Detection of Inflammatory Processes during Various Diseases by the Method of Flow Cytofluorometry

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Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 149, No. 4, pp. 471-475, April 2010
Original article submitted September 4, 2008

Oxidative (respiratory) burst is an important manifestation of inflammation. Precise quantitative assessment of this reaction by flow cytometry made it possible to record and evaluate the severity of the inflammatory processes in a wide spectrum of diseases including diphtheria, hepatitis, pneumonia, bronchial asthma, arthritis, vasculitis, postoperative complications, tuberculosis, psoriasis, rheumatoid arthritis, systemic lupus erythematosus, and myocardial infarction. This approach can be employed as a highly sensitive method of detection of inflammatory reactions and monitoring of their course in various pathological processes.

Key Words: *respiratory burst; inflammation; flow cytometry*

The inflammatory reactions accompany various pathological processes. Timely detection of inflammation can prevent severe consequences of various diseases, while quantitative recording of the inflammatory processes can be efficient prognostic tool helping to control the therapeutic course. Inflammation is a complex multicomponent process resulting from many elementary molecular, cellular, tissue, and organism reactions. In this concerted performance, not every particular element can be obligatorily involved in all versatile inflammatory manifestations, but it can take a part in the processes not considered as inflammatory. The methods of detection of oxidative respiratory burst (RB) in neutrophils and monocytes (HCT-test, luminal chemiluminescence) are used for detection of the inflammatory processes in combination with routine techniques that assess the intensity of inflammatory reactions (C-reactive protein, leukocytosis, ESR, *etc*.). RB is an important defensive mechanism of the organism consisting in production of superoxide radicals by specialized protein complexes located on leukocyte membranes. Then, these radicals are converted into other reactive oxygen species (ROS). Presently, the methods using flow cytometry for quantitative assessments of RB are actively developed [1-7, 9-11]. However, in many cases the reaction in neutrophils isolated from the blood with dichlorofluorescein or dihydrorhodamine dyes, which can be oxidized not only by superoxide radical, but also by its derivatives, cannot yield unequivocal results [10,11], because RB greatly differs in various healthy donors.

We previously developed a version of the flow cytometry method for evaluation of RB in neutrophils without their isolation from the blood and with hydroethidine as a dye, which made it possible to study the inflammatory processes involved in various diseases [2]. This method detects RB in the whole peripheral blood. In contrast to other methods used in clinical practice, flow cytometry can measure RB in a large number of individual cells (>10,000) with high efficiency and accuracy. During inflammation of any location and nature, a certain fraction of neutrophils demonstrates changed RB potency. Analysis of cell distribution by this potency can reveal the presence and intensity of inflammatory processes in the organism and trace its development.

The aim of this study was to assess the potency of our original method to evaluate the inflammatory processes of various nature.

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MATERIALS AND METHODS

RB was assessed no later than 8 h after drawing the blood. Storage of blood samples during this period did not affect the results of the tests. The blood samples (1 ml) were placed into tubes containing 40 U heparin. Before incubation, the blood samples were diluted (1:1) with standard phosphate buffer (pH 7.4) and supplemented with hydroethidine (100 μg/ml). RB was provoked by adding 10 ng/ml phorbol myristate acetate (PMA, Sigma). The reaction was carried out for 60 min at 37°C. PMA was not added to control part of the blood specimen. Hydroethidine was synthesized at St. Petersburg Institute of Nuclear Physics by reduction of ethidium bromide with sodium borhydrate according to the previously published method [8].

Distribution of fluorescence intensity was measured immediately after induction of RB using a standard flow cytofluorometer with argon laser as a light source (λ=488 nm). This device was calibrated with the standard fluorescent microspheres from Immuno-Brite Standards Kit (Beckman Coulter). The peak of fluorescence of microspheres from Level IV flask (Med-Hi) corresponded to channel 120. A typical histogram of fluorescent intensity after induction of RB is shown (Fig. 1, *a*). The mean fluorescent intensity of the stimulated cells was used as a measure of RB-producing capacity of neutrophils. Table 1 shows the mean values of RB for neutrophils from healthy donors and patients with various diseases.

