Several studies suggest that cytokines and neutrophils play an important role in the pathogenesis of acute pancreatitis (AP). The aim of the study was to assess the systemic release of proinflammatory cytokines and WBC (white blood cells) count with differential in patients with acute alcoholic pancreatitis (AAP) and to characterize the differences between patients with mild and severe forms of the disease.

Material and methods. Thirty-five patients with the mild form of acute alcoholic pancreatitis (MAAP) were compared to 11 patients with severe acute alcoholic pancreatitis (SAAP). Serum levels of IL-6, IL-8, IL-12p40 and WBC differential count were measured every second day during the first week after admission.

Results. During the course of the study, the average level of IL-6 was significantly (p<0.05) higher in patients with SAAP than in patients with the mild form of the disease (MAAP). Serum levels of IL-8, IL-12p40 and WBC differential count were measured every second day during the first week after admission.

Conclusions. Patients with SAAP had significantly higher proinflammatory cytokine IL-6 levels and neutrophil counts than patients with MAAP. The results suggest that proliferation and overstimulation of this subset of leukocytes might contribute to the development of the systemic inflammatory response in patients with SAAP.

Key words: acute alcoholic pancreatitis, proinflammatory cytokines, leukocytes

Alcohol abuse and gallstones are major causes of acute pancreatitis (AP) in developed countries. The incidence of alcohol etiology of AP varies markedly in different countries. About one-third of AP cases and between 60-90% of chronic pancreatitis cases in the USA are alcohol-induced (1). Alcohol is a rare cause of AP in Saudi Arabia where its consumption is prohibited for religious reasons. Alcohol was the inducing agent of AP in only 3.1% of patients (2).

The pathophysiology of acute alcohol pancreatitis (AAP) is still not fully understood. The amount of alcohol consumed may be an important factor in the severity of the first attack of AAP which would suggest a direct toxic effect of alcohol on the pancreas (3, 4). However, alcoholic pancreatitis develops in a
minority (about 5%) of people who abuse alcohol indicating the presence of specific susceptibility or trigger factors in some individuals (4). As Norback et al. aptly put it, it is unclear where the limits of alcohol consumption should be set to allow acceptance of the term alcoholic pancreatitis (5).

There is evidence that alcohol and its oxidative and nonoxidative metabolites (e.g. fatty acid ethyl esters – FAEEs) cause damage to the acinar cells of the pancreas predispose to the release of autodigestive factors and initiation of inflammatory response (6, 7). MAAP develops in approximately 80% of patients with AP and the mortality rate in this group of patients is less than 1%. (8). On the other hand, about 20% of patients with AP have a clinically severe form with a much higher mortality rate of 10% with sterile pancreatic necrosis and 25% with infected necrosis (8). Attacks of AAP recur after the first episode in about 50% of alcoholics who continue alcohol abuse (9).

As early as 1957 Thal et al. (10) reported that in 42 cases of fatal AP (necrotizing and interstitial) most of the patients had a WBC count greater than 15 000/uL. In 1974, Ranson et al. (11) noted that a WBC count greater than 16 000/uL on admission was poor prognostic factor in AP. It is to be stressed that they mentioned total WBC count only, without characterizing subsets of white blood cells (that is, WBC differential count).

Later, Rinderknecht et al. (12) suggested that fatal pancreatitis is the consequence of excessive leukocyte stimulation and an overaggressive general immune system response. Damage to the pancreatic tissue is accompanied by increased production of proinflammatory cytokines: interleukin 1β (IL-1β), interleukin 6 (IL-6), interleukin 8 (IL-8), tumour necrosis factor (TNF-α), and platelet activating factor (PAF) and massive mobilization of leukocytes (13, 14). Activated leukocytes discharge, damaging enzymes and superoxide radicals which can contribute to the development of systemic inflammatory response syndrome (SIRS) (14). Dissemination and amplification of this process in severe cases may lead to multiple organ dysfunction syndrome (MODS) and death (14).

The aim of the present study is to investigate the changes in proinflammatory cytokine levels and WBC differential count in patients with acute alcoholic pancreatitis.

