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• *RAPID COMMUNICATION* •

Phagocytic and oxidative burst activity of neutrophils in the end stage of liver cirrhosis

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Abstract

AIM: To evaluate the phagocytic activity and neutrophil oxidative burst in liver cirrhosis.

METHODS: In 45 patients with advanced postalcoholic liver cirrhosis (aged 45±14 years) and in 25 healthy volunteers (aged 38±5 years), the percentage of phagocytizing cells after *in vitro* incubation with *E. coli* (Phagotest Kit), phagocytic activity (mean intensity of fluorescence, MIF) and the percentage of neutrophil oxidative burst (Bursttest Kit), and the level of free oxygen radical production (MIF of Rodamine 123) were analyzed by flow cytometry. The levels of soluble sICAM-1, sVCAM-1, sP-selectin, sE-selectin, sL-selectin, and TNF-α were determined in blood serum.

RESULTS: The percentage of *E. coli* phagocytizing neutrophils in liver cirrhosis patients was comparable to that in healthy subjects. MIF of neutrophil - ingested *E. coli* was higher in patients with liver cirrhosis. The oxidative burst in *E. coli* phagocytizing neutrophils generated less amount of active oxygen compounds in liver cirrhosis patients (MIF of R123: 24.7±7.1 and 29.7±6.6 in healthy, $P<0.01$). Phorbol myristate acetate (PMA) - stimulated neutrophilsproduced less reactive oxidants in liver cirrhosis patients than in healthy subjects (MIF of R123: 42.7± 14.6 *vs* 50.2±13.3, $P<0.01$). A negative correlation was observed between oxidative burst MIF of PMA-stimulated neutrophils and ALT and AST levels (r -0.35, $P<0.05$; r-0.4, $P<0.03$). sVCAM-1, sICAM-1, sE-selectin concentrations correlated negatively with the oxygen free radical production (MIF of R123) in neutrophils after PMA stimulation in liver cirrhosis patients (r-0.45, $P<0.05$; r-0.41, $P<0.05$; r-0.39, $P<0.05$, respectively).

CONCLUSION: Neutrophil metabolic activity diminishes together with the intensification of liver failure. The metabolic potential of phagocytizing neutrophils is significantly lower in liver cirrhosis patients, which can be one of the causes of immune mechanism damage. The evaluation of oxygen metabolism of *E. coli*-stimulated neutrophils reveals that the amount of released oxygen metabolites is smaller in liver cirrhosis patients than in healthy subjects.

Key words: Neutrophil; Phagocytosis; Oxidative burst; Liver cirrhosis; Flow cytometry

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INTRODUCTION

Neutrophils play an important role in non-specific immune response and organism resistance, specifically in anti-bacterial resistance as effectors, inducing and regulating cells[1,2]. They reveal many features which are crucial in organism immunity: to produce and adhere towards vascular endothelial cells, migrate to inflammatory sites through the vessel walls, recognize and phagocytize opsonized molecules, and degradate and release proteins from granules[3,4]. These features are possible due to the presence of receptors distributed on the surface and inside of the cells such as cytokine-, neuromediator-, autocoid-, hormone receptors. Neutrophil chemotaxis occurs towards stimulus gradient, in response to chemotactic factors (chemotaxins) produced at the inflammatory site. Moreover, chemotaxins increase neutrophil metabolism, aggregation, and bactericidal abilities[5-7].

Phagocytosis is facilitated by specific (immunoglobulins IgG) and non-specific (complement components) opsonins, circulating in the plasma. On their surface, granulocytes have receptors that bind to Fc fragment of IgG1 and IgG2 immunoglobulins, and complement C3b fragment receptors[8]. Absorbed opsonized microorganisms are killed through both oxygen-dependent and independent mechanisms. Free oxygen radicals kill

absorbed bacteria in phagosomes and partially are released into the environment, intensifying killing microorganisms and simultaneously injuring the surrounding tissues. It is specifically intensified in acute inflammation, less in chronic course of the disease[1]. Microorganism killing is also possible by proteins present in azurophil granules, such as cathepsin G, lysozyme, interferons, and others.

