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BASIC STUDIES

Bacterial translocation increases phagocytic activity of polymorphonuclear leucocytes in portal hypertension: priming independent of liver cirrhosis

Hermann Neugebauer, Pia Hartmann, Sebastian Krenn, Thomas Glück, Jürgen Schölmerich, Rainer Straub and Reiner Wiest

Department of Internal Medicine, School of Medicine, University Hospital, Regensburg 93042, Germany

Keywords
bacterial translocation – leucocytes – liver cirrhosis – phagocytosis – portal hypertension

Abbreviations
BT, bacterial translocation; LC, liver cirrhosis; MLN, mesenteric lymph nodes; PMNL, polymorphonuclear leucocytes; PVL, portal vein ligation; SBP, spontaneous bacterial peritonitis.

Correspondence
Reiner Wiest, MD, Department of Internal Medicine I, University Hospital, 93042 Regensburg, Germany
Tel: +49 941 944 7104
Fax: +49 941 944 7073
e-mail: reiner.wiest@klinik.uni-regensburg.de

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Abstract

Aim: Bacterial translocation (BT) to mesenteric lymph nodes (MLN) in cirrhosis has been linked to impaired host defence. Phagocytosis by polymorphonuclear leucocytes (PMNLs) is the primary event in the killing of bacteria but has not been investigated in relation to the presence of BT. *Methods:* Mesenteric lymph nodes were harvested sterile and assessed for BT by culture techniques. Study groups included ascitic cirrhotic rats (LC), healthy controls (Con) as well as portal-vein-ligated (PVL) rats 2 days (acute PVL with and without norfloxacin) or 3 weeks after surgery (chronic PVL). PMNLs were isolated from systemic blood and the capacity to phagocytose opsonized *Escherichia coli* was evaluated by FACS analysis. *Results:* No BT was observed in Con and chronic PVL animals but 11/20 LC (55%) and six out of six acute PVL (100%) presented with BT. In the presence of BT, PMNL from PVL as well as LC rats showed significantly increased phagocytic activity as compared with controls. In contrast, PMNL from animals without BT, whether PVL or LC, exhibited phagocytic activity similar to those from control rats. The number of PMNLs involved in the phagocytic process was significantly increased only in portal-hypertensive rats with but not without BT as compared with controls. Norfloxacin did prevent BT in acute PVL animals, thereby correcting the increase in phagocytic capacity in PMNL. *Conclusions:* Cirrhosis *per se* is not associated with alterations of the phagocytic capacity of PMNL. The occurrence of BT, however, increases the phagocytic capacity of PMNL, being observed likewise in prehepatic portal hypertension, indicating an *in vivo* 'priming' of PMNL by BT independent of cirrhosis.

Bacterial infection is an often life-threatening event in patients with liver cirrhosis that commonly occurs with an incidence of 20–47% (1). Infections frequently precipitate decompensation of the underlying liver disease and thus are associated with an excessive mortality rate of up to 60% (2). The increased susceptibility to infections in patients with liver cirrhosis has been attributed to a number of alterations in the humoral and cellular immune responses. These include defective opsonization, e.g. via deficiencies in complement C3 (3, 4), defective chemotaxis (5), reduced production of superoxide anion, oxidative burst and bacterial killing capacity (6, 7). Advances in antibiotic treatment and hospital care have failed to decrease the fatality rate of bacterial infections in advanced cirrhosis, pointing out the need for a better understanding of the underlying mechanisms of the disease, in order to explore new treatment strategies. Neutrophils are the first line of host defence against bacterial infection and phagocytosis is one of their key mechanisms in order to kill invading microorganisms. Indeed, decreased phagocytic activity of polymorphonuclear leucocytes (PMNLs), whether inherited or acquired in the setting of different chronic or acute diseases, increases the incidence and morbidity of infections (8–10). Several investigations indicate that PMNL phagocytic function is impaired in liver cirrhosis (7, 11, 12).

