Effect of Glycolipid *Rhodococcus* Biosurfactant on Secretory Activity of Neutrophils *In Vitro*

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Glycolipid biosurfactant synthesized by nonpathogenic strain *Rhodococcus ruber* IEGM231 modulated the production of ROS and IL-8 by peripheral blood neutrophils in spontaneous and stimulated cultures. Secretion of IL-1β и TNF-α by neutrophils in the presence of biosurfactant changed insignificantly.

Key Words: *glycolipid; Rhodococcus ruber; neutrophils; cytokines; reactive oxygen species*

Development of drugs based on bioactive substances produced by bacteria is a promising area of pharmaceutical biotechnology. Glycolipid biosurfactants (GLB), an extensive group of surface-active microbial biosynthesis products combining high immunological and surfactant activity and anti-adhesion properties [4,5,12], are of great industrial interest. GLB are complexes based on mono- and disaccharides (rhamnose, sophorose, mannose, trehalose, *etc*.) linked with fatty acids through an ester bond. It is believed that immunological activity of these complexes is determined by the structure of their lipid components, in particular long chain fatty (mycolic) acids [13]. Mycolic acid from *Mycobacterium tuberculosis* was the first lipid antigen, for which the process of antigen presentation to T cells in CD1β molecule was described [6]. MINCLE lectin receptors abundantly expressed on monocyte-macrophage system cells and neutrophilic granulocytes also play an important role in the immunomodulatory effects of glycolipid complexes. In particular, this was shown for trehalose dimycolate (TDM), the best studied glycolipid component of the cell wall of *M. tuberculosis* characterized by pronounced immunogenicity [10]. However, the presence of long-chain mycolic acids (C_{74}-C_{89}) in TDM synthesized by *M. tuberculosis* cause high toxicity of this glycolipid [13,14].

We have previously demonstrated that actinobacteria *Rhodococcus ruber* produce nontoxic GLB with high immunomodulatory activity comprising TDM, monoacyl trehalose, and diacyl trehalose [9]. GLB from *R. ruber* are found to modulate the production of pro-inflammatory (IL-1β, IL-6, and TNF-α) [2,5] and anti-inflammatory (IL-10, IL-12, and IL-18) [3] cytokines by peripheral blood mononuclear cell. At the same time, the effect of trehalose lipids on functional activity of neutrophilic granulocytes, the first line of defense against acute infection, was never studied. Modulation of functional activity of polymorphonuclear leukocytes, namely the generation of reactive oxidants, is an important pathogenetic factor. Its assessment is relevant for both experimental and practical medicine, because it reflects the microbicidal potential of neutrophils and the risk damaging for the own tissues of the organism.

Here we studied the effects of bioactive *Rhodococcus* biosurfactant on microbicidal and secretory activity of peripheral neutrophil granulocytes.

MATERIALS AND METHODS

The work was carried out on non-fractionated leukocyte suspension and purified fraction of peripheral

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blood neutrophils taken from volunteer donors aged 18-35 years. Non-fractionated leukocyte suspension was obtained by blood sedimentation for 2 h at 37°C. After sedimentation, the upper layer of plasma with leukocytes was removed and centrifuged at 1500 rpm for 20 min. The precipitate was resuspended in 2 ml complete culture medium and used for culturing.

For purification of neutrophil fraction, a portion of leukocyte suspension was layered on ficoll-verografin (ρ=1.077) and centrifuged at 1500 rpm for 40 min. The precipitate (90-95% granulocytes) was washed twice in medium 199 for subsequent evaluation of cytokine production or Hanks solution for the reaction of luminol-dependent chemiluminescence (LCDL) and incubated at 4°C for 1 h. The purity of neutrophil suspension evaluated microscopically was 90-95%. Cell viability after separation by density gradient was assessed using 0.1% trypan blue solution and was 98%.

ROS production was estimated from LCDL. The cell suspension was treated with erythrocyte lysing buffer for 30 sec. The cells were then transferred to 96-well flat-bottom plates (Microlite) at a concentration of 10^5 cells/0.1 ml Hanks solution in triplicates. For induction of the respiratory burst, opsonized zymosan (OZ) in concentrations of 15 and 150 μg/ml was added to the wells. GLB obtained as previously described [5] was added to cultures in concentrations of 100, 10, 1, and 0.1 μg/ml. Working concentrations were prepared in Hanks solution as a water/oil emulsion by ultrasonic treatment (23 kHz, 30 sec), because glycolipid complexes require a certain orientation on the surface of microbial cells for the manifestation of the proinflammatory or immunoregulatory properties [15]. Luminol (10^{-5} M), which luminescence is nonselective to oxygen-containing radicals, served as a marker of the intensity of LCDL reaction. The results were recorded for 1 h at 5-min intervals on TECAN multifunctional spectrophotometer.