The pathologies were diagnosed in the following clinics: St. Petersburg Pediatry Academy (allergy and postoperative complications in the patients with peritonitis and appendicitis), St. Petersburg Research Institute of Children Diseases (diphtheria and its consequences), Russian Cardiology Labor Reserves Sanatorium (cardiopathies), St. Petersburg Medical University (bronchial asthma), and Department of Military Field Therapy of St. Petersburg Military Medical Academy (tumor diseases). These institutions provided us with the corresponding blood specimens.

RESULTS

Hydroethidine enters the plasma of cells unimpededly, where it is oxidized by superoxide radicals during RB. The amount of oxidized dye and consequently, the measured fluorescence intensity in the stained neutrophils is directly proportional to RB potency of these cells.

We previously demonstrated that RB reactions of PMA-stimulated neutrophils are approximately equal in various healthy donors [2]. The difference in RB reaction in these tests did not exceed 8.4%. In contrast, the diseases with pronounced inflammatory processes are associated with reduced RB in response to *in vitro* stimulation of peripheral blood neutrophils. In flow cytometry histogram, it is reflected as a shift of the distribution to the left. The optimal numerical assessment of changes in this distribution needs further study to elaborate the adequate calculation algorithms. Here

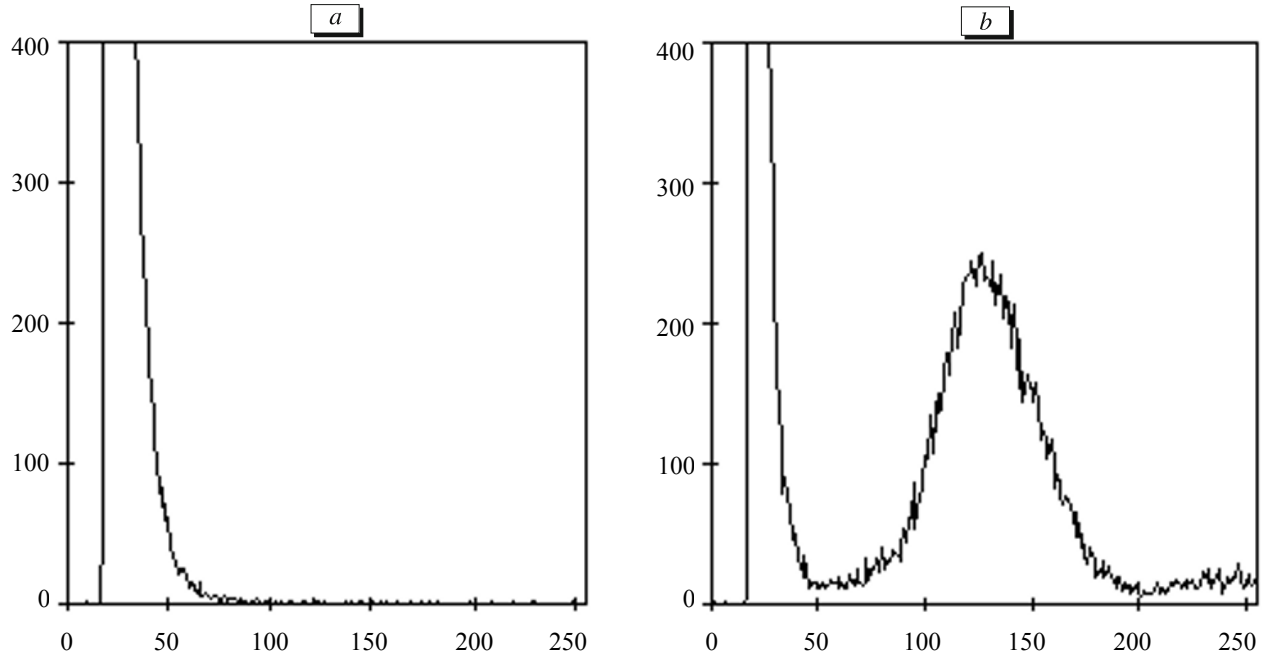

Fig. 1. Typical distribution of non-stimulated (*a*) and PMA-stimulated (*b*) peripheral blood neutrophils of healthy donors by the potency to RB. Here and in Figures 2-3: abscissa, fluorescence intensity, rel. units; ordinate, number of cells.

