

Innovative Technologies in the Evaluation of the Neutrophil Functional Activity in Sepsis

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The study purpose was to evaluate the possibility of using indicators of the functional activity of neutrophils in automated hematological analysis in critically ill patients for the diagnosis of septic complications.

Methods. Twenty patients were examined (14/6 men/women, 56 ± 3.8 years). Patients with documented sepsis were enrolled in the main group ($n = 10$). The comparison group consisted of patients without sepsis ($n = 10$). As control values, the results of the examination as part of medical checkup of 20 conditionally healthy people aged 45 to 60 years were used.

Samples of peripheral venous blood were examined on a hematological analyser Sysmex XN-1000 (Sysmex Co., Japan). The main parameters including leukocyte count, absolute and relative number of neutrophils, immature granulocytes (IG), and also advanced parameters of inflammation (NEUT-GI - neutrophil granularity intensity, NEUT-RI - neutrophil reactivity) were assessed. Concentrations of C-reactive protein (CRP) and procalcitonin (PCT) were determined using the Cobas 6000 (Roche, Switzerland).

Statistical processing of the results was carried out using the IBM SPSS Statistics 20 software.

Results. Analysis of the results of the expanded parameters of inflammation showed that NEUT-RI in the patients of the main group significantly exceeded both the control values and those observed in the comparison group (by 44.2%, $p < 0.001$ and 31.2%, $p < 0.001$, respectively). Subsequent correlation analysis revealed a strong direct correlation between the levels of NEUT-RI and generally recognized biomarkers of sepsis including PCT, CRP (Spearman's rank correlation coefficients were, respectively, $\rho = 0.683$, $p = 0.001$ and $\rho = 0.714$, $p < 0.001$) in patients in critical condition, which indicates the potential significance of NEUT-RI as a marker of sepsis.

Conclusion. Thus, significant differences in neutrophil fluorescence intensity (NEUT-RI) in critically ill patients with sepsis and without sepsis have been established, which allows considering this parameter as a promising diagnostic marker of sepsis. Simplicity, accessibility and speed of obtaining the result in a standard blood test open up opportunities for its widespread clinical use.

Key Words

Sepsis, the Functional Activity of Neutrophils, Neutrophil Granularity Intensity (NEUT-GI), Neutrophil Reactivity (NEUT-RI), C-Reactive Protein, Procalcitonin.

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This article presents a possibility to use the innovative technology of automated hematological analysis in assessing the neutrophil functional activity. Simplicity, accessibility and speed of obtaining the result in a standard blood test open up opportunities for widespread clinical use of advanced inflammatory parameters in the diagnosis of sepsis.

INTRODUCTION

Sepsis is one of the key problems of modern medicine due to its widespread prevalence in the world and high mortality^{1,2}. Thus, according to M. M. Levy et al. (2012),

hospital mortality of patients with sepsis in North America and Europe ranges from 28.3 to 41.1%³. At the same time, every hour of delay of the therapy start results in an increase in sepsis-related mortality by about 8%⁴, and in many patients sepsis may even be undiagnosed⁵.

According to the Sepsis-3 definition of 2016, sepsis is a life-threatening organ dysfunction caused by a dysregulated response of the body to infection⁶. Neutrophils play the key role in protecting against microbial infections, which is due to the presence of a large number of proteolytic enzymes and rapid production of reactive oxygen species to reduce pathogens. If these lytic factors or pro-inflammatory

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NEUT-GI – neutrophil granularity intensity, NEUT-RI – neutrophil reactivity intensity, these items are available only in European market.

cytokines are released extracellularly from the neutrophil-infiltrated tissue, this results in the local lesion. In sepsis, a local infection is accompanied by systemic activation of neutrophils.

Physiology and pathophysiology

Neutrophils develop in the bone marrow for 14 days: after a 7-day mitotic stage (myeloblast → promyelocyte → myelocyte), a 7-day maturation stage (myelocyte → metamyelocyte → stab neutrophil → mature segmented neutrophil) follows. Mature neutrophils are retained in the storage pools before they enter the bloodstream, where they transfer from the pool of circulating cells to the pool of leukocytes accumulation within 12-14 hours (contact with the walls of the blood vessels – marginal pool).

Further, if there are no bacterial infections, neutrophils enter the reticuloendothelial organs, for example, liver, or return to the bone marrow to undergo apoptosis followed by phagocytosis with local macrophages, thereby preventing tissue damage by lytic factors released from aging cells.