Inflammatory mediators, cytokines (e.g. TNFα) and selectins increase the ability of granulocytes to localize at the site of inflammation. Phagocytic ability is elevated by intensification of hydroxylic radical production and lysosomal enzyme release[9,10]. TNFα is an important factor strengthening granulocyte phagocytic and cytotoxic activity. Activated granulocytes secrete cytokines. IL-1, which stimulates monocytes, endothelial cells, and fibroblasts to secrete IL-8, in turn increases the expression of CD11b/CD18 adhesive molecules and granulocyte oxygen metabolism[11]. Interferon gamma is a strong cytokine, which intensifies Fc receptor expression, stimulates oxygen changes, and strengthens granulocytic granule release.

The aim of the study was to evaluate the metabolic activity of oxidative burst *in vitro* and the production of active oxygen compounds stimulated by *E. coli* and phorbol myristate acetate (PMA). Determination of the percentage of phagocytizing cells and their ability to phagocytize opsonized bacteria is of great importance in the estimation of neutrophil functioning. We evaluated the neutrophil ability to phagocytize *E. coli in vitro* in advanced liver cirrhosis. We also tried to establish the relationship between the neutrophil ability and the concentration of soluble adhesive molecules in peripheral blood.

MATERIALS AND METHODS

Patients

The studies were conducted in the group of 45 patients with advanced postalcoholic liver cirrhosis (30 men and 15 women, aged 45±14 years) (Table 1). Liver cirrhosis was confirmed clinically and histologically. Patients with acute viral or bacterial disease and those in the course of corticosteroid therapy who did not drink alcohol for at least 6 mo were excluded from the study. Patients with liver cirrhosis were divided into groups B and C in accordance with the classification of liver failure according to Child-Pugh[12]. The control group consisted of 25 healthy volunteers (12 women and 13 men, aged 38±5 years), who had never suffered from liver diseases and those without registered immunity disorders. Ethical approval for research was obtained from local Ethics Committee in the Medical University.

Methods

Blood was collected in plastic testing tubes with EDTA K2 and the absolute number of leukocytes and neutrophils was evaluated. The blood was collected in lithium heparin plastic testing tubes for the evaluation of phagocytizing cells and phagocytic activity and the percentage of bursting cells as well as neutrophil oxygen metabolism.

Table 1 Clinical characteristics of patients with liver cirrhosis (mean±SD)

	Child–Pugh B	Child–Pugh C
Age, year	43±12	46±13
Male/female	15/10	15/5
Albumin, g/dL	3.2±0.3	2.8±0.6
AST/ALT, IU/mL	98±65/87±98	110±45/97±45
Bilirubin, mg/dL	4.5±3.8	9.7±6.5

The assessment of phagocytosis was performed using the Phagotest Kit (ORPEGEN Pharma, Germany) containing fluorescein-labeled opsonized Escherichia coli (*E. coli* - FITC). Samples of 100 μL of blood with heparin were cooled in an ice bath for 15 min mixed with 2×10^7 FITC-labeled *E. coli* and then put in a chamber thermostat at 37 °C for 10 min. Simultaneously, the control samples were put into an ice bath to inhibit phagocytosis. Afterwards, 100 μL of brilliant blue (Quenching solution) was added in order to suppress the fluorescence of bacteria connected to the leukocyte surface. After two washing steps (with 2 mL of washing solution, centrifuged at 2 000 r/min, supernatant was pumped out), erythrocytes were lysed using lysis fluid for 20 min at room temperature. At the end, 50 μL propidyne iodide was added to stain leukocytes and bacterial DNA.

Oxidative burst

Granulocyte oxidative burst was determined quantitatively with Bursttest Kit (ORPEGEN Pharma, Germany). Fresh heparinized blood was put in a water bath for 15 min. Then, four testing tubes were filled with 100 μL of blood each and 2×10^7 unlabeled opsonized bacteria *E. coli*, 20 μL of substrate solution (negative control), 20 μL fMLP (peptide *N*-formyl-MetLeuPhe) as chemotactic low physiological stimulus (low control) and 20. μL phorbol 12-myristate 13-acetate (PMA), a strong non-receptor activator (high control). All the samples were incubated for 10 min at 37.0 °C in a water bath, dihydrorhodamine (DHR) 123 as a fluorogenic substrate was added and incubated again in the same conditions. The oxidative burst occurred with the production of reactive oxygen substrates (ROS) (superoxide anion, hydrogen peroxide) in granulocytes stimulated *in vitro*. In ROS-stimulated granulocytes, nonfluorescent DHR 123 underwent conversion to fluorescent rhodamine (R) 123 registered in the flow cytometer. Erythrocytes were removed using lysing solution for 20 min at room temperature, centrifuged (5 min, 250 r/min, 4 °C), and supernatant was discarded. Samples were washed again (washing solution), centrifuged (5 min, 250 r/min, 4 °C) and the supernatant was decanted. An amount of 200 μL of DNA staining solution (centrifuged and incubated for 10 min at 0 °C in a dark place) was added to discriminate and exclude aggregation artifacts of bacteria and/or cells in cytometric flow analysis.