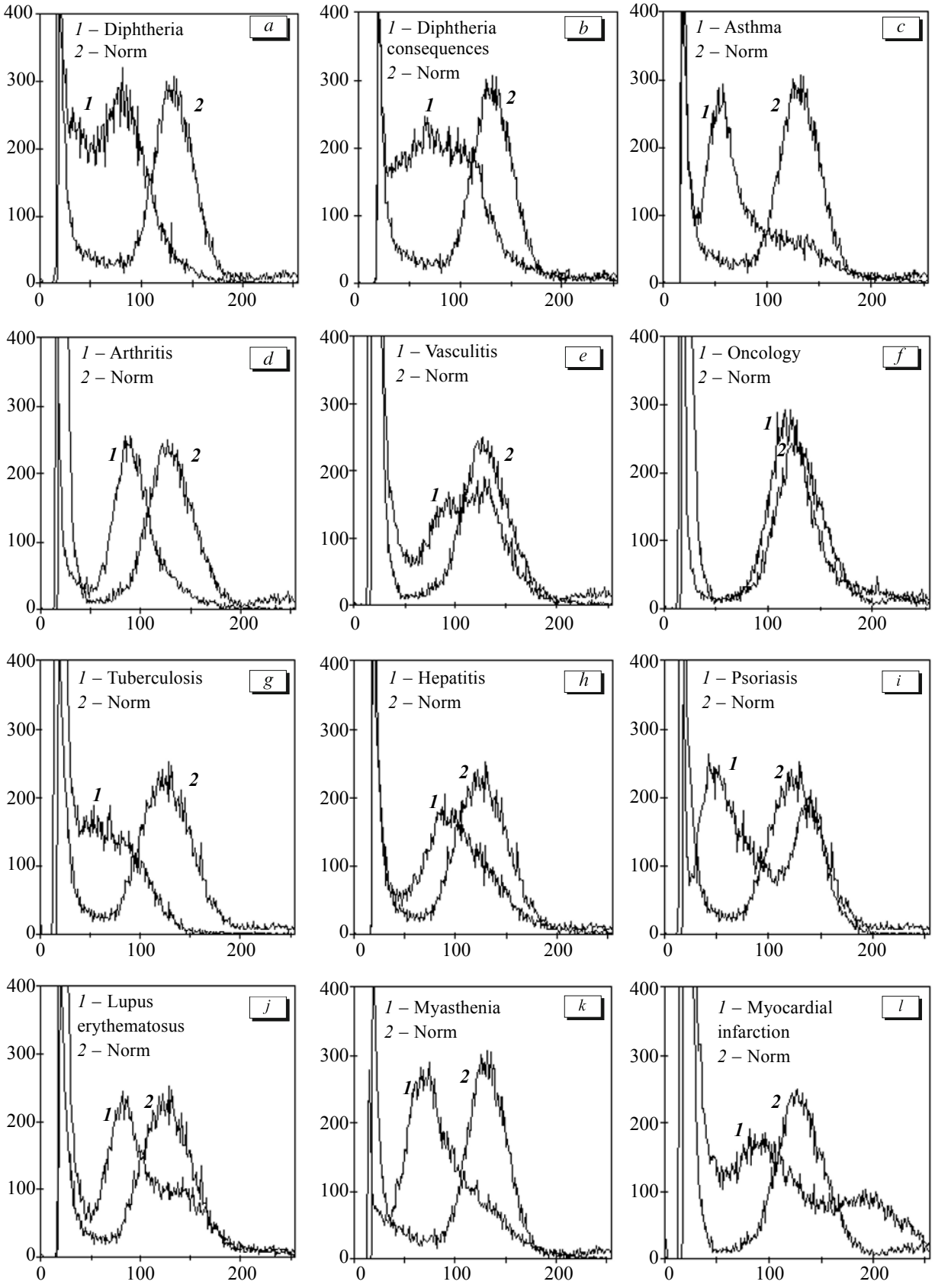

Fig. 2. Distribution of peripheral blood neutrophils by the potency to RB for the healthy donors and the individuals with various diseases.