Elimination of neutrophils through apoptosis is a homeostatic mechanism that prevents damage to healthy tissues. This process is key to the prevention and resolution of inflammation. Apoptosis of neutrophils is suppressed in patients with systemic inflammation, systemic infections, severe sepsis and in those at risk of multiple organ dysfunction, which is due to the activity of circulating factors (lipopolysaccharide, lipoteichoic acid and pro-inflammatory cytokines)⁷⁾. The binding of neutrophils to the endothelium, activated by pro-inflammatory cytokines, increases the lifespan of neutrophils as compared to the unstimulated endothelium and accelerates cell death.

In sepsis, the survival of neutrophils in the tissue can be further increased by the action of local anti-apoptotic factors. Inhibition of apoptosis occurs through the deregulation of a complex intracellular signaling network and functions of organelles. The long lifespan of neutrophils in patients with sepsis contrasts with increased lymphocyte apoptosis in the lymphoid tissue and subsequent immunoparesis⁸⁾.

Thus, high blood neutrophil concentrations can be associated with suppression of apoptosis, the return of marginal cells to the pool of circulating cells and excessive bone marrow production. The release of neutrophils from the bone marrow is controlled by several factors^{9,10,11)}. The most important are granulocyte colony-stimulating factor (GCF) and colony-stimulating granulocyte-macrophage factor (CSMF), which increase the number of circulating neutrophils, stimulate their maturation and activation, and increase the lifespan of neutrophils. In healthy people, the concentration of GCF in the blood is very low, whereas in the acute phase of infection its several-fold increase is observed with the subsequent increase in the number of neutrophils.

At the same time, there are data showing that a large pathogenic role in severe sepsis is not due to the total number of neutrophils in the bloodstream but the presence

of a cellular subpopulation whose phenotype and activation level stimulate tissue damage¹²⁾.

Neutrophils exist in three states: resting state (unstimulated), excited (a collision with an inflammatory agonist or a substance that attenuates the threshold stimuli necessary for activation) and activated (passage of a specific function). Transition of neutrophils from the resting state in the circulation to the activated state in the infection site is performed by a predetermined sequence of signals from motivating stimuli (for example, complementary peptide C5a, leukotriene B, platelet activating factor, bacterial peptide formyl-methionyl-leuco-phenylalanine and interleukin-8). Circulating factors (interleukin-8, TNF, etc.) may also be responsible for the functional status of blood neutrophils in sepsis. By increasing the lifespan of neutrophils and suppressing their migration through the vasculature, circulating factors increase neutrophil-endothelial cell interactions and increase vascular damage. That is, the dysfunction of neutrophils in severe sepsis is not the primary mechanism but is a consequence of systemic activation.

In turn, neutrophils are an important source of pro-inflammatory cytokines. Secretion of cytokines by neutrophils bound to the walls of blood vessels can alter the non-thrombogenic properties of the endothelium to a procoagulant state with activation of disseminated intravascular coagulation, and also stimulate production of nitric oxide in endothelial and smooth muscle cells. In addition to the development of septic shock hypotension, the release of nitric oxide can disrupt the metabolism in tissues through the inhibition of mitochondrial enzymes¹³⁾. Stimulated polymorphonuclear leukocytes of circulating blood bind to adhesion molecules on the surface of endothelial cells in organs, removed from the initially damaged tissue. In the binding sites, they secrete proteolytic enzymes and oxygen metabolites, which leads to endothelium damage and endothelial barrier disruption, increased capillary permeability, and infiltration of plasma and inflammatory mediators of the parenchyma of organs.

Thus, the function of neutrophils is to eliminate foreign bacteria, but under certain circumstances their activity can lead to organ failure^{14,15)}. On the other hand, persistent inflammation can lead to a decrease in the sensitivity of neutrophils to the components of complement, which, in turn, can promote the spread of infection¹⁶⁾.

These facts show the importance of timely evaluation of the functional activity of neutrophils, but the direct methods available to date for studying the function of these cells are time- and labour-consuming and are subject to bias. To date, a new series of hematological analyzers has been developed that allows not only to count and differentiate different populations of leukocytes quickly, but also to quantify their degree of maturity and activity according to the intensity of the fluorescent signal and the degree of light scattering. The first publications have appeared showing the usefulness of such an assessment for sepsis^{17,18,19,20)}. However, the clinical and diagnostic significance of hematological parameters of neutrophil activation has not been fully determined.

