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Experimental acute alcohol pancreatitis-related liver damage and endotoxemia: synbiotics but not metronidazole have a protective effect

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OBJECTIVE: The aim of this study was to test the effect of gut manipulation by either novel synbiotics or by metronidazole on either endotoxemia or the severity of liver damage in the course of acute pancreatitis from alcohol ingestion.

METHODS: Sprague–Dawley rats were fed for 1 week through an intragastric tube a liquid diet with either: (i) 1 mL t.i.d. of a mixture of synbiotics (*Lactobacillus acidophilus*, *Lactobacillus helveticus* and *Bifidobacterium* in an enriched medium); (ii) 20 mg/kg t.i.d. metronidazole; or (iii) standard diet. Then, acute pancreatitis was induced by caerulein and when the disease was full-blown, rats were fed an alcohol-rich diet. Synbiotic and metronidazole treatment was given for a further 2 weeks. Transaminase and endotoxemia levels were measured before treatment, after 6 h, after 24 h and 2 weeks later, at the time the rats were killed. Liver samples were obtained for histological analysis.

RESULTS: Synbiotics but not metronidazole improved the acute pancreatitis-induced increase in endotoxemia and transaminase levels. The addition of alcohol worsened these variables to a limited extent in the synbiotic-treated group, while metronidazole had a negative effect on liver damage.

CONCLUSIONS: Gut flora pretreatment with synbiotics was able to effectively protect against endotoxin/bacterial translocation, as well as liver damage in the course of acute pancreatitis and concomitant heavy alcohol consumption. The beneficial effect of synbiotics on liver histology seems to be correlated with endotoxemia. Metronidazole did not produce such a beneficial effect; in fact, it further worsened liver damage when alcohol was added to the background of ongoing acute pancreatic inflammation.

KEY WORDS: acute pancreatitis, alcohol-related liver disease, metronidazole, synbiotic.

INTRODUCTION

Marotta *et al.*[1] have recently shown that in the course of experimental acute pancreatitis, a significant bacterial translocation with either hematogenous and lymphatic systemic spread occurs. This phenomenon can be significantly improved by performing bowel cleansing with disaccharides. Further, we and other groups have demonstrated that the acute pancreatitis process is associated with a certain degree of liver dysfunction.[2–4] On the other hand, clinical and experimental evidence suggests that acute and chronic ethanol ingestion per se, without any specific gastrointestinal disease, are able to promote the systemic translocation of gut-derived endotoxins,[5,6] possibly due to increased mucosal permeability. Endotoxins represent a fundamental lipopolysaccharide component of the Gram-negative cell wall, and a large number of experimental investigations

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have demonstrated that endotoxins, by switching on the activation of the pro-inflammatory cascade,[7] play an important role in the pathogenesis of liver disease.[8] Bhagwandeen *et al*. have shown that endotoxins promote liver necrosis in rats fed an alcohol-enriched diet.[9] More recently, data supporting such a relationship between endotoxemia, pro-inflammatory mediators and ethanol-induced liver damage have been obtained.[7] Therefore, bacterial translocation represents an epiphenomenon of a multifaceted interplay between factors such as diet, microbial antagonism, mucosal physiology and turnover, intestinal motility, bilio-pancreatic secretion and local and systemic immune status. By employing a novel probiotic, we have very recently demonstrated that alcohol-induced endotoxin translocation and liver damage can be significantly improved.[10] The aim of the present study was to test the effect of metronidazole, an antibiotic active against anaerobe flora, as well as a probiotic mixture on both endotoxemia and on the severity of liver damage in the course of acute pancreatitis, followed by alcohol ingestion.

