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BRIEF ARTICLE

Vigorous, but differential mononuclear cell response of cirrhotic patients to bacterial ligands

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Abstract

AIM: To study the role of gram-positive and gram-negative bacteria in the pathogenesis of liver injury, specifically the activation of inflammatory mediators.

METHODS: Peripheral blood mononuclear cells of 20 out-patients were studied, 10 of them with cirrhosis. Peripheral blood mononuclear cells were isolated and exposed to lipopolysaccharide or lipoteichoic acid. CD14, Toll-like receptor 2 and 4 expression was determined by flow cytometry, and tumor necrosis factor (TNF) α, interleukin (IL)-1β, IL-6, IL-12 and IL-10 secretion in supernatants was determined by ELISA.

RESULTS: Higher CD14, Toll-like receptor 2 and 4 expression was observed in peripheral blood mononuclear cells from cirrhotic patients, ($P < 0.01$, $P < 0.006$, $P < 0.111$) respectively. Lipopolysaccharide and lipoteichoic acid induced a further increase in CD14 expression ($P < 0.111$ lipopolysaccharide, $P < 0.013$ lipoteichoic acid), and a decrease in Toll-like receptor 2 ($P < 0.008$ lipopolysaccharide, $P < 0.008$ lipoteichoic acid) and Toll-like receptor 4 ($P < 0.008$ lipopolysaccharide, $P < 0.028$ lipoteichoic acid) expression. With the exception of TNFα, absolute cytokine secretion of peripheral blood mononuclear cells was lower in cirrhotic patients under non-exposure conditions ($P < 0.070$ IL-6, $P < 0.009$ IL-1β, $P < 0.022$ IL-12). Once exposed to lipopolysaccharide or lipoteichoic acid, absolute cytokine secretion of peripheral blood mononuclear cells was similar in cirrhotic and non-cirrhotic patients, determining a more vigorous response in the former ($P < 0.005$ TNFα, IL-1β, IL-6, IL-2 and IL-10 lipopolysaccharide; $P < 0.037$ TNFα; $P < 0.006$ IL-1β; $P < 0.005$ IL-6; $P < 0.007$ IL-12; $P < 0.014$ IL-10 lipoteichoic acid). Response of peripheral blood mononuclear cells was more intense after lipopolysaccharide than after lipoteichoic acid exposure.

CONCLUSION: Peripheral blood mononuclear cells of cirrhotic patients are able to respond to a sudden bacterial ligand exposure, particularly lipopolysaccharide, suggesting that immune regulation mechanisms are still present.

Key words: Liver cirrhosis; Toll-like receptors; Cytokines; Lipopolysaccharide; Lipoteichoic acid

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INTRODUCTION

Patients with cirrhosis frequently present with intestinal bacterial overgrowth of both gram-negative and gram-positive bacteria. Coexisting increased intestinal permeability facilitates bacterial translocation into the portal vein[1]. The resulting bacteremia and endotoxemia can not be efficiently cleared by the injured liver[2], leading to a rise of systemic proinflammatory cytokines[3]. This is thought to aggravate the underlying liver damage. The role of gram-negative bacteria in the pathogenesis of liver injury has been extensively studied. As to gram-positive bacteria, a similar deleterious role has been proposed[4], but still remains to be proven.

It is known that bacterial cell wall products, such as lipopolysaccharide (LPS), lipoteichoic acid (LTA) and peptidoglycan (PGN) fragments, trigger monocyte expression of many inflammatory cytokines. LPS, also known as endotoxin, a major constituent of the outer membrane of gram-negative bacteria, elicits an immune reaction which is responsible for many of the harmful effects seen in septic shock patients. LPS binds to the LPS-binding protein (LBP), a member of a binding and transport protein family. It requires either mCD14 or sCD14 receptors to be transferred to the toll-like receptor 4 (TLR4), a transmembrane signaling receptor, and translocated into the hydrophobic pocket of myeloid differentiation factor-2 (MD-2)[5]. This signaling pathway activates a variety of transcription factors such as nuclear factor (NF)-κB (p50/p65) and AP-1 (c-Fos/c-Jun), which induce the production of many inflammatory mediators[6].

Nowadays, it has become clear that LPS can not reproduce all clinical features of sepsis. This emphasizes the participation of other contributing factors. Gram-positive bacteria, which lack LPS, are responsible today for a substantial part of sepsis incidence. The rapid transmission and acquisition of antibiotic-resistance genes among gram-positive bacteria, and their propensity to adhere and persist on vascular catheter surfaces and other implantable medical devices have contributed to an increasing incidence of gram-positive pathogens as a cause of sepsis[7]. The major wall components of gram-positive bacteria, LTA and PGN, are thought to contribute to the development of sepsis, septic shock[8] and multiple organ dysfunction syndrome (MODS)[9]. Like LPS, LTA can interact with CD14 to initiate signal transduction pathways that lead to NF-κB activation[10]. It has been observed recently, that LTA is recognized by TLR2, which heterodimerises with either TLR1 or TLR6[11,12]. Activation of the TLR2/6 heterodimer is greatly facilitated by CD36 in a similar way as TLR4 by CD14[13].

