ASSOCIATION OF ACUTE PANCREATITIS (AP) OR CHRONIC PANCREATITIS (CP) WITH INFLAMMATORY BOWEL DISEASE (IBD) has been reported in the literature. Several cases of AP were related to biliary lithiasis or to side-effects of drugs, including 5 ASA, sulfasalazine, corticosteroids, azathioprine and 6 mercaptopurine [1, 2]. However, a recent study found a standardized incidence ratio for AP of 4.3 in patients with Crohn’s disease (CD) and 2.1 in those with ulcerative colitis [3]. Side effects of medical treatment or biliary lithiasis could hardly account for such a high risk, suggesting that, in some cases, there is a pathogenetic relationship between IB and pancreaticitis. Two recent reports [4, 5] supported that hypothesis, but experimental evidence of a direct relationship is still lacking. Several animal models of colitis have recently been developed and proved useful in studies on the pathophysiology of IB. We chose the well-established model of 2,4,6-trinitrobenezene sulfonic acid (TNBS)-induced colitis in the mouse to investigate the consequences of IB on pancreas. This was done by monitoring histological changes but, because clinical data suggest that pancreatic alterations that accompany IB could be limited, we also monitored the expression in pancreatic tissue of the pancreatitis-associated protein (PAP). PAP is not detectable in normal pancreas, but its expression increases quickly and strongly upon pancreatic injury [6, 7]. In addition, pancreatic mRNA levels of PAP, IL-1β, TNFα, IL-10 were assessed.

Results — All mice treated with TNBS and none of the controls had colitis. Macroscopic and microscopic examination of the pancreas of the 4 control mice was normal, whereas in 5 out of the 8 TNBS-treated mice histological changes were observed, with inflammatory infiltrate and fibrin aggregates at the periphery of the gland. PAP immunohistochemistry was negative in all control mice and positive in all mice with TNBS-induced colitis, with a patchy distribution of staining. PAP immunolocalized to the cytoplasm of acinar cells, duct cells and islets of Langherans being negative. PAP and IL-1β mRNA levels in the pancreas were significantly increased but the increase in TNFα mRNA level did not reach statistical significance (P = 0.06). IL-10 mRNA levels did not show any significant modification.

Conclusion — PAP overexpression in pancreas demonstrates that inflammatory stress early occurs in the mouse pancreas during the course of TNBS-induced colitis. The concomitant pancreatic overexpression of IL-1β and, to a lesser extent, of TNFα, two proinflammatory cytokines also associated with the intestinal lesions of colitis, supported a pancreatic inflammatory mechanism mediated by cytokines.
Assessment of pancreatic damages and immunohistological staining procedures

The pancreas were carefully dissected and fixed in a 10% formaldehyde solution. The specimens were embedded in paraffin blocks. Five-µm thick sections were obtained and stained with hematoxylin and eosin and safran (HES) for routine microscopic examination.

Antibodies to PAP diluted at 1: 200 in 1% bovine serum albumin in PBS were used on paraffin sections (5-µm thick). An automated immunostaining procedure was used with avidin-biotin-peroxidase complex in a Ventana 230 device (Ventana Systems, Tucson, Arizona, USA) with Ventana kits (Ventana Systems, Strasbourg, France), using an aminoethyl carbazol reagent. Sections were counterstained with hematoxylin and mounted in glycerol.

Evaluation of histological changes and interpretation of PAP immunohistological stainings were made by an investigator unaware of the status of the corresponding animals.

Quantification of pancreatic TNFα and β-actin mRNAs by RT-competitive PCR and semi-quantification of PAP, IL-10 and IL-1β mRNAs by RT-PCR

Pancreatic samples were dissected, immediately frozen in liquid nitrogen and stored at –80°C. After incubation of samples in Trizol, total RNA was reverse transcribed into cDNA as previously described [10]. After treatment at 37°C for 30 min with 20-50 units of RNase-free DNase I, total RNA was reverse transcribed into cDNA. The RT reaction mixture was amplified by PCR using sense and antisense primers specific for TNFα, PAP, IL-10, IL-1β and β-actin as internal control. The samples were subjected to 40 PCR cycles and quantification of cDNA was performed by electrophoresis on a 3% agarose gel. Samples showing a β-actin mRNA level lower than 300.106 molecules were excluded. When the pancreatic concentration of β-actin mRNA was unaffected in our experimental conditions, for each sample TNFα mRNA level was expressed relative to the β-actin mRNA level, whereas PAP, IL-10 and IL-1β mRNA levels were expressed as the optical density ratios of their amplified cDNAs to that of β-actin cDNA.

