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TECHNICAL NOTE

In vitro cytokine stimulation assay for glycolipid biosurfactant from *Rhodococcus ruber*: role of monocyte adhesion

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Abstract Glycolipid biosurfactant (GLB) from *Rhodococcus ruber* IEGM 231 was found to stimulate tumor necrosis factor-α (TNF-α), interleukin (IL)-1β and IL-6 production when applied as an ultrasonic emulsion to the adherent human peripheral blood monocyte culture. However, a lack of cytokine-stimulating activity was registered with the GLB applied as a hydrophobic film coating in 24-well culture plates, indicating that it may have been due to its inhibitory effect on monocyte adhesion. The mode of GLB application may therefore play an important role in in vitro assay of immunostimulatory activity of this compound as well as other bacterial glycolipids. Additionally, GLB from *R. ruber* displayed no cytotoxicity against human lymphocytes and therefore could be proposed as a potential immunomodulating and antitumor agent.

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Keywords Glycolipid biosurfactant · *Rhodococcus* · Cytokine-stimulating activity · Monocyte adhesion · Toxicity

Abbreviations

GLB	Glycolipid biosurfactant
TNF	Tumor necrosis factor
IL	Interleukin
TDM	Trehalose-6,6'-dimycolate
IEGM	Institute of Ecology and Genetics of Microorganisms
ELISA	Enzyme-linked immunosorbent assay
LPS	Lipopolysaccharide

Introduction

Microbial glycolipid surfactants show biological activities towards various organisms ranging from prokaryotes to mammals. Such activities are determined by complicated but naturally selected structures of glycolipid biosurfactants allowing them to display more versatile properties compared to conventional chemical surfactants (Kitamoto et al. 2002). Amphiphilic molecules of glycolipids contain various carbohydrates (e.g. rhamnose, sophorose, mannose, and trehalose) as a hydrophilic moiety attached to a hydrophobic moiety represented by various fatty acids, e.g. saturated, unsaturated, branched, or hydroxylated.

Microbial glycolipids such as rhamnolipids from *Pseudomonas aeruginosa* and mannosylerythritol

lipids from *Pseudozyma candida* have been investigated for their possible biomedical applications (Rodrigues et al. 2006). Mycolata group actinobacteria, particularly members of the genera *Corynebacterium*, *Mycobacterium*, *Nocardia*, *Gordonia* and *Rhodococcus*, are characterized by high cell lipid concentration (30–60% of dry cell mass) and the presence of long-chain α-branched β-hydroxylated fatty (mycolic) acids (Chun et al. 1996). Mycolic acids are present in the cell envelope in a free state and as components of glycolipids. Surface-active trehalose di- and monomycolates of pathogenic (*M. tuberculosis*, *C. diphtheriae*) and opportunistic (*M. avium—M. intracellulare* group, *N. asteroids*, *C. matruchotii*, *C. xerosis*) mycolata play a key role in the pathogenesis of infections caused by these actinobacteria and are characterized by pronounced immunomodulating effects (Ryll et al. 2001). Biological activity of these glycolipid biosurfactants (GLBs) is significant, but apparent or potential pathogenicity of the producer strains and high toxicity of produced glycolipids hinder their biomedical applications (Sakaguchi et al. 2000; Franzetti et al. 2010). Therefore, the search for GLB producers among nonpathogenic actinobacteria is of importance. In our earlier studies, the GLB produced by a nonpathogenic *Rhodococcus ruber* strain was obtained; its chemical structure and surfactant properties were investigated (Kuyukina et al. 2001; Philp et al. 2002). It was found that the GLB from *R. ruber* IEGM 231 stimulates in vivo and in vitro immune response (Kuyukina et al. 2007; Chereshnev et al. 2010).