Cytometric analysis

The flow cytometer EPICS XL (Coulter, USA) equipped

with 488 nm argon-ion laser was used. The apparatus was calibrated every day using DNA check. Neutrophil populations were identified by the use of forward and right angle light scatter, and the fluorescence emission of 10^4 cells per sample was recorded on a logarithmic scale. Fluorescent measurements were conducted with identical settings as for the standard determination of cell phenotype with fluorochrome-stained mAb.

Phagocytic activity was determined as the percentage of phagocytizing neutrophils (one or more bacteria ingestion) and as the mean intensity of fluorescence (MIF) value, which equaled the mean number of bacteria phagocytized by the cells.

Granulocyte oxygen metabolism was determined with the percentage of cells phagocytizing *E. coli* producing reactive oxidants (cells undergoing bursts, the change from DHR 123 to R 123), and with the evaluation of granulocyte enzymatic activity (the amount of released active oxygen compounds – the amount of MIF R 123 per cell).

Adhesive molecules and TNFα

ELISA tests were used to determine the level of sICAM-1 (Human sICAM-1, R&D, UK), sVCAM-1 (Human sVCAM-1, R&D), and the level of tumor necrosis factor alpha (Human TNFα, R&D) in blood serum. Soluble forms of P- (Human soluble P-Selectin, R&D), E- (Human sE-Selectin, R&D), and L-selectins (Human sL-Selectin, R&D) were determined by ELISA tests simultaneously in blood serum.

Statistical analysis

The results were presented as mean±SD. Statistical analysis was performed by non-parametrical *U* (Mann-Whitney) test. The result of correlation was calculated by Spearman's correlation test. $P<0.05$ was considered statistically significant.

RESULTS

Phagocytic activity of neutrophils

The ability of neutrophils to phagocytize opsonized *E. coli* was assessed. No significant differences were found in phagocytizing neutrophils both in liver cirrhosis patients and in healthy subjects (Table 2). The MIF of absorbed *E. coli* was slightly higher in patients with liver cirrhosis (differences being statistically insignificant). A positive correlation was observed between the percentage of neutrophils-phagocytized *E. coli* and the percentage of neutrophils with oxidative burst after *E. coli* stimulation *in vitro* ($r = 0.37$, $P<0.05$, Table 3).

Neutrophil oxidative burst

Stimulation *in vitro* with a strong activator PMA causes a markedly lower production of reactive oxidants in neutrophils in liver cirrhosis patients than in healthy individuals (MIF 42.7±14.6 *vs* 50.2±13.3, $P<0.01$, Table 2). Incubation of neutrophils with non-opsonized *E. coli* induced oxidative burst in more neutrophils in patients with liver cirrhosis than in the control group. However, neutrophils phagocytizing *E. coli* showed markedly lower metabolic potential in liver cirrhosis patients than that in healthy subjects. The oxidative burst in neutrophils phagocytizing *E. coli* caused generation of smaller amounts of active oxygen compounds in the cells of patients with liver cirrhosis. Neutrophils with oxidative burst (MIF of rhodamine 123) were statistically lower in liver cirrhosis patients (24.7±7.1) than in healthy subjects (29.7±6.6, $P<0.01$). PMA neutrophil stimulation *in vitro* was more effective than a direct contact with *E. coli*.

The decrease in neutrophil metabolic and phagocytic activities was observed together with the intensification of liver damage. We noted a negative correlation of MIF of neutrophil oxidative burst after PMA stimulation and ALT and AST, which was $r = -0.35$, $P<0.05$ and $r = -0.4$, $P<0.03$, respectively.