This study compares, in cirrhotic and non-cirrhotic patients, the ability to activate inflammatory pathways of both gram-negative and gram-positive bacteria ligands. We therefore assessed the response of peripheral blood mononuclear cells (PBMC) of cirrhotic and non-cirrhotic patients to LPS and LTA exposure in terms of receptor expression (CD14, TLR2 and TLR4) and cytokine secretion [tumor necrosis factor (TNF) α, interleukin (IL)-1β, IL-6, IL-12 and IL-10].

Table 1 Biochemical characteristics of non-cirrhotic and cirrhotic patients

	Non-Cirrhotic (n = 10)	Cirrhotic (n = 10)
Bilirubin (mg/dL)	0.8 (0.6-1.2)	1.2 (0.5-28.5)
Albumin (g/dL)	4.0 (1.8-4.2)	3.3 (1.2-4.1)
PT (sec/ctl)	11.8 (9.7-15.3)	11.6 (10.2-18.4)
ALT (IU/L)	23 (15-61)	32.5 (19-57)
AST (IU/L)	23 (17-48)	43 (28-180)
Alkaline phosphatase (IU/L)	78 (56-204)	145.5 (56-479)

Data are expressed as median (minimum -maximum) values. PT: Prothrombin time; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase.

MATERIALS AND METHODS

Patients

Twenty out-patients were studied, ten of them with cirrhosis. Diagnosis of cirrhosis was supported clinically, by laboratory tests and ultrasound. Cirrhosis was due to alcohol in 4 patients, cryptogenic in 5, and due to portal thrombosis in 1. Child-Pugh classification was A in 5 patients, B in 3, and C in 2. Male:female ratio was 1:1 and the median age was 56.5 (36-79) years. Coexisting disorders were diabetes in 2 patients, hypertension in 1 and systemic sclerosis in 1. Laboratory tests are summarized in Table 1. Non-cirrhotic controls were patients with dyslipidemia (4), peptic ulcer disease (3), hypothyroidism (2), major depression (1), diabetes (1), hypertension (1), and achalasia (1). Their male:female ratio was 1:4 and median age 54.5 (41-75) years. At the time of inclusion, subjects neither had a concurrent infectious disorder, nor were receiving antibiotic or immune-modulating therapy. They all signed an informed consent before entry. The protocol of the study was approved by the Human Biomedical Research Committee of the Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán.

Isolation and stimulation of PBMC

Peripheral blood mononuclear cells were used as experimental units, given that they represent well-suited low-cost proxy-measures of monocytic response[14]. Peripheral venous blood was collected with heparinized sterile pyrogen-free disposable syringes (Becton Dickinson). PBMC were isolated from blood samples on a lymphoprep gradient (Axis Shield). After washing, PBMC were adjusted to 10^6 cells/mL in RPMI 1640 (Life Technologies, Invitrogen), and supplemented with 10% heat-inactivated fetal bovine serum (GIBCO, Invitrogen) and 1% penicillin-streptomycin 500 U/mL-500 μg/mL (GIBCO, Invitrogen). Then, 3×10^6 cells were plated on 2 mL media in 6-well round bottom tissue culture plates (NUNC). After stabilization at 37℃ and 5% CO_2, cells were stimulated (duplicate experiments) with either 0.1 μg/mL ultra-purified *Escherichia coli* endotoxin (Sigma Chemical Co.) or 0.1 μg/mL *Streptococcus faecalis* lipoteichoic acid (Sigma Chemical Co.). In order to establish the optimal concentration of activation, PBMC from blood donors were cultured with LPS or LTA at different concentrations such as 0.01, 0.1, 1 and 10 μg/mL and 0.1, 1.0, 10 and 20 pg/mL, respectively. Cultures were incubated for 24 h before supernatant harvest and TNFα concentration measurement. TNFα levels were found highest with a concentration of 0.1 pg/mL. Also, to establish the optimal time of activation, normal PBMC were cultured with 0.1 pg/mL of LPS or LTA, and supernatants harvested after 6, 24 and 48 h. TNFα levels were highest after 24 h (data not shown). We therefore used 0.1 pg/mL of LPS or LTA for a 24-h exposure. Supernatants were harvested after 24 h and stored at -70℃ until analysis.

CD14, TLR2 and TLR4 expression

5×10^5 freshly isolated or cultured PBMC were kept unexposed (NE), or were treated with LPS or LTA for 24 h. The expression of CD14, TLR2 or TLR4 was determined by flow cytometry. Briefly, treated PBMC were resuspended at 5×10^5 cells/mL in blocking buffer (PBS containing 2% FBS, 2% rabbit serum, 5 mM EDTA and 0.1% sodium azide) and incubated on ice for 30 min. Cell suspension was centrifuged and stained with fluorescein isothiocyanate (FITC)-conjugated anti-human CD14 (Santa Cruz Biotechnology), phycoeritrin (PE)-conjugated anti-human TLR2 (Santa Cruz Biotechnology), and PE-conjugated anti-human TLR4 (Santa Cruz Biotechnology). Isotype-matched nonbinding control goat anti-mouse IgG_{2a} (Santa Cruz Biotechnology) was used. The cells were incubated for 15 min in the dark, washed twice with FACS buffer (PBS containing 2% FBS, 5 mmol/L EDTA and 0.1% sodium azide) and fixed with 4% paraformaldehide in PBS (pH 7.2) for 30 min and analyzed on an EPICS-ALTRA (Beckman-Coulter). A total of 20 000 events was obtained for each sample. Data were analyzed with WinMDI 2.8 software. CD14, TLR2 and TLR4 values were expressed as % fluorescence.

