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Assessment of Neutrophil Function in Patients with Septic Shock: Comparison of Methods

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Patients with septic shock are shown to have decreased neutrophil phagocytic function by multiple assays, and their assessment by whole-blood assays (fluorescence-activated cell sorter analysis) correlates with assays requiring isolated neutrophils (microscopic and spectrophotometric assays). For patients with similar underlying conditions but without septic shock, this correlation does not occur.

Various neutrophil functions have been described as being reduced in sepsis, including adherence (21), chemotaxis (2), degranulation (20), phagocytosis (17), and production of reactive oxygen intermediates (ROI) (19, 24, 25). However, other studies have reported enhanced neutrophil chemotaxis and respiratory burst activities, particularly in patients without underlying conditions (8, 10, 12).

Neutrophils are considered fragile cells that are easily damaged by improper handling. Some tests of neutrophil function require isolation procedures to distinguish the effects of neutrophils on test results from those of other leukocyte types. These isolation procedures may be harmful to the cells or preactivate them.

In this study, we compared whole-blood flow cytometry assays (22) with fluorescence microscopy (13) and a cytochrome *c* reduction (5, 14) assay using Ficoll-Paque-separated neutrophils (11) for the assessment of neutrophil function in patients with septic shock and control subjects. In addition, killing capacity, levels of intracellular Ca^{2+}, chemokinesis, and chemotaxis of neutrophils were evaluated.

The cases of 13 patients with septic shock (5 with APACHE II scores between 25 and 34) and of 13 subjects ranging in age from 26 to 84 years (mean ± standard deviation of 55 ± 18 years) with the same underlying conditions (coronary heart disease [n = 1], bladder carcinoma [n = 1], diabetes mellitus [n = 1], intravenous drug abuse [n = 2], chronic renal failure [n = 1], pancreatitis [n = 1], antiphospholipid antibody syndrome [n = 1], liver cirrhosis [n = 2], renal calculi [n = 1], abdominal trauma [n = 1], or no underlying condition [n = 1]) were investigated. No human immunodeficiency virus-positive patients were studied. Blood sampling was performed prior to antimicrobial, adrenergic, or steroid therapy. On routine differential counts, the septic neutrophils were all positive for toxic granulations.

All assays were performed blinded and were interpreted by S. Patruta and K. Stich. There was a >90% agreement between their readings.

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Neutrophils were isolated from venous blood as described by Nauseef et al. (15) and Metcalf et al. (13). Phagocytosis and intracellular killing of opsonized *Escherichia coli* organisms were performed as described by Moiola (14), using *E. coli* strain ATCC 25922. Data are expressed as the percentage of bacteria phagocytized and killed by neutrophils. ROI production by neutrophils was determined by measuring superoxide dismutase-inhibitable reduction of cytochrome *c* according to the method of Nauseef et al. (15). Data are expressed as nanomoles of O^{2-} produced by 2×10^5 cells. The calculation was made using the molar extinction coefficient of 29.9×10^3 mol/liter. The levels of intracellular Ca^{2+} in neutrophils were measured with Fura-2 AM, using fluorometer, model LS 5B (Perkin-Elmer, Norwalk, Conn.) according to the method of Alexiewicz et al. (1). Data are expressed as nanomoles per liter. Chemotaxis and chemokinesis levels were assessed using the under-agarose method (6). Levels of phagocytosis and ROI production by neutrophils were determined by flow cytometry according to the method of Wenisch and Graninger (22). All tests were performed in duplicate.

Differences between groups were calculated using the Student t test. Pearson's correlation coefficient was used. All the analyses were two-sided, and differences with a P value of less than 0.01 were considered significant.

In patients with septicemia, the percentage of phagocytized bacteria, the number of *E. coli* organisms per neutrophil, and the percentage of killed bacteria were reduced (Table 1). In these patients, we found significant correlations between the level of phagocytosis, measured by fluorescence-activated cell sorting (FACS), and the percentage of phagocytized bacteria, measured by microscopic examination, (r = 0.784), and between the level of phagocytosis and the number of *E. coli* isolates per neutrophil (r = 0.748). The number of phagocytized bacteria that were killed was related to the level of stimulated ROI production, measured by the cytochrome *c* reduction assay (r = 0.735). No correlation between the FACS analysis results and the microscopic evaluations was seen for control subjects.

