EFFECT OF LETHAL Bacillus anthracis TOXIN ON PHAGOCYTOSIS AND DYNAMICS OF ENZYME ACTIVITY OF THE ANTIOXIDATIVE SYSTEM OF PERITONEAL MONONUCLEAR PHAGOCYTES OF MICE DIFFERING IN INBORN IMMUNITY TO ANTHRAX

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UDC 616.98:579.852.11]-092.19-07:[616.155.33-02:615.919:579.852.11

KEY WORDS: anthrax toxin; phagocytosis; antioxidative system; mononuclear phagocytes.

Differences in susceptibility of mice to anthrax infection are controlled by a single locus [2, 15]. Ability of the toxin to potentiate anthrax infection in mice is evdently determined by the genotype [2] and is determined mainly by the lethal toxin of Bacillus anthracis [3]. At the same time, it has been shown that the toxin selectively injures mononuclear phagocytes (MPh) [1, 6, 10]. These facts suggested that the mechanism of inborn resistance to anthrax is realized through the action of the toxin on MPh [2].

The phagocytic activity and intracellular level of reactive oxygen intermediates ($OH^{\bullet}$, $O_2^{\bullet}$, H_2O_2) are important components of the antimicrobial activity of MPh. The physiological concentration of oxidative radicals is controlled by enzymes of the antioxidative system. Various toxic effects on the cell are determined as a rule by accumulation of oxidative radicals and, consequently, they affect activity of enzymes of the antioxidative system [8, 9].

In view of the data described above, it was decided to study in vitro the action of purified lethal toxin (PA + LF) on MPh of BALB/c and CBA/lac mice, which differ in their susceptibility to anthrax infection, and in particular, on phagocytosis of sheep's red blood cells (SRBC) and on the dynamics of enzyme activity of the antioxidative system.

EXPERIMENTAL METHOD

Experiments were carried out on male mice of inbred lines BALB/c and CBA/lac, weighing 18-20 g, obtained from the "Stolbovaya" nursery, Academy of Medical Sciences of the USSR.

Purified components of B. anthracis toxin were used: protective antigen (PA) and lethal factor (LF); they were isolated from the toxic cultural filtrate by successive chromatography on porous glass, DEAE-Sephadex, hydroxyapatite, and blue sepharose. On polyacrylamide gel electrophoresis with sodium dodecylsulfate PA moved as a single polypeptide chain with mol. wt.of 87,000 D. LF contained impurities. Neither preparation possessed adenylate cyclase activity, and neither gave cross precipitation in Ouchterlony's double diffusion test with polyspecific antianthrax globulin. Intravenous injection of 40 μg of a PA + LF mixture (ratio 3:1) led to death of the BALB/c mice. PA and LF preparations injected separately had no lethal activity.

Peritoneal cells were obtained 4 days after intraperitoneal injection of 1 ml of sterile 10% proteose peptone. The cells were sedimented by centrifugation for 20 min at 330g (4°C) and suspended in RPMI 1640 culture medium with HEPES ("Flow Laboratories," England) with 10% fetal calf serum and 25 μg/ml of gentamicin.

To investigate enzymes of the antioxidative system, a suspension containing $1.5 \cdot 10^6$ cells/ml was poured in volumes of 0.5 ml into the wells of a 24-well panel ("Flow Laboratories") and incubated for 2 h at 37°C in a humid atmosphere containing 5% CO_2 (CO_2 incubator). Nonadherent cells were washed 3 times with Hanks' solution (the liquid was collected in centrifuge tubes) and fresh culture medium was added. The number of adherent cells in a well (about $0.68 \cdot 10^6$) was determined as the difference between the number added to the well ($0.75 \cdot 10^6$) and the number of cells washed out ($0.07 \cdot 10^6$).

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(Presented by Academician of the Academy of Medical Sciences of the USSR V. I. Pokrovskii) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 107, No. 3, pp. 288-291, March, 1989. Original article submitted December 18, 1987.

