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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*

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Sulfatides have been established recently as ligands for L-selectin, and we investigated whether they trigger transmembrane signals through ligation of L-selectin. We found that sulfatides trigger the increase of cytosolic free calcium in neutrophils and that this effect was strictly dependent on sulfation of the galactose ring, as non-sulfated galactocerebrosides were not stimulatory. Chymotrypsin and phorbol 12-myristate 13-acetate treatment of neutrophils caused shedding of L-selectin, but not of class I major histocompatibility complex antigens or β2 integrins, and blunted the capability of neutrophils to respond to sulfatides with an increase of cytosolic free calcium. Four different anti-L-selectin antibodies (DREG-200, LAM1/3, LAM1/6, and LAM1/10), but not four control antibodies directed against different surface molecules of neutrophils, also triggered an increase of cytosolic free calcium. The anti-L-selectin antibodies were stimulatory both if used in a soluble form, after cross-linking with anti-mouse $F(ab')_2$ fragments, and immobilized to protein A of *Staphylococcus aureus* through the Fc fragment. With immobilized antibodies, an increase of cytosolic free calcium was found also by plating neutrophils on antibodies bound to protein A-coated coverslips and monitoring the increase of cytosolic free calcium by fluorescence microscopy. Both sulfatides and anti-L-selectin antibody effects were not inhibited by pertussis toxin, thus indicating that a pertussis toxin-sensitive GTP-binding protein was not involved in signal transduction. Sulfatides also triggered an increase of tumor necrosis factor-α and interleukin-8 mRNAs in neutrophils. Also to act as stimuli of cytokine mRNA expression, sulfatides required sulfation of the galactose ring, as non-sulfated galactocerebrosides were not stimulatory, and depended on expression of L-selectin, as shedding of this molecules induced by chymotrypsin blunted their effects. These findings suggest that L-selectin can transduce signals activating selective cell function.

Migration of leukocytes into inflamed tissues is preceded by adhesive interaction between leukocytes and the vascular endothelium. Leukocyte-endothelium interaction is now viewed as a phenomenon occurring in distinct steps and mediated by distinct leukocyte surface molecules which recognize counter-receptors expressed by the endothelial cell (reviewed in Refs. 1 and 2). At first, leukocytes roll along the endothelium due to adhesive interaction mediated by selectins. The subsequent activation of integrins expressed by leukocytes promotes a firm adhesion to the endothelial cell mediated by ligation of members of the immunoglobulin supergene family and expressed by endothelial cells.

Leukocyte adhesion molecules involved in the interaction with the vascular endothelium may also participate in adhesive interaction in the extravascular space. This has been well established for the integrins; in fact, members of the β1 or β2 subfamilies of integrins recognize extracellular matrix proteins or counter-receptors expressed by cells present in sites of inflammation (see Ref. 3). Some evidence exists that leukocyte L-selectin also may participate in adhesive interaction in the extravascular space. For example, L-selectin was shown to mediate lymphocyte attachment to myelinated regions of the central nervous system (4), and, interestingly, some carbohydrate-based inhibitors of L-selectin inhibit the induction of experimental allergic encephalomyelitis in rats (5, 6).

That adhesive receptors expressed by leukocytes may, besides participating in adhesive interaction, transmit signals activating selective leukocyte functions has been addressed only recently. Both LFA-1 (CD11a/CD18), and VLA-4 (CD49d/CD29) were shown to provide costimulatory signals for lymphocyte activation (7–13). Studies by us (14) and other investigators (15) implicated β2 integrins in triggering of neutrophil respiratory burst and spreading upon adhesion to different ligands. Finally, VLA-4 was shown to trigger cytokine gene transcription in monocytes (16) and respiratory burst and spreading in eosinophils (17).

In contrast with the accumulating evidence that integrins can trigger leukocyte functions, no information exists on a possible role of leukocyte L-selectin as a signaling molecule. However, early studies showed that sulfatides can trigger neutrophil-selective functions (18). As sulfatides have been recently established as ligands for L-selectin (19–23) we addressed the question whether sulfatides trigger generation of intracellular messengers in neutrophils and whether ligation of L-selectin might be involved in this event. We show in this study that sulfatides increase cytosolic free calcium in neutrophils, and their effects are blunted by shedding of L-selectin; also soluble and cross-linked, or immobilized, anti-L-selectin antibodies have a triggering effect. We also provide evidence that sulfa-

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tides induce expression of interleukin (IL)[1]–8, and tumor necrosis factor (TNF)-α mRNAs.