For the analysis of cytokine secretion, granulocytes were cultured in round-bottom 96-well plates (Medpolimer) during 24 h. Each culture contained 2×10^5 cells/0.2 ml complete culture medium, which was prepared *ex tempore* on the basis of medium 199 (BioloT) supplemented with 10 mM HEPES (Sigma), 2 mM L-glutamine (Sigma), 100 μg/ml penicillin and 100 μg/ml streptomycin, and 10% FBS (BioloT). LPS *E. coli* B55:O5 (1 μg/ml; Sigma) was used as the inductor. Working GLB concentrations of 100, 10, 1, and 0.1 μg/ml were prepared in 199 medium. Cell culture supernatants were stored frozen at -20°C. The concentrations of TNF-α, IL-1β, and IL-8 were measured using Vector-Best kits according to manufacturer's instructions.

The results were analyzed by one-way ANOVA and LSD test for post-hock comparisons.

RESULTS

Neutrophils actively participate in antimicrobial protection releasing cytotoxic and bactericidal factors, in particular ROS, as the first line of defense against acute infections. ROS generation by blood neutrophils in the respiratory burst is one of the stages phagocytosis required for providing unspecific immunity. GLB at 100 μg/ml exerted statistically significant dose-dependent effect on spontaneous ROS production by neutrophil granulocytes (Fig. 1, *a*). A similar pattern was observed after simultaneous addition of GLB and OZ in a concentration of 150 μg/ml to neutrophils (Fig. 1, *c*). Generation of oxygen metabolites by neutrophils in cultures containing 100 μg/ml GLB was significantly enhanced throughout the observation period compared to the control cultures containing OZ alone. It should be noted that the peak of LCDL for neutrophil granulocytes simultaneously stimulated by two microbial products 3-fold surpassed the corresponding values in cultures stimulated by GLB alone (Fig. 1, *a*, *c*). At the same time, addition of 10 μg/ml GLB in the presence of 150 μg/ml OZ also significantly stimulated neutrophilic production of free radicals on the 5th, 10th, and 20th minutes of the experiment (Fig. 1, *c*). A somewhat different picture was observed at lower (15 μg/ml) concentrations of OZ (Fig. 1, *b*). Under these conditions, GLB in a concentration of 0.1 μg/ml significantly reduced ROS production by neutrophils throughout the observation period and in concentration of 10 μg/ml on minutes 30-60.

In the leukocyte fraction (60% neutrophils), GLB in a concentration of 100 μg/ml stimulated ROS production by spontaneous cultures on minutes 10-60 and in a concentration of 10 μg/ml on minutes 25-45 (Fig. 2). GLB had virtually no effect on OZ-induced LCDL of leukocytes. Thus, changes in the intensity of free radical oxidation under the influence of the glycolipid *Rhodococcus* biosurfactant in short-time cultures of neutrophils were opposite and depended on inductor concentration.

It is now well known that neutrophilic granulocytes secrete pro-inflammatory cytokines [1]. We studied production of IL-1β, TNF-α, and IL-8 by neutrophils, because this cytokine that controls the process of neutrophil chemotaxis is one of the most intense immune factors secreted by granulocytes. The secretion of IL-1β and TNF-α in the entire range of GLB concentrations did not change significantly in both spontaneous and LPS-stimulated cultures of neutrophils (Fig. 3). LPS added to cells alone (1 μg/ml) had no effect on the production of the studied cytokines. At the same time, secretion of IL-8 by neutrophils was significantly activated in the presence of GLB, which significantly stimulated the synthesis of this

