

Sulphatides trigger polymorphonuclear granulocyte spreading on collagen-coated surfaces and inhibit subsequent activation of 5-lipoxygenase

Galina F. SUD'INA*¹, Thomas G. BROCK†, Marina A. PUSHKAREVA*, Svetlana I. GALKINA*, Denis V. TURUTIN*, Marc PETERS-GOLDEN† and Volker ULLRICH‡

*A. N. Belozersky Institute of Physico-Chemical Biology, Moscow State University, Moscow 119899, Russia, †Pulmonary and Critical Care Medicine Division, University of Michigan, Ann Arbor, MI 48109, U.S.A., and ‡Faculty of Biology, University of Konstanz, 78457 Konstanz, Germany

Sulphatides are sulphate esters of galactocerebrosides that are present on the surfaces of many cell types and act as specific ligands to selectins. The present study was undertaken to investigate the effect of sulphatides on polymorphonuclear granulocyte (PMN) attachment, spreading and 5-lipoxygenase (5-LO) metabolism. Sulphatides, but not non-sulphated galactocerebrosides, dose-dependently enhanced attachment to collagen, as measured by the myeloperoxidase assay. Studies with blocking antibodies indicated that the increased attachment was mediated by CD11b/CD18 (Mac-1) β 2 integrin. Scanning electron microscopy indicated that sulphatides also greatly enhanced the degree of cell spreading. In PMNs treated in suspension, sulphatides had no effect on the ionophore A23187-stimulated release of arachidonic acid and the synthesis of 5-LO metabolites. In contrast, in PMNs attached to collagen, the enzymic conversion of arachidonic acid by 5-LO was inhibited by sulphatides.

Inhibition of 5-LO metabolism by sulphatides was observed even in the presence of exogenous substrate, suggesting that sulphatides directly inhibited 5-LO action. Consistent with this, sulphatides interfered with ionophore-induced translocation of the 5-LO to the nuclear envelope. Substances competing with sulphatide binding to cells, like dextran sulphate, or a strong inhibitor of cell spreading, like the actin-polymerizing agent jasplakinolide, prevented the effects of sulphatides on PMN attachment and spreading and leukotriene synthesis. We conclude that shape changes occurring in response to sulphatides specifically impair PMN leukotriene synthesis by inhibiting translocation of 5-LO.

Key words: adhesion, arachidonic acid, jasplakinolide, leukotriene, neutrophil.

INTRODUCTION

The sulphated forms of galactocerebrosides (sulphatides) have been established as ligands for the selectin family of adhesion molecules [1]. Sulphatides, which are expressed on the surface of many cell types, are 3-sulphated galactosyl ceramides that bear heterogeneous fatty acyl substitution on the sphingosine moiety [2]. The galactolipids galactocerebroside and sulphatide are among the most prevalent molecules in the myelin sheath. Sulphatides are expressed on the plasma membrane and excreted by both myeloid and tumour cells [3].

Sulphatides have been shown to avidly bind to selectins. The physiological significance of ligation of sulphatides and selectins is not clear. Previously it was shown that sulphatides induce reactive oxygen species (ROS) formation in polymorphonuclear granulocytes (PMNs) [4]. Sulphatides are known to enhance the expression of tumour necrosis factor, interleukin 8 and interleukin-1 β in human monocytes [5]. Sulphatides trigger an increase of cytosolic calcium in human PMNs [6], but without additional stimuli they were unable to induce leukotriene (LT) synthesis (G. F. Sud'ina and M. A. Pushkareva, unpublished work) in the cells. In this report, activation of L-selectins by sulphatides changed the further responses of the PMNs. We found that sulphatides dramatically and specifically increased attachment and spreading of PMNs on collagen-coated surfaces, interfered with ionophore-induced translocation of 5-lipoxygenase (5-LO)

to the nuclear envelope and inhibited Ca ionophore A23187-stimulated 5-LO metabolism in PMNs.

MATERIALS AND METHODS

BSA, fraction V and fibronectin were from Calbiochem (La Jolla, CA, U.S.A.). Acid-soluble collagen type I was from Boehringer Mannheim (Mannheim, Germany). Monoclonal anti-human CD11a (clone 38), anti-human CD11b (clone ICRF44), anti-human CD11c (clone 3.9), anti-human CD18 (clone IB4), anti-human CD29 (clone 4B7R) and anti-human CD50 (clone 186-2G9) antibodies and monoclonal mouse IgG1 (clone MOPC31C) were from Alexis (Laufelfingen, Switzerland). Monoclonal anti-human L-selectin antibody (clone DREG.55) was from Bender MedSystems (Vienna, Austria). [¹⁴C]Arachidonic acid ([¹⁴C]AA) was purchased from Amersham (Little Chalfont, Bucks., U.K.). Ficoll-Paque was purchased from Pharmacia (Uppsala, Sweden). Sulphatides, Hepes and *o*-phenylenediamine were from Fluka (Deisenhofen, Germany). Sulphatides were dissolved at 50 mg/ml in a mixture of ethanol/DMSO (1:3). Jasplakinolide (Mo Bi Tec, Göttingen, Germany) was dissolved in ethanol at 1 mM. Hank's balanced salt solution with calcium and magnesium but without Phenol Red and sodium hydrogen carbonate (HBSS), Dulbecco's PBS

Abbreviations used: PMN, polymorphonuclear granulocyte; 5-LO, 5-lipoxygenase; LT, leukotriene; AA, arachidonic acid; ROS, reactive oxygen species; HSA, human serum albumin; HBSS, Hank's balanced salt solution; LTB₄, leukotriene B₄; 5-HETE, 5-hydroxyeicosatetraenoic acid; ω -OH-LTB₄, 20-hydroxy-LTB₄; ω -COOH-LTB₄, 20-carboxy-LTB₄; iso-LTB₄, 5(S),12(S,R)-dihydroxy-all-*trans*-eicosatetraenoic acids.

¹ To whom correspondence should be addressed (e-mail sudina@genebee.msu.su).

with magnesium but without calcium, human serum albumin (HSA) and cytochrome *c* were purchased from Sigma (Deisenhofen, Germany).

PMN leucocyte isolation

PMNs were isolated from freshly drawn citrate-anti-coagulated donor blood. Leucocyte-rich plasma was prepared by 0.75% dextran T-500 sedimentation of erythrocytes at room temperature. Granulocytes were prepared from leucocyte-rich plasma by density-gradient centrifugation on a bilayer gradient of Ficoll-Paque (densities, 1.077 and 1.123 g/ml). Granulocytes were washed twice in PBS, resuspended at 10^7 /ml in Dulbecco's PBS without CaCl_2 , and stored at room temperature.

Preparation of collagen or fibronectin surfaces for leucocyte adhesion

Plastic tissue-culture 24-well plates or 75 cm² culture flasks were coated with 75 $\mu\text{g}/\text{ml}$ type I collagen or 15 $\mu\text{g}/\text{ml}$ fibronectin for 24 h. Before use they were incubated for 1.5 h in PBS with 0.2% HSA, and then were thoroughly washed with PBS.

Scanning electron microscopy

PMNs on collagen or fibronectin were incubated for 30 min in HBSS/10 mM Hepes medium with the compound tested (50 $\mu\text{g}/\text{ml}$ sulphatides and/or 1 μM jasplakinolide) or without additives (controls). For scanning electron microscopy the cells were fixed in 2.5% glutaraldehyde, dehydrated in graded ethanol (10–100%) and processed by conventional scanning-electron-microscopic techniques.

