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Liver Failure Induces a Systemic Inflammatory Response

Prevention by Recombinant N-Terminal Bactericidal/ Permeability-Increasing Protein

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The observed increased susceptibility of patients with fulminant hepatic failure for local and systemic infections has been hypothesized to be due to a failure of the hepatic clearance function and subsequent leaking of endogenous endotoxins into the systemic circulation. However, experimental evidence for such a systemic inflammation during liver failure due to endogenous endotoxemia is lacking. Therefore, we designed a study to clarify whether circulating endotoxins due to liver failure could lead to the development of systemic inflammation. In a rat model for liver failure induced by a two-thirds partial hepatectomy, we evaluated the course of circulating tumor necrosis factor and interleukin-6, changes in blood chemistry and hemodynamics, and histopathological changes in the lungs. Partially hepatectomized animals, but not sham-operated animals, demonstrated cardiac failure, increased levels of creatinin and urea, metabolic acidosis, high plasma levels of tumor necrosis factor and interleukin-6, and an influx of PMNs in the lungs–together indicating the development of a systemic inflammatory response. Continuous infusion of recombinant N-terminal bactericidal/permeability-increasing protein ($rBPI_{23}$), a well described endotoxin-neutralizing protein, prevented these inflammatory reactions. **Ex vivo** ***experiments with rat plasma samples confirmed the presence of circulating endotoxins in partially hepatectomized rats as opposed to those treated with $rBPI_{23}$. Thus, our results indicate that the early phase of liver failure induces a systemic inflammatory response triggered by circulating endotoxins, which can be prevented by perioperative infusion of $rBPI_{23}$. (Am J Pathol 1995, 147:1428–1440)***

Apart from its involvement in numerous metabolic and homeostatic processes, the liver plays a predominant role in the regulation of inflammatory responses, because it produces acute phase reactants, generates and metabolically inactivates inflammatory mediators, and clears bacteria and their products.[1] Up to 80 to 90% of the systemic phagocytic capacity consists of the phagocytic cells lining the liver sinusoids, the Kupffer cells.[2] Consequently, a decrease in hepatic phagocytic function significantly impairs the removal of circulating particles, in spite of a compensatory increase in the splenic and lung phagocytic capacity.[3–6] The important role of the liver in the host defense is illustrated by observations that patients with liver failure are susceptible to local and systemic infections,[7–10] frequently have a systemic endotoxemia,[7–9,11,12] and may even develop clinical signs of sepsis.[13]

Earlier studies have found that hepatic failure can lead to leaking of endogenous endotoxins into the systemic circulation.[14] The inflammatory sequelae of administration of a known amount of endotoxin have

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been described in numerous animal models. However, little is known about the systemic consequences of endogenously released endotoxins during liver failure. First, the exact nature of a supposed inflammatory host response after experimental liver failure has never been characterized. Second, it remains to be established to what extent endogenous endotoxins in models of liver failure contribute to hemodynamic and metabolic changes. For example, it is well known that patients with bacteremia do not necessarily suffer from sepsis, ie, the inflammatory response syndrome associated with clinical bacterial infection. Thus, the presence of circulating endotoxins as such does not inevitably lead to clinical manifestations.

Here we studied the development, extent, and prevention of an inflammatory host response and its clinical consequences in a rat model of liver failure induced by a two-thirds liver resection.[1] Various inflammatory and clinical parameters were analyzed in rats that received either continuous i.v. administration of the recombinant N-terminal fragment ($rBPI_{23}$)[15] of bactericidal/permeability-increasing protein (BPI),[16,17] a well described protein that specifically binds to and neutralizes lipopolysaccharides (LPS) *in vitro*[15,18,19] and *in vivo*,[20,21] or an inactive control protein (thaumatin) as opposed to sham-operated rats;[2] *ex vivo* experiments with rat plasma were used to detect the presence of circulating endotoxins in these rats. The results indicate that acute liver failure due to partial hepatectomy (phx) induces systemic inflammatory reactions in experimental rats, and that these reactions are significantly blunted by perioperative infusion of $rBPI_{23}$.

Materials and Methods

Animals

This study was carried out in accordance with the recommendations of the Guide for the Care of Laboratory Animals used in our institute. Male Wistar rats (n = 84, 230 to 250 g, Harlan CPB, Zeist, The Netherlands) were allowed to acclimatize to the laboratory environment for 5 days, during which time they had free access to water and rat chow (SRM-A; Hope Farms, Woerden, The Netherlands). The animals were housed under standard environmental conditions with a 12-hour light/dark cycle. Chow was withdrawn on the evening before surgery.

Experimental Protocol

The animals were randomized to receive a two-thirds phx or a sham operation, and a continuous i.v. infusion of either recombinant N-terminal bactericidal/permeability-increasing protein ($rBPI_{23}$; XOMA Corp., Berkeley, CA) or thaumatin (XOMA Corp.), as an isotypical (isoelectric, iso-kd) inactive control protein. This resulted in four groups of animals: 1) sham-thaumatin (SH-CON, n = 20);[2] sham-$rBPI_{23}$ (SH-BPI, n = 20);[3] phx-thaumatin (PHX-CON, n = 22); and[4] phx-$rBPI_{23}$ (PHX-BPI, n = 22). Before the start of the treatment and surgical procedures, the animals were anesthetized using ether and placed in the supine position on a heating pad. First, a loading dose of the assigned treatment drug was given via the tail vein: 1 mg/kg $rBPI_{23}$ or 1 mg/kg thaumatin in 0.5 ml buffer solution (containing 20 mmol/L sodium citrate, 150 mmol/L sodium chloride; pH 5). Then, a polyethylene catheter (PE-50; Fisher Scientific, Springfield, NY) was placed via the right jugular vein into the superior caval vein, and subcutaneously tunneled to the interscapular region. Through a springwire (Instech Laboratories, Plymouth Meeting, MA), this i.v. line was connected to a swivel (Instech Laboratories) and a micro-infusion pump (Harvard Apparatus, Boston, MA). Placement of the catheter was done under aseptic conditions in a flow chamber using sterile equipment. Once the connection was established, a second loading dose of the assigned treatment drug, containing 2 mg/kg $rBPI_{23}$ or thaumatin in 1 ml (0.5 ml buffer solution plus 0.5 ml 0.9% (w/v) NaCl), was injected slowly into the i.v. line. Immediately thereafter, a continuous infusion of 0.2 mg/kg/hour $rBPI_{23}$ or thaumatin was started at an infusion rate of 500 μl/hour. The dose and treatment regime used were based on previous studies in rats: 3 mg/kg $rBPI_{23}$ infused simultaneously (via a different vein) with 250 mg/kg LPS over 30 minutes, completely blocked induced cardiovascular effects.[21] Based on previous direct measurements of plasma levels of endotoxin in partially hepatectomized rats,[14] we estimated these levels to be <1 ng/ml. Therefore, the dose of $rBPI_{23}$ used in the present study was expected to be sufficient. Because of the rapid clearance of $rBPI_{23}$ from the circulation, equivalent to that of cytokines, we used two consecutive loading doses to reach sufficient plasma levels, one before placement of the infusion catheter and the other just before the operation, followed by a continuous i.v. infusion to maintain these levels during the entire observation period.