Bacterial translocation (BT) is defined as the passage of intestinal bacteria and/or bacterial products from the intestinal lumen to mesenteric lymph nodes (MLN) and/or extraintestinal sites (13). BT is now

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recognized as the initiating event in the pathophysiology of spontaneous bacterial peritonitis (SBP), bacteraemia and most likely endotoxinaemia in liver cirrhosis (14, 15). Moreover, the development of increased rates of BT to MLN in cirrhosis seems to impact on the number, distribution and function of immune cells (16). In this context, bacteria translocating most readily are aerobic gram-negative bacteria, e.g. *Escherichia coli*. In fact, gram-negative bacteria, particularly *E. coli*, are the most common agents isolated in bacterial infections such as SBP and bacteraemia (2) in cirrhosis.

Therefore, we investigated phagocytosis of *E. coli* by circulating PMNL in the presence as well as in the absence of BT in experimental cirrhosis. Moreover, we used an additional rat model of prehepatic portal hypertension induced by partial portal vein ligation (PVL) (17). This model has been used in our laboratory extensively and is characterized by BT to MLN in the very early phase after surgery but absence of BT in the chronic phase of portal hypertension 2–3 weeks after PVL (18). Finally, this model of portal hypertension and BT presents with almost normal liver and immune function. Using this approach, we aimed to differentiate the impact of portal hypertension *per se* on the phagocytic capacity of PMNL from that of liver damage.

Materials and methods

Animal models

Carbon tetrachloride-induced liver cirrhosis

Cirrhosis was induced in male pathogen-free CD rats (Charles River, 50–80 g initial weight) by inhalation of carbon tetrachloride (CCl_4) along with phenobarbital (0.35 g/L) in the drinking water, as described previously (15, 19). The CCl_4 administration was started three times a week over 1 min and increased every other week by 1 min to a maximum of 5 min, depending on the change in body weight of the animal. After 12–16 weeks, this approach induced micronodular liver cirrhosis with ascites. Seven days before experimental procedures, application of CCl_4 as well as phenobarbital was stopped. Only cirrhotic animals with decompensation of liver function and thus presence of ascites were used because previous experiments showed that BT is increased only in the presence but not in the absence of ascites (19). Phenobarbital-treated age- and sex-matched rats were used as the control group.

Portal vein ligation model

For these experiments, the same CD stem was used only with a higher initial body weight of 250 g in order to achieve comparable body weights and age by the time of the experiment. Portal hypertension was induced surgically under aseptic conditions as described previously (17). Briefly, rats were anaesthetized with ketamine hydrochloride (Ketalar, 100 mg/kg body weight; Parke, Davis, Avon, CT, USA). After a midline abdominal incision, the portal vein was freed from the surrounding tissue. A ligature (silk gut 3-0) was placed around a 20-gauge blunt-tipped needle lying alongside the portal vein. Subsequent removal of the needle yielded a calibrated stenosis of the portal vein. After the operation, the animals were housed in plastic cages and allowed free access to food and water. All studies were performed in 12–18 h-fasted animals and performed according to the German Physiological Society principles for the care and use of laboratory animals (Granted permission number 621-2531.1-23/00, Government of Bavaria).

Experimental protocols

This study was conducted in three separate protocols. The first was designed to establish whether BT in cirrhotic rats with ascites ($n = 20$) associates with changes in phagocytic activity of PMNL. In the second protocol, we examined whether the influence on phagocytosis is solely related to the phenomenon of BT or whether changes seen in the setting of liver cirrhosis are mediated by liver cirrhosis *per se*. For this purpose, the PVL model was chosen because it is known to lack hepatic parenchymal cell damage as well as Kupffer cell dysfunction. Moreover, this prehepatic portal-hypertensive animal model presents with an extraordinarily high rate of BT to MLN at 2 days after PVL whereas in the chronic phase (two and more weeks after PVL) no increase in BT is observed anymore (18). In this protocol, six rats with acute and chronic PVL, respectively, were included. Finally, in a third protocol it was assessed whether BT is causative for changes in the phagocytic capacity of PMNL in PVL animals by utililzing norfloxacin for selective gut decontamination. Norfloxacin was given by gavage (daily 20 mg/kg diluted in 2 ml isotonic saline, $n = 5$) for 7 days before the experimental procedures. In parallel, a placebo group of acute PVL animals was treated with placebo (daily isotonic saline 2 ml, $n = 5$).