THE STUDY OBJECTIVE

The study objective was to evaluate the possibility of using indicators of the functional activity of neutrophils in automated hematological analysis (NEUT-RI – intensity of reactivity, and NEUT-GI – intensity of granularity) in critically ill patients for the diagnosis of septic complications.

MATERIALS AND METHODS

Twenty patients were examined (men – 14 (70.6%), women – 6 (29.4%), average age 56 ± 3.8 years) from the intensive care unit of the Regional Clinical Center of Miners' Health Protection, Leninsk-Kuznetsky.

Patients were divided into groups according to the presence of signs of sepsis, which were detected according to the criteria of Sepsis-1²¹⁾ and Sepsis-3⁶⁾. Patients with documented sepsis were enrolled in the main group (n = 10). The comparison group consisted of patients without sepsis (n = 10). As control values, the results of the examination as part of medical checkup of 20 conditionally healthy people aged 45 to 60 years were used.

The study program was implemented with the use of laboratory methods of investigation on days 1 – 3 after admission of patients to the intensive care unit.

Samples of peripheral venous blood collected in tubes with an anticoagulant K₃EDTA (Becton Dickinson) were examined on a hematological analyzer Sysmex XN-1000 (Sysmex Co., Japan) for 2 hours after collection of samples. The main parameters including leukocyte count, absolute and relative numbers of neutrophils, immature granulocytes (IG), and also advanced parameters of inflammation (NEUT-GI – neutrophil granularity intensity, NEUT-RI – neutrophil reactivity) were assessed.

Concentrations of C-reactive protein (CRP) by immunoturbidimetric method and procalcitonin (PCT) by immunochemical method using the Cobas 6000 (Roche, Switzerland) were determined simultaneously in the serum samples obtained.

Statistical processing of the results was carried out using the IBM SPSS Statistics 20 software. Distribution of the values was estimated using visual evaluation of the frequency histograms. Since most of the data had a distribution different from normal, the results are presented as (Me) (LQ-UQ), where Me is the median, (LQ-UQ) is the interquartile range (LQ is 25%, UQ is 75%).

Differences between groups were detected using nonparametric Mann – Whitney criteria (for two groups) or Kruskal-Wallis (for three groups, followed by the procedure of multiple T3 Dunnet comparisons). Differences were considered statistically significant at $p < 0.05$. The correlation between the indicators was described using the Spearman rank correlation coefficient (ρ).

RESULTS AND DISCUSSION

In the course of the study, there were found regular

differences between groups of patients in the critical state according to the levels of PCT and CRP: in patients of the main group, the serum biomarker concentrations in 6.5 ($p = 0.001$) and 2.7 ($p < 0.001$) times, respectively, exceeded those in the comparison group, which confirmed the established diagnosis of sepsis (**Table 1**). At the same time, these groups did not differ in the total counts of leukocytes, neutrophils and immature granulocytes. On the average, the development of the inflammatory reaction in the patients of the intensive care unit was characterized by leukocytosis with absolute and relative neutrophilia, with the presence of immature forms in the blood.

Analysis of the results of the extended parameters of inflammation showed that the intensity of neutrophil reactivity (NEUT-RI) in the patients of the main group significantly exceeded both the control values and those observed in the comparison group (by 44.2%, $p < 0.001$ and 31.2%, $p < 0.001$, respectively). There were no statistically significant differences between the groups by neutrophil granularity intensity (NEUT-GI).

In the study of R. J. Dinsdale et al. similar results were obtained in patients with burns: a significant increase in NEUT-RI in the development of septic complications and a low ability to distinguish between septic and nonseptic patients using NEUT-GI¹⁷⁾.

Subsequent correlation analysis revealed a strong direct correlation between the levels of NEUT-RI and generally recognized biomarkers of sepsis PCT, CRP (Spearman's rank correlation coefficients were, respectively, $p = 0.683$, $p = 0.001$ and $p = 0.714$, $p < 0.001$) in patients in critical condition, which indicates the potential significance of NEUT-RI as a marker of sepsis.

