World J. Surg. 29, 766–770 (2005)
DOI: 10.1007/s00268-005-7409-9

WORLD Journal of SURGERY

Effect of Intraperitoneal Application of an Endotoxin Inhibitor on Survival Time in a Laparoscopic Model of Peritonitis in Rats

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Published Online: May 19, 2005

Abstract. Gram-negative sepsis due to fecal peritonitis is a hazardous disease with a high percentage having a lethal course. The inflammatory effects are induced by endotoxin release. We performed this study to evaluate the potential of direct intraperitoneal application of an endotoxin inhibitor in a laparoscopic peritonitis model in rats. The human feces specimen was prepared, and a standard fecal specimen (0.5 ml/kg b.w.) was applied via minilaparotomy. The rats were randomized to two studies. First, rats were randomized to three groups to define the survival time: (1) rats without further manipulation; (2) rats with laparoscopic lavage using NaCl; (3) rats with laparoscopic lavage using endotoxin inhibitor. Second, rats underwent the same procedure used in the first part of the study and an additional group with only NaCl lavage without peritonitis was randomized. To evaluate the immunologic or biochemical effects, animals were killed at a standard time of 20 hours until the postmortem examination was established. Interleukins 6 and 10 (IL-6, IL-10), malondialdehyde, and protein carbonyl group levels in plasma and particularly in peritoneal fluid were assayed. The first part of the experiment showed significantly increased survival after endotoxin inhibitor lavage. In the second part, administration of endotoxin inhibitor intraperitoneally caused a significant reduction of IL-6 in the peritoneal fluid, in contrast to that in the other groups. Laparoscopic application of endotoxin inhibitor intraperitoneally thus produced a beneficial effect on survival and reduction of IL-6 in peritoneal fluid. Hence, it is possible to influence the inflammation cascade by causing intraperitoneal endotoxin inhibition.

Sepsis resulting from peritonitis is a hazardous complication causing multiorgan failure and a high mortality rate (up to 47%) [1]. Various innovative treatments with anti-cytokine antibodies and soluble receptor or receptor antagonists have been studied in experimental and clinical generalized inflammation with controversial results [2–4].

Endotoxin a product of gram-negative bacteria, is an extremely potent toxin that leads to multiple organ failure in the presence of gram-negative sepsis and peritonitis. Endotoxin induces the release of a variety of mediators, including tumor necrosis factor -α (TNF-α), the interleukins (IL), platelet-activating factor (PAF), interferon-γ leukotrienes, and thromboxane A_2 [5]. A systemic inflammatory reaction follows, with subsequent organ failure. Systemic administration of an endotoxin inhibitor shows no clinical benefit [2]. Monoclonal antibodies against endotoxin may have a significant advantage in subgroups of treated patients [6]. The potential of endotoxin inhibition has not been completely evaluated, especially whether direct intraperitoneal application of the endotoxin inhibitor can inhibit the intraperitoneal endotoxin effects in peritonitis. The aim of the current study was to evaluate the effect of intraabdominal application of an endotoxin inhibitor in the presence of severe fecal peritonitis in an experimental laparoscopic model in rats.

Methods

Endotoxin Inhibitor (H-Lys-Thr-Lys-Cys-Lys-PHE-Leu-Lys-Lys-Cys-OH)

Endotoxin inhibitor (BACHEM Biochemica GmbH, Heidelberg, Germany) was applied intraperitoneally and intravenously. A 10-mg portion of endotoxin inhibitor (40 mg/kg b.w.) was dissolved in 1 ml phosphate-buffered saline (PBS), and 10 ml of this solution was injected intraperitoneally.

The defined specimen of human feces was prepared as described elsewhere [7]. All the experimental animals received the identical spectrum of organisms and a comparable number of pathogens. The fecal specimens were investigated microbiologically to determine the viability of the pathogens. Male Wistar rats weighing 280–330 g (Harlan Deutschland, Borchen) were acclimated to a climate- and light cycle-controlled environment for at least 7 days prior to the investigation. The experimental protocol was approved by the responsible committee of animal use and care (Regierungspräsidium Sachsen-Anhalt, Dessau).

