In vitro production of cytokines is influenced by sulfatide and its precursor galactosylceramide

Lone Frier Bovin[a,*], Pam Fredman[b], Jan-Eric Månsson[b], Karsten Buschard[c], Klaus Bendtzen[a]

[a] *Institute for Inflammation Research, IIR 7521, H:S Rigshospitalet National University Hospital, Tagensvej 20, DK-2200 Copenhagen, Denmark*
[b] *Institute of Clinical Neuroscience, Section of Experimental Neuroscience, University of Göteborg, Mölndal, Sweden*
[c] *Bartholin Institute, H:S Kommunehospitalet, Copenhagen, Denmark*

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Abstract Effects of sulfatide and its precursor galactosylceramide (gal-cer) on the kinetics of production of cytokines were studied. In human mononuclear leucocytes, gal-cer but not sulfatide induced significantly increased amounts of interleukin (IL)-1β, IL-6 and tumor necrosis factor (TNF) mRNA. In phytohemagglutinin-stimulated cultures, gal-cer increased the levels of IL-1β and IL-6 mRNA and secreted IL-1β and IL-6, while sulfatide decreased the amounts of IL-6 mRNA and secreted IL-6. Gal-cer also increased TNF secretion. In lipopolysaccharide-stimulated cells, sulfatide but not gal-cer decreased the secretion of IL-1β and IL-10, a potent suppressor of production of many cytokines. Thus, sulfatide and gal-cer affect cytokine production differently, most likely at the level of gene expression. This may have implications in diseases where inflammatory cytokines play a pathogenic role.

Key words: Sulfatide; Galactosylceramide; Interleukin; Tumor necrosis factor; Cytokine mRNA; Gene expression

1. Introduction

The effector and regulatory functions of mononuclear leucocytes (MNCs) are to a large extent related to their capacity to secrete inflammatory cytokines such as interleukin (IL)-1β, tumor necrosis factor (TNF) and IL-6 and anti-inflammatory cytokines such as IL-10. In inflamed tissues, an array of molecules induces cytokine secretion from MNCs. However, apart from cytokines themselves, little is known of other cytokine-modulating molecules expressed by tissues undergoing pathologic alterations.

Glycolipids are ubiquitous structural components of the lipid bilayer in most eukaryote biomembranes [1,2]. Sulfatide (galactosylceramide (gal-cer)-3-*O*-sulfate) is physiologically found in relatively large amounts in central and peripheral nervous tissues, mainly in connection with myelin [3]. Sulfatide is also expressed in pancreatic islets of Langerhans and in specific areas in the kidney, the choroid layer of the eye, the ovum and circulating granulocytes [4–6]. The immediate metabolic precursor of sulfatide, gal-cer, is also found in myelin and in the islets of Langerhans in amounts similar to sulfatide [2]. In myelin sheets, sulfatide and gal-cer are important in maintaining the integrity of the sheets, but other possible roles of sulfatide and gal-cer are largely unknown [2,7].

Sulfatide is a ligand for the adhesion molecule L-selectin and has reported to be involved in intercellular signalling through increased levels of cytosolic free calcium in human neutrophils and monocytes [8,9]. Sulfatide and gal-cer have been reported to influence the production of several cytokines thought to be involved in inflammatory disease processes [8–10], whereas GM1 is a ganglioside (sialic acid-containing glycosphingolipid) with a mild immunosuppressive activity when tested on the T-lymphocyte function [11,12].

In a previous in vitro study, we found that exogenously added sulfatide and gal-cer affected cytokine secretion from phytohemagglutinin (PHA)- and lipopolysaccharide (LPS)-stimulated MNCs [10]. The aim of this study was to further elucidate the effects of sulfatide and gal-cer on the in vitro production of both pro- and anti-inflammatory cytokines.