Cytokine assays

After activation, cell-free culture supernatants were harvested and concentrations of TNFα, IL-1β, IL-6, IL-12 and IL-10 were measured by enzyme-linked immunosorbent assay (ELISA) (OptEIA™, BD Pharmingen, San Diego, CA) according to the manufacturer's instructions. Detection limits for each assay were 4 pg/mL for TNFα, IL-1β, IL-6, and IL-10, and 15 pg/mL for IL-12. In each patient, every test was run in duplicate.

Data are summarized as median (minimum and maximum) values. Taking the NE condition as reference, absolute and relative (%) differences were determined for LPS or LTA exposed PBMC of cirrhotic and non-cirrhotic patients. The Mann-Whitney test was used to analyze differences between cirrhotic and non-cirrhotic groups, and the Wilcoxon sign-rank test to analyze differences between exposure and non-exposure to LPS or LTA. A P value < 0.05 was considered as statistically significant, and a $P < 0.10$ as tendency towards significance. The Stata v7 statistical package was used.

RESULTS

CD14, TLR2 and TLR4 expression

Expression of CD14, TLR2 and TLR4 by NE PBMC was higher in cirrhotic than non-cirrhotic patients. Median CD14 expression was 13.3% (8.9-34.6) *vs* 6.7% (3.5-27.5) ($P < 0.01$), median TLR2 expression was 9% (4.8-19.5) *vs* 4.8% (1.7-7.9) ($P < 0.006$), and median TLR4 expression was 26.9% (5.9-36.4) *vs* 8.5% (1.2-30) ($P < 0.111$), respectively. (Figure 1A-C) Non-exposure (NE), LPS or LTA exposure, bars represent median values.

After exposure to LPS, CD14 expression by PBMC of non-cirrhotic patients [5.6% (2-28.2)] was not significantly different from corresponding NE values [6.7% (3.5-27.5), NS], but TLR2 and TLR4 expressions were significantly lower [2% (1-6.5) *vs* 4.8% (1.7-7.9), $P < 0.047$, and 3.5% (0.9-26.1) *vs* 8.5% (1.2-30), $P < 0.028$]. PBMC of cirrhotic patients showed, after the same exposure, an increased CD14 expression [23.2% (3.2-48.5) *vs* 13.3% (8.9-34.6), $P < 0.111$], and significantly decreased TLR2 [4.9% (1.1-10.8) *vs* 9% (4.8-19.5), $P < 0.008$] and TLR4 [14.8% (1.2-32) *vs* 26.9% (5.9-36.4), $P < 0.008$] expression (Figure 1A-C). Taking the NE condition as 100% reference, the median relative difference in CD14 expression tended to be higher in cirrhotic than non-cirrhotic patients after LPS exposure ($P < 0.096$). As to TLR2 and TLR4 expression, LPS exposure induced a non-significant trend towards larger median relative differences in cirrhotic than non-cirrhotic patients (Table 2).

LTA exposure did not affect significantly CD14 expression in non-cirrhotic patients [6.5% (3.6-26.9)] when compared to NE conditions [6.7% (3.5-27.5), NS], neither did it affect TLR2 [2.1% (1-8.2) *vs* 4.8% (1.7-7.9), NS] expression. TLR4 expression was, however, significantly decreased [2.7% (0.7-28.8) *vs* 8.5% (1.2-30), $P < 0.013$]. LTA challenged PBMC of cirrhotic patients showed sig-

