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Original Contribution

PRODUCTION OF PRO- AND ANTIINFLAMATORY CYTOKINES, APOPTOSIS AND OXIDATIVE STRESS ARE ESSENTIAL FOR THE OUTCOME IN SEVERE SEPSIS

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ABSTRACT

To investigate in vitro secretion of cytokines from stimulated peripheral blood mononuclear cells (PBMC) in the early phase of severe sepsis (septic phase) and one month later in postseptic phase, when the patient is stabilized (healthy stage). The quantity determinations of TNF-α, IL-6, IL-12 and IL-10 were performed in culture supernatant by ELISA. The level of apoptosis of cultured cells was determined as well. The plasma level of lipid peroxidation products (MDA) as a marker of oxidative stress and the activity of the erythrocyte antioxidant enzyme catalase (CAT) in the patient during the septic stage were also explored. We observed no significant difference in the secreted quantity of TNF and IL-6 upon LPS, C3bgp, PHA and PWM stimulation alone by the PBMC of the patient. The IL-12 production depended on the individual stimuli applied. Further, the patients' PBMC in post septic stage exhibited a suppressed IL-12 production. A well expressed hyporeactivity of PBMC isolated from septic stage towards the production of IL-10 upon stimulation, was also observed while the production of IL-10 in post-septic stage in different quantities depended on the stimuli used. At the same time the rate of apoptosis has clear prevalence in septic stage and is greatly influenced by different stimuli.

Key words: Sepsis, Cytokine, PBMC, IL-12, IL-10

INTRODUCTION

Septic shock and its frequent precursor, severe sepsis, are the most common causes of morbidity and mortality in ICU today. An acquired immunodeficiency which may occur in septic patients is related to a modified state of monocytes and increased lymphocyte (predominantly T-cell) apoptosis. The sepsis syndrome is associated also with an exacerbated production of pro and anti-inflammatory cytokines as assessed by their increased level in the blood. Paradoxically, the hyporesponsiveness of peripheral blood lymphocytes and monocytes to in vitro produced cytokines from septic patients as compared to cells from healthy controls has been regularly reported (1,2). Reduced cytokine production has been observed with LPS stimulation and other stimuli. The production of IL-1, TNF-α and IL-6 by peripheral blood mononuclear cell (PBMC) was significantly reduced upon lipopolysaccharide (LPS) stimulation, compared to healthy control (3). Thus overall suppression of cytokine production by T cells and monocytes was already observed at the beginning of postoperative sepsis. Moreover persistence of depressed cytokine secretion correlated with lethality and recent clinical trials failed to demonstrate beneficial effects of anti-inflammatory sepsis therapy (4).

In a study performed in septic patients, some authors confirmed the importance of the nature of the activating signal to demonstrate the presence of cellular hyporeactivity (5). Muret et al. (5) showed that PHA stimulation is associated with an unmodified production of T-cell derived cytokines as compared to healthy controls whereas concanavalin A stimulation is associated with a significant reduction of IL-2, IL-5 and IL-10 cytokines. When PMA was used as a triggering agent,

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TNF and IL-1 production by monocytes from patients with severe sepsis were not significantly lower than in healthy control (6).

Despite the fact that sepsis is merely associated with a reduced capacity of monocytes to produce cytokines in response to LPS, there are few examples that demonstrate an unaltered reactivity of monocytes to certain stimuli. Further insight will be require to understand whether these discrepancies are associated with the nature of the stimuli used for in vitro triggering and whether they reflect on the outcome of the disease.

MATERIALS AND METHODS

Isolation of human PBMC and culture. The PBMC were isolated from the healthy donors and patient's peripheral blood and were cultured in vitro for 24 hours with various stimuli. All cultures were carried out in RPMI 1640 media (Sigma Chemical Co.) supplemented with penecillin G (200 U/ml), gentamycin (10 μg/ml) and L-glutamine (0.3 mg/ml) .

The following triggering/activating agents were employed for the in vitro stimulation: lypopolysaccharide (LPS) - 30μg/ml; phytohemagglutinin (PHA) -20μg/ml; C3 binging glycoprotein from Cuscuta europea (C3bgp) -30μg/ml; pokeweed mitogen (PWM) -30μg/ml and dexamethasone (DM) - 500μg/ml.