Cell spreading

Cell spreading was characterized by cell area [7,8] and determined by the measurement of mean diameter of PMNs on phase-contrast microscopic and scanning electron microscopic images of the cells.

Indirect immunofluorescent microscopy

PMNs on collagen were incubated for 30 min in HBSS/10 mM Hepes medium with the compound tested (50 $\mu\text{g}/\text{ml}$ sulphatides and/or 1 μM jasplakinolide) or with vehicle (controls). For staining, cells were prepared as described in [9]. Briefly, cells were fixed in 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 in PBS, blocked with blocking solution (10% non-immune goat serum and 1% BSA in PBS) and probed with a rabbit polyclonal antibody raised against purified human leucocyte 5-LO (a generous gift from Dr J. Evans, Merck Frosst Center for Therapeutic Research, Dorval, Quebec, Canada) [10]. After washing with blocking solution the primary antibody was labelled with FITC-conjugated goat anti-rabbit antibody and DNA was stained with diamidino-2-phenylindole. Fluorescence was detected with a Nikon Eclipse E600 microscope equipped for epifluorescence and imaged with a SPOT RT digital camera.

Incubations for studies of AA metabolism

Unlabelled or [¹⁴C]AA-labelled PMNs were incubated with collagen- or fibronectin-coated plastic surfaces. Labelled PMNs were prepared by 2 h incubations of cells at 5×10^6 cells/ml in Dulbecco's PBS with 2.5 μCi of [¹⁴C]AA in 25 ml. At the end of the incubation an equal volume of 0.2% HSA in Dulbecco's PBS was added, the cells were centrifuged, washed twice

with PBS, resuspended at 10^7 cells/ml in Dulbecco's PBS and diluted with HBSS/10 mM Hepes. To protein-coated vessels (75 cm²) were added medium and sulphatides, jasplakinolide or dextran sulphate, where indicated, then cells were added ($1.5 \times 10^7/10$ ml) and incubated for 30 min at 37 °C to establish adhesive contacts. Next cells were stimulated with A23187 in the presence or absence of exogenous AA for 20 min, and incubation was stopped by addition of an equal volume of methanol at –20 °C with prostaglandin B₂ as an internal standard. The samples were stored at –20 °C. The denatured cell suspension was centrifuged, yielding supernatants designated as water/methanol extracts.

Lipoxygenase product analysis

The water/methanol extracts were purified by solid-phase extraction using C₁₈ Sep-Paks. The purified samples were injected into a 5 μm nucleosil C₁₈ column (250 mm \times 4.6 mm; Bischoff Chromatography, Leonberg, Germany) and chromatographed by reversed-phase HPLC. The products were eluted at 0.7 ml/min in a linear gradient from 30 to 100% solvent B: the eluents consisted of methanol/acetonitrile/water/acetic acid/triethylamine in the ratios (solvent A) 10/10/80/0.05/0.08 and (solvent B) 50/50/0/0.05/0.08, and elution was monitored using a UV detector at 280 nm and 250 nm as well as a radiochemical detector with solid scintillator. Products of the 5-LO pathway that were measured included leukotriene B₄ (LTB₄), 5-hydroxyeicosatetraenoic acid (5-HETE), 20-hydroxy-LTB₄ (ω -OH-LTB₄), 20-carboxy-LTB₄ (ω -COOH-LTB₄) and iso-LTB₄ [5(S),12(S,R)-dihydroxy-all-*trans*-eicosatetraenoic acids], identified by their co-elution with authentic standards.

PMN adhesion assay

Myeloperoxidase activity was used to measure PMN attachment under static conditions to collagen or fibronectin adsorbed on to plastic surfaces. PMNs (5×10^5 /well) were added to a protein-coated 24-well culture plate in 500 μl of HBSS/Hepes medium. After 30 min of incubation in a CO₂ incubator at 37 °C to allow leucocyte adherence, wells were washed twice with 500 μl of PBS solution, for removal of non-adherent PMNs. The extent of adherence was measured after the addition of detergent and a myeloperoxidase substrate, as described in [11,12]. A solution (300 μl) of 5.5 mM *o*-phenylenediamine and 4 mM H₂O₂ in buffer (67 mM Na₂HPO₄, 35 mM citric acid and 0.1% Triton X-100, pH 5) was added to each well, and after 4 min the reaction was stopped by the addition of an equal volume of 1 M H₂SO₄. Standard dilutions of PMNs with or without tested compounds were used for calibration.

For monoclonal-antibody-inhibition experiments, PMNs were pre-incubated with 5% heat-inactivated human serum to block Fc receptors. This improved inhibition of attachment with blocking monoclonal antibodies but did not affect attachment itself (results not shown). PMNs at 10^7 /ml in Dulbecco's PBS with 0.2% heat-inactivated HSA were incubated with antibodies (20 $\mu\text{g}/\text{ml}$) for 30 min at room temperature prior to their dilution with medium (HBSS with 0.2% HSA) and addition to the microtitre wells.

Superoxide measurement

PMN incubations on collagen- and fibronectin-coated surfaces were performed as described for PMN adhesion. Before addition of PMNs 50 μM cytochrome *c* was added to the medium, and the plates were incubated at 37 °C for 60 min. The incubation was stopped by cooling to +4 °C, and cytochrome *c* reduction

was measured as the increase in $\Delta 550/535$ absorbance. Reduction of $10 \mu\text{M}$ cytochrome *c* produced an increase in $\Delta 550/535$ absorbance (the change in the ratio of absorbances at 550 and 535 nm) of 0.18 absorbance unit.

RESULTS

Effects of sulphatides were investigated in PMN incubations on collagen- and fibronectin-coated plastic surfaces. Equal numbers of PMNs were added to culture flasks coated by the proteins. After 30 min of incubation at 37°C a different degree of cell attachment was observed depending on the surface (Figure 1). Sulphatides dramatically increased attachment of PMNs to collagen. Attachment was enhanced in a concentration-dependent manner and also specifically; non-sulphated galactocerebrosides had no effect on attachment (Figure 1). Sulphatides increased PMN attachment in all cases; when added to the medium in culture dishes before PMNs, when added to the cells being in contact with the surface and when PMNs were separately pretreated with sulphatides before their addition to the surface (Table 1). When sulphatides were added to cells preloaded on to a collagen-coated plate (see scheme 2 in Table 1), the effects of the sulphatides were time-dependent, with 5 min being sub-optimal. So, sulphatides binding to PMNs but not to the collagen-coated surface constitutes the critical step in the sulphatides' effects.

The effect of sulphatides on PMN attachment to the surfaces depended on the presence of extracellular matrix substrates. Sulphatides had no effect on the adhesion of PMNs to plastic.

Table 1 Effect of $50 \mu\text{g/ml}$ sulphatides on PMN attachment to collagen

Three different schemes of sulphatide addition were used. Scheme 1, sulphatides added to the HBSS medium in a collagen-coated culture plate before the addition of PMNs; scheme 2, sulphatides added to the cells loaded on to a collagen-coated culture plate; scheme 3, PMNs pretreated separately with sulphatides (15 min at 37°C) before the addition of the cells to a collagen-coated plate. The percentage of adherent cells was determined after 30 min of incubation of PMNs at 37°C on collagen and washing of non-adherent cells.

