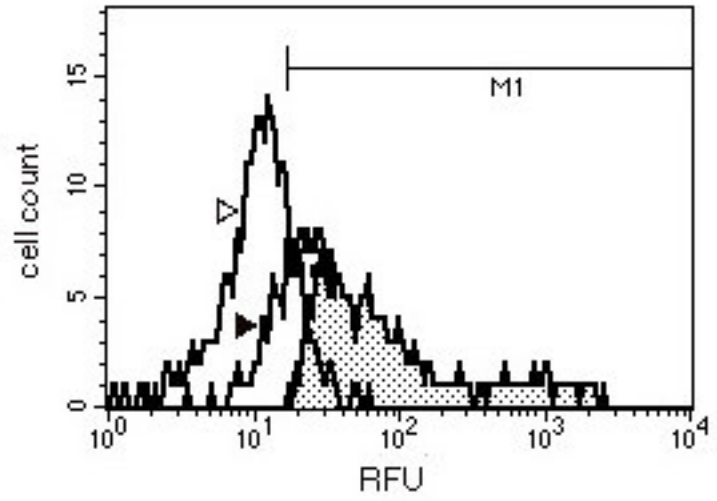

Figure 2. Flow cytometric determination of proportion of HLA-DR-positive monocytes by channel-by-channel subtraction. The control histogram (▷) was overlaid by and subtracted from the HLA-DR histogram (▶). An electronic gate (M1) was set at the intersection. The differential histogram (dotted area) indicates the proportion of HLA-DR-positive monocytes (65%). RFU, relative fluorescence units.

3.2.5 Statistical analysis

Results were expressed as median and inter-quartile range (IQR). For comparisons of three groups, the Kruskall-Wallis test (II, III), Cuzik's test for trend (II), or the Jonkheere-Terpstra test for trend (III) were used. Comparisons between two groups were done with the Mann-Whitney U test (I, III, IV). P-values were adjusted by the Bonferroni method; P-values less than 0.05 were considered significant (I-IV). The time course of inflammatory mediators was tested with Friedman's test (I, III), with Dunn's test used in *post hoc* comparisons (I). Fisher's exact test (I) or chi-square tests (III) were used to compare proportions of patients between two groups. Study of correlations between cytokine levels and monocyte HLA-DR expression was done by Pearson correlation and linear regression analysis (III). The Spearman rank correlation coefficient served in determining the relationship between percentage of HLA-DR-positive monocytes and HLA-DR fluorescence intensity (II), and in studying correlations between marker levels and interval to development of organ failure (IV). Agreement of the proportion of HLA-DR-positive monocytes between repeated measurements by the threshold method and between the threshold method and channel-by-channel subtraction method were evaluated with the intraclass correlation coefficient and its 95% confidence interval (CI) (II). Logarithmic

transformation or exponential transformation allowed normalisation of distributions of predictors when necessary (III, IV). Logistic regression analysis was used to find independent predictors (III, IV). To study whether combined markers provided better prognostic accuracy, the markers were combined with the Boolean operator 'or' (III, IV).

Optimal cut-off values for markers, with the corresponding sensitivities, specificities, and positive and negative likelihood ratios with 95% CI were determined by receiver operating characteristic (ROC) curves (II, III, IV). Areas under the ROC curves (AUC) (II, III, IV) with corresponding 95% CI were calculated (II, IV). The diagnostic odds ratio with 95% CI (IV) or accuracy (III) was determined for each predictor at the optimal cut-off level. The optimal cut-off point of a ROC curve is the point at which the slope R satisfies the equation $R = C/B \times (1 - P)/P$, where C is net cost of treating non-diseased individuals, B is net benefit of treating diseased individuals, and P is prevalence of disease. An estimation of C/B ratio was made, and the cut-off point for each marker was selected at which R = 1, which is equivalent to the point where the sum of sensitivity and specificity was maximized (IV) (Cantor et al. 1999).

Comparison of sensitivities and specificities was done by the method described by Newcombe (2001) (IV). This method displays point and 95% CI of differences in sensitivity and specificity (Δ) between two paired tests weighted by mixing parameter λ (range 0–1) ($\lambda = 1/(1 + R)$), which takes into account the prevalence together with C/B ratio.

3.3 Results

3.3.1 Mast cell tryptase, VEGF, and bFGF (I)

Of the 70 patients studied, tryptase levels were within normal limits (<13.5 µg/l) in 65 (93%) patients at all time points. However, the peak tryptase level was above the upper normal reference limit in 4 of 21 AP patients with organ failure but in only one of 49 patients without organ failure (P=0.026). Peak levels of tryptase were significantly higher in patients with organ failure (6.6 µg/l, IQR 4.8 to 12.6) than in

patients not developing organ failure (4.0 µg/l, IQR 2.7 to 6.2) (P=0.018). Furthermore, at day 2 after symptom onset, tryptase level was higher in organ-failure patients (6.0 µg/l, IQR 4.5 to 7.4) than in patients who did not develop organ failure (3.4 µg/l, IQR 2.3 to 4.7) (P=0.006). Serum concentrations of VEGF and bFGF were within normal limits at admission, but levels increased towards the end of the observation period (P<0.001), and in post hoc comparisons, day 7 concentrations were significantly higher than concentrations on days 0, 1, and 2 (P<0.001 and P<0.05, respectively). The time-course of VEGF and VEGF levels for each day in organ-failure patients and other patients were similar.

3.3.2 Monocyte HLA-DR expression in predicting organ failure (II)

The two methods, the channel-by-channel subtraction method and the threshold method, were evaluated for proportion of HLA-DR-positive monocytes. The intraclass correlation coefficient for the two assays was 0.96 (95% CI 0.95-0.97). The channel-by-channel subtraction method was preferred to the threshold method and used in further analysis, because it resulted in more reproducible values than did the threshold method. Monocyte HLA-DR expression decreased significantly with increased disease severity, as shown in Table 5. The proportion of HLA-DR-positive monocytes correlated with HLA-DR fluorescence intensity (r=0.89; 95% CI 0.86-0.91).