Cytokine determination. The quantity determinations of TNF-α, IL-6, IL-12 and IL-10 were performed in sera and culture supernatant by ELISA with commercially available kits purchased from BioSource, Austria, following the instruction of manufacturer. Color reaction developed was measured as OD units at 450 nm on an ELISA reader (Rosys Anthos 2010, Austria) The concentration of cytokines was expressed in pg/ml by kit's standard curve.

Assessment of apoptosis. The level of apoptosis of cultured cells was determined as percent of total cell number. For nuclear morphology staining, the harvested cells were washed in PBS. To this 20 μl of a 1:1 mixture of ethidium bromide (100 μg/ml; Sigma) was added. The cell suspension was incubated in the dark at room temperature for 5 min and was mounted on a slide. The nuclear morphology was examined by a fluorescence microscope. At least 500 cells per slide were counted. Normal cell morphology was defined as normal cells appearing large with multilobed yellow-stained nuclei. Cells with condensed or fragmented nuclei were considered as apoptotic cells.

Cell viability assay. The cell viability was determined by MTT assay. 100 μl MTT stock solution (5mg/ml) was added to 1 ml of each culture and incubated for 3h at 37^0C. After cultured was added 1ml 0.1N HCl in absolute isopropanol and absorbance of converted dye was measured at 570 nm.

Analyses of oxidative stress-related parameters. Total amount of lipid peroxidation products in the plasma of 150 healthy volunteers and the patient was estimated using the thiobarbituric acid (TBA) method, which measures the malondialdehyde (MDA) reactive products (7). The intensity of the pink color of the obtained fraction product was read at 532 nm. Erythrocyte CAT activity was determined following the method of Beers and Sizer (8). Briefly, hydrogen peroxide was used as a substrate and the decrease in its concentration at 22^oC in phosphate buffer (pH 7.0) was followed spectroscopically at 240 nm.

RESULTS

The present study investigated immune cell functions in a patient with postoperative sepsis due to *Pseudomonas aeruginosa* infection. The evidence for bacterial infection was provided by microbiological investigations. In this patient sepsis was proven by positive blood culture and clinical criteria for SIRS. Criteria for the diagnosis of SIRS were: hyperthermia (> 38^0 C); heart rate > 90 bpm; respiration rate > 20/min and minute ventilation > 10 l/min; WBC > 12.10^9 G/l and immature forms > 10%.

In vitro functions of stimulated peripheral blood mononuclear cells (PBMC) were investigated in early phase of severe sepsis (septic phase) and one month later, when patient was stabilized (postseptic or healthy phase). It should be noted that different triggering /activating agents were employed in this study.

Lipid peroxidation and Antioxidant enzyme activity.

We measured malondialdehyde (MDA) concentration, as a marker of oxygen-derived free radical-madiated lipid peroxidation, and the activity of one of the main antioxidant enzymes, catalase (CAT), as an index of antioxidant defenses. MDA in the plasma of the patient in septic stage was increased significantly and erythrocyte CAT activity of the patient in septic stage was also significantly increased compared to the

controls (for MDA: 5.00 μmol/l vs mean 1.86 ± 0.56 μmol/l, p<0.001; for CAT: 50 028 U/gHb vs mean 15346.51 ± 9905.77 U/gHb, p<0.001).

Cytokine production upon stimulation.
To determine the hypo or hyperreactivity of patient's PBMC to produce cytokine the following triggering agent for cultures were used: lypopolysaccharide (LPS); phytohemagglutinin (PHA); C3 binging glycoprotein from *Cuscuta europea* (C3bgp); pokeweed mitogen (PWM) and dexamethasone (DM). The cytokine quantities produced in cultured supernatant after 24 hours, were detected by ELISA (Biosource International, inc. Belgium) and the results are shown in Figure 1. The data on Figure 1a and 1b show that C3bgp, LPS, PHA and PWM are potent stimulators of proinflamatory cytokines. The released quantities of TNF and IL-6 are approximately equal upon LPS, C3bgp, PHA and PWM stimulation alone. We observed that the secreted quantities of IL-6 and TNF in culture supernatants were not influenced by the stage of the condition of the patient when cells were isolated.

As well known in immune cells, proinflamatory cytokine gene expression is downregulated by glucocorticoids and our results demonstrated a decreased quantity of IL-6 and TNF. The downregulating effect of DM is partially compensated in coculture with C3bgp. We recently demonstrated a similar effect of immunostimulation of C3bgp in mice, treated with cyclophosphamide (9).

