Characterization of the binding epitope of a monoclonal antibody to sulphatide

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An IgG1 monoclonal antibody, Sulph I, reacting with sulphatide (3'-sulphogalactosylceramide), was produced by immunizing Balb/c mice with that glycolipid coated on *Salmonella minnesota* bacterial membrane. Radioimmunoassay of the binding of the monoclonal antibody to structurally related glycolipids adsorbed to microtitre plates or chromatographed on thin-layer plates was used to determine its binding epitope. The antibody showed similar binding avidity to three sulphated glycolipids: sulphatide, sulpholactosylceramide and seminolipid. Lysosulphatide did bind the antibody, but, compared with sulphatide, 30 times more antigen was needed for half-maximal binding. Bis(sulphogangliosyl)ceramide and bis-sulphogangliosyltetraacylceramide did not bind the antibody. These results suggest that terminal galactose-3-O-sulphate and part of the hydrophobic region of the glycolipid are recognized by the Sulph I antibody.

INTRODUCTION

Sulphatide (3'-sulphogalactosylceramide) is an acidic glycosphingolipid located at the cell membrane. It is an early marker for the differentiation of oligodendrocytes and Schwann cells (Zalc & Baumann, 1982) and becomes highly enriched in the myelin. In the adult human brain myelin system it constitutes 4–5 mol % of the total lipids (Svennerholm *et al.*, 1978). Antibodies to sulphatide could be useful tools in studies concerning myelination and demyelinating disorders. The usefulness of such antibodies is, however, dependent on their binding specificity. Polyclonal antibodies are therefore less suitable, and of the monoclonal antibodies produced so far (Schachner, 1982; Hofstetter *et al.*, 1984; Goujet-Zalc *et al.*, 1986), only one has been characterized with regard to its antigenic determinants (Goujet-Zalc *et al.*, 1986). Our aim was to produce an antibody to sulphatide that was specific enough to be used for the quantitative assay of sulphatide in tissues and body fluids in demyelinating disorders. The present paper describes such a monoclonal antibody to sulphatide, Sulph I, and its binding specificity.

MATERIALS AND METHODS

Glycolipids and allied compounds

All non-sulphated glycolipids used in the present study were isolated previously from human tissue and were structurally characterized in the Department of Psychiatry and Neurochemistry, University of Gothenburg. Lactosylceramide-3-sulphate and sulphatide (3'-sulphogalactosylceramide) were isolated from the kidney of a patient who died from metachromatic leuko-

dystrophy (MLD). Sulphatide isolated from human adult brain was also used in this study. Seminolipid, lyso-seminolipid from mammalian testis and bis-sulphogangliosyltetraacylceramide (SB1a) from rat kidney were isolated and characterized at the Department of Biochemistry, Teikyo University School of Medicine. Keratan sulphate was a gift from Dr. D. Heinegård, Department of Physiological Chemistry, University of Lund, Lund, Sweden. All other mucopolysaccharides, dermatan sulphate, chondroitin 4-sulphate and heparin were purchased from Miles Research, Stoke Poges, Slough, Berks., U.K.

Preparation of lysosulphatide was performed as follows. Sulphatide was dissolved in 1 M-KOH in methanol to a concentration of 0.4 μ mol/ml in a vial with a Teflon-lined screw cap, and the deacylation was performed at 102 °C for 20 h. After neutralization with 1 M-HCl, the solution was evaporated, redissolved in chloroform/methanol/water (40:20:3, by vol.) and de-salted on a Sephadex G-25 column (Wells & Dittmer, 1963). The lysosulphatide was then isolated by preparative t.l.c., with chloroform/methanol/water (60:32:7, by vol.) as the developing solvent. Lysosulphatide was eluted from the gel with chloroform/methanol/water (3:6:2, by vol.).

³H-labelling of lysosulphatide was performed by the following procedure. Lysosulphatide (corresponding to 5 μ mol) was dissolved in 2 ml of distilled water and 2 ml of tetrahydrofuran. The solution was saturated with N₂, after which 1.9 GBq of NaB³H₄ (specific radioactivity 185–370 GBq/mmol) in 200 μ l of 2 M-KOH was added. A solution of 10 mg of palladium acetate in 100 μ l of 2-methoxyethanol was added and the tube shaken for

Abbreviations used: h.p.t.l.c. high-performance t.l.c.; OPD, *o*-phenylenediamine; e.l.i.s.a., enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline (for composition and pH, see the text); BSA, bovine serum albumin; SB2, bis-sulphogangliosyltetraacylceramide; SB1a, bis-sulphogangliosyltetraacylceramide; MLD, metachromatic leukodystrophy.