Mode of sulphatide addition	PMN attachment (% of added cells)
Control, no sulphatides	7 ± 2
Scheme 1	68 ± 8
Scheme 2	
30 min with sulphatides	63 ± 7
10 min on collagen before sulphatides + 20 min with sulphatides	58 ± 8
15 min on collagen before sulphatides + 15 min with sulphatides	56 ± 5
25 min on collagen before sulphatides + 5 min with sulphatides	31 ± 5
Scheme 3	57 ± 6

Without extracellular matrix proteins, a very high degree (80–90%) of PMN attachment to the surface was observed regardless of the presence or absence of sulphatides in the medium (results not shown). Collagen and fibronectin protected PMNs from activation by the plastic surface.

To assess the effect of sulphatides on cell spreading, we employed scanning electron microscopy of the cells. Neutrophils did not spread on to a collagen-coated surface. Sulphatides induced strong spreading with many extensions overlapping the

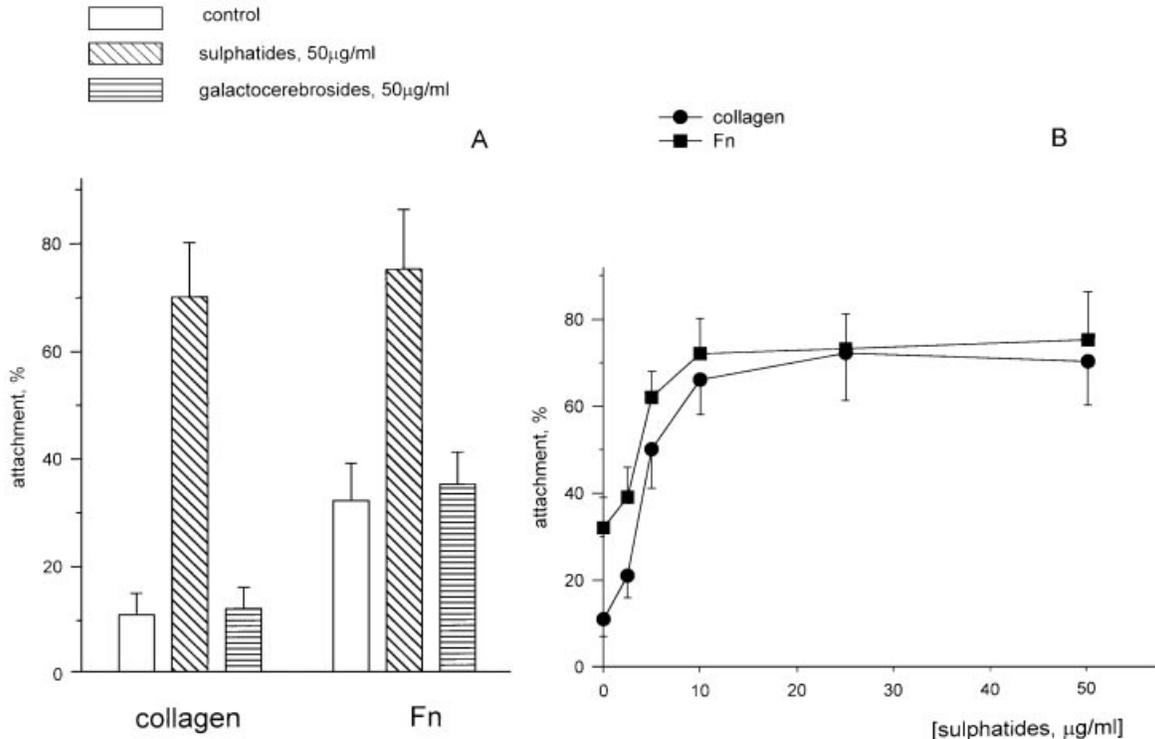


Figure 1 Sulphatides stimulate PMN attachment to collagen and fibronectin

(A) The effect of sulphatides and galactocerebrosides on PMN attachment to collagen- and fibronectin (Fn)-coated plastic surface. Suspensions of PMNs ($10^6/\text{ml}$) were incubated on various biological surfaces with or without $50 \mu\text{g/ml}$ sulphatides or $50 \mu\text{g/ml}$ galactocerebrosides in incubation medium. After 30 min at 37°C , non-adherent leucocytes were washed out by rinsing. PMN attachment has been expressed as a percentage of PMNs adhered in relation to the total number of PMNs added. (B) Suspensions of PMNs ($10^6/\text{ml}$) incubated on collagen- or fibronectin-coated surfaces with different concentrations of sulphatides in incubation medium.

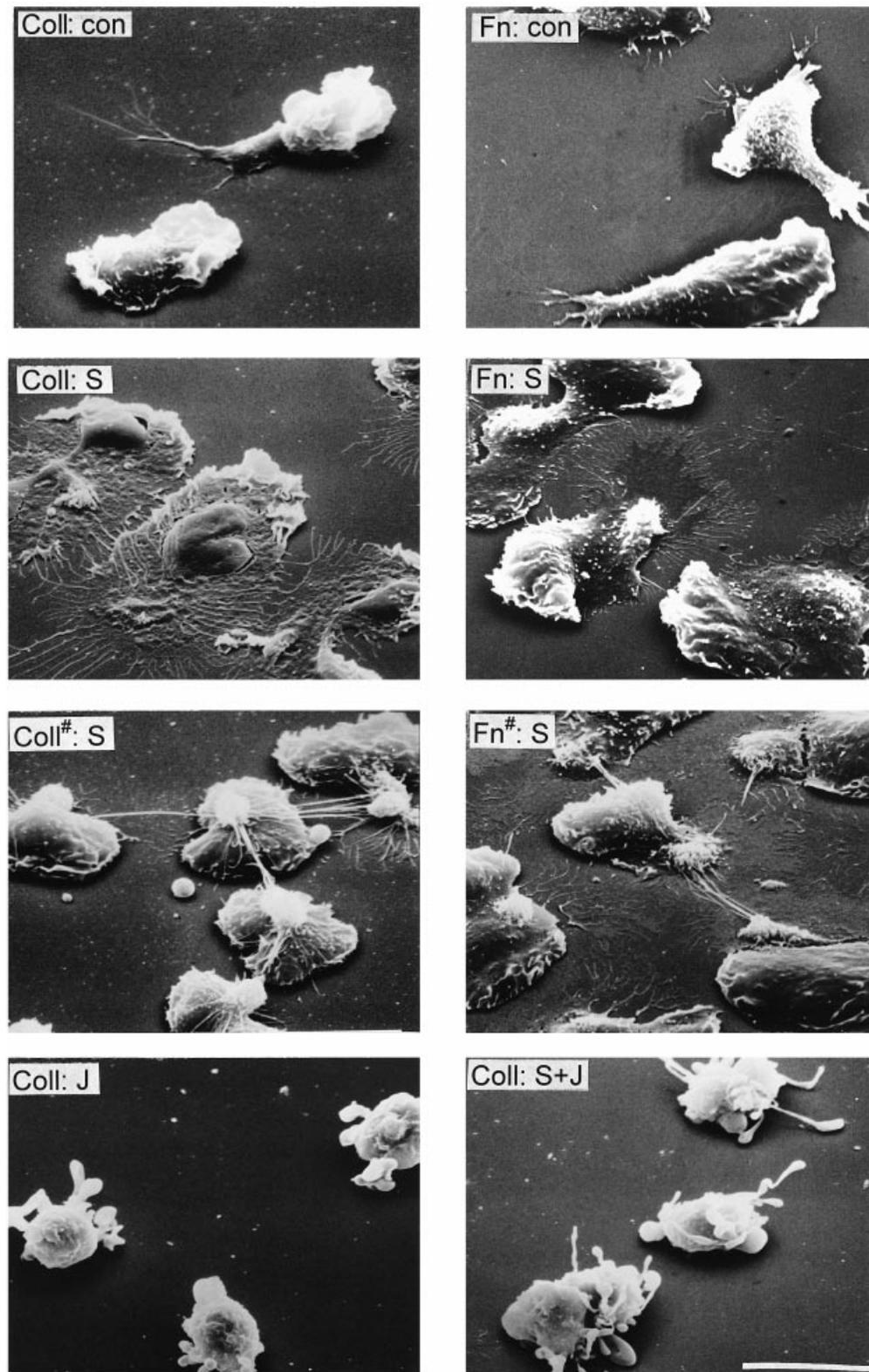


Figure 2 Scanning electron microscopy appearance of PMNs on collagen (Coll) and fibronectin (Fn)

