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Are Soluble Factors Relevant for Polymorphonuclear Leukocyte Dysregulation in Septicemia?

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Polymorphonuclear leukocytes (PMNs) of twelve patients with gram-negative septicemia exhibited a decreased capacity to phagocytize *Escherichia coli* and generate reactive oxygen products which normalized within 7 days of treatment. Ex vivo exchange of plasma from age-, sex-, and blood-group-identical normal controls resulted in an increase of both phagocytic capacity and reactive oxygen intermediate generation in PMNs of septicemic patients and transiently reduced phagocytosis and reactive oxygen intermediate production in PMNs of normal controls. These results suggest that extrinsic factors are crucial for PMN function.

Polymorphonuclear leukocytes (PMNs) contribute to the septic autoinjury process via synthesis and release of various prostanoids, enzymes, and reactive oxygen intermediates (ROIs) (3, 30, 33, 43, 47). A reduction of various PMN functions including adherence (41), chemotaxis (16), depressed degranulation (36), phagocytosis (29), and production of ROIs (30, 40, 49) has been demonstrated during septicemia. Plasma isolated from patients with septic shock and incubated with normal PMNs significantly inhibited superoxide anion production (48).

The current study was designed to assess, by flow cytometry, the phagocytic capacity and generation of ROIs of PMNs in patients with gram-negative septicemia prior to, during, and after antimicrobial therapy (5, 6, 8–12, 14, 17, 31, 37, 38). To determine whether factors intrinsic or extrinsic to the PMNs are likely to influence PMN function, plasma from septicemic patients was added to PMNs of age-, sex-, and blood-group-identical normal controls and plasma from normal controls was added to PMNs of septicemic patients.

Twelve patients (five females and seven males) ranging in age from 16 to 65 years (mean, 35 ± 6 years) and 12 age-, sex-, and blood-group-identical healthy subjects were investigated. All patients fulfilled previously published criteria for sepsis syndrome: clinical signs of infection, fever (>38.3°C) or hypothermia (<35.6°C) rectally, tachycardia (>90 beats per min), and tachypnea (>20 breaths per min while breathing spontaneously). They also showed at least one of the following manifestations of inadequate organ perfusion or dysfunction: deterioration from baseline mentation, hypoxemia (O_2 <75 mm Hg [ca. 10 kPa] on room air in the absence of pulmonary disease), elevated lactate levels, and oliguria (>30 ml/h or 0.5 ml/kg/h) (7). The pathogens isolated in multiple blood cultures prior to antimicrobial therapy were *Escherichia coli* (n = 10), *Morganella morganii*, and *Klebsiella pneumoniae.* After the blood and urine cultures were obtained, patients were treated with empirical antimicrobial therapy. In all cases patients or their relatives gave informed consent. Blood samples were obtained prior to antimicrobial therapy and 1, 3, 7, 14, 21, and 28 days after initiation of treatment. All assays were performed within 4 h of obtaining the blood. In order to examine the effect of septic and normal plasma on the ROI-generating capability and phagocytic capacity of PMNs, paired mixing experiments were performed (Fig. 1). Mixed samples were analyzed immediately and incubated for 1 h at room temperature to determine whether these effects were reversible. In addition, heat-inactivated plasma (30 min at 56°C) of normal controls was incubated with PMNs of patients with gram-negative septicemia to determine whether heat-labile factors (e.g., complement) are involved in the modulation of PMN function of PMNs of septicemic patients. All assays were repeated eight times. A flow cytometric method was used to study phagocytic capacity and generation of ROIs of PMNs (5, 6, 8–12, 17, 31, 35, 37, 38). Heat-killed *E. coli* ATCC 25922 organisms (10^8/ml) were labeled with fluorescein isothiocyanate as previously described (14). Dihydrorhodamine 123 was purchased from Molecular Probes Inc. (Eugene, Ore.) and dissolved in *N*,*N*-dimethylformamide (Sigma Chemicals, Munich, Germany) at a concentration of 3 μg/ml. The phagocytic capacity was assessed by adding 10 μl of precooled fluorescein isothiocyanate-labeled *E. coli* to 100 μl of heparinized whole blood and incubating for 10 min at 37°C. Thereafter, 100 μl of precooled quenching solution (Orpegen, Heidelberg, Germany) was added, and the samples were washed twice in phosphate-buffered saline (PBS; pH 7.4). Finally, 2 ml of fluorescence-activated cell sorter (FACS) lysing solution (Becton Dickinson) was added. After 20 min, the samples were washed again, resuspended in 100 μl of PBS containing propidium iodide at a concentration of 50 μg/ml for DNA staining, and kept on ice until analysis. For analysis of ROI production, blood samples were stimulated with 25 μl of *E. coli* ATCC 25922 (10^8/ml; unlabeled) at 37°C. After 10 min, 25 μl of the dihydrorhodamine solution was added. After another 10 min at 37°C, 2 ml of FACS lysing solution was added, and the mixture was incubated for 20 min at room temperature. Thereafter, the samples were washed in PBS and resuspended in 100 μl of PBS containing propidium iodide at a final concentration of 50 μg/ml for DNA staining. The cells were analyzed on a standard FACScan flow cytometer (Becton Dickinson). For each measurement, 10,000 events were collected. To exclude cell debris and nonphagocytized bacteria, a live gate was set on propidium iodide-stained leukocytes during acquisition in fluorescence channel 2 (FL2). For analysis of ROI production, the shift to the right in FL1 (green) was determined. The amount of cleaved substrate was estimated by the mean fluorescence, using the statistical option of the FACScan software. Similarly, the amount of phagocytized bacteria was assessed by a shift in mean fluorescence to the right (FL1). The mean fluorescence

