

PRODUCTION OF MONOCLONAL ANTIBODIES SPECIFIC FOR
TWO DISTINCT STERIC PORTIONS OF THE GLYCOLIPID
GANGLIO-*N*-TRIOSYLCERAMIDE (ASIALO GM₂)*

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Cell surface glycosphingolipids represent a large variety of antigens (1–3) and have been implicated as regulators of the cell cycle and of cell growth (4) and also as receptors for toxins and hormones (5). Plant lectins have been widely used for probing cell surface carbohydrates; nevertheless, anti-carbohydrate antibodies have several advantages as compared to lectins. These include better definition of specificity, ease of preparing the monovalent form (Fab), and relative lack of toxicity. Antibodies directed to a pure glycolipid (6–8) or an oligosaccharide-carrier protein complex (9, 10) have been prepared for studies of carbohydrate distribution in tissues and cells (7), as well as changes of carbohydrates associated with the cell cycle (11, 12), oncogenic transformation (13, 14), cell contact (14), and their functional modification (15). However, those anti-carbohydrate antibodies (6–10) were polyclonal and contained a variety of specificities. Therefore, to obtain monoclonal anti-carbohydrate antibodies, we have utilized the hybridoma technique developed by Kohler and Milstein (16, 17) to produce continuous cultures of hybrid cells that secrete monoclonal antibodies of predefined specificity.

We describe here the isolation of two cell lines each of which produces an antibody specific for distinct steric portions of the carbohydrate moiety of ganglio-*N*-triosylceramide (asialo GM₂)¹ (see structure in Table I). This carbohydrate structure was chosen because we previously demonstrated that asialo GM₂ represents a potential tumor-associated cell surface marker for BALB/c 3T3 cells transformed by the Kirsten strain of murine sarcoma virus (18, 19).

Materials and Methods

Antisera. Rabbit antiserum against mouse IgM, IgG, and IgA was purchased from Behring Diagnostics, American Hoechst Corp, Somerville, N. J. Goat antisera against purified mouse IgM and IgG1 were purchased from Meloy Laboratories Inc., Springfield, Va.; anti-IgG1 was absorbed with purified mouse IgG3 as previously described (20). Rabbit antisera against

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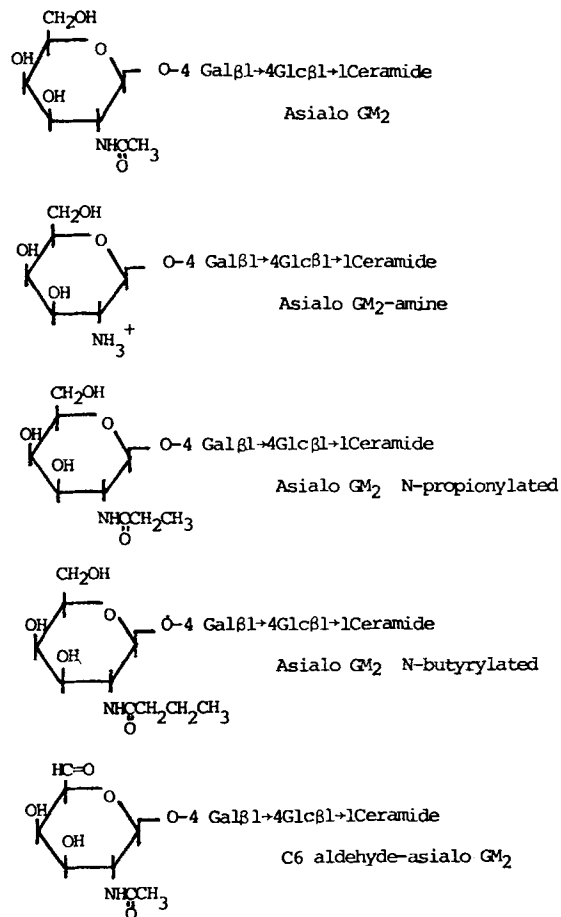
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¹ *Abbreviations used in this paper:* asialo GM₂, ganglio-*n*-triosylceramide; BSA, bovine serum albumin; IPA, ¹²⁵I-protein A from *Staphylococcus aureus*; 4 MeUmP, 4-methyl umbelliferone phosphate; PBS, phosphate-buffered saline 140 mM NaCl, 1 mM CaCl₂, 0.5 mM MgCl₂, 10 mM NaPO₄, pH 7.4; TLC, thin-layer chromatography.

