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Enhancement of FcγR- and CR3-Mediated Neutrophil Phagocytosis by Cerebrosides

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There is increasing evidence that the ligation of adhesion molecules such as L-selectin can activate phagocytes to their full inflammatory potential. Sulfatide has been established as ligand for L-selectin and shown to trigger intracellular signals in human neutrophils. However, it remains unclear whether the ligation of L-selectin with sulfatide affects neutrophil phagocytosis. We studied the effects of sulfatide upon FcγR- and CR3-mediated human neutrophil phagocytosis. Adhesion of the cells to a sulfatide-coated surface resulted in a dose-dependent enhancement of phagocytosis mediated via FcγR or CR3, or both receptors. Galactocerebroside, but not glucocerebroside, also enhanced phagocytosis by neutrophils; therefore, galactose residue is thought to be required on ceramide molecules for the activation. Chymotrypsin-treated neutrophils, from which most L-selectin had been removed, reacted with sulfatide and galactocerebroside to enhance phagocytosis. These results suggest that an unidentified receptor for these cerebrosides exists on neutrophils and participates in the enhancement of phagocytosis.

Key Words: **cerebroside; complement; Fc receptors; L-selectin; neutrophils; phagocytosis.**

There is increasing evidence suggesting that L-selectin, a cell surface glycoprotein participating in the adhesive interaction of leukocytes with the vascular endothelium, is capable of triggering transmembrane signals. Ligation of L-selectin on neutrophils by sulfatide triggers a rise in cytosolic cellular calcium, an increase in tyrosine phosphorylation of several cellular proteins and enhances the production of cytokines (1, 2). Sulfatide is expressed on the surface of many cell types including neutrophils, and is excreted from neutrophils (3, 4).

Neutrophils play an important role in the clearance of immune complexes (IC) and iC3b-IC, an opsonized IC formed by complement activation. IC are recognized by two Fc receptors, FcγRIIA (CD32) (5) and FcγRIIIB (CD16) (6), and iC3b-IC are recognized by both FcγR and a complement receptor, CR3 (CD11b/CD18, αMβ2 integrin), which is a member of the leukocyte β2-integrin family (7). When binding to IC and iC3b-IC through their receptors, neutrophils exert effector functions; e.g., phagocytosis and the generation of reactive oxygen. In a previous study, we assessed the role of phagocytic receptors of human neutrophils by utilizing three ligands, IC for FcγR, iC3b-IC for both FcγR and CR3, and iC3b-IC[$F(ab)_2$] for CR3, and showed that phagocytosis of iC3b-IC is mediated via FcγRIIA and CR3, while phagocytosis of IC is mainly mediated via FcγRIIIB (8).

Although sulfatide has been shown to induce intracellular signals and the generation of reactive oxygen (1, 2, 9, 10), its effects upon the phagocytic responses of neutrophils remain unclear. In this study, we attempted to clarify whether the ligation of L-selectin with sulfatide affects the phagocytic responses of human neutrophils mediated via FcγR or CR3, or both receptors. We show here that sulfatide enhances the FcγR- and CR3-mediated phagocytic activities of neutrophils. Galactocerebroside, an unsulfated analogue of sulfatide, also enhances neutrophil phagocytosis, while other sphingolipids such as glucocerebroside have no effect. We also show that in addition to L-selectin, an unidentified cerebroside receptor exists on the cell surface of neutrophils and both receptors are involved in the enhancement of phagocytic activities of human neutrophils.

Abbreviations used: FcγR, Fc gamma receptor; CR3, complement receptor type 3; IC, immune complexes; IC[$F(ab')_2$], IC made from Ag and the $F(ab')_2$ fragment of Ab; iC3b-IC, iC3b-opsonized IC; FIC, FITC-labeled IC; HFA-ceramide, hydroxy fatty acid ceramide; NHFA-ceramide, non-hydroxy fatty acid ceramide; KRPB, Krebs-Ringer phosphate buffer.