Table 5. Monocyte HLA-DR expression from 310 patients with AP at admission to hospital

HLA-DR expression	Group I (n=194)	Group II (n=87)	Group III (n =29)	P value
Positive monocytes (%)	90 (80, 95)	84 (66, 91)	62 (49, 76)	< 0.001
Density (RFU)	90 (53, 143)	65 (34, 115)	20 (13, 49)	< 0.001

Values are median (IQR). P values were obtained by Cuzik's test for trend and adjusted by Hommel's method. Group I, patients with mild AP; Group II, patients with severe AP, but no organ failure; Group III, patients with severe AP and subsequent organ failure. RFU, relative fluorescence units.

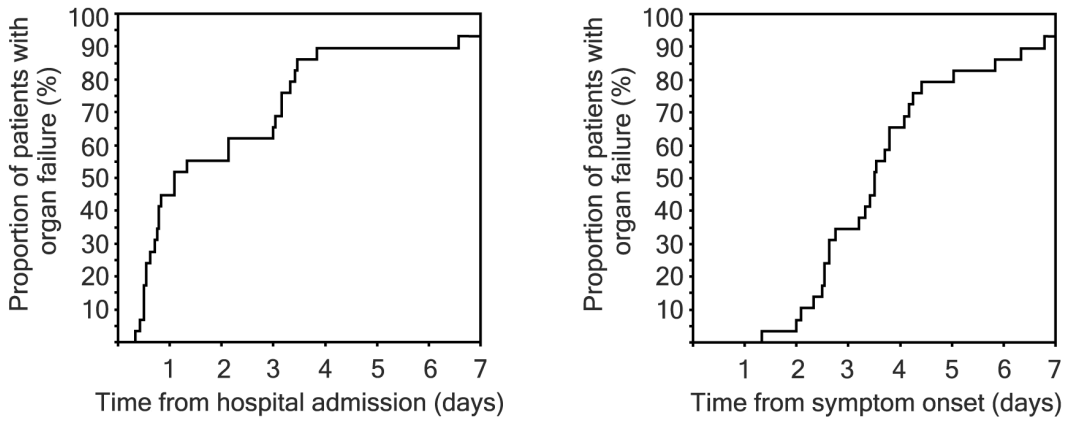

Figure 3. Development of organ failure during the first 7 days after hospital admission and after symptom onset in 29 patients with severe AP and subsequent organ failure.

Of the 29 patients with organ failure, in 27 (93%) it developed within the first week and in 13 (45%) within 24 h of admission (Figure 3). In predicting organ failure at admission, areas under the ROC curve for the percentage of HLA-DR-positive monocytes, HLA-DR fluorescence intensity, APACHE II, and CRP were a respective 0.78 (95% CI 0.66-0.87), 0.81 (0.71-0.89), 0.79 (0.69-0.86), and 0.80 (0.69-0.88). Table 6 shows optimal cut-off values with corresponding sensitivities, specificities, and positive likelihood ratios for the variables.

Table 6. Performance of the variables in predicting organ failure in patients with acute pancreatitis at admission to hospital

Measurement	Cut-off value	Sensitivity (%)	Specificity (%)	PLR
HLA-DR expression				
Positive monocytes	≤ 78%	83 (64 - 94)	72 (67 - 77)	3.0 (2.2 - 3.8)
Density	≤ 33 RFU	69 (49 - 85)	84 (79 - 88)	4.3 (2.9 - 6.1)
APACHE II score	≥ 7	76 (56 - 90)	73 (67 - 78)	2.8 (2.0 - 3.6)
CRP	≥ 57 mg/l	83 (64 - 94)	74 (68 - 79)	3.2 (2.5 - 4.1)

Values in parentheses are 95% CI; PLR, positive likelihood ratio; RFU relative fluorescence units

3.3.3 Anti-inflammatory response and immunosuppression (III)

Plasma concentrations of IL-6 (Figure 4A), IL-10 (Figure 4B), and IL-1ra (Figure 4C) increased significantly along with increasing disease severity at days 0, 1, 2, 3, and 7. During follow-up, plasma concentrations of IL-10 in Groups II and III were highest at

day 0 and then decreased by day 3 (P<0.001, Friedman's test), whereas the respective IL-6 and IL-1ra concentrations did not differ significantly between days 0, 1, 2, and 3. IL-4, -11, and -13 levels were below the detection limit in most of the samples (data not shown). Monocyte HLA-DR expression decreased significantly along with increasing disease severity at days 0, 1, 2, 3, 7, and 14 (Figure 4D).

