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Enhanced IgG- and complement-independent phagocytosis of sulfatide-enriched human erythrocytes by human monocytes

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Phagocytosis by adherent human monocytes of human erythrocytes (RBC), sulfatide-enriched by incubation with 10^{-12} to 10^{-9} M cerebroside sulfate, was enhanced approx. 6-fold. Increased phagocytosis was observed only in RBC opsonized with fresh plasma, and not in non-opsonized or serum-opsonized RBC. Increased phagocytosis was immunoglobulin- and complement independent. Thrombospondin and von Willebrand factor, present in plasma but not in serum, and binding selectively to sulfatides, are likely mediators of the enhanced phagocytosis.

Cerebroside sulfate; Sulfatide; Sulfated glycolipid; Erythrocyte; Phagocytosis; Thrombospondin; von Willebrand factor

1. INTRODUCTION

Sulfatides (sulfated glycolipids) are constitutive elements of RBC membrane complex glycolipids, together with gangliosides and fucoglycolipids [1]. According to their percent distribution (approx. 0.16% of total complex lipids) [1,2] they rank in the lowest range, if compared to PS (13%), PE (27–31%) or sphingolipids (24–27% of total complex lipids) [2]. Sulfatides are confined to the external leaflet of the RBC bilayer [1,2], play a role in cell adhesion in different cell types [4], and bind specifically and with high affinity to laminin [5], thrombospondin [6] and von Willebrand factor [7]. We report here that addition of minute amounts of sulfatides to the RBC membrane remarkably increases RBC phagocytosis by adherent human monocytes. Previous opsonization of sulfatide-enriched RBC in plasma, but not in serum, is necessary and binding of IgG or complement does not occur. Binding of thrombospondin or von Willebrand factor to the sulfatide-enriched RBC represents a likely trigger for phagocytosis.

2. EXPERIMENTAL

2.1. *Materials*

Sulfatide (cerebroside sulfate from bovine brain), PC, PS, alkaline phosphatase-conjugated rabbit anti-human IgG, cell culture products, buffers and other standard chemicals were from Sigma (St. Louis, MO). Tritiated cerebroside sulfate was prepared according to [8] using [^{3}H]borhydride from Amersham International (Bucks, UK).

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Abbreviations: BSA, bovine serum albumin, type V; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IgG, immunoglobulin G; PBS, phosphate-buffered saline; PBS-glucose, PBS supplemented with 10 mM glucose; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; RBC, erythrocyte(s).

2.2. *Enrichment of RBC with sulfatides*

RBC (group 0, Rh positive) were isolated from venous blood freshly drawn from healthy volunteers of both sexes. Informed consent was obtained from each donor. Blood was anticoagulated with 6 IU/ml heparin. After removal of plasma and buffy coat by aspiration and 3 washes in HEPES–saline buffer (NaCl 140 mM, HEPES 5 mM, glucose 5.5 mM, pH 7.4), RBC were enriched with sulfatides. Cerebroside sulfate was presented as a dispersion obtained by sonicating sulfatide suspensions (10^{-5} to 10^{-12} M in RPMI 1640, pH 7.4) for 5 × 20 s with 60 s cooling intervals between each sonication cycle. Immediately after sonication, RBC were added to RPMI 1640 medium containing the dispersed sulfatide at 1% hematocrit for 60 min at 37°C.

2.3. *Quantitation of RBC-associated added sulfatide*

RBC-associated sulfatide was quantitated after treatment with [^{3}H]sulfatide (8×10^5 cpm/μg sulfatide), presented to the RBC as sulfatide dispersion in RPMI 1640 medium. After 60 min incubation at 37°C, RBC were washed 4 times in HEPES–saline buffer and hypotonic hemoglobin-free ghosts were prepared [9]. After protein determination by the bicinchoninic acid method (Pierce) and liquid scintillation counting, RBC-associated sulfatide was expressed as cpm per μg ghost protein.

2.4. *Opsonization of RBC*

To a volume of packed, washed RBC was added an equal volume of fresh autologous serum, complement-inactivated (30 min at 56°C) serum or plasma, and the cell suspension was incubated for 30 min at 37°C.

2.5. *Preparation of adherent human monocytes*

Mononuclear cells were separated from fresh human blood collected in heparin (6 IU/ml blood) [10]. Separated lympho-monocytes were washed 3 times in PBS, resuspended in RPMI 1640 medium supplemented with 10% (v/v) autologous serum and plated at $1\text{–}2 \cdot 10^6$ cells/well in 24-well plates. After 1 h incubation in a humidified incubator (5% CO_2/95% air, 37°C), non-adherent cells were removed by

