Acta Biochimica Polonica

Vol. 45 No. 2/1998

493–499

QUARTERLY

Contradictory functions of sulfatide in the blood coagulation system as coagulant and anticoagulant*°

Mamoru Kyogashima[1]✉, Junichi Onaya[1], Atsushi Hara[2] and Tamotsu Taketomi[2]

[1]*Seikagaku Corporation Tokyo Research Institute Higashiyamato-shi, Tokyo 207-0021, Japan, and* [2]*Research Centre on Aging and Adaptation, Shinshu University School of Medicine, Matsumoto, Nagano 390-0802, Japan*

Sulfatide (galactosylceramide I^3-sulfate) has been reported to activate blood coagulation factor XII (Hageman factor), which suggests that it exhibits coagulant activity (Fujikama *et al.*, 1980 *Biochemistry* 19, 1322–1330) However, sulfatide administered into animals as a bolus shot without subsequent thrombus formation, prolonged conventional clotting times and bleeding time (Hara *et al.*, 1996 *Glycoconjugate J.* 13, 187–194). These findings suggest that it may exhibit anticoagulant rather than coagulant activity. Following this suggestion we found *in vitro* that binding of sulfatide to fibrinogen resulted in disturbance of fibrin formation. To examine a possible pharmacological effect of sulfatide on blood coagulation *in vivo* we continuously infused sulfatide into rats through plastic cannulae and found formation of giant thrombi around the tips of the cannulae. These data suggest that sulfatide may exhibit contradictory functions in the blood coagulation system.

In 1961 Wago suggested possible anticoagulant activity of sulfatide following its administration to rabbits [1] whereas in 1980, Fujikawa *et al.* [2] reported for the first time that sulfatide could activate blood coagulation factor XII *in vitro*. Since then, sulfatide has been believed to be one of the important factors in the initiation of the intrinsic coagulation pathway [3–5] although the physiological importance of the intrinsic pathway itself still remains unclear. On the other hand, we found that Watanabe hereditable hyperlipidemic

*Paper presented at the International Symposium on Structure, Metabolism and Functions of Glycosphingolipids and Sphingolipids, held in Warsaw in September, 1997.

°This work was supported in part by a Grant-in-Aid for Scientific Research (No. 07670141) from the Ministry of Education, Science, Sports and Culture of Japan.

✉Correspondence to: Dr. Mamoru Kyogashima, Seikagaku Co. Tokyo Research Institute 1253 Tateno 3-chome, Higashiyamato-shi, Tokyo 207-0021, Japan; tel: (+81 42) 563 5823; fax: (+81 42) 563 5846; e-mail: kyogashimamo@ma4.justnet.ne.jp

Abbreviations: I^3 SO_3-GalCer, sulfatide; II^3 NeuAcα-Gg_4Cer; ganglioside GM1; II^3NeuAcα_2-LacCer, ganglioside G_{D3}; WHHL, Watanabe hereditable hyperlipidemia; ATIII, antithrombin III; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline, pH 7.4; OA, ovalbumin.

(WHHL) rabbits treated with sulfatide (10 mg/kg) as a bolus shot every other day for three months showed no definite symptoms or pathological changes related to thrombotic disorders during the treatment and at autopsy [6]. In addition we recently found that sulfatide could prolong clotting times and bleeding time in rabbits [7–9]. These findings lead to the hypothesis that sulfatide may act as an anticoagulant rather than a coagulant. With respect to the above items, sulfatide may be similar to heparin which is a representative of anticoagulants and is widely used in clinical practice over the world. Heparin can prolong clotting time as well as bleeding time [10, 11] and it was also reported to be able to activate blood coagulation factor XII *in vitro* [13]. The precise mechanism of heparin acting as an anticoagulant depends on its binding to antithrombin III (ATIII) through its known pentasaccharide domain to enhance the activities both of ATIII towards thrombin and of anti-blood coagulation – factor Xa [10, 12]. A different mechanism has been suggested for sulfatide as an anticoagulant since sulfatide does not accelerate the ATIII activity [9]. Therefore we have studied its anticoagulant mechanism to find out that sulfatide by binding to fibrinogen lead to disturbances of fibrin formation. However, when continuously infused into rats, sulfatide induced giant thrombi at the tips of cannulae. The data presented suggest contradictory functions of sulfatide in the blood coagulation system.