MATERIALS AND METHODS

Seventy-two Sprague–Dawley rats (210–240 g) were used. Animals were housed in an environmentally controlled vivarium (with temperature, ventilation, humidity and light-dark cycle controlled), with free access to deionized water and non-nutrient fibers. Rats were fed a liquid diet that provided the daily caloric intake through an intragastric tube, which had been previously positioned under ketamine anesthesia, as described elsewhere.[11] The diet was supplemented with either: (i) 1 mL t.i.d. of a probiotic mixture (*Lactobacillus acidophilus*, *Lactobacillus helveticus* and *Bifidobacterium* suspended in an ion- and vitamin-enriched medium [Microflorana-F, Named SRL, Lesmo, Italy]) (24 rats); (ii) 20 mg/kg t.i.d. metronidazole (24 rats); or (iii) an equivalent amount of saline (24 rats). After 1 week, acute pancreatitis was produced by a 6-h i.v. infusion of caerulein at a dosage of 10 μg/kg. Six hours later, when the pancreatitis was full-blown, the diet composition was changed as follows: 25% fat, 21% protein, 12% carbohydrates and 42% alcohol. The diet was designed to maintain a blood alcohol concentration of more than 150 mg/dL, as measured by regular blood sampling. To do so, alcohol content was modulated from 10 g/kg per day to 15 g/kg per day. Venous blood samples were taken at entry into the study, and after 6 h (at the time of alcohol addition), after 24 h, and 2 weeks later at the time the rats were killed in order to determine the transaminase and endotoxemia levels. The latter parameter was measured by using modified Fukui's method.[12,13] Further, at the time the rats were killed, liver and pancreatic samples were obtained for histological analysis. The severity of liver damage was assessed blindly for five samples by arbitrarily grading steatosis (percentage of lipid-containing hepatocytes: 1+, 0–25%; 2+, 26–50%; 3+, 51–75%; 4+, >75%), necrosis and inflammatory infiltrate (each parameter separately: 1+, one focus per field; 2+, two-three foci per field; 3+: three or more foci per field). Healthy rats that were intragastrically administered a balanced liquid diet devoid of alcohol served as controls.

Statistical analysis

For the statistical analysis, one-way analysis of variance was used and significance between the experimental and control groups was determined by Bonferroni's method. A difference of $P < 0.05$ was considered significant. Results are expressed as mean ± SD.

RESULTS

Rats treated either with metronidazole or with Microflorana-F did not show any detectable change in bowel habits and, in particular, no diarrhea occurred. No deaths occurred during the study period. As compared with healthy controls, rats with acute pancreatitis had a significant increase in endotoxemia and transaminase levels ($P < 0.05$) (Figures 1, 2). The addition of metronidazole did not affect these variables, whereas treatment with the probiotics reverted both variables to normal ($P < 0.05$). The addition of alcohol to the rats' diet brought about a significant increase in endotoxemia and transaminase levels. Dietary supplementation with Microflorana-F caused a statistically significant decrease in both endotoxemia and transaminase levels ($P < 0.01$), although the levels were still significantly higher than those in the healthy control group ($P < 0.05$). The main histological features related to

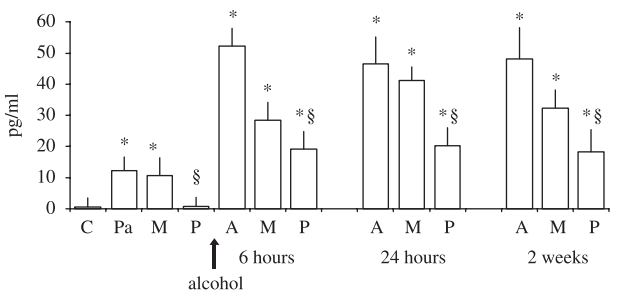

Figure 1. Plasma endotoxin concentration: time course assessment. C, control; Pa, pancreatitis; M, metronidazole; P, probiotic; A, alcohol. *$P < 0.001$ *vs* controls; §$P < 0.01$ *vs* alcohol ± metronidazole.

