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Sulfatides trigger cytokine gene expression and secretion in human monocytes

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Abstract

We investigated whether sulfatides are able to trigger transmembrane signals and activation of selective cell functions in human monocytes. Sulfatides stimulated an increase in cytosolic free-calcium in monocytes, and this depended on the release of calcium from intracellular stores. Non-sulfated galactocerebrosides had no effect on monocyte cytosolic free calcium. Sulfatides enhanced expression of tumor necrosis factor, interleukin-8, and interleukin-1β, but not interleukin-12/natural killer cell stimulating factor mRNAs. Sulfatides also triggered secretion of cytokines into the extracellular medium, although they were much less effective than lypopolysaccharide. Both enhanced expression of cytokine mRNAs and secretion by sulfatides required sulfation of the galactose ring of the glycolipid as non-sulfated galactocerebrosides had no effect. These findings suggest that sulfatides that are released at sites of inflammation can amplify the inflammatory reaction triggering cytokine expression in, and release by, monocytes.

Key words: Monocyte; Cytokine; Sulfatide; L-Selectin

1. Introduction

The effector and regulatory role of mononuclear phagocytes in inflammation is largely due to their capability to secrete cytokines. Cytokine gene expression in, and secretion by, mononuclear phagocytes occurs upon interaction with a wide array of molecules present in the inflamed interstitium. Apart from cytokines themselves, identification of molecules released by tissues undergoing pathologic alterations, and potentially able to trigger directly cytokine expression, is still limited.

In the course of investigations on the role of mononuclear phagocytes in the effector phase, and amplification of demyelinating diseases it was shown that the myelin P2 protein triggers secretion of tumor necrosis factor, and other proinflammatory cytokines [1]. A major constituent of the myelin sheet are glycolipids, including sulfated, and non-sulfated galactocrebrosides [2]. The sulfated forms of galactocerebrosides (sulfatides) have recently attracted the attention of different groups of investigators as ligands for members of the selectin family of adhesion molecules [3–6]. Evidence has also been presented that sulfatides expressed on the plasma membrane of, and excreted by, both myeloid and tumor cells represent ligands for P-selectin [7]. Although the physiological significance of ligation of sulfatides by selectins is not known, we have started to address whether sulfatides can act as signalling molecules able to trigger selective leukocyte functions.

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We show in this paper that sulfatides, but not non-sulfated galactocerebrosides, are able to transduce signals in human monocytes, and enhance cytokine gene expression and secretion. Our findings suggest that sulfatides released at sites of inflammation, as for example in the course of demyelinating diseases, can contribute to amplification and persistance of inflammatory reactions stimulating cytokine secretion by monocytes.

2. Materials and methods

2.1. Purification and cultivation of monocytes

Peripheral blood mononuclear cells were obtained from blood buffy coats of healthy donors by centrifugation over a Ficoll-Hypaque gradients. For purification of monocytes as a suspension of cells for the measurement of cytosolic free calcium, mononuclear cells were resuspended at 10^7/ml in RPMI 1640 medium supplemented with 2 mM glutamine, 10% foetal calf serum (FCS) and 4 mM HEPES, and previously adjusted to an osmolarity of 285 mOsmol/l with distilled water (iso-osmotic medium). Monocyte suspensions were layared over 46% Percoll (Pharmacia Biotech Europe, Brussels, Belgium; adjusted to an osmolarity of 285 mOsmol/l with 10 × phosphate-buffered saline), and then centrifuged at 550 × *g* for 30 min at room temperature. Monocytes were collected at the interface between the iso-osmotic medium and the Percoll, washed in PBS, and suspended in Hank's balanced salt solution supplemented with 0.5 mM $CaCl_2$ and 5.5 mM glucose (HCaG) at a density of 10×10^6 cells/ml. Cytocentrifuge preparations of the monocyte suspensions stained by May-Grunwald-Giemsa staining, or for non-specific esterase activity showed that these preparations consisted of more than 95% monocytes.