MATERIALS AND METHODS

Antibodies and Other Reagents—Anti-L-selectin antibodies were kindly donated by Dr. T. K. Kishimoto, Boehringer Mannheim (DREG-200) (24) and Dr. O. Spertini, Division d'Hematologie, Centre Hospitalier Universitarie Vaudois, Lausanne, Switzerland (LAM1/3, LAM1/6, LAM1/10) (25). B9.12.1, directed against HLA-A, B, C antigens (26), was kindly donated by Dr. R. Accolla, Istituto di Scienze Immunologiche, Verona, Italy, and G1-15, directed against CD45R (27), by Dr. E. Plata, Oncogene, Seattle, WA. IOL44, directed against CD44, was purchased from a commercial source (Immunotech, Marseilles, France). The anti-FcRγIII antibody, 3G8, was obtained from American Type Culture Collection, Bethesda, MD, and purified from ascitic fluid. Goat anti-mouse Fab_2 fragments were from Organon Teknika Corporation, Cappel Research Products, Durham, NC. Antibodies were used as soluble reagents, immobilized to *Staphylococcus aureus* (Pansorbin; Calbiochem) or protein A purified from culture medium of a protein A-secreting *S. aureus* (Sigma). In the former case, 50 μl of a 10% suspension of *S. aureus* were washed twice with PBS, incubated for 2 h with 50 μg of the antibody in PBS, and, after three washes with PBS, resuspended in 50 μl of PBS. Antibodies were immobilized to glass coverslips coated with protein A exactly as previously described (14). Sulfatides and cerebrosides used in these studies were obtained from Sigma, Fluka, or Chemika-BioChemika (Buchs, Switzerland). Immediately before use they were dissolved in PBS at 5 mg/ml and sonicated for 3 min. The mannose 6-phosphate-rich phosphomannan from *Hansenula hostii* known as PPME was a kind gift of Dr. E. Butcher, Stanford University School of Medicine, Stanford, CA. Fucoidan from *Fucus vesiculosus* was from Sigma. All of the reagents used for these studies were prepared in endotoxin-free water for clinical use.

Isolation of Neutrophils and Measurement of Cytosolic Free Calcium—Neutrophils were isolated by standard procedures after sedimentation of erythrocytes by dextran sulfate and centrifugation of leukocytes over Ficoll-Hypaque gradients (14). After lysis of contaminating erythrocytes with hypotonic saline, neutrophils were washed and resuspended 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. Methods for loading of Fura-2/AM and monitoring cytosolic free-calcium changes in cell suspensions were as previously described (28). For fluorescence microscopy, coverslips coated with protein A and antibodies, as described above, were mounted in a PDMI-2 perfusion chamber (Medical System, Greenvale, NY) thermostated at 37 °C. Fura-2/AM-loaded neutrophils were added to the chamber and alternatively illuminated at 340- and 380-nm excitation wavelengths. Emitted light, coming from 10–15 cells, was filtered with a 505-nm long pass filter and detected by a R928S Hamamatsu photomultiplier tube (see Dechecchi *et al.* (29) for further details).

Chymotrypsin and PMA Treatment of Neutrophils and FACS Analysis—For chymotrypsin or PMA treatment, neutrophils (10×10^6/ml of HCaG) were treated with 100 units/ml chymotrypsin (Sigma) or 20 ng/ml PMA for 5 min at 37 °C, then diluted 20-fold, and washed. For the measurement of the increase of cytosolic free calcium, chymotrypsin or PMA treatment was performed after loading of neutrophils with Fura-2, and, after washing, cells were resuspended in HCaG. For Northern blot analysis (see below), after chymotrypsin treatment and washing, cells were resuspended in RPMI 1640 medium supplemeted with 10% fetal bovine serum. Expressions of L-selectin, HLA-A, B, C antigens or CD18 were analyzed by FACS analysis after staining with the anti-L-selectin antibody DREG-200, the anti-HLA-A, B, C antibody B9.12.1, or the anti-CD18 antibody TS1/18, followed by fluorescein isothiocyanate-labeled goat anti-mouse $F(ab')_2$.