TABLE IB
Structural Analogues of Asialo GM₂

Glucosyl ceramide	Glcβ1→1Ceramide
Ceramide dihexoside	Galβ1→4Glcβ1→1Ceramide
Asialo GM ₂	GalNAcβ1→4Galβ1→4Glcβ1→1Ceramide
GM ₂ ganglioside	GalNAcβ1→4Galβ1→4Glcβ1→1Ceramide
	3
	↑
	2
	NANA
Asialo GM ₁	Galβ1→3GalNAcβ1→4Galβ1→4Glcβ1→1Ceramide
GM ₁ ganglioside	Galβ1→3GalNAcβ1→4Galβ1→4Glcβ1→1Ceramide
	3
	↑
	2
	NANA
Ceramide trihexoside	Galα1→4Galβ1→4Glcβ1→1Ceramide
Globoside	GalNAcβ1→3Galα1→4Galβ1→4Glcβ1→1Ceramide
Lacto-N-triosylceramide	GlcNAcβ1→3Galβ1→4Glcβ1→1Ceramide

TABLE IB
Structural Analogues of Asialo GM₂



purified mouse IgG2a, IgG2b, IgG3, κ -light chain and λ -light chain were purchased from Miles Laboratories Inc., Miles Research Products, Elkhart, Ind.

Rabbit antiserum against the purified glycolipid asialo GM₂ was produced by immunizing a New Zealand white rabbit with an emulsion containing asialo GM₂, bovine serum albumin, and complete Freund's adjuvant as previously described (18). IgM and IgG fractions were separated on a Sepharose CL 6B column (2.5 × 93 cm) (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J.) and concentrated with a collodion bag apparatus (Schleicher & Scheull, Inc., Keene, N. H.).

Glycolipids and Derivatives. Glycolipids were obtained from the following sources and purified as previously described (21): asialo GM₂ from guinea pig erythrocytes; glucosyl ceramide, ceramide dihexoside, ceramide trihexoside, and globoside from human erythrocytes; GM₂ ganglioside from the brain of a Tay-Sachs patient; and GM₁ ganglioside from bovine brain.

Lacto-*N*-triosylceramide was prepared from purified sialyl paralogoside of bovine erythrocytes by stepwise removal of the sialic acid by mild acid hydrolysis followed by cleavage of the terminal galactose with β -galactosidase. Asialo GM₁ was prepared by mild acid hydrolysis of GM₁ ganglioside.

The C6 position of terminal GalNAc of asialo GM₂ was oxidized to an aldehyde by galactose oxidase according to the method of Suzuki and Suzuki (22) and purified by thin-layer chromatography (TLC).

The acetyl group in amide linkage to the GalNAc residue of asialo GM₂ was removed by heating at 80°C for 5 h in the presence of hydrazine hydrate. The de-*N*-acetylated asialo GM₂ product, which was TLC purified in the solvent chloroform:methanol:water (60:35:8, vol/vol), had an R_f of 0.29 compared with that of 0.43 for asialo GM₂. 10 μ g of the free amino derivative gave a strong fluorescent spot when the TLC plate was sprayed with 0.03% fluorescamine (23) in acetone followed by viewing under an ultraviolet light.

The free amino asialo GM₂ was re-*N*-acylated by dissolving 0.5-mg aliquots of the glycolipid in 0.5 ml MeOH plus 0.2 ml pyridine followed by adding 0.05 ml of either acetic anhydride (Mallinckrodt Inc., St. Louis, Mo.), propionic anhydride (Aldrich Chemical Co., Inc., Milwaukee, Wis.), or butyric anhydride (Aldrich Chemical Co., Inc.). The reactions proceeded for 2 h at room temperature with 0.05-ml aliquots of each respective anhydride being added every 15 min. Solvents were removed under N₂ stream in the presence of excess toluene. Re-*N*-acetylated asialo GM₂ comigrated with native asialo GM₂ on TLC in chloroform:methanol:water (60:35:8, vol/vol) with R_f of 0.43; *N*-propionylated asialo GM₂ had an R_f of 0.48, and *N*-butyrylated had an R_f of 0.51. The methyl ester of GM₂ ganglioside was formed by incubation in the presence of Dowex-50 (H⁺ form) (Bio-Rad Laboratories, Richmond, Calif.) in methanol for 3 d at room temperature.