2. Materials and methods

2.1. Isolation and characterization of sulfatide, gal-cer and GM1

The monosialoganglioside GM1 served as a negatively charged glycosphingolipid control. Sulfatide, gal-cer and GM1 were isolated by high performance liquid chromatography from bovine brain as previously described and their purity was controlled by mass spectrometry [13]. Endotoxin was measured by a limulus amebocyte lysate (LAL) assay (QCL-1000, Bio-Whittaker, Walkersville, MD, USA).

2.2. Acid treatment of sulfatide

Removal of the sulfate group from sulfatide was performed as follows. Sulfatide, 2 μmol, was incubated with 2 ml 0.05 M H_2SO_4 for 20 h at room temperature. Addition of 1 ml 0.1 M NaOH stopped the reaction and 4 ml chloroform was added for phase partition. The lower phase was washed twice with methanol/water 1:1 (v/v). The organic phase was evaporated under N_2 and the acidic treatment was repeated. After a second washing procedure, the gal-cer was isolated as detailed elsewhere [13].

2.3. Cell cultures

MNCs from healthy adult blood donors were prepared by centrifugation of citrate blood buffy coats on Lymphoprep (Nycomed, Oslo, Norway). The cells were cultured in RPMI 1640 (Biological Industries, Kibbutz Beit Haemek, Israel) containing 2% fetal calf serum (FCS) (HyClone, Logan, UT, USA) and 25 μg/ml gentamicin (ICN Flow, Irvine, UK). MNCs, 2×10^6 cells/ml, were incubated with 30 μM gal-cer, GM1 or sulfatide, together with 0.1 μg/ml LPS (*Escherichia coli* 055:B5, Difco Laboratories, Detroit, USA) or 5 μg/ml PHA (Difco). After incubation, the supernatants were frozen at −20°C and the cell pellets were kept at −80°C for a maximum of 2 days before RNA extraction. The LAL assay did not reveal endotoxin contamination in the culture medium or the FCS.

2.4. Enzyme-linked immunosorbent assay (ELISA) for human cytokines

IL-1β, IL-2, IL-6, IL-10 and TNF were measured by a double-sandwich ELISA as described for IL-6 [14]. Briefly, Immuno-Maxisorb plates (Nunc, Roskilde, Denmark) were coated with protein-A affinity-purified unconjugated rabbit IgG raised against the purified recombinant cytokines. Non-attached sites were blocked with 1% (v/v)

*Corresponding author. Fax: (45) (35) 45 75 54.
E-mail: bovin@post7.tele.dk

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human serum albumin (State Serum Institute, Copenhagen, Denmark) and 4% (w/v) milk (Irma, Rødovre, Denmark) in phosphate-buffered saline. Biotinylated polyclonal rabbit antibodies to the cytokines were used for detection along with streptavidin-peroxidase (Kirkegaard and Perry, Gaithersburg, MD, USA). 1,2-Phenylenediamine (DAKO, Glostrup, Denmark) was used as substrate and the plates were read at 492 nm. Inter- and intra-assay coefficients of variations were below 10%. The sensitivity limit of the ELISAs was 10–15 pg/ml and the concentration range for the assays was up to 2 ng/ml. The different ELISAs were calibrated with the corresponding international standards (National Institute for Biological Standards and Controls, Potters bar, Hertfordshire, UK). The antibodies used in the five ELISAs did not cross-react or detect a number of other human cytokines, including recombinant IL-1α, IL-1 receptor antagonist and lymphotoxin (also termed TNFβ).

2.5. RNA purification and hybridization

RNA was purified from frozen cell pellets (10^7 cells per RNA preparation) and hybridized as previously described [15]. RNA was separated on 1% (w/v) agarose gels using glyoxal and acridine orange [16]. cDNA probes for IL-6 (*Taq*I/*Ban*II insert), TNF (*Eco*RI insert), IL-1β (*Eco*RI/*Pst*I insert) and for the household gene GAPDH (*Pst*I insert) were isolated from plasmids. After hybridization, the membranes were exposed to a Fuji imaging plate (type BAS-III) and scanned in a Fujix bio-imaging analyzer BAS 2000 (Raytest Isotopenmessgeräte, Straubenhardt, Germany). Excitation was quantitated by densitometry (Tina, Raytest). The amounts of IL-1β, IL-6 and TNF mRNA were calculated relative to the amount of the corresponding GAPDH mRNA.

