Glycosphingolipids Covalently Linked to Agarose Gel or Glass Beads

USE OF THE COMPOUNDS FOR PURIFICATION OF ANTIBODIES DIRECTED AGAINST GLOBOSIDE AND HEMATOSIDE*

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SUMMARY

Oxidative ozonolysis of the olefinic bond of the sphingosine moiety of either globoside (βGalNAc1→3αGal1→4βGal1→4βGlc→ceramide), or of the methyl ester of hematoside (αN-glycolylneuraminyl2→3βGal1→4βGlc→ceramide), and coupling of the carboxyl-bearing product to aminoethylagarose or to amino group-bearing glass beads in the presence of a carbodiimide resulted in globoside or hematoside covalently linked to agarose or glass beads. These compounds were used for purification of anti-glycosphingolipid antibodies from serum of immunized rabbits. The antibodies bound to the substrate were released by 1 M sodium iodide and their immunological properties were studied. Anti-globoside is directed toward the terminal β-(N-acetyl)galactosaminoyl-(1→3)α-galactopyranosyl structure, while anti-hematoside is directed predominantly toward the sialosyl residue of hematoside.

Glycosphingolipids are components of plasma membranes (2–6), whose carbohydrate moieties are exposed to the outer environment (7). In view of their changes in carbohydrate composition on cell to cell contact (8, 9), during the mitotic cell cycle, and on malignant transformation (10, 11), they may play some role in control of cellular interaction and proliferation (12). Some of them may serve as sites for ion transport (13, 14) and some role in control of cellular interaction and proliferation (12).

Lectins have been used with increasing popularity to study various cell surface properties related to carbohydrates (20, 21); nevertheless, most of their specificities are ill defined, and they show various kinds of “toxicity” to animal cells. They are not simple sugar-binding reagents but have multiple binding sites which could induce a series of alterations in membrane properties (see under “Discussion”).

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1 C. G. Gahmberg and S. Hakomori, unpublished observation.

If antibodies directed against a single glycosphingolipid with defined carbohydrate structure were purified, they would be useful reagents to study various phases of surface function related to specified carbohydrate structure.

This paper describes methods for preparation of globoside and hematoside covalently linked to agarose gel (Sepharose) or to glass beads for affinity columns which enable purification of antibodies directed against those glycosphingolipids. In addition, the immunological properties of the purified antibodies are examined.

MATERIALS AND METHODS

Glycolipid Preparations—Globoside, ceramide trihexoside, lactosylceramide, and “paragloboside” (lacto-N-neotetraacylceramide) were prepared from human erythrocyte ghosts as described previously (22, 23) and purified by an acetylation procedure (24). N-Glycolylhematoside (N-glycolyneuraminyl12→3αGal1→4βGlc→Ceramide) and N-glycolyl-O-acetylhematoside (N-glycoly-O-acetyleneuraminyl12→3αGal1→4βGlc→ceramide) were prepared from horse erythrocytes according to the established procedures (25, 26). N-Acetylhematoside (N-acetyleneuraminyl12→3αGal1→4βGlc→ceramide) was prepared from human spleen as described by Svennerholm (27). Methyl ester of N-glycolyneuraminyl12→3αGal1→4βGlc→ceramide was prepared according to the procedure of Yu and Ledeen (28).

Twenty milligrams of hematoside were dissolved in 20 ml of dry methanol, 200 mg of Dowex 50 H+ were added, and the reaction mixture was stirred at room temperature for 30 hours. The reaction mixture was filtered from Dowex 50, evaporated in a rotary evaporator, and applied onto a column of DEAE-Sephadex equilibrated in chloroform-methanol-water (35:60:8). The esterified product was eluted with the same solvent, and the unesterified hematoside was retained on the column (29). RP value of the...
methyl ester of the hematoside on thin layer Silica Gel H was 0.65, while free hematoside gave 0.30 in the solvent system chloroform-methanol-water (60:35:8). Cytolipin R \((\beta\text{GalaNAC} \cdot 3\alpha\text{Gal} \cdot 3\beta\text{Galt} \cdot 4\beta\text{Glc} \cdot \text{ceramide})\) (30) was donated by Doctors Rapport and Kisic of the New York State Psychiatric Institute. Forsmann glycolipid was prepared from goat erythrocyte membrane (31). Tritiated \([H]\)globside was prepared according to the method of Suzuki et al. (32) using galactose oxidase in a tetrahydrofuran-water mixture and with tritiated borohydride.