Study design

All experiments were performed under strict sterile conditions. Anaesthesia was induced with ketamine hydrochloride (Ketalar, 100 mg/kg body weight). Rats were shaved and skin disinfected with alcohol. Ascitic rats underwent paracentesis and samples of ascites were inoculated in aerobic and anaerobic blood

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culture bottles. Subsequently, after midline laparotomy, MLN draining lymph nodes from the terminal ileum, cecum and ascending colon were dissected, removed and weighed. Also, tissue samples of the liver and spleen were removed. MLN, liver and spleen specimens were diluted in phosphate-buffered saline (0.1 ml per 0.1 g), homogenized and 100 µl of suspension was cultured on McConkey, Mueller Hinton and whole blood agar for 48 h. Any growth of bacteria was considered as evidence of BT. Blood samples were obtained from the aorta abdominalis and collected into heparin-containing ice-chilled tubes and fractions inoculated in aerobic and anaerobic blood cultures for 7 days. Any positive blood culture was regarded as evidence of bacteraemia.

Determination of phagocytosis

The phagocytic activity of granulocytes was assessed by a commercial kit, Phagotest® (Orpegen Pharma, Heidelberg, Germany), according to the manufacturer's instructions as described previously (20, 21). Briefly, 100 µl heparinized whole blood was incubated in an ice bath for 10 min. Twenty microlitres of precooled fluorescein isothiocyanate (FITC)-labelled *E. coli* bacteria (*E. coli*, 2×10^7) opsonized with immunoglobulin and complement of pooled sera were added and incubated at 37 °C for 10 min. A control (baseline) was kept on ice. The phagocytic process was stopped on ice and 100 µl of ice-cold Quenching Solution® (Orpegen Pharma, Heidelberg, Germany), was added to each probe to quench the FITC fluorescence of bacteria bound to the cell surface, leaving the fluorescence of the internalized bacteria unaltered. After two washing steps (5 min, 250*g*, 4 °C), each probe was incubated at room temperature for 20 min, after addition of 2 ml of the kits Lysing Solution for removal of erythrocytes and fixation of phagocytes. After a final washing step, the probes were incubated with 200 µl DNA Staining Solution at 0 °C for 10 min. Cell suspensions were then measured by flow cytometry (Fig. 1) (10 000 events) within 60 min.

Flow cytometry analysis

Phagocytic activity was measured by flow cytometry (Facscalibur, Becton Dickinson, Heidelberg, Germany). Granulocytes were identified with the cellular forward and sideward scatter (FSC/SSC) upon the distribution of resting cells (4 °C) within the FSC/SSC window. They were discriminated by a gate from other cells, and only cells within the granulocyte gate were analysed. The green fluorescence (FL 1) of granulocytes induced by FITC and the red fluorescence (FL 2) of the PI-stained DNA of dead cells were analysed simultaneously with the cellular FSC/SSC with an argon laser tuned to 488 nm. FL 1 was obtained through a 525-nm band pass filter and was compensated for a spectral overlap by 2.5% of the signal from FL 2. The FL 2 was obtained through a 635-nm band pass filter, compensated by 25.1% from the signal of FL 1. Both FL 1 and FL 2 fluorescence were measured on a four-decade logarithmic scale. Granulocytes were considered as active in the phagocytic process and oxygen radical production if FL 1 was $> 10^1$. Thus, active cells within the granulocyte gate were determined by a marker M(a).