Measurement of the functional activity of cells on the Sysmex XN hematological analyzer is based on the change in the intensity of the fluorescence signals (SFL) depending on the number of nucleic acids in the cell and the lateral light scattering (SSC) depending on the complexity of the cell structures (the shape of the nucleus, the shape and size of the granules, vacuoles). Thus, an increase in the activity of neutrophils is accompanied by an increase in the metabolism with an increase in the number of nucleic acids that are more intensely stained with a fluorescent dye, which consequently results in an increase in the fluorescent signal and the magnitude of NEUT-RI. In addition, activated neutrophils are characterized by an increase in the number of secretory granules and vacuoles, leading to an increase in SSC. On the other hand, in inflammatory processes, there is an accelerated replenishment of the pool of circulating neutrophils with an increase in the number of immature and stab-shaped forms, which are characterized by weak light scattering. Apparently, therefore, the presented study did not reveal any significant differences between the groups according to the NEUT-GI index. Obviously, this indicator needs to be interpreted individually in each specific case, taking into account the dynamics and morphological features of neutrophils established during microscopic examination of the blood smear.

Table 1 Laboratory inflammatory values in critically ill patients

	Main Group – sepsis patients (n = 10)	Comparison Group – non-sepsis patients (n = 10)	Control Group – healthy (n = 20)
WBC, cells - 10 ⁹ /L	10.9 (7.57–12.70) P ₁ = 0.045 P ₂ = 0.49	14.4 (12.46–16.88) P ₁ < 0.001	6.7 (5.92–7.20)
Neutrophils, cells - 10 ⁹ /L	9.82 (6.63–10.95) P ₁ = 0.008 P ₂ = 0.77	11.4 (9.55–14.38) P ₁ < 0.001	4.0 (3.20–4.20)
Neutrophils, %	88 (86.0–90.2) P ₁ < 0.001 P ₂ = 0.07	83 (78.3–87.7) P ₁ < 0.001	57.9 (52.89–61.65)
IG, cells - 10 ⁹ /L	0.15 (0.092–0.242) P ₁ = 0.16 P ₂ = 0.77	0.12 (0.080–0.187) P ₁ = 0.014	0
IG, %	1.5 (0.87–2.40) P ₁ = 0.007 P ₂ = 0.35	0.8 (0.57–1.15) P ₁ = 0.011	0
NEUT-RI, FI	70 (66.2–75.0) P ₁ < 0.001 P ₂ < 0.001	53 (51.8–55.0) P ₁ < 0.001	49 (46.5–50.1)
NEUT-GI, SI	155 (149.2–158.4) P ₁ = 0.27 P ₂ = 0.94	155 (151.2–160.2) P ₁ = 0.055	152 (149.1–153.9)
PCT, ng/mL	4.2 (1.82–19.34) P ₂ = 0.001	0.6 (0.32–0.79)	nd
CRP, mg/L	274 (213.2–370.3) P ₂ < 0.001	103 (96.5–111.1)	nd

P₁ – significance of differences with the control group

P₂ – significance of differences between the main group and the comparison group

IG – immature granulocytes

NEUT-RI – neutrophil reactivity intensity

NEUT-GI – neutrophil granularity intensity

FI – fluorescence intensity

SI – scattering intensity

PCT – procalcitonin

CRP – C-reactive protein

nd – not determined

Clinical case

Patient B., 61 y. o., was admitted to the surgical department No. 1 of the Regional Clinical Center of Miners' Health Protection, Leninsk-Kuznetsky with a clinical pattern of acute purulent pelviorectal paraproctitis. On admission, laboratory leukocytosis was noted with relative and absolute neutrophilia (89% and $14.92 \times 10^9/L$, respectively), hyperbilirubinemia (total bilirubin $42.3 \mu\text{mol/L}$), hyperazotemia (creatinine $160 \mu\text{mol/L}$, urea – 15.2 mmol/L). The following operation was performed: autopsy, revision and drainage of the abscess; antibacterial therapy was prescribed (ciprofloxacin 0.4 g, 3 tpd). Subsequently surgical treatment was performed repeatedly. Despite the ongoing treatment, the patient's condition worsened with reported arterial hypotension, decreased dyspnea, diuresis less than 50 mL/h, gastrointestinal paresis, anemia, and episodic hyperthermia. On day 5, neutrophilia was preserved according to laboratory data with an increase in the number of immature forms and a significant increase in neutrophil reactivity (NEUT-RI) to 76.4 FI.

Based on the clinical picture and the examination findings, the patient was diagnosed with sepsis, and was transferred for further treatment to the intensive care unit. Later, the diagnosis was confirmed by the levels of procalcitonin (2.12 ng/mL) and CRP (199 mg/L). Since metropenem-sensitive culture of *E. coli* was isolated in the bacteriological study of the wound discharge, this antibiotic (1 g, 3 tpd) was prescribed, which was subsequently replaced by a

combination of sulfadiazone (4 g, 2 tpd) + amikacin (1.5 g, 1 tpd).