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24 h at room temperature. The reaction was stopped by adding 250 μ l of 2 M-acetic acid. Precipitated material was removed by filtration through glass wool in a Pasteur pipette. After desalting on Sephadex G-25 (Wells & Dittmer, 1963), the labelled lysosulphatide was purified by preparative t.l.c.

Other materials

The h.p.t.l.c. plates (Kisegel 60 backed on aluminium or glass plates) and Silica-Gel 70-230 mesh for column chromatography were from Merck A.G., Darmstadt, Germany. The ion-exchange resin (Spherosil-DEAE-dextran) was a gift from the Institut Merieux, Lyon, France, and was prepared as described previously (Fredman *et al.*, 1980). Protein A-Sepharose CL4B and Sephadex G-25 were from Pharmacia Chemicals, Uppsala, Sweden. Microtitre plates (Titertek) were from Flow Laboratories, and the X-ray film used for autoradiography was Kodak XAR-5. Second antibodies (125 I-labelled goat anti-mouse Ig) was purchased from New England Nuclear (Boston, MA, U.S.A.), and horseradish-peroxidase-conjugated goat anti-mouse Ig was from Dakopatts a/s, Glostrup, Denmark. OPD used as substrate for the e.l.i.s.a. was from Sigma. NaB^3H_4 was from Amersham International, Amersham, Bucks., U.K.

Production of monoclonal antibody

Sulphatide coated on *Salmonella minnesota* bacterial membrane as described by Galanos *et al.* (1971) and Young *et al.* (1979), in a sulphatide/membrane ratio of 2:25 (w/w), in PBS (0.01 M-phosphate buffer/0.14 M-NaCl, pH 7.4) was used as immunogen. Balb/c mice were injected subcutaneously with immunogen (corresponding to 5 μ g of sulphatide) mixed 1:1 with Freund's complete adjuvant. Booster doses, consisting of the immunogen mixed 1:1 with Freund's incomplete adjuvant, were given intraperitoneally. The first and second booster doses (5 μ g of sulphatide) were given after 4 and 6 weeks respectively, and the third, comprising 50 μ g of sulphatide, after 8 weeks. A final booster dose of 25 μ g of sulphatide was given after 10 weeks, and the fusion of the spleen cells with mouse myeloma cells (Sp 2/0) (de St. Groth & Scheidegger, 1980) was performed 3 days later. The hybridomas were grown in Iscove's medium containing 10% foetal-calf serum, screened against sulphatide with the e.l.i.s.a. method described below, and positive hybrids were cloned by limiting dilution. Production of monoclonal antibodies to sulphatide was performed by culturing the hybridomas in dialysis tubing according to the procedure described by Sjögren-Jansson & Jeansson (1985).

The subclass of the antibody was determined by immunofixation (Arnaud *et al.*, 1977) and found to be IgG1. Antibody concentration was assayed by using the single-radial-immunodiffusion method of Mancini *et al.* (1965).

Radioimmunoassay of glycolipid antigens adsorbed to microtitre wells

The procedure developed by Young *et al.* (1979) was performed as described by Brockhaus *et al.* (1981). The glycolipid dissolved in methanol was adsorbed to microtitre wells, whereafter the Sulph I antibody (generally 1.5 μ g/ml) was added, and subsequently a detecting second antibody, 125 I-labelled goat anti-mouse

Ig, was also added. The antibodies were diluted in Tris/BSA (0.05 M-Tris/HCl, pH 8.0, containing 0.14 M-NaCl, 1% BSA and 0.01% merthiolate).

E.l.i.s.a. screening for antibody-producing clones

This procedure was essentially the same as the radioimmunoassay described above. The clone media containing the produced antibodies was diluted 1:2-1:5 in Tris/BSA. Horseradish-peroxidase-conjugated rabbit anti-mouse Ig was used as the second antibody. OPD [5 mg in 10 ml of 0.1 M-citrate buffer, pH 4.5, containing 4 μ l of H_2O_2 (30% stock solution)] was used as the substrate, and incubation was performed for 5-10 min at room temperature. The developed colour was read at 450 nm in a Titertek Multiskan instrument.