For the measurement of cytokine expression, mononuclear cells collected from Ficoll-Hypaque gradients were washed in PBS, resuspended in RPMI 1640 medium supplemented with 2 mM glutamine, and heat-inactivated 5% human serum, pooled from 10–15 healthy donors, and plated in 6-well tissue culture plates (Nunc, Roskilde, Denmark) at 15×10^6/ml/well. After 1 h at 37°C, non-adherent cells were removed by several washings with PBS, and the remaining adherent cells (between 95 and 98% positive for non-specific esterase staining), were stimulated immediately, or after 1 day of culture at 37°C in

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95% air/5% CO_2. Cell-free supernatants were harvested, spun at 8000 × g and stored at −20°C. Adherent cells were extracted for total RNA. All the solutions used in these studies were prepared in lypopolysaccharide-free water for clinical use.

2.2. Monocyte stimulation

Monocytes were stimulated with sulfatides (Sigma Diagnostics, St. Louis, MO, or Fluka Chemika-Biochemika, Buchs, Switzerland), galactocerebrosides (Sigma), concanavalin A (Sigma), formyl-methionyl-leucyl-phenilalanine (Sigma), or lypopolysaccharide (LPS from *E. coli*, serotype 026.B6; Sigma). Sulfatides and galactocerebrosides were dissolved in PBS at 5 mg/ml and sonicated for 3 min.

2.3. Measurement of cytosolic free calcium in human purified monocytes

This was performed on suspensions of Fura-2/AM-loaded monocytes. Loading of monocytes with Fura-2/AM, and measurement of cytosolic free calcium was exactly as described in [8].

2.4. RNA isolation and Northern blot analysis

Total RNA was extracted and analysed as previously described [9]. Equal amounts of total RNA (10 μg) were loaded on each gel lane. mRNAs for TNF, IL-1β, IL-8, natural killer stimulating factor/IL-12/p40 (NKSF/IL-12/p40) and actin were detected by hybridization of nylon filters (Schleicher and Schuell, Dassel, Denmark) with ^{32}P-labeled cDNA fragments (Ready to go DNA labelling kit; Pharmacia Biotech Europe, Brussels, Belgium). Autoradiographs were quantitated by using a laser densitometer (LKB Instruments, TX), and the values were normalized with those of the uninduced cells on the basis of hybridization with actin probe.

2.5. Cytokine assays

Antigenic TNF was determined by using a double-ligand immunoassay (50 pg/ml detection limit), developed in our laboratory [10]. Extracellular antigenic IL-8 was measured by a specific ELISA with a detection limit of 20 pg/ml [11]. IL-1β was determined by a double-determinant RIA developed with Mab 609 and 206 (50 pg/ml detection limit) kindly donated by Dr. M.A. Cousin [12]. IL-6 was determined with a specific ELISA kit purchased from BioSource International (Camarillo, CA, USA, detection limit of 15 pg/ml). NKSF/IL-12 was determined by a double-determinant RIA detecting both the heterodimer and the p40 free chain with a detection limit of 10 pg/ml as described [13].

2.6. Statistical analysis

Data are expressed as means ± S.E.M. The statistical evaluation of the data was performed by Student's *t*-test for paired data and considered significant if $P < 0.05$.

3. Results and discussion

3.1. Sulfatides trigger increase of cytosolic free calcium in human monocytes

As shown in Fig. 1, sulfatides trigger an increase in cytosolic free calcium in monocytes. Sulfatides effect were detectable at 100 μg/ml, and increased up to 400 μg/ml (panel A). Several observations indicate that this is not merely a consequence of alterations of plasma membrane permeability due to partition of these glycolipids in the membrane bilayer. Firstly, as shown in panel B, non-sulfated galactocerebrosides were uncapable of triggering an increase in cytosolic free calcium. Secondly, the response to sulfatides was modulated by agents which have been partially characterized as able to affect transmembrane signalling pathways activated by agonists of distinct surface receptors. In fact, depolimerization of filamentous actin by cytochalasin B enhanced