Northern Blot Analysis—Neutrophils were suspended in RPMI 1640 medium (BioWhittaker, Inc., Walkersville, MD) supplemented with 10% fetal bovine serum (Biochrom Beteilingungs GmbH & Co., Berlin, Germany). 7 ml of cell suspensions (10^7/ml) were dispensed in T75 tissue culture flasks (Greiner GmbH, Frickenhausen, Germany), and the flasks left at 37 °C in 95% air, 5% CO_2 for the times indicated. Total RNA was extracted exactly as described by Cassatella *et al.* (30). Equal amounts of total RNA (10 μg) were loaded on each gel lane, and this was controlled by hybridization with a probe for β-actin. mRNAs for human TNF, IL-8, and actin were detected by autoradiography after hybridization of nylon fibers (Schleier Schuell) with ^{32}P-labeled cDNA fragments (Ready to Go DNA labeling kit, Pharmacia LKB Biotechnology Inc.).

[1] The abbreviations used are: IL, interleukin; TNF, tumor necrosis factor; PBS, phosphate-buffered saline; HCaG, Hanks' balanced salt solution supplemented with $CaCl_2$ and glucose; PPME, phosphomannan; PMA, phorbol 12-myristate 13-acetate; fMLP, formyl-methionyl-leucyl-phenylalanine; FACS, fluorescence activated cell sorting.

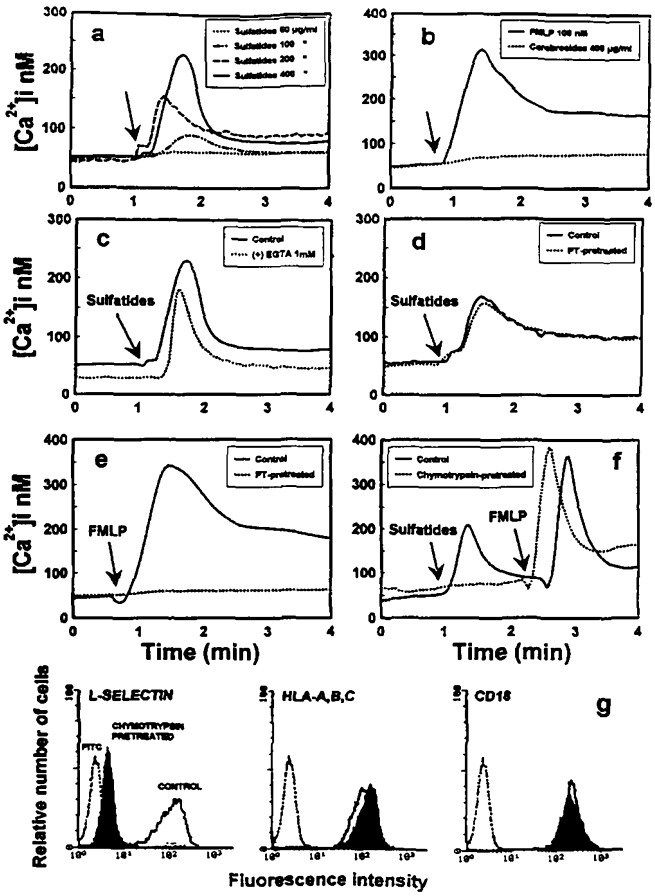

FIG. 1. **Sulfatides increase cytosolic free calcium in neutrophils, and shedding of L-selectin blunts the response to sulfatides.** *a* and *b*, the indicated amounts of sulfatides, galactocerebrosides, or the chemotactic agonist fMLP were added at the point indicated by an *arrow* to Fura-2/AM-loaded neutrophils. *c*, sulfatides (400 μg/ml) were added to neutrophils suspended in Hanks' balanced salt solution without (*Control*) or with 1 mM EGTA. *d* and *e*, neutrophils were incubated for 1 h at 37 °C with (*PT-pretreated*) or without (*Control*) 1 μg/ml pertussis toxin, and, after Fura-2/AM loading, stimulated with sulfatides (200 μg/ml), or fMLP (100 nM). *f*, Fura-2/AM-loaded neutrophils were incubated at 37 °C for 5 min in the absence (*Control*) or the presence of 100 units/ml chymotrypsin. After washing, neutrophils were stimulated with 400 μg/ml sulfatides, followed by 100 nM fMLP. *g*, FACS analysis of L-selectin, HLA-A, B, C and CD18 expressions of control neutrophils or neutrophils treated with chymotrypsin (*black histograms*) as described in *f*. Representative experiments which were repeated four to seven times with equal results are reported.