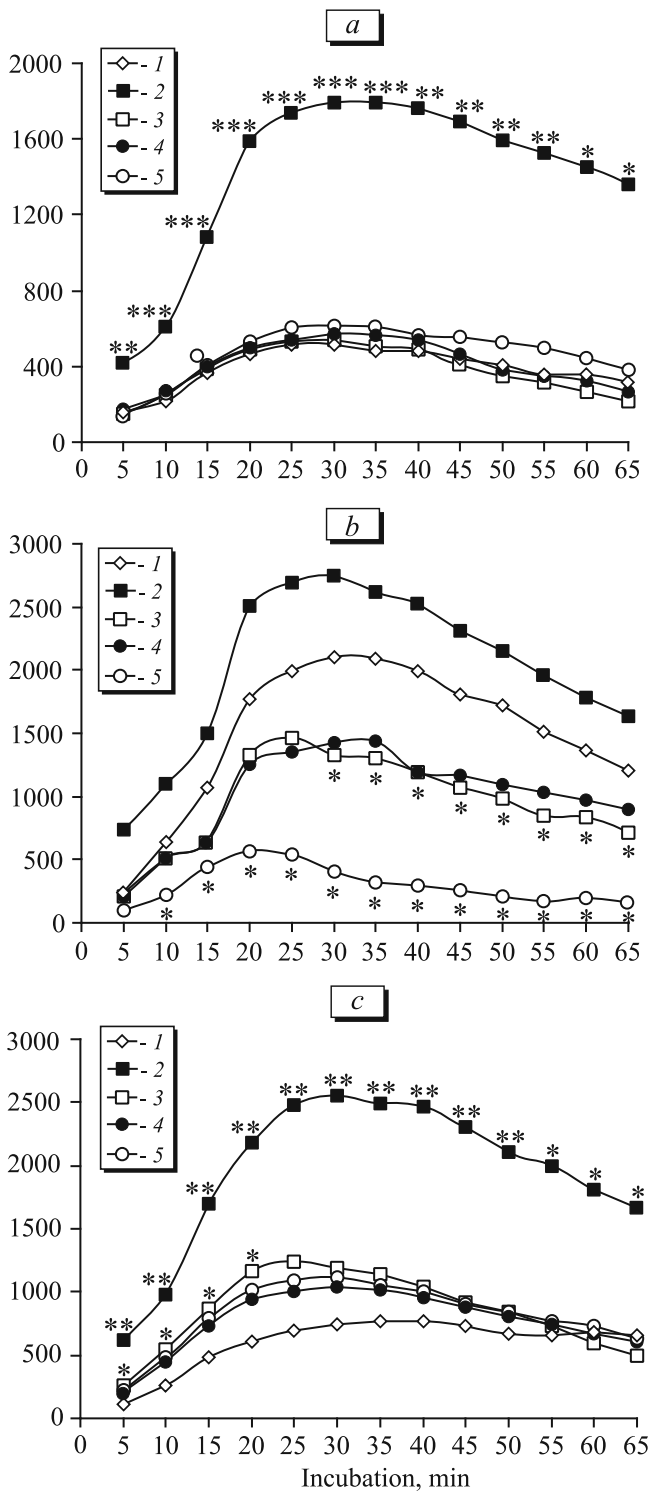

Fig. 1. Effects of GLB on the kinetics of LCDL in spontaneous (*a*) and stimulated by 15 μg/ml (*b*) and 150 μg/ml (*c*) OZ cultures of neutrophilic granulocytes. *a*: control (*1*), 100 μg/ml GLB (*2*), 10 μg/ml GLB (*3*), 1 μg/ml GLB (*4*), 0.1 μg/ml GLB (*5*); *b*: 15 μg/ml OZ (*1*), 100 μg/ml GLB+OZ (*2*), 10 μg/ml GLB+OZ (*3*), 1 μg/ml GLB+OZ (*4*), 0.1 μg/ml GLB+OZ (*5*); *c*: 150 μg/ml OZ (*1*), 100 μg/ml GLB+OZ (*2*), 10 μg/ml GLB+OZ (*3*), 1 μg/ml GLB+OZ (*4*), 0.1 μg/ml GLB+OZ (*5*). Here and in Fig. 2: Ordinate, rel. units of luminescence. *$p \le 0.05$, **$p \le 0.01$, and ***$p \le 0.001$ in comparison with the control.