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MATERIALS AND METHODS

Antibodies and reagents. Human anti-tetanus toxoid IgG was purchased from Yoshitomi Pharmaceutical Industries, Ltd. (Osaka, Japan). The $F(ab')_2$ fragment of human anti-tetanus toxoid IgG was prepared as previously described (8). Tetanus toxoid was provided by Dr. Taro Kinoshita (Research Institute for Microbial Disease, Osaka University). Sulfatide, galactocerebroside, glucocerebroside, hydroxy fatty acid ceramide (HFA-ceramide), non-hydroxy fatty acid ceramide (NHFA-ceramide), chymotrypsin and FITC were purchased from Sigma Chemical Co. (St. Louis, MO). Mouse anti-human L-selectin IgG1 (DREG-56) was purchased from Endogen (Cambridge, MA). The F(ab′) fragment of mouse anti-human FcγRII IgG2b (IV3) and the $F(ab')_2$ fragment of mouse anti-human FcγRIII IgG1 (3G8) were purchased from Medarex (Annandale, NJ); mouse anti-human CR3 IgG1 (44) from Leinco Technologies, Inc. (Ballwin, MO); and the $F(ab')_2$ fragment of FITC-conjugated sheep anti-mouse IgG from ICN biomedicals, Inc. (Aurora, OH). Ficoll-Paque was obtained from Amersham Pharmacia Biotech. (Bucks, UK); and Dextran from Nacalai Tesque Inc. (Kyoto, Japan). Other chemicals were of special grade.

Preparation of human neutrophils. Human peripheral blood was collected from healthy volunteers in acid citrate-dextrose. Neutrophils were isolated by sedimentation using Dextran and Ficoll-Paque, followed by hypotonic lysis of contaminating erythrocytes as reported previously (11, 12).

Preparation of FITC-labeled IC, iC3b-IC and iC3b-IC[$F(ab')_2$]. Tetanus toxoid was labeled with FITC by the method of Goding (13). FITC-labeled IC (FIC) and FITC-labeled IC[$F(ab')_2$] (FIC[$F(ab')_2$]) were prepared as reported previously (8, 14), with FITC-labeled tetanus toxoid and anti-tetanus toxoid IgG or anti-tetanus toxoid $F(ab')_2$.

Opsonization of FIC or FIC[$F(ab')_2$] with iC3b was performed as described previously (8). Briefly, 50 μg of FIC or FIC[$F(ab')_2$] was incubated with 300 μl of human serum for 1 h at 37°C, resulting in the covalent binding of iC3b through activation of the complement pathway. The apparent molar ratio of IgG and iC3b was assumed to be 1:1, as previously reported (8).

Chymotrypsin treatment of neutrophils. To shed L-selectin from the cell membrane, neutrophils in PBS (2.5×10^6 cells/ml) were treated with chymotrypsin (50 μg/ml) for 10 min at 37°C. After incubation, the cells were washed with cold PBS twice and resuspended in the same buffer.

Assay of phagocytosis. Sphingolipid-coated 96-well plates were prepared as described below. Briefly, sulfatide and other sphingolipids were dissolved at various concentrations in methanol and methanol/chloroform (7:3, v/v), respectively. One hundred μl of each solution was added to the wells of a 96-well plate (Nalge Nunc International, Denmark) and allowed to dry overnight at room temperature.

Phagocytosis of FIC or its derivatives (iC3b-FIC and iC3b-FIC[$F(ab')_2$]) was monitored by a fluorescence-quenching technique that enables the discrimination between ingested and extracellular surface-bound ligands. Neutrophils (1×10^5 cells) in 100 μl of KRPB were added to the sphingolipid-coated 96-well plate and allowed to adhere to the lipid-coated surface by centrifugation. The plate was incubated in a stationary position at 37°C for 5 min, and then each ligand was added at a final concentration of 30 μg/ml. After incubation for 60 min, ice-cold PBS was added to the wells to stop the reaction. After resuspension, the cells were recovered from each well and washed twice with cold PBS, then suspended in 20 mM sodium acetate buffer (pH 4.5) containing 150 mM NaCl and 0.5 mg/ml of Trypan blue to quench the fluorescence at the cell surface. The unquenchable fluorescence due to internalized FIC or its derivatives was measured using FACSort (Becton Dickinson, San Jose, CA).