Data management

Data were entered in a database software (Excel 5.0, Microsoft, USA) then transferred to a statistical software (Staview 4.5, USA). Simultaneous comparisons of three samples (controls saline, controls alcohol and TNBS) were performed using a Kruskal-Wallis (non parametric) test. The statistical significance threshold was set at P = 0.05. Results were expressed as median values.

Results

Colonic damages

The 4 Balb/c control mice which did not receive TNBS but intrarectal ethanol or saline solution did not develop colitis. Examination of their colons always resulted in a score of 0/10 according to Wallace and 0/6 according to Ameho. The 8 TNBS-treated mice developed colitis, as evidenced by their scoring from 3/10 to 10/10 according to Wallace and from 3/6 to 6/6 according to Ameho (table I).

Pancreatic damages

Macroscopic and microscopic examination of the pancreas showed normal histology in the 4 control mice. Histological alterations were observed in 5 of the 8 mice with TNBS-induced colitis. Yet, pancreatic lesions with inflammatory infiltrate and fibrin aggregates were scarce and mostly located at the periphery of the gland. Fat was also infiltrated by inflammatory cells (lymphocytes, granular cells, neutrophils). These cells seldom infiltrated peripheral pancreatic lobules with limited glandular necrosis (figure 1). No vascular lesions or haemorrhage were seen.

Immunohistochemical staining for PAP was negative in all control mice and positive in the 8 mice with TNBS-induced colitis. Staining distribution was patchy, some lobules being strongly positive whereas others were negative. The PAP-positive areas

Table I – Histological scores of colonic and pancreatic damages, and PAP immunohistochemical results in 4 control mice (saline, alcohol) and 8 mice with TNBS-induced colitis.

<table>
<thead>
<tr>
<th>CONTROL / TNBS MICE</th>
<th>Microscopic colonic score</th>
<th>Macroscopic colonic score</th>
<th>Pancreatic histological lesions</th>
<th>PAP immunohistochemistry</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline 1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Saline 2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Alcohol 1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Alcohol 2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>TNBS 1</td>
<td>4/6</td>
<td>5/10</td>
<td>0</td>
<td>+</td>
</tr>
<tr>
<td>TNBS 2</td>
<td>3/6</td>
<td>3/10</td>
<td>0</td>
<td>+</td>
</tr>
<tr>
<td>TNBS 3</td>
<td>4/6</td>
<td>7/10</td>
<td>Minimal, peripheric</td>
<td>+</td>
</tr>
<tr>
<td>TNBS 4</td>
<td>6/6</td>
<td>6/10</td>
<td>Minimal, peripheric</td>
<td>+</td>
</tr>
<tr>
<td>TNBS 5</td>
<td>6/6</td>
<td>5/10</td>
<td>Minimal, peripheric</td>
<td>+</td>
</tr>
<tr>
<td>TNBS 6</td>
<td>5/6</td>
<td>10/10</td>
<td>Minimal, peripheric</td>
<td>+</td>
</tr>
<tr>
<td>TNBS 7</td>
<td>6/6</td>
<td>6/10</td>
<td>Minimal, peripheric</td>
<td>+</td>
</tr>
<tr>
<td>TNBS 8</td>
<td>6/6</td>
<td>6/10</td>
<td>0</td>
<td>+</td>
</tr>
</tbody>
</table>

PAP: Protein-associated-pancreatitis. Microscopic colonic score according to Ameho et al. (9). Macroscopic colonic score according to Wallace et al. (8).

Fig. 1 – Microscopic examination of the TNBS-treated mouse pancreas showing minimal and peripheric changes.

ABBREVIATIONS:

AP : Acute pancreatitis
CD : Crohn’s disease
CP : Chronic pancreatitis
IBP : Inflammatory bowel disease
PAP : Pancreatitis-associated-protein
TNBS : 2,4, 6-trinitrobenzene sulfonic acid
were not related to areas showing histological lesions. Positive lobules also showed variation in the intensity of staining. PAP immunolocalization exhibited a granular cytoplasmic staining (figure 2). In cases of strong PAP expression, staining was also present in acinar lumen and sometimes in intralobular ducts (figure 3). No PAP immunoreactivity was seen in duct cells or islets of Langerhans.

Quantification of pancreatic TNFα and β-actin mRNA by RT-competitive PCR and semi-quantification of PAP, IL-10 and IL-1β mRNAs by RT-PCR

Results of mRNA quantifications in pancreas are shown in table II. Three mice with TNBS-induced colitis were excluded because the amplified β-actin mRNA level was low, reflecting mRNA degradation during processing. Control mice were divided in two groups according to the intrarectal instillation, either saline or alcohol.