RESULTS

As shown in Fig. 1, sulfatides increased neutrophil cytosolic free calcium. The effect of sulfatides was dose-dependent up to 400 μg/ml (Fig. 1*a*), and, at optimal concentrations, they increased cytosolic free calcium to an extent comparable to that induced by the chemotactic agonist formyl-methionyl-leucyl-phenylalanine (fMLP) (Fig. 1*b*). As shown for binding to L-selectin (19–22) the effect of sulfatides was strictly dependent on sulfation of galactose since non-sulfated galactocerebrosides had no effect (Fig. 1*b*). The sulfatide-induced increase of cytosolic free calcium derived from release of calcium from intracellular store; in fact, chelation of extracellular calcium by EGTA did not decrease cytosolic free calcium spikes (Fig. 1*c*). Chelation of extracellular calcium is known to have no inhibitory effect on binding of sulfatides to L-selectin (20, 22, 23).

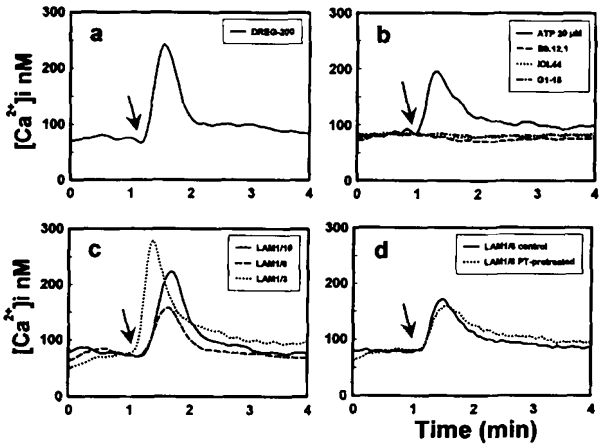

FIG. 2. **Soluble and cross-linked anti-L-selectin antibodies trigger increase of cytosolic free-calcium in neutrophils.** *a–d*, Fura-2/AM-loaded neutrophils (10 × 10^6/ml) were incubated at 4 °C for 1 h with 10 μg/ml of the indicated antibodies and, after two washings, resuspended in Hanks' balanced salt solution. After 60–80 s of stirring in the cuvette of a Perkin-Elmer LS-50 luminescence spectrometer, thermostated at 37 °C, goat anti-mouse $F(ab')_2$ fragments, at a final concentration of 25 μg/ml, were added (*arrow*). Treatment with pertussis toxin was as described in the legend to Fig. 1.

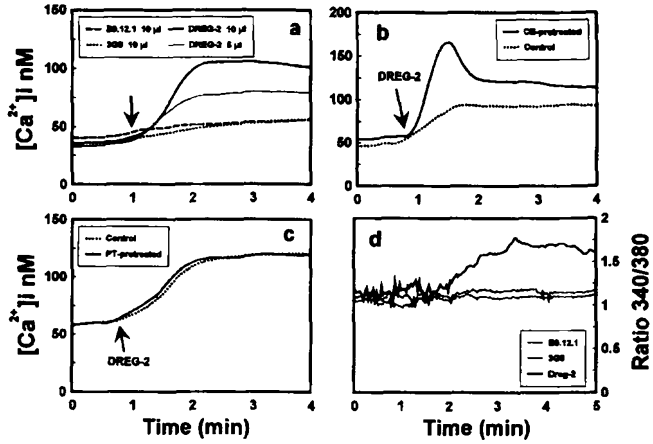

FIG. 3. **Immobilized anti-L-selectin antibodies trigger an increase of cytosolic free calcium in neutrophils.** *a*, the indicated antibodies were immobilized to *S. aureus* as described in "Materials and Methods" and added to neutrophil suspensions at the time indicated by an *arrow*. *b*, neutrophils were challenged with DREG-200 immobilized to *S. aureus* in the presence or the absence of 5 μg/ml cytochalasin B (*CB*). *c*, neutrophils were treated with pertussis toxin as described in the legend to Fig. 1 and then challenged with DREG-200 immobilized to *S. aureus*. *d*, Fura-2/AM-loaded neutrophils were plated on DREG-200, B9.12.1, or 3G8 immobilized to glass coverslips (see "Material and Methods"), and fluorescence changes were monitored with a Nikon TMD inverted microscope interfaced with an SLM 8000C spectrofluorometer. Representative experiments which were repeated three to five times with equal results are reported.