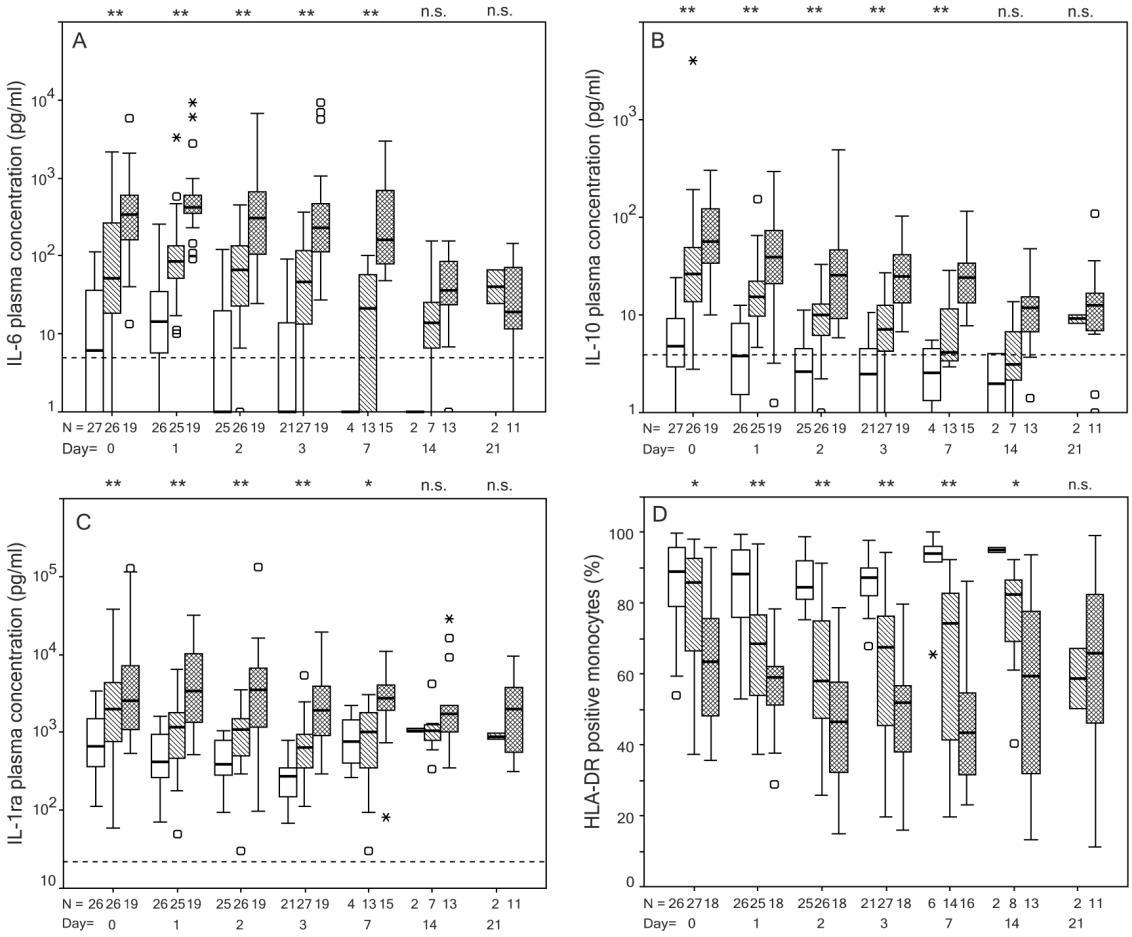

Figure 4. Plasma concentrations of IL-6 (A), IL-10 (B), and IL-1ra (C) and percentage of HLA-DR-positive monocytes (D) at days 0, 1, 2, 3, 7, 14, and 21 after hospital admission. Patients with mild AP (Group I) ☐, severe AP patients not developing organ failure (Group II) ▨, and organ-failure patients (Group III) ▩. Box-whisker plots show median, interquartile range, highest and lowest values, outliers, and extremes. Dashed line is sensitivity level of the assay. N is number of patients. Statistical significance for trend between the three groups at different time-points: * P < 0.01, ** P < 0.001, n.s. = not significant.

The proportion of HLA-DR-positive monocytes at day 2 correlated with peak IL-1ra, IL-6, and IL-10 concentrations during days 0, 1, and 2 (r = –0.47, r= –0.65, r= –0.67, respectively; P<0.001 each). In stepwise linear regression analysis, both IL-6 and

IL-10 were independent predictors of monocyte HLA-DR expression (P=0.013 and P=0.003, respectively), and these explained 50% of the variability of HLA-DR expression. The prevalence of severely depressed monocyte HLA-DR expression increased along with disease severity and was highest among patients with secondary infections (Table 7). For each of the nine patients with infectious complications, a control patient was chosen. Controls were matched by length of hospital stay and by group. The percentage of HLA-DR-positive monocytes value did not differ significantly at its nadir between infected patients and controls: median 27% (IQR 16%-45%) versus 36% (24%-50%), P=0.354, but it did differ at day 14: 32% (23%-61%) versus 65% (40%-80%), P=0.035; n=7, and at day 21: 49% (36%-69%) versus 83% (62%-96%), P=0.025; n=6.

Table 7. Clinical outcome in relation to lowest level of monocyte HLA-DR expression. Number of patients (%).

Outcome (number of patients)	Level of monocyte HLA-DR expression (Percentage of HLA-DR positive monocytes)		
	Severe depression (<50%)	Mild depression (50-80%)	Normal (>80%)
Mild AP (n=27)	0 (0%)	13 (48%)	14 (52%)
Severe AP, no organ failure (n=27)	13 (48%)	13 (48%)	1 (4%)
Severe AP with organ failure (n=20)	16 (80%)	4 (20%)	0 (0%)
Infectious complication (n=9)	8 (89%)	1 (11%)	0 (0%)

By stepwise logistic regression analysis, both IL-6 and IL-10 were independent predictors of organ failure at admission. IL-6 >130 pg/ml or IL-10 >39 pg/ml predicted development of organ failure at hospital admission with a sensitivity of 95%, and specificity of 88%. In diagnosing severe AP according to the Atlanta classification, independent predictors by logistic regression analysis were IL-10 and APACHE II. Admission IL-10 >12 pg/ml had a sensitivity of 91%, specificity of 89%, and post-test probability of 84% in diagnosing severe AP. In patients with organ dysfunction, peak levels of IL-10 and IL-1ra were significantly higher in the six non-survivors than in the fourteen survivors: median 122 pg/ml (IQR 110-342) versus 54 pg/ml (29-117), P=0.017 and median 65.9 ng/ml (IQR 7.6-129.9) versus 5.7 ng/ml (3.1-9.4), P=0.032, respectively. The nadir proportion of HLA-DR-positive monocytes in organ-failure patients was 34% (IQR 23-47%), which did not differ significantly between non-survivors and survivors.