### MATERIAL AND METHODS

Thirty-five patients with the mild form (MAAP) and 11 with the severe form (SAAP) of AAP were included in the study. The diagnosis of AP was based on clinical symptoms, elevated serum amylase activity (more than 3 times above the reference limit), abdominal ultrasonography (USG), and abdominal computed tomography (CT).

The severity of AP was determined in accordance with clinical and laboratory parameters. AP classification met the Atlanta criteria (15), Ranson’s criteria (11), and APACHE II criteria (16). The degree of organ dysfunction was measured using the MOD score (Multiple Organ Dysfunction score) (17).

In a few patients with SAAP, the CT scan was performed more than once. The etiology of AAP was determined on the basis of clinical history and the absence of stones in the gallbladder or in the common bile duct. We used interviews with the patients and/or relatives to assess frequency and amount of drinking.

<table>
<thead>
<tr>
<th>Number of patients</th>
<th>MAAP</th>
<th>SAAP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age years mean (range)</td>
<td>58 (24-88)</td>
<td>59 (47-80)</td>
</tr>
<tr>
<td>Sex male/female (n)</td>
<td>31/4</td>
<td>10/1</td>
</tr>
<tr>
<td>Ranson score (range)</td>
<td>1-2</td>
<td>4-6</td>
</tr>
<tr>
<td>APACHE II score (mean)</td>
<td>1.32</td>
<td>13.27</td>
</tr>
<tr>
<td>CT Grade-Balthazar score</td>
<td>A – 19</td>
<td>C – 2</td>
</tr>
<tr>
<td></td>
<td>B – 16</td>
<td>D – 6</td>
</tr>
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<td></td>
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<td>E – 3</td>
</tr>
</tbody>
</table>

MAAP – mild acute alcoholic pancreatitis; SAAP – severe acute alcoholic pancreatitis; CT – computed tomography
All the patients reported regular alcohol intake exceeded 300 g/weekly for at least one year prior to admission. The progression of morphological changes within the pancreas was evaluated by ultrasonography and the Becker scale (18). In each of the patients, USG was performed every day during the study allowing analysis of the evolution of the inflammatory changes within the gland and the surrounding areas. The CT scans allowed us to evaluate the necrotic changes of the gland parenchyma along with the pathological changes in the retroperitoneal and peritoneal spaces using a Balthazar score (19).

Venous blood samples were taken on the 1st, 3rd, 5th, and 7th days after admission. Plasma concentrations of IL-6, IL-8, and IL-12p40 were determined by enzyme-linked immunoassay (ELISA) kits from BioSource according to the manufacturer’s instructions. All serum supernatant samples were stored at -70 °C and were assayed at the same time by the same ELISA kit to avoid variation of assay condition. The automated hematology analyzer Sysmex K800 was used to process the WBC with differential.

Statistical analysis

The data were analyzed by means of the two-way factorial ANOVA model with repeated measurements. The studied factors were: groups (SAAP vs. MAAP) and time (four measurements taken every two days). The influence of time was assessed by assuming a linear trend. The analysis consisted of two steps. First, all data were included and possible outliers were identified for subsequent removal from the final analysis. An observation was skipped if the value of its standardized residual was greater than 3.5. The output of the second step – which is the proper analysis – was presented in the results.

No differences in the average age of patients from the two studied groups was found ($F_{1,73} = 0.41; p=0.5248$). Nevertheless, one could prefer to estimate the influence of form of pancreatitis on condition of the immune system depending on age. Including this variable in a model would hardly change the conclusions, so a more parsimonious model was used.

RESULTS

Serum levels of proinflammatory cytokine

1. Interleukin 6

Patients with SAAP had significantly higher levels of IL-6 during the course of the study than patients with MAAP ($F_{1,19} = 5.28; p=0.0332$) (fig. 1A). This difference decreased with time since in cases of SAAP there was a linear decreasing trend ($F_{1,19} = 10.96; p=0.0037$), while the level of IL-6 in the other group showed no sign of change over the studied period of time (linear trend $F_{1,19} = 0.49; p=0.4898$). Simultaneously the interaction of those two linear trends was significant ($F_{1,19} = 6.48; p=0.0197$).