MATERIALS AND METHODS

Glycosphingolipids and glycosaminoglycans. Sulfatide (an acidic glycosphingolipid, sulfuric ester of galactosylceramide at the C3 of galactose residue) and galactosylceramide were prepared from pig spinal cord in our laboratory [8]. Ganglioside G_{M1} and G_{D3} were purchased from Sigma (St. Louis, MO, U.S.A.) and Waco (Osaka, Japan), respectively. Heparin and chondroitin sulfate E were from Wako and Seikagaku Co. (Tokyo, Japan), respectively.

Effect of sulfatide on bleeding time. After sulfatide dissolved in PBS (5 mg/ml) was injected into mice (20 mg/kg) through tail veins, tail tips of the mice were cut periodically with a scalpel. The tail was immediately placed in PBS at 37°C and bleeding time was measured.

Enzyme-linked immunosorbent assay (ELISA). Glycosphingolipids dissolved in 100 μl of methanol were dried in wells of a 96 well microtiter polystyrene plate (Corning 25801, Corning NY, U.S.A.). The wells were filled with PBS containing 5% ovalbumin (PBS-OA). After 2 h, the wells were washed and fibrinogen (1 μg) in 100 μl of PBS-OA was added to each well. After another 2 h, the wells were washed and horseradish peroxidase-conjugated goat anti-human fibrinogen antibody (Organon-Technika, NC, U.S.A.) was added. After 1 h, the wells were washed and binding activities were detected by *o*-phenylenediamine/hydrogen peroxide treatment.

Inhibition of fibrin gel formation by sulfatide. Various amounts of sulfatide in 80 μl of 0.05 M Tris/HCl, pH 8.4, containing 0.075 M Na_2EDTA, 0.175 M NaCl, and 0.5% bovine serum albumin (Tris/BSA) were incubated for 3 min at 37°C. After adding thrombin (0.72 U, Sigma, MO, U.S.A.) in 20 μl of Tris/BSA to each sample, fibrinogen (1 mg) in 300 μl of 0.075 M Tris/HCl, pH 7.15 was added and clotting time was measured.

Continuous infusion of sulfatide into rats. Under anesthesia, the rats were incised at the left thigh and a polyethylene cannula (PE-10, Imamura, Tokyo, Japan) was retrogradingly inserted 4 cm into the abdominal *vena cava* by way of the left femoral vein. Sulfatide or gangliosides were continuously infused at a dose of 1.25 mg/kg per h. After 4 h, rats were killed under anesthesia and examined.

Kinetic analysis of plasma coagulation by turbidometry using a 96 well microtiter plate. This analysis was originally described for Limulus test to determine bacterial endo-

toxins [14]. One volume of plasma was gently mixed with the same volume of PBS (PBS-plasma). Various amounts of glycosphingolipids dissolved in 50 μl of methanol or chloroform/methanol (1:1, v/v) were added to wells of a 96 microtiter plastic plate. After complete evaporation of organic solvent, 100 μl of PBS-plasma were added to the wells. As glycosaminoglycans, 100 μl of PBS-plasma containing various amounts of heparin or chondroitin sulfate E were added to lipids-uncoated wells of the plate, respectively. The plate was installed in a microplate reader (Wellreader SK601, Seikagaku Co. Tokyo, Japan) and kept at 37°C during the analysis. The increase of absorbance by the increased turbidity due to fibrin formation was monitored at 405 nm and recorded every 15 s. The onset time of fibrin formation in this analysis was defined as the first of three sequential records when the absorbance value in each well was elevated more than 0.02 from the base value.

RESULTS AND DISCUSSION

Effect of sulfatide on bleeding time

Bleeding time were prolonged after injection of sulfatide and its maximal effect was obseved at 30 min after the injection (Fig.1).

