Expression of activation, adhesion molecules and intracellular cytokines in acute pancreatitis

A. Bhatnagar a, J.D. Wig b, S. Majumdar a,*

a Department of Experimental Medicine and Biotechnology, Postgraduate Institute of Medical Education and Research, Chandigarh 160 012, India
b Department of Surgery, Postgraduate Institute of Medical Education and Research, Chandigarh 160 012, India

Received 3 November 2000; received in revised form 25 January 2001; accepted 8 March 2001

Abstract

Adhesion and activation molecules as well as cytokines play an important role in an immune scenario. In acute pancreatitis, we have studied some of these in order to evaluate dysregulation. For this we took peripheral blood mononuclear cells and pancreatitis tissue cells. We analysed activation markers like CD69, CD25 and HLA-DR and found a marked elevation of CD69 as well as CD25 in both peripheral blood cells and tissue mononuclear cells when compared to controls. In PBMC-CD69: P < 0.01 and CD25: P < 0.01; in tissue-CD69: P < 0.001 and CD25: P < 0.001. The HLA-DR levels, however, were reduced in the disease state (in acute pancreatitis patient blood (P < 0.01) and tissue cells (P < 0.001)). The adhesion molecules showed unanimous rise in the blood and the tissue samples. In blood samples, CD11a: P < 0.05 and CD11b: P < 0.05 and tissue samples CD11a: P < 0.01 and CD11b: P < 0.01 and CD54 in peripheral blood (P < 0.05) and tissue (P < 0.01) of AP was high as compared to controls. By simultaneous flowcytometric analysis, we determined the co-expression of a surface marker (CD4/CD8/CD14) and intracellular cytokine (TNF-α and IFN-γ) in individual cells. The IFN-γ producing CD8+ T cells were elevated in pancreatic tissue (P < 0.01). TNF-α producing cell numbers were significantly higher in tissue cells than in blood and also in CD8+ T cells (P < 0.001). We conclude that monocyte function is affected in AP as shown by reduced HLA-DR numbers and lowered TNF-α producing cells. Moreover, the CD8+ T cells appear to play an important role in cytokine synthesis at the effector site. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Intracellular cytokines; Activation and adhesion molecules; Acute pancreatitis

1. Introduction

Pancreatitis is a serious disease with causes that largely remain obscure and treatment of which is mainly supportive. The mechanism(s) by which the inflammation occurs is unclear and much remains to be investigated. Immune surveillance depends on the circulation of leukocytes between the bloodstream and the extravascular space where pathogens are encountered and local immune reactions are initiated. The initial stages of inflammation precede the recruitment of the T, B cells and antigen presenting cells (APC). The expression of early activation inducer molecule (CD69) occurs very early (even before other activation antigens such as IL-2 receptor) after T or B cell activation. It is also expressed by macrophages, but is absent in resting lymphocytes. The IL-2 receptor (CD25) is also expressed only on activated T, B and macrophage cells.

Migration of leucocytes from the peripheral blood to extravascular sites is a key feature of the inflammatory response [1]. At the site of injury, immigrant leucocytes induce a prompt inflammatory response that is crucial for the early control of infection and for initiation of local immune responses. It is when the cells get activated that they synthesise inflammatory mediators that include interleukins, enzymes released from polymorphonuclear leucocytes, oxygen free radicals platelet activating factor and adhesion molecules [2]. Subsequent influx of T cells to sites of chronic inflammation is promoted by up-regulation of adhesion molecules on endothelial cells at the site of inflammation [3]. Intercel-

* Corresponding author. Tel.: +91-172-747403; fax: +91-172-747403.
E-mail address: majumdar@glide.net.in (S. Majumdar).
lular adhesion molecule-1 (ICAM-1, CD54) is one such molecule known to mediate the adhesion of T cells with APCs and is involved in T-cell/T-cell and T/B cell interactions. It binds to the integrins LFA-1 (CD11a/CD18) and Mac-1 (CD11b/CD18). ICAM-1 plays a central role in immune cell interactions. Activation of T cell requires interaction between T cell receptor (TCR) and specific antigen presented in the form of processed peptide in association with major histocompatibility complex II (MHC II) molecules. In addition to signals through TCR, costimulatory signals are required for complete T cell activation. Interactions between several adhesion molecules on T cells and their ligands on APCs provide costimulatory signals that result in cytokine production and T cell proliferation [4]. Apart from CD28/B7 pair, LFA-1/ICAM-1 costimulation has been known to take part in T cell activation [5].