3.3.4 Early prediction of organ failure with combined markers (IV)

Of the conventional markers, serum calcium, glucose, and creatinine were identified as the factors with independent significance (Table 8). Likewise, of the markers of inflammation, IL-10 and IL-6 independently predicted organ failure (Table 8). Finally, when these five predictors were applied to stepwise backward logistic regression analysis, only IL-10, glucose, and calcium were independent predictors of organ failure (Table 8).

Table 8. Independent predictors of organ failure by logistic regression analysis

Predictors	Odds ratio	P
Conventional markers*		
Calcium[†]	0.63 (0.5, 0.8)	0.001
Glucose[‡]	12 (2.7, 58)	0.001
Creatinine[§]	31 (2.6, 375)	0.007
Markers of inflammation**		
IL-6[§]	4.7 (1.8, 12)	0.002
IL-10[§]	5.5 (1.6, 18)	0.006
Independent markers[††]		
Calcium[†]	0.63 (0.5, 0.8)	<0.001
IL-10[§]	8.1 (2.4, 28)	<0.001
Glucose[‡]	6.2 (1.3, 30)	0.024

Values in parentheses are 95% confidence intervals. [*]CRP, LDH, AST, creatinine, urea, glucose, calcium, and platelet count; [**]monocyte HLA-DR and CD11b expression, neutrophil CD11b expression, IL-1β, IL-1ra, IL-6, IL-10, sIL-2R, and procalcitonin; [††]Calcium, glucose, creatinine, IL-6, and IL-10 were included in the analysis; [†]exp-transformed, [‡]natural log-transformed, [§]10-base log-transformed

The regression model: $g(X) = -4.117 + 2.097 \times \log10(X_1) - 0.47 \times \exp(X_2) + 1.821 \times \ln(X_3)$, where X_1 is plasma IL-10 concentration, X_2 is serum calcium concentration, and X_3 is serum glucose concentration had a larger AUC (0.91; 95% CI 0.85 - 0.98) in ROC analysis than did IL-10, calcium or APACHE II (Figure 5). IL-10 >50 pg/ml or calcium <1.65 mmol/l was the best marker combination (Figure 5, Table 9). This combination had a positive predictive value of 56% and a negative predictive value of 99%. Respective values for the logistic regression model at the optimal cut-off point were 51% and 98%. The combination of IL-10 and calcium had significantly better diagnostic accuracy than IL-6 (when cost/benefit ratio, C/B, was less than 0.18), IL-10 (when C/B was more than 0.044), calcium (when C/B was less than 0.17) or APACHE II (when C/B was more than 0.028) (Figure 6).

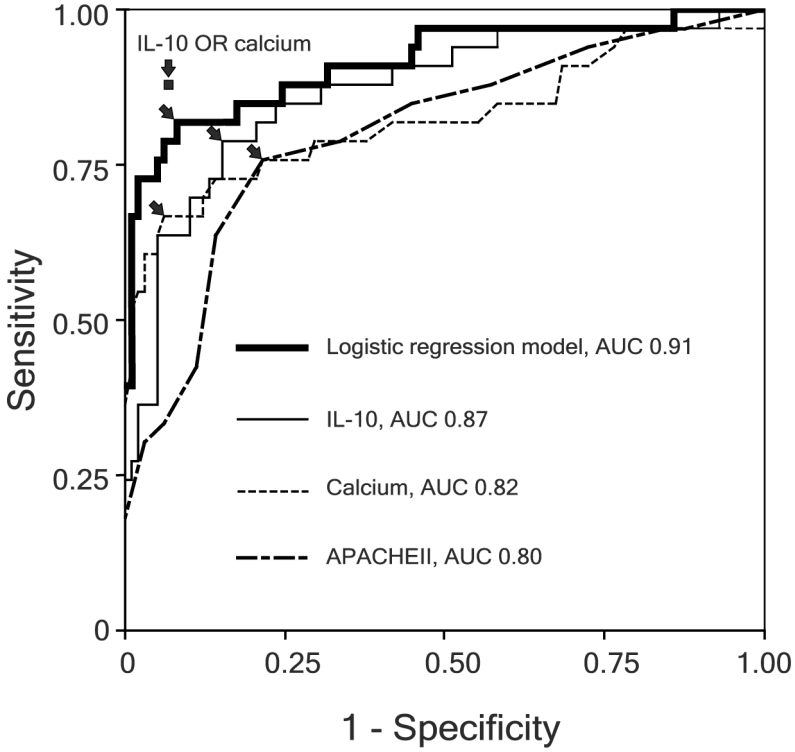

Figure 5. ROC curves for the regression model with IL-10, calcium, and glucose, and for IL-10, calcium, and APACHE II. The symbol represents sensitivity and specificity of a combination of IL-10 >50 pg/ml or calcium <1.65 mmol/l. Arrows show optimal cut-off points for the regression model (−0.55), IL-10 (>27.2 pg/ml), calcium (<1.82 mmol/l) and APACHE II (≥7). AUC, area under curve.

