Modulation of Cytokine Secretion and Oxidative Metabolism of Innate Immune Effectors by *Rhodococcus* Biosurfactant

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The glycolipid biosurfactant complex from *Rhodococcus ruber* IEGM 231 had a stimulatory effect on the production of IL-12, IL-18, and reactive oxygen species by cells of the innate immunity. This effect depended on the composition of cell cultures and presence of LPS. It was primarily observed in non-stimulated cultures. The glycolipid biosurfactant complex had little effect on IL-10 secretion by monocytes and mononuclear cells.

Key Words: *Rhodococcus rubber; glycolipid biosurfactant; interleukin-10, interleukin-18, and interleukin-12; chemiluminescence*

Increasing industrial interest in microbial biosurfactants is related to a variety of chemical structures and biological functions of these compounds. In recent years, microbial biosurfactants are considered as possible ingredients of new multipurpose drugs in pharmaceutical industry. It is associated with high immunomodulatory activity of biosurfactants [9]. The glycolipid biosurfactant complexes (GLB) consisting of monosaccharides and disaccharides that are conjugated with α-branched β-hydroxylated fatty (mycolic) acids hold much promise for biomedical researches [9,11]. The content of these structures is high in glycolipids of the outer membrane in coryneform and nocardioform actinobacteria. The structure and percentage of mycolic acids are now believed to determine the toxicity, persistence and immunogenicity of some microorganisms (*e.g.*, mycobacteria, nocardioform, and coryneform bacteria). Published data show that GLB modulate the innate, early acquired, cellular, and humoral immune response [15]. Glycolipids of pathogenic bacteria (*Mycobacterium tuberculosis*, *Corynebacterium diphtheria*, *etc*.) are well-studied in this respect. However, the existing or potential hazard of producing strains and high toxicity of glycolipids limit their use in practice [15]. Nonpathogenic actinobacteria of the *Rhodococcus* genus with unique biological properties and wide catabolic activity can be used for the screening of biosurfactant producers [8].

In 2000, the glycolipid biosurfactant product was obtained from nonpathogenic actinobacteria *Rhodococcus ruber* by means of induced biosynthesis (Institute for Ecology and Genetics of Microorganisms) [10]. Chemical structure and surfactant activity of this product were evaluated [10,14].

TLR-2 receptors that are mainly expressed on myelomonocytic cells serve as a main target for microbial glycolipid complexes [3]. Monocytes and neutrophils are activated during the interaction with bacterial cell wall components, which results in the increased secretion of pro-inflammatory cytokines (IL-1β, TNF-α, IL-6, IL-8, IL-12, and IL-18), cationic proteins, lysozyme, and reactive oxygen species. It is followed by an increase in the inflammatory response and destruction of intracellular pathogens.

Our previous studies revealed that the biosurfactant product from *R. ruber* has a modulatory effect on

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the production of pro-inflammatory cytokines IL-1β, IL-6, and TNF-α [1,2].

Here we studied the *in vitro* effect of biologically active GLB from *Rhodococcus ruber* IEGM 231 on the production of anti-inflammatory cytokine IL-10 and major Th1-polarizing factors (IL-12 and IL-18) by peripheral blood mononuclear cells and monocytes. We also evaluated the effect of GLB on reactive oxygen species generation by effector cells of innate immunity.

MATERIALS AND METHODS

The peripheral blood was sampled from donors of 18-35 years. The mononuclear cell fraction was obtained by centrifugation of peripheral blood plasma in a Ficoll-urografin density gradient (ρ=1.077). The monocyte fraction was isolated mechanically in Petri dishes. Cooled suspension of mononuclear cells was put in a glass Petri dish and incubated in a thermostat at 37°C for 1 h. Adherent cells were taken with a scraper, suspended in RPMI-1640 medium (ICN), and washed twice with this medium. Mononuclear cells and monocytes (10^6 cells/ml) were maintained at 4°C for 1 h and cultured in RPMI-1640 medium (ICN) containing 10 mM HEPES (Sigma), 2 mM L-glutamine (Sigma), 100 μg/ml gentamicin, and 10% FBS (ICN) for 1 day. GLB from cells of *R. ruber* strain IEGM 231 (Regional Profiled Collection of Aalkanotrophic Microorganisms; acronym IEGM, www.iegm.ru/iegmcol) is a mixture of nonpolar (80%) and polar lipids (20%). Polar lipids mainly consist (~15%) of three major glycolipids (trehalose dimycolate, diacyl trehalose, and monoacyl trehalose) [14]. GLB in doses of 100, 10, 1, 0.1 μg/ml was added to cultures. The working concentrations of GLB product were prepared in physiological saline or RPMI-1640 nutrient medium (ICN). A water/oil emulsion was obtained by ultrasonic disintegration (23 kHz, 30 sec). LPS in a dose of 1 μg/ml was added to some cultures simultaneously with GLB. The supernatants of cell cultures were frozen and stored at -20°C. The concentrations of IL-12, IL-18, and IL-10 in cell culture supernatants were measured by solid-phase enzyme-linked immunoassay with kits (Biosourse and Vector-Best) kits in accordance to the manufacturer's instructions.