For the surgical procedures, the animals were anesthetized by intraperitoneal injection of ketamine hydrochloride) 80 mg/kg b.w. (Ketanest, Parke Davis, Berlin, Germany) and an intramuscular injection of xylazine 12 mg/kg b.w. (Rompun, Bayer, Leverkusen, Germany). The standard fecal specimen (0.5 ml/kg b.w.) was applied via minilaparotomy [8]. The incision wound was then closed in two layers. Thereafter, the animals were randomized to two studies. A central venous catheter was additionally implanted via the vena jugularis interna.

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First Experiment: Survival Study; Experimental Model

Survival time and mortality were evaluated.

Group 1.1 (Control Group, n = 10). After induction of peritonitis, further surgical manipulation followed in group 1.1.

Group 2.1 (Laparoscopic Lavage, n = 10). In group 2.1 the median laparotomy wound was opened and a 5 mm trocar inserted. Via this trocar, pneumoperitoneum with a flow of 0.4 l/min and a maximum intraperitoneal pressure of 6 mmHg was established, and a 300 telescope (Karl Storz GmbH, Tuttlingen, Germany) was introduced. After exploration of the abdominal cavity, a Veress needle was passed through the abdominal wall in the right lateral quadrant, through which the abdominal cavity was twice lavaged with 2 ml physiological saline solution. The pneumoperitoneum was maintained for 20 minutes. Thereafter, the trocar incision was closed with a suture.

Group 3.1. (Laparoscopic Lavage and Endotoxin Inhibition, n = 10). The procedure for group 3.1 was similar to that for group 2.1. After NaCl lavage, 1 ml of the dissolved endotoxin inhibitor was applied intraperitoneally and left in the abdominal cavity.

Comment. The survival time was of interest and was defined as the interval from the primary surgical manipulation to the time of death. As supportive therapy, a central venous infusion with Ringer's lactate (1 ml/hr; Serum-Werk, Bernburg, Germany) and an antibiotic infusion with piperacillin/tazobactam 20 mg 96 h (Tazobac; Lederle Arzneimittel GmbH, Münster, Germany) was applied.

Second Experiment: Evaluation of Immunologic and Biochemical Effects

Forty male Wistar rats were used under conditions identical to those in the first experiment. To evaluate the immunologic or biochemical effects, a standard time for the postmarten examination was established. The results of the first experiment established this time length of to be 18 hours after surgical manipulation; this interval was then used to determine the median length of survival after intraperitoneal fecal implantation and laparoscopic lavage.

- Group 1.2: procedure identical to that for group 1.1; autopsy was performed 18 hours after surgical manipulation
- Group 2.2: procedure identical to that for group 2.1; autopsy was performed 18 hours after surgical manipulation
- Group 3.2: procedure identical to that for group 3.1; autopsy was performed 18 hours after surgical manipulation
- Group 4.2: peritonitis induction; lavage was performed at $t = 0$

During the postmortem examination peritoneal fluid, blood, and organs (kidney, lung, pancreas, liver) were removed. Thereafter, organ portions and peritoneal fluid were stored at −80°C in pyrogen-free and endotoxin-free cups for subsequent analysis. Blood was collected into a heparinized syringe via a sterile cardiac puncture. This sample was used for microbiologic studies and to obtain a differential blood count. The rest of the blood was centrifuged at 6000 rpm for 10 minutes and the plasma supernatant thus obtained stored at −80°C. For determination of oxidative stress markers, lung, liver, kidney, and pancreatic tissues were kept on ice, and 500 mg samples were homogenized in 2 ml of 50 mM Tris pH 7.6, 2 mM EDTA, 5 mM butylated hydroxytoluene (BHT), and 2 mM phenylmethylsulfonyl-fluoride (PMSF) using a Potter homogenizer. The supernatant and the pellet obtained by $800 \times g$ centrifugation was stored in aliquots at −80°C and used for further analyses.

Biochemical Methods

The IL-6 and IL-10 levels in plasma and peritoneal fluid were detected by commercial enzyme-linked immunosorbent assay (ELISA) kits (Laboserv, Staufenberg, Germany). Malondialdehyde (MDA), a marker of lipid peroxidation, was measured by derivatization with thiobarbituric acid and photometric detection at 532 nm after C18 reversed phase high-performance liquid chromatography (HPLC). Measurement of oxidative protein modification was based on the derivatization of protein-bound carbonyl groups with 2,4-dinitrophenylhydrazine (DNPH) and subsequent detection of the DNP moiety by a specific antibody (Sigma, Deisenhofen, Germany). This principle was employed in an ELISA [9].

