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CONCENTRATIONS OF LIPOPOLYSACCHARIDE-BINDING PROTEIN, BACTERICIDAL/PERMEABILITY-INCREASING PROTEIN, SOLUBLE CD14 AND PLASMA LIPIDS IN RELATION TO ENDOTOXAEMIA IN PATIENTS WITH ALCOHOLIC LIVER DISEASE†

CHRISTIAN SCHÄFER*, ALEXANDR PARLESAK[1], CHRISTINE SCHÜTT[2], J. CHRISTIAN BODE and CHRISTIANE BODE[1]

Division of Gastroenterology, Department of Internal Medicine, Robert-Bosch-Krankenhaus, 70376 Stuttgart, [1]Department of Physiology of Nutrition, Hohenheim University, 70599 Stuttgart and [2]Department of Immunology, University of Greifswald, 17489 Greifswald, Germany

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Abstract — There is increasing evidence that gut leakage in persons with chronic alcohol misuse leads to endotoxaemia, which might contribute to the development of alcoholic hepatitis or cirrhosis. In addition, it was recently shown that the endotoxin-binding capacity of whole blood is reduced in these patients. To analyse this phenomenon, we measured the concentration of functionally important endotoxin-binding plasma components which modify the action of endotoxin. In patients with minimal (n = 10), intermediate (n = 9), and cirrhotic alcoholic liver disease (n = 11), and healthy controls (n = 11), plasma endotoxin was determined in a limulus assay. The concentration of lipoproteins was assessed by measuring apolipoproteins, the other factors were directly measured in immunoassays. In the entire group of alcoholics, endotoxin and the concentration of binding factors that are involved in the action of endotoxin on its target cells (LPS-binding protein and sCD14) were increased. Endotoxin antagonists, such as bactericidal/permeability-increasing protein and high-density lipoprotein, were increased in the pre-cirrhotic stages, whereas a significant reduction of the latter was observed in cirrhosis. Low-density lipoprotein remained unchanged. The elevation of binding factors in the pre-cirrhotic stages of alcoholic liver disease might attenuate the effects of endotoxaemia, whereas in cirrhosis the reduction of high density lipoprotein, to which large quantities of endotoxin bind, may contribute to its pro-inflammatory effects.

INTRODUCTION

An increasing body of evidence indicates that elevated concentrations of endotoxin in plasma — due to alcohol-induced damage of the gastrointestinal mucosal barrier — might contribute to the pathogenesis of alcoholic liver disease (ALD) (Nolan, 1989; Watson *et al*., 1994). Endotoxin, a lipopolysaccharide (LPS) derived from the cell wall of gram-negative bacteria, is a potent stimulus for monocytes and macrophages, but also for non-phagocytic targets (e.g. endothelial cells). It is bound by various plasma components, which may either enhance or attenuate its biological activity (Tobias and Ulevitch, 1994; Viriyakosol and Kirkland, 1995; Ingalls *et al*., 1999).

LPS-binding protein (LBP), a type 1 acute phase reactant of ~60 kDa, is an LPS carrier molecule that disaggregates the lipophilic LPS from native micelles and facilitates its binding to humoral binding factors or CD14, a receptor on monocytes or macrophages (Tobias and Ulevitch, 1994). Another factor which enhances the effects of LPS is the soluble isoform of the CD14 receptor, sCD14, which enables the activation of LPS-responsive cells devoid of membranous CD14, such as endothelial cells, but also of CD14-bearing cells (Hailman *et al*., 1996). Bactericidal/permeability-increasing protein (BPI), on the other hand, a cationic protein of 50–60 kDa released from neutrophils, is a potent LPS-antagonist (Marra *et al*., 1990; Elsbach, 1998).

Due to its lipophilic structure, LPS also adheres to plasma lipoproteins, particularly HDL, as shown by incubation experiments with radiolabelled LPS (Roth *et al*., 1993). Both HDL and LDL attenuate the action of LPS (Flegel *et al*., 1993; Levine *et al*., 1993). The functional importance of lipoproteins for LPS binding is supported by studies showing that LBP transfers LPS to HDL and that apolipoprotein (Apo) A_1 plays the key role in the interaction of HDL with LBP (Park and Wright, 1996; Massamiri *et al*., 1997).

In a previously published study (Schäfer *et al*., 1997), we had measured the overall endotoxin-binding capacity of whole blood and found that blood from healthy controls and patients with minor alcoholic liver damage binds large amounts of LPS. Patients with advanced ALD and alcoholic cirrhosis had a significantly decreased endotoxin-binding capacity, which might result in an increase of the portion of unbound and, possibly more toxic, endotoxin. In the present study on patients with alcohol misuse, we measured the plasma concentration of several factors — LBP, sCD14, BPI, LDL and HDL — that might modulate the endotoxin-binding capacity of blood and, thus, play a role in the inflammatory process leading to advanced ALD.