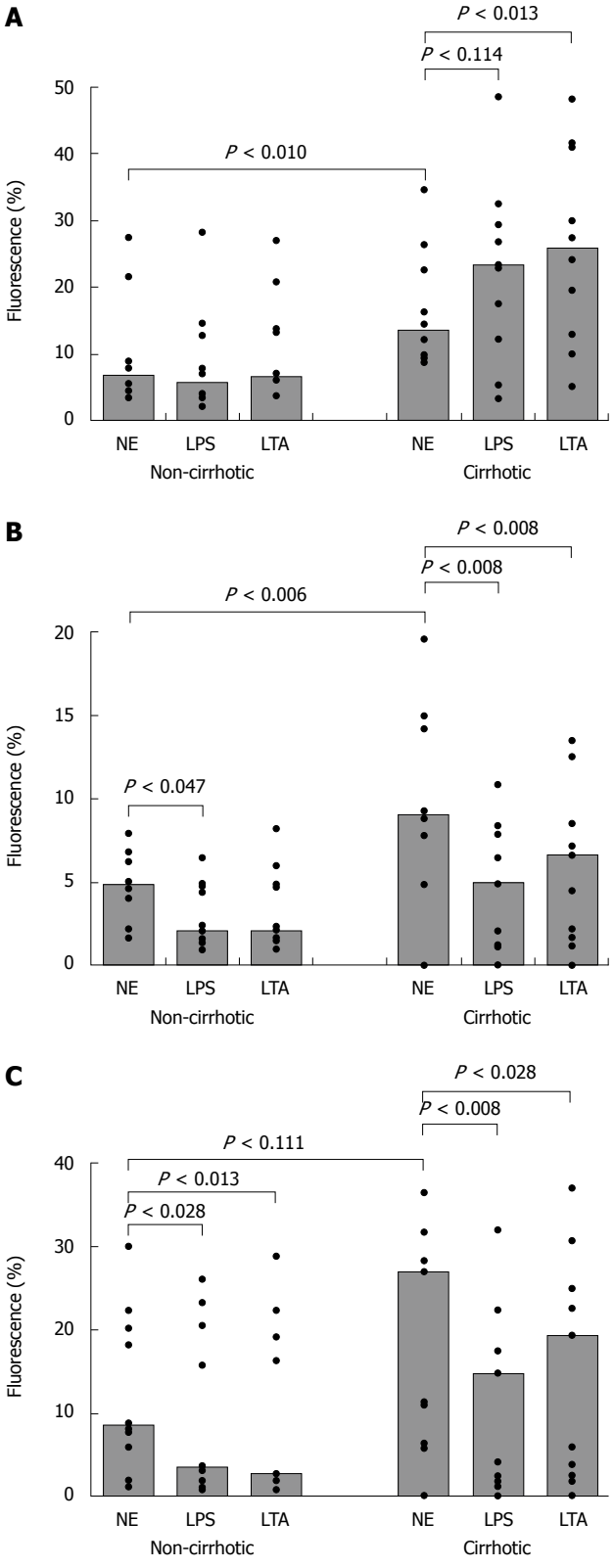

Figure 1 Receptor expression from peripheral blood mononuclear cells of non-cirrhotic and cirrhotic patients under conditions of non-exposure, lipopolysaccharide or lipoteichoic acid exposure for CD14, toll-like receptor 2 and toll-like receptor 4 expression. Bars represent median values. $P < 0.05$ denotes statistical significance and $P < 0.10$ denotes tendency to statistical significance. A: CD14 expression; B: TLR2 expression; C: TLR4 expression. NE: Non-exposure; LPS: Lipopolysaccharide; LTA: Lipoteichoic acid.

nificantly increased CD14 expression [25.7% (5-48.2) *vs* 13.3% (8.9-34.6), $P < 0.013$], and decreased TLR2 [6.6% (1.2-13.4) *vs* 9% (4.8-19.5), $P < 0.008$] and TLR4 [19.4% (1.9-37) *vs* 26.9% (5.9-36.4), $P < 0.028$] expression (Figure 1A-C). LTA induced median relative differences in CD14, TLR2 and TLR4 expression were similar to those induced by LPS (Table 2).

Table 2 Median relative difference[1] in receptor expression and cytokine secretion by peripheral blood mononuclear cells of non-cirrhotic and cirrhotic patients after exposure to lipopolysaccharide and lipoteichoic acid

		Non-cirrhotic (%) $n = 10$	Cirrhotic (%) $n = 10$	*P* value
Expression				
CD14	LPS	-9	+39	< 0.096[b]
	LTA	-3.50	+55	< 0.028[a]
TLR2	LPS	-10	-60	< 0.121
	LTA	0	-42	< 0.289
TLR4	LPS	30	-53	< 0.221
	LTA	-19.50	-29	< 0.935
Secretion				
TNFα	LPS	+7400	+8770	< 0.940
	LTA	+190	+360	< 0.970
IL-1β	LPS	+70.50	+1164	< 0.019[a]
	LTA	-6	+71	< 0.049[a]
IL-6	LPS	+91	+319	< 0.174
	LTA	+125	+246	< 0.326
IL-12	LPS	+3324	+6219	< 0.151
	LTA	+503	+1786	< 0.227
IL-10	LPS	+1768	+5844	< 0.364
	LTA	+50	+415	< 0.571

[1]Difference with the non-exposure value (considered as the reference or 100%). A negative value reflects a decrease, whereas a positive value reflects an increase. [a]Denotes statistically significant ($P < 0.05$) differences between non-cirrhotic and cirrhotic patients. [b]Denotes tendency towards statistically significant ($P < 0.10$) differences between non-cirrhotic and cirrhotic patients. LPS: Lipopolysaccharide; LTA: Lipoteichoic acid; TLR: Toll-like receptor; IL: Interleukin; TNF: Tumor necrosis factor.

TNFα, IL-1β, IL-6, IL-12 and IL-10 secretion

NE PBMC of non-cirrhotic *vs* cirrhotic patients secreted similar amounts of TNFα [≤ 4 pg/mL (≤ 4-143) *vs* ≤ 4 pg/mL (≤ 4-42), NS] and IL-10 [26 pg/mL (≤ 4-275) *vs* 6 pg/mL (≤ 4-72), NS]. Secretion of IL-6 tended to be higher in non-cirrhotic [401 pg/mL (12-1530)] than cirrhotic [168 pg/mL (5-459)], patients ($P < 0.070$). Secretion of IL-1β and IL-12 was significantly higher in non-cirrhotic [26 pg/mL (≤ 4-159) and 19 pg/mL (≤ 15-959)] than cirrhotic [≤ 4 pg/mL (≤ 4-10) and ≤ 15 pg/mL (≤ 15-38)] patients ($P < 0.009$ and < 0.022) (Figure 2A-E).

