Lysosomal-Cationic Test and NBT Reduction Test Just Partially Reflect the Completeness of Phagocytic Process in Human Granulocytes

G. A. Belokrylov and O. Ya. Popova

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The results of NBT test correlated with the index of phagocytosis completeness in only 43% of 21 clinically healthy volunteers. The level of the lysosomal-cationic test was significantly reduced only if the phagocytosis completeness index was markedly decreased. The latter is an integral value reflecting the bactericidal activity of granulocytes.

Key Words: *granulocytes; complete phagocytosis; lysosomal-cationic test; NBT test*

Death and disintegration of bacterial cells in granulocytes (complete phagocytosis) are determined by bactericidal activity of compounds participating in oxygen-dependent [5,10,12] and oxygen-independent [8] processes. Bactericidal agents participating in oxygen-dependent processes (respiratory burst products) are detected in the NBT reduction test [4,14], while compounds responsible for oxygen-independent bactericidal activity (defensins and structurally related peptides [3]) are detected in the lysosomal-cationic test (LCT) [7]. However, the correlation between the results of NBT test and LCT, on the one hand, and completeness of phagocytic process, on the other, has never been evaluated.

We evaluated the correlation between the results of NBT test and LCT and the index of completeness of phagocytosis (PCI) of bacteria by human neutrophilic granulocytes.

MATERIALS AND METHODS

Clinically healthy volunteers (n=21) were examined (5 women aged 22-57 years and 16 men aged 22-60 years).

Institute of Experimental Medicine, Russian Academy of Medical Sciences, St. Petersburg

Blood (5 ml) was collected under sterile conditions from the ulnar vein, stabilized with heparin (Biochemie, 32 U/ml), and centrifuged at 800 rpm for 5 min. The leukocyte layer consisting mainly from neutrophilic granulocytes (80-85%) was carefully harvested with a Pasteur pipette, and cell viability was evaluated with 0.2% trypan blue (Sigma). This parameter was usually 97-98%.

The percentage of granulocytes reducing NBT into diformazane (percentage of diformazan-positive cells) was evaluated by the cytochemical method [4, 14]. NBT reduction was induced by *Bact. prodigiosum* LPS (50 μg/ml), most actively stimulating NBT reduction in diformazan granules [4]. Spontaneous and induced NBT reduction and reserve coefficient (RC), calculated as the ratio of induced to spontaneous reduction, were evaluated. For evaluation of enzymatic activity (NBT test) and phagocytic activity of granulocytes, all ingredients were diluted with apyrogenic saline (Polfa).

The content of defensins and structurally related peptides was measured by staining with fast green in methanol [7]. The results were expressed in arbitrary units [7].

Granulocyte phagocytic activity was evaluated by a modified method [1,9]. The test bacterium was *St. albus* (strain 9198) in a concentration of 2.5×10^7 bacterial cells/ml incubation medium. Staphylococ-

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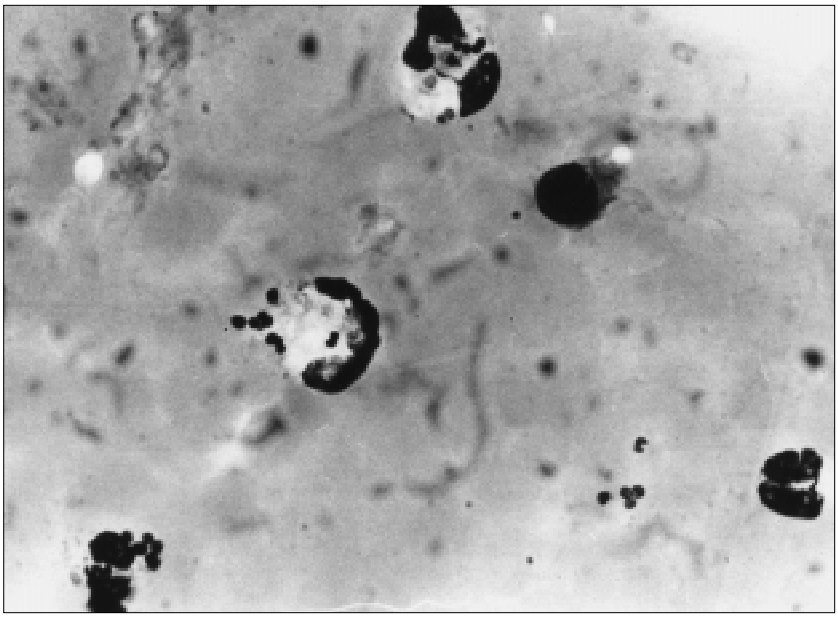

Fig. 1. Impression (stained after Romanowskii—Giemsa) 3 h after leukocyte-bacterial suspension culturing in agar, ×1000. Staphylococcus ghosts in phagocytes (complete phagocytosis) and dark viable bacterial cells.

cus growth was evaluated by 3-h culturing of the leukocyte-bacterium suspension in 1.8% meet-peptone agar and prints were analyzed.

The phagocytic index (percentage of granulocytes participating in phagocytosis), phagocytic number (arithmetic mean of bacteria phagocytosed by one phagocyte), and PCP (the percent ratio of the number of leukocytes with signs of complete phagocytosis to total number of leukocytes with phagocytosed bacteria) were evaluated.

At least 300 cells were counted in each experiment. Experiments were repeated twice.

RESULTS

The correlation between the NBT test values and phagocytosis completeness was observed in only 9 (43%) examinees. The correlation manifested as relatively low (12.2±1.0%) spontaneous reduction of NBT, essential (3.6 times) increase of RC, and high PCP (63.0±1.5%). In 5 (33.3%) subjects high PCP (60.3±1.5%) was observed in the presence of similar values (18.6±1.2%) of spontaneous NBT reduction and notably reduced (to 11.2±1.0%) stimulated value. In 7 (23.7%) subjects PCP was low (19.9±1.3%) in the presence of high spontaneous NBT test (35.5±1.5%) and relatively high RC (1.8). The phagocytic index and phagocytic number had a trend to an increase (Table 1).