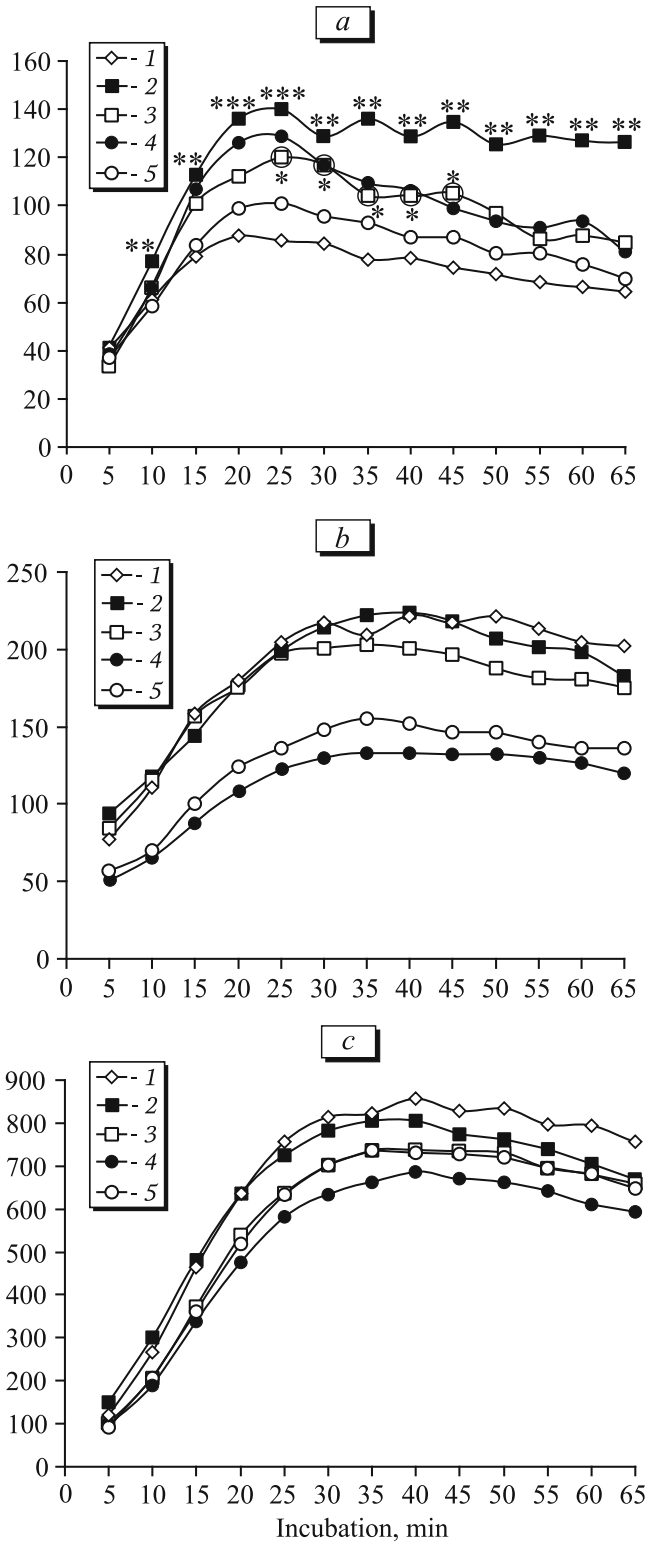

Fig. 2. Effects of GLB on the kinetics of LCDL **in** spontaneous (*a*) and stimulated by 15 μg/ml (*b*) and 150 μg/ml (*c*) OZ cultures of leucocytes. *a*: control (*1*), 100 μg/ml GLB (*2*), 10 μg/ml GLB (*3*), 1 μg/ml GLB (*4*), 0.1 μg/ml GLB (*5*); *b*: 150 μg/ml OZ (*1*), 100 μg/ml GLB+OZ (*2*), 10 μg/ml GLB+OZ (*3*), 1 μg/ml GLB+OZ (*4*), 0.1 μg/ml GLB+OZ (*5*); *c*: 15 μg/ml OZ (*1*), 100 μg/ml GLB+OZ (*2*), 10 μg/ml GLB+OZ (*3*), 1 μg/ml GLB+OZ (*4*), 0.1 μg/ml GLB+OZ (*5*).