Table 9. Diagnostic performance of predictors

Predictor	Cut-off	Sensitivity (%) (95% CI)	Specificity (%) (95% CI)	PLR (95% CI)	NLR (95% CI)	Odds ratio (95% CI)
IL-10 or calcium	>50 pg/ml or <1.65 mmol/l	88 (71, 96)	93 (85, 97)	12 (6.0, 25)	0.13 (0.05, 0.33)	94 (26, 345)
Regression model*	>−0.55	82 (64, 92)	92 (84, 96)	10 (5.0, 20)	0.20 (0.10, 0.41)	51 (16, 159)
Calcium	<1.82 mmol/l	67 (48, 81)	94 (87, 97)	11 (4.8, 25)	0.36 (0.22, 0.58)	31 (10, 92)
IL-6	>132 pg/ml	73 (54, 86)	91 (83, 95)	8.0 (4.1, 15)	0.30 (0.17, 0.52)	27 (10, 75)
LDH	>750 U/l	77 (58, 90)	86 (77, 92)	5.7 (3.3, 9.7)	0.26 (0.14, 0.50)	22 (8, 60)
IL-10	>27.2 pg/ml	79 (61, 90)	85 (76, 91)	5.2 (3.2, 8.6)	0.25 (0.13, 0.48)	21 (8, 56)
Monocyte HLA-DR	<78%	82 (64, 92)	75 (65, 83)	3.2 (2.2, 4.7)	0.24 (0.12, 0.50)	13 (5, 36)
Glucose	>8 mmol/l	79 (61, 90)	78 (68, 85)	3.5 (2.4, 5.3)	0.27 (0.14, 0.53)	13 (5, 34)
Monocyte CD11b	>210 RFU	87 (68, 96)	66 (55, 75)	2.5 (1.8, 3.5)	0.20 (0.08, 0.51)	12 (4, 39)
APACHE II	≥7	76 (57, 88)	78 (68, 85)	3.4 (2.2, 5.2)	0.31 (0.17, 0.57)	11 (4, 28)
CRP	>108 mg/l	70 (51, 84)	83 (74, 89)	4.1 (2.5, 6.6)	0.37 (0.22, 0.62)	11 (4, 27)
Creatinine	>98 μmol/l	70 (51, 84)	83 (74, 89)	4.1 (2.5, 6.6)	0.37 (0.22, 0.62)	11 (4, 27)
Urea	>5 mmol/l	84 (66, 94)	66 (56, 75)	2.5 (1.8, 3.4)	0.24 (0.11, 0.55)	10 (4, 29)
sIL-2R	>710 U/ml	85 (67, 94)	62 (51, 71)	2.2 (1.7, 2.9)	0.25 (0.11, 0.56)	9 (3, 25)
IL-1β	>2.65 pg/ml	79 (61, 90)	69 (58, 77)	2.5 (1.8, 3.5)	0.31 (0.16, 0.60)	8 (3, 21)
AST	>45 U/l	88 (71, 96)	51 (40, 61)	1.8 (1.4, 2.2)	0.24 (0.09, 0.61)	7 (2, 23)
Platelet count	<152 x10^9/l	58 (39, 74)	83 (74, 89)	3.4 (2.0, 5.7)	0.51 (0.34, 0.77)	7 (3, 16)
Procalcitonin	>1.1 ng/ml	73 (54, 86)	72 (62, 80)	2.6 (1.8, 3.7)	0.38 (0.22, 0.67)	7 (3, 16)
Neutrophil CD11b	>260 RFU	77 (57, 89)	62 (51, 72)	2.0 (1.4, 2.8)	0.38 (0.19, 0.73)	5 (2, 14)
IL-1ra	>930 pg/ml	82 (64, 92)	53 (42, 63)	1.7 (1.3, 2.2)	0.35 (0.16, 0.73)	5 (2, 13)

*Logistic regression model of IL-10, calcium, and glucose; PLR, positive likelihood ratio; NLR, negative likelihood ratio

Only calcium level correlated significantly with onset of organ failure (Spearman´s rank correlation coefficient = 0.449, P=0.026). In the subgroup of patients with symptoms for less than 24 hours before hospital admission, levels of IL-6 and IL-10 differed significantly between organ failure cases and control patients: median 57 (IQR: 36-588) versus 11 (5-48) pg/ml for IL-6 (P=0.035), and median 65 (30-196) versus 8 (4-18) pg/ml for IL-10 (P=0.002), respectively.