Lethal toxin (PA + LF = 4:1) was added in a volume of 0.5 ml to each well, and incubation was repeated for 2 h at 37°C in a humid atmosphere with 5% CO_2. One concentration of the toxin was added to 12 wells. Each sample for investigation consisted of four wells, with three samples for each dose. At the end of incubation the cell monolayer was washed twice with Hanks' solution, after which 0.4 ml of 0.05 M potassium-phosphate buffer, pH 7.4, was added in a volume of 0.4 ml to each well (1.6 ml per sample), and this was followed by freezing and thawing 3 times in cold ethanol at −80°C.

The disintegrated cells were sedimented by centrifugation at 400g for 15 min and the supernatant was withdrawn and kept for 2 weeks at −40°C. Superoxide dismutase (SOD) activity was determined by the method in [7] at 30°C; glutathione transferase (GT) was determined to 1-Cl-2,4-dinitrobenzene at 37°C [12], and activity of glutathione peroxidases (to hydrogen peroxide − GP-H_2O_2, and to tert-butyl hydroperoxides − GP-TBH) was determined at 30°C [14]. Glutathione reductase (GR) activity was measured at 30°C, as described in [4]. The protein concentration in the test material was determined by Lowry's method.

The results were subjected to two- and one-factor dispersion analysis for a small group of data and by Student's test [5].

To investigate phagocytosis, a monolayer of macrophages was obtained in a 96-well panel ("Nunc," Denmark) by the scheme described above (0.1 ml of a suspension containing $0.75 \cdot 10^6$ peritoneal cells was introduced into each well). To five experimental wells of each row (five rows for BALB cells, five for CBA cells, two for calibration of the reaction for the pseudoperoxidase activity of hemoglobin) 0.01 ml of one of the doses of toxin was added (no toxin added to the control wells), and the panels were incubated again. Phagocytosis by MPh of SRBC, sensitized by rabbit antibodies (IgG) to SRBC ($SRBC_S$), was estimated photometrically, using the pseudoperoxidase activity of hemoglobin of the phagocytosed $SRBC_S$ [11]. Phagocytic activity of MPh in the presence of the toxin was expressed as a percentage of the control.

EXPERIMENTAL RESULTS

Phagocytic activity of MPh of BALB and CBA mice in the control was identical (BALB 1175 ± 26; CBA 1180 ± 44 optical density units), evidence of equivalence of the macrophagal monolayers of BALB and CBA mice, so that it is possible to compare the effects of the toxin on their functional activity.

Lethal toxin was found to inhibit phagocytic activity of BALB mice, resistant to infection, more strongly than MPh of CBA mice, which are susceptible to anthrax (Fig. 1).