2. Interleukin 8

There were no significant differences in the average levels of IL-8 between the two groups ($F_{1,23} = 1.43; p=0.2439$) (fig. 1B). The trend of changes in both groups was decreasing in the course of time in what appeared to be a linear trend (severe $F_{1,23} = 2.79; p=0.1086$, mild $F_{1,23} = 6.35; p=0.0191$, the trend for both groups combined $F_{1,23} = 6.86; p=0.0153$, the assumption about parallelism of both trends seems to be correct: interaction of trends $F_{1,23} = 0.13; p=0.7175$).

3. Interleukin 12p40

The levels of IL-12 did not significantly differ in the two groups (fig. 1C). There was no clear trend within a group (groups: $F_{1,23} = 1.67; p=0.2061$, linear trends in time: severe $F_{1,23} = 2.84; p=0.1028$, mild $F_{1,23} = 2.85; p=0.1020$). There were some signs of a general trend for both groups combined $F_{1,23} = 5.22; p=0.0298$.

White Blood Cell Count (WBC) and differential

1. Total WBC count

The average total WBC count (fig. 2A) calculated from pooled measurements taken during all 7 days of observations was higher in the group of patients with SAAP ($F_{1,31} = 8.14; p=0.0076$). Nevertheless, on the 1st and the 3rd day of the study there were no significant dif-
Mammotome biopsy in the diagnostics and treatment of nodular breast lesions

Fig. 1. Serum levels of proinflammatory interleukins (A: IL-6, B: IL-8, C: IL12p40) on days 1, 3, 5 and 7 of hospitalization in patients with SAAP (n=11, solid line) versus MAAP (n=35, dashed line) *p<0.05; n=numbers of patients; MAAP – mild acute alcoholic pancreatitis; SAAP – severe acute alcoholic pancreatitis

ferences between the two groups of patients in total WBC’s (1st day $F_{1,31}=0.11; p=0.7316$, 3rd day $F_{1,31}=0.39; p=0.5382$). We observed two opposite linear trends in both groups; increasing in the SAAP group ($F_{1,31}=6.44; p=0.0164$) and decreasing in the MAAP group of patients ($F_{1,31}=29.12; p<<0.0001$).

2. Neutrophil count

The average number of neutrophils was higher in the SAAP group than in the MAAP group (fig. 2B) ($F_{1,32}=5.19; p=0.0296$). The first two measurements showed a similar neutrophil count in the two groups – later the average number of neutrophiles increased in a linear fashion ($F_{1,32}=4.49; p=0.0420$) in SAAP group while showing decreasing linear trend in MAAP group ($F_{1,32}=26.66; p<<0.0001$).

3. Lymphocyte count

We did not find any differences in the average lymphocyte level between the two groups. (SAAP vs. MAAP $F_{1,30}=0.10; p=0.7496$, time $F_{3,90}=1.69; p=0.1738$, interaction of time and group $F_{3,90}=2.05; p=0.1125$ (fig. 2C).

4. Monocyte count

The average monocyte count is slightly higher in the SAAP group but the difference is borderline insignificant ($F_{1,30}=3.36; p=0.0766$). We observed that the amount of monocytes increased in a quadratic fashion ($F_{1,30}=4.40; p=0.0445$) (fig. 2D).

5. Eosinophil count

No significant differences in the average eosinophil numbers and trends were noted between the two groups of patients (SAAP vs. MAAP $F_{1,30}=0.50; p=0.4946$, time $F_{3,90}=0.48; p=0.6969$, interaction of time and group $F_{3,90}=2.35; p=0.0779$) (fig. 2E).

**DISCUSSION**

Several clinical and experimental studies confirmed the central role of cytokines and
leukocytes in the development and clinical course of AP (13, 14, 20). Alcohol or its toxic metabolites initiate injury to pancreatic acinar cells triggering the autodigestive process and release of chemokines and cytokines (21). Cytokines attract inflammatory cells into the pancreas – among them, leukocytes which are essential determinants of local and remote organ injury in AP and are related to the severity of the disease (22). The white blood cell (WBC) count is a simple, inexpensive, and commonly performed test in patients with AP.