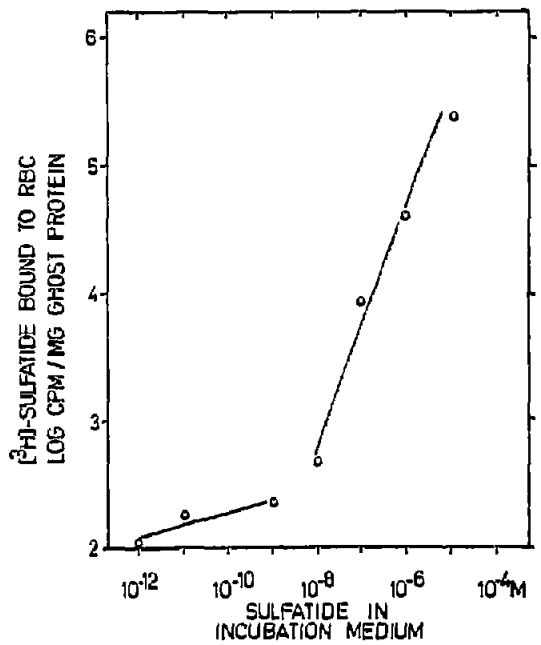

Fig. 1. Relationship between sulfatide concentration in the incubation medium and sulfatide associated to the RBC membrane. Typical experiment out of four with similar results.

washing the wells with lukewarm RPMI 1640 medium. Adherent monocytes were reincubated with the same medium for 5–10 h until starting the phagocytosis experiments.

2.6. *Quantitation of phagocytosis*

Fifteen μl opsonized RBC (hematocrit 10%) were added to each well containing approx. 50,000 monocytes. The ratio RBC/monocyte was approx. 300. After 1 h incubation at 37°C in a humidified incubator (see above) non-ingested/non-adherent RBC were removed by aspiration and 3 washes with PBS. Non-ingested, adherent RBC were osmotically lysed by addition of ice-cold distilled water for 20 s, followed by an additional wash with PBS to remove debris and free hemoglobin. After solubilization of monocytes containing ingested RBC by adding to each well 1 ml solubilizing solution (0.1 N NaOH, 0.025% (v/v) Triton X-100), phagocytosis was quantified by measuring ingested hemoglobin by luminol-elicited luminescence in the presence of *tert*-butylhydroperoxide as an electron donor [11].

2.7. *Quantitation of bound IgG*

After opsonization with serum or plasma, RBC were washed 5 times with RPMI 1640 medium at 4°C and suspended at 10% hematocrit in PBS-G supplemented with 2% (w/v) BSA and alkaline phosphatase-conjugated rabbit anti-human IgG, diluted 1:1,000 (v/v). After 2 h incubation at room temperature, RBC were washed 5 times with PBS-G supplemented with 2% BSA (w/v) and hypotonic ghosts prepared [9]. After ghost solubilization in 0.5% (v/v) Triton X-100, rabbit alkaline phosphatase-conjugated anti-human IgGs were quantitated spectrophotometrically at 405 nm [12].

3. RESULTS

The relationship between sulfatide concentration in the incubation medium and exogenous sulfatide associated to the RBC membrane is shown in Fig. 1. Incubation of RBC in a medium containing 10^{-12} to 10^{-9} M sulfatide (presented as sonicated sulfatide suspension) causes a modest increase in sulfatide association to the RBC membrane. A thousand-fold increase in sulfatide concentration in the incubation medium from 10^{-12} to 10^{-9} M sulfatide brought about a 2-fold increase in sulfatide associated to RBC membrane. Above 10^{-9} M sulfatide concentration in the incubation medium, the curve becomes much steeper, indicating a linear relationship between sulfatide concentration in the incubation medium and sulfatide associated to the RBC membrane.

Enrichment of RBC membrane with sulfatide increases RBC phagocytosis by adherent monocytes (Figs. 2 and 3). The increase is small (1.7-fold), non-significant and not related to the amount of RBC-associated sulfatide, when sulfatide-enriched RBC were not opsonized (Fig. 3). When sulfatide-enriched RBC were opsonized with autologous plasma, enhancement of phagocytosis was very marked. Maximum increase was approx. 6-fold over non-enriched controls when enrichment was performed by pre-incubating RBC at 10^{-9} M sulfatide (Fig. 2). Increase in phagocytosis was not modified when RBC were opsonized with complement-inactivated plasma, but dropped to control values when opsonization was carried out with serum instead of plasma (Figs. 2 and 3).

Dependence between enhancement of phagocytosis and the amount of RBC-associated sulfatide was also studied. As shown in Fig. 2, there is a narrow range of sulfatide enrichment which is associated with 5- to 6.5-