3. Results

In the first series of experiments, we examined the dose response of sulfatide, gal-cer and GM1 on the production of cytokines by MNC. IL-1β, IL-6 and TNF mRNA were measured, together with the levels of secreted IL-1β, IL-2, IL-6, TNF and IL-10. The glycolipids were tested at concentrations from 0.1 μM to 300 μM on MNCs co-incubated with 0.1 μg/ml LPS or with 5 μg/ml PHA. Generally, sulfatide, gal-cer and GM1 did not substantially affect cytokine mRNA production or secretion below 10 μM (data not shown).

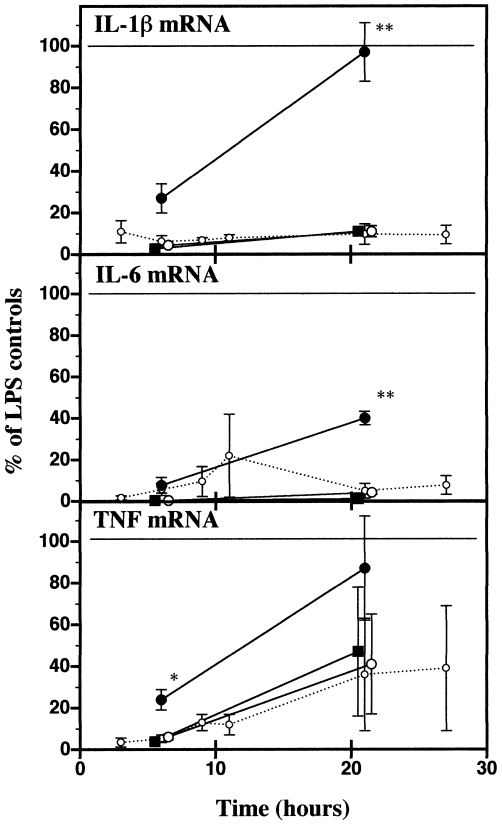

Fig. 1. Cytokine mRNA production. MNCs were cultivated in medium alone (○, dashed lines) or in medium with 30 μM of each of the glycolipids: sulfatide (■), gal-cer (●) and GM1 (○). Data are shown as percentages of LPS controls ± S.E.M., $n = 5$. * $P = 0.04$, ** $P = 0.02$; the Mann-Whitney rank sum test.

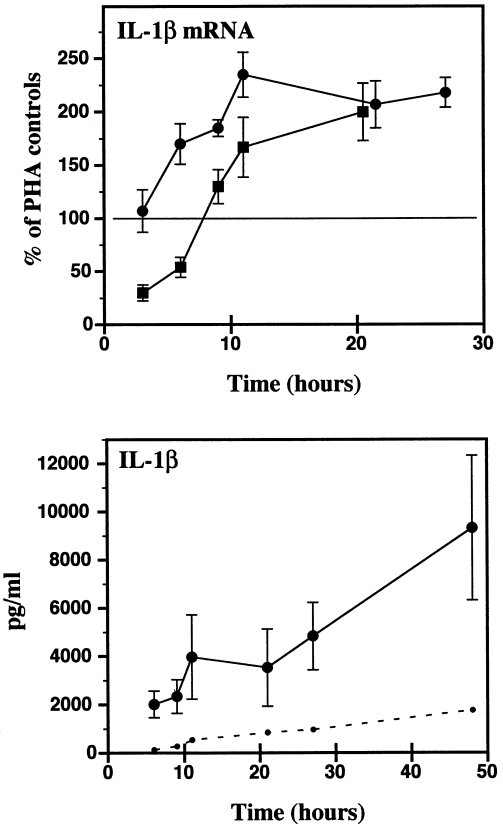

Fig. 2. PHA-induced IL-1β mRNA/protein production. Effects of sulfatide and gal-cer on IL-1β mRNA production (upper panel) and IL-1β secretion (lower panel). MNCs were cultivated with 5 μg/ml PHA and 30 μM glycolipid. Symbols are as in Fig. 1. Data are shown as percentages of PHA controls ± S.E.M., $n = 4$ (upper panel) and as pg IL-1β/ml ± S.E.M., $n = 4$ (lower panel). The broken line shows the PHA control.