Subsequently, the rats received a two-thirds phx or a sham operation. All surgical procedures were performed between 9:00 AM and 11:00 AM. phx was performed according to the method of Higgins and Anderson,[22] consisting of resection of the median and left hepatic lobes. The resection was performed

using a single vicryl ligature, which was carefully placed around the pedicles of these lobes using cotton wool sticks to prevent bleeding from the liver bed. Utilizing this method, no blood loss occurred during and after the operation. Sham animals underwent a midline laparotomy, and gentle manipulation and exteriorization of the median and left lobes, but no actual resection was performed. The incision was closed in two layers by vicryl sutures. The animals were then allowed to regain consciousness, and within 20 minutes they were able to move freely, with exception of the animals that were used for frequent blood sampling (see Blood Sampling section). All animals continued to receive their i.v. infusions. In addition, the animals remained fasted and received no fluids orally during the experiment.

Six animals of each group were used to obtain serial blood samples during a 4-hour period, after which they were sacrificed by a lethal injection of sodium pentobarbital (100 mg/kg). Eight animals of each group were included in the protocol for assessment of hemodynamic parameters, measured at 4 hours after the operation. Frequent blood sampling was not done in these animals, as this may interfere with hemodynamic parameters. In the remaining animals of each group (SH-CON and SH-BPI, each n = 6; PHX-CON and PHX-BPI, each n = 8) treatment was continued for a 24-hour period. After 24 hours, these animals were reanesthetized using ketamine HCL (50 mg/kg i.p.) and a small arterial blood sample (200 μl) was taken for comparative interleukin-6 (IL-6) analysis. Thereafter, the lungs were removed for histological studies. Rats were killed by an overdose of sodium pentobarbital as described.

Blood Sampling

From six animals of each group, small quantities of arterial blood (250 μl) were drawn before and 1, 2, 3, and 4 hours after the operation for determination of plasma levels of cytokines, hematocrit (at $t = 4$ hours only) and, additionally, for stimulation experiments of human whole blood. For frequent blood sampling, anesthesia was maintained using 50 mg/kg ketamine HCL i.p. and a small arterial line was placed in the femoral artery. Heparinized blood samples were immediately placed on ice, and plasma was obtained by centrifugation of blood for 15 minutes at 1500 × *g* at 4°C. All plasma samples were harvested in a laminar flow cabinet to prevent contamination, and stored in aliquots at −70°C until tested.

The eight animals of each group that were randomized for the hemodynamic measurements were only sampled after these experiments, ie, at 4 hours after sham operation or phx. Arterial blood samples were taken for chemical blood analysis as well as determination of plasma levels of tumor necrosis factor (TNF) and IL-6. Cytokine levels assessed in these animals were subsequently compared with those of the animals from which samples had been taken more frequently.

Hemodynamic Measurements

For hemodynamic measurements, animals were anesthetized using ketamine HCL (50 mg/kg i.p.) and placed in the supine position on a heating pad that maintained rectal temperature at 37°C. The trachea was intubated with a small polyethylene tube (PE-240; Fisher Scientific) to facilitate breathing. The right carotid artery and left femoral artery were cannulated using PE-50 tubing. Both catheters were connected to P23Db Statham pressure transducers to monitor placement of the carotid catheter into the left cardiac ventricle and to measure femoral artery blood pressure. After these procedures, rats were allowed to stabilize for 20 minutes. Before the hemodynamic measurements, 150 ml of blood was drawn from the femoral artery for hematological, pH, and blood gas analysis. Thereafter, mean arterial blood pressure (MAP) and heart rate were recorded continuously during 1 minutes.

Cardiac output (CO) measurements were performed as part of an organ blood flow study (to be published separately) using the radiolabeled microsphere method, as previously described.[23,24] Briefly, 0.7 ml of a 0.9% NaCl suspension containing 1.0 to 1.5×10^5 microspheres labeled with ^{46}Sc (New England Nuclear, Boston, MA) were injected into the left ventricle over a period of ~20 seconds. A reference blood sample was obtained from the femoral artery by a calibrated roller pump starting 5 seconds before microsphere injection, at a rate of 0.50 ml/minute for 90 seconds. After the microsphere injection, arterial blood pressure was recorded once more, to assure that the procedure had not been performed during significant hemodynamic changes nor had caused such changes. Thereafter, blood was drawn from the femoral artery, and the animals were killed by an intra-arterial injection of pentobarbital sodium (100 mg/kg). After verification of the position of the left ventricular catheter, the heart was dissected free and weighed. Radioactivity was counted in a well type γ counter (LKB-1280, Ultrogamma, Wallac, Turku, Finland). Count rates were corrected for natural background and counter dead time. CO was calculated according to the equation $CO = Fa\ (Q_{tot}/Q_a)$, where Fa is reference flow, Q_{tot} is

the total injected radioactivity, and Q_a is the radioactivity in the reference sample. Reference flow was computed from the weight of blood in the sample syringe (assuming a whole blood density of 1.069 g/ml) and the duration of withdrawal. Arterial blood flow to the heart (coronary arteries) was calculated using the equation $F = Fa\ (Q_o/Q_a)$, where Q_o is the count rate in the organ to be measured.

Hematological and Chemical Blood Analysis

To determine acid–base balance, pH was measured as part of a blood gas analysis using a commercial blood gas analyzer (ABL 330, Radiometer, Copenhagen, Denmark). Hemoglobin, hematocrit, total white blood cell (WBC) numbers, and chemical parameters were analyzed by automated laboratory analysis. Ammonia levels were assayed by a standard enzymatic method.