Preparation of Hybrid Cell Lines. BALB/c mice were immunized with asialo GM₂ noncovalently adsorbed to naked *Salmonella minnesota* by the procedure of Galanos et al. (24). Each mouse received the following intravenous injections of the glycolipid-bacterial complex in saline (weight ratio of glycolipid to bacteria of 1:4): 5 μ g asialo GM₂ on day 0, 10 μ g on day 4, 15 μ g on day 7, and 20 μ g on day 12. After a rest period of at least 2 wk the mice received a final booster injection (20 μ g asialo GM₂) 4 d before the spleens were harvested.

Spleen cells were fused according to the method of Kohler and Milstein (16, 17) by polyethylene glycol with the mutant BALB/c myeloma cell line MOPC21 NSI/1, which was kindly provided by C. Milstein (Molecular Research Council, Cambridge). Hybrids producing anti-asialo GM₂ were screened, minicloned, and cloned in microtiter wells according to the method of Nowinski et al. (20). Briefly, the screening assay utilized the finding of Holmgren (25) that glycolipids are adsorbed from an aqueous medium onto plastic surfaces. Asialo GM₂ (20 μ g/ml in water plus sodium azide) was incubated in Microtest II wells (Falcon Labware, Div. of Becton, Dickinson, & Co., Oxnard, Calif.) overnight at 37°. Nonspecific binding of antibodies to the plate was then blocked by incubation with 5% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 2 h at 37°C.

The antibody binding assay was performed in three steps: (a) 50 μ l of culture fluid was incubated in each of the glycolipid-adsorbed wells for 45 min at 37°C. Nonbound immunoglobulin was then removed from the wells by washing three times with PBS containing 1% BSA. (b) 100,000 cpm of ¹²⁵I-protein A from *Staphylococcus aureus* (IPA) in 50 μ l of PBS was added to each well for 45 min at 37°C. The residual nonbound IPA was then removed from

the wells by washing with PBS. (c) The immune reactions were detected by overnight autoradiography of the IPA-treated Microtest plates on Kodak NS-2T film (Eastman Kodak Co., Rochester, N. Y.) with enhancement by x-ray intensifying screens (26). To detect mouse immunoglobulin classes that did not react directly with IPA, between steps (a) and (b) above, the wells were incubated with rabbit anti-mouse immunoglobulin for 45 min at 37°C. The nonbound rabbit antibodies were removed by washing with PBS.

After completion of the above protocol, large amounts of concentrated antibodies from cloned hybridomas were prepared either by ammonium sulfate precipitation (40% saturation) of culture fluids or by intraperitoneal inoculation of hybridoma cells into BALB/c mice that had been primed with 2,6,10,11-tetramethylpentadecane (pristane, Aldrich Chemical Co.). The ascites fluid and sera from such sera were analyzed by electrophoresis on cellulose acetate membranes (Microzomal System, Beckman Instruments Inc., Fullerton, Calif.).

Determining Antibody Specificity. Asialo GM₂ is the major glycolipid of guinea pig erythrocyte membranes (27), and these cells are readily agglutinated by anti-asialo GM₂. Inhibition of hemagglutination was tested using lipid vesicles with mole ratio of egg lecithin:cholesterol:glycolipid of 1:1:0.1. The vesicles were diluted in microtiter plates and then preincubated with three hemagglutination doses of antibody solution at 4°C for 1 h. After the addition of a 2% suspension of guinea pig erythrocytes, the incubation was continued for 1 h at 4°C.