Recent studies have indicated that ICAM-1 levels may be upregulated during inflammation [6]. It is broadly expressed in response to proinflammatory cytokines such as IL-1β, TNF-α and IFN-γ [7]. Tumour necrosis factor-α (TNF-α) is produced in large amounts in several organs during severe acute pancreatitis and is believed to mediate pathophysiological changes [8]. According to Werner et al., upregulation of ICAM-1 and subsequent leukocyte infiltration appear to be significant mediators of pancreatic and pulmonary injury in pancreatitis [10].

These results suggest that the inflammatory mediators produced and released during pancreatitis (acute/chronic) may not just be the markers of disease severity but could also be pieces of pathogenesis and pathophysiological puzzle.

In this article, we report the results of studies that examined the numbers of cells expressing activation markers (i.e. CD69, HLA-DR and CD25) as well as adhesion molecules (i.e. ICAM-1, CD11a and CD11b) on cells derived from the pancreatic tissue excised during necrosectomy and PBMC. Also, by simultaneous flow cytometric analysis, we have determined the co-expression of a surface marker (CD4/CD8/CD14) and intracellular cytokine (TNF-α and IFN-γ) in individual cells.

2. Materials and methods

2.1. Subjects

The study included 34 patients admitted to the Nehru Hospital (at Postgraduate Institute of Medical Education and Research, Chandigarh, India) with a first attack of acute pancreatitis (mean age = 39.26 years ranging from 25 to 53 years). There were 20 males and 14 females. The diagnosis was based on (i) characteristic signs and symptoms, (ii) increased α-amylase (enzymatic colorimetric test) in serum and (iii) characteristic sonographic and/or computed tomographic (CT) findings. Patients with prior acute pancreatitis (AP) or episodes of unexplained abdominal pain were excluded from the study.

2.2. Clinical material

Patients were classified by aetiology, acute physiological and chronic health evaluation (APACHE II score) and computed tomography (amount of pancreatic necrosis and CT severity index). Only intravenous contrast enhanced CT scans were used for classification. All the patients were initially managed with supportive treatment — nasogastric decompression, intravenous fluid and antibiotics. The patients were operated upon when there was either progression of sepsis (presence of frank pus or positive aspirate on image guided fine needle aspiration) and organ dysfunction despite maximum conservative treatment.

The pancreas was exposed by division of gastroduodenal ligament. Blunt debridement of intrapancreatic and extrapancreatic necrotic tissue was carried out. As much of the necrotic pancreatic and peripancreatic tissue as possible was removed. The whole peritoneal cavity was inspected, with special attention to subphrenic and paracolic spaces. Abscesses or fluid collections were opened. Closed lesser sac lavage was instituted. A feeding jejunostomy tube was placed routinely for enteral nutrition.

Pancreatic necrosis was identified in all patients. While 14 patients had necrosis involving less than 30% of pancreas, 20 patients had necrosis involving more than 50% of the organ. The mean time gap between admission and intervention was 8.4 days. The study showed a high incidence of systemic complication and distal organ failure. While sepsis was seen in 17 patients (50%), 18 patients developed adult respiratory distress syndrome (56%) and 10 patients developed acute renal failure (31%). Six patients developed more than two organ systems failure (18%). The mortality in the study was 25% and the cause of death was distant organ failure.