Histological Studies

Twenty-four hours after phx or sham operation the lungs were removed. Sections of the tissues were made by conventional methods and chloroacetate-esterase (Lederstain) was used for staining of polymorphonuclear neutrophils (PMNs). All sections were examined in a blinded fashion.

TNF and IL-6 Bioassays

Biological TNF activity was measured as described by Espevik and Nissen-Meyer,[25] using the murine fibrosarcoma WEHI 164 clone 13 cell line. Briefly, 4×10^4 WEHI cells/100 μl were incubated in RPMI 1640 containing 100 U/ml penicillin, 100 μl/ml streptomycin, L-glutamine, 10% (v/v) fetal calf serum (FCS), and 1 μg/ml actinomycin D in the presence of serial dilutions of samples to be tested. After 18 hours at 37°C, cytotoxicity was assessed with the MTT (3-(4,5-dimethylthazol-2-yl)-2,5-diphenyl tetrazolium bromide) method, as previously described.[26] Serial dilutions of samples to be tested were compared to a standard curve of recombinant mouse TNF and expressed as units per ml. One unit per ml is the amount of TNF that kills 50% of the cells. The lower detection limit of the assay at the dilutions of samples used was 1 U/ml.

IL-6 bioactivity was measured with the murine hybridoma B cell line B9, as described previously.[27,28] In short, 5000 B9 cells in 200 μl flat-bottomed wells were grown in Iscove's modified Dulbecco's medium (IMDM) containing 100 U/ml penicillin, 100 μg/ml streptomycin, 5×10^{-5} mol/L 2-mercaptoethanol, and 5% (v/v) FCS in the presence of serial dilutions of samples to be tested. After 68 to 72 hours cells were labeled with 7.4 kBq of [^{3}H]thymidine (74 GBq/mmol), and thereafter the radioactivity incorporated in the nuclei was counted. Results were compared with a standard curve of natural IL-6 from cultured human monocytes stimulated with endotoxin, and expressed as units/ml. One unit/ml of IL-6 is the concentration that induces half-maximal thymidine incorporation in the assay. The lower detection limit of the assay at the dilutions of samples used was 10 U/ml.

Endotoxin-Induced Stimulation of Human Whole Blood Cultured With ex Vivo *Rat Plasma*

To assess the presence of biologically active endotoxins in the plasma of each rat during the treatment regime, plasma samples from each group of animals, taken before and 1 and 2 hours after surgery, were tested for their potency to induce the release of pro-inflammatory cytokines by human mononuclear cells. From each group, samples from six animals taken at the indicated time points were assayed. The results of each animal were compared to its own baseline ($t = 0$ hours) value.

Human whole blood of healthy volunteers was cultured at a final dilution of 1:10 in 200 μl flat-bottom wells in endotoxin-free IMDM containing 100 U/ml penicillin, 100 μl/ml streptomycin, and 0.1% (v/v) endotoxin-free FCS, in the presence of serial dilutions of rat plasma samples to be tested. Each sample was tested alone and in the presence of either 5 μg/ml αCD14 MAb (αCD14.22; Central Laboratory of the Netherlands Red Cross Blood Transfusion Services (CLB), Amsterdam, The Netherlands), 2 μg/ml rBPI$_{23}$ or 2 ng/ml polymyxin B (Sigma Chemical Co., St. Louis, MO). After incubation for 18 hours at 37°C, culture supernatants were harvested and stored at −70°C until tested for the presence of cytokines. As a control, normal rat plasma containing LPS (*Escherichia coli* 055:B5) at concentrations of 0.1 to 1000 pg/ml, was repeatedly tested under similar conditions and in the same assay as the experimental samples to validate the results. In another control experiment, the effect of αCD14 MAb, rBPI$_{23}$, polymyxin B, and polyclonal αTNF (CLB, αTNF-514) antibody on cytokine induction by recombinant human TNF-α (courtesy of Dr. A. Creasey, Chiron Corp., Emeryville, CA) was assessed in human whole blood in the presence of normal rat plasma.

TNF-α in the supernatant was measured by a sandwich-type enzyme-linked immunosorbent assay

(ELISA) using two monoclonal antibodies (MAbs, CLB, Department of Immune Reagents) raised against recombinant human TNF-α. One MAb (MAb CLB-TNFα-7) was used for coating at a concentration of 2 μg per ml. The other MAb (MAb CLB-TNFα-5) was biotinylated and used in combination with streptavidin poly-horseradish peroxidase conjugate (CLB, Department of Immune Reagents) to detect bound TNF-α. Stimulated human mononuclear cell supernatant was used as a standard for the assay. The amount of TNF-α present in this supernatant was assessed by comparison with purified recombinant human TNF-α. Results were expressed as pg per ml by reference to this standard.

IL-6 concentration in the supernatant was quantified with an ELISA modified from that described in detail before.[29] Briefly, purified monoclonal anti-human-IL-6 antibody (MAb CLB-IL6–16) was used as a capture antibody, and biotinylated sheep antibodies in combination with streptavidin poly-horseradish peroxidase conjugate were used to detect bound IL-6. Results were expressed as pg per ml by reference to a standard consisting of recombinant human IL-6.[30]

IL-8 was measured with a sandwich-type ELISA modified from that described previously[31]; monoclonal anti-human-IL-8 antibody (MAb CLB-IL8/1) and biotinylated affinity purified sheep anti-IL-8 antibodies were used as capture and detecting antibodies, respectively. Polymerized horseradish peroxidase conjugated to streptavidin was used to quantify bound biotinylated antibodies. Results were compared with those obtained with dilutions of recombinant human IL-8 and expressed as pg per ml.

All samples were assayed in duplicate in the same culture experiment using blood from one healthy donor, and this was repeated using blood from another donor, to assess donor-dependent variability. All ELISAs used had interassay variation coefficients of <15%, as was estimated from the variation of dose–response curves obtained on at least three different days over a three-month period. All supernatants were tested within one assay procedure to minimize the interassay variation for each cytokine.