The level of LCT did not change in 19 examinees and was 1.6-1.7 arb. units. In only 2 (40%) of 5 subjects with markedly decreased PCP (12.8± 1.2%) LCT also decreased (to 1.20±0.03 arb. units, $p<0.01$) (data not presented).

Analysis of the results of LCT, NBT test, and PCP showed no sex- and age-associated differences.

In the 1970s the level of spontaneous NBT reduction in normal subjects did not surpass 10% [4]. In our study, similarly as in other recent studies [2], the maximum level of spontaneous NBT reduction reached 35%. However, the induced value reached 64.5±1.5% ($p<0.01$). On the other hand, the induced parameter decreased to 11.2±1.0% ($p<0.01$, PK<1) the level of the spontaneous parameter was

TABLE 1. Comparative Characteristics of NBT Test and Phagocytosis Completeness Parameters (*M*±*m*)

Parameter		Group 1 (*n*=9)	Group 2 (*n*=5)	Group 3 (*n*=7)
NBT test	spontaneous, %	12.2±1.0	35.5±1.5	18.6±1.2
	induced, %	44.5±1.6*	64.5±1.5*	11.2±1.0*
RC		3.6	1.8	<1
Phagocytic index, %		48.9±1.6	61.4±1.5	52.9±1.6
Phagocytic number		5.0±0.8	7.5±2.6	4.7±0.8
PCP, %		63.0±1.9	19.9±1.3	60.3±1.5

Note. *$p<0.01$ *vs.* spontaneous level.

relatively low (18.6±1.2%). Hence, the level of LPS-induced NBT reduction was not always determined by its spontaneous value.

Comparison of the results of NBT test and LCT with PCP just partially confirmed their correlation, repeatedly described before [5,8,10,12]. The results of NBT test correlated with PCP in only 43% cases. Bactericidal activity of substances functionally related to the respiratory burst also did not reflect general bactericidal activity of granulocytes in [11,13, 15]. The myeloperoxidase (MPO) system, including MPO, H_2O_2, and cofactors I^-, CL^-, CNS^- [3] and possessing bactericidal activity towards bacteria, fungi, mycoplasma, and viruses [3,12], is not found in intact chicken pseudoeosinophils [11] and neutrophils of patients with chronic granulomatous disease [15]. However, the capacity to bacterial killing and disintegration is completely retained in chickens [11] and is reduced in only one-third of patients with chronic granulomatous disease [15]. Donor granulocytes retain the capacity to bacterial killing and disintegration after inhibition of MPO system by ascorbic acid [13].

LCT was less sensitive: its level in the present study and in our previous investigation [1] decreased only in the presence of pronounced decrease of PCP paralleled by bacterial growth inside and outside phagocytes. A lower sensitivity of LCT in comparison with NBT test is confirmed by published reports [6].

Hence, the best integral indicator of bactericidal activity is PCP reflecting cell death and disintegration inside phagocytes (Fig. 1). The use of *St. albus* strain 9198 as the test microbe (stained after Romanowskii-Giemsa, this bacterium after death changes its dark violet color to pink) helps to accurately evaluate the degree of phagocytosis completeness.

We should like to draw special attention of clinical immunologists to interpretation of the results of LCT and NBT test as PCP, because these tests only partially reflect bactericidal activity of human granulocytes.

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REFERENCES

1. G. A. Belokrylov, O. Ya. Popova, and O. N. Derevnina, *Byull. Eksp. Biol. Med.*, **126**, No. 10, 430-432 (1998).
2. E. N. Kirillova, K. N. Muksinova, E. D. Drugova, *et al., Immunologiya*, No. 1, 30-35 (2001).
3. V. N. Kokryakov, *Biology of Antibiotics of Animal Origin* [in Russian], St. Petersburg (1999).
4. A. N. Mayanskii, M. E. Viksman, P. N. Kotel'nikov, and I. V. Molchanova, *Zh. Mikrobiol.*, No. 6, 108-111 (1977).
5. D. Novak, M. Prozinski, T. Pietras, *et al., Ter. Arkh.*, **73**, No. 3, 40-44 (2001).
6. V. S. Novikov, V. V. Goranchuk, and E. B. Shustov, *Physiology of Critical States* [in Russian], St. Petersburg (1998).
7. V. E. Pigarevskii and Yu. A. Mazing, *Lab. Delo*, No. 10, 579-582 (1981).
8. V. E. Pigarevskii, *Clinical Morphology of Neutrophilic Granulocytes* [in Russian], Leningrad (1988), pp. 3-11.
9. V. M. Shubik and M. Ya. Levin, *Immunological Reactivity of Young Athletes* [in Russian], Moscow (1982).
10. B. M. Babior, *N. Engl. J. Med.*, **298**, 659-667 (1978).
11. K. Brune, M. S. Leffel, and J. K. Spitznagel, *Infect. Immunol.*, **5**, 283-287 (1972).
12. S. J. Klebanoff, *Inflammation. Basic Principles and Clinical Correlates*, New York (1992), pp. 541-588.
13. C. B. McColl, R. DeChatelet, M. R. Cooper, and P. Ashburn, *J. Infect. Dis.*, **124**, 194-198 (1971).
14. B. N. Park, S. M. Pikrig, and E. M. Smithwik, *Lancet*, **11**, 532-534 (1968).
15. E. Undriz, *Blut*, **14**, 129-136 (1968).