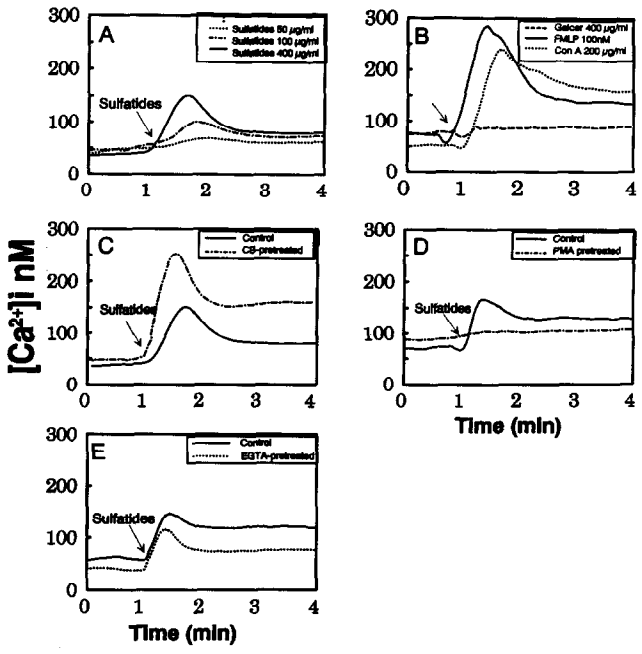

Fig. 1. Sulfatides trigger increase in cytosolic free calcium in monocytes. Monocytes were purified as described in section 2 and loaded with Fura-2/AM. 2×10^6 cells in 2 ml of Hank' s balanced salt solution supplemented with 0.5 mM $CaCl_2$, and 5.5 mM glucose were stirred in the cuvette of a Perkin Elmer LS 50 Luminescence Spectrometer, and stimuli added at the point indicated by an arrow. In panel C, monocytes were pretreated for 3 min with 5 μg/ml cytochalasin B (CB) before addition of 400 μg/ml sulfatides. In panel D, monocytes were pretreated for 3 min with 50 ng/ml phorbol myristate acetate (PMA) before addition of 400 μg/ml sulaftides. Panel E shows the increase in cytosolic free calcium in response to 400 μg/ml sulaftides in the presence and in the absence of 1 mM EGTA. Representative results of experiments which were repeated 4–5 times are reported.

(panel C) while pretreatment of the cell with PMA suppressed (panel D) the monocyte response to sulfatides. Cytochalasin B is known to potentiate signalling by chemotactic agonists, and activation of protein kinase C by PMA inhibits receptor-mediated activation of phospholipase C by affecting G protein-phospholipase C coupling [14]. Finally, the increase in cytosolic free calcium depended on release from intracellular stores; in fact, chelation of extracellular calcium by 1 mM EGTA did not affect the response to sulfatides (panel E).

Taken together, these findings point to the existence of a mechanism of action of sulfatides based on binding to distinct receptors. As sulfatides bind to L-selectin [3–6] it is legitimate to speculate that, as we previously demonstrated in neutrophils [15], sulfatides' effects might depend on ligation of L-selectin also in monocytes.

3.2. Sulfatides augments the expression of cytokine mRNA in monocytes

As shown in Fig. 2 exposure of monocytes to sulfatides for 6 h enhanced the expression of TNF, IL-1β, and IL-8 mRNAs. At 6 h sulfatides enhanced expression of TNF,