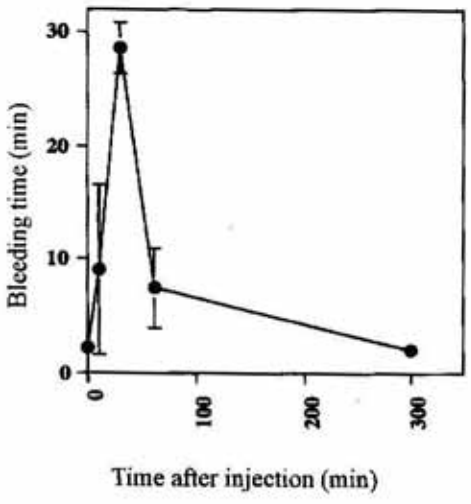

Figure 1. Effect of sulfatide on bleeding time.

Interaction of sulfatide with fibrinogen

Since our earlier results showed that sulfatide inhibited neither thrombin nor factor Xa activity [9], we examined the action of sulfatide on fibrinogen in a system where only sulfatide, fibrinogen, thrombin and albumin were involved but the other plasma proteins including many factors related to blood coagulation and fibrinolysis were excluded. As shown in Fig. 2a, sulfatide effectively inhibited the formation of fibrin gel in a dose-dependent manner suggesting that sulfatide may act on fibrinogen. To further confirm this suggestion we tested by ELISA whether sulfatide binds to fibrinogen. As shown in Fig. 2b, sulfatide but not GalCer bound to fibrinogen

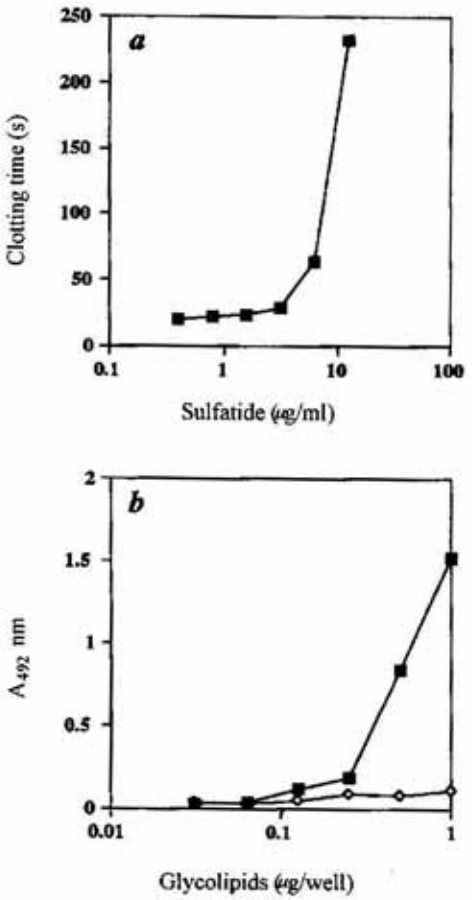

Figure 2. The interaction of sulfatide with fibrinogen.

a, Prolongation of the clotting; b, binding of fibrinogen to sulfatide and galactosylceramide; ■, sulfatide; ◇, GalCer.

