3. I. A. Eryukhin, V. Ya. Belyi, and V. K. Vagner, *Inflammation as a General Biological Reaction in a Model of Experimental Acute Peritonitis* [in Russian], Leningrad (1989).
4. G. S. Ivanova, V. Ya. Glumov, A. K. Kranchev, *et al.*, *Arkh. Patol.*, № 7, 51-57 (1988).
5. A. G. Kriger and A. A. Lindenberg, *Vestn. Khir.*, № 3, 130-133 (1985).
6. B. L. Lur'e, A. I. Lobakov, and I. M. Kaliman, *Lab. Delo*, № 2, 95-98 (1986).
7. V. E. Pigarevskii and Yu. A. Mazing, *ibid.*, № 10, 579-587 (1981).
8. V. A. Popov, *Peritonitis* [in Russian], Leningrad (1985).
9. S. S. Remennik, *Zdravookhr. Turkmenii*, № 7, 21-25 (1965).
10. S. A. Shishkin, *Laboratory Methods for Clinical Assessment of Endogenous Intoxication* [in Russian], Izhevsk (1992).

Oxidative Metabolism of Neutrophilic Polymorphonuclear Leukocytes in Experimental Massive Pulmonary Embolism

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UDC 616.127-091+611.018.74.001

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 118, № 12, pp. 640-644, December, 1994
Original article submitted April 5, 1994

Oxidative metabolism of neutrophilic polymorphonuclear leukocytes circulating in the blood was studied by chemiluminescent analysis and the nitroblue tetrazolium reduction test. Pronounced activation of oxidative metabolism of polymorphonuclear leukocytes was observed by the third and sixth hours of massive embolism of the pulmonary arteries. Comparison of the examined parameters of oxidative metabolism of leukocytes isolated simultaneously from venous and arterial blood indicates a delay of the most active cell fraction in pulmonary vessels during massive embolism. The transfer of functionally active leukocytes from the circulating to the marginal pool of the lungs suggests their participation in damaging the pulmonary microvascular endothelium and in increasing its permeability.

Key Words: *massive pulmonary embolism; neutrophilic polymorphonuclear leukocytes; lung vessels; endothelium*

Damage to the endothelial lining of microvessels is thought to be the principal factor in the development of the respiratory distress syndrome in adults with various diseases [17]. Numerous reports attest that endothelial damage is associated with the activation of oxidative metabolism in neutrophilic polymorphonuclear leukocytes (NPMNL) and the production of reactive oxygen species (ROS) by them [11,13,14,21]. We have shown that acute massive embolism of pulmonary arteries (MEPA) is paralleled by neutrophil accumulation in the lumen of alveolar capillaries, by numerous local impairments of the endothelium, and by manifest interstitial edema [2]. The aim of this work was to investigate NPMNL oxidative metabolism and the possible role of ROS generated by these cells in damage to the endothelium of pulmonary microvessels in experimental MEPA.

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MATERIALS AND METHODS

Mongrel dogs weighing 15 to 20 kg were used in experiments which were carried out under conditions of closed chest and spontaneous respiration. For

0007-4888/94/0012-1332$12.50

premedication promedol was injected intramuscularly in a dose of 10 mg/kg; narcosis during the experiment was achieved by fractional intravenous infusion of sodium thiopental in a dose of 20 mg/kg. Catheterization of the heart and vessels and acute MEPA modeling were performed as described previously [2]. The experimental group consisted of 14 animals; blood samples were collected immediately after catheterization of the heart and then 3 and 6 h after MEPA reproduction. The control group consisted of 13 animals from which blood samples were collected at the same times. In all cases, blood for analysis was collected simultaneously from the right and left ventricles of the heart. Euthanasia was performed by intravenous infusion of a lethal dose of sodium thiopental in both groups of animals.