In vitro detection of immunomodulatory activity of bacterial glycolipids is complicated by the relatively high hydrophobicity of their molecules. For immunological analysis, water insoluble glycolipids are dissolved in organic solvents, e.g. chloroform, methanol, or oil-in-water emulsions are prepared with the mineral oil and chemical surfactants, e.g., Tween 80 (Tsuji et al. 2000; Uehori et al. 2003; Novik et al. 2005). As was shown by Yarkoni and Rapp (1978), the toxicity and biological activity of trehalose-6,6'-dimycolate (TDM) from *M. tuberculosis* depend on the size distribution of the emulsion oil droplets. Therefore, we have used an ultrasonic emulsion of the GLB from *R. ruber* in water to provide a highly dispersed emulsion with uniform oil droplets (Kuyukina et al. 2007). Due to emulsifying activity of the crude GLB and the presence of neutral lipids in its composition (Kuyukina et al. 2001), it was possible to obtain a stable oil-in-water emulsion without mineral oil, solvent, chemical surfactant, or other potentially toxic reagent additions.

In some new in vitro protocols, to study the cytokine-stimulating activity of TDM, murine neutrophils and macrophages were incubated in TDM-coated microplates (Sakaguchi et al. 2000; Okamoto et al. 2006; Ozeki et al. 2006) and onto TDM-coated coverslips (Lima et al. 2001). This method avoids the emulsification procedure and allows preparation of stock TDM-coated microplates or coverslips, which could be stored and used in mass immunological tests. We supposed, though, that an application of TDM or other glycolipids as a hydrophobic film on the surface of microplates or coverslips could prevent the adhesion of human or animal cells tested and therefore influence their cytokine production. Numerous in vitro experiments (Hofsli et al. 1988; Petit-Bertron et al. 2003; Pomorski et al. 2004) have reported that monocyte adherence induces expression of cytokine (TNF, IL-1β, IL-8) genes and potentiates monocyte responsiveness to activating signals. It was also reported (Rodrigues et al. 2006) that microbial glycolipid biosurfactants possess anti-adhesive activity against various bacteria and yeasts. To verify the proposal suggested, we have studied monocyte adhesion to the GLB-covered and uncovered microplates and also compared their spontaneous and LPS-induced cytokine production.

The purpose of the present study was to compare two methods for in vitro assay of GLB immunostimulatory activity using 24-well plates, particularly, hydrophobic film coating and ultrasonic emulsion application. The cytotoxicity of the GLB for human lymphocytes was also investigated.

Materials and methods

GLB preparation

The GLB was extracted from *n*-dodecane-grown culture of *R. ruber* IEGM 231 with methyl tertiary-butyl ether and partly purified as described elsewhere (Kuyukina et al. 2001). Structural elucidation of the GLB revealed a complex of trehalose mycolates with various alkyl chain lengths (Philp et al. 2002). The

GLB crude extract contained, apart from glycolipids, 44% non-polar lipids including acylglycerols and free fatty acids (Kuyukina et al. 2001). The GLB samples were kept at −20 °C under nitrogen until used. Ultrasonic emulsions containing various concentrations of the GLB were prepared in RPMI 1640 medium (ICN) by sonication (23 kHz, 10 s).

Isolation of monocyte-enriched peripheral blood cells

Venous blood from healthy male volunteers aged from 22 to 30 was centrifuged in a ficoll-verografin density gradient. Peripheral blood mononuclear cells were then washed and resuspended in RPMI 1640 medium. The cell suspension was incubated for 45 min at 37 °C in a plastic dish at the cell density of 10^6 cells/mL. Nonadherent cells were then removed, and the adherent cells were used as monocyte-enriched peripheral blood mononuclear cells. These cells were suspended in RPMI 1640 supplemented with 10 mM HEPES (Sigma), 2 mM L-glutamine (Sigma), 100 μg/mL gentamicin and 10% fetal calf serum (FCS, ICN).