The necrotic pancreatic tissue was removed during necrosectomy and placed in RPMI-1640 medium containing penicillin and streptomycin and 5% FCS. Normal donors for peripheral blood (n = 20) and autopsy cases (n = 15) with no overt infection and with no known pancreatic pathology/history for normal pancreatic tissue were included as controls. Informed consent was obtained from patients, blood donors as well as nearest kin of normal pancreatic tissue donors.
2.3. Cell preparation

Mononuclear cells were isolated from the resected pancreatic tissue specimens from AP patients. In brief, small pieces of resected tissue were washed with and then incubated in RPMI-1640 with serum in 25 cm² culture flask. The tissue was then incubated with collagenase (Sigma) at a final concentration of 200 units/ml. Monensin (3 μM) (Sigma, St. Louis, MO) was added to the culture for the last 4 h of the incubation in order to disrupt intracellular protein transport. The supernatant cells were collected after allowing clusters to settle, and centrifuged at 50–100 g for 3 min. The cell suspension so obtained was centrifuged for 10 min at 100 g. The cells were washed twice with PBS. The cells were adjusted to a concentration of 2 × 10⁶ cells/ml in RPMI-1640 supplemented with 10% heat inactivated FCS and 2 mM L-glutamine. Mononuclear cells were stimulated for cytokine production for 8 h at 37°C in the presence of 5% CO₂ with 1 μg/ml LPS. Single cell suspensions of pancreatic cells and peripheral blood mononuclear cells (PBMC) were stained with FITC labelled and anti-human CD69, CD25, CD54, CD11a, CD11b and HLA-DR (Becton Dickinson, Mountain View, CA) for 20 min at 4°C. In order to determine the T cell sub-population responsible for the cytokine production, a two colour staining technique was performed. The cells were pooled and washed in PBS and 0.01% sodium azide. Hundred microlitres of cellular suspension was stained with a fluorochrome (fluorescein isothiocyanate, FITC) conjugated antibody specific for a cell surface antigen i.e. anti-human CD4/CD8/CD14 FITC (Pharmingen, San Diego CA 92121) for 15 min at 4°C. Thereafter, the cells were washed, fixed and permeabilised according to the manufacturer’s instructions. The cells were then incubated with an optimal dose of an anti-human fluorochrome (Phycocerythrin, PE) conjugated antibody for intracellular staining of TNF-α and IFN-γ. As negative controls, aliquots of cell suspension were incubated with an irrelevant isotype matched monoclonal antibody conjugated to the same fluorochrome as the sample and processed. Cells were incubated for 30 min at 4°C and washed with PBS-azide. The cells were resuspended in buffer and acquired on the FACScan flow cytometer (Becton Dickinson, Mountain View, CA). Live gates were set for lymphocytes using FSC/SSC characteristics. Ten thousand gated events were recorded for each cell surface marker and gated cells were analysed with Cell Quest Software (Becton Dickenson) for intracellular cytokine expression on CD4+, CD8+ and CD14+ cells. The FL1 and FL2 channels were set up and compensated on a tight lymphocyte/monocyte gate using tubes with a single labelled strongly positive antibody. Dead cells were excluded by forward and side scatter gating. Statistical markers were set using the irrelevant isotype matched controls as reference.

Results are expressed as the percentage of cells expressing a particular cell surface marker/cytokine in a population of specified cells.

2.4. Statistical analysis

The results reported are expressed as mean ± SD values obtained from duplicate determinations of each sample. In all figures, the vertical bars denote SD values. The significance of changes was evaluated using the Student t-test as the data consisted of only two groups. A P value of ≤ 0.05 indicates a significant difference.

3. Results

3.1. Clinical data of subjects in the study

The clinical data of AP patients and donors are summarised in Table 1. A total of 34 patients were included in the study; the ages ranged from 24 to 53 years. Patient characteristics such as aetiology, complications and fatal outcome are listed in Table 1. The distribution of gender was 20 males and 14 females in acute pancreatitis category. The control group consisted of 20 healthy donors (12 males, 8 females) ranging from 24 to 40 years of age. Control pancreatic tissue samples were obtained from 15 autopsy cases who died in accidents and had no pancreatic complications.