Complement fixation was determined in microtiter plates using a minor modification of a previously described method (28), using lipid vesicles with a mole ratio of egg lecithin:cholesterol:glycolipid of 1:1:0.03.

Antibody-complement mediated damage to glycolipid liposomes was determined according to the method of Six et al. (29). 4-methyl umbelliferone phosphate (4Me-UmP) was trapped inside multicompartiment liposomes prepared with a mole ratio of dimyristoyl phosphatidylcholine:cholesterol:dicetyl phosphate:glycolipid of 1:0.75:0.1:0.04. Release of the trapped marker from the liposomes by appropriate antibody plus complement was detected fluorometrically by the conversion of the fluorogenic substrate to the highly fluorescent 4-methyl umbelliferone by alkaline phosphatase. Each assay tube contained alkaline phosphatase (5 μl, 2.5 μg/ml), 25 μl guinea pig serum as the complement source, and the indicated volume of antibody, in a total vol of 990 μl. The reaction was started by the addition of 10 μl of liposomes (2 mM dimyristoyl phosphatidylcholine).

Results

Production of Anti-Asialo GM₂ Hybridomas. The first fusion of the NS-I/1 myeloma with spleen cells from three BALB/c mice immunized against asialo GM₂ yielded 72 culture wells from which one anti-asialo GM₂-producing clone (2D4) was obtained. In immunodiffusion tests the concentrated culture fluid was shown to contain an IgM immunoglobulin with κ-light chains. In a second fusion using two mouse spleens, the supernate from 2 of 300 culture wells reacted with asialo GM₂ in the initial antibody binding assay. Only one of these resulted in a stable clone (D11G10) which produced an IgG3 antibody, also with κ-light chains. Interestingly, these results represent another example of the findings of Perlmutter et al. (30) that mouse antibodies specific for carbohydrates are, in addition to IgM, primarily of the IgG3 subclass.

The levels of anti-asialo GM₂ antibodies were initially compared by hemagglutination of guinea pig erythrocytes. The highest hemagglutination titer in the sera of mice immunized against asialo GM₂ was 1:32-1:64. As shown in Table II the titer of the medium from a dense culture of the IgM-producing clone was comparable to this peak serum titer. The tremendous amplification of antibody production that can be achieved by the hybridoma technique is indicated by the titer of the ascites fluid from mice bearing the IgM-producing hybridoma which was nearly 1,000 times stronger than immune serum; in addition an average of ~6-8 ml ascites fluid was obtained from each mouse. Animals inoculated with the IgG3-producing cells yielded only ~2-

TABLE II
 Characteristics of Anti-Asialo GM₂ Hybridomas

	Clone 2D4	Clone D11G10
Immunoglobulin:		
Heavy chain type	μ	γ3
Light chain type	κ	κ
Hemagglutination titer:*		
Culture medium	1:32-1:64	1:4-1:8
Ascites fluid	1:4,000-1:32,000	1:256-1:512
Immunoglobulin concentration in ascites fluid, mg/ml	30-40	10-15

* Hemagglutination of 2% guinea pig erythrocytes at 4°C.

4 ml ascites fluid. Cellulose acetate electrophoresis indicated that ascites fluids from mice bearing each hybridoma contained monoclonal gamma globulins (Fig. 1), with approximate immunoglobulin concentrations as indicated in Table II.

Antibody Specificity Determined by Hemagglutination Inhibition. The specificity of the anti-asialo GM₂ hybridoma antibodies was determined using hybridoma culture medium which had been concentrated 30-fold by ammonium sulfate precipitation. Several purified glycolipids were tested initially for their ability to inhibit the agglutination of guinea pig erythrocytes. Asialo GM₂ completely inhibited the hemagglutination caused by each hybridoma antibody (Table III); none of the other glycolipids tested caused any inhibition even at 100 μg/ml. These glycolipids included ceramide dihexoside (see structures in Table I), indicating that the major immunodeterminant for both antibodies was the terminal *N*-acetylgalactosamine and not the penultimate galactose residue. GM₂ ganglioside, which differs from asialo GM₂ only by the presence of sialic acid attached to C3 of the galactose residue, also did not inhibit. To rule out the possibility that GM₂ was not recognized simply because of the negative charge of the sialic acid carboxyl group, the GM₂ methyl ester was tested and found to lack inhibitory activity as well. Thus, both antibodies failed to recognize the GalNAcβ1→4Gal structure when a bulky group was attached to C3 of the galactose. In addition, asialo GM₁ did not inhibit either antibody indicating that the C3 hydroxyl of the terminal GalNAc must be free. Finally, the fact that globoside did not inhibit indicated that both antibodies were specific for the GalNAcβ1→4Gal linkage rather than GalNAcβ1→3Gal.