Chemiluminescent analysis and the nitroblue tetrazolium (NBT) reduction test are widely used in studies of the functional activity of NPMNL and of ROS production by them [3,5]. For the NBT test, a drop of incubation medium (0.2 ml 0.2 M NaH_2PO_4, 0.8 ml 0.2 M Na_2HPO_4, 2 mg crystal stain NBT, pH 7.2) was added to a drop of blood on a slide and mixed with a glass rod. The mixture was incubated for 65-75 min in a humid chamber at 37°C and then cooled at room temperature. Smears were prepared, dried, and fixed with ethyl alcohol for 3 min. NPMNL nuclei were then additionally stained with a 1% aqueous solution of methyl green for 10 min. Counts of NPMNL with different contents of diformazan granules in the cytoplasm (Table 1) were assessed as described previously [4] and the mean cytochemical index of activity per 100 cells was estimated: the number of the group was multiplied by the number of NPMNL in this group, the products were summed and divided by 100. Chemiluminescent analysis was carried out using a 1251 luminometer (LKB). Hanks solution, pH 7.39, luminol, cells, and activator (opsonized zymosan) in final concentrations of 2.0×10^{-5} M, 0.25×10^{6}/ml, and 0.1 mg/ml, respectively, were placed in the luminometer cuvette. The luminescence light sum per 10 sec was measured during constant stirring at 37°C. Chemiluminescence was assessed before and after the addition of activator to cells. This value corresponded to the difference of the peaks of the curves reflecting activated and spontaneous chemiluminescence. The data were processed by mathematical statistics using Student's *t* test.

RESULTS

The cytochemical index of activity of NPMNL circulating in the blood was found to rise by the third and sixth hours of the experiment in the control group (Fig. 1). There were no differences between the changes in this parameter in blood samples collected from the right and left ventricles. The rise of the cytochemical index occurred due to an increased share of NPMNL with a high level of oxidative metabolism (Table 1). Specific counts of functionally active NPMNL were virtually the same in blood samples from both ventricles. Chemiluminescent analysis did not reveal appreciable changes in spontaneous or activated chemiluminescence of NPMNL in the blood flowing from the lungs (Fig. 2). There was a tendency toward a decrease in the intensity of both types of chemiluminescence of NPMNL isolated from mixed venous blood.

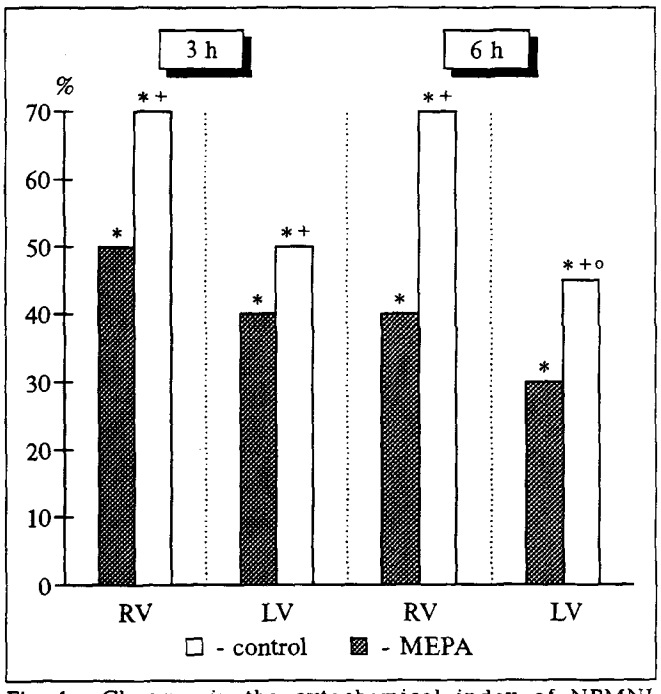

Fig. 1. Changes in the cytochemical index of NPMNL activity during the third and sixth hours of control and experimental (MEPA) studies. Ordinates: changes in the cytochemical index of NPMNL activity in % of initial values. Blood from right (*RV*) and left (*LV*) ventricles. Differences reliable ($p<0.05$) *vs.* the initial (*) and control (+) values and between *LV* and *RV* (°).

Hence, a moderate increase of the functional activity of NPMNL circulating in the blood was observed in the controls, which might be due to some factors associated with the experimental conditions (narcosis, catheterization of the heart, prolonged immobilization of animals). The mean cytochemical index of NPMNL activity and the specific count of blood NPMNL with a high level of oxidative metabolism were the same before and after blood passage through the lungs. This rules out a delay of the NPMNL fraction most active

with respect of ROS production in the pulmonary microvessels in the control, an assumption which is confirmed by the results of chemiluminescent analysis, which did not reveal a reduction of NPMNL capacity to generate ROS after these cells had passed through the lungs.