Statistical Methods

The data are expressed as means ± SEM. The nonparametric Mann-Whitney *U* Test was used to assess the significance of differences between groups. Analysis of variance for repeated measures was used to assess significant changes in parameters in the course of the observation period. A difference was considered significant at $P < 0.05$ (two-tailed). The Mann-Whitney *U* Test was then used to determine the significance of the differences between groups. Data were analyzed with a commercial statistical package (Stat-View; Abacus Concepts, Inc., Berkeley, CA).

Results

Systemic Hemodynamics

Repeated pilot experiments involving continuous measurement of MAP, heart rate, and CO during the first 6 hours after surgery and periodically over the next 18 hours, revealed that the nadir of hemodynamic changes occurred 4 hours after liver resection. In these pilot experiments, an arterial pressure transducer instead of the microsphere method was used to assess CO, enabling us to perform frequent measurements in the same animals. Decreases in MAP, heart rate and CO were initiated after 2 hours, reaching a nadir after 4 hours, and had almost disappeared after 6 to 8 hours.

Rats receiving a two-thirds phx showed significant decreases of MAP and heart rate of 27% and 19%, respectively (both $P < 0.005$) at 4 hours after surgery, whereas CO decreased by 43% ($P < 0.005$) as compared with sham animals. $rBPI_{23}$ treatment in phx rats prevented these changes in MAP, heart rate, and CO, resulting in values equivalent to those of sham animals. $rBPI_{23}$ had no effect on these parameters in sham rats (Figure 1).

The blood supply to the heart tissue remained unchanged after phx: 4.76 ± 1.22 and 4.93 ± 0.67 ml · minutes^{-1} in sham and phx animals, respectively. When the blood flow was calculated per gram of heart tissue, again no difference was observed: 6.07 ± 1.61 and 6.72 ± 0.88 ml · minutes^{-1} · g^{-1} in sham and phx animals, respectively. Infusion of $rBPI_{23}$ had no significant effect on these parameters in either sham or phx animals. The hypotension in the phx animals apparently had induced a compensatory redistribution of blood flow, because these animals showed a 62% increase in the proportion of CO that was distributed to the heart tissue via the coronary arteries ($P < 0.05$). In $rBPI_{23}$-treated phx animals, CO distribution to the heart tissue was similar to that of the sham group.

Histological Studies

The lungs are frequently involved in systemic inflammatory reactions. We therefore investigated possible inflammatory changes in this organ. All phx animals showed a significant number of PMNs infiltrating the

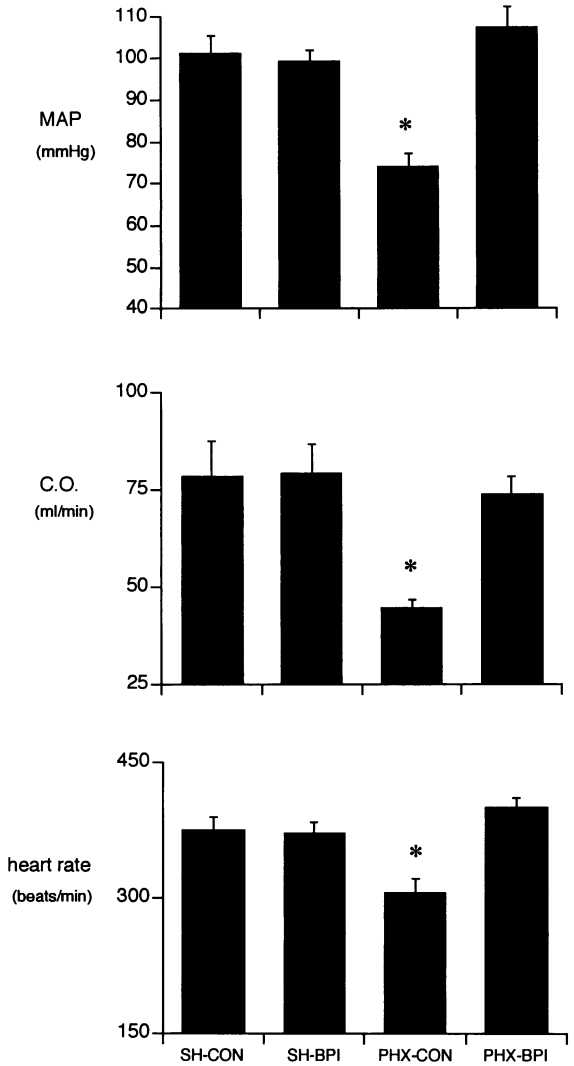

Figure 1. *Cardiac function. Hemodynamic parameters of partially hepatectomized and sham rats treated with rBPI$_{23}$ or an isotypical inactive control protein (thaumatin). Results are expressed as means ± SEM. *P < 0.005, by the Mann-Whitney U Test.*

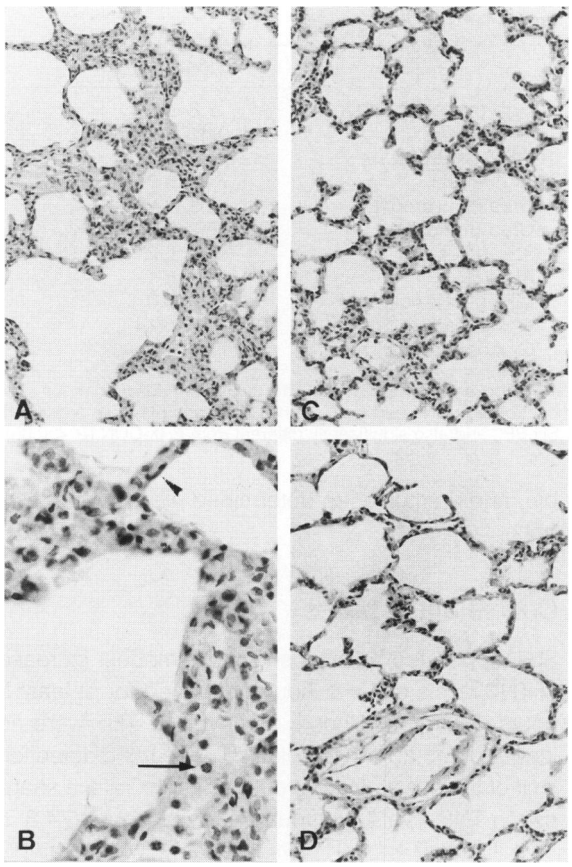

Figure 2. *Lung morphology. Histological appearance of the lungs obtained 24 hours after phx.* (**A**) *Section of the lung from a control-treated hepatectomized rat, showing PMN accumulation and neutrophil aggregates (PHX-CON; 155×).* (**B**) *Higher magnification (410×) of* **A**, *showing neutrophil margination* (**arrowhead**) *and intravascular accumulation of PMNs* (**arrow**). (**C**) *Section of the lung from a rBPI$_{23}$-treated hepatectomized rat, showing mainly erythrocyten in the alveolar capillaries (PHX-BPI; 155×).* (**D**) *Section of the lung from a sham operated rat with normal lung morphology (SH-CON; 155×).*