To further explore the specificity of these antibodies, several structural analogues of asialo GM₂ were tested (Table IB). Removal of the acetyl group of the terminal GalNAc by hydrazinolysis produced a free amino asialo GM₂ which was not recognized by either antibody (Table III). Re-acylation of the amino function with chains of increasing lengths produced the following striking results. The IgM antibody recognized the re-*N*-acetylated, *N*-propionylated, and *N*-butyrylated structures, but the IgG3 was inhibited only by the first two analogues and completely failed to recognize the *N*-butyrylated structure. These results strongly suggested that the terminal GalNAc including the C2-moiety of asialo GM₂ represented a major immunodeterminant for the IgG3 antibody.

An additional derivative was prepared by oxidizing the C6 hydroxyl function of the terminal GalNAc of asialo GM₂ to an aldehyde group by galactose oxidase. Although this structure inhibited agglutination by the IgG3 to the same extent as

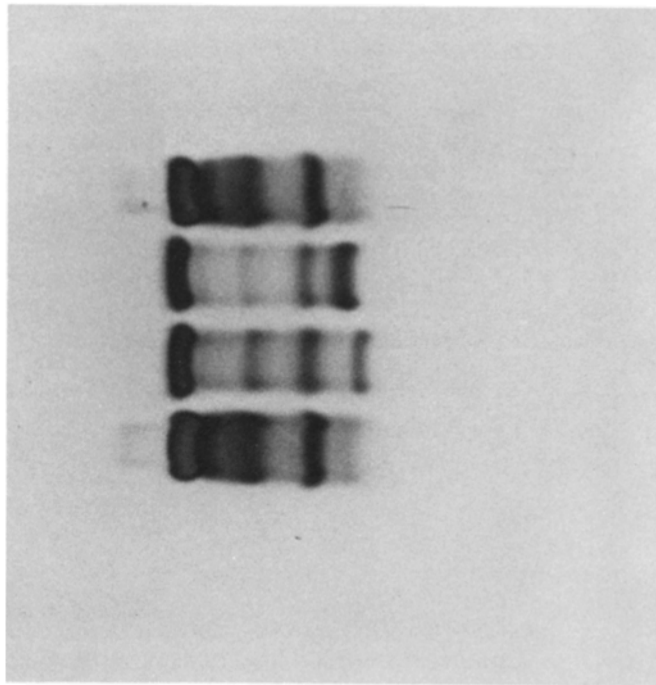


FIG. 1. Electrophoretic separation of proteins in the ascites fluid of mice bearing anti-asialo GM₂-secreting hybridomas. Electrophoresis on cellulose acetate membranes was performed with (from top to bottom): a. normal mouse serum; b. ascites fluid from IgM secreting hybridoma; c. ascites fluid from IgG3 secreting hybridoma; d. normal mouse serum.

native asialo GM₂, it was not recognized by the IgM antibody in this assay. Therefore, the terminal GalNAc of asialo GM₂ including C6 portion was identified as a major immunodeterminant for the IgM antibody.

A rabbit antiserum specific for asialo GM₂ was also tested for its ability to bind analogs of asialo GM₂ (Table III). Agglutination of guinea pig erythrocytes by the rabbit IgG fraction, unlike IgG3 antibody, was not inhibited by asialo GM₂ C6 aldehyde, and it also failed to recognize *N*-butyrylated asialo GM₂. The fact that asialo GM₂ *N*-propionylated caused only partial inhibition even at the highest concentration (100 μg/ml) indicates that the rabbit IgG antibodies consisted of a mixture of specificities, only some of which reacted with this derivative. Thus, it was difficult to define immunodominant portions of asialo GM₂ for the rabbit IgG fraction although the data indicate that both the C2 and C6 portion of the terminal GalNAc may influence IgG antibody binding. Similarly, the rabbit IgM fraction was also heterogeneous with respect to binding specificity, as it was only partially inhibited by asialo GM₂ *N*-butyrylated and asialo GM₂ C6 aldehyde.