SUBJECTS AND METHODS

We examined plasma and serum samples obtained from a randomly chosen set of alcohol-misusing patients and control subjects. The study design and blood sampling were carried out in accordance with the Declaration of Helsinki and with

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*Author to whom correspondence should be addressed at: Division of Gastroenterology, Department of Internal Medicine, Robert-Bosch-Krankenhaus Stuttgart, P. O. Box 50 11 20, D-70341 Stuttgart, Germany.

the permission of the institutional Ethical Review Board. All subjects gave informed consent.

Study subjects

Patient group and subgroups. The entire patient group comprised a total of 30 patients with chronic alcohol misuse (>60 g alcohol per day over ≥2 years), which were divided into three subgroups according to the following criteria: 10 patients had biochemical signs of 'minimal ALD', as indicated by a bilirubin level of ≤1.5 mg/dl and aspartate aminotransferase (ASAT) of ≤45 U/l; nine patients whose ASAT and bilirubin levels were above these limits were assigned to a second subgroup termed 'intermediate ALD'. In the subgroup 'cirrhotic ALD' ($n = 11$), cirrhosis was either confirmed by liver biopsy, or the following criteria had to be met: (1) presence of ascites, oesophageal varices and typical findings of the ultrasonic image of the liver; (2) presence of two of the following three criteria: prothrombin time according to the international normalized ratio (INR) >1.4, bilirubin >3.5 mg/dl, and serum albumin <25 g/l.

Exclusion criteria for the entire patient group were: abstinence from alcohol for more than 3 days prior to admission, chronic viral hepatitis B or C, haemochromatosis, Wilson's disease, α_1-antitrypsin deficiency, autoimmune hepatitis, as well as major surgical procedures less than 2 weeks previously, clinically overt gastrointestinal bleeding, obvious signs of bacterial infection or gram-negative sepsis, acute pancreatitis, acute renal failure, malignancies, rheumatic disease and recent intake of corticosteroids. Additionally, cirrhotic patients were excluded if they had undergone any type of therapeutic portosystemic shunting.

Control group. Eleven age-matched healthy persons (alcohol consumption <20 g/day) served as controls.

Blood sampling

Peripheral venous blood was collected within 24 h after admission.

Assay of endotoxin plasma levels

Plasma endotoxin levels were determined in an improved limulus amoebocyte lysate (LAL) assay (Coatest; Chromogenix, Mölndal, Sweden) using chromogenic substrate and individual standard curves for each sample, as previously described (Fukui *et al*., 1989).

Measurement of endotoxin-binding factors

Plasma BPI was determined in a sandwich enzyme-linked immunosorbent assay (ELISA) (White *et al*., 1994). LBP was quantified using a similar assay format and identical reagent solutions. The only differences were that affinity-purified rabbit anti-LBP was used for coating the plates (2 μg/ml) and biotinylated anti-LBP was used for detection (1:2000). Similarly, soluble CD14 in plasma was measured in a two-site ELISA (Grunwald *et al*., 1992). This assay had a sensitivity of 1 ng/ml.

The plasma concentration of HDL and LDL was assessed by determining the serum concentrations of Apo A_1 and A_2 as markers of plasma HDL concentration and Apo B as a marker of LDL, using the BN-100 immunoassay kit (Dade Behring, Marburg, Germany).

Statistics

Values are expressed as means ± SEM. Differences between alcohol misusers vs controls (and the subgroups vs controls) were evaluated for significance using the Mann–Whitney *U*-test. Correlations were calculated using Pearson regression analysis.

RESULTS

Patient characteristics

The clinical and laboratory characteristics of the individuals participating in the study are listed in Table 1. The control group comprised slightly more females than males, whereas in the patient groups two-thirds of the patients were male,