**Preparation of Antiserum**—An emulsion was prepared consisting of 2.2 ml of Freund's incomplete adjuvant (Difco Biol. Ltd.), 5 mg of tubercle bacilli (Difco Biol. Ltd.), 5 mg of bovine serum albumin (Sigma Chemical Co.), and 2.2 ml of physiological saline in which 2.5 mg of glycolipid had been dissolved by warming and sonicating. Aliquots of 0.3 to 0.4 ml of this emulsion were injected into six loci of New Zealand albino rabbits (5 to 6 kg), intramuscularly, subcutaneously, and into the foot pads. After 2 weeks the injection was repeated and serum was collected every 10 days and stored at -70°C. This schedule was the same for preparation of both anti-globoside and anti-hematoside and is essentially the same as described previously (33).

**Preparation of 2-Hydroxy-S-(\(N\)-lignocerylamo)-4-\((O-N\)-acetylglactosaminyl\) \(\rightarrow 3\) galactosyl \(\rightarrow 4\) galactosyl \(\rightarrow 4\) glucopyranosyl\) - oxybutyric Acid (\("\text{globo-N-tetraosylceramide acid}\"\) )—The following scheme is outlined in Fig. 1. Three hundred milligrams of globoside were acetylated in 20 ml of acetic anhydride-pyridine (1:1) for 2 hours at 80°C. After cooling, reagents were removed in vacuo with the addition of excess toluene until the odor of acetic anhydride was absent. Residual vapors were removed under a stream of nitrogen. The fully acetylated globoside was dissolved in methylene chloride-methanol (1:1) in a volume of 20 ml, and an ozone-air mixture (produced by "OREC Ozonator" model 03V1; instrument settings 0.9 amp for power output and 5 to 6 settings for air flow rotameter) was bubbled through the solution until excess ozone could be detected by starch-iodide paper. The products were examined by thin layer chromatography in 1,2-dichloroethane-methanol (9:1) using Silica Gel G.

The compound had a mobility of 0.65 in relation to the migration rate of the original compound. This reaction mixture, containing the ozonide of acetylated globoside was evaporated to dryness in vacuo and the residue was dissolved in 15 ml of a mixture of glacial acetic acid and an aqueous solution of 30% hydrogen peroxide (1:1 v/v), in a screw-capped tube with Teflon-lined cap and was allowed to stand at 37°C with gentle shaking for 72 hours (34). Excess hydrogen peroxide was destroyed by addition of 10 mg of platinum oxide. After foaming ceased, the water and acetic acid were removed in vacuo, using toluene to assist evaporation. The dried residue was dissolved in chloroform-methanol (2:1) and filtered. To 4 ml of this clear solution, 1 ml of 0.5% sodium methoxide was added and deacetylation was allowed to proceed at 25°C for 30 min. Dowex 50-H+ was added with stirring until the mixture was neutral to pH paper, and the solution was quickly filtered and dried to preclude methyl ester formation.

**Identification of 2-Hydroxy-S-(\(N\)-lignocerylamo)-4-\((O-N\)-
**Acetylgalactosaminyli +3 galactosyll +4 galactosyll +4 glucosyl oxybutyric acid** ("GlobO-N-tetraosylceramide Acid")—Methanolyis of 0.5 mg of the product in 2 ml of 1 N anhydrous methanolic HCl at 80° for 18 hours was followed by extraction three times with hexane to remove fatty acids and then by addition of silver carbonate (10 mg) with stirring until a neutral pH was obtained. Acetic anhydride was added (0.2 ml), and N-acetylation was allowed to proceed for 6 hours at 25°. After filtration, the clear solution was dried under nitrogen and 100 µl of a mixture of pyridine-hexamethyldisilazane-trimethylchlorosilane (5:2:1) were added with care to avoid moisture. Gas-liquid chromatography was performed on a Hewlett-Packard F&M instrument equipped with a column, 2 m × 3 mm, of 3% SE-30 on Supelcoport (80 to 100 mesh). The temperature was programmed from 140-200° at 3° per min. Mass spectrometry was performed on a Finnigan quadrupole instrument equipped with a glass molecular separator and glass-lined transfer tubing. The ionizing electron energy was 70 e.v., ion source temperature 250°, and separator temperature 250°, gas-liquid chromatography column conditions were as described above.