In the present case, increased in vitro production of proinflamatory cytokine – IL-12 depends on the stimuli used was detected. In response to LPS, C3bgp, PHA and PWM alone, production of this cytokine is significantly lower compared to costimulation with C3bgp (Figure 1d).

Production of antiinflamatory cytokine IL-10 was altered, with different kinetics being selectively elevated during the late postseptic course (Figure 1c). The quantity of secreted IL-10 depends on the stimuli used and was the greater after PWM stimulation, followed by PHA, C3bgp and LPS**.** It was also observed considered hyporeactivity of PBMC isolated from septic stage towards production of this cytokine upon stimulation

We also determined the cytokine level in serum of the patient (Figure 1e). It was detected the significantly difference in concentration of IL-6 only, depends on the health stage. The quantity of IL-6 was approximately 10 times higher in septic stage, compared to late post-septic phase.

Cell Proliferation and apoptosis rate.We analyzed whether cellular survival and apoptosis rate are associated with septic and healthy stage of in vitro cultured PBMC. After incubation for 24 hours cell survival was measured by colorimetric assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma). As shown in fig. 2a, data obtained by MTT assay demonstrated that the ability of cell survival was generally higher in septic stage compared to the post-septic stage in respect of the stimuli used. The main difference was observed between mono- and coculture, where the survival of PBMC varied depends on the patient's health condition.

Apoptosis was detected by morphological criteria and by DNA fragmentation in ladder form after agarose gel electrophoresis. In the same experiment the determined rate of apoptosis clearly prevails in septic stage and is greatly influenced by different stimuli (Figure 2b). LPS and PHA enhanced the level of apoptosis, while C3bgp (alone and in coculture), DM and PWM decreased this level.

DISCUSSION

Functional alterations of the innate and adaptive immune system have been studied most extensively in patients with severe sepsis. Some of them, demonstrated reduced secretion of interleukin TNF-α, IL-1 and IL-6 by LPS stimulated whole blood from septic patients (3). Mitov et all reported that in a severely infected patient the cytokine responses of periferal blood cells to LPS may be suppressed, while the response to other bacterial components is enhanced (10). Other authors report that proinflamatory cytokines production is different in septic survivors and nonsurvivors (11). Adamik et al. reported that the ability of PBMC from the patients to produce IL-6 in culture, stimulated with PHA or LPS is depressed in nonsurvivor septic patients. More profound differences were found with respect to TNF-α production, which was deeply depressed in septic nonsurvivors (11). Moreover, attempts to suppress proinflamatory mediators have failed to improve an outcome in sepsis (4). To determine the production of pro- and anti-inflammatory cytokines, their dynamics and relationship to the outcome of sepsis we investigate a patient in the early phase of severe sepsis and one month later when the patient was stabilized (postseptic, healthy stage).