Dauphine et al. demonstrated that white blood cell count and glucose levels are useful predictors for development of major systemic complications and/or mortality in patients with first-time alcoholic pancreatitis (23). Moreno-
Osset et al. utilized labeled leukocyte scintigraphy (LLS) and measured the C-reactive protein (CRP) in patients with AP (24). The results indicated that an increase of leukocyte concentration and CRP correlates with local pancreatic damage and the systemic inflammatory reaction. Werner et al. used Technetium-99m-labeled leukocytes in acute experimental pancreatitis induced in rats (25). He concluded that mild pancreatitis was characterized by a low degree of leukocyte activation and infiltration in the pancreas while severe pancreatitis was marked by leukocyte concentration in both the pancreas and the lung. The activity of leukocytes in AP was also assessed by measuring the level of polymorphonuclear (PMN) elastase — a hydrolytic enzyme released by activated PMN granulocytes. Again, the studies (26) showed that PMN-elastase serum levels are significantly higher in severe AP than in mild AP during the first 12-24 hours of the disease. Neutrophils from patients suffering from acute pancreatitis cause more severe in-vitro endothelial damage compared with neutrophils from healthy donors and are differently regulated by endothelins (27). T cells, natural killer (NK) cells, and monocytes/macrophages are major sources of cytokines, but it has been shown that neutrophils also have the ability to synthesize and release immunoregulatory cytokines (28). Data presented in this paper showed that neutrophils significantly elevated in SAAP patients when compared to patients with MAAP.

There are several studies suggesting the clinical importance of IL-6 in AP. Suva et al. (29, 30) have shown that IL-6 shortens the transit time of neutrophils through the marrow and accelerates their release into circulation. IL-6 also induces neutrophil sequestration in the lung and delays neutrophil apoptosis, thereby inhibiting the resolution of inflammation (31). Raised IL-6 serum levels correlated with the clinical severity of AP (32) and the combined use of serum lipase and IL-6 was useful in simultaneously establishing both the diagnosis and prognosis of AP (33). Increased serum level of IL-6 was the only parameter that significantly predicted complicated acute pancreatitis, a result that could not be foreseen with either IL-8 or IL-10 or even three prognostic scoring systems — Ranson, Glasgow and APACHE II (34). A recent study suggested that increased serum levels of IL-6 both predicted organ failure with severe pancreatitis and suggested its pathophysiological significance in AP (35).

In the present study, the highest levels of IL-6 were noted on the day of admission in patients with the mild form of AAP as well as with the severe form of AAP. During the whole course of the study, serum levels of IL-6 in patients with SAAP were significantly higher when compared to patients with MAAP. In our previous study, we showed that the levels of IL-6 were also significantly higher throughout the study in patients with the severe form of acute biliary pancreatitis than in patients with the mild form of the disease (36).

IL-8 is a proinflammatory cytokine produced by macrophages and epithelial cells exerting chemotactic activity on neutrophils. In turn, activated neutrophils can be a significant source of IL-8 (37). Patients with complicated pancreatitis had significantly higher mean values of IL-8 and neutrophil elastase than patients with the uncomplicated disease (38). Serum levels of IL-8 positively correlated with increased alcohol consumption and occurred more frequently in females than males (39).

IL-12p40 is an agonistic cytokine acting as a chemoattractant for macrophages (40). In a study by Pezzilli et al., patients with AP had markedly higher serum concentrations of IL-12p40 than those of healthy subjects, which might be responsible for the patient’s increased susceptibility to infection (41).

CONCLUSIONS

1. Mean serum levels of IL-8 and IL-12p40 were higher on admission in the SAAP group than in the MAAP group although the differences were not significant.
2. Our present and previous studies alike showed that significantly increased serum levels of IL-6 were found in both severe acute alcohol pancreatitis and severe acute biliary pancreatitis when compared to the mild forms of the diseases.
3. Out of all the peripheral white blood cells, neutrophils the most significantly increased in SAAP when compared to MAAP.
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Address correspondence: 31-501 Kraków, ul. Kopernika 21