lungs at 24 hours after the operation (Figure 2 A and B), which was largely abrogated by rBPI$_{23}$ treatment (Figure 2C). Furthermore, in the phx group some marginating neutrophils and intravascular accumulation of PMNs were found in the lungs as well as some edema of the alveolar septa, whereas all rBPI$_{23}$-treated phx rats showed virtually none of these morphological changes. Neither rat of the control group (sham-operated with or without rBPI$_{23}$) had an inflammatory infiltrate in the lungs (Figure 2D).

Blood Chemistry

Chemistry data as measured 4 hours after the surgical procedure are shown in Table 1. After phx, hemoglobin concentration and hematocrit both increased ($P < 0.05$). In addition, arterial blood pH and glucose levels had significantly declined as compared with sham values ($P < 0.05$ and $P < 0.01$, respectively). Levels of creatinin and urea (both $P < 0.05$), as well as of hepatic enzymes (aspartate aminotransferase and alanine aminotransferase), bilirubin, and ammonia, and total WBC numbers (all $P < 0.005$) were increased at 4 hours after liver resection. rBPI$_{23}$ treatment in phx animals resulted in significantly reduced creatinin levels and total WBC numbers, although the latter were still significantly higher than sham levels. rBPI$_{23}$ also reduced the fall in glucose levels and prevented the fall of pH in phx animals. Conversely, the pH in these animals was slightly higher than in sham animals. Urea levels in PHX-BPI animals tended to be lower than those of the PHX-CON group. rBPI$_{23}$ treatment had no significant effect on changes in hepatic enzymes, biliru-

Table 1. *Blood Chemistry*

	SH-CON	SH-BPI	PHX-CON	PHX-BPI
Hgb (mmol/L)	7.8 ± 0.4	7.6 ± 0.2	9.0 ± 0.3*	7.8 ± 0.3
Hct	0.41 ± 0.02	0.40 ± 0.01	0.45 ± 0.02*	0.40 ± 0.01
WBC (×10E9/L)	4.9 ± 0.9	5.2 ± 0.5	12.0 ± 1.5**	8.0 ± 0.9†
Arterial pH	7.33 ± 0.01	7.31 ± 0.01	7.26 ± 0.03*	7.39 ± 0.02†
Creatinin (μmol/L)	33 ± 2	34 ± 2	43 ± 4*	31 ± 2
Urea (mmol/L)	6.2 ± 0.6	5.7 ± 0.5	9.4 ± 0.5*	8.0 ± 0.4
AST (U/L)	66 ± 2	73 ± 4	622 ± 64**	689 ± 90**
ALT (U/L)	21 ± 1	28 ± 3	443 ± 69**	462 ± 128**
Bilirubin (μmol/L)	2 ± 0.1	2 ± 0.3	9 ± 1.4**	10 ± 2.7**
Ammonia (μmol/L)	104 ± 14	92 ± 7	227 ± 16**	198 ± 13**
Glucose (mmol/L)	8.3 ± 0.4	8.1 ± 0.4	6.6 ± 0.4*	7.3 ± 0.5

Blood analysis at 4 hours after a partial hepatectomy or sham operation of animals treated with either $rBPI_{23}$ or an isotypical inactive control protein (thaumatin). *$P < 0.05$ *versus* SH-CON or SH-BPI; **$P < 0.005$ *versus* SH-CON and SH-BPI; †$P < 0.05$ *versus* SH-CON or SH-BPI, and also significantly different from PHX-CON ($P < 0.05$).

bin, and ammonia, as determined 4 hours after surgery.

Course of Cytokines

Sham-operated animals showed a modest increase in TNF levels at $t = 1$ hour that lasted for at least 2 hours. From $t = 4$ hours on, levels of TNF were no longer detectable in the plasma or near the detection limit of the assay. In control-treated phx rats, a sharp rise in TNF levels was found at $t = 2$ hours (74.8 ± 16.1 U/ml; $P < 0.01$ *versus* sham), which rapidly declined thereafter to reach values of 6.8 ± 2.3 U/ml 4 hours after the resection (Figure 3A). Conversely, in the $rBPI_{23}$-treated phx animals, plasma levels of TNF were undetectable except in two animals, which had detectable TNF concentrations at only one time point ($t = 3$ hours; 1.9 and 2.9 U/ml). The difference in TNF concentrations between $rBPI_{23}$-treated and control-treated phx animals was highly significant at $t = 2$ hours ($P < 0.001$) and significant at subsequent time points ($P < 0.01$). During the entire observation period, TNF levels of $rBPI_{23}$-treated phx rats also were lower than those of sham rats. In contrast, $rBPI_{23}$ treatment had no effect on the low TNF levels found in sham-operated animals (Figure 3A).

After the sham procedure, an early although moderate rise of IL-6 was found from $t = 1$ hour on. These levels were not affected by $rBPI_{23}$ treatment in sham animals. In control-treated phx rats, IL-6 levels demonstrated a steady increase from $t = 2$ hours on, reaching peak levels of 2119 ± 396 U/ml at $t = 4$ hours ($P < 0.005$ *versus* sham). $rBPI_{23}$ treatment in phx rats yielded significantly lower levels of IL-6 compared with control treatment ($P < 0.005$ at $t = 4$ hours). Only at $t = 3$ hours, IL-6 levels in $rBPI_{23}$-treated phx rats were higher compared with sham rats ($P < 0.05$; Figure 3B).