Specificity Determined by Complement-dependent Assays. The specificity of the two BALB/c monoclonal antibodies was also tested by complement fixation (Fig. 2). The IgM antibody failed to recognize the free amino-asialo GM₂ but reacted with asialo GM₂ and with the three re-*N*-acylated structures to the same degree. There was a weak but detectable reaction of the IgM with asialo GM₂ C6-aldehyde which was not seen in the hemagglutination inhibition assay.

TABLE III
Inhibition by Glycolipid of Guinea Pig Erythrocyte Hemagglutination Caused by Anti-Asialo GM₂ Antibodies

Glycolipid inhibitor	Concentrated hybridoma supernate:		Rabbit anti-asialo GM ₂	
	2D4 (IgM)	D11G10 (IgG3)	IgM	IgG
Asialo GM ₂	3.1*	3.1	1.6	12.5
Asialo GM ₂ -NH ₂	>100	>100	ND	ND
Asialo GM ₂ -re- <i>N</i> -acetylated	1.6	6.3	1.6	12.5
Asialo GM ₂ - <i>N</i> -propionylated	1.6	3.1	3.2	12.5‡
Asialo GM ₂ - <i>N</i> -butyrylated	0.8	>100	6.4‡	>100
Asialo GM ₂ -C6 aldehyde	>100	6.2	12.5‡	>100
Glucosylceramide	} >100	} >100	} ND	} ND
Ceramide dihexoside				
GM ₂ , GM ₂ methyl ester				
GM ₁ , asialo GM ₁ , globoside				
Ceramide trihexoside				
Lacto- <i>N</i> -triacylceramide				

ND, not determined.

* Minimum dose of glycolipid in $\mu\text{g/ml}$ which inhibits three hemagglutination doses of antibody. Glycolipids were tested in the presence of auxiliary lipids (Materials and Methods).

‡ Inhibition even at the highest glycolipid concentrations was not complete.

Although IgG3 Fc fragments do not appear to bind complement (31), IgG3 anti-asialo GM₂ did fix complement in agreement with the description of another anti-carbohydrate IgG3 (32). The IgG3 anti-asialo GM₂ culture supernate concentrated 30-fold had a complement fixation titer of only 1:8. Nevertheless, this antibody reacted to an equal extent with asialo GM₂, the C6 aldehyde structure, and the re-*N*-acetylated and *N*-propionylated forms, but failed to recognize either the free amino derivative or *N*-butyrylated asialo GM₂.

Lysis of multicompartiment glycolipid liposomes by the monoclonal antibodies plus complement produced specificity patterns in agreement with the preceding assays. Because the complement fixing activity of the IgM antibody was so strong, it was possible to analyze unconcentrated culture fluid in this assay (Fig. 3A). The IgM reacted with asialo GM₂ and the three re-*N*-acylated structures to the same extent. Reaction with liposomes containing asialo GM₂ C6-aldehyde was very weak; only 9.6% of the trapped marker was released in the presence of 150 μl culture fluid.

The concentrated IgG3 antibody in the presence of complement produced significant marker release from liposomes prepared with asialo GM₂, the C6-aldehyde structure and the re-*N*-acetylated and *N*-propionylated derivatives (Fig. 3B), but failed to react with the asialo GM₂ *N*-butyrylated liposomes. In addition to the data shown, neither the IgM nor IgG3 antibodies reacted with the following glycolipids by complement fixation or liposome lysis: ceramide dihexoside, GM₂ ganglioside, GM₂ methyl ester, GM₁ ganglioside, or globoside.