Contrary to fMLP, sulfatides increased cytosolic free calcium also in pertussis toxin-treated neutrophils (Fig. 1, *d* and *e*). This finding indicates that the transduction sequence triggered by sulfatides does not require the integrity of pertussis toxin-sensitive GTP-binding proteins; insensitivity to pertussis toxin was also found when cytosolic free calcium was triggered by anti-L-selectin antibodies (see Fig. 2*d*).

The capability of sulfatides to increase cytosolic free calcium required expression of L-selectin (Fig. 1, *f* and *g*). Treatment of neutrophils with chymotrypsin induced shedding of L-selectin (31) (Fig. 1*g*). We confirmed that shedding of L-selectin displays some selectivity as the expression of HLA-A, B, C antigens and β_2 integrins was not decreased by treatment with chymotrypsin (Fig. 1*g*). Chymotrypsin-treated neutrophils did not respond to sulfatides with an increase of cytosolic free calcium; however, they still responded to fMLP (Fig. 1*f*). Also shedding of L-selectin induced by PMA (32) (see "Materials and Methods") blunted the sulfatide-induced increase of cytosolic free calcium (not shown).

We also tested whether two known ligands of L-selectin were able to increase cytosolic free calcium. Neither fucoidan up to 400 μg/ml nor PPME up to 100 μg/ml triggered a cytosolic free calcium increase. The possible reasons for the different effect of sulfatides compared to fucoidan and PPME will be addressed under "Discussion." As these L-selectin ligands gave negative results, we challenged the indication deriving from studies with sulfatides by analyzing the capability of anti-L-selectin antibodies to trigger an increase in cytosolic free calcium.

Cross-linking of L-selectin by antibodies also increased cytosolic free calcium (Fig. 2). Binding of the anti-L-selectin antibodies DREG-200 (IgG1), LAM1/10 (IgG1), LAM1/6 (IgG1), and LAM1/3 (IgG1), followed by cross-linking with anti-immunoglobulin $F(ab')_2$ fragments triggered cytosolic free calcium spikes (Fig. 2, *a* and *c*) to an extent comparable to that of a known agonist of calcium signaling such as ATP (Fig. 2*b*). Three different antibodies reacting with other neutrophil surface molecules B9.12.1 (IgG2a), anti-HLA-A, B, C, IOL44 (IgG1), anti-CD44, G1-15 (IgG1), anti-CD45R did not trigger any response upon cross-linking (Fig. 2*b*). As found with sulfatides, pertussis toxin did not inhibit the increase of cytosolic free calcium induced by cross-linked anti-L-selectin antibodies (Fig. 2*d*).

We also analyzed the effect of anti-L-selectin antibodies immobilized through the Fc fragment (Fig. 3). DREG-2 immobilized to *S. aureus* triggered an increase of cytosolic free calcium (Fig. 3*a*). Compared to soluble antibodies, immobilized DREG-2 caused an increase of cytosolic free calcium which was lower but sustained up to 3 min from its addition. The B9.12.1 antibody or the anti-FcγRIII antibody 3G8 (IgG1) immobilized to *S. aureus* were not stimulatory (Fig 3*a*). The action of *S. aureus*-immobilized DREG-2 was insensitive to pertussis toxin (Fig 3*c*) and was potentiated by the microfilament-disrupting agent cytochalasin B (Fig 3*b*). This last finding makes it unlikely that internalization of *S. aureus* was in part responsible for triggering the increase of cytosolic free calcium and suggests that this correlates with the extent of cross-linking of L-selectin as it also emerged from the experiments with soluble antibodies (Fig. 2).

The anti-L-selectin antibody DREG-2 was also immobilized trough the Fc fragment to protein A-coated coverslips, and the increase in cytosolic free-calcium was monitored by fluorescence microscopy (Fig. 3*d*). Emission of light by Fura-2-loaded neutrophils plated on DREG-2 and bound to immobilized protein A increased at 340 nm and decreased at 380 nm as expected from the increase of cytosolic free calcium. Light emission at these two wavelengths by Fura-2-loaded neutrophils plated on anti-HLA-A, B, C or anti-FcγRIII antibodies bound to immobilized protein A did not change (Fig. 3*d*).