Table 1. Clinical characteristics and laboratory values of patients and controls

			Alcoholic liver disease		
Parameter	Controls ($n = 11$)	All patients ($n = 30$)	Minimal ($n = 10$)	Intermediate ($n = 9$)	Cirrhotic ($n = 11$)
Sex ratio (male/female)	5/6	22/8	9/1	7/2	6/5
Age (years)	40 ± 3	47 ± 2	43 ± 4	46 ± 4	52 ± 4**
Oesophageal varices	0	8	0	0	8
Ascites	0	5	0	0	5
ASAT (U/l)	11 ± 1	46 ± 6***	22 ± 2***	65 ± 11***	54 ± 11**
ALAT (U/l)	14 ± 1	32 ± 4*	23 ± 3*	51 ± 10**	23 ± 4*
γ-GT (U/l)	10 ± 1	375 ± 66**	137 ± 50*	604 ± 146***	404 ± 93***
AP (U/l)	78 ± 5	192 ± 21**	121 ± 10***	157 ± 34*	278 ± 33***
Bilirubin (mg/dl)	0.9 ± 0.1	2.53 ± 0.8	1.4 ± 0.2	1.7 ± 0.2**	4.3 ± 2.0
Prothrombin time (INR)	1.0 ± 0	1.11 ± 0.03	1.02 ± 0.01	1.05 ± 0.03	1.3 ± 0.1**
Albumin (g/dl)	4.6 ± 0.1	4.6 ± 0.1	4.5 ± 0.2	4.4 ± 0.2	3.7 ± 0.2***
WBC (1000/μl)	5.75 ± 0.4	6.7 ± 0.4	7.5 ± 0.8	5.9 ± 0.7	6.6 ± 0.6
Transferrin (mg/dl)	280 ± 18	215 ± 14*	237 ± 20	195 ± 22*	209 ± 28*
C-reactive protein (mg/dl)	<0.5	1.7 ± 0.3*	1.5 ± 0.5	1.3 ± 0.4	2.3 ± 0.4***

All results are given as means ± SEM.
Significance (patients vs controls): *$P < 0.05$, **$P < 0.005$, ***$P < 0.0005$.
ASAT, aspartate aminotransferase; ALAT, alanine aminotransferase; γ-GT, gamma-glutamyltransferase; AP, alkaline phosphatase; INR, international normalized ratio; WBC, white blood count.

predominantly in the subgroups 'minimal' and 'intermediate ALD' (see also comment below). The mean age of the control subjects did not differ significantly from the age of the entire patient group or the two subgroups 'minimal ALD' and 'intermediate ALD', whereas the seniority of the third subgroup (12 years above the age of the control group) reflects the longer course of ALD. The differences in the clinical features and in the liver test results are due to the criteria used for (sub)group selection. Patients with alcohol misuse had only discrete signs of inflammation, as indicated by normal white blood cell counts; only the C-reactive protein was slightly elevated, the highest values being measured in the subgroup 'cirrhotic ALD'.

Plasma endotoxin levels

Plasma endotoxin levels (Table 2) were significantly elevated in patients with alcohol misuse. This increase was independent of the degree of ALD.

Endotoxin-binding factors

LBP: In alcohol-misusing patients, the plasma concentrations of the acute-phase protein LBP were increased by more than 2-fold, which also holds true for the three subgroups (Fig. 1). The highest LBP concentrations were observed in patients with cirrhotic ALD.

Soluble CD14: Subjects with alcohol misuse had a significantly elevated level compared to controls, though this difference was less pronounced compared to the LBP levels. In the individual subgroups, this difference did not reach levels of significance at $P < 0.05$ (Table 2).

BPI: The LPS-antagonist BPI showed a marked elevation in patients with alcohol misuse. Sub-group analysis showed that patients with minimal ALD had significantly elevated levels, whereas in the two other subgroups (intermediate and cirrhotic ALD) the increase of BPI was less pronounced and did not reach statistical significance (Table 2).

Plasma lipoproteins

Apo A_1 and Apo A_2, as markers of HDL, showed no change for the total group of alcohol misusers. In the subgroups, however, we observed a biphasic pattern, with an elevation in patients with non-cirrhotic ALD and a drop of the HDL markers in cirrhotics. Apo B as a marker of LDL showed no change (Table 2).

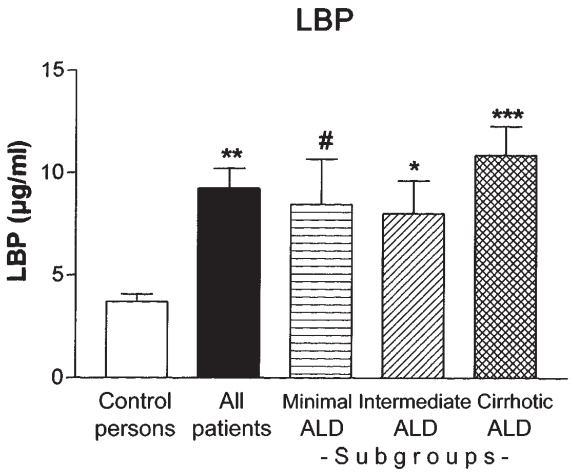

Fig. 1. Plasma concentrations of lipopolysaccharide-binding protein (LBP) in patients with chronic alcohol misuse.
Significant differences (patients vs controls): *$P < 0.075$, **$P < 0.005$, ***$P < 0.0005$, #$P = 0.086$. ALD, alcoholic liver disease.