Production of reactive oxygen species in cultures of peripheral blood leukocytes was estimated from the intensity of luminol-dependent chemiluminescence (LDCL). The cells were obtained after sedimentation of blood plasma at 37°C for 2 h. The concentration of cells was brought to 10^6 cells/ml. GLB in doses of 100, 10, 1, and 0.1 μg/ml was added to cultures. Luminol (10^{-5} M) served as a marker LDCL. Luminescence of this agent is nonselective to oxygen-containing radicals [13]. The results were recorded on a Guardian liquid scintillation counter (Wallac) for 1 h.

The results were analyzed by one-way analysis of variance for paired samples and paired *t*-test. The data in figures are presented as means and standard errors ($M \pm m$).

RESULTS

GLB had little effect on spontaneous and LPS-induced secretion of IL-12 (p70) by mononuclear cells (Fig. 1). Spontaneous secretion of IL-12 (p70) by cultured monocytes was significantly increased after addition of GLB in doses of 10 and 1 μg/ml to the culture medium (Fig. 1). LPS increased the production of IL-12 (p70) by monocytes as compared to the baseline level. However, simulatenous addition of two products of microbial biosynthesis (LPS and GLB) had no effect on the secretion of this cytokine (as distinct from LPS-treated culture of monocytes; Fig. 1).

Analysis of the effect of GLB on the production of IL-18, which is functionally similar to IL-12 and serves as an IL-12 synergist, produced the following results. GLB significantly increased spontaneous (100 μg/ml) and LPS-induced secretion of IL-18 by mononuclear cells (100 and 10 μg/ml; Fig. 1). The stimulatory effect of the biosurfactant was less pronounced in monocyte cultures (Fig. 1).

GLB had no significant effect on spontaneous and induced secretion of IL-10 by monocytes and mononuclear cells (Fig. 2, *a*, *b*).

Studying the effect of GLB on oxidative metabolism of innate immune effectors showed that this agent in all doses (except for 10 μg/ml) has a strong stimulatory effect on generation of reactive oxygen species by the leukocyte suspension. This effect depended on the incubation time (Fig. 3). The product at high concentration (100 μg/ml) significantly increased the oxidative metabolism by the 10th minute of incubation. The maximum level of LDCL was observed earlier than in control (non-treated) samples. After treatment with GLB in lower doses (1 and 0.1 μg/ml), the peak intensity of LDCL coincided with the maximum level of chemiluminescence in control samples. However, the intensity of LDCL was much higher after addition of GLB to leukocytes. The most significant effect was observed after treatment with GLB in a dose of 1 μg/ml. Addition of GLB in this concentration to leukocytes was accompanied by the increased production of oxygen-containing radicals.

Our results indicate that GLB has a modulatory effect on secretion of IL-12 and IL-18 by *Rhodococcus ruber* IEGM 231 and production of reactive oxygen species by innate immune effectors. This effect depended on cellular composition of cultures and pres-