TABLE 1. Potency of Neutrophils to RB in Patients with Various Diseases (*M*±*m*)

Group of patients	Number of examinees	RB, arb. units
Healthy donors	100	130±11
Asthma	73	84±29
Arthritis	23	74±26
Vasculitis	19	112±15

we used rough assessment of the mean fluorescence intensity distribution. To assess informative value of this approach, it was tested in clinical settings.

One hundred male and female healthy donors of different age (10-40 years) were examined. All the examinees had RB of the same type, which we considered as the norm (Table 1). Typical distributions of cells by fluorescence intensity are shown (Fig. 1, *a*, *b*).

To compare our data with clinical observations, we examined the diseases accompanied by inflammatory reactions of various nature.

Blood specimens from five adults and two children with diphtheria were examined using the above-described method. At the onset of the disease, *in vitro* production of superoxide radicals in response to PMA-stimulation was reduced by 40-80% depending on the severity of the disease course. As the patients recovered, the distribution of neutrophil fluorescence intensity slowly approached the distribution characteristic of healthy donors; the differences did not exceed 10-30% (Fig. 2, *a*). The patients with post-diphtheria complications demonstrated significant deviations of fluorescence intensity distribution from the norm (Fig. 2, *b*). Analysis of the blood drawn from patients with infectious diseases such as tuberculosis and hepatitis B and patients with pneumonia revealed pronounced inflammatory processes accompanying these diseases (Fig. 2, *g, h*). However, PMA-induced RB decreased in all these patients by 50-60%.

We examined 73 patients with bronchial asthma (Table 1). Twenty-seven of these patients were examined repeatedly during remission. In 80% patients, acute attack of the disease was associated with a 30-40% decrease in RB and in 20% patients this decrease attained 50-60% (Fig. 2, *c*). During remission, RB reaction returned to normal in 17 patients, while in other patients it decreased by only 10-20%.

Analysis of blood specimens taken from 24 children with arthritis of various nosological forms showed that in 23 children RB decreased by 40-50%, while in one case the decrease was 80% (Table 1). The typical distribution is shown (Fig. 2, *d*). Analysis of the blood drawn from children with vasculitis (*n*=19, 3 cases repeatedly) revealed weak inflammation in most cases (*n*=15) characterized by inhibition of RB by 10-15% (Fig. 2, *e*). In 4 cases, production of ROS decreased by 40%.

Previous studies showed that PMA-stimulated RB considerably decreased in patients operated for appendicitis and peritonitis during the postoperation period and gradually returned to normal during recovery [2] (Fig. 3). If the course of the postoperation period was