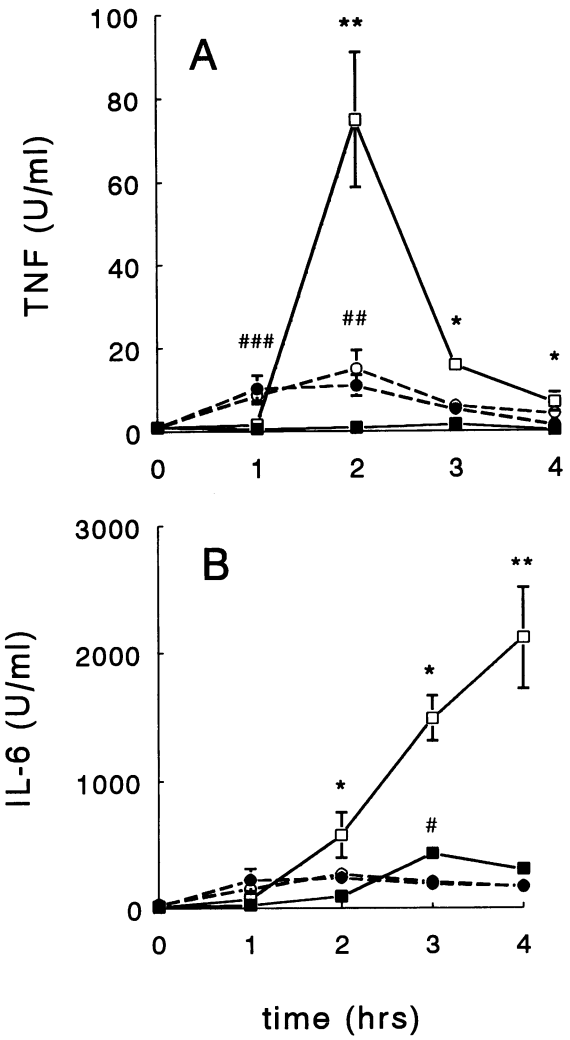

Figure 3. *Cytokine levels. Plasma levels of TNF* (**A**) *and IL-6* (**B**) *as assessed by WEHI and B9 bioassay, respectively. Results are expressed as means ± SEM: PHX-CON* (□), *PHX-BPI* (■), *SH-CON* (○), *SH-BPI* (●). **PHX-CON* versus *PHX-BPI: P < 0.01 for TNF and P < 0.05 for IL-6;* ***PHX-CON* versus *PHX-BPI: P < 0.001 for TNF and P < 0.005 for IL-6; #SH-CON or SH-BPI* versus *PHX-BPI: P < 0.05; ##SH-CON or SH-BPI* versus *PHX-BPI: P < 0.005; ###SH-CON or SH-BPI* versus *PHX-CON or PHX-BPI: P < 0.01, by the Mann-Whitney U Test.*

Stimulation of Human Whole Blood by Rat Plasma

To assess whether the observed changes after phx were due to circulating endotoxins, we examined the potency of rat plasma, taken before or after the hepatectomy or sham operation, to induce the release of cytokines in human whole blood. Using the experimental conditions described in Materials and Methods, 1 and 100 pg/ml LPS added to human whole blood resulted in a TNF-α concentration in the supernatant of 145 ± 38 and 634 ± 112 pg/ml, respectively, in the presence of 15% (v/v) normal rat plasma. This volume of normal rat plasma was equal to that used in the *in vitro* experiments with plasma from the experimental animals. Addition of αCD14 MAb, rBPI$_{23}$, and to a lesser extent polymyxin B (used at the concentrations described in Materials and Methods) resulted in an almost complete inhibition of TNF-α, IL-6, and IL-8 levels in the supernatant induced by LPS concentrations up to 100 pg/ml (data not shown). Incubation of human whole blood with plasma of sham rats gave rise to TNF-α levels in the supernatant ranging from 117 to 255 pg/ml. When αCD14 MAb, rBPI$_{23}$, or polymyxin B were added to cultures with sham plasma, levels of TNF-α as well as those of IL-6 and IL-8 in the supernatant were not significantly affected. Supernatant cytokine levels generated by rTNF-α added to cultures in the presence of normal rat plasma were not affected by αCD14 MAb, rBPI$_{23}$, or polymyxin B as opposed to polyclonal αTNF, consistent with the specific anti-LPS activities of the former three reagents.

Incubation of human whole blood with plasma of control-treated phx rats taken 1 and 2 hours after the resection, yielded significantly higher levels of human TNF-α in the supernatant as compared with plasma taken before the resection (Figure 4; both $P < 0.05$) or plasma of sham rats (both $P < 0.05$). TNF-α levels induced by phx plasma of $t = 1$ hour were consistently higher than those of phx plasma taken at $t = 2$ hours (Figure 4). In contrast, no significant increase in supernatant TNF-α levels was measured when plasma of rBPI$_{23}$-treated rats, taken at $t = 1$ (Figure 4) or $t = 2$ hours (data not shown) was tested, levels being the same as obtained with their preoperative plasma, and nearly equal to the levels found with plasma of sham-operated rats (both P = NS).

When αCD14 MAb, rBPI$_{23}$, and polymyxin B was added to the cultures and incubated with plasma of control-treated phx rats taken at 1 or 2 hours, the supernatant TNF-α concentrations were reduced to levels equal ($P < 0.05$ for αCD14 MAb and rBPI$_{23}$,

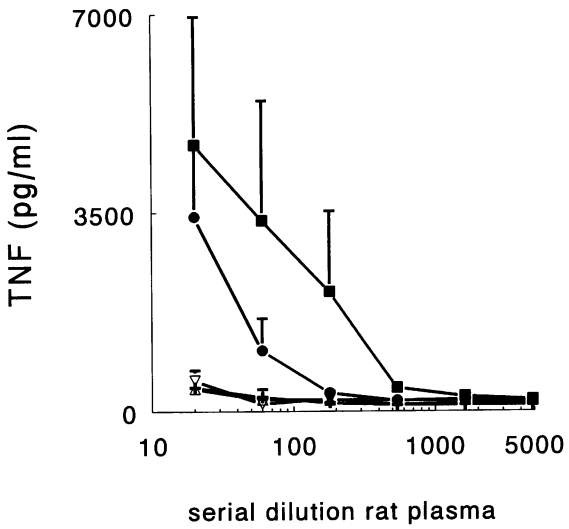

Figure 4. *Endotoxin-induced stimulation of human whole blood cultured with* ex vivo *rat plasma. Induction of TNF-α in cultures of human whole blood by plasma of control-treated or rBPI$_{23}$-treated phx rats taken before and 1 or 2 hours after the resection. TNF-α induced by PHX-CON plasma of* t = *1 and* t = *2 hours are significantly higher than that of plasma taken before the resection and plasma of PHX-BPI animals at any given time point (both* P < *0.05, by analysis of variance for repeated measures). PHX-CON,* t = *0* (+), t = *1* (■), *and* t = *2 hours* (○); *PHX-BPI,* t = *0* (Δ) *and* t = *1 hour* (▼).

compared with levels induced by phx plasma alone) or nearly equal ($P = 0.06$ for polymyxin B) to those induced by baseline ($t = 0$ hours) plasma (Figure 5A). In cultures with plasma of rBPI$_{23}$-treated phx rats, αCD14 MAb, rBPI$_{23}$, and polymyxin B did not significantly alter the levels of TNF-α (Figure 5B).