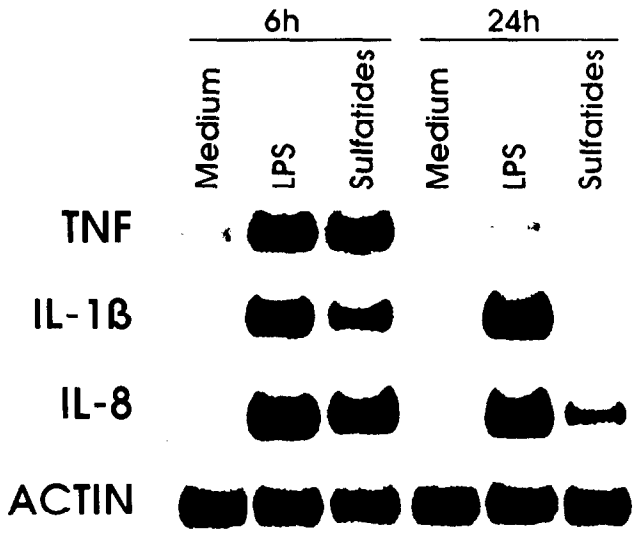

Fig. 2. Sulfatides enhance expression of cytokine mRNAs in monocytes. Monocytes were purified by adherence as described in section 2 and cultivated for6 or 24 h, as indicated, in the presence of no stimulus (medium), LPS (10 ng/ml), or sulfatides (400 μg/ml). Northern blots of total RNA (10 μg/lane) were hybridized with the indicated cDNA probes. Representative results of experiments which were repeated three times are reported.

and IL-8 at levels comparable to those found in monocytes treated with 10 ng/ml of LPS, but they were less effective than LPS in enhancing IL-1β mRNA expression. After 24 h of treatment with sulfatides cytokine mRNAs expression decreased substantially (by 63, 50 and 76% for TNF, IL-1β and IL-8, respectively, as revealed by densitometric analysis). Also when LPS was used as a stimulus, TNF mRNA expression decreased by about 60% after 24 h of treatment; however, expression of IL-1β and IL-8 did not change. The different kinetics of induction of IL-1β and IL-8 mRNA expression by sulfatides and LPS indicates that the sulfatides effects did not depend on contamination with LPS.

These findings show that sulfatides, which are simple constituents of the membrane of cells, which can be released in the course of tissue injury can trigger cytokine mRNA expression and, in particular, of TNF and IL-8. These two cytokines play a central role in recruitment of inflammatory cell, and alterations of the endothelium involved in leukocyte adhesion and transmigration. The only stimuli which were previously shown to induce IL-8 mRNA in monocytes are LPS, cytokines, and particles opsonised with IgG [11,16]

3.3. *Production of cytokines by monocytes stimulated with sulfatides*

Sulfatides also trigger release of cytokines in the extracellular medium (Fig. 3). After both 6 and 24 h of incubation with sulfatides we detected a significant increase in TNF, IL-1β, IL-8, as well as IL-6, in the extracellular medium. At 6 h, cytokine secretion in response to sulfatides was much lower than in response to LPS. In fact, LPS-induced secretion was 7-, 30-, 3- and 200-fold higher than sulfatide-induced secretion for TNF, IL-1β, IL-8 and IL-6, respectively. Cytokine secretion after 24 h of challenge was also much higher in response to LPS than to sulfatides. Significantly, LPS, but not sulfatides, stimulated a high level of secretion of IL-12/NKSF, thus excluding the possibility that the described effect of sulfatides was due to contamination with LPS.

The capability of sulfatides to trigger cytokine release was dose-dependent (not shown). A significant increase ($P < 0.05$, $n = 3$) of cytokine secretion was detected at 100 μg/ml sulfatides with TNF, and IL-1β, at 400 μg/ml with IL-6, and at 10 μg/ml with IL-8. Also induction of TNF, and IL-8 mRNAs by sulfatides was dose-dependent (not shown). Independent of the concentrations used, sulfatides did not trigger any significant release of IL-12/NKSF, while, in accord with previous results [1,13], LPS induced this cytokine.

In order to obtain further evidence that the sulfatides' effects were not due to contaminating LPS, we exploited the findings that at 100 μg/ml sulfatides also triggered