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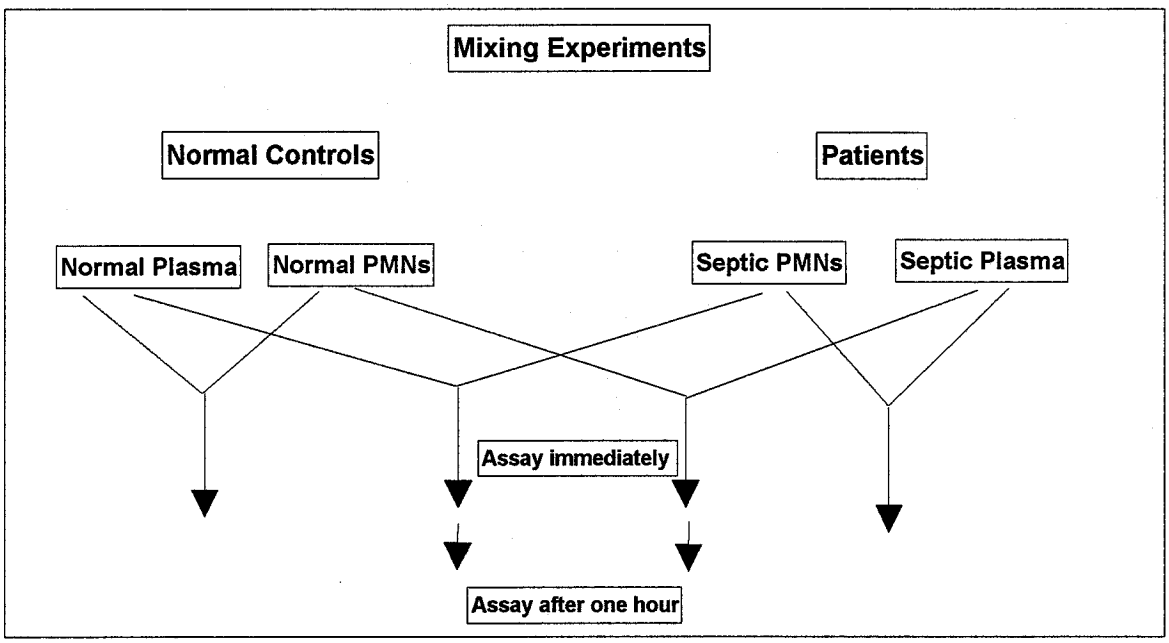