Cytokine production stimulation assay

In one experiment, 100-μL aliquots of freshly prepared GLB (1, 10, 100 and 1,000 μg/mL) ultrasonic emulsions in RPMI 1640 were placed into 24-well, flat-bottom polystyrene culture plates (Orange Scientific, Belgium) containing adherent monocyte cultures (10^6 cells/mL, 900 μL). As controls, 100-μL aliquots of RPMI 1640 medium were added to monocyte-containing plates.

In another experiment, the GLB was applied as a hydrophobic film coating in 24-well plates. GLB-coated plates were prepared as described by Sakaguchi et al. (2000). Namely, the GLB was dissolved in isopropanol to the concentrations of 1, 10, 100 and 1,000 μg/mL (similar to those used for emulsion preparation), and 100-μL aliquots were placed into wells. The plates were allowed to dry in a sterile atmosphere overnight. The monocytes (10^6 cells/mL, 1 mL) were then added to GLB-coated and uncoated (control) plates.

Some monocyte cultures were simultaneously incubated with GLB and lipopolysaccharide (LPS). The LPS from *Salmonella enterica* serotype *typhimurium* (Sigma, Cat. #L6143) was added in a concentration of 1 μg/mL either with GLB-emulsions to the adhered monocytes or with a monocyte suspension to the GLB-coated plates.

All plates were incubated in humidified atmosphere with 5% CO_2 at 37 °C for 24 h. The supernatants were collected and stored at −20 °C. Cytokines released in the supernatants were measured using ELISA kits for IL-1, IL-6 and TNF-α (Vector-Best, Novosibirsk, Russia).

Adhesion test

For the adherence assay, the GLB was applied as a hydrophobic film coating in 96-well polystyrene flat-bottom microplates (Costar) as previously described for 24-well plates. Final GLB concentrations (1, 10 and 100 μg/mL) were similar to ones used in cytokine production assay. Uncoated microplates were used as controls. A monocyte suspension in RPMI 1640 medium (10^6 cells/mL, 100 μL) was added to microplate wells. Microplates were incubated in humidified atmosphere with 5% CO_2 at 37 °C for 24 h. After the RPMI 1640 medium with non-adherent cells was carefully removed from the wells, the adherent monocytes were fixed with 96% ethanol or 1% glutardialdehyde, stained with crystal violet for 20 min, washed with H_2O and dried. For the quantitative analysis of cell adhesion, the dye was dissolved in 100 μL of ethanol–water-acetic acid (50:49.9:0.1), and the microplates were examined with a microplate reader (Multiskan Ascent, Thermo Labsystems, Finland) at 450 nm.

Cytotoxicity test

In vitro toxicity of the GLB was assessed by measuring the inhibition of proliferative activity of cultured human peripheral blood lymphocytes. Lymphocytes were isolated from the same blood source as monocytes. After the erythrocytes in blood samples were allowed to sediment, the buffy coat layer was centrifuged at 1,500 rpm for 20 min, resuspended in RPMI 1640, and used for lymphocyte culture. Lymphocyte cultures (2×10^6 cells/mL, 100 μL) in RPMI 1640 with added 10 mM HEPES (Sigma), 2 mM L-glutamine (Sigma), 100 μg/mL gentamicin, and 10% FCS (ICN) were placed into 96-well polystyrene round-bottom microplates (Medpolymer, St.-Petersburg, Russia). Freshly prepared emulsions

(100 μL) of the GLB (adjusted to the final concentrations of 0.01, 1, 100 and 10,000 μg/mL) in RPMI 1640 were added to the cultures. Microplates were incubated in humidified atmosphere with 5% CO_2 at 37 °C for 72 h. The cells were labeled with 2 μCi of [^{3}H]thymidine (Isotope, St.-Petersburg, Russia) for an additional 18 h and measured using a Guardian liquid scintillation counter (Wallac).

Statistical analysis

Data in tables and figures are presented as means ± standard errors. Differences between groups were analyzed using the paired 2-way ANOVA and Student's *t* test. Results were considered significant at $P < 0.05$.