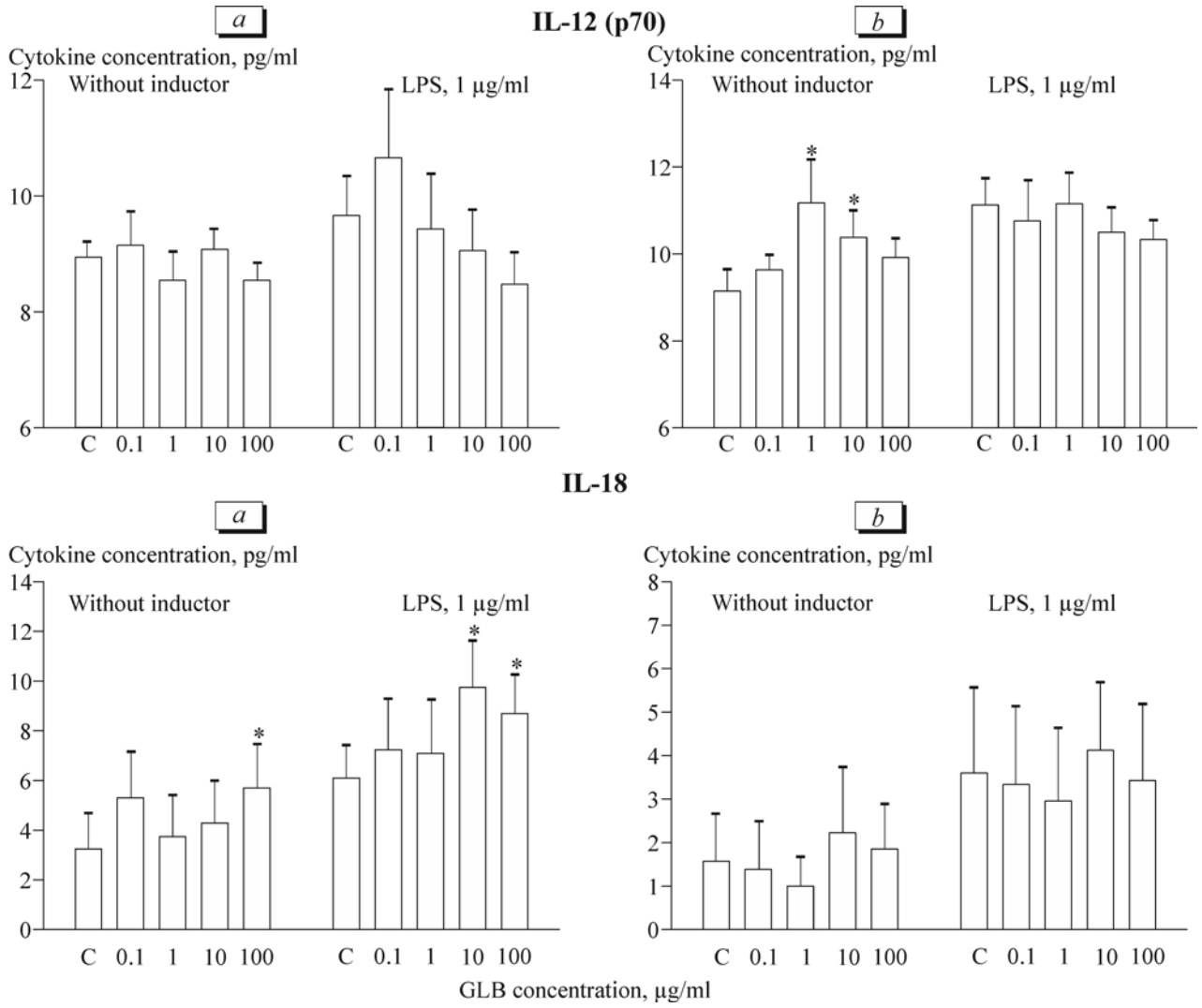

Fig. 1. Effects of GLB on the production of IL-12 (p70) and IL-18 by mononuclear cells (*a*) and monocytes (*b*) of the peripheral blood. Here and in Fig. 2: C, control. *$p<0.05$ compared to the control (Fisher's LSD test).

ence of the inducing agent. It was primarily observed in non-stimulated cultures. TLR-2 receptors serve as the major target for microbial glycolipids complexes. They belong to a family of Toll-like receptors, are mainly expressed on myelomonocytic cells [3], and induce a cascade of inflammatory reactions. Studying the pro-inflammatory activity of GLB showed that this agent has a stimulatory effect on the secretion of IL-1β and TNF-α in the monocyte fraction [1,2]. These effects were not observed in the mononuclear cell culture. It was probably associated with the ability of lymphocytes to secrete some suppressor molecules (particularly IL-4 and IL-10) under the influence of GLB [6,12]. We showed that GLB increases the secretion of IL-18 during combined culturing of monocytes and lymphocytes. The additional stimulation of cells by LPS has an important role under these conditions. In the absence of costimulatory signals from lymphocytes, GLB stimulates the secretion of IL-1β, TNF-α [1,2], and IL-12 by monocytes. On the one hand, the course and outcome of various infectious diseases depend on the ability of a causative agent, its components, and products to induce the synthesis of IL-12, which potentiates the production of IFN-γ and promotes Th1 polarization of the immune response. These changes result in the effective and self-limiting defense of cells from a causative agent. On the other hand, IL-12 is considered as a promising antitumor agent [4]. Some authors reported that the irresponsiveness of T lymphocytes to standard mitogens and low level of induced production of IL-2 and IFN-γ during chronic inflammation can be corrected *in vitro* by addition of IL-12 to the culture of blood leukocytes [5]. The adverse consequence of long-term treatment with high concentrations of IL-12 is a toxic effect. Therefore, it is important to search for endogenous

