IMMUNOLOGY AND MICROBIOLOGY

In Vitro Immunomodulating Activity of Biosurfactant Glycolipid Complex from *Rhodococcus Ruber*

M. S. Kuyukina, I. B. Ivshina, S. V. Gein, T. A. Baeva, and V. A. Chereshnev

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 144, No. 9, pp. 301-305, September, 2007
Original article submitted February 8, 2007

The biosurfactant glycolipid complex synthesized by *Rhodococcus ruber* actinobacteria is not toxic and exhibits no appreciable effect on proliferative activity of peripheral blood leukocytes. In the monocyte fraction, the biosurfactant activates the production of IL-1β and TNF-α cytokines without modifying the production of IL-6. In the mononuclear fraction, the glycolipid biosurfactant exhibited no effects on the production of IL-1β, TNF-α, and IL-6. These results indicate good prospects for further studies of immunomodulating and antitumor activities of biosurfactant drug.

Key Words: *actinobacteria; Rhodococcus ruber; biosurfactant; cytokines; toxicity*

Bacterial biosurfactants attract attention as possible objects of biomedicine [5] due to biological activity not detected in synthetic analogs, and because of increasing need of pharmacological industry for multipurpose bioactive substances with antibacterial, immunomodulating, and antitumor activities. Glycolipid biosurfactants (GLB) representing complexes on the basis of mono- and disaccharides connected via ester bonds with fatty acids (Pseudomonas rhamnolipids, yeast mannosylerythritol- and sophorolipids) [5,8]. The coryneform and nocardioform actinobacteria are characterized by high (30-60%) content of lipids, predominantly high-molecular-weight α-branched β-hydroxylated fatty (mycolic) acids present in a free state and as components of cell membrane glycolipids. Surface trehalose corynomycolates, known as cord factor and liso-cord factor, first detected in *Mycobacterium tuberculosis*, are studied in detail. Trehalose di- and monocorynomycolates were described for other pathogenic mycobacteria (*M. avium*, *M. intracellulare*), nocardia (*N. asteroides*), and corynebacteria (*C. diphtheriae*, *C. matruchotii*, *C. xerosis*) [10,12]. Surface-active trehalose dicorynomycolates (TDM) play the key role in the pathogenesis of infections caused by these actinobacteria and are characterized by pronounced immunomodulating effects: they stimulate congenital and acquired immune response by inducing the production of cytokines (IL-12, IFN-γ, TNF-α, IL-4, -6, -10) and chemokines (MCP-1, MIP-1α, IL-8) [12,14]. Though purified TDM preparations exhibit immunotropic effect, crude macromolecular complexes of mycobacterial cell walls are characterized by maximum activity [14]. For example, crude extract of *M. bovis* cell walls stimulates the immunopresentation process by modulating the maturation and activation of dendritic cells, while purified preparations of TDM, lipoarabinomannan, and other individual lipid components do not activate dendritic cells [14].

Surfactant and biological activity of TDM is biotechnologically significant, but apparent or po-

Institute of Ecology and Genetics of Microorganisms, Ural Division of Russian Academy of Sciences, Perm; Perm State University. ***Address for correspondence:*** kuyukina@iegm.ru. Kuyukina M.S.

tential pathogenicity of the producer strains and high toxicity of produced glycolipids limit their use [13]. Therefore, the search for GLB producers among representatives of nonpathogenic actinobacteria is an important problem.

GLB preparation was obtained as a result of induced biosynthesis on the basis of apathogenic *Rhodococcus ruber* actinobacteria [6]. Molecular structure of the glycolipid component (~15% of biosurfactant) contains trehalose dimycolate, diacyltrehalose, and monoacyltrehalose [11], characterized by higher polarity in comparison with that of TDM from pathogenic coryne- and mycobacteria. We previously detected an immunomodulating effect of crude GLB on humoral and cellular immune response [1].