Results

Human peripheral blood monocytes were analyzed in vitro for spontaneous and LPS-stimulated cytokine production in response to GLB applied as an ultrasonic emulsion or a hydrophobic film (Fig. 1a). It was found that monocytes incubated with GLB emulsions released significantly higher levels of cytokines compared to control (no GLB) monocyte cultures. Similar stimulatory effects were observed with three different GLB concentrations (0.1, 1 and 10 μg/mL), while the higher (100 μg/mL) concentration tested resulted in decrease of TNF-α and IL-1β levels almost to the control values.

In contrast, the GLB applied as a hydrophobic film in all tested concentrations exhibited the lack of stimulatory effect on cytokine production by monocytes. Cells released significantly lower TNF-α, IL-1β and IL-6 levels when incubated in GLB-coated microplates compared to cells incubated in the presence of GLB emulsions. Moreover, the cytokine levels produced by monocytes in GLB-coated microplates were as low as those produced by control (no GLB) cells.

Simultaneous monocyte incubation with both GLB and LPS had no significant effects on cytokine secretion (Fig. 1b) compared to the effect of LPS alone. However, control values for LPS-stimulated cytokine production were significantly higher than that of non-stimulated monocytes (see Fig. 1a), suggesting high monocyte activation by cell-wall components of the pathogenic bacterium *S. enterica*. The mode of GLB application, either as film coating or emulsion, seemed to have no effect on LPS-stimulated cells, except the significant increase in TNF-α production of monocytes incubated with 0.1 μg/mL of GLB emulsion compared to GLB film. Also, the GLB in concentration of 100 μg/mL inhibited LPS-induced production of TNF-α when applied as an emulsion, and inhibited LPS-induced production of IL-1β when applied as a film coating or emulsion.

Adhesion has been shown to play an important role in monocyte and macrophage cellular activity and to induce their in vitro and in vivo cytokine production (Petit-Bertron et al. 2003). We have studied the influence of GLB hydrophobic film on monocyte adhesion to 96-well microplates. As shown in Fig. 2, there was an evident inhibition of cell adhesion to GLB-covered wells; clear free of cells zones appeared alongside with dark spots of conglutinated mass of monocytes. In contrast, homogenous layers of monocytes adherent to plastic surfaces were observed in control (no GLB) wells. Furthermore, GLB emulsion added in similar concentration did not affect smooth monocyte layer adherent to plastic (data not shown). The GLB-inhibiting effect on monocyte adhesion was found to be concentration-dependant (Fig. 3). Particularly, 50–60% decrease in cell adhesion was registered when the GLB applied in concentrations 1–10 μg/mL (similar to concentrations used in cytokine production experiments), compared to control (no GLB addition) monocyte cultures.

In vitro toxicity of the GLB was assessed from the inhibition of human peripheral blood lymphocyte growth. Results on cultured lymphocyte proliferative activity in the lymphocyte blast transformation test (Fig. 4) showed no inhibitory effect of the GLB (10^{-2} to 10^4 μg/mL) on ^{3}H-thymidine incorporation in cell cultures. Furthermore, small but statistically insignificant lymphocyte stimulation was observed with the maximum tested GLB concentration (10 mg/mL). These data confirmed our previous results (Kuyukina et al. 2007) showing the lack of in vivo toxicity of the GLB.

Discussion

We found that GLB from *Rhodococcus ruber* had stimulated TNF-α, IL-1β and IL-6 production when applied as an ultrasonic emulsion to an adherent