By the third and sixth hours of MEPA the cytochemical index of NPMNL activity in venous and arterial blood was noticeably increased, surpassing the baseline and control levels (Fig. 1), although the values of this index were less changed in samples collected from the left ventricle than from the right. The rise of the cytochemical index occurs due to an increase in the percent content of NPMNL with a high level of oxidative metabolism (Table 1). In parallel with this, the share of NPMNL with low functional activity drops. The specific share of the most active NPMNL (groups 4 and 5) is smaller in the blood samples from the left than from the right ventricle. Chemiluminescent analysis revealed increased spontaneous and activated chemiluminescence of NPMNL isolated from the venous pool (Fig. 2). The right to left ventricle ratio increases by the third and sixth hours of MEPA as regards activated chemiluminescence of NPMNL (2.35±0.59 and 1.45±0.29), reliably surpassing the control (0.85±0.09 and 0.68±0.12, respectively, $p<0.05$).

Hence, MEPA is associated with a more pronounced increase of the functional activity of NPMNL circulating in the blood and of their capacity to produce ROS, in comparison with the control. Changes in NPMNL and in vascular endothelium are known to be closely related and interdependent. Activation of the endothelial layer induces a cascade of reactions which boost oxidative metabolism of NPMNL [1]. Various factors may influence the status of vascular endotheliocytes, such as changes in hemodynamics, gaseous and biochemical composition of the blood, and activity of the neurohumoral regulatory systems, which are involved in the complex of pathophysiological reactions in MEPA [2,6,9]. Comparison of the examined parameters of oxidative metabolism in NPMNL isolated simultaneously from venous and arterial blood demonstrated a retention of the cells most actively producing ROS in pulmonary vessels in experimental MEPA. The following circumstance may be conducive to this. Embolic occlusion of a lung vessel is associated with a pressure drop and blood flow disorders in vessels distal to the site of involvement [6]. Since the massive variant of experimental embolic occlusion involves bilateral damage to quite a number of lobar arteries [2], the observed circulatory changes may have an appreciable impact on the size and

TABLE 1. Percent Content of NPMNL with Different Levels of Oxidative Metabolism in the Blood of Control and Experimental Animals (NBT Reduction Test) (M±m)

Number of diformazan granules in NPMNL cytoplasm	Control group						Experimental group (MEPA)					
	0 h		3 h		6 h		0 h		3 h		6 h	
	RV	LV	RV	LV	RV	LV	RV	LV	RV	LV	RV	LV
No granules (group 0)	21±3	16±3	10±3*	10±3	16±4	14±4	21±4	16±5	7±3*	7±3	12±4	17±4
5-7 granules (group 1)	27±3	31±4	13±3*	14±2*	18±4*	18±3*	30±3	32±2	11±2*	11±2*	19±3*	14±3*
Granules fill up to 30% of cytoplasm (group 2)	29±3	27±2	25±2	26±3	22±3	26±3	26±2	30±4	18±3	23±4	15±5	17±5
Granules fill 30-50% of cytoplasm (group 3)	16±1	18±2	31±2*	29±2*	23±3*	21±3	14±2	18±2	27±3*	30±4*	18±3	25±4*
Granules fill 50–100% of cytoplasm; nucleus discernible (group 4)	6±1	7±1	14±2*	14±1*	10±3	12±1*	5±1	5±1	22±3*+	16±2*	20±2*+	13±2*
Granules fill up to 100% of cytoplasm; nuclei indiscernible (group 5)	2±1	2±1	8±1*	8±1*	11±1*	10±2*	2±1	2±1	14±2*+	10±2*	16±4*	14±3*

Note. RV: right, LV: left ventricle. Reliable differences ($p<0.05$) *vs.* initial (*) and control (+) values.