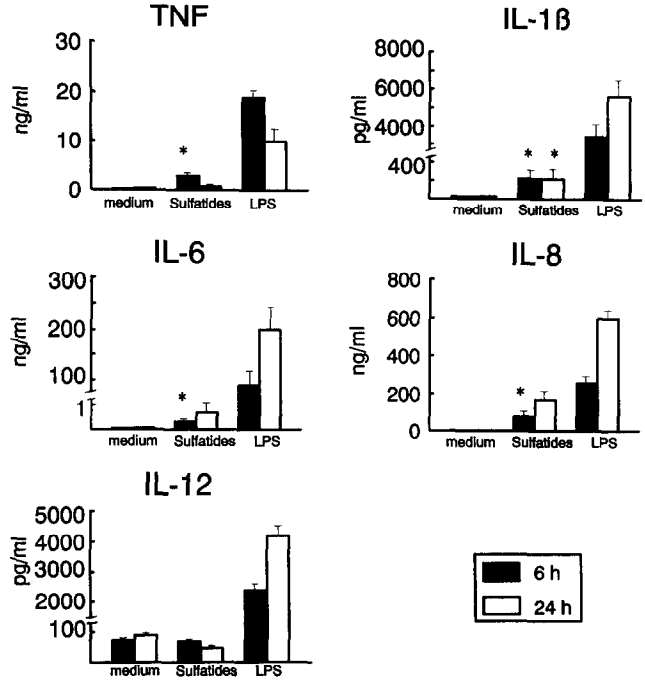

Fig. 3. Sulfatides trigger cytokine secretion by monocytes. Monocytes were purified by adherence as described in section 2 and cultivated for 6 or 24 h, as indicated, in the presence of no stimulus (Medium), LPS (10 ng/ml), or sulfatides (400 μg/ml). Cell-free supernatants were prepared and analysed for the presence of cytokines as described in section 2. The figure shows the means ± S.E.M. of duplicate assays performed with supernatants collected and pooled from three wells for each condition. Data derived from six experiments for TNF, IL-8 and IL-6, and seven for IL-1β. *Indicates a P value (0.05) of statistically significant difference between sulfatide-treated and untreated cells.

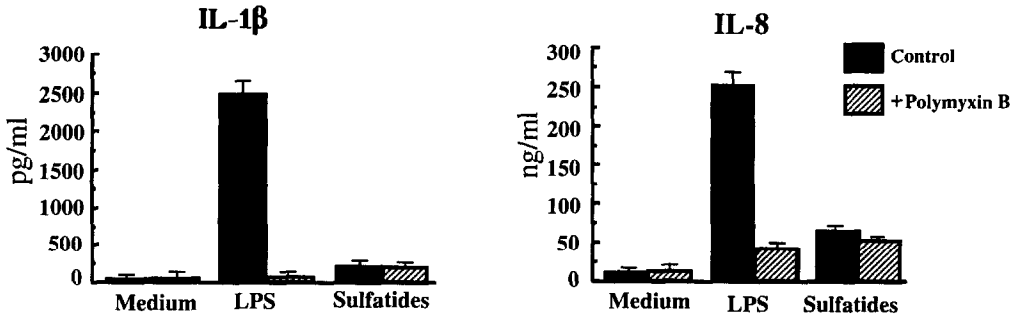

Fig. 4. Insensitivity of sulfatide effects to polymixin B. Monocytes were purified by adherence as described in section 2 and cultivated for 6 h in the presence of no stimulus (Medium), 10 ng/ml LPS, or 100 μg/ml sulfatides in the absence or presence of 10 μg/ml polymixin B. Cell-free supernatants were prepared and analysed for the presence of cytokines as described in section 2. Mean results of triplicate assays ± S.D. of one representative of three experiments is reported.

secretion of IL-1β and IL-8. As reported in Fig. 4, inhibition of LPS-stimulated IL-1β or IL-8 secretion by polymixin B, which prevents LPS' biological activities [17], was by 97, and 84%, respectively, but inhibition of sulfatide-stimulated IL-1β or IL-8 secretion by polymixin B was by 12 and 19% respectively.

Comparing the effect of LPS, and sulfatides on cytokine mRNA expression and secretion it emerges that sulfatides are more effective in triggering signals involved in mRNA accumulation than in mRNA translation and/ or protein secretion. These findings suggest that the main action of sulfatides is to prime monocytes, activating mechanisms leading to enhanced expression of cytokine mRNAs. Other factors present in the inflammatory site can synergise with sulfatides in triggering effective translation and secretion of cytokines in the extracellular milieau.