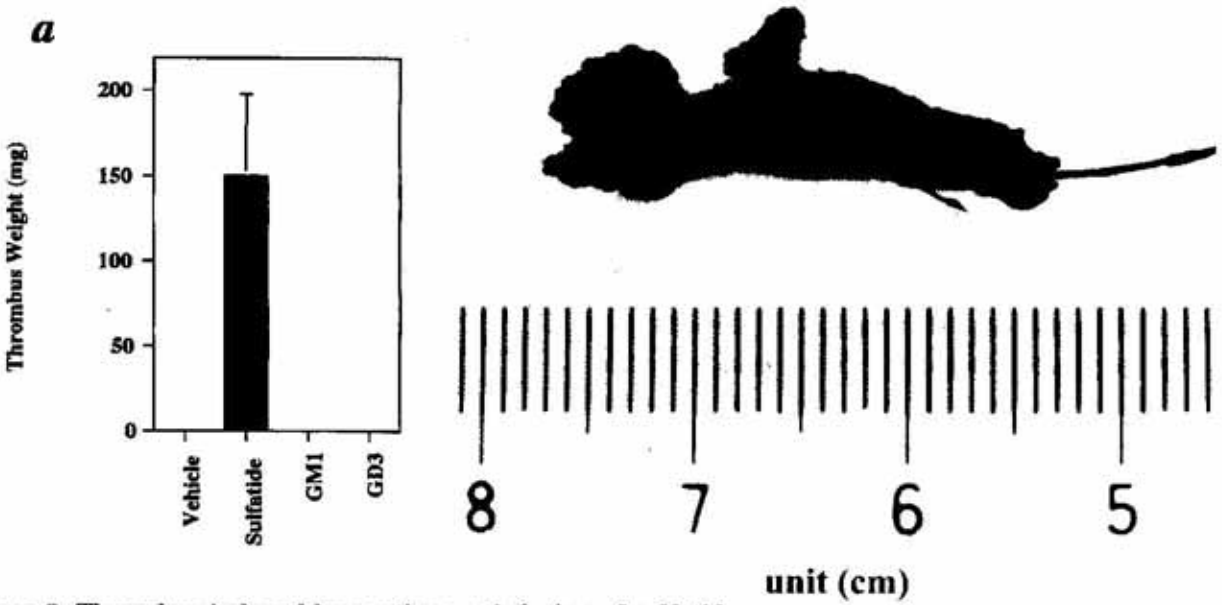

Figure 3. Thrombus induced by continuous infusion of sulfatide.

in a dose-dependent manner, suggesting that a sulfated moiety is important for the binding.

Effect of sulfatide continuously infused into rats

After infusion of glycosphingolipids, we found giant thrombi at the tips of cannulae from all rats into which sulfatide was infused (Figs. 3a and 3b). The average weight of the thrombus was 150 mg. It should be noted that thrombi were formed only at the tips. On the contrary, no thrombi were induced at the tips in the rats into which ganglioside G_M1 or GD3 was infused.

Effect of plastic plate coated with sulfatide on plasma coagulation times in a 96 well microtiter plate

In order to evaluate coagulant activity of glycosphingolipids on animal plasma, we fixed glycosphingolipids on plastic wells a procedure which is commonly used to test binding ability of glycosphingolipids to many carbohydrate binding proteins [15]. Plasma coagulation was simply initiated by $CaCl_2$. The benefit of this method is that it directly compares the effect of hydrophobic lipids with that of hydrophilic ones on plasma coagulation with-

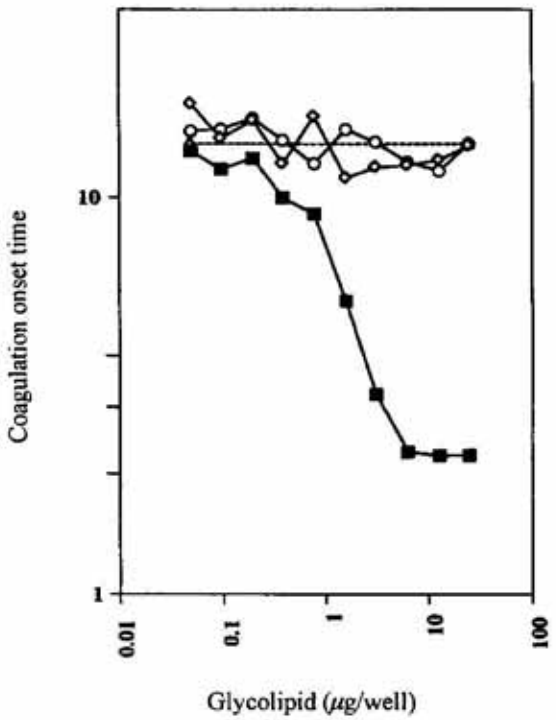

Figure 4. The relation between coagulation onset time [$\log_{10}$(min)] and sulfatide [$\log_{10}$(μg/well)] GalCer and monosialoganglioside G_{M1} (μg/well).

■, Sulfatide; ◇, G_{M1}; ○, GalCer; Δ, no glycolipid.