Statistical evaluation of the measurements was performed using SPSS version 13.0 (SPSS Inc., Chicago, IL, USA). As the percentage of active granulocytes varies individually and is dependent on the stimulant, the phagocytic activity (FL 1 active) was determined as mean FL $1 \times$ M(a) (%). To allow comparison of individual results, the phagocytosis index was calculated as the ratio of fluorescence upon phagocytosis and fluorescence at baseline (FL 1 active stim/FL 1 active baseline). While the product of cells (%) participating in the phagocytic process and their mean fluorescence (FL 1) determine the individual phagocytic capacity, the number of fluorescent cells (%) itself as determined by M(a) indicates the individual readiness of PMNL to participate in the phagocytic process. In order to assess this readiness of PMNL, we also compared the percentage of actively phagocytosing cells within the granulocyte gate of control rats with that of PVL and cirrhotic rats with and without BT.

Statistical analysis

Results were expressed as median ± SE. Where indicated, confidence intervals are presented. Statistical analysis was performed using ANOVA (two-way, with repeated measurements) or paired and unpaired Student's *t*-test if appropriate. The statistical significance level was $P < 0.05$ (after Bonferroni correction where applicable).

Results

Animals

There were no differences in body weight between acute (with and without norfloxacin) and chronic PVL rats as well as control rats (320 ± 9 g and 325 ± 3 g and 336 ± 11 g vs. 340 ± 10 g respectively). However, cirrhotic rats with BT were found to present with lower body weight as compared with cirrhotic rats without BT (306 ± 14 g vs. 345 ± 16 g respectively). Cirrhotic

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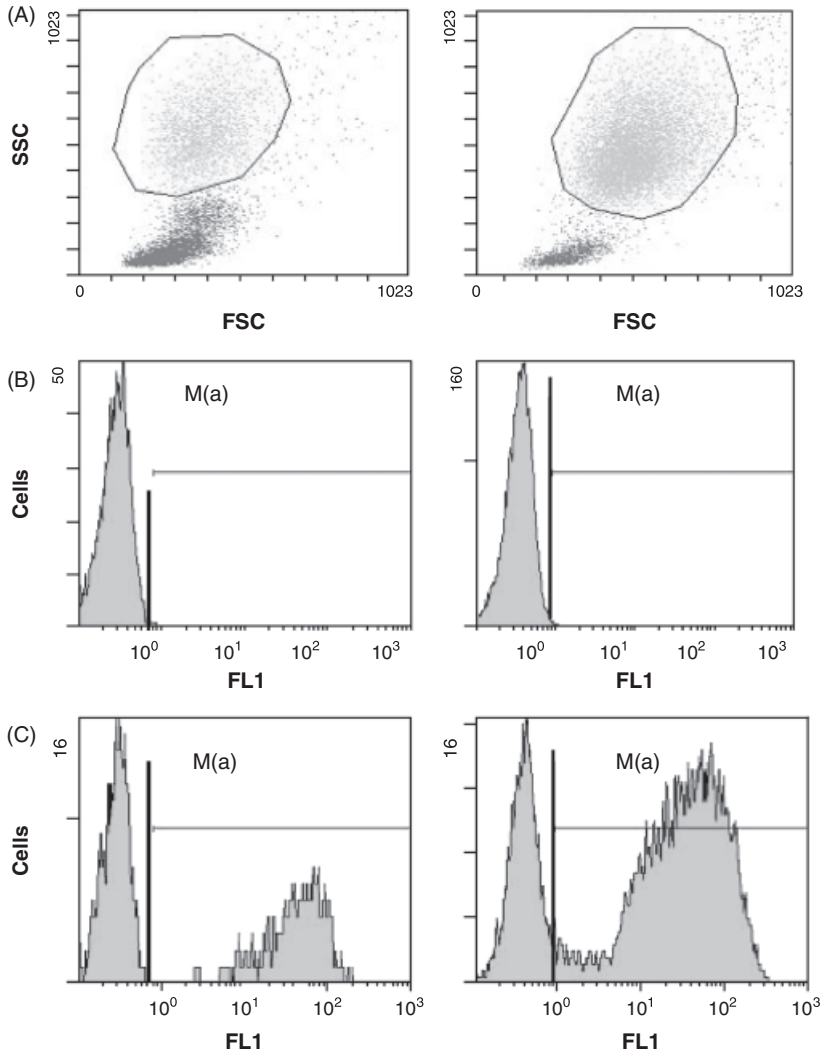