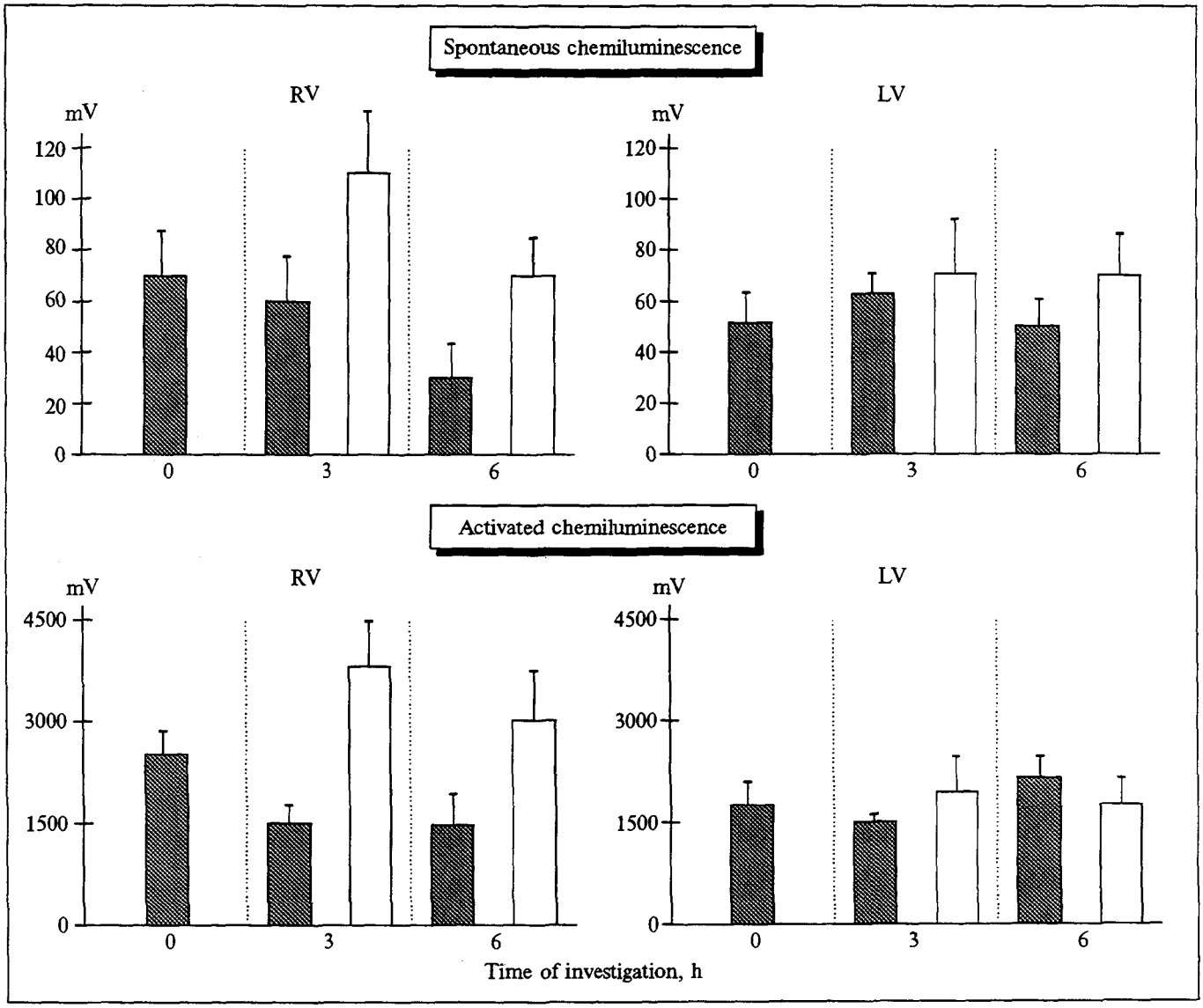

Fig. 2. Spontaneous and activated chemiluminescence of NPMNL in control and experimental (MEPA) studies. Notation as in Fig. 1.

composition of the marginal pool in the lungs. The shift of functionally active NPMNL from the circulating to the marginal pulmonary pool suggests that they play a role in damaging the vascular endothelium [1,20]. Our previous ultrasound study of pulmonary microvascular endothelium in MEPA [2] revealed signs of its impairment (clearing of the cytoplasm, destruction of mitochondria, appearance of lamellar membrane structures, transformation of transport communications, development of local membrane defects) which were most frequently observed at sites of endotheliocyte contact with activated NPMNL. The results of the present study permit us to attribute the endotheliocyte damage detected in experimental MEPA to reactions involving ROS generated by functionally active NPMNL.

When assessing the possible changes in oxidative metabolism of NPMNL and their role in impairing the pulmonary microvascular endothelium in patients with massive pulmonary artery thromboembolism, one should bear in mind several factors. On the one hand, changes in the hemostasis system leading to the development of embologenous venous thromboses are observed in this category of patients [6], but are absent in experimental animals. The processes of clot formation are associated with the production of numerous bioactive substances stimulating NPMNL [1]. This has been demonstrated, for example, for arachidonic acid derivatives [12,15,20]. The data presented permit us to expect more intensive activation of NPMNL circulating in the blood in patients with massive pulmonary artery thromboembolism than in experi-

mental MEPA. On the other hand, hydrocortisone levels are appreciably increased in the blood of patients with acute massive pulmonary thromboembolism, which is never observed in experimental MEPA [9]. Glucocorticoids are known to prevent injury to the vascular endothelium and an increase of its permeability, including in cases following reduced adhesion, aggregation, and chemotaxis of NPMNL, inhibition of phosphorylase A_2 and NADPH oxidase [10,16,18,19,22]. The latter factor is responsible for the reduction of ROS production, this being paralleled by a reduction of NPMNL chemiluminescence under the effect of glucocorticoids [23]. Hence, the degree of oxidative metabolism activation of NPMNL and their role in the genesis of injury to the pulmonary vascular endothelium in patients with MEPA depend on the ratio between stimulating and inhibitory factors, which is largely an individual matter.