Taking NE values as reference [≤ 4 pg/mL (≤ 4-143) and ≤ 4 pg/mL (≤ 4-42)], LPS exposure triggered significant increases in TNFα secretion by both non-cirrhotic [443 pg/mL (52-658), $P < 0.005$] and cirrhotic [355 pg/mL (52-713), $P < 0.005$] PBMC. Similar increases were observed for IL-1β, IL-6, IL-12 and IL-10 secretion. Specifically, IL-1β PBMC secretion increased from NE values of 26 pg/mL (≤ 4-159) in non-cirrhotic and ≤ 4 pg/mL (≤ 4-10) in cirrhotic patients, to 61 pg/mL (8-192) and 51 pg/mL (17-286) after LPS exposure, respectively ($P < 0.028$ and < 0.005). As for IL-6, secre-

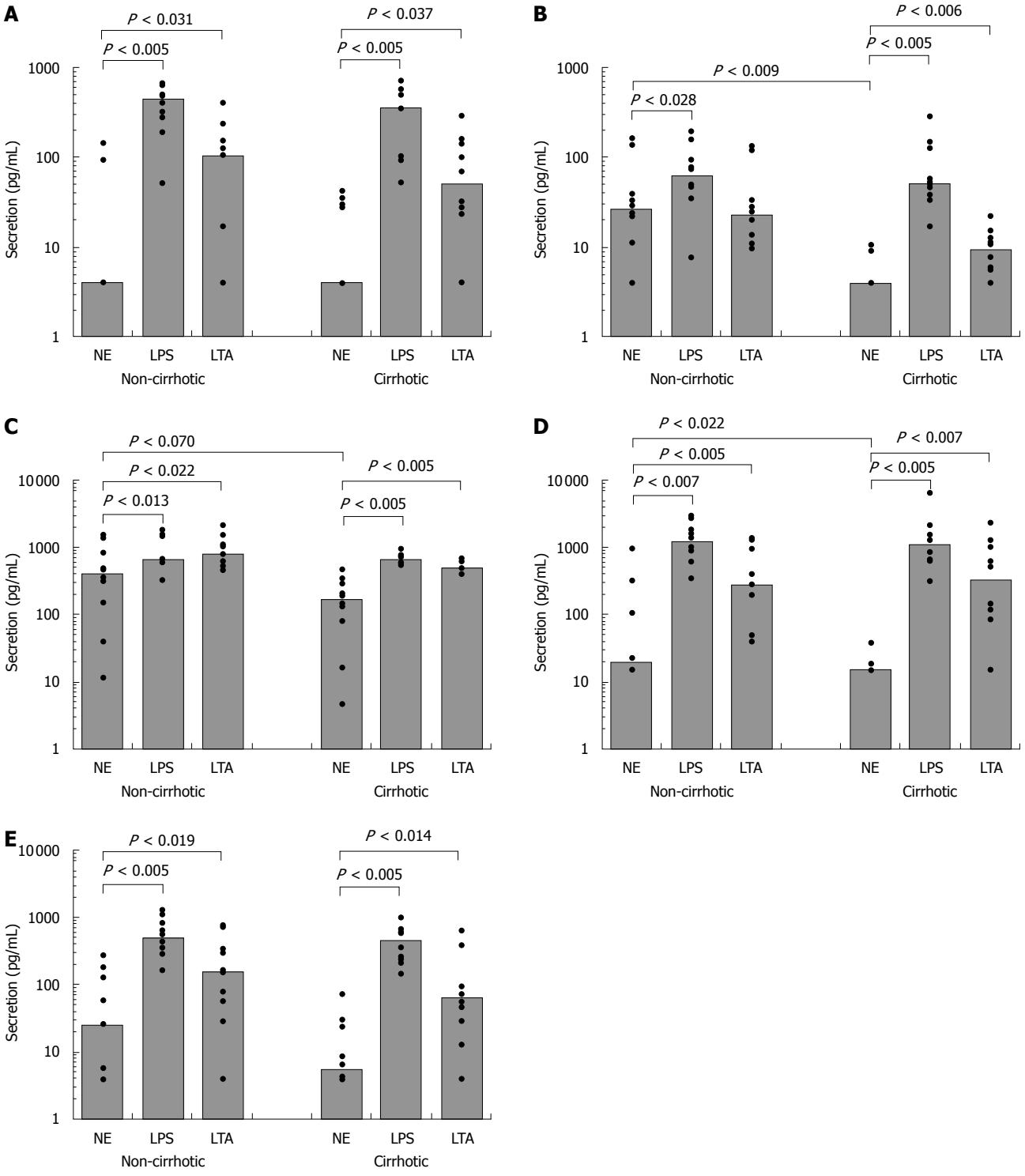

Figure 2 Cytokine secretion from peripheral blood mononuclear cells of non-cirrhotic and cirrhotic patients under conditions of non-exposure, lipopolysaccharide or lipoteichoic acid exposure for tumor necrosis factor α, interleukin-1β, interleukin-6, interleukin-12 and interleukin-10 secretion. Bars represent median values. $P < 0.05$ denotes statistical significance and $P < 0.10$ denotes tendency to statistical significance. A: Tumor necrosis factor α secretion; B: Interleukin (IL)-1β secretion; C: IL-6 secretion; D: IL-12 secretion; E: IL-10 secretion. NE: Non-exposure; LPS: Lipopolysaccharide; LTA: Lipoteichoic acid.

tion increased from NE values of 401 pg/mL (12-1530) and 168 pg/mL (5-459), to 645 pg/mL (325-1793) and 660 pg/mL (540-946), $P < 0.013$ and < 0.005. IL-2 secretion showed an increase from 19 pg/mL (≤ 15-959) and ≤ 15 pg/mL (≤ 15-38), to 1201 pg/mL (15-2850) and 1074 pg/mL (317-6397), $P < 0.007$ and < 0.005. IL-10 secretion was 26 pg/mL (≤ 4-275) and 6 pg/mL (≤ 4-72) under NE conditions, and 498 pg/mL (163-1292) and 464 pg/mL (146-1010) after LPS exposure, $P < 0.005$ and < 0.005. Median relative difference in cytokine secretion between LPS exposure and NE tended to be higher in cirrhotic than non-cirrhotic patients, reaching statistical

significance in IL-1β only ($P < 0.019$) (Table 2).

To a lesser degree than LPS, LTA exposure also induced increases in cytokine secretion. TNFα secreted by PBMC of non-cirrhotic and cirrhotic patients increased from NE values of ≤ 4 pg/mL (≤ 4-143) and ≤ 4 pg/mL (≤ 4-42), to 105 pg/mL (4-409) and 51 pg/mL (4-288), $P < 0.031$ and < 0.037. IL-1β secretion was 26 pg/mL (≤ 4-159) and ≤ 4 pg/mL (≤ 4-10) under NE conditions, and 23 pg/mL (10-133) and 9 pg/mL (4-22) after LTA exposure, NS and $P < 0.006$. IL-6 secretion increased from 401 pg/mL (12-1530) and 168 pg/mL (5-459), to 802 pg/mL (454-2155) and 509 pg/mL (397-705), $P < 0.022$ and < 0.005. IL-12 secretion increased from 19 pg/mL (≤ 15-959) and ≤ 15 pg/mL (≤ 15-38), to 275 pg/mL (40-1385) and 334 pg/mL (≤ 15-2339), $P < 0.005$ and < 0.007. IL-10 secretion increased from 26 pg/mL (≤ 4-275) and 6 pg/mL (≤ 4-72), to 157 pg/mL (≤ 4-756) and 64 pg/mL (≤ 4-638), $P < 0.019$ and < 0.014 (Figure 2A-E). Median LTA-induced relative differences in cytokine secretion tended to be more vigorous in cirrhotic than in non-cirrhotic patients, reaching statistical significance in IL-1β only ($P < 0.049$) (Table 2).

DISCUSSION

In this study, higher PBMC CD14, TLR2 and TLR4 expression was observed in cirrhotic patients under NE and LPS/LTA exposure conditions. LPS and LTA exposure induced an increase in CD14 expression in cirrhotic patients, and a decrease in TLR2 and TLR4 expression in both non-cirrhotic and cirrhotic patients. With the exception of TNFα, PBMC absolute cytokine secretion was lower in cirrhotic patients under NE conditions. However, once exposed to LPS or LTA, cytokine secretion was similar in both non-cirrhotic and cirrhotic patients, determining a more vigorous response in the latter, as shown by the corresponding relative differences. As to LPS, and with the exception of IL-6 secretion, this bacterial ligand triggers a more vigorous cytokine response than LTA.

CD14, TLR2 and TLR4 expression

Higher PBMC CD14 expression in cirrhotic patients under NE conditions reflects a state of hyperactivation, conditioned probably by a long-standing exposure to intestinal microorganisms and their products. This hyperactivation leads to vigorous reactions with any further bacterial stimuli[15]. It should be kept in mind that PBMC expression in our study is summarized as percentage of control baseline fluorescence conditions. In terms of the mean fluorescence intensity (MFI)[16-18], no significant differences in CD14, TLR2 or TLR4 expression among cirrhotic and non-cirrhotic PBMC before and after exposure to LPS and LTA were observed (data not shown). This means that the herein reported differences in PBMC expression reflect differences in the number of activated cells, not in the amount of antibody bound per cell.

Chronic increase in circulating LPS, and the resulting state of PBMC hyperactivation has been associated to low levels of high-density lipoprotein (HDL), a well-known complication of cirrhosis. HDL is able to bind LPS and neutralize its bioactivity. HDL can also down-regulate monocyte CD14 expression, and has other anti-inflammatory properties[19]. Low HDL levels could explain the increased CD14 expression observed in PBMC of our cirrhotic patients under both NE and exposed conditions.