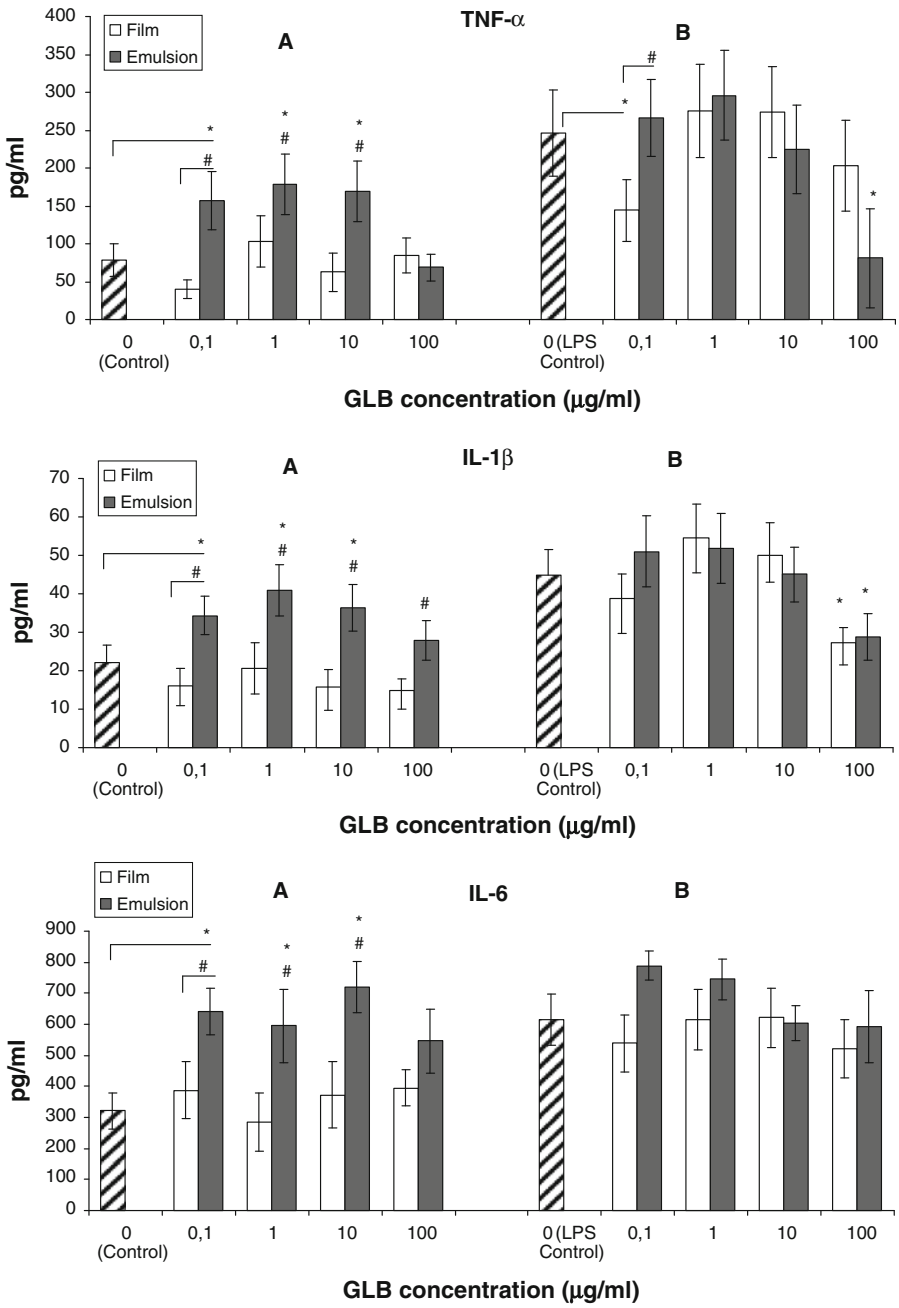

Fig. 1 Spontaneous (**a**) and LPS-stimulated (**b**) production of TNF-α, IL-1β and IL-6 by human blood monocytes cultivated in the presence of GLB applied as a hydrophobic film (*white columns*) or ultrasonic emulsion (*black columns*). Control (no GLB) monocyte cultures (*hatched columns*) were cultivated without LPS (**a**) or with 1 μg/mL LPS (**b**). GLB concentrations are 0.1, 1, 10 and 100 μg/mL. Data represent means ± SD from 6 experiments. Significant differences (*, $P < 0.05$) in cytokine production were found between GLB-stimulated and control monocyte cultures; significant differences (#, $P < 0.05$) in cytokine production were found between monocyte cultures incubated with GLB emulsion and hydrophobic film

human peripheral blood monocyte culture. These findings correlate with our previous results showing increased secretion of IL-1β, TNF-α (Kuyukina et al. 2007), and IL-12 (Chereshnev et al. 2010) by monocytes in the presence of GLB emulsion. However, lack of cytokine-stimulating activity was registered with