Radioimmunoassay of the binding of the Sulph I antibody to glycolipids on h.p.t.l.c. plates

This method, elaborated by Magnani *et al.* (1982), was used in slightly modified form. The glycolipids were applied to an aluminium-backed h.p.t.l.c. plate and chloroform/methanol/water (60:32:7, by vol.) was used as developing solvent. The plate was dipped in poly-(isobutyl metachrylate) [0.01% (w/v) in hexane] and incubated for 15 min with Tris/BSA at room temperature before being overlaid with the Sulph I antibody (1.5 μ g/ml of Tris/BSA). After 4 h the second antibody, 125 I-labelled goat anti-mouse Ig (10⁶ c.p.m./ml of Tris/BSA), was overlaid on to the plate for 16 h. The incubations with antibody was performed at +4 °C. Finally, the plate was exposed to X-ray film for 20 h.

Inhibition assay

The inhibitory effect of sulphated glycolipids on the binding of the antibody to 3'-sulphogalactosylceramide was performed as follows. Methanol solutions of the sulphatide or lysosulphatide were placed in glass tubes and evaporated. Thereafter 150 μ l of the Sulph I antibody, 1.5 μ g of Tris/BSA/ml, was added and the tube incubated at room temperature for 30 min. The remaining free antibody in the solution was quantified by the radioimmunoassay in microtitre wells as described above.

Immunoblotting analyses

Immunoblots were performed as described by Davidsson *et al.* (1987), an adaptation of previously described methods (Towbin *et al.*, 1979; Baitteger *et al.*, 1982) being used. Homogenates of brain white and grey matter were electrophoresed on SDS/polyacrylamide gels and then transferred electrophoretically to nitrocellulose sheets. The nitrocellulose sheet was exposed to Sulph I-antibody (1.5 μ g/ml), washed, and incubated in peroxidase-conjugated rabbit anti-mouse Ig used at 1:200 dilutions.

RESULTS

Epitope specificity of the Sulph I antibody

The epitope specificity of the antibody was determined by comparing its binding to glycolipids structurally related to sulphatide (Fig. 1) in the two solid-phase assays: glycolipids adsorbed to microtitre wells or chromatographed on h.p.t.l.c. plates. By using the former procedure, the Sulph I antibody was found to

Fig. 1. Chemical structures of the glycolipids used in the present study

have approximately the same binding affinity to three glycolipids: sulphatide, sulpholactosylceramide and seminolipid. Furthermore, sulphatide from human brain and from the kidney of a patient who died from MLD showed the same binding affinity. Half-maximal binding was obtained with 1–3 pmol of the glycolipid per well (Figs. 2 and 3). SB2 and SB1a (Fig. 3) and the non-sulphated glycolipids galactosylceramide and lactosylceramide did not bind the antibody.

Removal of the fatty acid from sulphatide and seminolipid, resulting in their corresponding lyso com-

pounds, gave a remarkable diminution in their binding to the antibody. Lysosulphatide showed the same binding-curve profile as sulphatide, but an approx. 30 times higher amount of the glycolipid was needed to obtain half-maximal binding (Fig. 4). Lysoseminolipid reacted differently from seminolipid (Fig. 3). The maximal-binding value was only half that of seminolipid. The binding curve did not have the ordinary S-form, which made comparison difficult. Whether this discrepancy was related to decreased adsorbance of the lyso compounds to the microtitre wells, and thereby a lowered amount of

Fig. 2. Radioimmunoassay of the binding of the Sulph 1 antibody to sulphatide (●) and lactosylceramide 3-sulphate (○) adsorbed to microtitre wells

The procedures are described in the Materials and methods section.

Fig. 3. Radioimmunoassay of the binding of the Sulph 1 antibody to seminolipid (■), lysoseminolipid (●), SB2 (□) and SB1a (○) adsorbed to microtitre wells

The procedures are described in the Materials and methods section.

antigen available for antibody binding, was tested by measuring the adsorbance of ¹⁴C-labelled sulphatide and ³H-labelled lysosulphatide. After the first incubation with Tris/BSA for 15 min, 73% of the sulphatide, but only 13% of the lysosulphatide, remained adsorbed to the wells. Further incubations, employing the procedure used for measuring the antibody binding, resulted in additional losses, and finally 36% of sulphatide and 7% of lysosulphatide remained attached.