In the second series of experiments, we examined the time course of co-stimulating LPS- or PHA-stimulated MNCs with 30 μM sulfatide, gal-cer and GM1.

3.1. Effect of glycolipids alone

As shown in Fig. 1, only small amounts of cytokine mRNA were produced in MNCs when cultivated in medium alone, in medium containing sulfatide or in medium containing GM1. A minor increase in the level of TNF mRNA was seen with prolonged incubation. An increased secretion of TNF, however, did not follow this.

In contrast, significantly increased amounts of IL-1β, IL-6 and TNF mRNA were found in cells from cultures containing gal-cer. In cultures containing sulfatide, the production of IL-6 mRNA was slightly but not significantly reduced compared to IL-6 mRNA production from MNCs cultured in medium alone.

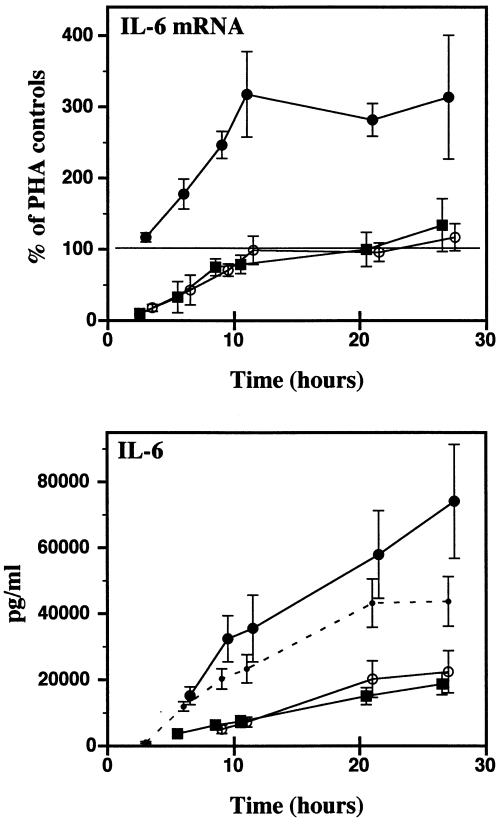

Fig. 3. PHA-induced IL-6 mRNA/protein production. Effects of sulfatide and gal-cer on IL-6 mRNA production (upper panel) and IL-6 secretion (lower panel). Data are shown as percentages of PHA controls ± S.E.M., $n=4$ (upper panel) and as pg IL-6/ml ± S.E.M., $n=4$ (lower panel). The broken line shows the PHA control. See legend to Figs. 1 and 2.

3.2. Effect on PHA-stimulated MNCs

PHA primarily functions as a non-specific activator of T-lymphocytes. Accordingly, PHA is only a weak inducer of the cytokines IL-1β and IL-6, which in MNCs cultures are primarily derived from the monocytes/macrophages (see legends to Figs. 2 and 3).

As shown in Fig. 2, both gal-cer and sulfatide increased the amount of stimulated IL-1β mRNA. However, gal-cer accelerated the formation of PHA-induced IL-1β mRNA compared with sulfatide. While only small amounts of IL-1β were secreted from MNCs stimulated with PHA alone and co-stimulation with sulfatide failed to release cytokines at measurable levels, co-stimulation with gal-cer increased the amounts of IL-1β in the supernatants.

As shown in Fig. 3, sulfatide initially decreased the amounts of IL-6 mRNA and secreted IL-6 from PHA-stimulated MNCs. GM1 had a similar effect. In contrast, gal-cer substantially increased the amounts of IL-6 mRNA and secreted IL-6. In PHA-stimulated MNCs, sulfatide induced a minor increase in the secretion of IL-2 and a decrease in IL-10. GM1, but not gal-cer, had similar effects (not shown). IL-2 and IL-10 mRNA levels were not examined.