Fig. 1. Representative FACS analysis of phagocytic activity of polymorphonuclear leucocytes (PMNL) from cirrhotic ascitic rats with (right panels) and without bacterial translocation (left panels) against *Escherichia coli in vitro*. Dot plot (A) of forward scatter (FS; indicating size) and side scatter (SS, indicating granularity) was used to gate on PMNL in the sample. Phagocytic activity of the gated population was then analysed by histogram of cell number vs. fluorescent intensity (FL 1 log). Cells unexposed to bacteria (B) were used to set a marker on the histogram to exclude background cell autofluorescence. Cells incubated with preopsonized, fluorescein–isothiocyanate-labelled *E. coli* (C) were then analysed for percentage of cells to the right of the marker (indicating they had ingested *E. coli*) and the mean FL 1 of ingesting cells (relative number of bacteria/engulfed cell).

rats as well as chronic but not acute PVL rats (with or without norfloxacin) showed elevated spleen weights, expressed as percentage of body weight as compared with control rats (3.7 ± 0.24 mg/kgbw; 3.4 ± 0.7 g/kgbw; 2.3 ± 1 g/kgbw or 2.2 ± 1 g/kgbw vs. 1.9 ± 0.05 g/kgbw, $P < 0.001$ respectively).

Bacterial translocation to mesenteric lymph nodes

None of the control rats or the chronic PVL animals presented with BT to MLN. In contrast, in every PVL rat studied 2 days after PVL, MLN culture was found to be positive. Norfloxacin treatment prevented BT to MLN because none of the treated acute PVL animals displayed positive MLN culture. Among cirrhotic rats, in 11/20 cases (55%), BT was detected. Bacteraemia and SBP were detected in five out of 11 cirrhotic rats with BT but in none of the cirrhotic rats without BT, or in PVL or control rats.

In vitro phagocytosis

Autofluorescence of PMNLs was $< 10^1$ fluorescence intensity. No difference in mean fluorescence intensity

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Table 1. FACS data after incubation with *Escherichia coli* in polymorphonuclear leucocytes

		PVL		LC	
Study group	Con	Without BT	With BT	Without BT	With BT
Mean fluorescence	21.5 (11.1/44.5)	15.5 (6.9/33.6)	18.8 (2/48.4)	25.8 (9.3/43.8)	27.9 (21.3/37.3)

Data are given as median and 95% confidence interval and corrected by values obtained in the control assay.
BT, bacterial translocation; Con, healthy controls; LC, ascitic cirrhotic rats; PVL, portal-vein-ligated.