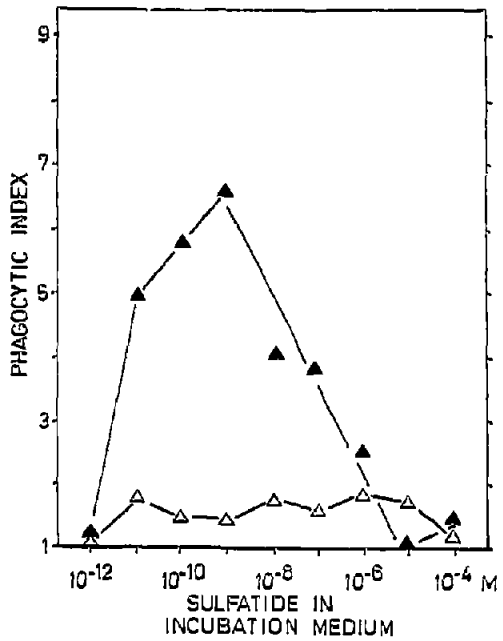

Fig. 2. Relationship between sulfatide concentration in incubation medium and RBC phagocytosis by adherent human monocytes. ▲, RBC opsonized in plasma; △, RBC opsonized in serum. Phagocytic index, ratio between number of phagocytosed sulfatide-enriched RBC vs. control RBC/monocyte. Each experimental value is the average of four distinct phagocytosis wells. Typical experiment out of three with similar results.

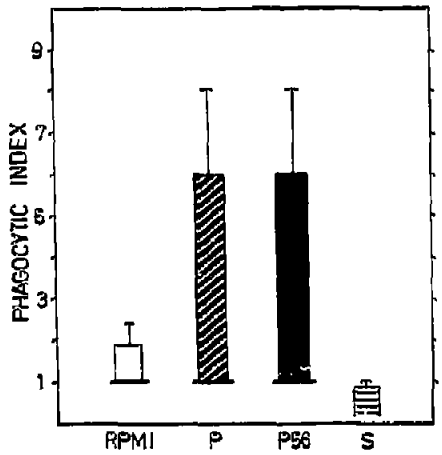

Fig. 3. Dependence of phagocytosis of sulfatide-enriched RBC from opsonization. Sulfatide-enriched RBC were opsonized in RPMI 1640 medium (RPMI), in fresh autologous plasma (P), in complement-inactivated plasma (P56) and in fresh autologous serum (S). Phagocytic index, ratio between number of phagocytosed sulfatide-enriched RBC vs. control RBC/monocyte. Mean values ± S.D. of four experiments. Each experiment is the average of four distinct phagocytosis wells.

fold enhancement of phagocytosis. This range is obtained by incubating RBC in a 10^{-12} to 10^{-9} M sulfatide suspension. Further increase in sulfatide was accompanied by a sharp decrease in phagocytosis. Phagocytosis was back to control values when RBC were sulfatide-enriched by incubating RBC in sulfatide suspensions at 10^{-8} sulfatide. Interestingly, the diminution in phagocytosis corresponds with the steep portion of the biphasic curve in Fig. 1, and with the association of large numbers of sulfatide molecules to the RBC.

Binding of IgG was monitored by phosphatase-conjugated anti-human IgG (not shown). Repeated assays performed on RBC enriched with different amounts of sulfatide and opsonized by incubation in autologous plasma or serum for 1 h at 37°C constantly gave negative results, indicating that RBC-associated sulfatides did not cause increased deposition of IgG.

4. DISCUSSION

We show here that addition of small amounts of sulfatides to the membrane of human RBC remarkably increased their phagocytosis by adherent human monocytes. The highest increase in phagocytosis was observed after incubation of RBC in sulfatide dispersions at 10^{-12} to 10^{-9} M sulfatide. We observed proportionality between added sulfatide and increased phagocytosis only in a narrow range of sulfatide supplementation. Incubation of RBC at higher sulfatide concentrations led to a decrease in phagocytosis. This behavior was observed at sulfatide concentrations corresponding to or surpassing the micellar point concentration (B. Cestaro, personal communication).

The increase in phagocytosis was not mediated by increased deposition of autologous IgG and was not complement-dependent. Therefore, autoantibodies directed against sulfatides or autoantibodies bound unspecifically to RBC regions with increased densities of negative charges can be excluded from playing a role. Also, we can exclude that exogenously associated sulfatides may activate the alternative complement pathway and mediate a complement-dependent phagocytosis.

Opsonization with fresh, platelet-containing plasma was necessary in order to obtain phagocytosis stimulation. Opsonization with fresh autologous serum was ineffective. Thus, some plasma component which is no longer present in serum could mediate increased phagocytosis. Considering proteins known to bind to sulfated glycolipids with high affinity, laminin is only present in basal membranes [13], whereas thrombospondin and von Willebrand factor are both present in plasma [14,15]. Thrombospondin is produced by platelets, monocytes, endothelial cells and a number of other cell lines and is present in plasma at concentrations varying between 97 and 163 ng/ml [14]. Von Willebrand factor is not produced by platelets, and is present in plasma at the concentration of approx. 20 ng/ml [15]. Both factors are depleted in serum following platelet aggregation and binding of thrombospondin to fibrinogen [14,15]. Both factors bind to sulfated glycolipids with high affinity, but thrombospondin has 3 times higher affinity than von Willebrand factor [4]. For these considerations, thrombospondin seems to be a likely candidate as a mediator for increased phagocytosis.

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