FIG. 1. Schematic depiction of exchange experiments with normal and septic PMNs and with normal and septic plasma.

of each assay was compared with that of unstimulated controls. Daily alignment and calibration of the instrument were done with fluorescence beads (Calibrite; Becton Dickinson). The beads were put into the same histogram channel every day. Differences between groups were calculated by using the Mann-Whitney U test. The ROI production and phagocytic capacity of patients' PMNs during the follow-up period were compared with those of controls by using the Wilcoxon rank sum test for dependent samples. Spearman correlation was used. All analyses were two sided, and differences with a P value of less that 0.05 were considered significant.

The most common diagnosis was urosepsis (8 of 14 patients). Underlying diseases were renal stones, prostate hyperplasia, liver cirrhosis, and ileus. Fever subsided, on average, after 7 days of therapy; leukocytosis and serum levels of C-reactive protein were within the normal ranges after 14 days of treatment, whereas blood sedimentation rate and serum levels of fibrinogen were within the normal ranges 28 days after initiation of therapy. No steroids were used.

Figure 2 demonstrates the phagocytic capacity and generation of ROIs in PMNs of patients with gram-negative septicemia compared with normal controls. Prior to treatment, both phagocytosis and ROI production were decreased. ROI production and phagocytic capacity increased significantly during the first days of therapy and reached normal values on day 7. Thereafter, no significant alterations in phagocyte function occurred. The generation of ROIs was correlated with phagocytic capacity of PMNs ($r = 0.683$; $n = 12$; $P > 0.05$). No relation between phagocytic capacity or ROI production and the number of band forms in peripheral blood (PMNs, $[12 \pm 4] \times 10^9$/liter; $7\% \pm 3\%$ band forms), serum levels of acute-phase reactants, blood sedimentation rate, leukocyte counts, or fever was found.

Figure 3 demonstrates the effect of plasma exchange and inactivation on PMN function of septicemic patients and normal controls. Exposure of normal PMNs to septic plasma resulted in decreased phagocytic capacity and generation of ROIs after immediate analysis. However, after a 1-h incubation, phagocytic capacity normalized and ROI production increased. Exposure of PMNs of septicemic patients to normal plasma reversed the depressed function after immediate analysis and resulted in a significant increased phagocytic capacity and ROI generation of PMNs after a 1-h incubation. However, incubation of septic PMNs with heat-inactivated plasma of normal controls did not enhance either ROI generation or phagocytic capacity and did not influence PMN function in normal controls.

Although "sepsis" is initiated by microbes and their related toxins, the resultant humoral and cellular inflammatory reactions actually define the magnitude of the septic response. Feedback loops are thought to define leukocyte-cytokine networking in septicemia (3, 16, 25, 29, 30, 33, 41, 43, 47). Many of the humoral mediators elicited early in the course of sepsis, such as endotoxin, tumor necrosis factor, interleukin-1, interleukin-8, gamma-interferon, activated complement components, granulocyte colony-stimulating factor, and platelet-activating factor (13, 18, 22, 23, 26–28, 44), are known to prime neutrophils for an enhanced respiratory burst (1, 4, 21, 34, 39, 42). However, endotoxin itself may exhibit a direct inhibitory effect on neutrophil superoxide anion synthesis (45).

Previous findings (48) and the current study demonstrate that plasma isolated from septicemic patients apparently contains factors capable of suppressing neutrophil superoxide anion production. After a 1-h incubation, PMN function normalized again, which suggests that these factors have a short half-life. Conversely, normal plasma significantly enhanced phagocytic capacity and ROI generation in PMNs of septicemic patients after 1 h of incubation. The incubation with heat-inactivated normal plasma, however, could not reverse depressed PMN function. Whether this effect is due to cytokine depletion, reduction of levels of free proteases, and/or the presence of activatable complement in normal plasma remains to be determined.