We also addressed whether sulfatides can signal enhanced expression of cytokines genes. As shown in Fig. 4, sulfatides induced expression of mRNA for TNF-α and IL-8. The effect of sulfatides was dose-dependent and evident at a concentration of 100 μg/ml. Densitometric analysis showed that at 100 μg/ml sulfatides increased TNF and IL-8 mRNAs 5- and 3-fold, respectively, compared to untreated cells. Maximally stimulatory doses of sulfatides (400 μg/ml) increased expression of TNF-α and IL-8 mRNAs to an extent comparable to lypopolysaccharide (Fig. 4*a*); in fact, as revealed by densitometric analysis, TNF mRNA expression was increased 20-fold by both LPS and 400 μg/ml sulfatides, and IL-8 mRNA expression was increased 4.5-fold by LPS and 5.5-fold by 400 μg/ml sulfatides. As found

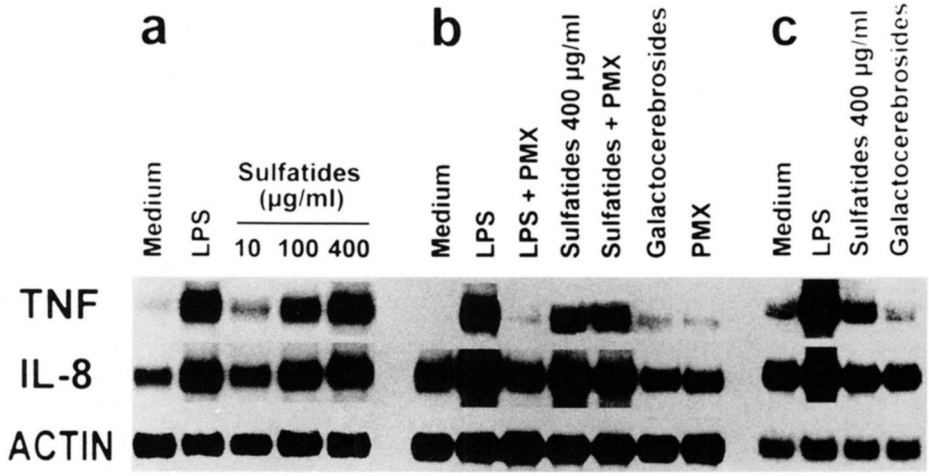

FIG. 4. **Sulfatides induce expression of TNF-α and IL-8 mRNAs.** *a*, neutrophils were incubated for 6 h in RPMI 1640 medium containing antibiotics and supplemented with 10% fetal bovine serum in the absence (*Medium*) or the presence of 100 ng/ml of LPS (from *Escherichia coli*, serotype O26.B6, Sigma) or sulfatides. Northern blots of total RNA (10 μg/lane) were hybridized with the indicated cDNA probes. *b*, neutrophils were incubated for 3 h as described above in the absence or the presence of 10 μg/ml polymixin B sulfate (Sigma) and without (*Medium*) or with 100 ng/ml LPS, 400 μg/ml sulfatides, or galactocerebrosides. *c*, as *a*, but with 30-min incubation. Representative experiments which were repeated three to five times with equal results are reported.

with the increase in cytosolic free calcium, the effect of sulfatides depended on sulfation of the galactose ring because galactocerebrosides did not enhance expression of mRNAs for TNF-α and IL-8 (Fig. 4*b*). As shown in Fig. 4*b*, polymixin B, which was shown to prevent most of the biological activities of LPS (33) and effectively inhibit the response to LPS in our assay conditions (75 and 70% inhibition of the LPS-induced expression of TNF and IL-8 mRNAs, respectively, as revealed by densitometric analysis), had only a negligible effect (7 and 3% inhibition of the sulfatide-induced expression of TNF and IL-8 mRNA, respectively, as revealed by densitometric analysis) on the action of the sulfatides preparations we used. This finding excludes a possible effect of contaminating dose of LPS. As shown in Fig. 4*c*, exposure to sulfatides for times as short as 30 min enhanced expression of TNF, but not IL-8 mRNA. After 30 min of treatment, the sulfatide effects on TNF mRNA expression were lower than those of LPS (2.5-fold increase *versus* 14-fold increase as revealed by densitometric analysis). As previously reported (34), neutrophils expressed constitutively high levels of IL-8 mRNA after 30 min of incubation in medium. LPS, but not sulfatides, enhanced this constitutive expression (Fig. 4*c*). That sulfatides did not induce IL-8 mRNA after 30 min of incubation further excludes a possible effect of contaminating doses of LPS; in fact, LPS enhanced IL-8 mRNA by about 2-fold within 30 min of treatment (Fig. 4*c*).