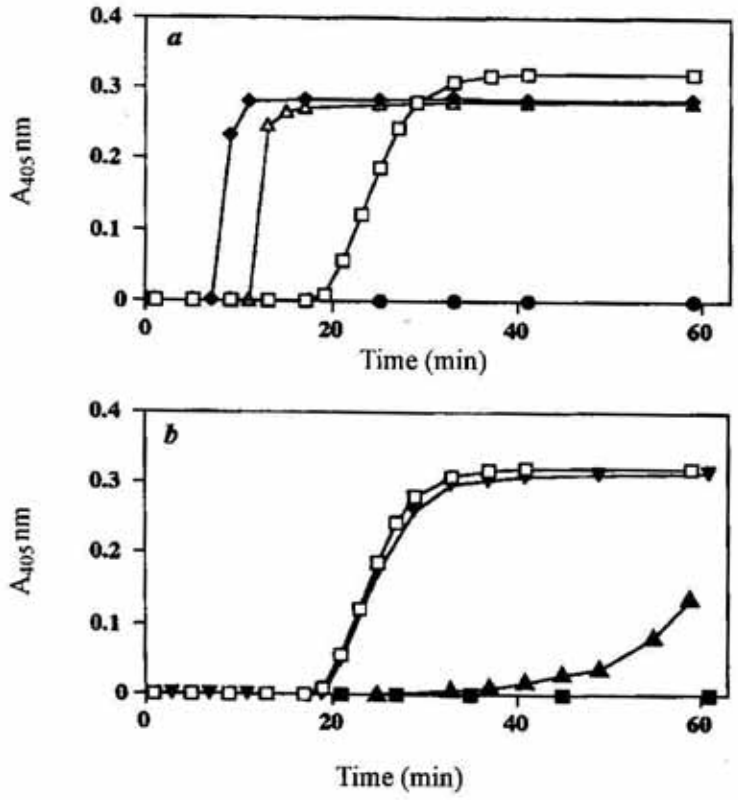

Figure 5. Effect of sulfatide (a) and heparin and chondroitin sulphate E (b) on coagulation of human plasma lacking factor XII and a mixture (the same volume) of normal and factor XII-deficient plasma were examined by kinetic assay using a 96 well microtiter plate.

□, Normal plasma control; ◆, normal plasma with sulfatide (6.25 μg/well); Δ, normal/factor-XII deficient plasma (1:1) with sulfatide (6.25 μg/well); ▲, normal plasma with chondroitin sulfate (6.25 μg/well); ■, normal plasma with heparin (more than 0.4 μg/ml) or chondroitin sulfate E (more than 94 μg/ml); ▼, normal plasma with heparin (less than 0.1 μg/ml or chondroitin sulfate E (less than 5.9 μg/ml).

out the necessity of adding additional detergent or proteins to solve the lipids. Figure 4 shows the relationships between plasma coagulation onset time and glycosphingolipids in rat plasma, indicating that sulfatide could accelerate coagulation at an amount so little as 0.39 μg/well, with a maximal effect at 6.25 μg/well. On the other hand, GalCer as well as G_{M1} did not exhibit such activity suggesting that as previously reported [2] a sulfated moiety is important. The result observed in this *in vitro* experiment appears to be consistent with that *in vivo* (Figs. 3a and 3b), since sulfatide but neither galactocerebroside (GalCer) and ganglioside G_{M1} could induce thrombi only at the tip of plastic cannula *in vivo*. On the other hand, sulfatide but not GalCer nor ganglioside coated on plastic plate could accelerate plasma coagulation. Sulfatide infused through a plastic cannula into rat may coat the tip of the cannula in a short time.

In 1980 Fujikawa *et al.* [2] reported that sulfatide could activate blood coagulation factor XII *in vitro*. Later this has also been confirmed in our system by the use of normal and factor XII-deficient human plasma. The factor XII-deficient plasma-PBS did not coagulate either in the presence or absence of any amount of sulfatide. However, when normal and the deficient plasma were mixed, the coagulation was rapid in the presence of sulfatide, as in the case of normal plasma (Fig. 5a) Both heparin and chondroitin sulfate E are highly sulfated glycosaminoglycans and were reported to activate factor XII *in vitro* [13]. However, neither of them did accelerate, but they rather prolonged normal plasma coagulation onset times in the system applied (Fig. 5b). Generally, the hemostatic system acting *via* the extrinsic pathway (starting from the tissue factor/factor VII) is believed to be physiologically important [16], but the role of the system *via* the intrinsic pathway in which factor XII is involved seems to be questionable because there are no bleeding symptoms in patients lacking this factor [3–5]. Also the question as to the role of the intrinsic pathway could have been raised because even heparin, a typical anticoagulant widely used in the current clinical practice, was reported to activate factor

XII *in vitro* [13], but it is not to enhance thrombogenesis *via* factor XII [11]. However, the possible importance of the intrinsic pathway should be taken into account because, in addition to the present report, we observed enhancement of thrombogenesis by sulfatide *in vivo* in the experimental thrombosis model [17].