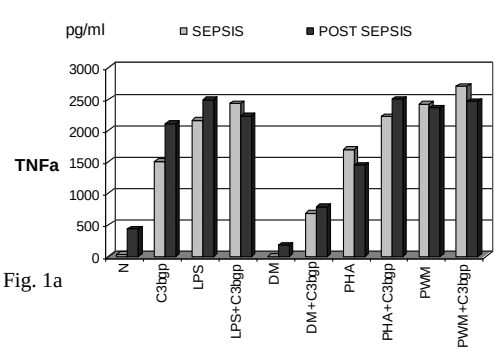

Fig. 1a

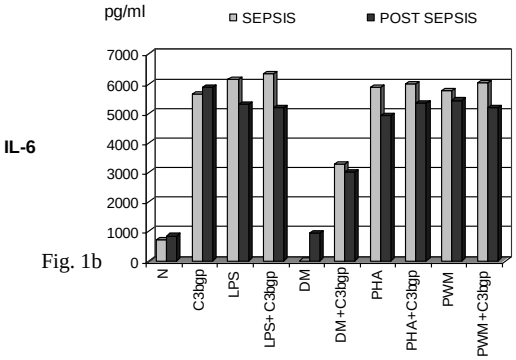

Fig. 1b

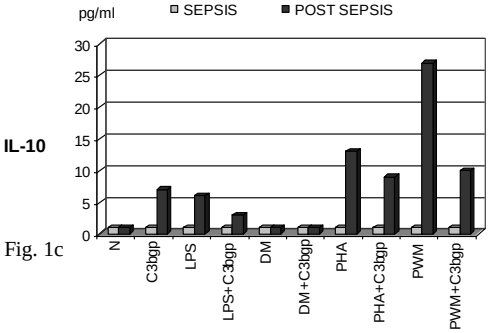

Fig. 1c

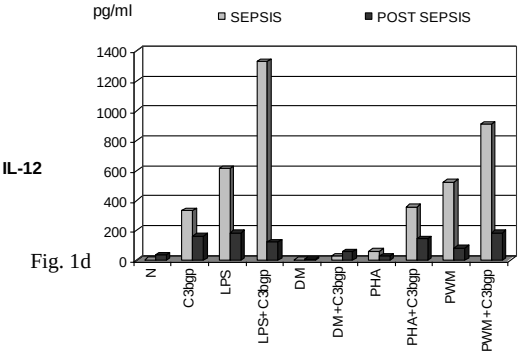

Fig. 1d

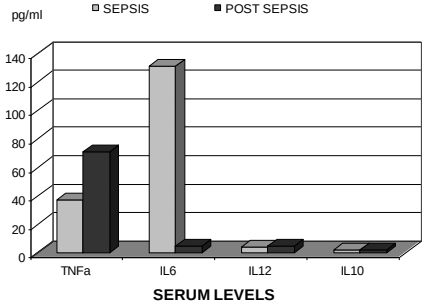

Fig. 1e

Figure1. Cytokine levels, produced by cultured PBMC (a-d) and in patient's serum (e). Isolated PBMC from patient in septic and postseptic stage were cultured 24 hour with indicated triggering agents: lypopolysaccharide (LPS-20µl/ml); phytohemagglutinin (PHA-20µl/ml); C3 binging glycoprotein from *Cuscuta europea* (C3bgp-20µl/ml); pokeweed mitogen (PWM-20µl/ml) and dexametason (DM-2mg/ml). The secreted quantity of TNFα (a); IL-6 (b); IL-10 (c) and IL-12 (d) were determined by ELISA test (Biosource International, inc. Belgium).

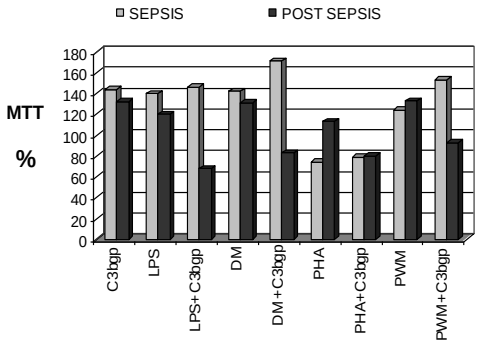

Fig. 2a

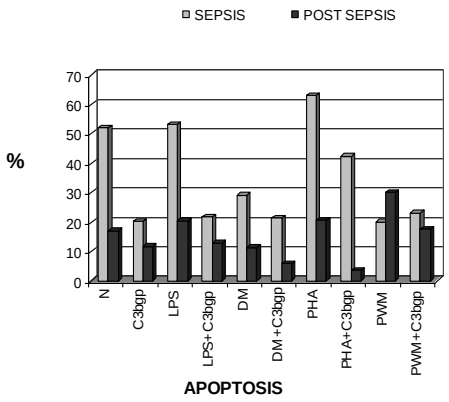

Fig. 2b

Figure2. Cell survival, determined by MTT assay (a), and apoptosis, determined by light microscopy of cultured PBMC (b).

Our results showed that the levels of secreted cytokines in stimulated cultures depended on the triggering agent, as well as on the health condition of patient when PBMC were isolated. In vitro cultured of peripheral blood mononuclear cells with either LPS, C3bgp, PHA or PWM for 24 hours led to a strongly or moderately enhancement of TNF-α and IL-6 production, approximately equal in septic and nonseptic stage. The smallest activity to induce TNF-α had PHA persisted over the stages of health condition. We observed a great differences between sepsis and postseptic stages when was determined the concentration of these cytokines in the patient's sera. The strongly elevated level of IL-6 was a mark only for sepsis stage. No relation was found between circulating plasma levels of investigated cytokines and levels after in vitro stimulation. In general decreased production of all determined cytokines after exposure to dexamethasone was detected. The main differences, we observed after PBMC stimulation, concern the concentration of IL-12 and IL-10 in sepsis and healthy phase.