In accordance with the results described above, the Sulph I antibody showed the highest affinity to sulphatide, sulpholactosylceramide and seminolipid also in the h.p.t.l.c. radioimmunoassay method (Fig. 5). The lowest detectable amount of sulphatide and sulpholactosylceramide was 5 pmol, whereas seminolipid could be detected when only 1 pmol was spotted. However, it should be noticed that the latter migrates in one band, whereas the other two migrate in two, owing to ceramide differences. The lyso compounds of seminolipid and

Fig. 4. Radioimmunoassay of the binding of sulphatide (●) and lysosulphatide (□) adsorbed to microtitre wells

The procedures are described in the Materials and methods section.

sulphatide chromatographed on h.p.t.l.c. plates showed, as they did in the microtitre wells, a very low affinity for the Sulph I antibody. A detectable binding was observed first when 1500 pmol was applied to the plates (Fig. 6). The sulphated glycolipids SB2 and SB1a, and the non-sulphated glycolipids galactosylceramide and lactosylceramide, showed no detectable binding of the antibody even when 1500 pmol of the respective compound was applied on the h.p.t.l.c. plate.

The inhibitory effect of lysosulphatide was compared with that of sulphatide, and the results are illustrated in Fig. 7. A detectable inhibitory effect of sulphatide was seen at 0.5 μ M concentration, whereas the corresponding value for lysosulphatide was 15 μ M. None of the glycolipids gave complete inhibition, and the maximum inhibition by sulphatide was approx. 70%, whereas the corresponding value for lysosulphatide was 40%. This assay was also used for measuring the inhibitory effect of sulphated mucopolysaccharides. The highest concentration of each respective substance (heparin, dermatan sulphate, chondroitin 4-sulphate and keratan sulphate) was calculated to contain 5 μ mol of sulphate groups/litre. None gave any measurable inhibition.

The binding of the antibody to sulphated glycoproteins was assayed with the immunoblotting technique. Delipidized homogenates corresponding to 0.14 mg of human brain cortex or 0.16 mg of white matter did not bind any detectable amount of antigen.

DISCUSSION

Immunization of Balb/c mice with sulphatide adsorbed on *Salmonella minnesota* (Galanos *et al.*, 1971; Young *et al.*, 1979) and subsequent fusion with mouse myeloma cells resulted in a hybridoma producing anti-sulphatide antibody. This antibody, which did not react with sulphated mucopolysaccharides or glycoproteins, was tested against glycolipids with structures closely related to that of sulphatide. Three glycolipids, namely sulphatide, sulpholactosylceramide and seminolipid, were found to have equal affinities for the antibody. They share the same terminal sequence, galactose 3-O-sulphate, which is included in the binding epitope. The

Fig. 5. Radioimmunodetection of the binding of the Sulph 1 antibody to glycolipids chromatographed on h.p.t.l.c. plates

The developing solvent used for sulphatide and lactosylceramide 3-sulphate was chloroform/methanol/water (60:32:7, by vol.) and for seminolipid it was chloroform/methanol/0.2% CaCl_2 (15:10:2, by vol.). Doublet spots are due to differences in fatty acid composition of the ceramide.

Fig. 6. Radioimmunodetection of the binding of the Sulph 1 antibody to glycolipids chromatographed on h.p.t.l.c. plates

Plate (a) was developed in chloroform/methanol/water (60:32:7, by vol.). The amount of sulphatide was 10 and 100 pmol, and of lysosulphatide, 15 and 1500 pmol. Plate (b) was developed in chloroform/methanol/0.2% CaCl_2 (15:10:2, by vol.), and the amount of lysoseminolipid was 800 pmol in lane 1 and 1500 pmol in lane 2. The lysoseminolipid was contaminated with minute amounts of seminolipid as indicated in lane 2. Procedures are as described in the Materials and methods section.

absence of detectable binding to galactosylceramide and lactosylceramide, the non-sulphated equivalents of sulphatide and sulpholactosylceramide, provides support for the involvement of the sulphate group.