<table>
<thead>
<tr>
<th>Features</th>
<th>Healthy donors</th>
<th>Autopsy cases</th>
<th>Acute pancreatitis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>20</td>
<td>15</td>
<td>34</td>
</tr>
<tr>
<td>Age (years)</td>
<td>32</td>
<td>38.92</td>
<td>39.26</td>
</tr>
<tr>
<td>Range</td>
<td>24–40</td>
<td>32–51</td>
<td>25–53</td>
</tr>
<tr>
<td>Gender</td>
<td>12M; 8F</td>
<td>8M; 7F</td>
<td>20M; 14F</td>
</tr>
<tr>
<td>Etiology:</td>
<td>Alcohol</td>
<td>17</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Gall stones</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Idiopathic</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Complications:</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>ARDS</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Renal failure</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fatal outcome</td>
<td>10</td>
<td></td>
</tr>
</tbody>
</table>
Fig. 1. Expression of activation markers in acute pancreatitis. Fluorescein isothiocyanate labeled anti-human antibodies against CD69, CD25 and HLA-DR were used to label the mononuclear cells from peripheral blood and pancreatic tissue. Cells were analysed on the FACS and results are denoted as percentage positive cells. **P < 0.01 and ***P < 0.001.

3.2. Expression of activation markers

Immunofluorescent staining of CD69 (early activation inducer molecule), CD25 (IL-2 receptor) and HLA-DR was carried out on cells obtained from peripheral blood cells as well as pancreatic tissue obtained from healthy donors and AP patients (Fig. 1). CD69 and CD25 expressing cells were significantly high in both PBMC and pancreatic tissue cells from AP patients. In PBMC — CD69: P < 0.01 and CD25: P < 0.01; in tissue — CD69: P < 0.001 and CD25: P < 0.001. In comparison, we find that cells expressing both these molecules were prominently elevated in tissue cells as compared to peripheral blood cells from the same patient population. Cells expressing HLA-DR molecules on cell surface were reduced in acute pancreatitis patient blood (P < 0.01) and tissue cells (P < 0.001).

3.3. Expression of adhesion molecules is increased following acute pancreatitis

In order to determine whether pancreatitis affects the expression of adhesion molecules, we analysed the peripheral blood as well as pancreatic tissue cells from control as well as affected group by flowcytometry (Fig. 2). Change in cell population expressing cell surface adhesion molecules CD54 (ICAM-1), CD11a and CD11b was determined by comparing the percentage of cells in all the cases. CD54 positive cells were considerably amplified in the peripheral blood (P < 0.05) and tissue (P < 0.01) of AP as compared to controls. CD11a and b also showed a similar trend where the blood (CD11a: P < 0.05 and CD11b: P < 0.05) and tissue samples (CD11a: P < 0.01 and CD11b: P < 0.01) from AP showed elevated number of cells expressing them.

3.4. Intracellular cytokines IFN-γ and TNF-α

After stimulation with LPS (1 μg/ml), CD4+, CD8+ and CD14+ cells were examined on the single cell level for cytoplasmic productions of IFN-γ and TNF-α.

3.4.1. IFN-γ production in CD4+ and CD8+ cells

Fig. 3 A and B shows that the proportion of IFN-γ expressing CD4 and CD8 cells was not increased in T cells from peripheral blood of AP patients as compared to normal cells of controls. The analysis of CD4+ and CD8+ T cells from the pancreatic tissue showed that CD8+ T cells from the tissue were the main secretors of IFN-γ (P < 0.01). The CD4+ T cells did not show any significant changes. Thus, the CD8+ T cells ac-
3.4.2. TNF-α production by CD14+ cells and T cells

To examine the potential role of TNF-α in AP, the peripheral blood as well as the cells derived from pancreatic tissue was analysed for the presence of TNF-α expression. The numbers of TNF-α positive cells from control subjects (both blood and tissue) remained very low. Therefore, CD4+ T, CD8+ T and CD14+ cells did not express a considerable amount of TNF-α (Fig. 4 A and B).