As to LTA, this ligand relies, at least in part, on CD14 to initiate signal transduction pathways[10,20]. It has been shown recently, that CD14 expression enhances markedly LTA binding to plasma cell membranes[21]. It seems, therefore, that increased CD14 expression in cirrhosis is due to high circulating levels of both LPS and LTA. Increased circulating levels of LPS and proinflammatory cytokines have been documented in patients with chronic liver disease, even in the absence of infection. However, no significant correlation between LPS and these inflammatory mediators has been shown, raising the possibility that other agents, besides LPS, may play a role. Recent studies on TLR expression in cirrhotic patients show that this might be in fact true. TLR4, in the presence of LPS, triggers the signal transduction that leads to TNFα production. When PGN and LTA are present, TLR2 is required for signaling and activation of the inflammatory cascade. Recently, PBMC expression of TLR2, but not TLR4 was shown to correlate significantly with circulating levels of both TNFα and anti-inflammatory soluble TNF receptors. These findings suggest that gram-positive microbial stimuli might be important in the proinflammatory state of chronic liver disease. If proven true, this would contraindicate the use of probiotic agents, such as gram-positive lactobacilli, in cirrhotic patients. Current evidence, however, shows that probiotic use is associated with a significant increase of fecal lactobacilli and a decrease of potentially pathogenic gram-positive and gram-negative bacterial species. Probiotics reverse bacterial overgrowth and improve minimal hepatic encephalopathy. They improve the Child-Pugh class at the expense of serum bilirubin, albumin and prothrombin. Also, serial ALT levels show a significantly reduced hepatic necroinflammatory activity, suggesting that probiotics can protect against hepatocellular damage[22].

In our study, PBMC of cirrhotic patients expressed more TLR2 and TLR4 under NE conditions than PBMC of non-cirrhotic patients. Exposure to LPS and LTA decreased expression of both receptors in all patients. (Figure 1B and C) A similar decrease in TLR2 expression was observed by Riordan et al. after exposing PBMC of cirrhotic patients to gram-positive bacteria products *in vitro*. However, *in vivo*, they observed an increased PBMC expression of TLR2, but not TLR4, in cirrhotic subjects[4]. It has been shown recently, that monocyte expression of TLR4 is down-regulated in cirrhotic patients with Child-Pugh class C, whereas TLR2 expression is equivalent to controls. In our study, we included patients with Child-Pugh class A or B mainly, or patients with a reasonably preserved liver function and immune competence. TLR4 down-regulation in advanced cirrhosis is associated with

LPS tolerance, enhanced bacterial translocation and portal venous endotoxemia[23]. In this context, endotoxin tolerance is viewed as a regulation mechanism that protects the cell from "over expression" or sustained activation. It is regarded as a protection mechanism that aims to limit tissue damage due to excessive immune response. Another explanatory mechanism of TLR down-regulation is receptor internalization, which has been shown for TLR2 and TLR4[24].

After exposure to LPS, PBMC of both cirrhotic and non-cirrhotic patients showed a lower TLR2 and TLR4 expression. A similar but smaller decrease was observed after LTA exposure, suggesting that these two TLRs might not be completely specific. It is well documented that TLR2 recognizes LPS as well as LTA, while TLR4 recognizes LPS mainly [5,25]. From our results, we can not exclude a cross-recognition of LPS and LTA that could lead to an "additive activation" of signaling pathways.

Differences and changes in CD14, TLR2 and TLR4 expression observed in our study support the so called hyperactivation state in cirrhotic patients which, compared to the non-cirrhotic patients, does not appear to be an uncontrolled response, but a process of cellular reprogramming or adaptation to bacteria or their products[26]. We should point out that our non-cirrhotic controls had dyslipidemia, peptic ulcer disease, hypothyroidism, major depression, diabetes, hypertension, and/or achalasia. It is known that some of these entities compromise, up to certain degree, the immune response. In spite of this, PBMC response to bacterial stimuli among cirrhotic patients was significantly different to their non-cirrhotic counterpart.

TNFα, IL-1β, IL-6, IL-12 and IL-10 secretion

Cytokines, chemokines, and growth factors such as TNFα, IL-1β, IL-6, interferon-γ, IL-8, macrophage inflammatory protein-1, macrophage chemoattractant factor-1, and transforming growth factor, are all upregulated in patients with cirrhosis[1]. This upregulation varies according to the degree of liver damage, or Child-Pugh score[4,19]. *In vitro*, PBMC exposure to bacterial and viral ligands results in an elevated production of inflammatory cytokines, particularly IL-1β, IL-6, IL-8, and TNFα, β[16]. In our study, PBMC exposure to LPS or LTA triggered a significant TNFα, IL-1β, IL-6, IL-12 and IL-10 secretion in both cirrhotic and non-cirrhotic patients. Due to sample size restrictions, no correlation with the Child-Pugh score was observed.