In conclusion, this investigation demonstrates the "protective" function of the lungs vis-a-vis other vascular regions in MEPA. Retention of the most activated NPMNL in pulmonary vessels, leading to a reduction of their activity and of their ability to produce ROS, results in the release of functionally less active NPMNL in the greater circulation. This, in turn, lessens the possibility that NPMNL and the ROS produced by them will be able to damage the vascular endothelium in other organs, primarily the myocardium. The latter circumstance is extremely important because the outcome of MEPA, namely the possible development of irreversible heart failure, depends to a great degree on the status of the myocardium, as was demonstrated in our previous studies [7,8].

REFERENCES

1. V. V. Banin and G. A. Alimov, *Morfologiya,* № 2, 10-34 (1992).
2. A. O. Virganskii, V. V. Banin, V. A. Klevtsov, *et al., Fiziol. Zh. SSSR,* **76**, № 10, 1355-1360 (1990).
3. Yu. A. Vladimirov, O. A. Azizova, A. I. Deev, *et al., Recent Developments in Science and Technology, Ser. Biofizika,* № 29, 9-19, 33-35, 168-172, Moscow (1991).
4. K.A.Voitkevich, *Lab. Delo.,* № 3, 147-148 (1977).
5. A. N. Mayanskii and D. N. Mayanskii, *Essays on the Neutrophil and Macrophage* [in Russian], Novosibirsk (1983).
6. V. S. Savel'ev, E. K. Yablokov, and A. I. Kirienko, *Massive Pulmonary Artery Embolism* [in Russian], Moscow (1990).
7. M. S. Tverskaya, V. V. Karpova, A. O. Virganskii, *et al., Byull. Eksp. Biol. Med.,* **113**, № 3, 327-329 (1992).
8. M. S. Tverskaya, V. V. Karpova, A. O. Virganskii, *et al., ibid.,* **114**, № 9, 319-322 (1992).
9. M. S. Tverskaya, L. D. Makarova, N. A. Sergeeva, *et al., ibid.,* **116**, № 7, 29-32 (1993).
10. J. Tepperman and H. Tepperman, *Metabolic and Endocrine Physiology,* Year BK. Med. Publ., Chicago (1987).
11. I. A. Clark, W. B. Cowden, and N. H. Hunt, *Med. Res. Rev.,* **5**, № 3, 297-332 (1985).
12. J. Doukas, H. B. Hechtman, and D. Shepro, *Blood,* **71**, № 3, 771-779 (1988).
13. J. C. Fantone and P. A. Ward, *Hum. Pathol.,* **16**, № 10, 973-978 (1985).
14. M. R. Flick, S. A. Milligan, J. M. Hoeffel, and I. M. Goldstein, *J. Appl. Physiol.,* **64**, № 3, 929-935 (1988).
15. G. Goldman, R. Welbourn, C. R. Valeri, *et al., Microvasc. Res.,* **41**, № 3, 367-375 (1991).
16. D. E. Hammerschmidt, J. G. White, P. R. Graddock, *et al., J. Clin. Invest.,* **63**, 798 (1979).
17. B. Hammond, H. A. Kontos, and M. L. Hess, *Canad. J. Physiol. Pharmacol.,* **63**, № 2, 173-187 (1985).
18. G. L. Mandell, W. Rubin, and E. W. Hook, *J. Clin. Invest.,* **49**, 1381 (1970).
19. P. R. McGregor, P. J. Spagnuolo, and A. L. Lentnek, *New Engl. J. Med.,* **291**, 642 (1974).
20. B. M. Perlman, A. Johnson, W. Jubiz, *et al., Circulat. Res.,* **64**, № 1, 62-65 (1989).
21. P. A. Ward, K. J. Johnson, and G. O. Till, *Acta Physiol. Scand.,* Suppl. 548, 79-85 (1986).
22. S. J. Wiener, R. Wiener, M. Univetzky, *et al., J. Clin. Invest.,* **56**, 679 (1975).
23. J. K. Wojcik, P. Derentowitz, P. Garlinski, *et al., Pneumonol. Pol.,* **55**, 154 (1987).