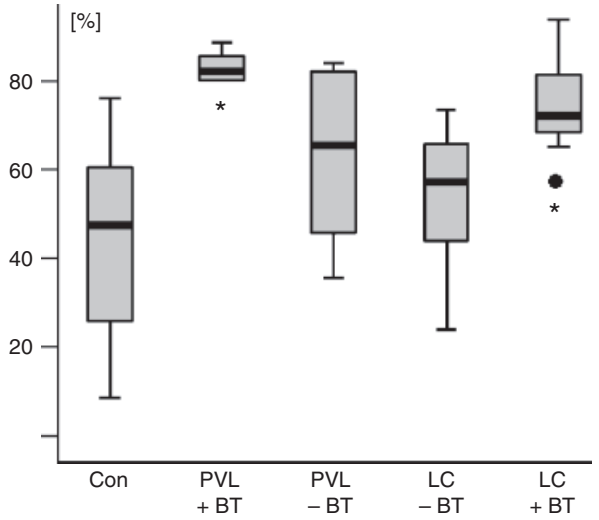

Fig. 2. Readiness of polymorphonuclear leucocytes of cirrhotic, portal-vein-ligated and control rats to phagocytose *Escherichia coli in vitro* depending on the presence of bacterial translocation (BT) *in vivo*. Data are given as box plots with the 5th, 10th, 50th (median), 90th and 95th percentile. $^{*}P < 0.05$ vs. Con; $^{\bullet}P < 0.05$ vs. LC without BT.

$> 10^1$ at baseline (4 °C) given by intracellular FITC-labelled *E. coli* was observed between the study groups. After an incubation period of 10 min at 37 °C, statistical comparison for mean fluorescence activity (FL 1) in PMNL between the study groups revealed no significant difference (Table 1).

However, the percentage of PMNL contributing to FL 1 as determined by M(a) was found to be significantly increased in animals presenting with BT, whether PVL or LC rats, as compared with control rats (Fig. 2). As for cirrhotic rats, PMNL of animals with BT also exhibited a significantly higher percentage of PMNL involved in the phagocytic processes as compared with rats without BT ($P < 0.05$). This so-called readiness to phagocytose was not significantly different between BT-positive cirrhotic ascitic rats with and without bacteraemia (Table 2). Readiness to phagocytose also tended to be higher in PMNL of PVL rats with BT as compared with PVL rats without BT, but this difference did not achieve significance ($P = 0.07$)

Table 2. Phagocytosis parameter in cirrhotic ascitic rats with bacterial translocation in dependency on presence of bacteraemia

Blood culture status	Readiness to phagocytose	Phagocytosis index
Positive ($n = 5$)	67.4 (62.0/82.8)	279 (215/389)
Negative ($n = 6$)	69.2 (53.4/83.9)	353 (264/475)

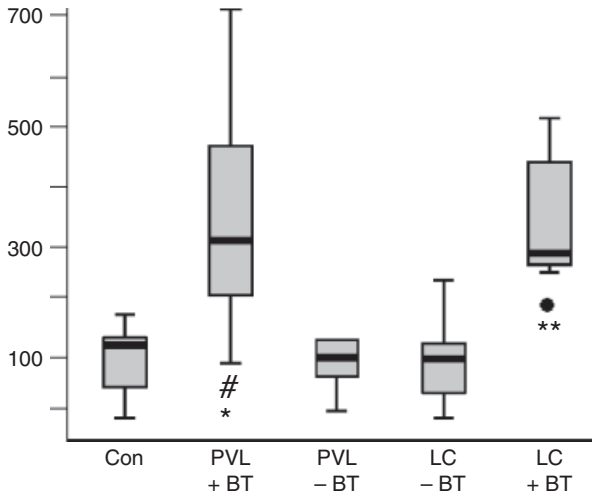

Fig. 3. Phogocytosis index in polymorphonuclear leucocytes of cirrhotic, portal-vein-ligated and control rats depending on the presence of bacterial translocation (BT). Data are given as box plots with the 5th, 10th, 50th (median), 90th and 95th percentile. $^{*}P < 0.05$ vs. Con. $^{**}P < 0.0001$ vs. Con. $^{\#}P < 0.05$ vs. PVL without BT; $^{\bullet}P < 0.001$ vs. LC without BT.

(Fig. 2). No difference in readiness to phagocytose could be detected between PMNL derived from LC and PVL rats with BT. In addition, the fraction of active PMNL was not different between control rats and PVL as well as LC rats without BT.