Adhesive molecules and TNFα

The concentrations of adhesive molecules sVCAM-1 and sICAM-1 in blood serum were several times higher in patients with liver cirrhosis ($P<0.01$, Table 2). Soluble E-selectin concentrations in liver cirrhosis patients were also significantly higher than in healthy subjects ($P<0.01$). However, significant differences of sL-selectin concentrations were not present, while sP-selectin concentrations were lower in liver cirrhosis patients. The level of sVCAM-1, sICAM-1, sE-selectin correlated negatively with the activity of oxygen radical production (MIF) after PMA neutrophil simulation ($r = -0.45$, $P<0.05$, $r = -0.41$, $P<0.05$, $r = -0.39$, $P<0.05$, respectively). The concentration of TNFα was markedly higher in liver cirrhosis patients than in healthy individuals ($P<0.01$). No correlation was observed between neutrophil metabolic and phagocytic activities and TNFα concentration. On the other hand, there was a positive correlation between sVCAM-1 and sE-selectin concentration ($r = 0.4$, $P<0.05$) and between sL-selectin concentration and the production of free oxygen radicals in neutrophils (MIF of R123) ($r = 0.35$, $P< 0.05$).

DISCUSSION

Infectious and toxic factors and over reactivity of the immune system are crucial in the pathogenesis of chronic liver diseases. The essence of the disease is accumulation of natural killer cells and inflammatory cells as well as intensified fibrosis in the liver. Chronic failure of endothelial cells of hepatic vessels increases expression and concentration of adhesive molecules. It facilitates inflammatory cell activation, margination and accumulation of leukocytes in the liver[4,13-15]. Neutrophils are professionally phagocytizing cells, which play an important role in immunological processes of the organism. They participate mainly in non-specific response as they do not possess the properties for precise recognition of antigens. Chemotactic factors and cytokines (IL-1, IL-8, TNFα, TGF-β) induce migration of granulocytes and other cells to the inflammatory site.

Table 2 Phagocytic and oxidative burst activity of neutrophils, level of soluble form of adhesive molecules and TNFα in liver cirrhosis patients (mean±SD)

		Liver cirrhosis		Healthy
	Total	Child–Pugh B	Child–Pugh C	
Oxidative burst				
Percentage of neutrophils with oxidative burst after PMA stimulation (%)	98.3±1.2	98.4±1.5	98.2±1.6	98.8±2.8
MIF oxidative burst after PMA stimulation	42.7±14.6[b]	44.3±10.1[b]	41.1±12.6[b]	50.2±13.3
Percentage of neutrophils with oxidative burst after *E. coli* stimulation (%)	94.0±4.8	93.0±4.1	94.0±3.8	92.2±3.7
MIF oxidative burst after *E. coli* stimulation	24.7±7.1[b]	25.1±8.1[b]	22.2±5.1[b]	29.7±6.6
Phagocytic activity				
Percentage of neutrophils phagocytizing of *E. coli*	93.0±3.3	92.9±4.3	93.8±2.3	92.5±4.3
MIF phagocytosis *E. coli*	20.0±3.9	19.8±4.1	21.0±2.8	19.0±5.8
Soluble form of adhesion molecules and TNFα				
sICAM-1 (ng/mL)	852±331[b]	788±343[b]	892±293[b]	254±74
sVCAM-1 (ng/mL)	2 937±1 591[b]	2 637±1 291[b]	3 297±1 490[b]	510±248
sP-selectin (ng/mL)	101±180[b]	98±140[b]	104±162[b]	124±58
sE-selectin (ng/mL)	136±89[b]	120±68[b]	148±58[b]	49±21
sL-selectin (ng/mL)	1 209±364	1 206±314	1 211±344	1 209±291
TNFα (pg/mL)	2.94±1.43[b]	2.64±1.33[b]	2.99±1.02[b]	1.58±0.22

[b]$P<0.01$ *vs* healthy subjects (Mann–Whitney *U* test).