Here we analyzed the proliferative response of lymphocytes and the production of TNF-α, IL-1β, and IL-6 (the main cytokines determining polarization of immune response development) *in vitro* in response to GLB produced by *R. ruber*.

MATERIALS AND METHODS

R. ruber strain IEGM 231 from Regional Specialized Collection of Alkanotrophic microorganisms (www.iegm.ru/iegmcol/strains) was cultured in mineral medium with *n*-dodecane [4]. Bacterial cells were cultured in Erlenmeyer flasks (250 ml) in 100 ml nutrient medium on an orbital shaker (160 *rpm*) for 3 days at 28°C.

GLB produced by rhodococcal cells was extracted with methyl-tert-butyl ether on an orbital shaker (150 rpm) for 4 h [6]. The extract was centrifuged for 10 min at 3000*g* and the lower hydrophobic fraction was collected. The solvent was removed using a rotor evaporator at 50°C and the extract was lyophilized. The content of residual hydrocarbon in the extract was measured with an Agilent 6890N gas chromatograph with an Agilent MSD 5973N quadrupole mass-spectrometer and HP-5 MS SN US 1518974-1 quartz column (Agilent). Lipid components of GLB were separated in Silica gel 60-140 mesh column (Merck) using systems of solvents with ascending polarity [6]. Qualitative analysis of lipid fractions was carried out, fatty acid composition and concentration of the glycolipid component were evaluated [6,11]. The preparation was stored in nitrogen at -20°C. Working concentrations of the drug were prepared in saline or nutrient medium 199 in the form of water-oil emulsion by ultrasonic (23 kHz, 30 sec) treatment.

Acute toxicity of the preparation was studied on outbred male albino mice (21-23 g). The animals were divided into 6 groups, 9 per group. Acute toxicity was evaluated after a single intraperitoneal injection of the studied preparation in doses of 1, 3, and 10 g/kg (fresh emulsion in 0.5% NaCl). Controls were injected with equivalent volumes of saline. General appearance of animals, changes in body weight and behavioral reactions were observed for 14 days after drug injection.

For evaluation of proliferative activity of lymphocytes, heparinized venous blood from healthy volunteers (male) aged 22-30 years was used. Leukocytes were cultured in plastic round-bottom 96-well plates (Medpolymer) in complete culture medium (medium 199 with 10 mM HEPES (Sigma), 2 mM L-glutamine (Sigma), 100 μg/ml gentamicin, and 10% FCS (ICN). Cell culture contained 2×10^5 cells in 0.2 ml complete culture medium. Phytohemagglutinin (PHA; Sigma) in concentrations of 1.25, 2.5, 5, 10, and 20 μg/ml served as the mitogen. Fresh emulsion of the studied preparation in medium 199 was added to cultures in concentrations of 10^{-2}-10^{-12} μg/ml. Culturing was carried out in a humid atmosphere with 5% CO_2 at 37°C for 72 h. ^{3}H-Methylthymidine (2 μCi; 10 μl) was added to each well 18 h before the end of culturing. Radioactivity of the samples was measured on a Guardian liquid scintillation counter (Wallac).

The monocyte fraction was isolated by the mechanical method. To this end, the mononuclear fraction was isolated in Ficoll-verograffin density gradient (ρ=1.077) at 400*g*, the cell suspension was then washed twice, suspended in RPMI-1640 (ICN), and left for 1 h at 4°C until termination of mononuclear activation caused by isolation procedure. Cold mononuclear suspension was put into a sterile glass Petri dish and incubated in a thermostat at 37°C for 1 h. Nonadherent cells (lymphocytes) were removed, while adherent cells were collected with a scraper, suspended in RPMI-1640 (ICN), washed twice in this medium, and left for 1 h at 4°C. The monocytes and mononuclear fraction in a concentration of 10^6 cell/ml were cultured for 24 and 48 h in RPMI-1640 (ICN) with 10 mM HEPES (Sigma), 2 mM glutamine (Sigma), 100 μg/ml gentamicin, and 10% FCS (ICN). Cell culture supernatants were stored at -20°C. The concentrations of TNF-α, IL-1β, IL-6 in supernatants were measured using commercial kits (Cytokine Company) according to manufacturer's instruction.