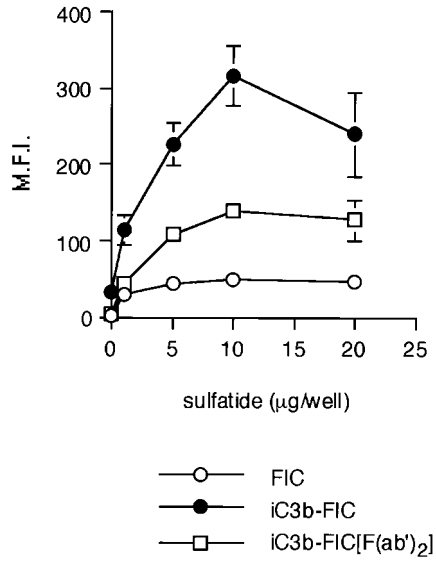

FIG. 1. Sulfatide enhances FcγR- and CR3-mediated phagocytic responses of human neutrophils. Neutrophils (1×10^5 cells) were allowed to adhere to plastic surfaces that had been coated with various amounts of sulfatide and incubated for 5 min at 37°C. After incubation, FIC, iC3b-FIC, or iC3b-FIC[$F(ab')_2$] were added at a final concentration of 30 μg/ml. After incubation for 60 min at 37°C, the cells were recovered from each well and suspended in 0.5 mg/ml of Trypan blue solution to quench the fluorescence at the cell surface. Internalized fluorescence of each cell was measured by flow cytometry and the results are shown as mean fluorescence intensity (M.F.I.).

FACS analysis. Expression of each receptor was analyzed by FACS after staining neutrophils (5×10^5 cells) with 0.2 μg of mouse anti-L-selectin mAb DREG-56, anti-FcγRII mAb IV3, anti-FcγRIII mAb 3G8, or anti-CR3 mAb 44, followed by 1 μg of FITC-conjugated sheep anti-mouse $F(ab')_2$.

RESULTS

Sulfatide enhances FcγR- and CR3-mediated phagocytic activities of human neutrophils. Human peripheral neutrophils were allowed to adhere to the wells of a 96-well plate coated with various amounts of sulfatide and their phagocytic activities were assessed with three ligands, FIC, iC3b-FIC, and iC3b-FIC[$F(ab)_2$]. As shown in Fig. 1, adhesion of the cells to sulfatide-coated surfaces resulted in a dose-dependent increase in phagocytosis, up to 10 μg/well. Under the optimum condition, phagocytosis of FIC and iC3b-FIC increased about 10-fold (3.61 and 38.78, respectively, by the cells adherent to the plastic surface and 43.52 and 311.38, respectively, by the cells adherent to the sulfatide-coated surface), and phagocytosis of iC3b-FIC[$F(ab)_2$] increased about 20-fold (5.45 by the cells adherent to the plastic surface and 124.31 by the cells adherent to the sulfatide-coated surface).

Galactose residue is required on ceramide molecules for the enhancement of phagocytic activities of human neutrophils. Next, we assessed whether the enhancement of phagocytic responses was specific for sulfatide