In conclusion, we propose contradictory functions for sulfatide in the blood coagulation system although physiological functions of sulfatide in blood coagulation are still uncertain. Prolongation of bleeding time by sulfatide (Fig. 1) and clotting time (Fig. 2) could be observed under inactivation of the intrinsic pathway, whereas, on the contrary, induction of thrombi by sulfatide probably might occur under activation of this pathway. In addition, it should be emphasized that sulfatide occurs naturally in serum as a component of lipoproteins [7, 18] and accumulates in the walls of atherosclerotic aortae of WHHL rabbits [19]. Sulfatide also occurs in plasma membranes of many cells, especially cells in the nervous system [20] where its presence may be related to cerebral thrombotic diseases. In addition sulfatide can bind many proteins such as laminin [21], thrombospondin [22], von Willebrand factor [23], antistasin [24], and properdin [25] almost all of which have been reported to be closely related to haemostasis and/or inflammation. Such characteristics may be important for evaluation of the roles of sulfatide in blood coagulation and atherosclerosis. Finally, recently anti-inflammatory actions of sulfatide have been reported and sulfatide may be expected to become a promising anti-inflammatory medicine [26, 27]. However, sulfatide should be carefully used because it may induce thrombosis under still unknown conditions.

We thank Dr. M. Waki of Seikagaku Co. Tokyo Research Institute for critical reading of the manuscript.

REFERENCES

1. Wago, K. (1961) Antiatherosclerotic and anticoagulant activity of cerebroside sulfate. *Jpn. Heart J.* **2**, 354–367.

2. Fujikawa, K., Heimark, R.L., Kurachi, K. & Davie, E.W. (1980) Activation of bovine factor XII (Hageman factor) by plasma kallikrein. *Biochemistry* **19**, 1322–1330.

3. Mann, K.G., Jenny, R.J. & Krishnaswamy, S. (1988) Cofactor proteins in the assembly and expression of blood clotting enzyme complexes. *Annu. Rev. Biochem.* **57**, 915–956.

4. Saito, H. (1994) The contact phase of blood coagulation; in *Haemostasis and Thrombosis* (Bloom, A.L., Forbes, C.D., Thomas, D.P. & Tuddenham, E.G.D., eds.) 3rd. edn., pp. 289–307, Churchill Livingstone, Edinburgh.

5. Schmaier, A.H. (1997) Contact activation: A revision. *Thromb. Haemost.* **78**, 101–107.

6. Taketomi, T., Hara, A., Uemura, K. & Kyogashima, N. (1989) Effect of long-term treatment with sulfatide on hyperlipidemia and progression of atherosclerosis in WHHL rabbits. *Jpn. J. Exp. Med.* **59**, 221–231.

7. Zhu, X., Hara, A. & Taketomi, T. (1991) The existence of galactosylceramide I^3-sulfate in serums of various mammals and its anticoagulant activity. *J. Biochem.* **110**, 241–245.

8. Hara, A., Uemura, K. & Taketomi, T. (1996) Sulfatide prolongs blood-coagulation time and bleeding time by forming a complex with fibrinogen. *Glycoconjugate J.* **13**, 187–194.

9. Hara, A., Kutsukake, Y., Uemura, K. & Taketomi, T. (1993) Anticoagulant activity of sulfatide and its anti-thrombotic effect in rabbit. *J. Biochem.* (Tokyo) **113**, 781–785.

10. Bjork, I., Olson, S.T. & Shore, J.D. (1989) Molecular mechanisms of the accelerating effect of heparin on the reactions between antithrombin and clotting proteinases; in *Heparin* (Lane, D.A. & Lindahl, U., eds.) pp. 229–255,

Edward Arnold, London, Melbourne, Auckland.

11. Thomson, J.M. & Poller, L. (1985) The activated partial thromboplastin time; *in Blood Coagulation and Haemostasis* (Thomson, J.M., ed.) pp. 301–339, Churchill Livingstone, Edinburgh, London, Melbourne, New York.