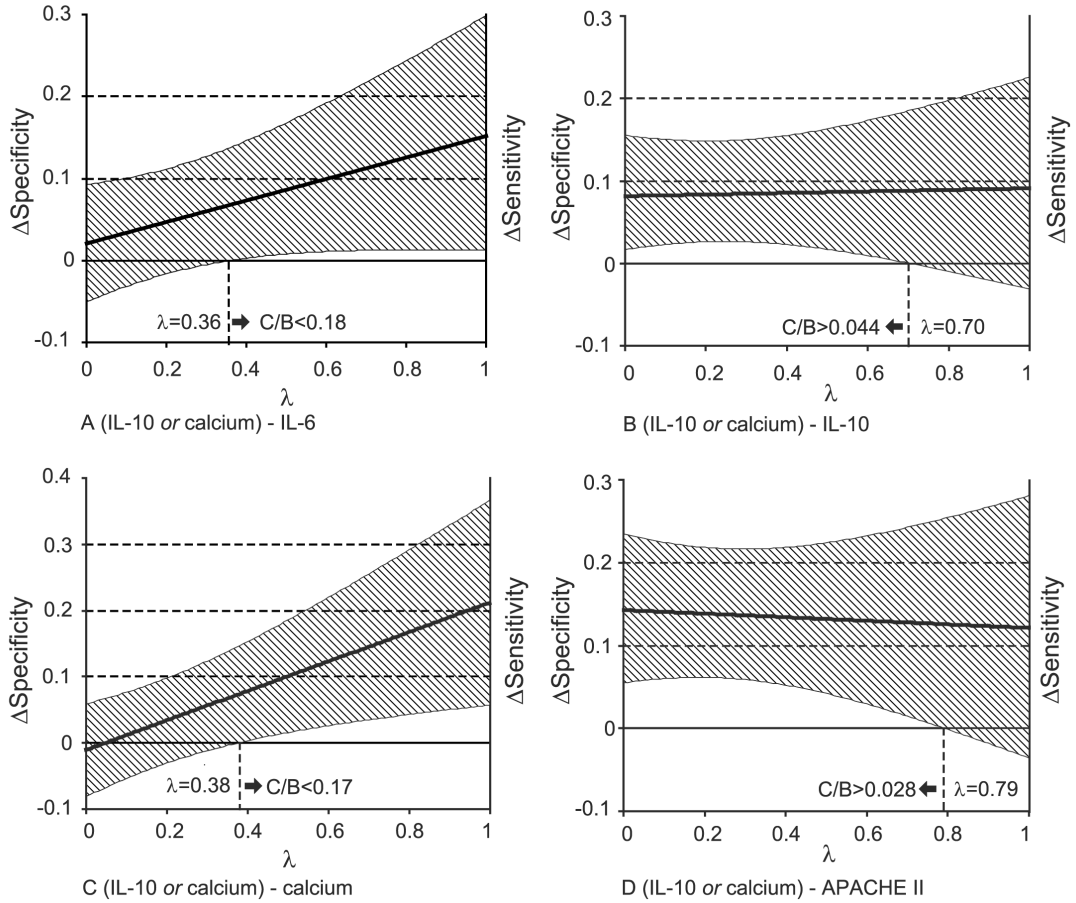

Figure 6. Difference (Δ) in sensitivity and specificity weighted by λ of two tests and its 95% confidence interval (shaded area). **A.** Difference between combination of IL-10 >50 pg/ml or calcium <1.65 mmol/l and IL-6 >132 pg/ml; **B.** Difference between combination of IL-10 >50 pg/ml or calcium <1.65 mmol/l and IL-10 >27.2 pg/ml; **C.** difference between combination of IL-10 >50 pg/ml or calcium <1.65 mmol/l and calcium <1.82 mmol/l; **D.** difference between combination of IL-10 >50 pg/ml or calcium <1.65 mmol/l and APACHE II ≥ 7. Dashed vertical line shows λ-levels where lower 95% confidence limit crosses zero. λ-level at intersection and corresponding cost/benefit ratios (C/B) where significant difference is present are shown.

3.4 Discussion

3.4.1 Mast cell tryptase, VEGF, and bFGF (I)

Increased vascular permeability and development of tissue oedema are pathognomonic features of AP. In mild AP, oedema is confined to the pancreas and peripancreatic tissues, whereas in severe AP complicated with organ dysfunction, the development of massive systemic oedema is common. Strong evidence has accumulated that mast cell activation may promote endothelial barrier dysfunction by releasing pro-inflammatory cytokines such as TNF-α (Thorlacius et al. 1994, Kubes and Granger 1996). In addition, mast cells upon activation release histamine, prostaglandins, and tryptase, all of which can promote the inflammatory reaction. Furthermore, activation of peripheral mast cells induces remote organ dysfunction in the lungs (Mukundan 2001), and in many models of experimental AP (Yonetci et al. 2001, Dib et al. 2002a, 2002b), mast cells have been implicated in the systemic manifestations of AP. In the present investigation, the peak tryptase levels were higher in patients with organ dysfunction than in patients with mild AP or severe AP with local complications only. Interestingly, these levels were also higher at day 2 after onset of symptoms in patients who later developed lung injury than they were in patients without subsequent remote organ dysfunction. This finding raises the question whether in AP, tryptase, a potent agonist of vascular endothelium (Molino et al. 1997, Coughlin 2000), contributes to the development of remote organ dysfunction. The origin of increased levels of tryptase is unclear. Tryptase may originate from the activated mast cells from inflamed pancreatic tissue or from activated lung mast cells in particular, as lung is a rich source of mast cells. Thus, severe AP may lead to activation of lung mast cells, which would release tryptase along with other pro-inflammatory mediators. However, whether the tryptase is derived from the pancreas or lung or both, the result would be increased permeability of the lung endothelium barrier, leading to organ damage.

Although increased serum VEGF levels in organ-failure patients were expected, we were unable to observe any relationship between VEGF levels and disease severity. However, a significant increase in VEGF concentration at day 7 after admission was evident, and the time-course of the increase resembled that of bFGF, indicating their

involvement in the pancreatic regeneration process. Since all patients with organ dysfunction experienced respiratory failure, and this developed earlier than did increase in VEGF concentrations, it is unlikely that systemic release of VEGF plays a major role in alteration of pulmonary microvascular permeability and development of the lung injury in AP.

The difference in tryptase levels between patient groups, although significant, appeared marginal and at hospital admission was not significant. In short, the results showed that for clinical AP neither serum tryptase nor VEGF seems to be a useful prognostic marker.

3.4.2 Anti-inflammatory response and immunosuppression (II, III)

At the early stage of severe AP, a reactive state in the blood develops, which is characterised by the co-occurrence of pro-inflammatory cytokines, anti-inflammatory cytokines, and emerging immune suppression, as defined by depressed monocyte HLA-DR expression. In the present investigation (III), an early and sustained elevation in IL-1ra, IL-6, and IL-10 plasma levels in patients with severe AP was in accordance with that of earlier studies (Brivet et al. 1999, Mayer et al. 2000). The highest level of IL-10 was detected at admission and decreased thereafter, whereas IL-1ra and IL-6 levels did not differ significantly during the first 4 days. In contrast to Chen and co-workers (1999), we detected no IL-11 in most of the patients; levels of IL-4 and IL-13 also proved to be low and showed no logical pattern in relation to AP severity.