The CD14+ cells from AP blood did exhibit TNF-α producing cells that was significant when compared to the same cells from healthy volunteers. On the analysis of these CD14+ cells from the pancreatic tissue we observed an elevation in the number of cytokine producing cells of AP patients. Next we measured TNF-α production by CD4 and CD8+ T cells from blood and pancreatic tissue as both T cells as well as mononuclear CD14+ cells produce it. In comparison to the controls, there was an enhanced number of TNF-α positive both CD4+ (P < 0.05) and CD8 T-cells (P < 0.01) from the peripheral blood of AP patients. A considerably intense signal was observed when TNF-α producing CD4+ and CD8+ cells from pancreatic tissue were analysed and here again, CD8+ T cells (P < 0.001) prevailed over the CD4+ T cells (P < 0.01) in TNF-α production.

4. Discussion

The degree to which the cellular immune function is disturbed in acute pancreatitis remains unclear. Previous studies suggest that early response to pancreatic inflammation involves predominantly the tissue macrophages and lymphocytes in and around the pancreas. The invading inflammatory cells that release cytokines play an important role in the pathogenesis of the disease, although the exact mechanisms that trigger the inflammatory and necrotising process are not well understood [11]. A variety of inflammatory mediators are released which are initially supposed to limit the local damage. It is therefore important to recognise that tissue levels and not serum levels are responsible for the...
Fig. 3. (Continued)
Fig. 4. (Continued)
vast majority of the biologic effects of cytokine and surface markers expressed on the cells. In this report, we demonstrate that AP is associated with the elevation of activation and adhesion molecules as well as of cytokines such as TNF-\(\alpha\) and IFN-\(\gamma\) in the pancreatic tissue.

A major advantage of this study is that it allowed phenotypic analysis of TNF-\(\alpha\) and IFN-\(\gamma\) producing cells in the pancreatic tissue. Upon injury, the leucocytes are sequestered within the affected areas. This begins with activation of the cells, involves adhesion of circulating activated cells to the injured area and finally inflammatory factors are released. CD69, an early activation inducer molecule is known to appear very early after T or B cell activation. It is also expressed by macrophages and NK cells, but is known to be absent in resting lymphocytes. Pancreatitis patients showed prominently high numbers of cells from pancreas expressing this molecule when compared to blood. The IL-2 receptor is also found on activated T, B and macrophage cells (inflammatory response). The expression of MHC class II antigens is a vital component of the antigen presentation mechanism. It is also a marker of monocyte activation, as the downregulation of MHC class II antigens correlates with clinical outcome. Recently, Richter et al. [12] confirmed this in acute pancreatitis patients where HLA-DR expression on CD14+ monocytes was grossly reduced in acute pancreatitis patients. Reduced number of HLA-DR expressing cells from the pancreas indicate disrupted cellular immune response and also suggest exposure to infection.

Next, we probed the expression of adhesion molecules such as CD54 (ICAM-1) and its counter receptors i.e. lymphocyte function associated antigen 1 (CD11a/CD18) and Mac-1 (CD11b/CD18). Several studies have been carried out to find out the level of ICAM-1 and its role in experimental pancreatitis [9,13,14]. We have found that intrapancreatic cellular expression of ICAM-1 and its subsequent receptors are markedly upregulated in AP. As is well known, leukocyte adhesion and infiltration, both mediated by ICAM-1 are central events in the pathogenesis of pancreatitis. Hence, upregulation of ICAM-1 expressing cells along with those expressing CD11a and CD11b appear to be significant mediators of pancreatic injury. ICAM-1 is broadly expressed in response to cytokines such as TNF-\(\alpha\) and IFN-\(\gamma\) [7]. By binding to its counter receptors, LFA-1 and Mac-1, ICAM-1 have been shown to mediate adhesion of leucocytes to endothelial cells [1,15]. While ICAM-1 expressing cells could not be detected at significant levels in normal tissue, increased cell numbers were observed.