12. Bourin, M.-C. & Lindahl, U. (1993) Glycosaminoglycans and the regulation of blood coagulation. *Biochem. J.* **289**, 313–330.

13. Hojima, Y., Cochrane, C.G., Wiggins, R.C., Austen, K.F. & Stevens, R.L. (1984) *In vitro* activation of the contact (Hageman factor) system of plasma by heparin and chondroitin sulfate E. *Blood* **63**, 1453–1459.

14. Tanaka, S. & Iwanaga, S. (1993) Limulus test for detecting bacterial endotoxins. *Methods Enzymol.* **223**, 358–364.

15. Magnani, J.L., Spitalnik, S.L. & Ginsburg, V. (1987) Antibodies against cell surface carbohydrates: Determination of antigen structure. *Methods Enzymol.* **138**, 195–207.

16. Broze, G.J., Jr. (1994) The tissue factor pathway of coagulation: Factor VII, tissue factor, and tissue factor pathway inhibitor; in *Haemostasis and Thrombosis* (Bloom, A.L., Forbes, C.D., Thomas, D.P. & Tuddenham, E.G.D., eds.) 3rd edn., pp. 349–377, Churchill Livingstone, Edinburgh.

17. Kyogashima, M., Onaya, I., Hara, A. & Taketomi, I. (1998) Sulfatide can markedly enhance thrombogenesis in rat deep vein thrombosis model. *Glycoconjugate J.* (in press).

18 Hara, A. & Taketomi, T. (1987) Occurrence of sulfatide as a major glycosphingolipid in WHHL rabbit serum lipoproteins. *J. Biochem.* **102**, 83–92.

19. Hara, A. & Taketomi, T. (1991) Characterization and changes of glycosphingolipids in the aorta of the Watanabe hereditable hyperlipidemic rabbit. *J. Biochem.* (Tokyo) **109**, 904–908.

20. Roberts, D.D. & Ginsburg, V. (1988) Sulfated glycolipids and cell adhesion. *Arch. Biochem. Biophys.* **267**, 405–415.

21. Roberts, D.D., Rao, C.N., Magnani, J.L., Spitalnik, S.L., Liotta, L.A. & Ginsburg, V. (1985) Laminin binds specifically to sulfated glycolipids. *Proc. Natl. Acad. Sci. U.S.A.* **82**, 1306–1310.

22. Roberts, D.D., Haverstick, D.M., Dixit, V.M., Frazier, W.A., Santoro, S.A. & Ginsburg, V. (1985) The platelet glycoprotein thrombospondin binds specifically to sulfated glycolipids. *J. Biol. Chem.* **260**, 9405–9411.

23. Roberts, D.D., Williams, S.B., Gralnick, H.R. & Ginsburg, V. (1986) von Willebrand factor binds specifically to sulfated glycolipids. *J. Biol. Chem.* **261**, 3306–3309.

24. Holt, G.D., Krivan, H.C., Gasic, G.J. & Ginsburg, V. (1989) Antistasin, an inhibitor of coagulation and metastasis, binds to sulfatide (Gal(3-SO_4)β1-1Cer) and has a sequence homology with other proteins that bind sulfated glycoconjugates. *J. Biol. Chem.* **264**, 12138–12140.

25. Holt, G.D., Pangburn, M.K. & Ginsburg, V. (1990) Properdin binds to sulfatide (Gal(3-SO_4) β1-1Cer) and has a sequence homology with other proteins that bind sulfated glycoconjugates. *J. Biol. Chem.* **265**, 2852–2855.

26. Suzuki., Y., Toda, Y., Tamatani, T., Kiso, M., Hasegawa, A., Tadano-Aritomi, K., Ishizuka, I. & Miyasaka, M. (1993) Sulfated glycolipids are ligands for a lymphocyte homing receptor, L-selectin (LECAM-l), binding epitope in sulfated sugar chain. *Biochem. Biophys. Res. Comm.* **190**, 426–434.

27. Higashi, H., Suzuki, Y., Mukaida, N., Takahashi, N., Miyamoto, D. & Matsushima, K. (1997) Intervention in endotoxin shock by sulfatide ($^3ISO_3$-GalCer) with a concomitant reduction in tumor necrosis factor alpha production. *Infect. Immun.* **65**, 1223–1227.