LPS elicited a more vigorous cytokine secretion than LTA, irrespective of the presence or absence of cirrhosis. This "attenuated" response to LTA has been observed by other investigators and attributed, *in vivo*, to serum components such as lipoproteins and LBP[19,27]. *In vitro*, to get a proinflammatory response in monocytes and hepatic stellate cells, the minimal active concentration of PGN or LTA needs to be 100 times higher than that of LPS[1]. We used 0.1 μg/mL of LTA and LPS based on dose-response experiments. With this exposure dosage, the highest TNFα secretion was obtained, which was quantitatively lower for LTA than LPS.

As to IL-6, a higher secretion was observed after LTA than after LPS exposure. This cytokine plays a pivotal role in the acute response to bacterial products. Wang et al. reported that whole human blood is a potent source of IL-6 production after stimulation with *S. aureus* LTA[28]. However, other investigators failed to induce IL-6 release from monocyte cultures[29]. This discrepant IL-6 secretion has been attributed to non-monocytic cells present in the whole blood, not well characterized paracrine factors absent in monocyte cultures[28], variable LTA exposure dosage[20], and inter- and intra-species LTA variations[29].

We should consider that, in cirrhosis, the innate immunity hyper-responsiveness observed in this and other studies do not occur in isolation to alterations in adaptive immunity. It is known that cirrhotic patients are prone to get frequent bacterial infections due to an immunosuppressed state. Contrary to the expected, their T lymphocytes are activated. The proportion of CD4+ T cells expressing CD25 and CD122 antigens is increased significantly, and so is the proportion of memory CD4+ and CD8+ T cells with characteristics of senescent cells. It is thought that repeated cycles of inflammation and damage lead to a continuous recruitment of effector leucocytes within the liver and amplify effector responses exerted by T cells, macrophages, natural killer cells or neutrophils[30]. The contribution of these immune derangements, separately and as a whole, to chronic liver injury remains to be documented.

PBMC of cirrhotic patients show a hyperactivation state in terms of CD14, TLR2 and TLR4 expression. Exposure to LPS or LTA decreases this expression in both cirrhotic and non-cirrhotic PBMC, suggesting that control mechanisms are still present in chronic liver disease. Given that PBMC receptor expression changed after exposure to both LPS and LTA, our data suggest a non-specific cross-activation. Decreased CD14, TLR2 and TLR4 expression is accompanied by an increased TNFα, IL-1β, IL-6, IL-12 and IL-10 secretion. This secretion is relatively higher in cirrhotic than non-cirrhotic patients. How this systemic hyperactivation relates to the progression of liver injury is still speculative. Both LPS and LTA elicit a PBMC response, but to a different degree. The impact of this differential response needs to be evaluated, particularly when potentially beneficial gram-positive bacteria (probiotics) are involved.

COMMENTS

Background

Liver diseases figure as the fifth cause of death in Mexico. They are the third cause of death in subjects between 35-44 years, and the fourth cause of death in subjects aged 45-64 years. Patients with advanced chronic liver disease or cirrhosis frequently present with intestinal bacterial overgrowth of both gram-negative and gram-positive bacteria. This leads to infectious complications such as spontaneous bacterial peritonitis or sepsis, and to a chronic proinflammatory state.

Research frontiers

The role of gram-negative bacteria in the pathogenesis of liver injury has been extensively studied. It involves intestinal bacterial translocation and decreased

liver clearance, leading to inflammation, tissue injury and, eventually, cirrhosis. As to gram-positive bacteria, a similar damaging role has been proposed, but still remains to be proven.

Innovations and breakthroughs

It became clear that lipopolysaccharide, a gram-negative bacterial cell wall product, cannot reproduce all the clinical features observed in sepsis. This emphasizes the participation of other contributing factors. Gram-positive bacteria, which lack lipopolysaccharide, are responsible today for a substantial part of sepsis incidence. Peripheral blood mononuclear cells of cirrhotic patients are able to respond to a sudden bacterial ligand exposure, particularly lipopolysaccharide, in terms of a decreased expression of CD14, Toll-like receptor 2 and 4, and an increased tumor necrosis factor α, interleukin (IL)-1β, IL-6, IL-12 and IL-10 secretion. The authors suggest that immune regulation mechanisms persist in chronic liver disease, at least in Child-Pugh A and B stages.

Applications

Both lipopolysaccharide and lipoteichoic acid elicit a peripheral blood mononuclear cells response, but to a different degree, suggesting that gram-positive microbial stimuli might be important in the proinflammatory state of chronic liver disease. The impact of this differential response needs to be evaluated, particularly when potentially beneficial gram-positive bacteria (probiotics) are involved. Current evidence shows that probiotic use is associated with a significant increase of fecal lactobacilli and a decrease of potentially pathogenic gram-positive and gram-negative bacterial species.

Terminology

Intestinal bacterial overgrowth is a major promoting factor of bacterial translocation in cirrhosis. It is defined as bacterial migration from the intestinal lumen to the mesenteric lymph nodes or other extra-intestinal sites. Sepsis is a common cause of death in cirrhotic patients. Toll-like receptors are transmembrane receptor proteins that play a critical role in the induction of innate immunity to microbial pathogens *via* recognition of conserved molecular patterns.

Peer review

The paper is very scientific, has copious data and is well written.

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