3.3. Effect on LPS-stimulated MNCs

LPS is a strong stimulator of monocyte/macrophage-derived IL-1β, IL-6 and TNF with a limited effect on T-lymphocyte-derived cytokines, such as IL-2. Hence, LPS induced 10 times more IL-6 mRNA and had a 6-fold stronger effect on IL-1β mRNA production than PHA (not shown). The amounts of secreted IL-1β, IL-6, TNF and IL-10 were also substantially increased compared to those released in response to PHA (not shown).

As shown in Fig. 4, co-incubation with sulfatide decreased the LPS-stimulated secretion of IL-1β and IL-10, while gal-cer increased the secretion of IL-1β, but not IL-10. GM1 inhibited the secretion of TNF.

3.4. Endotoxin (LPS) contamination of gal-cer

While the sulfatide and GM1 preparations contained less than 6 pg LPS/μmol, a LAL assay revealed a slight endotoxin contamination of the gal-cer preparation: 70 pg LPS/μmol gal-cer, resulting in LPS levels in the cell cultures up to 2 pg/ml. This, however, did not influence the cytokine mRNA induction or cytokine secretion (data not shown). To further examine whether the slight LPS contamination underlined the different effects of sulfatide and gal-cer on cytokine production, sulfatide was treated with acid. This removes the sulfate group and thus generates gal-cer and acid treatment does not affect LPS. As expected, acid-treated sulfatide, containing 50 pg LPS/μmol gal-cer, had the same effect on IL-6 mRNA production and IL-6 secretion as native gal-cer.

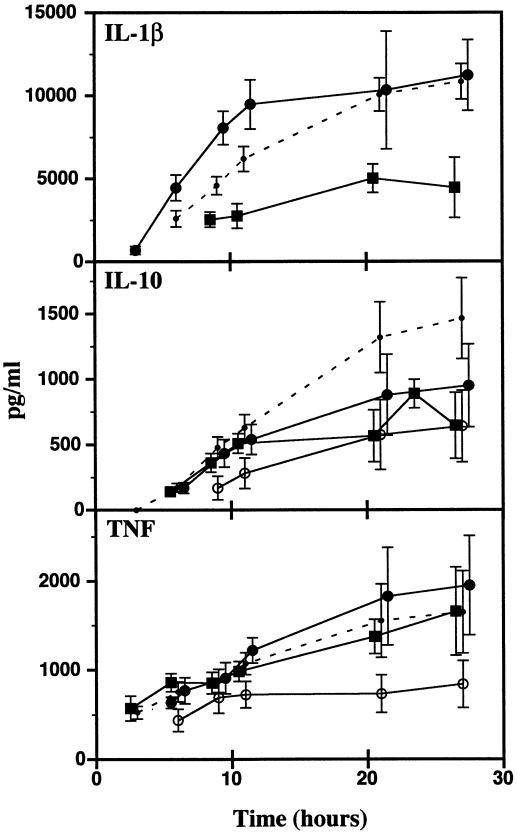

Fig. 4. LPS-induced cytokine production. MNCs were cultivated with 30 μM glycolipid and 0.1 μg/ml LPS. Data are shown as pg/ml ± S.E.M., $n=4$. The broken line shows the LPS control. Symbols are as in Fig. 1.

4. Discussion

Sulfatide alone failed to affect IL-1β or TNF mRNA but reduced the expression of IL-6 mRNA relative to its expression in MNCs cultured in medium alone. In contrast, gal-cer alone significantly enhanced the expression of IL-1β, TNF and IL-6 mRNA in MNCs. Thus, the cytokine modulatory effects of sulfatide and gal-cer are dependent on sulfation of the galactose ring. We could not confirm the pattern of cytokine release reported by Constantin et al. [9], most likely because Constantin et al. used 4–15 times higher levels of sulfatide to show significant effects on TNF, IL-1β and IL-6. Sulfatide forms micelles in aqueous solutions, the sizes of which are dependent on the sulfatide concentration.