During sepsis, various proteases such as elastase and collagenase are released into circulation as a result of neutrophil degranulation (20). Membrane-bound NADPH oxidoreduc-

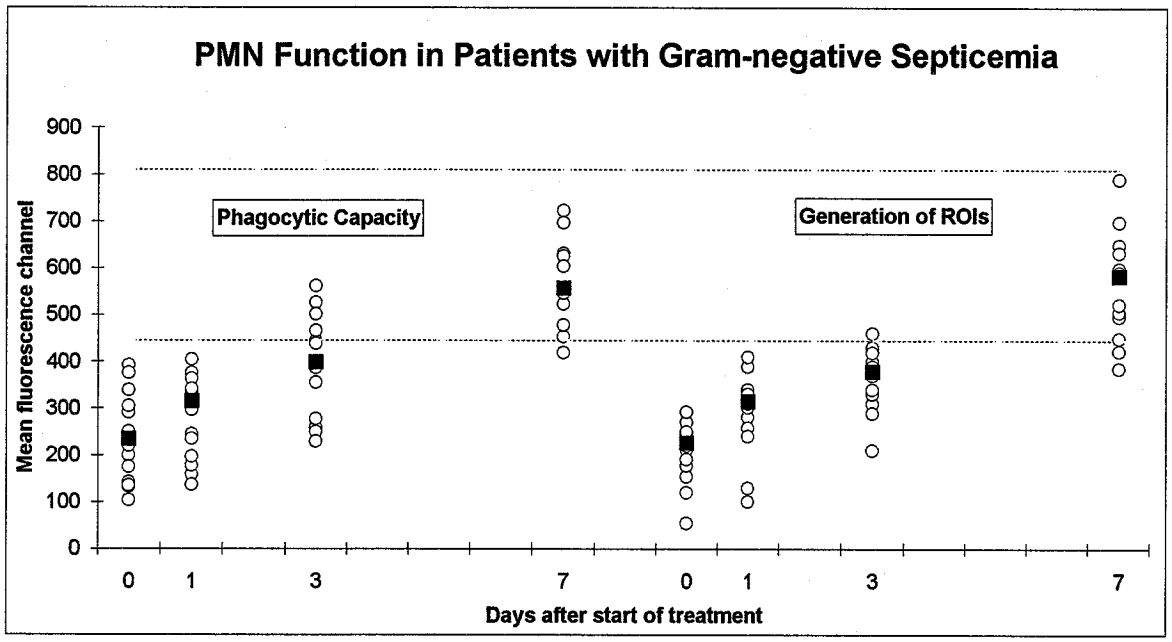

FIG. 2. The amount of ROI production is proportional to the conversion of dihydrorhodamine 123 to rhodamine 123 and is estimated by the mean fluorescence channel. Similarly, the amount of phagocytized fluorescein isothiocyanate-labelled bacteria is assessed by a shift in the mean fluorescence channel. The open circles reflect the phagocytic capacity and ROI generation of each patient's PMNs during therapy for septicemia, the dotted lines denote the normal range (mean fluorescence channel 446-810), and black squares are medians.

tase is known to be extremely vulnerable to proteases (24). In addition, it could be demonstrated that NADPH oxidoreductase may be inhibited in vitro by nitric oxide (15). Both mechanisms are likely to result in depressed oxidative metabolism during septicemia.

Another factor that may play a partial role in sepsis is the fact that immature granulocytes have a reduced superoxide anion synthetic capacity in vitro (46). However, in the current study, in neither patients nor controls could discrete PMN subpopulations which take up an unusually low or high number