The data were processed statistically using Student's paired *t* test.

RESULTS

Qualitative analysis of GLB from *R. ruber* IEGM 231 detected polar (20%) and nonpolar (80%) li-

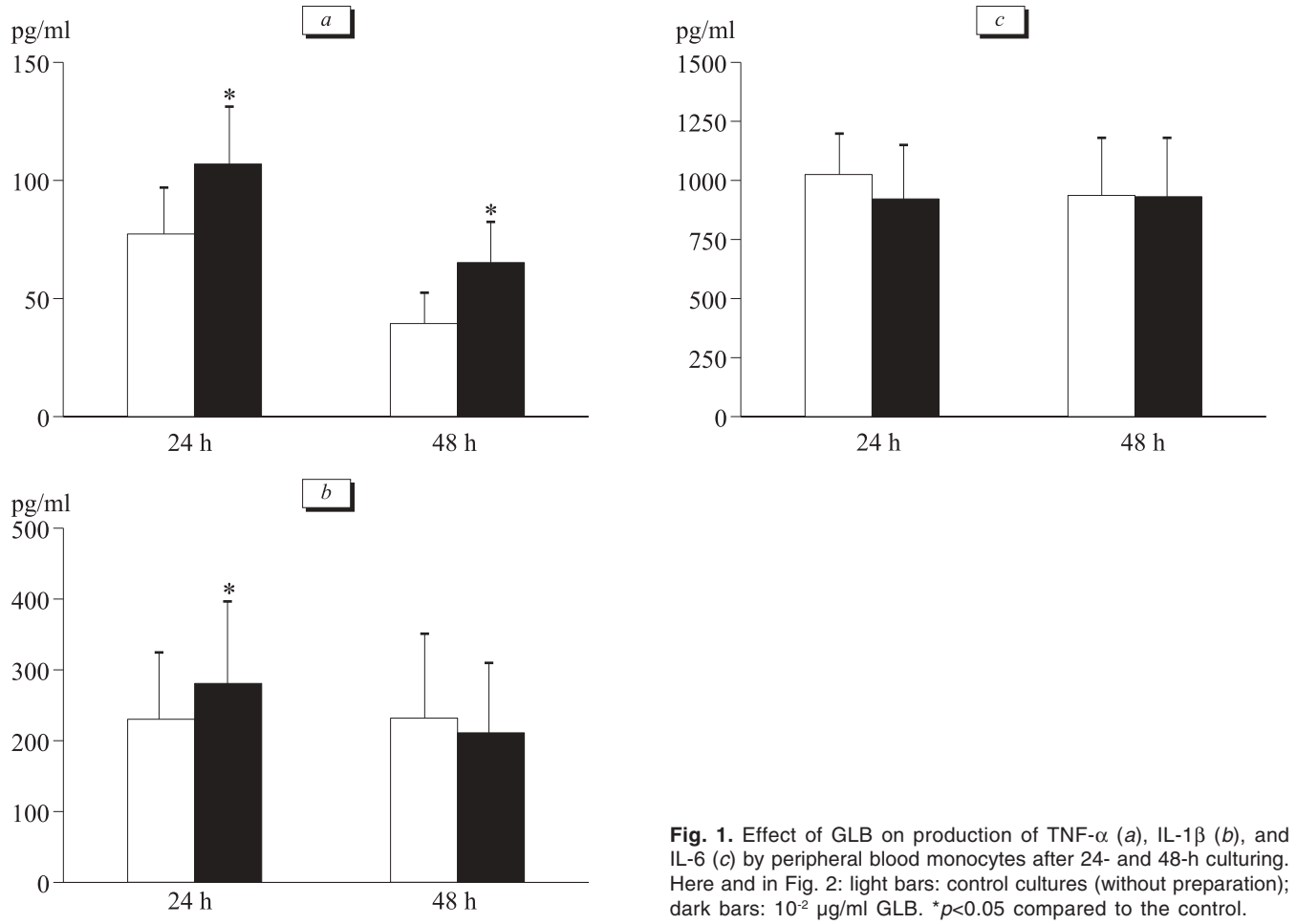

Fig. 1. Effect of GLB on production of TNF-α (*a*), IL-1β (*b*), and IL-6 (*c*) by peripheral blood monocytes after 24- and 48-h culturing. Here and in Fig. 2: light bars: control cultures (without preparation); dark bars: 10^{-2} μg/ml GLB. **p*<0.05 compared to the control.

pids. Nonpolar lipids were presented by acylglycerols and free fatty acids, mainly palmitic, oleic, dodecanoic, and tetradecanoic fatty acids. Thin-layer chromatography of the polar lipid fraction showed 3 main glycolipids and a negligible amount of cardiolipin. The GLB obtained by culturing of the producer strain with *n*-dodecaine was characterized by low (0.1%) content of residual hydrocarbon, due to which no additional purification of the preparation by column chromatography used for removal of residual hydrocarbon in traditional culturing of the producer in medium with *n*-hexadecane was needed [6].

Glycolipids for immunological studies are usually dissolved in organic solvents (chloroform, methanol) or oil-and-water emulsion is prepared with

TABLE 1. Effects of GLB on PHA-Induced Proliferative Response of Normal Human Peripheral Blood Lymphocytes (cpm; *M*±*m*)

GLB concentration, g/ml	Without PHA	PHA concentration, μg/ml				
		1.25	2.5	5	10	20
Control (without preparation)	241±70	1342±451	2455±758	6858±1934	17 110±6585	15 152±4569
10^{-2}	348±79	2041±704	3581±1019	7620±2074	24 926±7105	16 984±4385
10^{-4}	181±22	995±297	2242±701	6304±1957	18 550±4491	16 428±6657
10^{-6}	179±25	1377±464	2788±744	7849±2599	12 355±4236	18 554±5241
10^{-8}	225±51	1174±442	2472±808	7127±2220	18 569±4648	21 092±7017
10^{-10}	245±48	2000±879	2763±1080	6348±2122	13 262±3214	20 001±6134
10^{-12}	228±53	1460±533	2250±794	6152±1681	17 323±4408	19 315±6588

Note. Results of 7 observations are presented for each sampling.