There was a dramatic decrease in the binding affinity of the antibody for lysosulphatide and lysoseminolipid as compared with sulphatide and seminolipid respectively. Removal of the fatty acid from the glycolipids will influence such characteristics as packing and hydrophobicity. If packing phenomena were the reason for decreased binding, one would expect auxiliary lipids to eliminate such an effect. However, the addition of

Fig. 7. Inhibition of the Sulph 1 antibody with sulphatide (●) and lysosulphatide (□)

The antibody solution was preincubated with various concentrations of the inhibitor, after which the remaining binding capacity toward sulphatide adsorbed to microtitre wells was assayed. The procedures were as described in the Materials and methods section.

cholesterol and lecithin (phosphatidylcholine) to the microtitre wells did not increase the binding affinity of the antibody to lysosulphatide but that to sulphatide (results not shown). The diminished hydrophobicity lowers the adsorbance of the lyso compounds to the plastic wells, as was shown by using radiolabelled sulphatide and lysosulphatide. This to some extent explains the decreased amount of bound antibody, but an additional effect is that the hydrophobic region of the glycolipid is part of the binding epitope.

The fatty acid could be attached through either an amide bond, as in sulphatide or sulpholactosylceramide, or an ester bond, as in seminolipid. The hydroxy group at C-3 of the sphingosine moiety is not involved, since seminolipid, in which this group is absent, showed the same binding affinity as sulphatide. It is not possible to draw any conclusion about the importance of the carbonyl group of the fatty acid from the present study.

The absence of binding of the Sulph I antibody to SB2

and SB1a indicates that the additional sulpho-GalNAc and sulpho-Gal-GalNAc (Fig. 1) make too large a distance between the sulphogalactose, or possibly a sulpho-*N*-acetylgalactosamine, and the fatty acid. In addition, the rotational angle around the glycosidic bond of GalNAc is considerably restricted by the presence of a methyl group of the acetamide and C-6 hydroxy group in GalNAc, extending outward the pyranoside ring, and by the large hydrated sulphated group in galactose. However, there is no measurable difference in binding affinity of the antibody to sulphatide or sulpholactosylceramide, which has an extra glucose residue between the sulphate and the fatty acid. This might be explained by the fact that the rotational angle of the glycosidic bond (the C-1 carbon and the oxygen molecule bond to C-1) of galactose and glucose appears to be limited, whereas the rotation around the oxygen-C-1 bond of the sphingosine base is relatively large. If, then, one assumes a Hakomori (1986) L-shaped conformation for sulpholactosylceramide, the distance between sulphogalactose and the fatty acid is not very different from that in sulphatide. The lack of binding of Sulph I to SB1a reveals that a terminal SO₃-galactose moiety itself was not sufficient for binding. These results support the previous suggestion that the hydrophobic region of the glycolipid is also recognized by the antibody.

The monoclonal antibodies previously reported to react with sulphatide have been mostly characterized by their reaction with tissues or cells assumed to contain sulphatides (Schachner, 1982; Hofstetter *et al.*, 1984; Guerri *et al.*, 1986). Goujet-Zalc *et al.* (1986) performed a partial characterization of their monoclonal antibody, but there is no quantitative data for the binding affinity to various glycolipid antigens. The authors did not detect any binding of their antibody 224-58 to sulpholactosylceramide or lysosulphatide, which might suggest that the two antibodies have different binding epitopes. Nevertheless, further studies of 224-58 are necessary before such a conclusion can be drawn.

The usefulness of monoclonal antibodies depends on their epitope specificity, which makes binding-specificity studies of the outmost importance. A monoclonal antibody with unknown cross-reactivity, though very weak, might give a false picture when applied to tissues, cells, crude lipid extracts, etc. These may contain little or no antigen, but large amounts of the cross-reacting substance. The epitope specificity of the Sulph I antibody makes it a useful tool in the detection of sulphatide

in cerebrospinal fluid, as sulpholactosylceramide and seminolipid do not occur in human brain.

This study was supported by the Swedish Research Council (project no. 3X-627) and the National Swedish Board for Technical Development (project no. 84-4667).

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Received 7 July 1987/28 September 1987; accepted 17 November 1987