TNF-\(\alpha\) is an important effector molecule in acute inflammation. Several studies have examined the relationship between TNF and AP in humans [16,17]. They correlated TNF levels with disease severity or mortality. More recent studies, however, have produced conflicting results [18,19]. Hence, there was a need to assay TNF-\(\alpha\) levels at cellular level in AP. This study therefore highlights at site intracellular expression of cytokines. It is these and not the serum levels, which are responsible for the vast majority of the biological affects of the cytokines in the tissue. We report significantly high numbers of TNF-\(\alpha\) producing leukocytes from pancreatic tissue of AP cases. Since TNF-\(\alpha\) is known to play a major role in the initiation of an immune response, it is tempting to speculate that it is these TNF-\(\alpha\) producing cells which contribute during the early phase of the response by producing proinflammatory cytokines. Increased TNF-\(\alpha\) levels in several organs (during severe acute pancreatitis) are believed to mediate many of the pathophysiological changes [8,20]. Evidence suggests that this cytokine production within the pancreas and lung propagate pancreatic necrosis and the development of the adult respiratory distress syndrome (ARDS), both of which are major causes of morbidity and mortality [11,21].

Frossard et al. [6] report increased intra-pancreatic levels of ICAM-1 in severe secretagogue induced pancreatitis and diet induced pancreatitis. They suggest that ICAM-I play an important pro-inflammatory role in pancreatitis and lung injury. IFN-\(\gamma\) is also known to increase expression of MHC I and II antigens and ICAM. Our most prominent observation was that HLA-DR expression on CD14+ cell declined suggesting thereby reduced monocyte function. Our observation is supported by a study conducted on AP in Germany [12].

Our results point to disturbances of the monocytic function in acute pancreatitis. The TNF-\(\alpha\) and IFN-\(\gamma\) secretion in the affected areas is mainly due to the T cells and also CD8+ T cells. It is this subset of T cells which takes an edge over the other subtype in cytokine synthesis. To our surprise, we found that the Doyle et al. [22] report of activated CD8+ T cells that can

Fig. 4. Intracellular TNF-\(\alpha\) producing CD4+, CD8+ and CD14+ cells as determined by flow cytometry. Cells stained with FITC conjugated anti-human CD4/CD8/CD14 antibodies were permeabilised and stained with PE conjugated anti-human TNF-\(\alpha\) antibody. (A) All the samples were analysed by gating on the respective cell surface marker and only positive cells were then acquired with proper negative control as described in the legend to Fig. 3. FL1 on x-axis denotes FITC and FL2 on y-axis denotes PE fluorescence intensity. The dot blots shown here are representative of such experiments. In the figure, a, d, g and j are plots for CD4+ cells and TNF-\(\alpha\); b, e, h and k are plots for CD8+ cells with TNF-\(\alpha\) and c, f, i and l are CD14+ cells expressing TNF-\(\alpha\) (upper right quadrant = double positive cells). (B) The total result of the cytofluorometric analysis is plotted as histogram. Cells are expressed as percentage of cells expressing both fluorochromes, i.e. double positive. *\(P<0.05\), **\(P<0.01\) and ***\(P<0.001\).
respond flexibly to stimulation conditions in the periphery. This seems to be applicable to our study where the effector site retains the potential to alter the cytokine profile in response to local stimuli.

In conclusion, the current studies are consistent with the concept that expression of ICAM-1 in the pancreas during pancreatitis is a critical link in the development of tissue injury and organ dysfunction. Reduced expression of HLA-DR molecules on monocytes/macrophages seem to be part of immune deviation which is associated with the development of pancreatitis. Although overexpression of inflammatory mediators do play an important role in pathogenesis but the molecular mechanisms of the whole cascade i.e. cell surface molecules, signalling into the cell and resulting response of the cell has still not been worked out. Further studies are needed to clarify these queries.

References