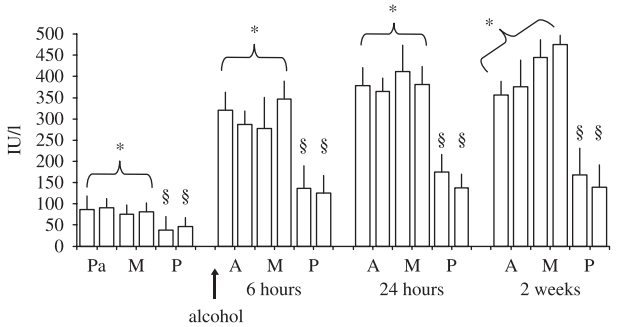

Figure 2. Plasma transaminase concentration aspartate aminotransferase-alanine amino transferase (AST-ALT): time course assessment. Control values for AST-ALT: >50 IU/L. Pa, pancreatitis; M, metronidazole; P, probiotic; A, alcohol. *$P < 0.001$ *vs* controls; §$P < 0.001$ *vs* alcohol ± metronidazole.

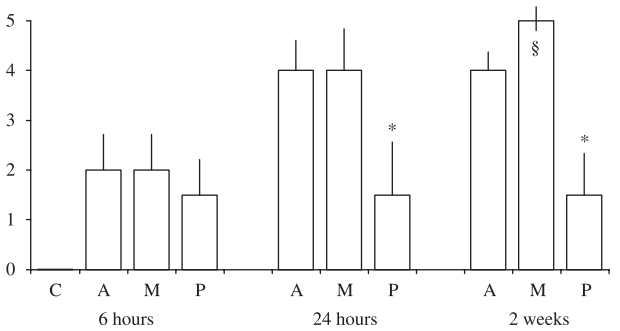

Figure 3. Necro-inflammatory score of the liver: time course assessment. C, control; M, metronidazole; P, probiotic; A, alcohol. *$P < 0.05$ *vs* alcohol ± metronidazole; *$P < 0.05$ *vs* alcohol.

the diet with alcohol were severe steatosis, with a mild to moderate grade of scattered inflammatory and necrotic foci ($P < 0.001$ *vs* healthy control). Concomitant treatment with metronidazole did not improve the situation, in fact a more marked necro-inflammatory score was recorded (Figure 3). Accordingly, the transaminase level remained constantly high ($P < 0.001$ *vs* healthy control). As compared with the alcohol- and alcohol plus metronidazole-fed rats, dietary supplementation with the probiotics significantly improved the histological score and, in particular, the necro-inflammatory assessment ($P < 0.001$; Figure 3). No relationship was found between the endotoxemia or transaminase level and the overall histological score. However, when these factors were examined separately, the necro-inflammatory score had a statistically significant relationship with endotoxemia (r: 0,61, $P < 0.05$; Figure 4). Neither the biochemical (trypsin and amylase) nor the histological assessment of the acute pancreatitis process showed any significant change, irrespective of the treatment employed (data not shown).

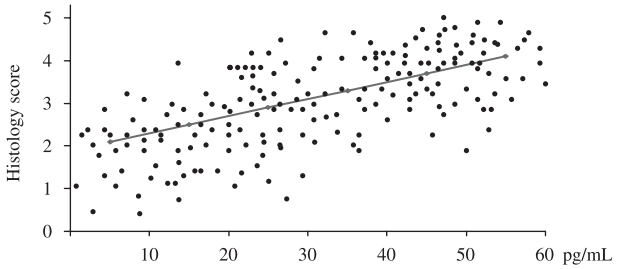

Figure 4. Correlation between blood endotoxin level and histological score (necrosis and inflammatory infiltration). $r = 0.61$; $P < 0.05$.