While PHA primarily stimulates T-lymphocytes to cytokine production, LPS, one of the principal envelope components of Gram-negative bacteria, stimulates cytokine production in monocytes/macrophages [17,18]. The most prominent findings in the case of PHA stimulation were the substantial secretion of IL-1β induced by gal-cer and the different effects on IL-6 mRNA production and IL-6 secretion induced by sulfatide and gal-cer. The different effects of sulfatide and gal-cer on cytokine production are unlikely due to endotoxin contamination. Sulfatide and gal-cer were isolated from bovine brain using a similar chromatographic equipment and separation steps. Furthermore, acid-treated sulfatide (i.e. gal-cer) had the same effect on IL-6 mRNA production and IL-6 secretion as native gal-cer. Gal-cer, while strongly inducing IL-1β, IL-6 and TNF in PHA-stimulated MNCs, did not modulate the production of IL-10 or IL-2. Since IL-10 is induced by LPS, one would expect an increased production of IL-10 if the effects of gal-cer were due to endotoxin contamination. The concentrations of LPS in cultures were always less than 2 pg/ml and at least 10-fold lower than the threshold level capable of activating MNC cytokine production (unpublished findings). The structural difference alone is therefore likely to account for the observed different effects of sulfatide and gal-cer on cytokine production.

Sulfatide and gal-cer appear to affect the PHA-induced cytokine production at the level of gene expression. Thus, the induction of IL-1β and IL-6 secretion from MNCs stimulated with PHA and gal-cer followed an increased production of IL-β and IL-6 mRNA. Sulfatide decreased the secretion of IL-6 and modulated the secretion of TNF from MNCs stimulated with PHA after similar changes in the mRNA levels. Finally, a minor increase in production of TNF mRNA was followed by a substantial increase of TNF secretion in MNCs co-incubated with PHA and gal-cer. In contrast, gal-cer and sulfatide modulated the secretion of IL-1β from LPS-stimulated cells without affecting the mRNA level. IL-1β, together with its close relative IL-1α, differs from most other cytokines by the lack of a signal sequence. This results in dissociation of synthesis and secretion of the cytokine and processing of the immature pro-IL-1β are thought to be subject to multiple levels of regulation [19–22]. Secretion of IL-1β is regulated differentially in different cell types and in monocytes/macrophages, IL-1β secretion occurs without preceding gene expression [20,21].

After challenge with LPS, MNCs produce and secrete inflammatory cytokines such as IL-1β, IL-6 and TNF [19,23,24]. Though less abundantly, these cytokines are also produced by activated T-lymphocytes and, secondary, after T-lymphocyte activation of monocytes/macrophages in MNC cultures. The substantial increase in IL-1β, IL-6 and TNF production and secretion from PHA-stimulated MNCs challenged with gal-cer is likely to originate primarily from activation of T-lymphocytes. Whether sulfatide suppresses cytokine production and secretion primarily through T-lymphocytes cannot be elucidated from this study. The effects of the glycolipids were seen most clearly when co-cultivated with PHA and often, the effects on the mRNA or the secreted cytokine from MNCs stimulated with LPS alone could not be distinguished from those with added glycolipid. It is possible that LPS induces near maximum production of cytokines and that additive effects of glycolipids are difficult to measure.

It is potentially interesting that gal-cer increases production of cytokines that are cytotoxic to islet β-cells [25]. For example, gal-cer enhanced IL-1β mRNA production by PHA-stimulated MNCs and this was followed by an intensified secretion of IL-1β at levels up to 10 000 pg/ml after 48 h. Since sulfatide and gal-cer are present in the islets of Langerhans and their expression is regulated by glucose [26], stressed β-cells might stimulate local infiltrating leucocytes to produce potentially β-cell-damaging mediators, including IL-1β.

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