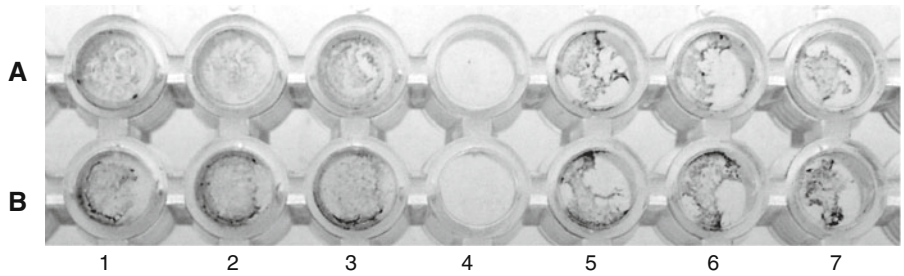

Fig. 2 Inhibition of monocyte adhesion by GLB applied as a hydrophobic film to 96-well microplates. Wells 1–3, control—no GLB addition; wells 4—blank; wells 5–7—GLB (1 μg/mL). Adherent monocytes were fixed with 1% glutardialdehyde (*A*) or 96% ethanol (*B*), stained with crystal violet, washed with distilled water, and dried

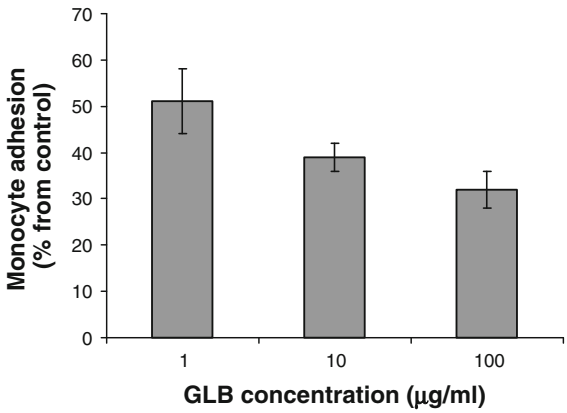

Fig. 3 Inhibition of monocyte adhesion by GLB. Monocytes were incubated in microplate wells coated with GLB-film or uncoated wells (control) for 24 h. After incubation, non-adherent monocytes were removed and adherent monocytes were fixed and stained with crystal violet as previously described. For quantitative analysis of cell adhesion, the dye was dissolved in ethanol–water-acetic acid (50:49.9:0.1) and the microplates examined with a microplate reader at 450 nm. Data are expressed as percentages from control (without GLB) optical density values, which were defined as 100%. Results are shown as percentage means ± SD from 12 microplate wells

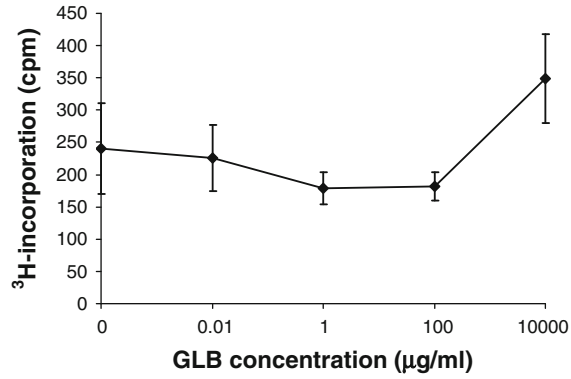

Fig. 4 Effect of GLB on human lymphocyte proliferation. Ultrasonic emulsions of GLB were added to lymphocyte cultures. Lymphocytes were incubated for 72 h, labeled for an additional 18 h with [^{3}H]thymidine, and measured using a Guardian liquid scintillation counter. ^{3}H-incorporation levels express proliferative activity of lymphocytes treated with different concentrations of GLB. Data represent means ± SD from 7 experiments