Statistics

Values were expressed as the mean and standard deviation (SD). Differences were tested for statistical significance by analysis of variance (ANOVA) for comparison of multiple groups. Differences found by ANOVA were confirmed by Student's *t*-test. The survival rate was tested by a log rank test. In all instances, $p < 0.05$ was considered to indicate significance.

Results

First Experiment

In all groups the mortality rate was 100% as expected. In the untreated group the survival time was a median of 13.87 ± 2.68 hours. In contrast, the treated group (laparoscopic lavage with NaCl) had a significantly longer survival time ($p < 0.01$) of 18.74 ± 3.87) hours. In the third group (with laparoscopic lavage and intraperitoneal application of endotoxin inhibitor) the rats survived a median of 25.1 ± 3.47 hours. In comparison to both of the other groups the significance was $p < 0.001$. Kaplan-Meier curves showed a significantly decreased survival course between the groups (Fig. 1).

Second Experiment

Results of the first experiment show increased survival time in lavage-treated rats with intraperitoneal infection. Additional intraperitoneal injection of endotoxin inhibitor produces an even more prolonged survival time. After induction of fecal peritonitis, there was a massive elevation of the IL-6 level (Fig. 2); but the level in the abdominal cavity was higher than that in the plasma measured at the same time. Administration of endotoxin inhibitor intraperitoneally caused a significant reduction in the IL-6 level in peritoneal fluid in contrast to that in the other groups (Fig. 3).

There was no difference in IL-10 levels between the control and peritonitis groups and no statistical variation between the plasma and peritoneal fluid levels.

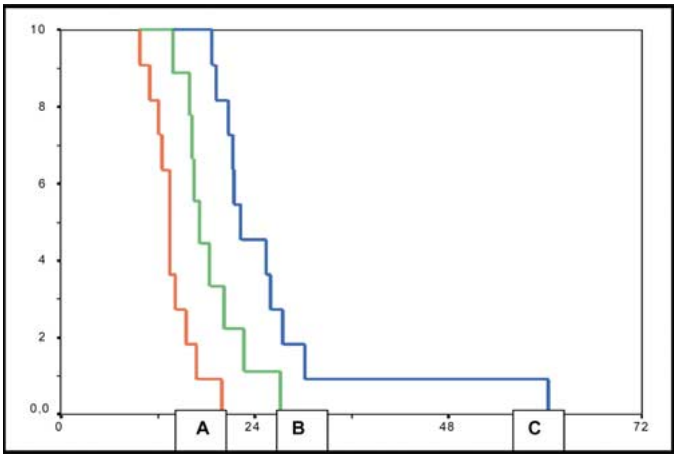

Fig. 1. Survival time (Kaplan-Meier survival analysis) after laparoscopic lavage with NaCl (B), laparoscopic lavage with NaCl and application of endotoxin inhibitor (C) versus the control without abdominal lavage (A). A: peritonitis no therapy; B: laparoscopic lavage with NaCl; C: laparoscopic lavage with NaCl and endotoxin inhibitor. Significance ($p < 0.001$; log-rank test) to A and B.

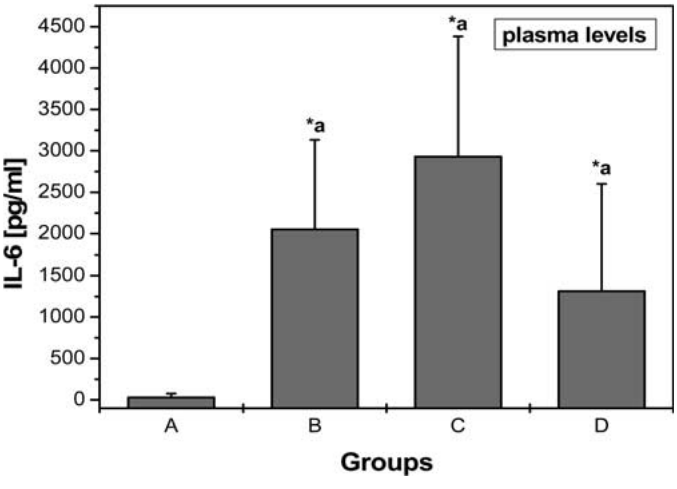

Fig. 2. Interleukin-6 (IL-6) concentration in plasma 20 hours after induction of intraperitoneal fecal infection and 18 hours after laparoscopic lavage. A: control group, no peritonitis only laparoscopic lavage; B: peritonitis without therapy; C: peritonitis and laparoscopic lavage with NaCl; D: peritonitis and laparoscopic lavage with NaCl and intraperitoneal endotoxin inhibitor; *a: significance: $p < 0.05$ between groups with and without peritonitis.