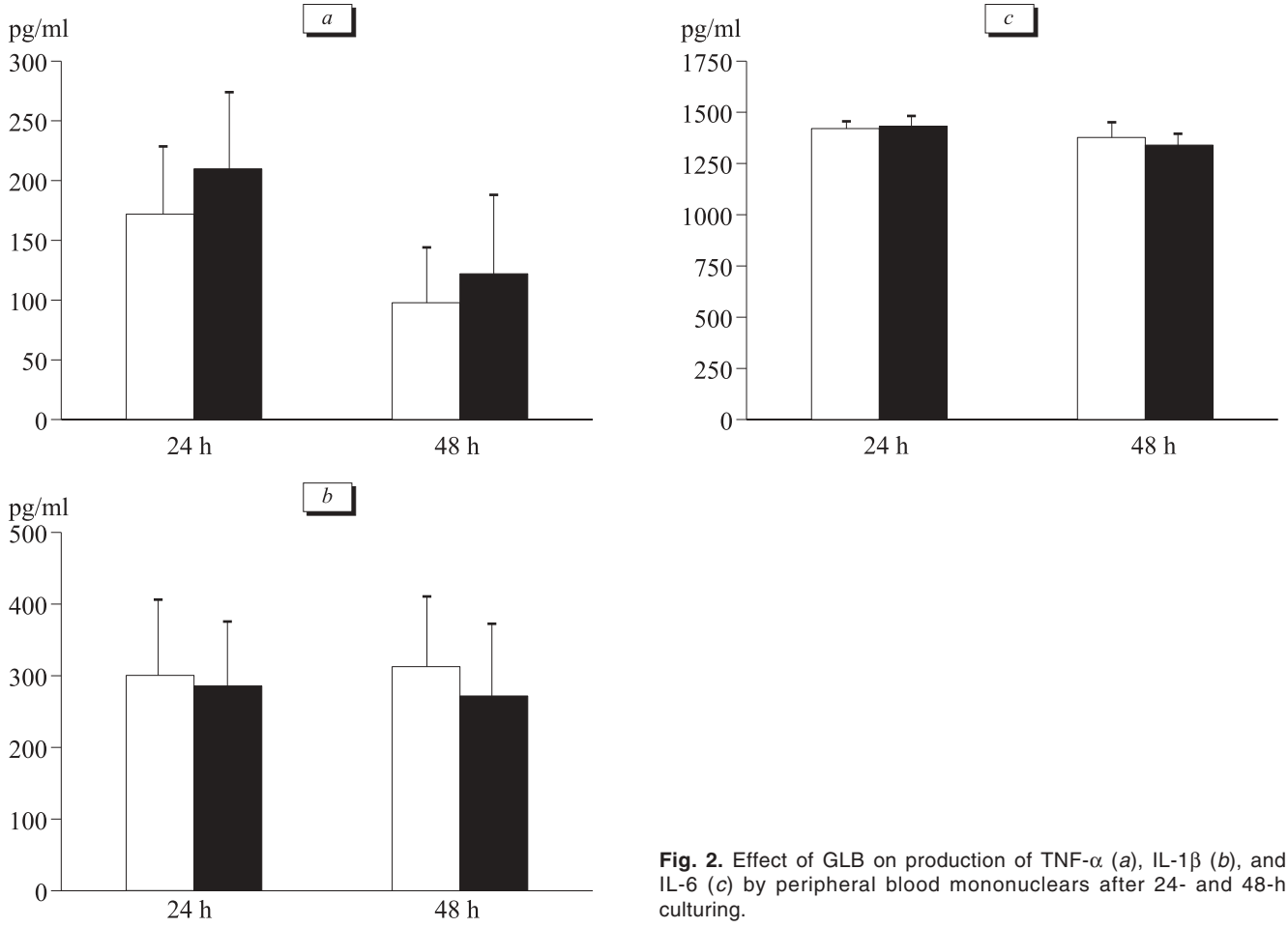

Fig. 2. Effect of GLB on production of TNF-α (*a*), IL-1β (*b*), and IL-6 (*c*) by peripheral blood mononuclears after 24- and 48-h culturing.

mineral oil and chemical surfactants, for example, Twin-80 [2,10]. Due to high emulsifying activity of the resultant GLB and the presence of neutral lipids in its composition, it became possible to obtain highly dispersed stable emulsion of the drug without solvents, chemical surfactants, and other toxic reagents.

Analysis of the toxicity of GLB complex showed that the preparation in the specified doses had no effect on CNS of experimental animals and caused no stimulation or inhibition of their behavioral activity. No deaths or body weight loss were observed over 14-day observation, *i.e.* the drug was not toxic. It is noteworthy that intravenous injection of 300 μg *M. tuberculosis* TDM to mice caused a 20% body weight loss [13]. These data are in line with the results of evaluation of *R. ruber* GLB toxicity on a Microtox toxicity analyzer with *Vibrio fischeri* bioluminescent bacteria as targets, which showed that the studied preparation was 2-10-fold less toxic than bacterial glycolipids from *R. erythropolis* and *Pseudomonas aeruginosa* [4].

Evaluation of the effects on lymphocyte proliferative activity in the lymphocyte blast transformation test (Table 1) showed no significant effect of GLB on ^{3}H-thymidine incorporation in spontaneous and PHA-induced cell cultures.