Evaluation of gender distribution

To control for the disparity in gender distribution, we calculated the means of the endotoxin levels and the six endotoxin-binding parameters separately for males and females in each group and subgroup [5 (sub)groups × 7 parameters × 2 genders = 70 mean values] and compared them with the results (means ± SEM) found in the mixed groups. Out of these 70 means, 66 values (i.e. 94%) were within the range of the mean ± 2 SEM, and 48 (69%) even in the range of the mean ± 1 SEM. In addition, the qualitative differences between patients (entire group and subgroups) and controls remained the same, no matter whether only males, only females or the mixed groups were compared, the only exception being the LBP levels in the subgroup of intermediate ALD, in which the mean of the two females was below that of the females in the control group, unlike the data calculated for the mixed group (see Fig. 1). Thus, there is no evidence for a confounding influence of gender distribution.

Correlations

In order to examine whether the endotoxaemia observed in alcoholics was associated with changes of plasma binding

Table 2. Concentrations of plasma endotoxin and endotoxin-binding factors in patients with chronic alcohol misuse

Parameter	Healthy controls	All patients	Alcoholic liver disease		
			Minimal	Intermediate	Cirrhotic
Endotoxin (pg/ml)	2.20 ± 0.70	8.53 ± 1.47**	8.13 ± 1.63***	8.73 ± 3.50*	8.77 ± 2.67*
sCD14 (µg/ml)	3.49 ± 0.27	4.48 ± 0.27*	4.50 ± 0.42#	4.24 ± 0.42	4.68 ± 0.56#
BPI (ng/ml)	18.7 ± 5.8	50.1 ± 11.1*	65.5 ± 17.3*	38.6 ± 14.5	46.9 ± 23.4
Apolipoprotein A_1 (mg/dl)	186 ± 8	175 ± 15	212 ± 19#	200 ± 17	118 ± 26#
Apolipoprotein A_2 (mg/dl)	48 ± 3	51 ± 5	65 ± 8#	65 ± 4*	27 ± 5*
Apolipoprotein B (mg/dl)	102 ± 8	117 ± 11	121 ± 22	106 ± 18	121 ± 19

All results are given as means ± SEM.
Significance (patients vs controls): *$P < 0.05$, **$P < 0.005$, ***$P < 0.0005$, #$P < 0.75$.
sCD14, soluble isoform of the CD14 receptor; BPI, bactericidal/permeability-increasing protein.

factors, we calculated the correlation of LPS levels vs the concentrations of plasma binding factors in all study subjects (Table 3). Only one significant correlation could be detected, namely between plasma endotoxin and LBP (r = 0.51, P = 0.0011).

DISCUSSION

Elevated plasma endotoxin concentrations are reproducibly observed in patients with chronic alcohol misuse, independently of the degree of liver disease (Bode *et al*., 1987; Fukui *et al*., 1991; Schäfer *et al*., 1995). Animal experiments provide evidence that endotoxaemia is not just an epiphenomenon, but rather contributes to the inflammatory process leading to alcoholic hepatitis or cirrhosis: In alcohol-fed rats, gut sterilization by antibiotics leads to reduced plasma endotoxin levels and a reduction of inflammatory changes in the liver (Adachi *et al*., 1995). Microcirculation studies in alcohol-fed mice showed changes in the hepatic sinusoids, e.g. leukocyte sticking, which were reduced in endotoxin-resistant animals (McCuskey *et al*., 1995). Since the response to LPS is largely dependent on humoral components, we analysed the concentration of various endotoxin-binding factors in patients with chronic alcohol misuse.

LBP is an acute phase protein with a normal serum level of 5–15 μg/ml which may, e.g. in severe sepsis, rise by up to 30-fold (Schumann and Zweigner, 1999). In our patients, we observed a less pronounced, but significant, elevation of serum LBP, which occurred even in patients with minimal ALD, suggesting that chronic alcohol consumption *per se* may induce LBP production — even in the absence of inflammation. This view is supported by experiments (Lukkari *et al*., 1999), in which an early and sustained increase of the expression of LBP mRNA was demonstrated in rats nourished on alcohol. The induction of LBP may be attributed to interleukin (IL)-1 and IL-6, two mediators which may be induced by the endotoxaemia that is associated with chronic alcohol misuse (McClain *et al*., 1993; Schumann and Zweigner, 1999).