Malondialdehyde a marker for lipid oxidation, did not change in the liver, lung, or pancreas. In the kidney, however, the difference between the untreated peritonitis group and all other groups was significant. Intraperitoneal application of endotoxin inhibitor produced a significant, though modest, increase in the MDA level compared to that in the other groups.

Oxidation-modified proteins originated by free radicals are verifiable by measuring protein-binding carbonyl groups. After 18 hours the protein-carbonyl concentrations in the kidney and pancreas were significantly different in rats without treatment and depending on the fecal infection in the treated animal groups. There was no difference in the levels in lavage-treated groups with or without endotoxin inhibitor.

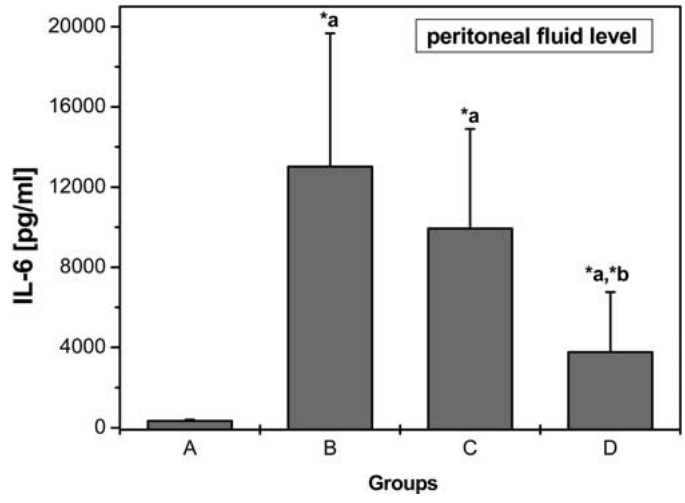

Fig. 3. Interleukin-6 (IL-6) concentration in peritoneal fluid 20 hours after induction of intraperitoneal fecal infection and 18 hours after laparoscopic lavage. A: control group, no peritonitis, only laparoscopic lavage; B: peritonitis without therapy; C: peritonitis and laparoscopic lavage with NaCl; D: peritonitis and laparoscopic lavage with NaCl and intraperitoneal endotoxin inhibitor; *a: significance: $p < 0.05$ between groups with and without peritonitis; *b: significance: $p < 0.05$ between groups with and without endotoxin inhibitor.

Discussion

Laparoscopic surgical procedures are being used increasingly for intraabdominal diseases complicated by inflammatory processes [10]. Reports in the literature describe the successful use of laparoscopic interventions to treat acute appendicitis, perforated gastric ulcer, and diverticulitis of the sigmoid colon [11–14].

We hypothesized that laparoscopic lavage and additional administration of endotoxin inhibitor would have beneficial effects on the outcome and clinical symptoms of severe fecal peritonitis in a rat model. There are now experimental data evaluating the direct intraabdominal influence of endotoxin inhibition. Other authors have demonstrated prolonged survival after fecal peritonitis in rats because of antibacterial substances [15]. The effect of intravenous endotoxin inhibitor application in patients with gram-negative sepsis is controversial. Although Greenman et al. [6] reported prolonged survival, others have seen no benefit in treated patients in randomized trials [2, 6, 16]. In contrast to these results, we found improved survival and paraclinical parameters in our experimental study. However, we did not compare intravenous and intraperitoneal application of endotoxin inhibitor in our experiments. We concluded that intraperitoneal application of endotoxin inhibitor is due to down-regulation of the inflammatory reaction in the abdominal cavity. Unfortunately, there are not sufficient clinical data in randomized trials, so assessment of a clinical benefit is not possible. Because of the unsatisfactory amount of data derived from interventional intravenous endotoxin inhibitor studies in patients, further experimental studies for local application of endotoxin inhibitor are needed.