**Covalent Attachment of "Globo-N-tetraosylceramide Acid" to Solid Substrate**—Products from oxidative ozonolysis were dissolved in 40 ml of dimethylformamide and mixed with 40 ml of packed aminoethyl-Sepharose (35). The pH was adjusted to 4.7 with 1 N HCl. Five hundred milligrams of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide were dissolved in 5 ml of water and added with mixing over 5 min. After 20 hours at room temperature, the Sepharose gel was washed with 2 liters of 50% dimethylformamide in water in the filter funnel, and then transferred to a column (25 mm diameter) for further washing with distilled water and finally with PBS, pH 7, until no more products were eluted. For storage, the PBS was added with sodium azide at 0.002 M concentration.

**Preparation of 2-Acetoxy-3-(N-lignocerylamido)-4-O-(1-carboxymethoxy-N-acetoglycolyl-3-deoxy-4,7,8,9-tetra-O-acetyl)-nonulosaminyl-2-(2,4,6-tri-O-acetyl)galactosyll 4-(2,3,6-tri-O-acetyl glucosyl))-oxybutyric acid** ("N-Glycolyl-l-carbomethoxy-neuraminyllactosylceramide acid deca-O-acetate") and Coupling to Amino-Glass Beads—The scheme for this reaction sequence is depicted in Fig. 2. The carboxyl group of hematoside was first esterified, followed by essentially the same procedure as described in preparation of "globo-N-ceramide acid." Deacetylation was not performed until after the coupling to glass beads. The methyl ester of N-glycolylhematoside was acetylated in pyridine and acetic anhydride as described before; the fully acetylated methyl ester of N-glycolylhematoside was subjected to ozonolysis and oxidative cleavage of ozonide to convert the double bond of sphingosine to a carboxyl group in the same procedure as described in the previous section.

The product as described under "N-Glycolyl-1-carbomethoxy-neuraminyllactosylceramide Acid Deca-O-acetate," derived from 20 mg of hematoside, was dissolved in 3.75 ml of dimethylformamide-tetrahydrofuran (1:1 v/v), in which 1 g of amino glass beads (α-aminopropyltriethoxysilane glass compound, Corning biomate-

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**Fig. 2. Outline for oxidative ozonolysis of equine N-glycolylhematoside with a protected carboxyl group and coupling of the newly formed carboxyl to amino group-bearing glass beads. DMF, dimethylformamide. DCC, dicyclohexylcarbodiimide.**
After 30 min at room temperature, the reaction was allowed to proceed overnight, and the product was washed by filtration with chloroform-methanol (2:1).

Preparation of N-Glycolylneuraminylactosylceramide-acyl-glass Beads Complex and Evidence for This Linkage—The final product described in the preceding section was suspended in 10 ml of chloroform-methanol (2:1), and 2 ml of 0.5% sodium methoxide in methanol (2:1), followed by washing with 100 ml of water, then with 50 ml of phosphate buffer, pH 7.0, and were stored in this buffer after addition of 2 ml of sodium azide.

One hundred milligrams of this compound were methanolyzed in 0.5% methanolic HCl at 70° for 18 hours, and the methanolyzed products were determined by gas chromatography as the trimethylsilyl derivatives.

Purification of Antibodies against Globoside and N-Glycolylneuraminylactosylceramide on Solid Substrate Glycolipid Column—Anti-globoside serum was mixed with 56 volume of saturated ammonium sulfate and the product was washed by filtration with chloroform-methanol (2:1); followed by washing with 100 ml of water, then with 50 ml of phosphate buffer, pH 7.0, and were stored in this buffer after addition of 2 ml of sodium azide.

If raw serum were used for the purification, heating at 56° for 30 min was performed to inactive complement. After a 5-min incubation period, the column was eluted with 15 ml of PBS. Sodium azide in 1 ml of sodium iodide was used to elute the bound antibody, followed by three washes with 3 ml of PBS. The resulting solution was dialyzed against PBS (three changes) and reduced in volume by reverse dialysis against polyethylene glycol 20,000 molecular weight. PBS was used to wash the column for recycling for the next application of anti-globoside. If the column were to be stored, 1,000 ml sodium azide was added. Absorption of anti-N-glycolylneuraminylactoside antibody was performed by the same procedure as described above. It is recommended, however, that anti-bovine serum albumin present in the crude anti-hematoside antiserum be removed by bovine serum albumin at the optimal ratio of precipitation. Forty microliters of the antiserum which had been treated with bovine serum albumin and which contained 2.6 mg of protein were applied onto 2.5 ml of the glass beads column matrix linked with N-glycolylhematoside.