In analogy with studies on the generation of cytosolic calcium transients (see Fig. 1), the sulfatide effects described in Fig. 4 were blunted by treatment of neutrophils with chymotrypsin (Fig. 5). Treatment of neutrophils with chymotrypsin in the same conditions used for the experiments reported in Fig. 1 caused shedding of L-selectin, as revealed by 90% inhibition of binding of anti-L-selectin antibodies, but not of HLA-A, B, C or CD18 antigens (not shown). As shown in Fig. 5, chymotrypsin treatment caused either a slight enhancement of the LPS capability to induce TNF mRNA (7% enhancement as revealed by densitometric analysis) or a negligible inhibition of the LPS capability to induce IL-8 mRNA (15% inhibition, as revealed by densitometric analysis). On the contrary, the effect of sulfatides was clearly inhibited (74 and 80% inhibition of the sulfatide-induced expression of TNF and IL-8 mRNAs, respectively, as revealed by densitometric analysis).

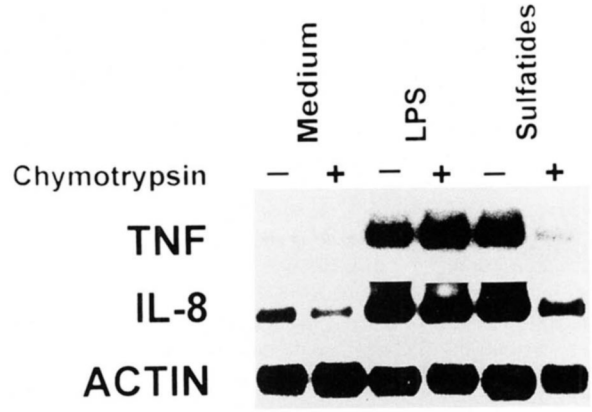

FIG. 5. **Treatment with chymotrypsin blunts the neutrophil capability to respond to sulfatides with enhanced expression of TNF and IL-8 mRNAs.** Neutrophils were treated with chymotrypsin as described in the legend to Fig. 1. After washing, cells were stimulated for 4 h with 100 ng/ml LPS or 400 μg/ml sulfatides. Northern blots of total RNA (10 μg/lane) were hybridized with the indicated cDNA probes. A representative experiment that was repeated three times with equal results is reported.

DISCUSSION

L-Selectin is one of the members of a family of lectin-like molecules that recognize carbohydrate-bearing ligands (35–38), and it participates in initial adhesion of leukocytes to, and rolling along, inflamed venular endothelium (39–42). It has been established that sulfatides are ligands for L-selectin (19–23). The findings presented in this study are the first to show that a ligand for L-selectin is capable of triggering transmembrane signals. The capability of sulfatides to have such an effect showed the same requirements characterized for binding to L-selectin (19–22). In fact, triggering of an increase of cytosolic free calcium and cytokine genes expression required sulfation of the galactose ring of galactocerebrosides, and non-sulfated analogues had no effect (Figs. 1 and 4). Shedding of L-selectin induced by treatment of neutrophils with chymotrypsin blunted their capability to respond to sulfatides with an increase of cytosolic free calcium and cytokine genes expression. We cannot exclude that chymotrypsin treatment caused shedding of other, yet unidentified, sulfatide-binding molecules. However, chymotrypsin-induced L-selectin shedding showed selectivity, at least as class I major histocompatibility complex antigen and β_2 integrin expression was concerned; also fMLP receptor expression was not affected by chymotrypsin treatment since chymotrypsin-treated neutrophils responded normally to fMLP. Furthermore, the response to sulfatides was also blunted by treating neutrophils with PMA which was shown to effectively shed L-selectin (32).

The capability of sulfatides to trigger an increase of cytosolic free calcium in a L-selectin-dependent way was mirrored by the effect of anti-L-selectin antibodies. Four different anti-L-selectin antibodies had such an effect. These triggered a cytosolic free calcium increase either when used in solution, after cross-linking with second antibody, or immobilized to protein A

through the Fc fragment. Although in the type of assay described in Fig. 3*d* we could not reproduce conditions resembling neutrophil rolling, it is of interest that settling of neutrophils on anti-L-selectin antibodies immobilized through the Fc fragment in an unstirred condition was accompanied by an increase of cytosolic free calcium. Four control antibodies reacting with different surface molecules expressed by neutrophils did not trigger an increase of cytosolic free calcium, demonstrating that transmembrane signals triggered by anti-L-selectin antibodies were not merely a consequence of membrane perturbation due to cross-linking of surface molecules. Although we could reproduce the effect of sulfatides with anti-L-selectin antibodies we could not block sulfatide effects with the antibodies we used.[2] This is not surprising in the light of the evidence that DREG-200 and the LAM/1 series of antibodies we used are restricted in binding to the lectin-like, calcium-dependent domain of L-selectin (24, 25), while binding of sulfatides to L-selectin is calcium-independent (20, 21, 23).