When intensive treatment was administered in the intensive care unit, a gradual improvement in the patient's condition was noted, and by day 20 the state was closer to the average severity level. Breathing was unassisted and adequate, hemodynamics were stable, diuresis was sufficient, and the phenomena of intestinal paresis was relieved.

The dynamics of the results of laboratory studies corresponded to the positive dynamics of the patient's condition. In particular, on day 20 there was a decrease in the level of PCT (1.43 ng/mL), C-reactive protein (92 mg/L), the number of leukocytes and immature granulocytes. At the same time, changes in the indices of functional activity of neutrophils were ambiguous. Thus, the level of NEUT-RI, analogous to C-RB and PKT, gradually decreased (On day 11 – 73.3 FI, 20 – 57.0 FI). At the same time, NEUT-GI noted a reverse trend: an increase from 159.7 SI (day 5) to 163.9 (11 days) and up to 167.2 SI (20 days), which can be associated with the appearance in the bloodstream of hypersegmented neutrophils possessing a high degree of lateral light scattering. **Fig. 1** and **Fig. 2** show the scattergrams of different leukocyte population distribution dynamics. This assumption was confirmed by microscopy. The presented example demonstrated that the indicator of the functional activity of neutrophils NEUT-RI can be useful in assessing the severity of the course of the purulent-septic process.

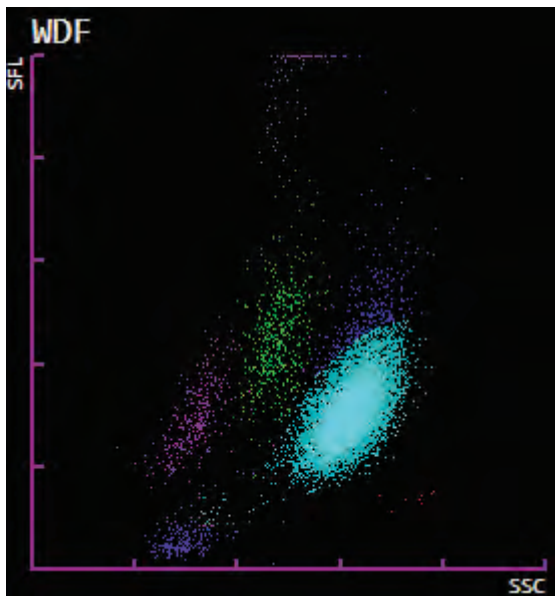


Fig. 1 Leukocyte population distribution scattergram of patient B. Day 3 of the disease.

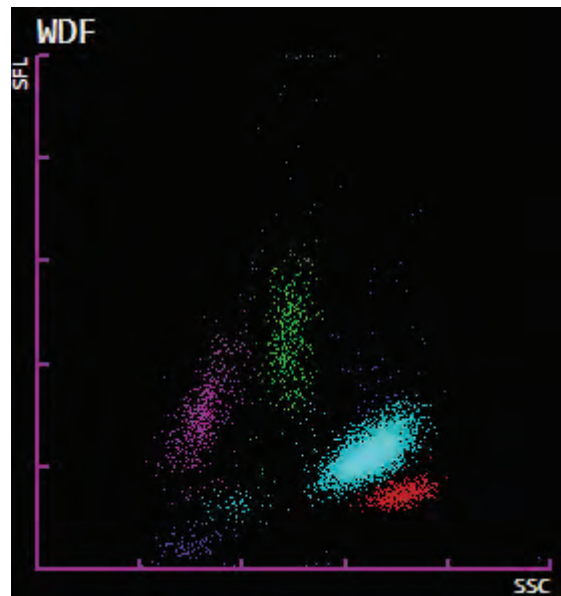


Fig. 2 Leukocyte population distribution scattergram of patient B. Day 13 of the disease.

CONCLUSION

Thus, significant differences in neutrophil fluorescence intensity (NEUT-RI) in critically ill patients with sepsis and without sepsis have been established, which allows the consideration of this parameter as a promising diagnostic marker of sepsis.

Simplicity, accessibility and speed of obtaining the result in a standard blood test open up opportunities for its widespread clinical use.

The limited nature of the study performed warrants further large-scale studies.

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