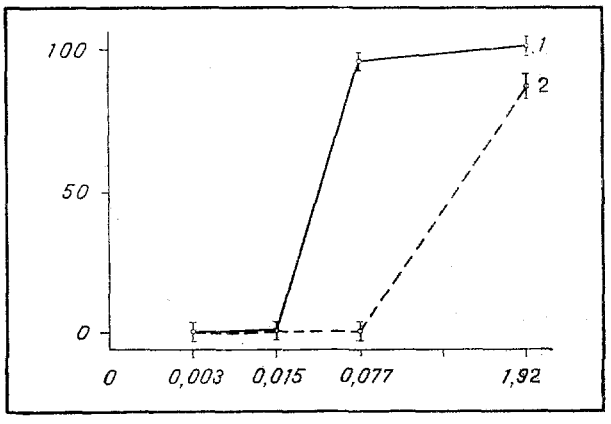

Fig. 1

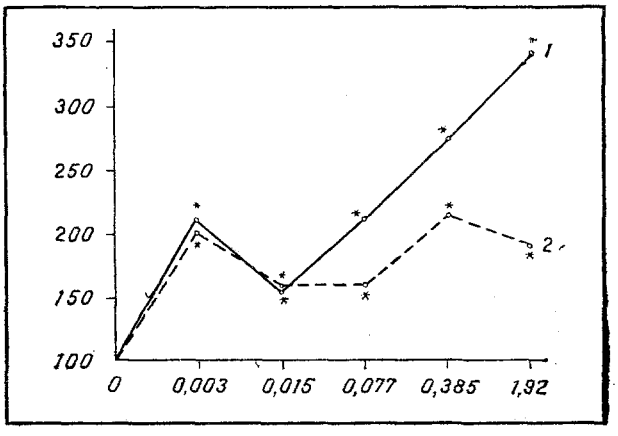

Fig. 2

Fig. 1. Effect of lethal toxin on phagocytic activity of MPh of BALB and CBA mice. Abscissa, $\log_5$ (concentration of toxin, μg/ml); ordinate, phagocytic activity (in % of control). 1) BALB, 2) CBA.

Fig. 2. Dynamics of GP-H_2O_2 activity in MPh of BALB and CBA mice, treated with lethal (PA + LF) toxin. Abscissa, $\log_5$ (concentration of toxin, μg/ml); ordinate, enzyme activity (in % of control, expressed per milligram protein). 1) BALB, 2) CBA. *$p < 0.05$ (for data of one-factor dispersion analysis) compared with control.

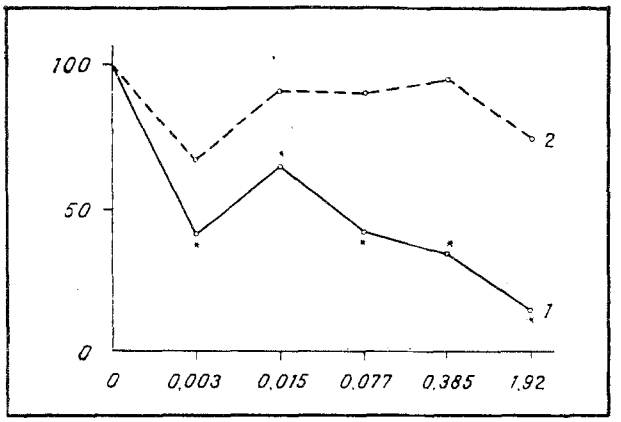

Fig. 3. Dynamics of GR/GP-H_2O_2 ratio in MPh of mice treated with lethal (PA + LF) toxin. Ordinate, ratio of enzyme activity (in % of control). Remainder of legend the same as to Fig. 2.

TABLE 1. Changes in Glutathione Reducing Potential (GR/(GT × GP-TBH·GP-H_2O_2) in MPh of CBA and BALB Mice under the Influence of Lethal (PA + LF) Anthrax Toxin (M ± m)

Dose of lethal factor, μg/ml	BALB	CBA
Control	5,48±0,77	2,6±0.37
0,003	2,73±0,77*	1,35±0.37*
0,015	4,12±0,77	3,34±0.37
0,077	3,05±0,77*	3,64±0.37
0,385	1,49±0,77*	2,29±0,37
1,92	0,58±0,77*	2,64±0.37

Legend. *$p < 0.05$ (data of one-factor dispersion analysis) compared with control.

The action of the lethal toxin on the phagocytic activity of MPh, like the ability of this toxin to kill animals when injected intravenously into them, is thus inversely proportional to the susceptibility of the animal to anthrax injection [2].

Analysis of the dynamics of SOD, GT, GR, GP-TBH, and GP-H_2O_2 activity of MPh of the two lines of mice after incubation with increasing doses of lethal anthrax toxin revealed significant interlinear differences only in the case of the dynamics of GP-H_2O_2. GR, GP-TBH, and GT activity of MPh of mice of both lines did not differ at any point of the investigation from the control values (data not given). With the majority of doses of the toxin no changes were observed in SOD activity.

Meanwhile, interlinear differences in the dynamics of GP-H_2O_2 activity were found in response to the toxin (Fig. 2). For instance, activity of this enzyme increased in MPh of BALB mice, and the increase was directly dependent on the dose of the toxin (within the dose range 0.077-1.92 μg/ml) on average by 50-66% ($p < 0.05$).