In more advanced ALD, especially in severe alcoholic hepatitis or cirrhosis, the perpetuated inflammatory process may stimulate the production of LBP independently of alcohol misuse, as shown in another study (Fujimoto *et al*., 2000). The functional significance, however, remains unclear. LBP is a prerequisite for the interaction of LPS with its binding receptors, CD14 and toll-like receptor 4, on target cells, i.e. monocytes and macrophages. On the other hand, LBP transfers LPS to lipoproteins (HDL) resulting in neutralization of LPS activity (Wurfel *et al*., 1994).

We also found an increase of the sCD14 levels in all patient groups with alcohol misuse. Similar observations were made by Oesterreicher *et al*. (1995) in patients with alcoholic cirrhosis, who, however, had been abstinent for 4 weeks, so that cirrhosis itself may induce high sCD14 levels. In our study, sCD14 elevation occurred in patients without evidence of cirrhosis (subgroups 'minimal ALD' and 'intermediate ALD'), suggesting that alcohol itself may initiate the expression of sCD14, which also has been directly demonstrated in the above-mentioned animal study (Lukkari *et al*., 1999). The elevation of sCD14 may be explained by increased shedding from monocytes (Schütt *et al*., 1992) or hepatocytes (Su *et al*., 1999). Elevated sCD14 levels have been observed in other disease states with endotoxaemia, such as septicaemia, burns with sepsis or haemodialysis patients (Krüger *et al*., 1991; Landmann *et al*., 1995; Nockher and Scherberich, 1995). In sepsis, high levels of sCD14 correlated with fatal outcome. However, there is a considerable overlap of sCD14 serum levels between septic patients — even those with severe disease — and healthy controls (Landmann *et al*., 1995).

Like LBP, sCD14 has an ambiguous effect regarding LPS action. It promotes the interaction of LPS with its targets, i.e. CD14-deficient cells (endothelial cells) but also CD14-bearing cells (Hailman *et al*., 1996). On the other hand, binding of LPS to HDL particles, mediated by sCD14, probably mitigates endotoxicity (Wurfel *et al*., 1995).

Three of the counter-regulatory mechanisms of LPS action, i.e. BPI, HDL (as determined by measurement of Apo A_1 and A_2) and LDL (as estimated by Apo B) were measured. An increase of BPI was observed in the entire patient group and particularly in the subgroup 'minimal ALD'. BPI is a potent anti-endotoxin released from neutrophils (Elsbach, 1998). Which pathways contribute to its elevation in ALD is unknown. The markers of HDL, on the other hand, showed an increase in the two pre-cirrhotic subgroups and a reduction in cirrhotics. The results for Apo B indicate that LDL remains unchanged in ALD. The increase of HDL is a well-known metabolic effect of alcohol consumption (Rimm *et al*., 1999) and has usually been interpreted with respect to the cardiovascular effects of alcohol, but it may also reflect an intact counter-regulation directed against the effects of LPS in the pre-cirrhotic stages, whereas the decrease of HDL in patients with cirrhotic ALD implies a failure of this mechanism. HDL might indeed be the serum factor to which most of the endotoxin binds, as suggested by an *in vitro* study using radiolabelled LPS (Roth *et al*., 1993). The protective capacity of high HDL plasma could be elegantly demonstrated in mouse experiments (Levine *et al*., 1993).

That the levels of LPS are similar in the three subgroups, despite differences in the concentration of HDL, seems paradoxical, but may be explained by the LPS measurement protocol, notably the heat pretreatment of the plasma samples, which makes bound LPS accessible for the LAL reaction

Table 3. Correlations between lipopolysaccharide (LPS) plasma levels and LPS-binding factors (LBP)

	LPS vs:					
	LBP	BPI	sCD14	Apo A_1	Apo A_2	Apo B
Pearson r	0.510	0.003	–0.194	–0.003	–0.072	–0.266
P value	0.0011	0.987	0.271	0.986	0.675	0.116

Apo, apolipoprotein; BPI, bactericidal/permeability-increasing protein; sCD14, soluble isoform of the CD14 receptor.

and, thus, does not discriminate between bound and unbound endotoxin. However, the drop in HDL may contribute to the diminished capacity of whole blood of patients with advanced ALD for neutralizing larger quantities of LPS (>1 ng/ml) added *in vitro*, as shown in a previous study. In these incubation experiments, active endotoxin was measured without prior heat treatment of the samples (Schäfer *et al*., 1997).

In summary, ALD is associated with changes of various functionally important endotoxin-binding factors. Whether these changes attenuate or, rather, reinforce the effects of endotoxaemia observed in patients with ALD remains to be determined by future investigations.

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