Direct lavage with physiologic NaCl shows an effect, but lavage with endotoxin inhibitor is more efficient. This suggests that direct inhibition of endotoxin produced by gram-negative bacteria via lavage is possible. The mechanism by which direct application offers protection in the rat model is unclear. Further studies are nessesary to determine whether lipopolysaccharide (LPS) release

macrophage migration, or release of inflammation mediators was diminished.

The inflammation cascade was triggered by the intraperitoneal infection and became evident intraabdominally and in plasma. IL-6 is a proinflammatory cytokine component of the inflammation cascade and can be produced by a number of cells [17, 18]. The IL-6 concentration was significant higher in peritoneal fluid than in plasma. This reflects an early inflammatory reaction of the peritoneum and inflammatory activation by mesotheliol cells, which is initially compartmentalized. This is shown by the relatively small increase in the plasma level. We therefore concluded that early bacteremia cannot be interpreted as sepsis. The lavage fluid revealed therapeutic potential in comparison with the untreated animals after 18 hours. Our data are in accordance with other published data [19]. In contrast to the plasma IL-6 levels the peritoneal fluid IL-6 level was lower than when NaCl lavage wasa applied. Lavage leads to a dilution effect, no toxin inhibition in the abdominal cavity, and limited local down-regulation of he inflammation in the abdominal cavity. Migration of inflammatory cells and release of cytokines with systemic inflammation is possible, however resulting in higher IL-6 plasma levels. Otherwise, IL-6 activation is an early process during inflammation. Therefore after 18 hours the IL-6 level may already be past its peak in the peritoneal fluid, and the inflammation cascade may explain the plasma IL-6 level. Another study showed an intermittent decrease in peritoneal fluid IL-6 after induction of peritonitis with subsequent persistently high levels of IL-6 in patients who developed secondary peritonitis [20]. We suggest that lavage alone is not sufficient to prevent local severe peritonitis and systemic sepsis, and IL-6 plasma levels indicate further severe inflammation. However, we showed that local application of endotoxin inhibitor is more efficient.

Otherwise, severe peritonitis is not controlled by the application of endotoxin inhibition in our study. Because there is no difference between the treatment groups and the peritoneal fluid and plasma IL-10 levels we believe that IL-6 is a more sensitive marker for early inflammation. This is in accord with the results of other studies [20, 21].

Lipid peroxidation results in the occurrence of relatively stable aldehydes, most notably MDA. The MDA levels in pancreas, liver, lung, and kidney were significantly decreased after endotoxin inhibition. This reflects diminished damage of biologic membranes (and therefore cell damage and tissue injury in the kidney) after intraperitoneal endotoxin inhibitor application[22].

The multisystem organ failure that occurs with sepsis is often ascribed to widespread hypotension and impaired oxygen delivery. This explanation is especially intuitive in the kidney, where hemodynamic forces drive glomerular filtration, and high metabolic activity exists in the transporting tubular epithelium. The presence of TNF is well known to lead to the release of multiple other cytokines into the circulation [23]. Among 52 patients with spontaneous bacterial peritonitis, significantly higher levels of TNF and IL-6 were found in the peritoneal fluid and plasma of the patients with renal failure [24].

Thus we suggest that cytokines such as TNF and IL-6 mediate LPS-induced acute renal failure indirectly by leading to hypotension by the release of other inflammatory mediators into the circulation, or both. We showed that blockade of endotoxin by direct inhibition reduces cytokine release and membrane damage. Renal failure may be later prevented or even a beneficial course of renal function during endotoxin sepsis is conceivable. It is unclear whether endotoxinemia has a direct influence on renal failure.

The protein carbonyl content is the most general indicator and the most used marker of oxidative stress. In this study, protein carbonyl group concentration was significant decreased after 20 hours in kidney and pancreatic tissue in the treatment groups. This shows an effect of lavage; but the influence of endotoxin application (if any) remains unclear because there was no significant difference in the lavaged groups with and without endotoxin.

Conclusions

In summary, we concluded that intraperitoneal application of endotoxin inhibitor is an effective treatment for fecal peritonitis. It may, in fact, interfere with the inflammation cascade.

Acknowledgments

This study was supported by Kultusministerium Sachsen-Anhalt grant 2355A/0085H.

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