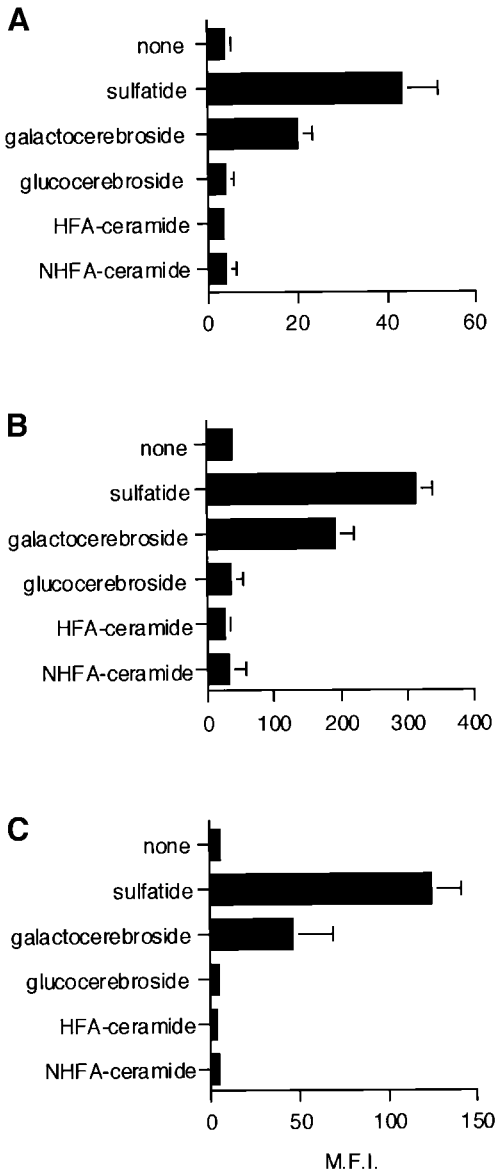

FIG. 2. Galactose residue is required on ceramide molecules for the enhancement of the phagocytic activities of human neutrophils. Neutrophils (1×10^5 cells) were allowed to adhere to plastic surfaces that had been coated with various sphingolipids (10 μg/well) and incubated for 5 min at 37°C. After incubation, FIC (A), iC3b-FIC (B), or iC3b-FIC[$F(ab')_2$] (C) were added at a final concentration of 30 μg/ml and incubated for 60 min. Phagocytosis of the three ligands was measured by flow cytometry and the results are shown as M.F.I.

or detectable with other ceramide analogues. Adhesion of neutrophils to surfaces coated with galactocerebroside, an unsulfated form of sulfatide, also triggered the enhancement of phagocytic responses to these ligands (Fig. 2). The galactocerebroside-induced increase in phagocytosis was about half of that induced by sulfatide. On the other hand, adhesion of the cells to surfaces coated with two kinds of ceramides (HFA-ceramide and NHFA-ceramide) or glucocerebroside did not produce an increase in the phagocytosis of these ligands (Fig. 2). These results suggest that galactose residue is required on ceramide molecules for the activation of neutrophils, and sulfation of the galactose residue resulted in the enhancement of its effect.

Sulfatide- and galactocerebroside-induced enhancement of phagocytic responses is mediated via L-selectin and other cell membrane components. We next investigated whether L-selectin plays a significant role in the sulfatide- and galactocerebroside-induced enhancement of phagocytic responses. Since L-selectin is shown to be sensitive to chymotrypsin (15), neutrophils were treated with chymotrypsin to shed L-selectin. Figure 3 shows that almost all L-selectin was shed upon treatment with chymotrypsin. On the other hand, the surface expression of FcγR and CR3 was not decreased by treatment with chymotrypsin (Fig. 3). Chymotrypsin-treated neutrophils still responded with sulfatide to enhance FcγR- and CR3-mediated phagocytosis. The increase in phagocytosis of FIC, iC3b-FIC and iC3b-FIC[$F(ab)_2$] was about 91.0, 62.8, and 35.7% of that by nontreated cells, respectively (Fig. 4). L-selectin is thought to be the only receptor for sulfatide that is expressed on the neutrophil cell surface (1, 2). However, these results suggest that in addition to L-selectin, other cellular components able to interact with sulfatide and induce the enhancement of phago-