Finally, in the presence of BT, whether PVL or LC rats, a significantly increased phagocytic index was detected as compared with control animals (Fig. 3). In the presence of BT, no difference was detected in the phagocytic index between PVL and LC rats. Moreover, in PVL as well as LC rats, the phagocytic index was significantly increased in animals with BT as compared

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Table 3. Phagocytosis parameter in acute portal-vein-ligated rats in dependency on selective gut decontamination

Study group	Readiness to phagocytose	Phagocytosis index
Acute PVL-norfloxacin ($n = 5$)	60.5 (42/80.2)*	90.3 (25/124)*
Acute PVL-placebo ($n = 5$)	76.7 (74.3/88.9)	310 (128/572)
Control ($n = 6$)	45.6 (19/66)*	137 (51/182)*

*$P < 0.05$ vs. Acute PVL-placebo.

with those without BT. No difference in the phagocytic index was observed between PVL or LC rats without BT and control rats. Phagocytic index as well as the readiness to phagocytose of PMNL were not significantly different between acute PVL animals treated with norfloxacin as compared with placebo-treated acute PVL rats or control rats (Table 3), demonstrating a normalization of phagocytic capacity by selective gut decontamination and associated prevention of BT.

Discussion

Cirrhosis increases the susceptibility to and mortality from bacterial infections in both humans and rats (1, 2, 22). Phagocytic cells, particularly PMNL, are of major importance for clearance of bacteria from the blood stream. Studies on PMNL function in cirrhosis have yielded conflicting results, (7, 11, 12, 23–27) and association with the presence of BT has not been investigated so far. Here we report for the first time that the phagocytic capacity of circulating PMNL is increased in portal-hypertensive rats in the presence of BT but is unaltered in the absence of BT. Thus, PMNL appear to be activated under conditions of BT.

These results appear to be conflicting with most previous investigations because the phagocytic capacity of PMNL in the setting of liver cirrhosis has been reported as being normal by some authors (23, 28) or decreased by others (7, 11, 12, 24–26). However, none of these investigations did assessed the presence of BT and/or bacterial infections. Moreover, findings of impaired phagocytic function have almost exclusively been attributed to be serum-dependent and thus to be caused by a lack of opsonization (3, 29). Our study design, however, used optimally opsonized *E. coli* and thus cannot be compared with most previous trials. Moreover, most studies in cirrhotic patients were performed in compensated patients (23) and not in advanced end-stage liver disease. In addition, many investigations are confounded by conditions such as continued ethanol abuse, (12, 25) well known to be associated with immunodepression and impaired neutrophil function, (30) independent of the severity of liver disease *per se*. Finally, major differences in the experimental methods applied have to be considered. For instance, it is worth noting that for *E. coli* a serum-independent reduction in phagocytosis by PMNL could only be detected at a certain time point of incubation and if selected bacteria to cell ratios were used (7). Furthermore, it is important to consider the source and compartment of PMNL being studied. Finally, increased function of circulating PMNL during portal hypertension has been reported beforehand. In a previous report by Feliu *et al.*(31), five of the 23 cirrhotic patients displayed an unexplained significant increase in the ingestion rate of heat-killed opsonized *Klebsiella* . Unfortunately, no data are available on the presence of BT and/or infections in these patients. Moreover, the same phagocytosis assay was used by Kirsch *et al*. (23). Although interpreted as normal phagocytic capacity in the overall population, 16/18 patients presenting with ascites actually displayed significantly increased *E. coli* phagocytosis as compared with individuals with extrahepatic portal hypertension and lack of chronic liver disease. Furthermore, Levy *et al*. (27) reported an increased phagocytic capacity of neutrophils in experimental portal hypertension induced by bile-duct ligation . Phagocytosis of zymosan opsonized with serum from control animals was significantly higher in bile-duct-ligated rats than that of control rats. This increase in phagocytic function was observed as early as 12 h after surgery. Considering the well-accepted occurrence of BT early after bile-duct ligation, (32) these results support our finding of enhanced phagocytosis under conditions of BT. Finally, in an animal model of intra-abdominal sepsis, a comparable increase in FcRIII-mediated phagocytosis by PMNL has been demonstrated (33).