Similar results were obtained when IL-6 or IL-8 were measured in the culture supernatants. Again, plasma of control-treated rats taken at $t = 1$ and $t = 2$ hours yielded significantly higher levels of these cytokines in the supernatant than those of their preoperative samples or phx plasma during rBPI$_{23}$ treatment. These elevated levels could be significantly diminished by adding αCD14 MAb, rBPI$_{23}$, and to a lesser extent polymyxin B to the culture (data not shown).

Discussion

More than 20 years ago, Gans et al[32] suggested the involvement of endogenous endotoxins in the development of hemodynamic alterations during acute liver failure. These authors reported an immediate hypotension accompanied by a decrease in CO and heart rate in dogs that underwent a radical liver resection. These changes were delayed by pretreating the animals with orally administered neomycin, a broad spectrum antibiotic.[32] Liver failure induced by

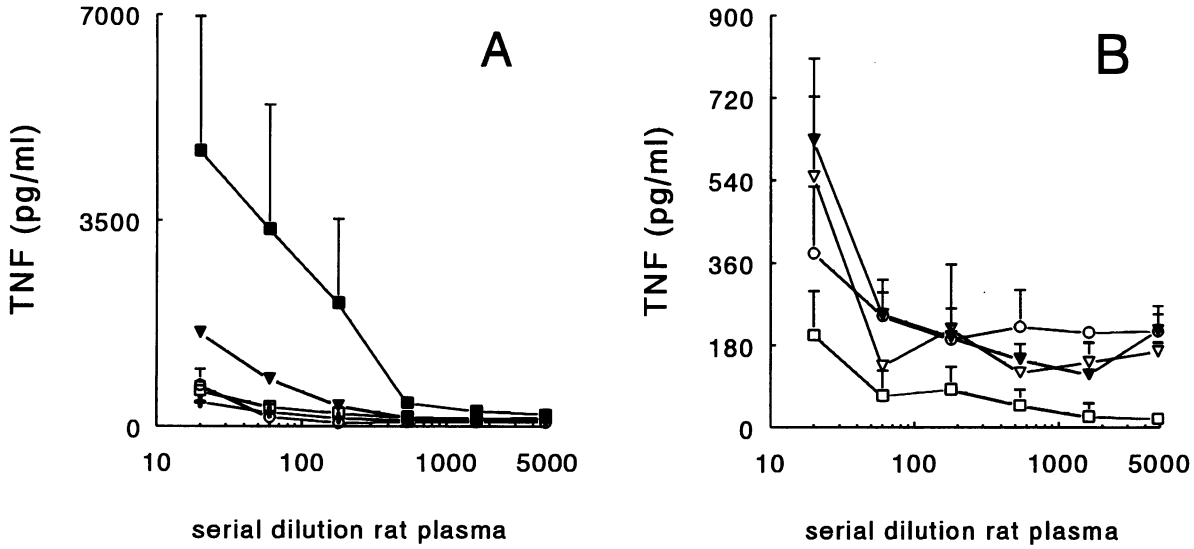

Figure 5. *Inhibition of endotoxin-induced stimulation of human whole blood cultured with* ex vivo *rat plasma. Induction of TNF-α in cultures of human whole blood by PHX-CON plasma obtained 1 hour after resection is inhibited by αCD14, $rBPI_{23}$, or polymyxin B.* (A) *Cultures of PHX-CON plasma taken at* t = *1 hour were incubated in the presence of 5 μg/ml αCD14 MAb* (○), *2 μg/ml $rBPI_{23}$* (□), *2 ng/ml polymyxin B* (▼), *or PBS* (■). *TNF-α levels obtained with baseline* (t = *0 hours*) *PHX-CON plasma are shown for comparison* (+). (B) *Plasma from $RBPI_{23}$-treated animals was incubated with human whole blood in the presence of αCD14 MAb* (○), *$rBPI_{23}$* (□), *polymyxin* B (▼), *or PBS* (▽). *Note the difference in scale of the y-axis of* A *and* B.

phx has previously been found to result in endotoxemia, as assessed using a limulus assay, which was prevented by enteral administration of nonspecific endotoxin-binding agents.[14] Others have suggested that endotoxemia secondary to failure of hepatic phagocytic function contributes to the pathogenesis of infectious complications[3,7,9] or has an additive effect on the pathology of the liver itself, and contributes to extrahepatic complications.[14,33] However, experimental evidence for this notion is lacking. Whether circulating endotoxins after liver failure may induce inflammatory responses leading to clinical manifestations of infection is not known.

In the present study, we show that liver failure due to phx in rats induces an inflammatory response, reflected by the course of plasma levels of TNF and IL-6, biochemical, and hemodynamic alterations, and morphological changes in the lungs. These inflammatory manifestations after liver failure were strongly reduced by intervention with $rBPI_{23}$, an endotoxin-neutralizing protein, suggesting this response is due to the appearance of endotoxins in the circulation. Based upon earlier observations in this model, these endotoxins most likely were derived from the gut.[14]

Development of a systemic inflammatory response during liver failure was evidenced by the appearance of proinflammatory cytokines in the circulation. After the induction of liver failure, TNF levels peaked at 2 hours, resembling the response after a bacterial or endotoxin challenge.[34–36] In these models, IL-6 reaches its nadir at 3 hours after a mild challenge, whereas after a more severe challenge such as a lethal dose of live *E. coli*, levels of this cytokine still increase after 6 hours.[37] In the present study, IL-6 levels at 4 hours after hepatectomy were still increasing, which would be consistent with a response after a severe challenge. During $rBPI_{23}$ treatment of hepatectomized rats, release of both TNF and IL-6 was effectively blunted. At 24 hours, the IL-6 levels that we measured in the group of hepatectomized animals that were sacrificed for histological studies still were significantly elevated compared with sham animals (423 ± 96 and 162 ± 27 U/ml, respectively; $P < 0.05$), suggesting ongoing IL-6 production beyond 4 hours after the induction of liver failure. In contrast, IL-6 levels in $rBPI_{23}$-treated hepatectomized animals (207 ± 30 U/ml; $P < 0.05$ *versus* PHX-CON and P = NS *versus* SHAM) were reduced to near sham levels.