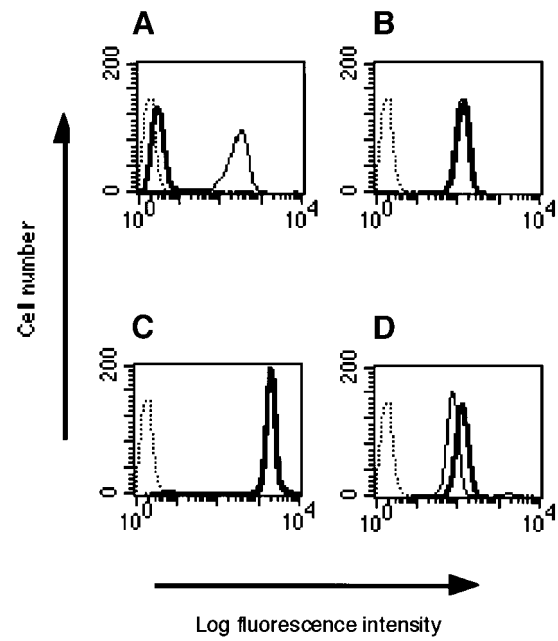

FIG. 3. L-selectin, but not FcγRIIA, FcγRIIIB, or CR3, is shed from the cell surface of neutrophils on treatment with chymotrypsin. Neutrophils (5×10^5 cells) were incubated with 50 μg/ml of chymotrypsin for 10 min at 37°C. The cells were washed with cold PBS and stained with mAb against L-selectin (A), FcγRIIA (B), FcγRIIIB (C), or CR3 (D), followed by the addition of FITC-conjugated sheep anti mouse $F(ab')_2$. The expression of each receptor was measured by flow cytometry. Thin line, control (nontreated cells); thick line, chymotrypsin-treated cells; broken line, no primary mAbs added.

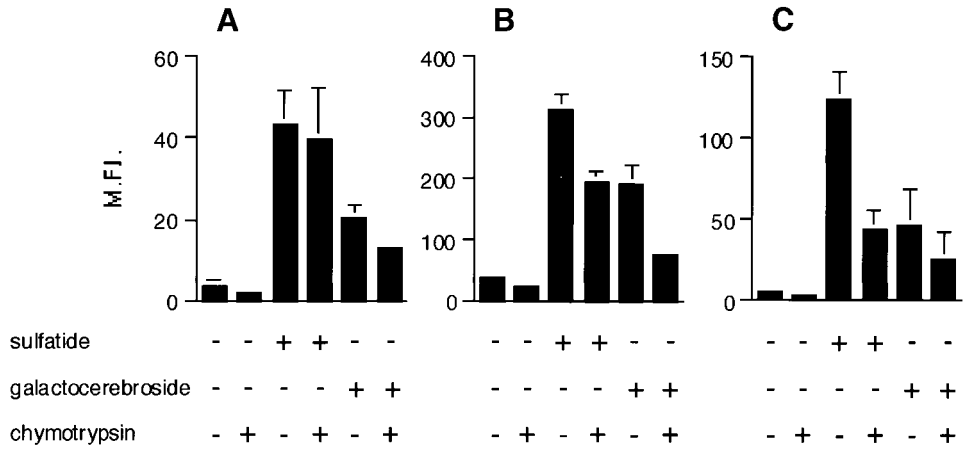

FIG. 4. Sulfatide- and galactocerebroside-induced enhancement of phagocytic responses is mediated via L-selectin and other cell membrane components. Neutrophils (1×10^5 cells) were incubated alone or with 50 μg/ml of chymotrypsin for 10 min at 37°C. After chymotrypsin treatment, the cells were allowed to adhere to plastic surfaces that had been coated with indicated cerebrosides (10 μg/well) and incubated for 5 min at 37°C. After incubation, 30 μg/ml of FIC (A), iC3b-FIC (B), or iC3b-FIC[$F(ab')_2$] (C) were added and incubated for 60 min at 37°C. Phagocytosis of the three ligands was measured by flow cytometry and the results are shown as M.F.I.

cytic responses exist on the cell surface of neutrophils. As well as sulfatide, galactocerebroside also enhanced phagocytic responses of chymotrypsin-treated cells, although the increase in phagocytosis was smaller (about 45–60%) than that of nontreated cells (Fig. 4).