Monocyte HLA-DR expression was depressed significantly along with increasing severity of AP (II, III). This early decrease in expression in patients with severe disease is in accordance with earlier findings (Ricter et al. 1999, Kylänpää-Bäck et al. 2001b). HLA-DR expression was already depressed at admission in patients with subsequent organ failure (II, III) and reached its nadir by day 2 after admission (III). Possible factors that reduce its expression may include IL-10, because IL-10 promotes intracellular retention of HLA-DR molecules in monocytes (Koppelman et al. 1997, Fumeaux and Pugin 2002). In other clinical disorders including sepsis and burn injury (Lin et al. 1994, Sachse et al. 1999), an inverse correlation occurs between circulating IL-10 level and monocyte HLA-DR expression. We found that in patients with AP,

HLA-DR expression was related inversely to IL-10 level, and, in addition, to the levels of IL-6 and IL-1ra; but only IL-6 and IL-10 independently predicted its expression in multiple linear regression analysis (III). Monocytes with low HLA-DR density show functional defects (Döcke et al. 1997, Wolk et al. 2000) which may lead to increased susceptibility to infections. Indeed, AP patients in a sustained anergic state are more susceptible to secondary infections than are non-anergic controls (Garcia-Sabrido et al. 1989). In accordance with this, we and others (Satoh et al. 2002) showed that failure to recover initially depressed monocyte HLA-DR expression by the end of the second week was related to secondary infection (III). Moreover, no secondary infections occurred in patients with normal expression (III). In contrast to previous findings, low monocyte HLA-DR expression was not related to fatal outcome in organ-failure patients (III).

Of the several methods used to measure monocyte HLA-DR expression (Livingston et al. 1988, Hershman et al. 1990, Wakefield et al. 1993, van den Berk et al. 1997, Ditschkowski et al. 1999, Sachse et al. 1999, Giannoudis et al. 1999), none has established a gold standard for measurement of the surface density of HLA-DR molecules. The threshold method, commonly used to determine the proportion of HLA-DR-positive monocytes with flow cytometry, is subjective, because the lowest level of positive fluorescence is set manually. In patients with decreased HLA-DR density, the control and test histograms have considerable overlap, rendering the threshold method unreliable (Overton 1988, Lampariello 1994, Watson 2001). The histogram subtraction method has thus been proposed as an objective option (Overton 1988). We showed that the proportion of HLA-DR-positive monocytes can be measured objectively with the histogram subtraction method and that the proportion correlated with HLA-DR fluorescence intensity, each of which show a similar correlation with clinical outcome (II). However, the correlation plot (II, Figure 3) revealed that a few samples show a considerable discrepancy between the HLA-DR-positive proportion of monocytes and HLA-DR fluorescence intensity. Thus, neither of the two methods used to define monocyte HLA-DR expression appears to be perfect.

Higher anti-inflammatory cytokine IL-6 and IL-10 levels appear in patients with fatal AP than in patients who recover (Brivet et al. 1999, Mayer et al. 2000). In accordance

with this, plasma IL-10 level at day 1 and peak IL-10 level were both significantly higher in non-survivors than in survivors (III). A similar difference was evident also in IL-6 levels, although was not significant. Furthermore, we found significantly higher IL-1ra levels in patients with fatal AP than in surviving patients (III). A number of experimental studies suggest that in AP, the anti-inflammatory cytokines IL-10, IL-6, and IL-1ra may have a protective effect (Norman et al. 1995a, Gloor et al. 1998, Cuzzocrea et al. 2002). The paradoxical relationship between high circulating levels of anti-inflammatory cytokines and severe disease can be explained by the compartmentalization of cytokine production: Anti-inflammatory activity takes place in circulating compartments, whereas in tissues such as the lungs, a pro-inflammatory state appears to prevail (Cavaillon et al. 2001, Dugernier et al. 2003). Because locally produced pro-inflammatory mediators induce an anti-inflammatory counter-reaction in the blood, circulating levels of anti-inflammatory cytokines may thus reflect the overall severity of the inflammatory response syndrome, whereas the pro-inflammatory reaction in tissues eventually determines patient outcome.

In summary, levels of circulating anti-inflammatory cytokines may reflect systemic activation of the inflammatory response, and their high levels are related to organ failure and possibly to fatal outcome. The initial inflammatory challenge in early severe AP is countered by the "compensatory anti-inflammatory response syndrome" (Bone 1996), which is characterised by a decrease in HLA-DR expression on peripheral blood monocytes and an impaired immune response. The anti-inflammatory state reflects the disease severity and is related to secondary infection in the later course of the disease, but is not related to early death from non-septic multiple organ failure.

3.4.3 Prediction of organ failure (II, III, IV)

Most organ failure develops during the first week of AP. We found it to begin to develop after the end of the second day after disease onset, and nearly half the organ failures manifested during the first day of hospital admission. This early development has important implications for the current use of severity markers for AP, for the search for novel predictors of organ dysfunction, and for the design of therapeutic studies aimed at altering the course of systemic inflammation in AP in order to prevent the development of organ failure (Johnson et al. 2001). Organ failure

predictors thus need to be measured within hours of admission, and preferably within the first 2 days after symptom onset.

Multi-factorial scoring systems like the Ranson score (Ranson et al. 1974) and Glasgow scores (Blamey et al. 1984) require follow-up for 48 hours, which negates their use in AP as predictors of organ failure. The advantage of APACHE II is that it requires no follow-up, but it may be too complex to accomplish in clinical practice on admission to hospital (Toh et al. 2000). In the present study, a number of single markers were equal to APACHE II, or were even better in predicting organ failure (II, III, IV).