PAP and IL-1β mRNA concentrations were significantly increased in TNBS-treated mice, compared to controls (P < 0.05). TNFα mRNA levels were also increased, although without reaching statistical significance (P = 0.06). The median mRNA levels of PAP, IL-1β and TNF in TNBS-treated mice were greater than in alcohol-treated mice, being also increased comparing to mRNA levels in saline-treated mice. By contrast, IL10 mRNA levels remained unaffected.

Discussion

The frequency of IBD-associated pancreatitis, 1.2–1.5% on clinical criteria, is probably underestimated because the disease is mainly silent [4, 11–19]. In fact, autopsy studies in ulcerative colitis and CD revealed the presence of macroscopic or microscopic pancreatic lesions in 14–53% and 38% of the cases, respectively [12, 13]. The pancreatic exocrine function assessed by the secretin-cerulein test, a Lundh meal test or the PABA test [14–16] was found altered in 21% to 80% of patients, without systematic association with pancreatic ductal changes [16].

Finally, in patients with IBD, the frequency of clinical pancreatitis differs markedly from the incidences of increased serum levels of amylase (5.8–15.8%) [16–19]. Increased serum levels of amylase or lipase could not be specific of pancreatic involvement [4, 18].

Its relationship to the extent and the severity of the disease led to a controversial conclusion in the recent literature [4, 18, 19]. Such data and the recent evaluation of standardized incidence ratios of pancreatitis, 4.3 for CD and 2.1 for ulcerative colitis [3], suggest that many cases of IBD-associated pancreatitis are extra-intestinal manifestations of the intestinal disease [4, 5]. A better understanding of the consequences of IBD on pancreatic pathophysiology should therefore lead to improved patient care.

Available information on the pathogenesis of IBD-associated pancreatitis is scarce. Based on histopathological features, mainly intra and interlobular fibrosis, marked acinar regression and intense inflammatory infiltrate but without pseudocyst or pancreatic stone, IBD-associated pancreatitis is reminiscent of autoimmune pancreatitis [4, 20–24]. Pancreatic autoantibodies have been found in 4% or 39% of patients in two studies, suggesting immune deregulation triggered by mucosal ulceration of the bowel, although artefactual cross-reactivity with other autoantibodies cannot be excluded [25–27].

Getting further insight into IBD-associated pancreatitis requires experimental studies on an animal model. Several models of experimental colitis are available, but few of them can reproduce the chronic, spontaneously relapsing inflammation usually associated with IBD [28]. We chose the model of TNBS-induced colitis in which acute and possibly chronic colitis is induced in unsensitized rats [29, 30]. TNBS acts as a hapten inducing an inflammatory response, possibly mediated by macrophage-mediated recognition and degradation of cells exposed to TNBS, triggering a T-cell mediated response [29–36].

In fact, instillation of TNBS into rat pancreatic ducts also induces chronic inflammation [37]. Therefore, TNBS seems to be a non-specific inducer of tissue inflammation. Because we are looking for pancreatic alterations associated with TNBS-induced colitis, the possibility that TNBS could leak from the rectum into the bloodstream and directly affect pancreas must be considered. Because TNBS-induced lesions are secondary to macrophage activation, pancreatic inflammation would require prolonged exposure to significant amounts of the hapten, as previously shown in colon and in experiments with intraductal TNBS instillation. However, because its poor solubility requires rectal instillation in a 50% ethanol solution, its diffusion through the rectal wall to build up significant serum concentrations is unlikely. In addition, serum amylase and lipase were not assessed in mice. Although data about amylasemia associated with inflammatory colitis are available in humans [16–19], serum level of amylase is not comparable in human and in experimental animal models since the former correlates with the severity of the disease although the latter does not.
### Table II  – Quantification of TNFα mRNA by competitive PCR and semi-quantification of PAP, IL-10 and IL-1β mRNAs in mouse pancreas (individual values).