DISCUSSION

L-Selectin, a lectin-like molecule recognizing carbohydrate-bearing ligands, has recently been shown to be able to transmit intracellular signals, including increased tyrosine phosphorylation and activation of MAP kinase (2). In addition, it was reported that the ligation of L-selectin with sulfatide generates intracellular oxygen radicals and increases cytokine mRNA expression (1, 10). These results suggest that the interaction between L-selectin and sulfatide modulates the effector functions of neutrophils. Phagocytosis is the critical effector function for host defense by neutrophils and in the present study we assessed the effects of sulfatide upon FcγR- and CR3-mediated phagocytic responses of human neutrophils. To assess FcγR- and CR3-mediated phagocytosis, we prepared three immune complexes; IC, iC3b-IC, and iC3b-IC[$F(ab)_2$]. IC induces the activation of the classical complement pathway to form C3b-IC, which is quickly processed into iC3b-IC, a possible natural ligand for both FcγR and CR3. Evaluation of CR3 function was performed using iC3b-IC[$F(ab)_2$].

The findings presented in this study are the first to show that sulfatide and galactocerebroside are capable of enhancing both FcγR- and CR3-mediated phagocytic activities of human neutrophils. The sulfatide-induced increase in phagocytosis was estimated to be about 10-fold depending upon the ligands, and the CR3-mediated phagocytic response to iC3b-IC[$F(ab)_2$] was estimated to have been enhanced about 20-fold. The ability of galactocerebroside to enhance neutrophil phagocytosis was smaller than that of sulfatide by approximately 50%. On the other hand, glucocerebroside, which differs from galactocerebroside only in the configuration of the 4-hydroxyl group on the carbohydrate, had no effect on neutrophil phagocytosis. These results suggest that the 4-hydroxyl configuration of the carbohydrate is important in increasing neutrophil phagocytosis, and the 3-sulfo group on the carbohydrate enhances this effect.

Treatment of neutrophils with chymotrypsin resulted in an almost complete shedding of L-selectin from the cell surface and a decrease in the sulfatide-induced enhancement of phagocytic responses to iC3b-IC and iC3b-IC[$F(ab')_2$]. Thus, the enhancement of phagocytosis is, at least in part, triggered by the interaction between sulfatide and L-selectin. CR3 is known to be stored as intracellular pools localized in peroxidase-negative granules in neutrophils, and Crokett-Torabi *et al.* reported that cross-linking of L-selectin using mAb induced an up-regulation of surface CR3 expression on neutrophils (16, 17). Therefore, it is possible that the adhesion of neutrophils to sulfatide-coated surfaces induces the ligation of L-selectin and up-regulation of surface CR3 expression, and finally induces enhancement of CR3-mediated phagocytic responses.

As chymotrypsin-treated neutrophils still responded fully with sulfatide to enhance phagocytosis of IC, it appears that L-selectin contributes little to the sulfatide-induced enhancement of FcγR-mediated phagocytosis. In addition, sulfatide also enhanced phagocytic responses to iC3b-IC and iC3b-IC[$F(ab')_2$] of chymotrypsin-treated

cells, although the degree of the increase in phagocytosis was smaller than that of non-treated cells. These results suggest that an unidentified sulfatide-binding protein, which is distinct from L-selectin and contributes to increases in both FcγR- and CR3-mediated phagocytosis, might be expressed on human neutrophils. Sulfatide is expressed not only in hemopoietic cells but also in some tissues such as the brain and kidney, and it has been reported that leukocytes recruited into tissue express extremely low levels, if any, of L-selectin (18). Therefore, if sulfatide serves as an activator for neutrophils that migrate into inflamed tissues, it is reasonable to speculate that another sulfatide receptor exists on the cell surface of neutrophils to enhance their effector functions.

Treatment of the cells with chymotrypsin also decreased the ability of galactocerebroside to enhance phagocytic responses, although it has been reported that L-selectin is not capable of interacting with galactocerebroside (19). In our experiments, neutrophils were forced to adhere to galactocerebroside-coated surfaces by centrifugation and we suppose that under such conditions, L-selectin might interact weakly with galactocerebroside. As well as sulfatide, a chymotrypsin-resistant galactocerebroside-binding protein seems to exist on neutrophils and contribute to the enhancement of phagocytic responses in addition to L-selectin. In this study, we could not elucidate whether the unidentified receptors for sulfatide and galactocerebroside are identical and we are currently investigating these receptors.

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