Neutrophils ($10^6/\text{ml}$) in HBSS/Hepes medium were plated on protein-coated coverslips and incubated for 30 min at 37 °C in the medium (con), or with 50 $\mu\text{g/ml}$ sulphatides (S), 1 μM jasplakinolide (J) or 50 $\mu\text{g/ml}$ sulphatides + 1 μM jasplakinolide (S+J). In the samples Coll# and Fn# neutrophils were plated on collagen and fibronectin at higher cell density, $2 \times 10^6/\text{ml}$. Scale bar, 10 μm .

Table 2 Effect of antibodies to integrins, L-selectin and ICAM-3 (intercellular cell-adhesion molecule 3) on PMN attachment to collagen-coated surface

The cells were pretreated with 20 $\mu\text{g/ml}$ of monoclonal non-immune IgG1 (control) or with 20 $\mu\text{g/ml}$ of monoclonal antibodies against integrins CD11a, CD11b, CD11c, CD18 (β_2), CD29 (β_1) and against L-selectin and ICAM-3. Non-immune antibodies, used as a negative control, had no effect on PMN attachment. Attachment was measured as described in the Materials and methods section. PMN attachment is expressed as the percentage of PMNs adhered in relation to the total number of PMNs added. Values shown are the means \pm S.D. from three experiments, each run in duplicate.

	Attachment to the surface			
	No sulphatides		Sulphatides added (50 $\mu\text{g/ml}$)	
	% of added cells	% relative to control	% of added cells	% relative to control
Control (IgG1 or no additives)	13 \pm 1.5	100	58 \pm 7	100
Anti-CD11a	5.9 \pm 1.5	45	52 \pm 7	90
Anti-CD11b	9 \pm 3	69	26 \pm 3	45
Anti-CD11c	12 \pm 2	92	56 \pm 4	97
Anti-CD18	1.5 \pm 0.6	12	16 \pm 3	28
Anti-CD29	9.3 \pm 1.8	72	56 \pm 5	97
Anti-L-selectin	13 \pm 1.0	100	26 \pm 2	45
Anti-ICAM-3	30 \pm 3	231	49 \pm 8	84
Anti-CD11a + anti-CD18	1.2 \pm 0.5	9.2	16 \pm 1.5	28
Anti-CD11b + anti-CD18	3.0 \pm 1.0	23	2.4 \pm 1.0	4.1
Anti-CD11c + anti-CD18	1.0 \pm 0.3	7.7	16 \pm 2	28
Anti-CD29 + anti-CD18	1.0 \pm 0.5	7.7	13 \pm 1.5	22

surface and forming long distance cell-cell contacts with neighbouring cells (Figure 2). Mean cell diameter increased from $8.8 \pm 0.5 \mu\text{m}$ without sulphatides to $15.5 \pm 0.5 \mu\text{m}$ with 50 $\mu\text{g/ml}$ sulphatides, which corresponded to an increase in cell area from $61 \mu\text{m}^2$ to $189 \mu\text{m}^2$. The actin-polymerizing agent jasplakinolide inhibited the effect of sulphatides on PMN spreading. On fibronectin-coated surfaces PMN attachment was higher, sulphatides increased the attachment (Figure 1), improved spreading (increasing cell diameter from $9.45 \pm 1.3 \mu\text{m}$ to $12.5 \pm 0.3 \mu\text{m}$) and induced formation of long-distance extensions to neighbouring cells (Figure 2).

Attachment of PMNs to collagen in the absence of sulphatides was inhibited by antibodies to CD11a, CD11b, CD29 (β_1 integrin) and CD18 but not L-selectin and CD11c, with the most potent inhibition achieved by anti-CD18 and anti-CD11 or anti-CD29 combined with anti-CD18 (Table 2). The sulphatide-induced spreading process resulted in binding that was inhibited by monoclonal antibodies to CD11b and CD18 and also L-selectin, but was insensitive to antibodies to CD11a, CD11c and CD29 (β_1 integrin; Table 2). Sulphatide-induced binding was very sensitive to anti-CD18 antibodies, and obviously was mediated by CD11b/CD18 (Mac-1) β_2 integrin. ICAM-3 (intercellular adhesion molecule 3) did not mediate sulphatide-induced attachment, although monoclonal antibodies to ICAM-3 stimulated adhesion to collagen in control treatments.

LT synthesis stimulated by the calcium ionophore A23187, evaluated as the sum of all products of the 5-LO pathway, including LTB_4 , 5-HETE, ω -OH- and ω -COOH- LTB_4 , and iso- LTB_4 , in PMNs was inhibited after treatment with sulphatides depending on the time of the treatment (Figure 3A). Sulphatides had the most substantial effects on incubations on collagen- or fibronectin-coated surfaces, with insignificant effects on PMNs in suspension (Figure 3B). In the incubations on a plastic surface, LT synthesis was markedly diminished and sulphatides had no effect on A23187-stimulated LT formation. Thus the effect of sulphatides depended on the presence of extracellular matrix substrates (Figure 3).

In PMN-collagen interactions, the release of the sum of labelled eicosanoids plus AA in sulphatide-treated cells was not inhibited (Figure 4A), but the formation of all individual LTs

was suppressed, with maximal inhibition of iso- LTB_4 formation (Table 3). Also, the addition of exogenous AA (24 μM) could not overcome the loss in the 5-LO-product-formation capacity observed after 30 min of incubation with sulphatides on a collagen-coated surface (Figure 4B). In the presence of exogenous AA, it appeared that the inhibition of 5-HETE synthesis was greater than that of the LTs. These results indicate that the enzymic conversion of AA by 5-LO (and possibly also by LTA_4 -hydrolase) was inhibited.

Next, we attempted to find pharmacological agents or treatments capable of selectively eliminating sulphatide-induced effects on PMN adhesion and LT synthesis in order to understand the biochemical basis of this activity. Sulphatides significantly increased the production of ROS, as indicated by an increase in cytochrome *c* reduction capacity (Table 4).

Decreased 5-LO product formation could result from oxidative degradation of some metabolites or inactivation of enzymes in this pathway, i.e. sulphatide-induced ROS formation in PMNs. We tested the abilities of diphenylene iodonium (DPI), adenosine, erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA), dextran sulphate and jasplakinolide to block the effects of sulphatides on PMN attachment, ROS formation and LT synthesis. DPI, adenosine and EHNA potently blocked sulphatide-induced ROS production, but we did not observe restoration of 5-LO activity by the inhibition of ROS formation (Table 4). Inhibition of LT synthesis obviously did not correlate with the degree of ROS formation. However, when sulphatide-induced PMN attachment was blocked by either dextran sulphate or jasplakinolide, the inhibition of LT synthesis by sulphatides was also blocked (Table 4).