<table>
<thead>
<tr>
<th></th>
<th>PAP</th>
<th>IL-10</th>
<th>IL-1β</th>
<th>TNFα</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Controls (saline)</td>
<td>Controls (alcohol)</td>
<td>TNBS</td>
<td>Controls (saline)</td>
</tr>
<tr>
<td>0.00</td>
<td>9.33</td>
<td>8.56</td>
<td>68.72</td>
<td>88.16</td>
</tr>
<tr>
<td>0.00</td>
<td>13.33</td>
<td>51.97</td>
<td>57.93</td>
<td>36.35</td>
</tr>
<tr>
<td>0.00</td>
<td>2.84</td>
<td>33.27</td>
<td>91.86</td>
<td>102.35</td>
</tr>
<tr>
<td>4.27</td>
<td>61.39</td>
<td>157.55</td>
<td>76.40</td>
<td>58.83</td>
</tr>
<tr>
<td>1.50</td>
<td>69.11</td>
<td>84.08</td>
<td>100.2</td>
<td>37.88</td>
</tr>
<tr>
<td>47.67</td>
<td>17.22</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>55.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Med 0</td>
<td>Med 13.3</td>
<td>Med 51.9</td>
<td>Med 76.4</td>
<td>Med 58.8</td>
</tr>
<tr>
<td>P = 0.007</td>
<td>P = 0.68</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P = 0.01</td>
<td>P = 0.06</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Control saline: intrarectal instillation of NaCl saline solution; control alcohol: intrarectal instillation of 50% ethanol; TNBS: intrarectal instillation of TNBS in 50% ethanol. mRNA concentrations were expressed as a function of the number of mRNA molecules in the same sample of α-actin as internal control. Statistical test: Kruskal-Wallis; med: median.

Macroscopic and microscopic lesions of the pancreas were found in 5/8 TNBS-treated mice, located at the periphery of the pancreatic gland in the area adjacent to the colon, the rest of the gland being histologically normal. Such lesions are likely due to local diffusion of inflammatory mediators from the colon rather than a consequence of systemic changes. By contrast, PAP expression in acinar cells evidenced by immunohistological staining was found in the whole gland, including areas without histological abnormalities, with a patchy distribution similar to the distribution of lesions in patients with IBD-associated pancreatitis [4]. PAP is not expressed in the normal pancreas, as confirmed by the absence of staining in control saline mice. PAP staining was not significant in control ethanol-treated animals despite the weak number of control alcohol-mice. However PAP is slightly increased in control alcohol mice compared to control saline mice but with a median value inferior to that of TNBS mice (table II), probably because a 50% alcohol solution can trigger intestinal mucosa damages. In fact, PAP is the most sensitive among stress proteins as marker of pancreatic alteration [6, 7]. For instance, serum PAP is already elevated in many alcoholics without clinical signs of pancreatitis [38]. Yet, the PAP mRNA median level in control alcohol mice was lower than in TNBS-treated mice (table II), the hapten-effect of TNBS increasing the mucosal damages caused by the alcoholic solution in which it is dissolved. Our data showing PAP overexpression in the pancreas of TNBS-treated mice, with a median value of PAP mRNA, normalized to β-actin, of 51.9 compared to 13.3 in control alcohol mice and 0 in control saline mice (table II), reflect the presence of a mild pancreatic stress, which may not be accompanied by significant histological alterations. Such lesions might eventually develop upon exposure to a prolonged stress associated with chronic colitis. Induction by colitis of a mild pancreatic stress would explain why IBD-associated pancreatitis is clinically silent. Pancreatic lesions might be triggered by early overexpression of cytokines. The role of cytokines in producing intestinal alterations is well-known [36]. Overexpression of TNFα and IL-1β mRNA was observed in the intestinal mucosa or submucosa with CD damages as well as in adjacent tissue not yet altered by the disease [36, 39]. To our knowledge, expression of cytokines involved in the CD pathway has not been performed in extra-intestinal locations of CD. Median pancreatic levels of IL-1β mRNA were significantly increased upon TNBS treatment from 33.6 in the control saline group and 76.2 in the control alcohol group to 92.1 (P = 0.01) (table II) suggesting involvement of these pro-inflammatory cytokines in the eventual development of pancreatic lesions associated with intestinal inflammatory diseases. Median pancreatic levels of TNFα mRNA were also increased but without reaching the threshold of statistical significance (P = 0.06) (table II). In that context, it is noteworthy that the mRNA levels of IL-10, a cytokine considered as anti-inflammatory, remained unchanged after TNBS-induced colitis.

In conclusion, experimental colitis in mice is associated with early pancreatic alterations, evidenced by PAP overexpression in acinar cells. The patchy distribution of PAP-expressing acini is very similar to that of pancreatic lesions observed in IBD-associated pancreatitis in human. It is noteworthy that intrarectal instillation of 50% ethanol already triggered pancreatic stress-response, suggesting that mucosal alteration rather than colitis itself is responsible for pancreatic response. Overexpression of TNFα and IL-1β two mediators involved in intestinal lesions, also occurred in pancreas during TNBS-induced colitis. Altogether, these results strongly suggest that pancreatitis should be considered as a possible extra-intestinal manifestation of IBD. Future experimental studies should aim at characterizing the molecular messengers sent by the colon, that induce pancreatic stress.

### REFERENCES