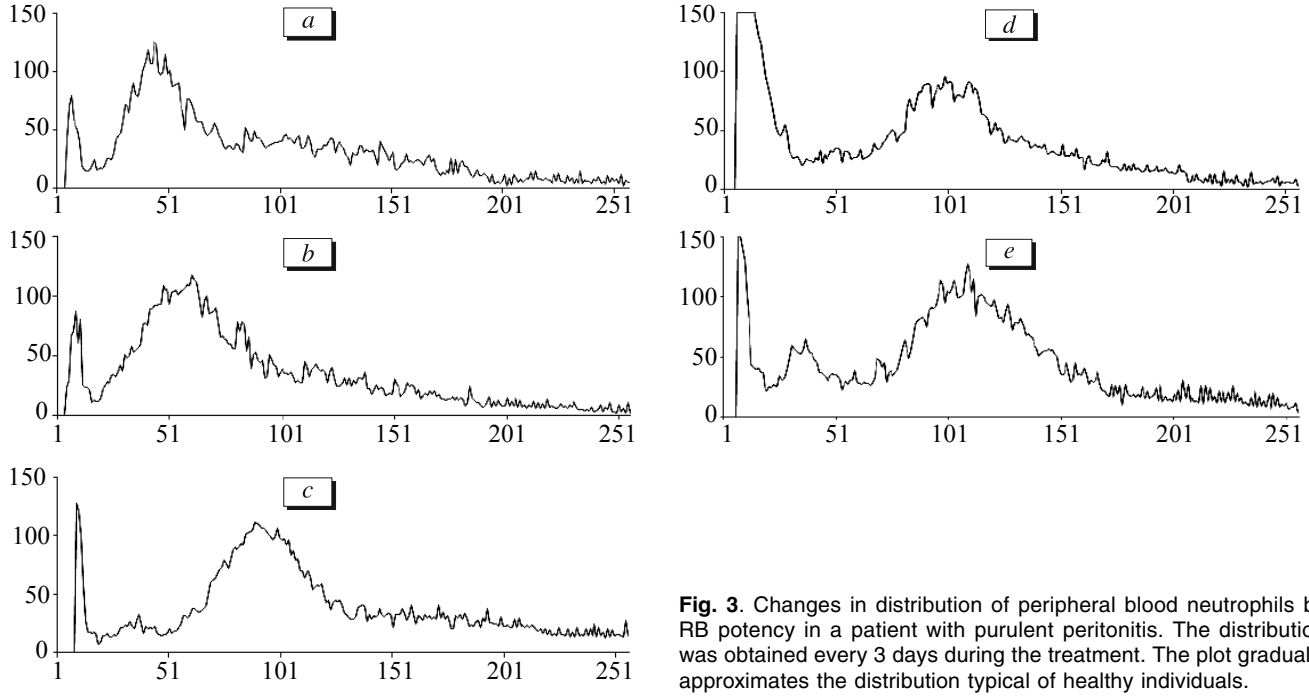

Fig. 3. Changes in distribution of peripheral blood neutrophils by RB potency in a patient with purulent peritonitis. The distribution was obtained every 3 days during the treatment. The plot gradually approximates the distribution typical of healthy individuals.

accompanied by suppurative complications, the reaction did not return to normal.

Paradoxically, analysis of the blood drawn from 25 cancer patients before surgery revealed no significant inflammation even in cases with pronounced necrotization of the tumor: in these blood specimens, RB decreased merely by 5-10% (Fig. 2, *f*).

In patients with autoimmune diseases, production of ROS by neutrophils was markedly suppressed. In patients with rheumatoid arthritis, RB was below the normal by 70%. In patients with psoriasis or systemic lupus erythematosus, neutrophils with normal ROS production and neutrophils with reduced RB response were found (Fig. 2, *i*, *j*). A dramatic drop (≤60%) in RB was also observed in patients with myasthenia (Fig. 2, *k*).

Myocardial infarction was accompanied by an inflammatory process, which was reliably detected by our method. On postinfarction day 3, RB significantly differed from the normal (Fig. 2, *l*). Preliminary data showed that RB decrease correlated with the severity of myocardial infarction.

Thus, the data on inflammatory processes obtained in the analysis of a wide spectrum of diseases showed that our version of flow cytometry developed to assess RB in neutrophils using hydroethidine as a sensitive fluorescence tag agree with the clinical findings. These data can be of diagnostic and prognostic importance.

REFERENCES

1. S. V. Dambaeva, D. V. Mazurov, and B. V. Pinegin, *Immunologiya*, **23**, No. 2, 87-93 (2002).
2. M. Filatov, E. Varfolomeeva, and E. Ivanov, *Biochem. Mol. Med.*, **55**, No. 2, 116-121 (1995).
3. O. C. Illoh, *Arch. Pathol. Lab. Med.*, **128**, No. 1, 23-31 (2004).
4. E. Matteucci and O. Giampietro, *Curr. Med. Chem.*, **15**, No. 6, 596-603 (2008).
5. M. A. Model, M. A. KuKuruga, and R. F. Todd 3rd, *J. Immunol. Methods*, **202**, No. 2, 105-111 (1997).
6. J. A. Smith and M. J. Weidmann, *Ibid.*, **162**, No. 2, 261-268 (1993).
7. S. Szucs, G. Vámosi, R. Póka, *et al.*, *Cytometry*, **33**, No. 1, 19-31 (1998).
8. G. Thomas and B. Rogues, *FEBS Lett.*, **26**, 169-175 (1972).
9. J. Vuorte, S. E. Jansson, and H. Repo, *Scand. J. Immunol.*, **43**, No. 3, 329-334 (1996).
10. S. Walrand, S. Valeix, C. Rodriguez, *et al.*, *Clin. Chim. Acta*, **331**, Nos. 1-2, 103-110 (2003).
11. J. Zhao, B. Juettner, D. Scheinichen, *et al.*, *Acta Anaesthesiol. Scand.*, **47**, No. 6, 702-706 (2003).