The experiments with jasplakinolide and dextran sulphate reversal of the sulphatide-induced inhibition of LT synthesis proved that the enzyme remained active. Substances competing with sulphatides in binding to cells, like dextran sulphate or the strong inhibitor of spreading jasplakinolide, prevented sulphatide-induced attachment and inhibition of LT synthesis (Table 4). There was a clear correlation with PMN spreading: the data on PMN interactions with fibronectin-coated surfaces showed substantial inhibition of LT synthesis by sulphatides on fibronectin-coated surfaces (Figure 3) and a moderate effect of

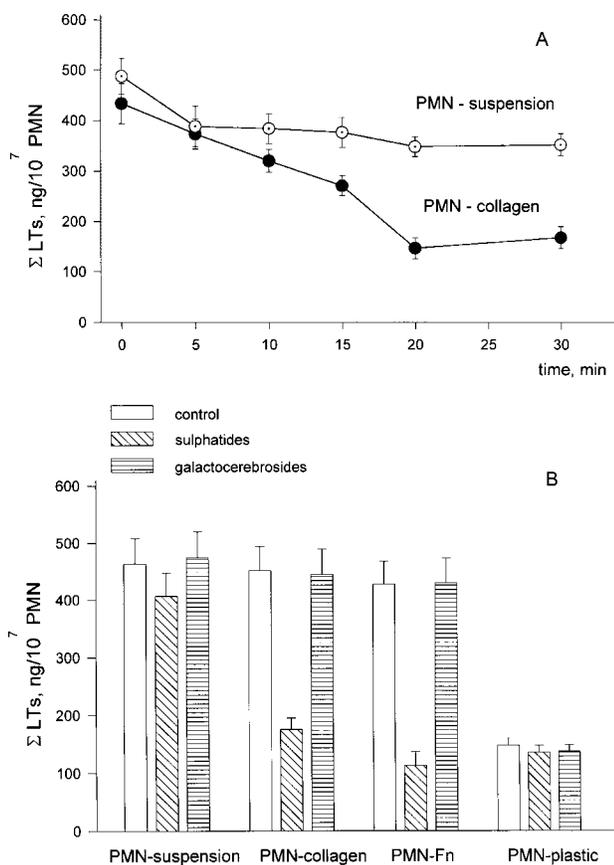


Figure 3 Effect of sulphatides on A23187-stimulated LT synthesis in PMNs in suspension and interacting with different surfaces

(A) Effect of sulphatides on LT synthesis in PMNs in suspension and in PMNs interacting with a collagen-coated surface. PMNs were pre-incubated for 30 min on collagen or in siliconized vessels with the addition of 50 μ g/ml sulphatides at different times during the pre-incubation period, and were then stimulated for 20 min with 2 μ M A23187. The data on LT synthesis are plotted versus the time of the sulphatide pre-treatment: the x axis indicates the length of time during the 30 min pre-incubation that the PMNs were incubated with sulphatides (e.g. the 20 min time point indicates 10 min of pre-incubation then 20 min of sulphatide treatment). (B) Effect of sulphatides and galactocerebrosides on LT synthesis in PMNs interacting with collagen-coated, fibronectin-coated (Fn) or plastic surfaces and in suspension in siliconized vessels. PMNs were incubated for 30 min on the indicated surface or in vessels with medium (control), 50 μ g/ml sulphatides or 50 μ g/ml galactocerebrosides, and then stimulated for 20 min with 2 μ M A23187.

sulphatides on PMN attachment (Figure 1), and obviously increased PMN spreading and induced cell-cell interactions (Figure 2).

To assess the effect of sulphatides on 5-LO localization in PMNs, cells were treated with sulphatides and the effect of this treatment on 5-LO localization and translocation in response to the stimulation by calcium ionophore A23187 was examined. In adherent PMNs, 5-LO is soluble and is found in the nucleoplasm [13]. There was no effect of sulphatides on 5-LO localization in resting PMNs (Figure 5). In PMNs stimulated with A23187, 5-LO was abundantly evident at the nuclear envelope (Figure 5). However, sulphatides blocked the movement of 5-LO to the nuclear membrane when the cells were stimulated with A23187. This type of 'translocation' is thought to be critical for 5-LO to get its substrate for metabolism. Jasplakinolide eliminated the effect of sulphatides on the 5-LO translocation, though

jasplakinolide induced an odd, distorted morphology in the cells (results not shown).

DISCUSSION

It has previously been shown that adherence alone can alter AA metabolism in PMNs and eosinophils [13,14]. Using substances influencing PMN attachment, it was found that the ecto-ATPase inhibitor and chemotherapeutic drug suramin stimulated PMN attachment to EAhy926 and ECV304 cell monolayers and to collagen-coated surfaces [15] and in parallel inhibited the biosynthesis of LTs [16]. In the present study we demonstrate that specific ligands for selectins, sulphatides, dramatically increase spreading of PMNs on a collagen-coated surface and suppress 5-LO metabolite generation in PMNs. Notably, the inhibitory effect of sulphatides on 5-LO metabolism was negligible in suspension, in the absence of a collagen-coated surface. Our data on sulphatide-induced inhibition of LT synthesis in PMNs are consistent with the inhibition of LTC₄ synthesis caused by adherence in human blood eosinophils [14]. It was demonstrated that, in eosinophils, the inhibitory effect of adherence on LTC₄ generation was at the level of 5-LO and not at the level of LTC₄ synthase or AA release. In eosinophils, this inhibition was attributable to a failure of 5-LO to translocate to the nuclear envelope following cell stimulation [14]. The present study has also shown that inhibition of LTB₄ synthesis, resulting here from sulphatide-induced spreading, can be attributed to inhibition of movement of 5-LO to the nuclear membrane when the cells were stimulated with A23187.

Our current study presents results on the reversibility of the effects of sulphatides, both on adhesion and 5-LO product formation. Our findings point to a correlation between the spreading and 5-LO inhibition. We used two kinds of effectors for this reversal: the sulphated polysaccharide dextran sulphate, a competitor of sulphatides binding to the cells, and the actin-polymerizing agent jasplakinolide. High-molecular-mass glucan sulphates are known to decrease cell adhesion to collagen [17] and to inhibit binding of ligands to L-selectin [18]. Thus dextran sulphate reversed the effects of sulphatides by blocking cell adhesion and spreading, and this block was achieved extracellularly at the point of cell-collagen contact.

The action of jasplakinolide on sulphatide-induced PMN adhesion may be explained in several ways. Jasplakinolide has several known activities: it induces an increase in actin polymers [19] and inhibits filament disassembly [20], with neutrophils becoming rigid and not adhesive in a flow-based adhesion assay [21]. Jasplakinolide can also induce protein ectodomain shedding from human neutrophils: the cell-surface expression of CD16, CD43, CD44 and L-selectin was down-regulated after induction of actin polymerization by jasplakinolide [22]. Sulphatides induced strong spreading of the cells on the surface, as confirmed by scanning electron microscopy. Jasplakinolide inhibited adhesion and spreading of PMNs on the surface, and also did it in sulphatide-treated cells.