3.4. *Sulfatide effects depend on the sulfation of the galactose ring*

The ability of sulfatides to enhance cytokine mRNA expression and secretion was strictly dependent on sulfation of the glycolipid galactose ring. As shown in Fig. 5, sulfatides reproducibly acted as stimuli of cytokine mRNA expression and secretion. However, treatement for 6 h (Fig. 5) or 24 h (not shown) with non-sulfated galactocerebrosides, at doses which were maximally stimulatory for sulfatides, caused neither secretion nor mRNA expression of TNF and IL-8. The data reported in Fig. 5A, relative to the expression of IL-12/NKSF, also strenghtens the conclusion, inferred from findings described above, that LPS possibly contaminating the sulfatides preparations we used does not contribute to the findings described in this paper. In fact, LPS, but not

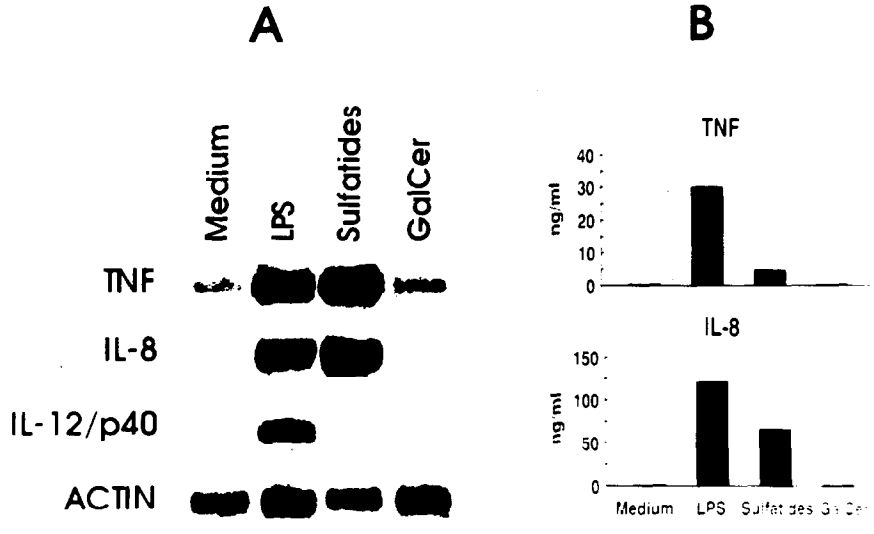

Fig. 5. Dependence of sulfatides effects on sulfation of the galactose ring of galactocerebrosides. Monocytes were purified by adherence as described in section 2 and cultivated for 6 h in the presence of no stimulus (Medium), 10 ng/ml LPS, or 400 μg/ml sulfatides, or galactocerebrosides (GalCer). (A) Cytokine mRNA expression. Northern blots of total RNA (10 μg/lane) were hybridized with the indicated cDNA probes. (B) Cytokine secretion. Cell-free supernatants were prepared and analysed for the presence of cytokines as described in section 2. Representative results of one experiment which was repeated three times are reported.

sulfatides, acted as a powerful inducer of IL-12/NKSF mRNA.

Sulfatides are simple constitutents of the cell membrane. The human myelin contains as much as 3.8%, in dry weight, of sulfatides [2]. Sulfatides were shown to be released by neutrophils and tumor cells [7]. The physiological, or pathological significance of triggering of monocyte functions by sulfatides remains to be elucidated. However, it is significant that monocytes are thought to play a central role in inflammatory damage in the course of demyelinating diseases [18,19]. Significantly, TNF has been shown to mediate myelin and olygodendrocyte damage in vitro [20,21], and to induce VCAM-1 and ICAM-1 on human neural cells [22]. It was also demonstrated that serum and cerebrospinal fluid of patients with actively progressive multiple sclerosis [23–25] and those with Guillain-Barré syndrome [23] contains increased concentrations of TNF and IL-1β. Modulation of TNF and IL-8 expression by sulfatides can therefore amplify damage at the central nervous system and recruitment of inflammatory cells.

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