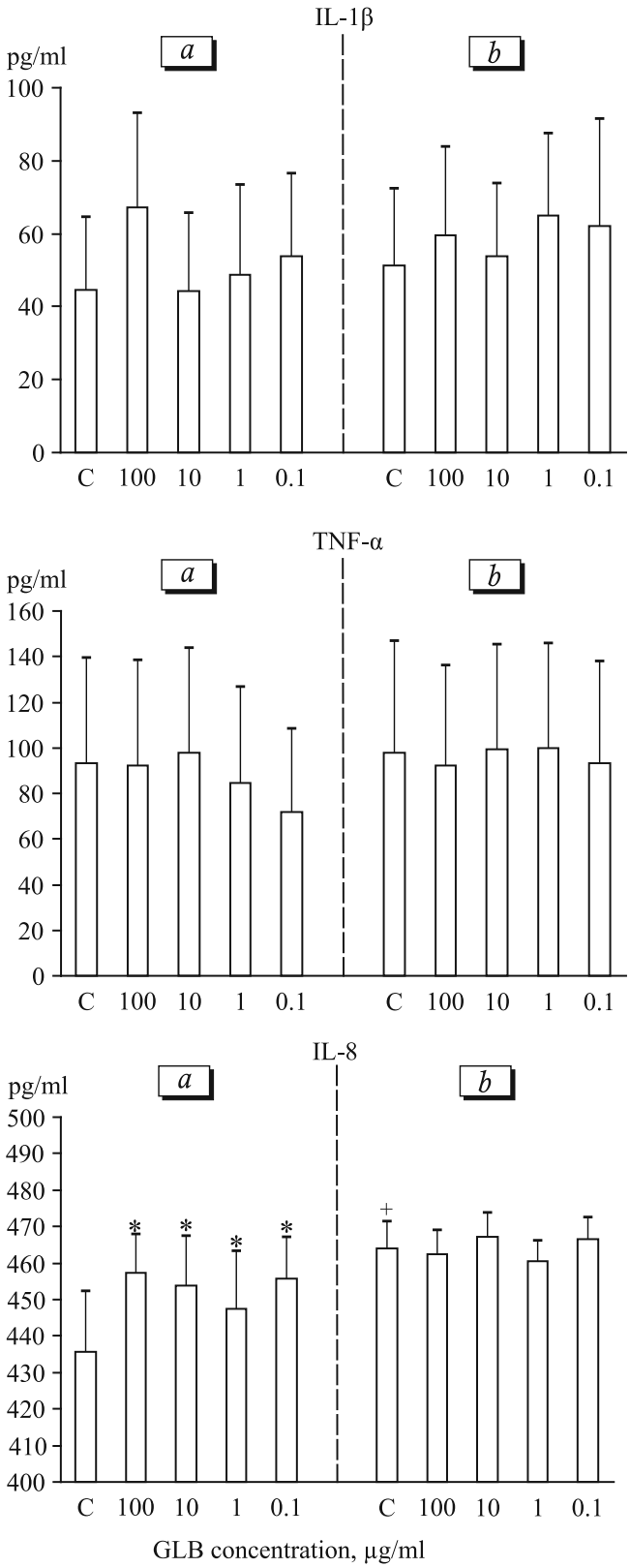

Fig. 3. Effects of GLB on spontaneous (*a*) and induced by 1 μg/ml LPS (*b*) secretion of IL-1β, TNF-α, and IL-8 by peripheral blood neutrophils. $^{+*}p \leq 0.05$ in comparison with the control (C).

cytokine throughout the range of the studied concentrations. Enhanced neutrophilic production of IL-8 was revealed along with LPS activation. However, GLB did not show significant stimulating activity under these conditions.

Thus, GLB from *R. ruber* IEGM 231 dose-dependent modulated secretory activity of peripheral neutrophil granulocytes. At the same time, the intensity and direction of GLB effects largely depended on the concentrations of OZ and the study object. Thus, in the leukocyte suspension (the most integral model in terms of physiology), biosurfactant intensified ROS production only in spontaneous cultures. In stimulated cultures, GLB had no effect despite the fact that OZ and analyzed biosurfactant interact with different surface receptors on innate immunity cells. OZ binds to TLR 2/6 [11], CR3 and CR4 receptors to iC3b component of complement [7]. Glycolipids bound to T cell receptor of γδ-T cells [8] and with CD1 molecule [6] and MINCLE lectin receptor on the surface of innate immunity cells [10].

The modulating capacity of GLB was greater in the fraction of neutrophils and manifested in both spontaneous and OZ-induced cultures. The preparation had stimulating and suppressive effects depending on the concentration of the inductor. This, in our opinion, is due to the lack of intercellular cooperation, regulatory and co-stimulatory signals from monocytes in the leukocyte suspension and, as a consequence, the earlier initiation of apoptosis [1]. Markedly stimulated neutrophilic secretion of IL-8, a factor responsible for enhanced infiltration of inflammatory focus with granulocytes shows GLB capacity to activate the neutrophil migration [1]. Our results indicate the prospects for further research of immunomodulatory activity of GLB from *Rhodococcus*.

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