The high incidence and lack of BT in acute and chronic portal-hypertensive PVL rats, respectively, confirms previous reports (18) and provides the opportunity to separate a potential influence of portal hypertension *per se* from that of BT. The PVL model is characterized by a lack of hepatocellular injury but extensive porto-systemic shunting and haemodynamic disturbances such as a hyperdynamic circulation closely resembling those seen in liver cirrhosis (17). Here we show that only in the presence of BT, PMNL from PVL rats exhibit an increased phagocytic activity as a consequence of the higher number of PMNL involved in the phagocytic process. In fact, norfloxacin treatment did prevent BT in acute PVL rats, leading to a normalization of phagocytic capacity of PMNL in these animals. Moreover, the presence of bacteraemia

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in addition to BT in cirrhotic ascitic rats was not associated with any further increase in the phagocytic function of PMNL. This indicates BT as the main trigger for upregulation of PMNL phagocytic function and argues against any significant role of portal hypertension and associated haemodynamic and neurohormonal changes in the observed changes in PMNL function.

The observed comparable increments in PMNL phagocytic function in cirrhotic rats as well as acute PVL animals in the presence of BT strongly suggest a priming phenomenon *in vivo* induced by BT. Potential factors known to trigger such priming of PMNL include lipopolysaccharides (LPS) and pro-inflammatory cytokines such as tumour necrosis factor (TNF) (34, 35). Most interestingly, we could show earlier significant endotoxaemia in the presence but not in the absence of BT in cirrhotic ascitic rats (15). Moreover, elevated levels of TNF have been reported in various models of rats with liver cirrhosis, (36) which appear to be most pronounced and mainly gut derived in the advanced stage (37), known to be associated with the presence of BT. It is important to emphasize that particularly in the early phase after PVL but not or far less in long-standing PVL animals' enhanced serum levels of TNF and endotoxins have been demonstrated (36, 38). It is therefore tempting to speculate that these high levels of, e.g., TNF and endotoxins released from the gut and its associated lymphatic tissue in response to BT are responsible for the increased PMNL phagocytic activity in portal-hypertensive animals. This is also in accordance with previous observations of PMNL priming in the intestinal circulation in response to ischaemic or traumatic injury (39). The potential cellular mechanisms responsible for such priming of PMNL, however, remain to be elucidated but may well be related to an up-regulation of receptors known to mediate internalization of bacteria such as integrins, e.g. CD11b/CD18. In fact, CD11b/CD18 expression has been demonstrated to be up-regulated by TNF and/or LPS (40, 41). Indeed, enhanced CD11b expression on circulating PMNL has been evidenced in cirrhotic patients (42).

The *in vivo* significance of our findings is not known. Nonetheless, these results suggest that PMNL phagocytic activity is not impaired during liver cirrhosis. Whether the observed increase in PMNL phagocytic activity in response to BT during portal hypertension is sufficient for triggering subsequent defence mechanisms and/or is normal in comparison with healthy conditions remains to be elucidated. However, the priming effects of LPS and TNF on opsonophagocytic function have been reported to be decreased in cirrhotic patients (4). Unfortunately, these authors did not present any data on the presence or absence of BT in the study population.

In conclusion, liver cirrhosis or portal hypertension with extensive porto-systemic shunting *per se* is not associated with a defect in phagocytosis in PMNL. BT may represent a triggering mechanism, inducing an increase in PMNL phagocytic activity under conditions of portal hypertension. This phenomenon appears to be independent of the presence of liver cirrhosis and most likely represents an *in vivo* 'priming' of PMNL by BT. Such increases in the phagocytic function of PMNL could represent a compensatory mechanism aiming to improve first-line defence mechanisms against invading bacteria during portal hypertension.

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