Table 3 Correlation (*r*) of phagocytic activity of neutrophils with their oxidative burst level and biochemical tests of liver dysfunction (Spearman test)

Correlations	*r*	*P*
MIF oxidative burst neutrophils after PMA stimulation with ALT level	-035	0.05
MIF phagocytizing *E. coli* with prothrombin time	-0.47	0.03
Percentage of neutrophils phagocytizing *E. coli* with leukocyte number	0.35	0.05
Percentage of oxidative burst neutrophils after *E. coli* stimulation with MIF phagocytosis *E. coli*	0.37	0.05

The study showed a significant impairment of neutrophil immune mechanisms in liver cirrhosis as well as oxidative burst damage with diminished amount of generated free oxygen form in neutrophils. Stimulation *in vitro* with the strong stimulant PMA and non-opsonized *E. coli* induces less amount of released active oxygen compounds in neutrophils in liver cirrhosis patients than in healthy subjects. It should be assumed that it is a result of permanent stimulation of neutrophils by inflammatory factors or lipopolysaccarides (LPS). Observed disorders can be treated as dysfunctions of exhausted neutrophils, and oxygen exchange impairment (processes being important in the maintenance of organism immunity) has been confirmed in other studies[16,17]. The higher release of oxygen metabolites observed after PMA is probably due to different stimulation mechanisms. The neutrophil phagocytic activity in both groups was comparable. Cytometric examinations of the percentage of phagocytizing neutrophils and MIF of absorbed opsonized *E. coli* did not show differences in both groups.

Vascular endothelium has many important functions, and is a barrier against pathogens as well as the site of immunological and inflammatory process initiation with participating neutrophils[18]. Neutrophil accumulation at the site of inflammation depends on the adhesive molecule expression in endothelial cells. Neutrophil stimulation leads to activation of L-selectin and other molecules (β2-integrines) that participate in adhesion to the endothelium. In addition, increase in adhesive molecule expression directly activates antibacterial mechanisms, granule secretion and neutrophil oxygen metabolism. Activation of neutrophils (the main generators of free oxygen radicals in inflammatory processes) leads to elevation of active oxygen metabolites responsible for killing microorganisms[19]. In patients with end-stage liver disease, the ability of neutrophils to migrate to the inflammatory sites is impaired. It was observed that neutrophils present there have their phagocytizing activity reduced[16].

Adhesive molecules (ICAM-1, VCAM-1, selectins) play an important role in keeping neutrophils in liver sinusoids. Antibodies against anti-ICAM-1 and anti-VCAM-1 inhibit neutrophil accumulation in liver tissues and diminish their damage[9,20]. P- and E-selectin expressions in endothelial cells and L-selectin – in leukocytes – lead to slower flow of leukocytes and blood platelets in bloodstream, activation, rolling, and adhesion of these cells. In liver cirrhosis patients, adhesive molecule expression increases due to tissue hypoxia, deposits of immunological complexes, endogenic toxic compounds, LPS, bacteria, and viruses[21-23].

Fiuza *et al*[16]. have suggested that, in the end-stage liver disease, neutrophils have injured ability to migrate to the inflammatory sites and their phagocytic activity is diminished, which in turn, correlates with the severity of liver disease. It has been shown that chronic intravascular peripheral neutrophil activation occurs in vessels. Their results point to the fact that antibacterial early inflammatory immune response is damaged in liver cirrhosis. The increase in bacterial translocation and endotoxin absorption from the bowel is a stimulus for the reticuloendothelial system to produce proinflammatory cytokines[24,25]. Neutrophil activity and phagocytic ability failure may result in ascitic fluid microinfection and

subclinical SBP. On the other hand, a persistent stimulation of neutrophils by inflammatory factors can lead to the exhaustion of their functional potential. Proinflammatory factor elimination from peripheral blood is impaired in liver cirrhosis which facilitates persistent stimulation of peripheral blood neutrophils[25].

We observed high concentrations of soluble adhesive molecules VCAM-1, ICAM-1, and E-selectin, which can account for a marked vascular endothelium damage in liver cirrhosis. Endothelial cell failure or stimulation can occur due to high levels of proinflammatory cytokines (e.g. TNFα). Chronic endotoxemia may also stimulate adhesive molecule expression. High concentrations of VCAM-1, ICAM-1 and E-selectin stimulate inflammatory site to accumulate in terminal capillaries of the liver and other organs. In our studies, the increased expression and concentration of soluble adhesive molecules might result in exhaustion of oxygen metabolism of neutrophils in liver cirrhosis.

Neutrophils are an important host immune barrier against bacterial infections. A persistent stimulation of leukocytes that flow through the liver affected by inflammation leads to the exhaustion of mechanisms responsible for their immune properties.

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