In AP, immunosuppression develops so early that monocyte HLA-DR expression can serve as a predictor of organ failure. Expression determined either by proportion of HLA-DR-positive cells or by HLA-DR fluorescence intensity predicted organ dysfunction at admission as well as did CRP or APACHE II (II, IV). However, the positive likelihood ratio was lower than 5 for these three markers, indicating only a small change in probability and little advantage in clinical decision-making (Windsor 2000). Furthermore, the optimal cut-off for proportion of HLA-DR-positive monocytes (≤78%) in predicting organ failure at admission was far from lowest level (median 34%). Similarly, cut-offs for CRP (≥57 mg/l in II or >108 mg/l in IV) at admission were not near peak levels (median 345 mg/l in patients with severe AP, Mayer et al. 2002). These levels appeared not infrequently after, and not before, occurrence of organ failure. Due to their relatively slow time course, HLA-DR and CRP thus may not be ideal markers in its prediction.

Time of presentation and possibly individual differences may affect levels of cytokines or other markers. Combining predictors may have reduced the impact of these effects; we found that combining markers improved prediction of organ failure. Among anti-inflammatory cytokines, the combined markers (IL-6 >130 pg/ml or IL-10 >39 pg/ml) provided better sensitivity than did any single cytokine (III). Among conventional markers of severity and inflammation markers together the best combined markers were IL-10 >50 pg/ml or calcium <1.65 mmol/l, a combination with a positive likelihood ratio (12), negative likelihood ratio (0.13), and odds ratio (94) better than any of the single markers. The combination of IL-10 and calcium had

significantly better sensitivity (88%) and specificity (93%) than did any of the single markers or APACHE II when compared together and weighted by a clinically relevant C/B ratio (IV). In clinical practice, this combined test may have a great impact on decision-making and may in future provide a means by which to select patients who might benefit from special treatment modalities.

Levels of cytokines rise early in the course of both experimental and clinical severe AP (Van Laethem et al. 1998, Mayer et al. 2000), which facilitates their use as early predictors of organ dysfunction. In the current study, patients who developed organ failure had significantly elevated IL-6 and IL-10 levels compared to levels of other patients with AP within the first 24 hours of symptom onset (IV). At admission, these tests predicted development of organ failure equally well, having positive likelihood ratios over 5 (III, IV). Furthermore, IL-10 was one of the independent predictors of organ failure in logistic regression analysis involving 19 prognostic markers (IV). In predicting severe AP according to the Atlanta classification, IL-10 >12 pg/ml at admission showed very good diagnostic accuracy (90%) and post-test probability (84%) (III). It may be that circulating IL-10 levels reflect the initial activation of systemic inflammation in AP that ultimately leads to organ dysfunction, and so IL-10 represented an early independent predictor of organ dysfunction.

Serum calcium level appeared to be the best single marker in predicting organ failure (odds ratio 31), but in accordance with earlier findings (Kawa 2000), subgroup analysis showed that this was not true until 24 hours after symptom onset. Calcium and other conventional markers predictive of clinical organ failure later in the course of AP may not be predictive in terms of pathophysiology but rather may reflect the presence of organ dysfunction. Massive vascular leakage is one of the main pathophysiological events preceding organ failure (Lehr et al. 2000). Increased vascular permeability facilitates sequestration of circulating calcium and albumin into the extracellular space, resulting in hypocalcaemia (Bhattacharya et al. 1985, Carlstedt et al. 2000). A correlation of calcium level with clinical onset of organ failure suggests that low calcium levels may denote microvascular dysfunction and therefore ongoing organ dysfunction that will soon manifest itself clinically.

In conclusion, early development of organ failure in AP suggests that its prediction should take place as early as upon hospital admission. The best single predictor of organ failure among a number of predictive tests available in routine clinical practice is serum calcium. Combined with serum calcium, IL-10, an early marker of systemic inflammation, is superior to single markers in predicting organ failure in AP, and this combination test could be of considerable value in clinical practice.

3.4.4 Importance of inflammatory state in future treatments

Despite increased knowledge of the pathogenesis of AP and of multiple organ dysfunction syndrome, little progress has occurred in treatment of patients with severe AP. To affect the clinical course of AP, we need to be able to identify the patient at risk for developing organ dysfunction very early, then to attempt to modulate the early pro-inflammatory response, then to abolish the subsequent immunosuppression later in the course of the disease. The current study presented a method with combined markers for early identification of these patients at risk. Although a number of anti-inflammatory mediators for modulation of early pro-inflammatory response are at present available, data from clinical trials involving AP are limited, and an optimal drug or combination of them for this purpose remains to be established. However, because a considerable number of patients with AP seek medical help only after a relatively long delay after symptom onset, the therapeutic window for modulating the early pro-inflammatory response may be too narrow (Johnson et al. 2001). Future trials with anti-inflammatory drugs should probably concentrate on those patients admitted within the first 24 hours after onset of AP, when organ dysfunction has not yet begun to develop. In addition, those patients who develop organ failure and survive the first week might be candidates for immunostimulatory therapy, aimed at preventing secondary infections and late mortality.

3.5 Conclusions

1. Mast cell activation may play a role in the development of remote organ dysfunction in patients with AP. However, in clinical AP, neither serum tryptase nor VEGF may serve as predictors of organ dysfunction.

2. Anti-inflammatory response in AP developed early and may in part be responsible for development of immunosuppression. A strong initial anti-inflammatory response was related to organ failure and fatal outcome. IL-6 and IL-10 may be useful in prediction of organ failure as early as within the first 24 hours of disease onset.

3. Organ failure developed early in the course of AP, not infrequently during the first day of hospital admission. Prediction of organ failure should therefore take place within hours of admission. At hospital admission, monocyte HLA-DR expression predicted organ failure as well as did CRP and APACHE II score, however, IL-10 combined with serum calcium was superior to single markers or APACHE II score in predicting organ failure in AP, and thus may offer valuable support in clinical decision-making.

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