In a static adhesion assay used in this work anti-L-selectin antibodies partly inhibited sulphatide-induced attachment. The adhesion was mostly inhibited by antibodies to β 2 integrins. β 1 Integrins are known as collagen and fibronectin receptors [23,24]. The importance of β 1 integrin-mediated binding to extracellular matrix molecules is well established for most types of leucocyte, with the exception of PMN. On neutrophils, the expression of β 1 was found to be very low [25], whereas the expression of β 2 integrins is abundant [26]. According to our findings, sulphatide-induced spreading on collagen was mediated by Mac-1 β 2

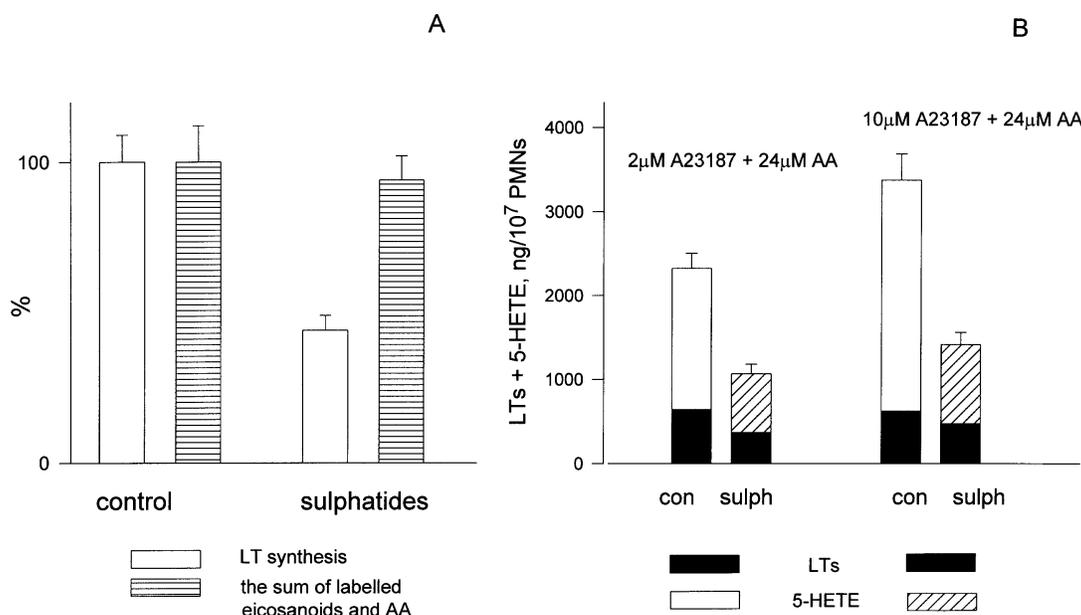


Figure 4 Effect of sulphatides on LT synthesis and on the sum of labelled eicosanoids and AA release

(A) [^{14}C]AA-prelabelled PMNs were incubated on a collagen-coated surface. The cells were pretreated for 30 min at 37 °C without (control) or with 50 $\mu\text{g}/\text{ml}$ sulphatides, and then stimulated for 20 min with 2 μM A23187. Radiolabelled eicosanoids were extracted from the medium and separated by HPLC. LT synthesis and eicosanoid release in the presence of sulphatides are presented as a percentage of the values in control samples (taken as 100%). (B) Effect of sulphatides on the synthesis of 5-LO metabolites (LTs and 5-HETE) in PMNs incubated on a collagen-coated surface. The cells were pretreated for 30 min at 37 °C without (con, control) or with 50 $\mu\text{g}/\text{ml}$ sulphatides (sulph), and then stimulated for 20 min with 2 or 10 μM A23187 and 24 μM AA.

Table 3 Effect of sulphatides on the formation of different LTs in A23187-stimulated PMNs on collagen-coated surfaces

Iso-LTB₄ represents the products of non-enzymic hydrolysis of LTA₄; sum of LTs represents the sum of all LTs, formed by enzymic conversion of LTA₄ (LTB₄, ω -OH-LTB₄ and ω -COOH-LTB₄) and non-enzymic LTA₄ metabolism (iso-LTB₄). The cells were pretreated for 30 min at 37 °C without (control) or with 50 $\mu\text{g}/\text{ml}$ sulphatides, and then stimulated for 20 min with 2 μM A23187.

	LT formation (ng/10 ⁷ PMNs)			
	LTB ₄	ω -OH- and ω -COOH-LTB ₄	Iso-LTB ₄	Sum of LTs
Control	113 ± 7	219 ± 17	70 ± 6	402 ± 33
Sulphatides	82 ± 6	106 ± 9	18 ± 4	206 ± 17

integrin, strongly inhibited by CD18 antibodies and totally inhibited by a combination of the antibodies to CD18 and CD11b. We cannot exclude a role for β 1 integrin: adhesion of PMNs induced via binding and activation of β 1 integrins is known to be dependent on β 2 integrins and can be prevented by blocking monoclonal antibodies to β 2 integrin CD11b/CD18 [27]. Sulphatide-induced L-selectin-mediated signalling directly stimulated neutrophil adhesive function via β 2 integrins. β 2 Integrins are constitutively expressed in a low-avidity state and require activation to mediate cell adhesion [28,29]. It has been observed that L-selectin signals induce exocytosis of granules containing CD11b/CD18 accompanied by an increase in the adhesive function of Mac-1 (CD11b/CD18) β 2 integrin [30]. Later it was shown that the ligation and clustering of L-selectin results in spatial colocalization of L-selectin and the β 2 integrin subunit CD18 [31], which results in an increased adhesive affinity of β 2 integrin.

An important finding of the current study is the link between sulphatide-induced spreading and the inhibition of LT synthesis. LT formation was inhibited after sulphatides were added, despite mobilization of AA being unaffected. Sulphatides interfered with ionophore-induced translocation of 5-LO to the nuclear envelope, but presumably not with ionophore-induced translocation of cytosolic phospholipase A₂. This suggests that the cytoskeleton most likely plays an important role in this effect. Spreading is associated with extensive rearrangement of the actin cytoskeleton. It is assumed that translocation of 5-LO, and hence LT synthesis, requires the interaction of the enzyme with the cytoskeleton. 5-LO contains a Src homology 3 binding motif, which enables *in vitro* interactions with cytoskeletal proteins [32]. However, additional studies will be required to elucidate relationships between 5-LO activity and interactions with cytoskeletal proteins.

A role for p38 kinase has been established in the phosphorylation and activation of the 5-LO enzyme [33]. Cross-linking of L-selectin induces phosphorylation of p38 mitogen-activated protein kinase (within several minutes) and enhances adhesive function of β 2 integrins [34]. It has been shown recently that mitogen-activated protein kinase phosphatase (MKP-1) plays an important role in the down-regulation of p38 mitogen-activated protein kinase [35]. The role of sulphatides in phosphatase activation was first shown by Zambrano et al. [36,37]. Spreading on a surface may further up-regulate phosphatases: it is known that cell-surface expression of receptor protein tyrosine phosphatase is enhanced in dense cell cultures, with shedding occurring in the absence of cell-cell contacts [38]. We can speculate that sulphatides down-regulate 5-LO activity and translocation via activation of cellular phosphatases. Preliminary data on the inhibition of phosphatases in neutrophils show partial restoration of LT synthesis in sulphatide-treated cells by the phosphatase inhibitor *o*-vanadate (results not shown).

Table 4 Effect of diphenylene iodonium (DPI; 10 μ M), adenosine (1 mM), erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA; 10 μ M), dextran sulphate (0.5 mg/ml) and jasplakinolide (1 μ M) on sulphatide (50 μ g/ml)-induced attachment, superoxide production and LT synthesis

The percentage of adherent cells was determined after 30 min of incubation of PMNs at 37 °C on collagen and washing of non-adherent cells. Cytochrome *c* reduction was measured after 60 min of incubation of 10⁶ PMNs/ml on a collagen-coated surface. For LT synthesis PMNs were incubated for 30 min on a collagen-coated surface without (control) or with 50 μ g/ml sulphatides and then stimulated for 20 min with 2 μ M A23187.