In a concentration of 10 μg/ml GLB stimulated TNF-α release by monocytes during 24- and 48-h culturing. A less pronounced, but statistically significant stimulatory effect of the preparation on the production of IL-1β was recorded in the supernatants obtained after 24-h incubation of monocytes. On the other hand, no effect on IL-6 production by monocytes was noted (Fig. 1).

A trend to an increase of TNF-α level in 24-h cultures was noted in the mononuclear fraction, but no significant stimulatory effect of GLB on the production of TNF-α, IL-1β, and IL-6 in the mononuclear cell suspension was detected (Fig. 2).

Hence, our findings suggest that the studied GLB activates monocytes and induces the production of IL-1β and TNF-α. Stimulation of TNF-α production was most pronounced, detected throughout the entire period of culturing and manifested as a trend in the mononuclear fraction. Presumably, lymphocytes present in the mononuclear fraction inhibit the synthesis of IL-1β and TNF-α by mono-

cytes in the presence of GLB. It was shown [3,7,9] that cytokines produced by lymphocytes, for example, IL-4, transforming growth factor-β, IL-10, IL-13, suppress the production of TNF-α, IL-1, -6, -8, and -18 by activated monocytes. Stimulation of TNF-α and IL-1β production by *R. ruber* GLB indicates the prospects for further investigation of its immunomodulating and antitumor activities.

The study was supported by the Program for Leading Scientific Schools Support (grant No. RI-112.0/001/290), MKB Program of the Board of the Russian Academy of Sciences, and Russian Foundation for Basic Research (grant No. 07-04-97612-r_ofi).

REFERENCES

1. M. S. Kuyukina, T. A. Yushkova, I. B. Ivshina, and V. V. Yushkov, *Microbiology and Biotechnology of the 21st Century* [in Russian], Minsk (2002), pp. 47-48.
2. G. I. Novik, N. I. Astapovich, M. Pastsyak, and A. Gam'yan, *Mikrobiologiya*, **74**, No. 6, 781-787 (2005).
3. W. L. Goff, W. C. Johnson, S. M. Parish, *et al.*, *Vet. Immunol. Immunopathol.*, **84**, No. 3-4, 237-251 (2002).
4. I. B. Ivshina, M. S. Kuyukina, J. C. Philp, and N. Christofi, *World J. Microbiol. Biotechnol.*, **14**, 711-717 (1998).
5. D. Kitamoto, H. Isoda, and T. Nakahara, *J. Biosci. Bioenerg.*, **94**, No. 3, 187-201 (2002).
6. M. S. Kuyukina, I. B. Ivshina, J. C. Philp, *et al.*, *J. Microbiol. Methods*, **46**, No. 2, 149-156 (2001).
7. M. K. Levings and J. W. Schrader, *J. Immunol.*, **162**, No. 9, 5224-5229 (1999).
8. R. M. Maier and G. Soberon-Chavez, *Appl. Microbiol. Biotechnol.*, **54**, No. 5, 625-633 (2000).
9. A. Nuntapraset, Y. Mori, Y. Muneta, *et al.*, *Comp. Immunol. Microbiol. Infect. Dis.*, **28**, No. 2, 83-101 (2005).
10. R. L. Perez, J. Roman, S. Roser, *et al.*, *J. Interferon Cytokine Res.*, **20**, No. 9, 795-804 (2000).
11. J. C. Philp, M. S. Kuyukina, I. B. Ivshina, *et al.*, *Appl. Microbiol. Biotechnol.*, **59**, No. 2-3, 318-324 (2002).
12. R. Ryll, Y. Kumazawa, and I. Yano, *Microbiol. Immunol.*, **45**, No. 12, 801-811 (2001).
13. I. Sakaguchi, N. Ikeda, M. Nakayama, *et al.*, *Infect. Immunity*, **68**, No. 4, 2043-2052 (2000).
14. S. Tsuji, M. Matsumoto, O. Takeuchi, *et al.*, *Ibid.*, No. 12, 6883-1890.