The rise ($p < 0.05$) of GP-H_2O_2 activity (by 93-117%) in MPh of the CBA mice was independent of the dose of toxin. The fact must be emphasized that activity of GP-H_2O_2 and GR in MPh of CBA mice was maintained at the same level irrespective of the dose of toxin, whereas GP-H_2O_2 activity in MPh of BALB mice increased in direct proportion to the dose of toxin, while the GR level remained unchanged. The dynamics of the GR/GP-H_2O_2 ratio in cells of both lines of mice depending on the dose of toxin was most demonstrative in this respect (Fig. 3). The GR/GP-H_2O_2 ratio in MPh of CBA mice was maintained within limits of the control values for all doses of toxin, but in MPh of BALB mice this parameter fell in direct proportion to the dose of toxin. It can accordingly be concluded that under the influence of lethal toxin, the balance is preserved in this antitoxic system in CBA mice, but the balance of this system is disturbed in BALB mice.

The results given in Table 1 indicate a fall in the value of the glutathione-reducing potential GR/(GT × GP-TBH × GP-H_2O_2) in BALB mice and the stability of this parameter in CBA mice. This suggests that lethal toxin induces a hereditarily determined deficit of reduced glutathione in BALB mice.

The use of two-factor dispersion analysis revealed the effect of the dose of the toxin and the hereditary features of the mouse MPh on GP-H_2O_2 activity and on the GR/GP-H_2O_2 ratio, whereas no such dependence was found for SOD, GR, GT, or GP-TBH, or the GR/GP-TBH, GR-GT, and SOD/GP-H_2O_2 ratios.

This is evidence of hereditary differences in the response of the antioxidative system of mouse MPh to lethal anthrax toxin.

Since changes in the system of antioxidative enzymes are induced by the level of components of free-radical oxidation, the results suggest that under the influence of lethal toxin, the H_2O_2 level in MPh of BALB mice rises more strongly than in MPh of CBA mice, and this may determine the greater anthracidal activity of these cells.

Thus purified lethal anthrax toxin (PA + LF) in vitro had a stronger inhibitory activity on phagocytosis and a stronger stimulating activity on glutathione peroxidase toward H_2O_2 in the mononuclear phagocytes of mice resistant to anthrax infection than in the same cells of susceptible mice.

LITERATURE CITED

1. V. A. Abalakin and P. V. Glan, Progress and Prospects in the Control of Anthrax in the USSR [in Russian], Moscow (1978), pp. 92-93.
2. V. A. Abalakin and B. L. Cherkasskii, Zh. Mikrobiol., No. 5, 45 (L986).
3. V. A. Abalakin, T. D. Cherkasova, and L. V. Sergeeva, Theoretical Problems in Present-Day Epidemiology and Immunology [in Russian], Nal'chik (1986), pp. 111-112.
4. A. M. Gerasimov, L. A. Koroleva, O. S. Brusov, et al., Vopr. Med. Khimii, No. 1, 89 (1976).
5. G. N. Zaitsev, Mathematical Statistics in Experimetnal Botany [in Russian], Moscow (1984), pp. 286-289.
6. Yu. M. Fedotova, Arkh. Patol., No. 11, 30 (1970).
7. C. Beauchamp and I. Fridovich, Anal. Biochem., 44, 276 (1971).
8. R. F. del Maestro and I. Alexander, The Inflammatory Process and Introduction to the Study of Cellular and Humoral Mechanisms, ed. by P. Venge and A. Lindbom, Stockholm (1981), pp. 113-143.
9. L. Flohe, Oxygen Free Radicals and Tissue Damage, ed. by D. W. Fitzsimons, Amsterdam (1979), pp. 95-122.
10. A. M. Friedlander, J. Biol. Chem., 261, No. 16, 7123 (1986).
11. T. W. Jungi, J. Immunol. Meth., 82, 143 (1985).
12. W. H. Habig, M. J. Pabst, and W. B. Jakoby, J. Biol. Chem., 249, 7130 (1974).