Immunological Assays—Activities of anti-globoside and anti-hematoside were titrated by hemagglutination on microtiter plates (Cooke Engineering Co., Alexandria, Va.) with a 1% suspension of trypsinized human erythrocytes for detection of anti-globoside and with a 1% human erythrocytes passively coated plates (Cooke Engineering Co., Alexandria, Va.) with a 1% suspension of trypsinized human erythrocytes for detection of anti-hematoside activity. Antibodies were also determined by microcomplement fixation employing reagents as described by Levins (37) and performed on “microtiter plates” according to the procedure as described by Lennette (38). Antigen glycolipids were mixed with 5 parts each of cholesterol and lecithin in chloroform-methanol (2:1).

Coupling of Spingolipid Derivatives to Solid Substrate—[3H]-Globoside was prepared by the galactose oxidase-sodium borohydride method of Suzuki (32) to a specific activity of 6 × 10^6 cpm per mg. After coupling of the globoside derivative to Sepharose according to Fig. 1, 1.62 × 10^5 cpm per ml or 21 μg per ml (16 nm) were bound.

The amount of hematoside bound to the glass bead column (Fig. 2) was determined by methanolysis of the product and comparison with an internal standard of mannitol on GLC. Four hundred ten nanomoles of N-glycolylhematoside were bound per g of the glass bead support. The carbonyl group from the oxime residue of hematoside attached to the column was titrated with 0.01 N sodium hydroxide under nitrogen. The titration curve showed a sigmoid between pH 6 and 9.

Absorption and Elution of Anti-Glycolipid Antibody to Sepharose or Glass Bead Complex—Results from the elution of a globoside-Sepharose column are shown in Fig. 4 (top). The passed fractions contained 25.8 mg of protein (98.5%) and 12% of the anti-globoside activity, as demonstrated by agglutination of trypanized human red cells (33). The fractions eluted with sodium iodide contained 0.42 mg of protein and 83% of the anti-globoside activity. In a control experiment with a column of aminocetyl-Sepharose, all anti-globoside activity was eluted with the breakthrough fraction. Anti-N-glycolylneuraminylactoside was purified on a N-glycolylneuraminylactoside-glass beads column in the same manner as described for anti-globoside Fig. 4 (lower). Specific activity of anti-hematoside, as measured by agglutination of human erythrocytes coated with the product was estimated at 35 to 45% for the degradation product from globoside containing compounds in the flame ionization detector, the yield was estimated at 35 to 45% for the degradation product from globoside.
Fig. 4. Top, elution pattern of anti-globoside from globoside-Sepharose column. Rabbit anti-globoside serum containing 26.2 mg of protein was applied to a 2.5 ml column of globoside-Sepharose, 5-min incubation time was allowed after application of the crude antibody, and the column was eluted with 15 ml of PBS, followed by elution with 1 m sodium iodide. Three-milliliter fractions were collected. Activity was measured by agglutination of a 1% trypsinized human erythrocyte suspension at 6°C. — — , elution pattern of protein; --- , agglutination unit per µg of protein. The unit member is the reciprocal of the maximal dilution of antibody that causes obvious hemagglutination at 4°C. Protein was determined by the Lowry method. Bottom, elution pattern of anti-N-glycolylhematoside from a hematoside-glass bead column. Column volume was 2.5 ml; antibody was allowed to incubate with column for 5 min and 3-ml fractions of PBS were collected. Bound antibody was removed with 1 m sodium iodide. Agglutination was passive with N-glycolylhematoside absorbed on human erythrocytes.

with N-glycolylhematoside (30) was increased 50 to 100 fold after purification through the hematoside-glass beads column. However, the amino-glass bead itself has a small, but measurable degree of capacity to absorb the antibody by nonspecific interaction. The degree of such capacity was practically negligible after the glass beads were coupled to hematoside, and the anti-hematoside antibody eluted from the hematoside-glass beads column by sodium iodide had no reaction with bovine serum albumin and showed high specificity to hematoside.