In septicemia, basal ROI production was increased, but the level of stimulated ROI production and the percentage of increase upon stimulation were decreased (Table 1). In septicemic patients, a correlation was seen between the basal ROI

TABLE 1. Neutrophil phagocytosis, killing, and ROI production in controls and patients with septicemia with the same underlying conditions

Test and parameter	Value (mean ± SD) for: Control patients	Sepsis patients	P value
Microscopic phagocytosis assays			
% Phagocytosis	89 ± 5	39.8 ± 14	<0.001
Number of *E. coli* per neutrophil	6.4 ± 1.6	2.9 ± 0.7	<0.001
% of killed *E. coli*	61.1 ± 3.4	39 ± 10.5	<0.001
FACS analysis (FITC[a]-*E. coli*), fluorescence channel	441 ± 93	212 ± 61	0.002
Cytochrome *c* reduction assays			
Basal nmol of $O^{2-}/10^6$ neutrophils	1.7 ± 1.3	3.6 ± 1.9	0.01
Stimulated nmol of $O^{2-}/10^6$ neutrophils	57.0 ± 13	29.3 ± 14	<0.001
FACS analysis (rhodamine fluorescence), fluorescence channel	78 ± 21	26 ± 12	<0.001

[a] FITC, fluorescein isothiocyanate.

production measured by the cytochrome *c* reduction assay and the FACS assay results ($r = 0.701$). The basal ROI production (cytochrome *c* assay) was related to chemotaxis ($r = 0.734$). Again, no relation between the FACS assay results and the cytochrome *c* reduction assay results was seen for controls.

The basal intracellular calcium levels were not different between sepsis patients and control subjects (26 ± 6 and 30 ± 7 nmol/liter, respectively). In addition, the levels of intracellular calcium after stimulation did not differ between the groups (331 ± 98 nmol/liter in controls versus 279 ± 56 nmol/liter in patients with septicemia. In control subjects, a negative relation between the basal and stimulated intracellular Ca^{2+} levels and the number of phagocytized *E. coli* isolates per neutrophil was observed ($r = 0.701$). In contrast, no correlations between intracellular Ca^{2+} and phagocytosis levels were seen in patients with septicemia.

Neutrophil chemokinesis was impaired in patients with septicemia (0.48 ± 0.1 mm in controls versus 0.38 ± 0.1 mm in sepsis patients [$P = 0.006$]). Similarly, neutrophil chemotaxis was decreased (4.8 ± 0.7 mm in controls versus 3.3 ± 1.1 mm in sepsis patients [$P < 0.001$]). No relation between chemotaxis and chemokinesis and other indices of neutrophil function was seen.

The present study confirms previous reports of decreased levels of chemotaxis (2), phagocytosis (20), ROI production (19, 22, 24, 25), and killing (16, 17) in neutrophils from septicemic subjects. For patients with septicemia, methods using whole blood and assays with isolated cells yielded similar results for neutrophil phagocytosis and ROI production. However, no correlation between these assays was seen for controls. This is of particular interest since isolation procedures have been shown to upregulate expression of plasma membrane receptors such as CD18/CD11b, CD32, and CD16 (7, 9). In septicemia, such an upregulation might already have occurred in vivo. Both separation of neutrophils from whole blood and temperature were shown to be important in the expression of C3 receptors (3). A spontaneous increase occurred with centrifugation, resuspension, and higher temperatures. In general, assays using purified neutrophils can be affected by multiple factors, including isolation procedures, erythrocyte contamination, temperature, anticoagulants, delay between blood sampling and analysis, and neutrophil count (4, 18, 23). These factors could provide additional explanations for the missing correlation between flow cytometry and microscopy and cytochrome *c* reduction assays for controls.

A prompt increase of intracellular Ca^{2+} in activated neutrophils is related to the level of neutrophil phagocytosis. In severe sepsis, no such relation has been seen, which could be explained by factors extrinsic (electrolytes and cytokines, etc.) and intrinsic (auto-oxidation, etc.) to the neutrophils (22).

Altogether, this study suggests that both methods (i.e., using isolated neutrophils and using whole-blood-derived neutrophils) should be applied for an accurate interpretation of neutrophil function, particularly for patients with nonsevere depressed function.

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