	PMN attachment (% of added cells)	Cytochrome <i>c</i> reduction (Δ 550/535)	LT synthesis (ng/10 ⁷ PMNs)
Control	22 \pm 4	0.273 \pm 0.03	427 \pm 47
Sulphatides	66 \pm 8	0.62 \pm 0.09	144 \pm 40
DPI	23 \pm 3	0	490 \pm 35
Sulphatides + DPI	65 \pm 7	0.099 \pm 0.01	187 \pm 21
Adenosine	59 \pm 8	0	412 \pm 33
Sulphatides + adenosine	69 \pm 7	0.112 \pm 0.01	156 \pm 30
EHNA	20 \pm 3	0.051 \pm 0.01	437 \pm 50
Sulphatides + EHNA	66 \pm 6	0.138 \pm 0.02	174 \pm 25
Dextran sulphate	4 \pm 2	0.088 \pm 0.02	513 \pm 50
Sulphatides + dextran sulphate	8 \pm 3	0.07 \pm 0.03	508 \pm 50
Jasplakinolide	1 \pm 0.5	0	727 \pm 90
Sulphatides + jasplakinolide	11 \pm 3	0.285 \pm 0.04	714 \pm 40

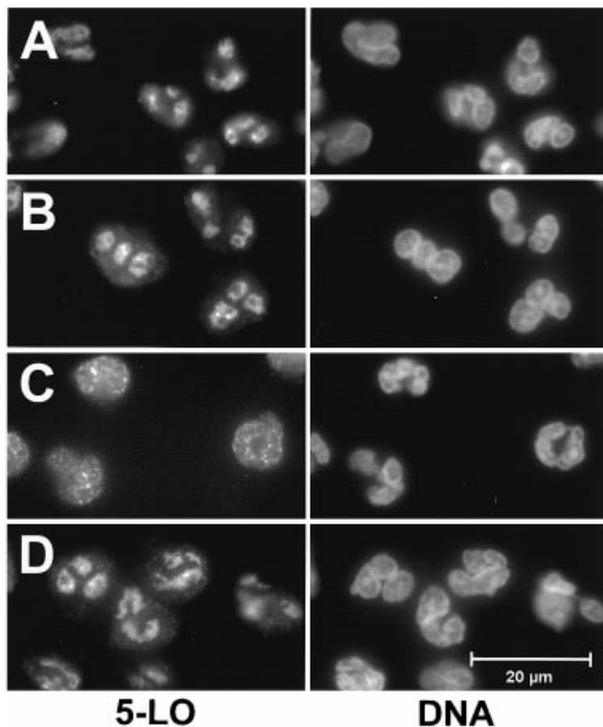


Figure 5 Effect of sulphatides on 5-LO localization in resting and activated PMNs on collagen-coated surfaces

Cells were incubated on collagen-coated glass coverslips without (A, C) or with (B, D) 50 μ g/ml sulphatides for 30 min at 37 °C. Cells were then either left unstimulated (A, B) or stimulated (C, D) with 2 μ M A23187 for 20 min at 37 °C. Staining for 5-LO and DNA was performed as described in the Materials and methods section.

Little is known about the physiological role of sulphatides in PMN function. Our data suggest that in association with effects on cell attachment and spreading, sulphatides reduce the formation of the potent chemoattractant and PMN activator, LTB₄. Such mechanisms may help to explain the diminished

accumulation of neutrophils in inflamed tissues that has been observed in studies *in vivo* with these substances [39–41].

This work was supported by INTAS (International Association, formed by the European Community; grant 97-11300), by the Deutsche Forschungsgemeinschaft (grant UI 36/24-1) and by the Russian Foundation for Basic Research (grant 98-04-48249).

REFERENCES

- Green, P. J., Tamatani, T., Watanabe, T., Miyasaka, M., Hasegawa, A., Kiso, M., Yuen, C. T., Stoll, M. S. and Feizi, T. (1992) High affinity binding of the leucocyte adhesion molecule L-selectin to 3'-sulphated-Le(a) and -Le(x) oligosaccharides and the predominance of sulphate in this interaction demonstrated by binding studies with a series of lipid-linked oligosaccharides. *Biochem. Biophys. Res. Commun.* **188**, 244–251
- Roberts, D. D. and Ginsburg, V. (1988) Sulfated glycolipids and cell adhesion. *Arch. Biochem. Biophys.* **267**, 405–415
- Aruffo, A., Kolanus, W., Walz, G., Fredman, P. and Seed, B. (1991) CD62/P-selection recognition of myeloid and tumor cell sulphatides. *Cell* **67**, 35–44
- Bengtsson, T., Grenegard, M., Olsson, A., Sjogren, F., Stendahl, O. and Zalavary, S. (1996) Sulfatide-induced L-selectin activation generates intracellular oxygen radicals in human neutrophils: modulation by extracellular adenosine. *Biochim. Biophys. Acta* **1313**, 119–129
- Constantin, G., Laudanna, C., Baron, P. and Berton, G. (1994) Sulfatides trigger cytokine gene expression and secretion in human monocytes. *FEBS Lett.* **350**, 66–70
- Laudanna, C., Constantin, G., Baron, P., Scarpini, E., Cabrini, G., Dececchi, C., Rossi, F., Cassatella, M. A. and Berton, G. (1994) Sulfatides trigger increase of cytosolic free calcium and enhanced expression of tumor necrosis factor- α and interleukin-8 mRNA in human neutrophils. Evidence for a role of L-selectin as a signaling molecule. *J. Biol. Chem.* **269**, 4021–4026
- Gallin, J. L., Seligmann, B. E., Cramer, E. B., Schiffmann, E. and Fletcher, M. P. (1982) Effects of vitamin K on human neutrophil function. *J. Immunol.* **128**, 1399–1408
- Roberts, R. L., Nath, J., Friedman, M. M. and Gallin, J. I. (1982) Effects of taxol on human neutrophils. *J. Immunol.* **129**, 2134–2141
- Healy, A. M., Peters-Golden, M., Yao, J. P. and Brock, T. G. (1999) Identification of a bipartite nuclear localization sequence necessary for nuclear import of 5-lipoxygenase. *J. Biol. Chem.* **274**, 29812–29818
- Woods, J. W., Evans, J. F., Ethier, D., Scott, S., Vickers, P. J., Hearn, L., Charleson, S., Heibein, J. A. and Singer, I. I. (1993) 5-Lipoxygenase and 5-lipoxygenase activating protein are localized in the nuclear envelope of activated human leukocytes. *J. Exp. Med.* **178**, 1935–1946
- Bath, P. M., Booth, R. F. and Hassall, D. G. (1989) Monocyte-lymphocyte discrimination in a new microtitre-based adhesion assay. *J. Immunol. Methods* **118**, 59–65