The action of sulfatides and anti-L-selectin antibodies as triggers of an increase of cytosolic free calcium is not restricted to neutrophils. Also peripheral blood lymphocytes and monocytes responded to this challenge with an increase of cytosolic free calcium,[3] thus indicating that the capability of L-selectin to behave as a signaling molecules is not cell-specific.

We could not reproduce the described effects of sulfatides with two other L-selectin ligands, *i.e.* fucoidan and PPME (19). The simplest explanation of this finding might be that sulfatides, which display the characteristic to bind to L-selectin in a calcium-independent way (20, 22, 23), interact with a distinct epitope whose binding triggers L-selectin-dependent signals. Alternatively, it is worth pointing out that sulfatides form micelles and thus are presented in an aggregated, insoluble-like form. As we could use fucoidan and PPME only in a soluble form it cannot be excluded that L-selectin ligands are able to trigger signals only if immobilized to a surface. Significantly, L-selectin ligands are expressed at the endothelial cell surface. Cellular ligands of L-selectin have just begun to be identified, and a strict sulfation requirement for binding to L-selectin has been demonstrated (43, 44).

The findings presented in this report suggest that the signaling capacity of L-selectin does not depend, as for example that of receptors for chemotactic agonist present on phagocytic cells (45), on a pertussis toxin-sensitive guanine nucleotide binding regulatory protein (G protein). In fact, both the effects of sulfatides (Fig. 1) and anti-L-selectin antibodies (Figs. 2 and 3) were not inhibited by conditions of treatment of the cells with pertussis toxin which totally blocked the response to fMLP. However, experiments of chelation of extracellular calcium by EGTA (Fig. 1) demonstrated that the increase of cytosolic free calcium induced by sulfatides is due to release from intracellular stores. Taken together, these findings suggest that ligation of L-selectin may activate phosphoinositide-specific phospholipases C generating inositol trisphosphate through mechanisms independent of heterotrimeric GTP-binding proteins.

Generation of transmembrane signals by ligation of L-selectin might be involved in complex events such as rolling, or enhancement of leukocyte-endothelial adhesion through an inside-out mechanism of increase of adhesive interaction. The use of experimental models appropriate to investigate leukocyte-endothelial interaction will provide important clues to understand the significance of our findings *in vivo*.

That sulfatides can also signal enhanced expression of cytokines suggests that L-selectin-dependent adhesion can prime neutrophils to fulfil their proinflammatory role once migrated into the extravascular space. A few hours of treatment with sulfatides induced TNF and IL-8 mRNAs at levels comparable to those induced by LPS. Also for cytokine mRNAs induction the effect depended on sulfation of the galactose ring of galactocerebrosides, and chymotrypsin-induced shedding of L-selectin was accompanied by a lack of the response to sulfatides (Figs. 4 and 5). We found that TNF, but not IL-8, mRNA is induced after times of exposure to sulfatides as short a 30 min. This finding suggests that sulfatides induce modulation of distinct cytokine mRNAs with a temporal sequentiality. TNF which exerts proinflammatory autocrine effects on the neutrophils itself and pleiotropic paracrine effects on endothelial and tissue cells is induced early by sulfatides. Although any extrapolation to an *in vivo* situation is premature, it is tempting to speculate that ligation of L-selectin might activate gene transcription, and once transmigrated into the extravascular space neutrophils are fully committed to secrete cytokines into the extracellular milieu. In the last few years evidence has been accumulated that neutrophils participate in the amplification of the inflammatory response through secretion of cytokines (34, 46–48).

The findings that sulfatides induce cytokine mRNA accumulation also points out a possible mechanisms of amplification of tissue damage. Sulfatides are a major constituent of the nervous system. Interestingly, lymphocyte binding to myelinated tracts of the central nervous system was shown to be mediated by L-selectin (4). Release of sulfatides in certain demyelinating diseases can therefore amplify tissue damage by induction of cytokine genes.

[2] C. Laudanna, F. Rossi, M. A. Cassatella, and G. Berton, unpublished observation.

[3] C. Laudanna, G. Constantin, and G. Berton, unpublished observation.

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