The observed dysfunction of the various organs during liver failure induced by phx was similar to those occurring in sepsis or sepsis-like conditions. We found that liver failure led to the development of a hypodynamic circulatory state as evidenced by lowering of blood pressure, CO, and heart rate. These derangements in systemic hemodynamics resembled those seen shortly after exogenously administered endotoxin[38,39] or during polymicrobial sepsis.[40] Also, an early increase in hematocrit was

observed, indicating hypovolemia presumably due to an increase in vasopermeability. Renal dysfunction, as demonstrated by rising creatinin and urea, changes in WBC numbers and glucose levels, and acidosis all are signs of a systemic inflammatory response syndrome and sepsis.[41–44] Administration of rBPI$_{23}$ preserved blood pressure, CO, and heart rate after liver resection. In addition, renal dysfunction; metabolic acidosis; and changes in total WBC numbers, hematocrit and glucose levels were not observed or diminished in hepatectomized animals that were treated with rBPI$_{23}$.

Signs of liver failure such as elevated plasma levels of hepatic enzymes and ammonia also could be due to this inflammatory response. However, in our model, liver failure more likely was primarily related to the surgical procedure, because an early rise in transaminases and ammonia was not affected by rBPI$_{23}$ treatment (Table 1). In additional experiments, we found that transaminase and ammonia levels 24 hours after liver resection were indeed reduced by rBPI$_{23}$, indicating superimposed liver failure related to endotoxins.[45]

The lungs are frequently affected during a systemic inflammatory response. We therefore performed histological studies of this organ to further substantiate such a response during liver failure. Accumulation of neutrophils in the pulmonary microcirculation is a prominent feature in the lungs during endotoxemia, and it is known to precede lung injury,[46,47] characterized by pulmonary edema and increased alveocapillary permeability.[48,49] The structural changes we found in the lungs 24 hours after the induction of liver failure resembled those seen 5 hours after *E. coli*-induced shock,[50] but were less severe than those seen 26 hours after a lethal endotoxin infusion,[51] suggesting less advanced although significant lung injury. Importantly, rBPI$_{23}$ infusion prevented these morphological changes in rat lungs after phx.

The inflammatory response as observed during hepatectomy-induced liver failure, was not likely related to mere surgery. Although minor or major surgery may increase levels of proinflammatory cytokines,[12,52] the sham-operated animals in our experiments did not show a severe systemic inflammatory response, as defined by significant changes in hemodynamics, organ dysfunction, and high circulating levels of inflammatory mediators. The early, mild increase in levels of TNF and IL-6, from $t = 1$ hour on, were not likely due to the effects of endotoxins, because rBPI$_{23}$ treatment in sham animals did not alter these cytokine levels. For IL-6, such an increase in its plasma levels after laparotomy has been described before, possibly related to a stress reaction and/or tissue injury.[53] We have no explanation for the observation that TNF levels in rBPI$_{23}$-treated phx rats were even lower than those in rBPI$_{23}$-treated sham rats. Possibly, this difference can be attributed to an endogenous anti-inflammatory response involving cytokines such as IL-10 and IL-1ra, which are known to be released after an endotoxin challenge.[54,55]

Besides evaluating the *in vivo* effects of rBPI$_{23}$ treatment, we performed *in vitro* experiments with rat plasma and human whole blood to substantiate the presence of endotoxins in the circulation. We aimed to assess the bioactive properties of circulating endotoxins by evaluating their capacity to induce the production of cytokines in blood from healthy humans. We used a human blood system, as we only had antibodies against human CD14, the receptor for endotoxin on monocytes, available, and not antibodies against its counterpart in rats. Moreover, the use of human blood allowed the use of highly sensitive immunosorbent assays that are specific for human cytokines and do not cross-react with rat cytokines. The results showed that after phx, *ex vivo* rat plasma induced high levels of TNF, IL-6, and IL-8 in human blood cultures. This stimulatory capacity of phx rat plasma was evident as early as 1 hour after the resection, thus preceding the endogenous cytokine response of these animals. Cytokine release in human whole blood cultures by hepatectomized rat plasma could be blocked by αCD14 MAb, polymyxin B, and rBPI$_{23}$, in agreement with endotoxins being present in hepatectomized rat plasma. This *in vitro* cytokine release induced by plasma of hepatectomized rats and subsequent blockade showed a similar pattern as that of LPS added to normal rat plasma. Further results were consistent with the *in vivo* experiments, as plasma of rBPI$_{23}$-treated animals induced low levels of cytokines in culture, equivalent to plasma of sham animals, which were near background levels. The observation that the stimulatory capacity of phx plasma taken 2 hours after the resection was less than that of plasma taken after 1 hour might suggest that the endotoxemia was limited to the first hours of liver failure. Because the clearance capacity of the liver is not restored at that time,[3] we postulate that translocation of enteric endotoxins is not a continuous but rather a pulsatile process. After 24 to 48 hours after a liver resection in rats, liver cell proliferation is maximal and the amount of functional liver tissue partly restored,[3,56] illustrating that in this model liver failure is self-limiting.

In conclusion, we have shown that liver failure due to phx induces a systemic endotoxemia leading to

the development of a systemic inflammatory response. This response and its clinical, hemodynamic, and biochemical sequelae were blunted by the administration of the recombinant N-terminal fragment of BPI. These findings may lead to a better understanding of extrahepatic complications during liver failure. Further studies are needed to establish whether clinical interventions based on this pathogenic mechanism may reduce the morbidity or mortality related to liver failure or liver surgery in humans.

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