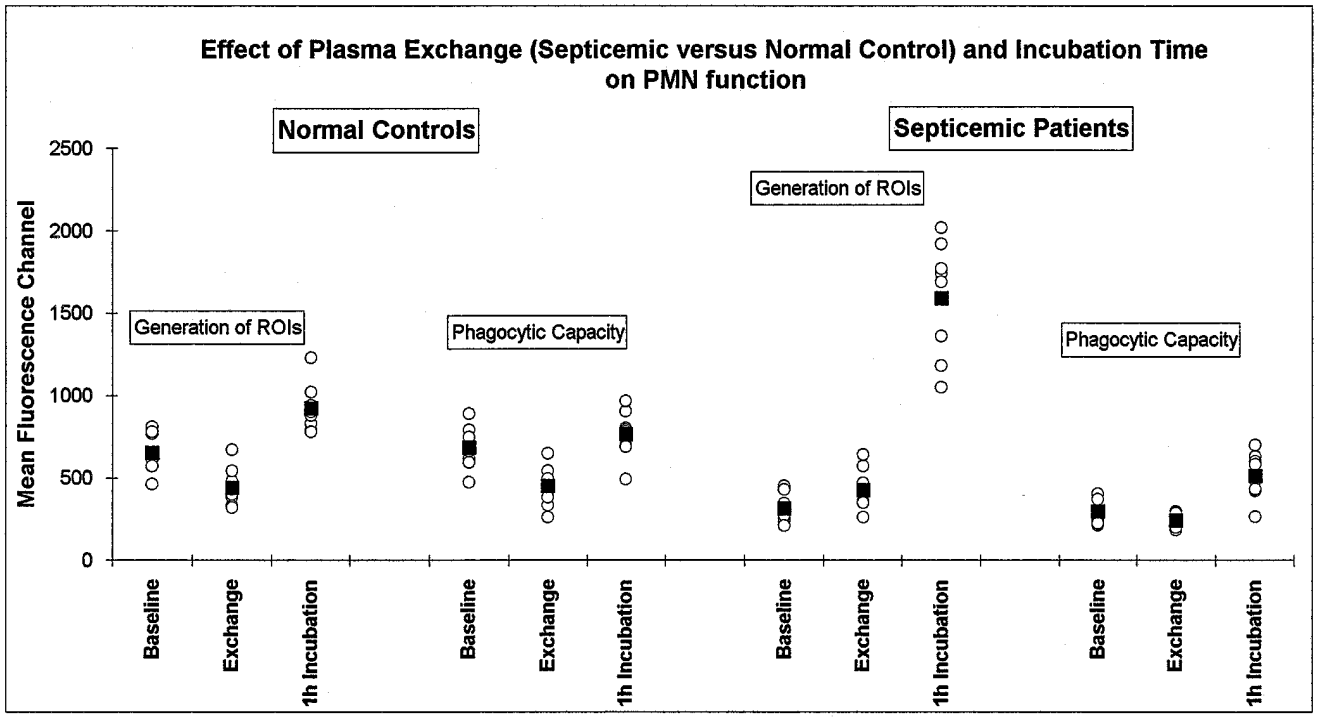

FIG. 3. Baseline denotes no plasma exchange. Exchange denotes plasma exchange, incubating normal plasma with septicemic PMNs and septic plasma with normal PMNs, and immediate analysis. 1 h incubation denotes performance of phagocytosis and ROI assay after 1 h of incubation of mixed samples (normal plasma and septic PMNs or septic plasma and normal PMNs). See the legend to Fig. 2 for explanation of symbols.

of microorganisms or exhibit an extraordinarily weak respiratory burst be found.

The pronounced phagocytic and respiratory responses of patient and control leukocytes observed in our study may also reflect the fact that, in contrast to previous studies, we did not separate blood leukocytes from their normal microenvironment before incubating them with bacteria. It has been demonstrated that isolation from whole blood can alter expression of cell surface antigens and likewise leukocyte functional behavior (42). Even so, because anticoagulation with heparin and temperature alterations were required for the experiments, the conditions were not entirely physiologic. However, it is unlikely that the addition of heparin or the temperature preferentially influences the phagocytic or oxidative radical-producing capacity of septic PMNs as opposed to normal donor leukocytes.

In conclusion, the results demonstrate a dynamic alteration of PMN activity during septicemia and subsequent convalescence. Further studies are needed to identify putative plasma proteins capable of influencing PMN function in septicemia. Altogether, the balance between pro- and anti-inflammatory mediators and substrates is likely to determine PMN function and/or clinical outcome.

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