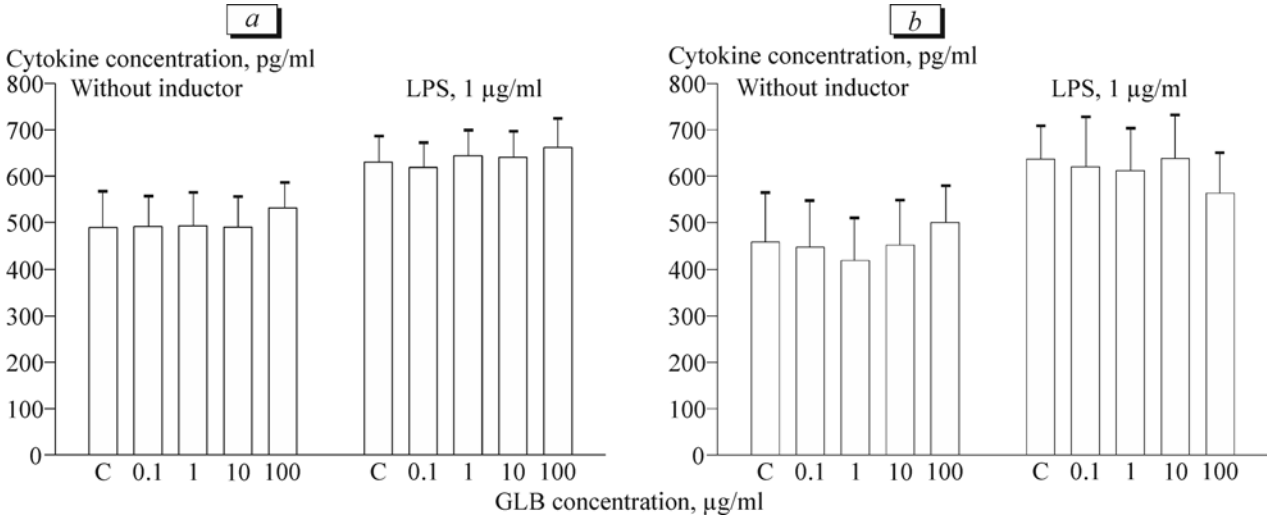

Fig. 2. Effect of GLB on IL-10 production by mononuclear leukocytes (*a*) and monocytes (*b*) of the peripheral blood.

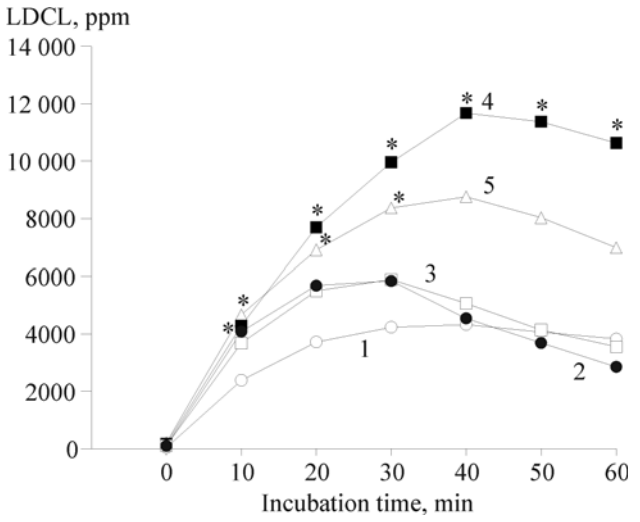

Fig. 3. Production of reactive oxygen species by peripheral blood leukocytes after addition of GLB to cultures. *1*, control (no preparation); *2*, GLB, 100 μg/ml; *3*, 10 μg/ml; *4*, 1 μg/ml; *5*, 0.1 μg/ml. *$p<0.05$ as compared to the control (Student's *t* test).

stimulators of IL-12 and synergistic compounds [4]. GLB has a stimulatory effect on IL-12 secretion only in the absence of lymphocytes in the culture medium. Hence, GLB can serve as an alternative stimulator of T cells during chronic infections associated with T cell deficiency. Under these conditions, GLB stimulates the production of IL-12 by monocytes and macrophages.

The production of biocidal oxygen radicals by phagocytes is an example, which illustrates the positive role of peroxidation processes. It determines not only the microbicidal and cytotoxic effect, but also the immunoregulatory function of activated phagocytes. Little is known about the effect of microorganism glycolypid compounds on oxidative metabolism of innate immune cells. The effect of a purified complex of mycolic acids and trehalose from the cell wall of pathogenic or opportunistic strains was described in all studies [15]. The content of NO^{2-} was high in cultures of murine peritoneal macrophages after treatment with trehalose dimycolate from the cell wall of *Mycobacterium tuberculosis*. However, NO^{2-} concentration was shown to decrease to the baseline in the presence of an NO synthase inhibitor [7,15]. We showed that a nonpurified macromolecular biosurfactant product from the nonpathogenic strain of *R. ruber* IEGM 231 has a stimulatory effect on oxidative metabolism in peripheral blood phagocytes. On the one hand, reactive oxygen species generation serves as a reliable criterion for functional activity of phagocytes in the blood and tissues. On the other hand, this process contributes to variations in phagocyte reactivity. The described changes play a key role in the pathogenesis of infections.

The immunomodulatory activity and mechanisms for action of GLB on the immune system should be evaluated in further researches.

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