GLB applied as a hydrophobic film to monocyte suspension. We have hypothesized that low cytokine production was due to GLB inhibitory effect on monocyte adhesion. Indeed, cytokine levels produced by monocytes incubated in GLB-coated microplates were as low as those produced by non-stimulated cells. It was also found that a GLB-covering film partly prevents monocyte adhesion to plastic. However, our findings are in controversy with the published results (Sakaguchi et al. 2000; Ozeki et al. 2006) showing increased TNF-α release by murine neutrophils and macrophages after the incubation in TDM-coated microplates, and with data of Lima et al. (2001) showing high cytokine (TNF-α, IL-6, 10, 12, IFN-γ) release by murine macrophages incubated in TDM-covered glass coverslips. One possible explanation for the difference between our and other authors' results lies in the fact that cytokine secretion by monocytes is a complex process requiring both cell adhesion and exposure to a chemical inducer such as bacterial antigen or endotoxin (Petit-Bertron et al. 2003). Since the GLB applied as a hydrophobic film was apparently less available for monocyte receptors compared to the GLB emulsion, it arose a weaker cytokine response. In contrast, the LPS introduced into the GLB-covered plates stimulated the cytokine production of monocytes at levels similar to those of control LPS-incubated cells (see Fig. 1B).

In addition, the GLB from *R. ruber* seems to be a relatively less active immunostimulatory agent compared to TDM from *M. tuberculosis* (Yasuda 1999), so even at high concentrations, its signaling effect could not be strong enough to induce cytokine production of non-adherent monocytes. Furthermore, dose-dependent TNF-α production by murine macrophages incubated in TDM-coated microplates decreased considerably at higher TDM concentrations (Okamoto et al. 2006; Ozeki et al. 2006) possibly due to both TDM cytotoxicity and inhibition of cell adhesion. In our experiments, the GLB applied at high concentration (100 μg/mL) significantly decreased TNF-α and IL-1β production of monocytes (particularly, in the presence of LPS), while it was not toxic for human peripheral blood lymphocytes in the range of 0–10 mg/mL. This fact could be explained by the combined inhibiting effect of LPS and GLB and needs further investigation.

There are only few reports on human monocyte/macrophage/neutrophil stimulation in vitro by TDM or other mycobacterial glycolipids using oil-in-water emulsions (Tsuji et al. 2000; Uehori et al. 2003; Franzetti et al. 2010) and glycolipid-coated polystyrene beads (Puissegur et al. 2007; Simons et al. 2007); however, there are no similar publications describing TDM-coated microplate application. As was shown by Perez et al. (2000), addition of TDM-coated polystyrene beads led to some interfering nonspecific stimulation of mice bone marrow-derived macrophages resulting in increased levels of TNF-α, IL-1β, IL-6 possibly induced by cell adhesion to the beads.

In conclusion, the present study reveals the importance of bacterial glycolipid application mode for in vitro cytokine-stimulation assay; this is especially important for biologically active glycolipids produced by non-pathogenic bacteria (e.g. GLB from *Rhodococcus ruber*), having low toxicity and moderate immunostimulatory activity. In our experiments, the GLB-in-water emulsion obtained by sonication without the use of surfactants or mineral oil was applied to the monocyte cultures resulting in statistically significant increase of TNF-α, IL-1β and IL-6 production. Thus, cytokine stimulating activity and lack of in vivo/vitro toxicity of GLB from *R. ruber* suggest its potential as an immunomodulatory and antitumor agent.

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