Specificities of Anti-Globoside and Anti-Hematoside—Since bovine serum albumin was used as a carrier of globoside to produce anti-globoside, the immune sera contained large amounts of anti-bovine serum albumin. An indication of purity of the affinity-retained anti-globoside was the lack of anti-bovine serum albumin in the purified fraction as shown by Ouchterlony immunodiffusion precipitation lines in Fig. 5 (top). Rabbit erythrocytes conjugated with bovine serum albumin by tannic acid (40) gave a negative reaction to agglutination by the purified antibody.

Quantitative microcomplement fixation with anti-globoside detected some cross-reaction of glycolipids structurally related to globoside. Cytolipin R, a close homolog differing only in the position of internal α-galactosyl-β-galactose linkage (30), gave cross-reaction with anti-globoside, which was, under the observed indication, indistinguishable from the reaction with globoside. At very high levels of antigen, both Forssman glycolipid and αGal1→4Gal1→4Glc-ceramide gave a low degree of cross-reaction, at least 100 fold less than globoside. Ceramide lactoside and lacto-N-neotetraosylceramide gave a negative reaction. These results are summarized in Fig 6 (left).

Ouchterlony immunoprecipitation gave a slightly different result. Cytolipin R showed much less cross reactivity than was evident in complement fixation, and CTH showed slight reactivity, but Forssman glycolipid gave no visible precipitin line (Fig. 5, lower left).

The purified anti-globoside contained a predominance of IgG and a small amount of IgM, as shown by immunoprecipitin lines with goat anti-rabbit IgG and IgM.

Reactivity of the purified anti-hematoside antibody was tested by complement fixation and Ouchterlony immunodiffusion with N-acetylgalactosamine, N-glycolyl-O-acetylgalactosamine, N-glycolyl-N-acetylgalactosamine, N-glycolyl-O-acetylmethyl ester, lactosylceramide, and gangliosides of human brain. Cross-reactivities for N-glycolyl-O-acetylated and N-glycolyl-O-methylated N-acetylgalactosamine were demonstrated by both complement fixation (Fig. 5, right) and immunodiffusion (Fig. 5). Ceramide lactoside and other gangliosides of brain gave completely negative results. Inhibition of passive agglutination with N-glycolylated N-acetylgalactosamide coated erythrocytes was tested with lactosylceramide, CTH, N-acetylgalactosamines, mixed human brain gangliosides, and N-glycolylated N-acetylgalactosamines (CTH and lactosylceramides were mixed with brain gangliosides for solubility). Only N-glycolylated N-acetylgalactosamide inhibited this agglutination. Ouchterlony gel immunodiffusion confirms this reactivity as shown in Fig. 6 (lower right). Methyl ester of N-glycolylated N-acetylgalactosamide showed, however, only a faint precipitation line while it gave a strong complement fixation.

The agglutinating property of the purified anti-globoside showed an enormous temperature sensitivity, agglutinating trypsinized human erythrocytes very strongly at 4°C, to a lesser extent at room temperature, and very weakly at 37°C. Such temperature response was not observed in agglutination caused by anti-hematoside.

DISCUSSION

The results clearly show that either neutral glycosphingolipids or gangliosides can be covalently linked to solid substrates carry-
Lectins have been used with increasing interest and popularity in studies of interaction of bacterial exotoxin and animal cells by Landsteiner (33). The procedure should be useful for higher oligomers of glycosphingolipids because of the sensitivity of the carbohydrate chains to alkali degradation. The presently described procedure should be useful for higher oligomers of glycosphingolipids, including those which contain sialic acid.

Recently, a ganglioside-Sepharose complex has been used for studies of interaction of bacterial exotoxin and animal cells by Cuatrecasas (58). In this compound, the carboxyl of sialyl group in monosialoganglioside was used for coupling to Sepharose. Such a complex may be useful for limited purposes, as this structural arrangement of gangliosides on Sepharose is not analogous to that on natural cell surfaces. Since the hematoside of the complex of hematoside-glass beads described in this paper is SO close to that on natural cell surfaces. Since the hematoside of the complex of hematoside-glass beads described in this paper is SO close to that on natural cell surfaces. Since the hematoside of the complex of hematoside-glass beads described in this paper is SO close to that on natural cell surfaces. Since the hematoside of the complex of hematoside-glass beads described in this paper is SO close to that on natural cell surfaces. 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A number of cell surface-mediated phenomena could be uniquely studied with the use of purified antibodies or their "Fab" communication) while some workers claimed to derivatize concanavalin A into monovalent form by trypsinization (59).
fractions directed against glycosphingolipids; some of these problems are under active pursuit in this laboratory.

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