- 12 Schienwagen, C., Bylund-Fellenius, A. C. and Lundberg, C. (1990) Improved method for quantitation of tissue PMN accumulation measured by myeloperoxidase activity. *J. Pharmacol. Meth.* **23**, 179–186
- 13 Brock, T. G., McNish, R. W., Bailie, M. B. and Peters-Golden, M. (1997) Rapid import of cytosolic 5-lipoxygenase into the nucleus of neutrophils after *in vivo* recruitment and *in vitro* adherence. *J. Biol. Chem.* **272**, 8276–8280
- 14 Brock, T. G., Anderson, J. A., Fries, F. P., Peters-Golden, M. and Sporn, P. H. S. (1999) Decreased leukotriene C4 synthesis accompanies adherence-dependent nuclear import of 5-lipoxygenase in human blood eosinophils. *J. Immunol.* **162**, 1669–1676
- 15 Sud'ina, G. F., Mirzoeva, O. K., Galkina, S. I., Pushkareva, M. A. and Ullrich, V. (1998) Involvement of ecto-ATPase and extracellular ATP in polymorphonuclear granulocyte–endothelial interactions. *FEBS Lett.* **423**, 243–248
- 16 Sud'ina, G. F., Pushkareva, M. A., Galkina, S. I., Surkov, S. A., Mehl, M. and Ullrich, V. (1999) Effects of suramin on PMN interactions with different surfaces. *Biosci. Rep.* **19**, 547–558
- 17 Klebe, R. J. and Mock, P. J. (1982) Effect of glycosaminoglycans on fibronectin-mediated cell attachment. *J. Cell Physiol.* **112**, 5–9
- 18 Yoshida, T., Fennie, C., Lasky, L. A. and Lee, Y. C. (1994) A liquid-phase binding analysis for L-selectin. A strong dependency on highly clustered sulfate groups. *Eur. J. Biochem.* **222**, 703–709
- 19 Bubb, M. R., Senderowicz, A. M. J., Sausville, E. A., Duncan, K. L. K. and Korn, E. D. (1994) Jaspilkinolide, a cytotoxic natural product, induces actin polymerization and competitively inhibits the binding of phalloidin to F-actin. *J. Biol. Chem.* **269**, 14869–14871
- 20 Cramer, L. P. (1999) Role of actin-filament disassembly in lamellipodium protrusion in motile cells revealed using the drug jaspilkinolide. *Curr. Biol.* **9**, 1095–1105
- 21 Sheikh, S., Gratzner, W. B., Pinder, J. C. and Nash, G. B. (1997) Actin polymerisation regulates integrin-mediated adhesion as well as rigidity of neutrophils. *Biochem. Biophys. Res. Commun.* **238**, 910–915
- 22 Middelhoven, P. J., van Buul, J. D., Roos, D. and Hordijk, P. L. (1999) Actin polymerization induces shedding of Fc γ RIIIb (CD16) from human neutrophils. *Biochem. Biophys. Res. Commun.* **255**, 568–574
- 23 Yamamoto, K. and Yamamoto, M. (1994) Cell adhesion receptors for native and denatured type I collagens and fibronectin in rabbit arterial smooth muscle cells in culture. *Exp. Cell Res.* **214**, 258–263
- 24 Dalton, S. L., Scharf, E., Briesewitz, R., Marcantonio, E. E. and Assoian, R. K. (1995) Cell adhesion to extracellular matrix regulates the life cycle of integrins. *Mol. Biol. Cell* **6**, 1781–1791
- 25 Rieu, P., Lesavre, P. and Halbwachs-Mecarelli, L. (1993) Evidence for integrins other than beta 2 on polymorphonuclear neutrophils: expression of alpha 6 beta 1 heterodimer. *J. Leukoc. Biol.* **53**, 576–582
- 26 Carlos, T. M. and Harlan, J. M. (1994) Leukocyte-endothelial adhesion molecules. *Blood* **84**, 2068–2101
- 27 van den Berg, J. M., Mul, F. P., Schippers, E., Weening, J. J., Roos, D. and Kuijpers, T. W. (2001) Beta1 integrin activation on human neutrophils promotes beta2 integrin-mediated adhesion to fibronectin. *Eur. J. Immunol.* **31**, 276–284
- 28 Ginsberg, M. H., Du, X. and Plow, E. F. (1992) Inside-out integrin signalling. *Curr. Opin. Cell Biol.* **4**, 766–771
- 29 Hogg, J. C. and Doerschuk, C. M. (1995) Leukocyte traffic in the lung. *Annu. Rev. Physiol.* **57**, 97–114
- 30 Simon, S. I., Burns, A. R., Taylor, A. D., Gopalan, P. K., Lynam, E. B., Sklar, L. A. and Smith, C. W. (1995) L-Selectin (CD62L) cross-linking signals neutrophil adhesive functions via the Mac-1 (CD11b/CD18) β 2 integrin. *J. Immunol.* **155**, 1502–1514
- 31 Simon, S. I., Cherepanov, V., Nadra, I., Waddell, T. K., Seo, S. M., Wang, Q., Doerschuk, C. M. and Downey, G. P. (1999) Signaling functions of L-selectin in neutrophils: alteration in the cytoskeleton and colocalization with CD18. *J. Immunol.* **163**, 2891–2901
- 32 Lepley, R. A. and Fitzpatrick, F. A. (1994) 5-Lipoxygenase contains a functional Src homology 3-binding motif that interacts with the Src homology 3 domain of Grb2 and cytoskeletal proteins. *J. Biol. Chem.* **269**, 24163–24168
- 33 Werz, O., Klemm, J., Samuelsson, B. and Radmark, O. (2000) 5-Lipoxygenase is phosphorylated by p38 kinase-dependent MAPKAP kinases. *Proc. Natl. Acad. Sci. U.S.A.* **97**, 5261–5266
- 34 Smolen, J. E., Petersen, T. K., Koch, C., O'Keefe, S. J., Hanlon, W. A., Seo, S., Pearson, D., Fossett, M. C. and Simon, S. I. (2000) L-Selectin signaling of neutrophil adhesion and degranulation involves p38 mitogen-activated protein kinase. *J. Biol. Chem.* **275**, 15876–15884
- 35 Franklin, C. C. and Kraft, A. S. (1997) Conditional expression of the mitogen-activated protein kinase (MAPK) phosphatase MKP-1 preferentially inhibits p38 MAPK and stress-activated protein kinase in U937 cells. *J. Biol. Chem.* **272**, 16917–16923
- 36 Gonzalez, E. and Zambrano, F. (1983) Possible role of sulphatide in the K⁺-activated phosphatase activity. *Biochim. Biophys. Acta* **728**, 66–72
- 37 Zambrano, F. and Rojas, M. (1987) Sulphatide content in a membrane fraction isolated from rabbit gastric mucosal: its possible role in the enzyme involved in H⁺ pumping. *Arch. Biochem. Biophys.* **253**, 87–93
- 38 Gebbink, M. F., Zondag, G. C., Koningstein, G. M., Feiken, E., Wubbolts, R. W. and Moolenaar, W. H. (1995) Cell surface expression of receptor protein tyrosine phosphatase RPTP mu is regulated by cell-cell contact. *J. Cell Biol.* **131**, 251–260
- 39 Mulligan, M. S., Warner, R. L., Lowe, J. B., Smith, P. L., Suzuki, Y., Miyasaka, M., Yamaguchi, S., Ohta, Y., Tsukada, Y., Kiso, M. et al. (1998) *In vitro* and *in vivo* selectin-blocking activities of sulfated lipids and sulfated sialyl compounds. *Int. Immunol.* **10**, 569–575
- 40 Ueda, K., Nozawa, M., Miyasaka, M., Akamatsu, J. and Tajima, S. (1998) Sulfatide protects rat skin flaps against ischemia-reperfusion injury. *J. Surg. Res.* **80**, 200–204
- 41 Squadrito, F., Bagnato, G., Altavilla, D., Ferlito, M., Campo, G. M., Squadrito, G., Urna, G., Sardella, A., Arlotta, M., Minutoli, L. et al. (1999) Effect of sulfatide on acute lung injury during endotoxemia in rats. *Life Sci.* **65**, 2541–2552