DISCUSSION

Most of the intestinal bacteria population in humans consists of obligate anaerobes whose luminal concentration is greatly increased relative to the aerobiotic flora that exist after the ileo-cecal valve. Under normal conditions, endotoxins pass through the intestinal wall, with transient detectable amounts in the portal circulation.[14] These endotoxins are then rapidly cleared by the liver parenchymal matrix and Kupffer cells.[15,16] Therefore, a number of pathological conditions affecting the gastrointestinal mucosa, such as inflammatory bowel diseases, have been shown to enhance gut permeability to bacterial toxins. Nonetheless, we have shown that in the course of diseases affecting visceral organs other than the gut wall, such as acute pancreatitis, a significant increase in peritoneal permeability,[17] as well as bacterial translocation, occurs.[1] We have demonstrated that this latter phenomenon is associated with an increased interglandular space in the gut mucosa, and concomitant motility disorders,[18] as well as splanchnic and gut villi ischemia,[19] are likely to take place, as suggested by other groups. The first finding in our study was that pretreatment with probiotics prevented gut-derived endotoxemia, together with causing the normalization of biohumoral markers of acute pancreatitis-associated liver disease. This result is particularly interesting when it is considered that acute pancreatitis per se negatively affects the clearance of intravenously infused labeled endotoxins.[20] However, enteral alcohol feeding, even without any concomitant gastrointestinal disease, is able to substantially enhance endotoxin absorption, possibly via a rise in gastrointestinal pH followed by bacterial overgrowth in the jejunum.[21] Endotoxins, in turn, can trigger a reactive liver chemotactic recruitment of neutrophils, and activate the resident macrophages, thus further worsening liver damage.[22,23] Therefore, supervening alcohol intoxication during the course of acute pancreatitis, as occurred in our model, represented a further deleterious factor

on both the promotion of endotoxin absorption from the gut, on its clearance from the liver and, in the final analysis, on overall liver damage. Accordingly, when the changes associated with steatosis are directly related to alcohol abuse, our study has confirmed a statistically significant direct correlation between endotoxemia and necro-inflammatory score of liver dysfunction. These data are in agreement with the recent observation in a population of patients with chronic liver disease that endotoxins are closely correlated with the degree of liver damage.[24] To date, the therapeutic measures so far used to limit bacterial translocations and liver damage have been either very aggressive surgical ones, such as colectomy in experimental models,[25] or conservative ones that use antibiotics,[26,27] with the latter choice being liable to possibly enhance *Escherichia coli* overgrowth and translocation through the mesenteric lymph nodes.[28] As a matter of fact, metronidazole was ineffective with respect to abnormalities of endotoxemia and biochemical liver parameters following acute pancreatitis. During the 2-week histological assessment of the liver, the groups treated with this antibiotic had an increased necro-inflammatory damage score. The reasons behind such a phenomenon are still unclear; however, it could be postulated that the selective inhibitory effect on anaerobic gut flora might have elicited a facultative bacterial translocation into the mesenteric lymph nodes, and a massive release of endotoxins, and both these suggestions are supported by the literature.[29,30] Interestingly, as we have already very recently demonstrated in a classical model of alcohol intoxication,[10] the present probiotic mixture has been found to play a role in the intestinal ecosystem, by mechanisms that are still awaiting further investigation, and which significantly limit endotoxin absorption and related negative consequences to the liver. It is possible that the probiotics might promote gut motility and/or interact directly with the mucosal lining. In this regard, it is noteworthy that *L. acidophilus* and *Bifidobacterium*, which were both in the probiotic mixture we used, have been long known to exert a barrier effect against *E. coli* translocation.[31] Local and systemic immune function might represent a further mechanism linking the effects of the probiotics and the two diseases encompassed in this model, that is, pancreatitis and alcohol intoxication. Indeed, we have already shown that in the course of experimental acute pancreatitis, the killing oxidative burst from macrophages is impaired,[32] which might partially explain our previously mentioned observation that liver clearance of endotoxins is impaired in this disease.[20] Moreover, alcoholics have a depressed reticulo-endothelial function,[33,34] which, even in the absence of overt liver failure, can cause detectable levels of endotoxins in the blood.[12] Although in the present study we did not address this issue, the probiotic mixture used has been recently demonstrated by our group to significantly promote splenocyte production of interferon-α and -γ, as well as enhancing the enzymatic activity, phagocytosis and oxidative burst generation of macrophages.[35,36]

In conclusion, our data suggest that gut flora pretreatment with probiotics is able to effectively protect against endotoxin/bacterial translocation, as well as liver damage in the course of acute pancreatitis and concomitant heavy alcohol consumption. Metronidazole had no positive impact on this phenomenon and, in fact further worsened liver damage when alcohol consumption was added to the background of ongoing acute pancreatic inflammation.

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