The levels of IL-10 detected in the blood of the patient in septic and non septic condition were similar. When the release of IL-10 was mesuared into stimulated PBMC, the concentration of this antiinflamatory reacting cytokine was significantly decreased by all the stimuli used during sepsis stage compared with non-septic stage of the patient. These results are in line with previous studies indicating reduced secretion of IL-10 by PBMC from critically ill patients and by splenocytes harvested from mice in a trauma-sepsis model (12).

A negative correlation between cell survival rate and IL-10 quantity (r=-0,653 and p=0.04, correlation analysis) was found. The elevated IL-10 quantity, established in culture in postseptic course well correlated with diminished apoptosis rate, compared to early septic stage. Moreover we observed statistically significant positive correlation between IL-10 quantity and rate of apoptosis in healthy stage (r=0.7, p=0.024, correlation analisys). Results are obtained in the opposite direction for IL-12 quantity.

In the recent case, increased in vitro production of proinflamatory cytokine – IL-12 triggered in response to LPS, C3bgp, PWM and costimulation with C3bgp were considered important for the outcome of gram-negative sepsis, because only PBMC isolated from septic stage produced this cytokine (Figure1d). IL-12 exerts protective effect during experimental endotoxemia trough upregulation of cellular immunity and phagocytic functions. Naïve T cell can be induced to express the Th1 phenotype by exposure to the cytokine IL-12. Most of the studies reported that secretion of IL-12 in stimulated whole blood from critically ill patients was significantly decreased compared to the control group (13). In the same study was reported that the secretion of IL-12 was less reduced when using isolated cultures of adherent cells. Our experiment with isolated PBMC cultures stimulated with LPS, PWM alone and all coculture with C3bgp in the septic shock produced increased quantity of IL-12. We considered that increased IL-12 appearance must be associated with a favorable outcome in this septic patient. Moreover, recent evidence suggest a selective preoperative defect in monocyte IL-12 production as a predictive factor for the lethal outcome of postoperative sepsis (14). IL-12 can protect cells from apoptosis induced by DNA-damaging UV radiation (15). Because apoptosis play a pivotal role in sepsis, we propose that enhanced IL-12 production supports survival of lymphocyte and implied in diminished apoptosis level, as demonstrated our results and affect the favorable disease outcome. The enhanced MDA levels compared to those of the controls demonstrated the presence of an increased oxidative stress in septic stage due to reactive oxygen species and an adaptive increase of CAT activity. Important sources of increased reactive oxygen species production might be activated monocyte during septic stage. These findings are in line with the result of several other studies describing that free radical formation during the septic stage is markedly enhanced (16).

Cytokines, and to a lesser extend reactive oxygen species, are the best identified candidates for mediating apoptosis of PBMC in sepsis. Wang et al. (17) demonstrated that apoptosis of thymocytes may account for thymic involution and T cell suppression in a mouse model of gram-negative or positive sepsis, and that thymocytes apoptosis may be mediated by TNF-α. Recently, Hotchkiss et al. (18), using the CLP mouse model of sepsis, demonstrated lymphocyte apoptosis in the thymus and spleen. Increased expression of the apoptosis inducing antigen and its ligand was also reported on the surface of mononuclear cells of septic patients (19). The role of cytokines in apoptosis in septic patients is indisputable, however investigation has failed to identify specific causal cytokines

(20). In the present case we observed well expressed PBMC apoptosis by microscopic and DNA electrophoresis methods in septic compared to healthy stage. The level of apoptosis decreased after incubation with DM or C3bgp and elevated after incubation with PHA. We suppose that the chances in apoptosis are not correlated with IL-6 and TNF levels. A relation was observed with increased level of IL-10 in healthy stage and decreased apoptosis of PBMC compared to septic stage. We suppose that apoptotic rates are increased in septic stage, as a result of secreted proinflamatory cytokine, mainly TNF-α; decreased secretion of IL-10 and enhanced oxidative stress. In this case cultured PBMC, isolated from septic patient has preserved capacity to produces proinflamatory cytokines. The level of produced IL-12, but not IL-6 and TNF, depends on the stimulus used, as well as on the physiological status (functional stage) of isolated PBMC.

Our data illustrates that the immune depression reported in septic patients, often revealed by a diminished capacity of leukocytes to respond to LPS is not a generalized phenomenon. The ability of stimulated PBMC in septic stage for producing enhanced quantity of IL-12 and diminished quantity of IL-10 may correlate with the outcome of postoperative sepsis and might be used as a predictive factor.

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