Discussion

Using the hybridoma procedure developed by Kohler and Milstein (16, 17) we have established continuous cultures of hybrid cells producing two monoclonal

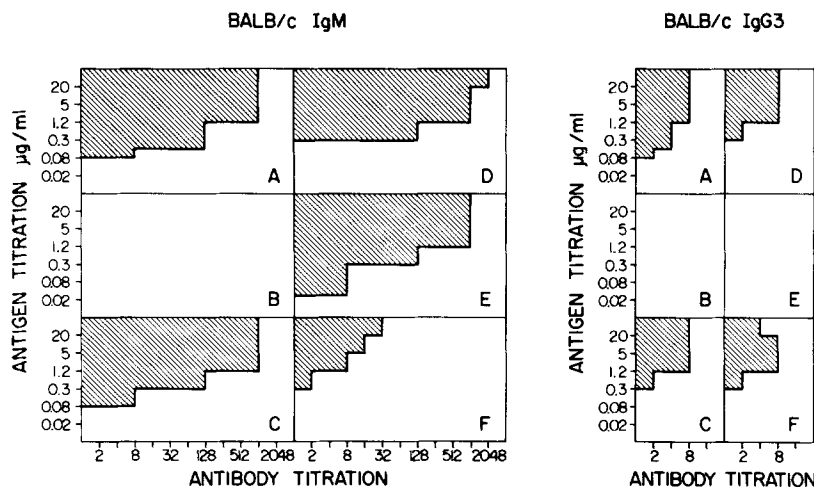


FIG. 2. Complement fixation patterns produced by anti-asialo GM₂ hybridoma antibodies of the IgM class (left) and IgG3 subclass (right) and various glycolipid derivatives. Abscissa, reciprocal of the antibody dilution; in both cases the antibody source was culture medium from the hybridoma cells which had been concentrated 30-fold by ammonium sulfate precipitation. Hatched areas indicate only 100% fixation. A, asialo GM₂; B, asialo GM₂-amine; C, asialo GM₂ re-*N*-acetylated; D, asialo GM₂ *N*-propionylated; E, asialo GM₂ *N*-butyrylated; and F, C6 aldehyde asialo GM₂.

antibodies specific for the glycolipid asialo GM₂. Using structural analogs of asialo GM₂, we identified the C6 hydroxyl-bearing group of the terminal *N*-acetylgalactosamine residue as a major immunodeterminant for one of these antibodies, which is of the IgM class (Table III and Figs. 2 and 3 A). In contrast the other hybridoma antibody, which is an IgG3, recognized the C2 *N*-acetyl group of the terminal sugar as a major determinant (Table III, Figs. 2 and 3 B). It is unlikely that these differences seen in reactivity of the two antibodies might simply be a result of some general difference between IgM and IgG, because rabbit IgM and IgG antibodies specific for asialo GM₂ did not display the same pattern of reactivity (Table III). Therefore, the simplest interpretation of our results is that the monoclonal IgM antibody is directed toward the C6 portion of terminal galactosamine, whereas the IgG3 antibody recognizes the C2 position, which is actually on the other side of the molecule as depicted in Fig. 4. Clearly, both antibodies have some additional recognition of the entire GalNAc β 1 \rightarrow 4Gal structure without sialosyl attachment at the penultimate Gal, as neither globoside nor GM₂ ganglioside reacted with either antibody.

Previous studies have indicated that anti-carbohydrate antibodies can possess such strict specificity. Natural monoclonal IgM antibodies present in patients with Waldenstrom macroglobulinemia have been known to possess anti-carbohydrate specificity. Some of these antibodies, collectively known as cold-reactive anti-I and anti-i antibodies, recognize the following specific domains of defined carbohydrate structures. The determinants recognized by 10 of 11 monoclonal anti-I antibodies are all located on various domains within the branched lacto-*N*-isooctaosyl structure (33, 34). The determinants recognized by four out of six monoclonal anti-i antibodies are located on various domains of the lacto-*N*-norhexaosyl structure, which is a linear chain with two repeating Gal β 1 \rightarrow 4GlcNAc residues (33-35). These data provide additional examples that specific domains within the same carbohydrate chain can be recognized by different monoclonal antibodies.

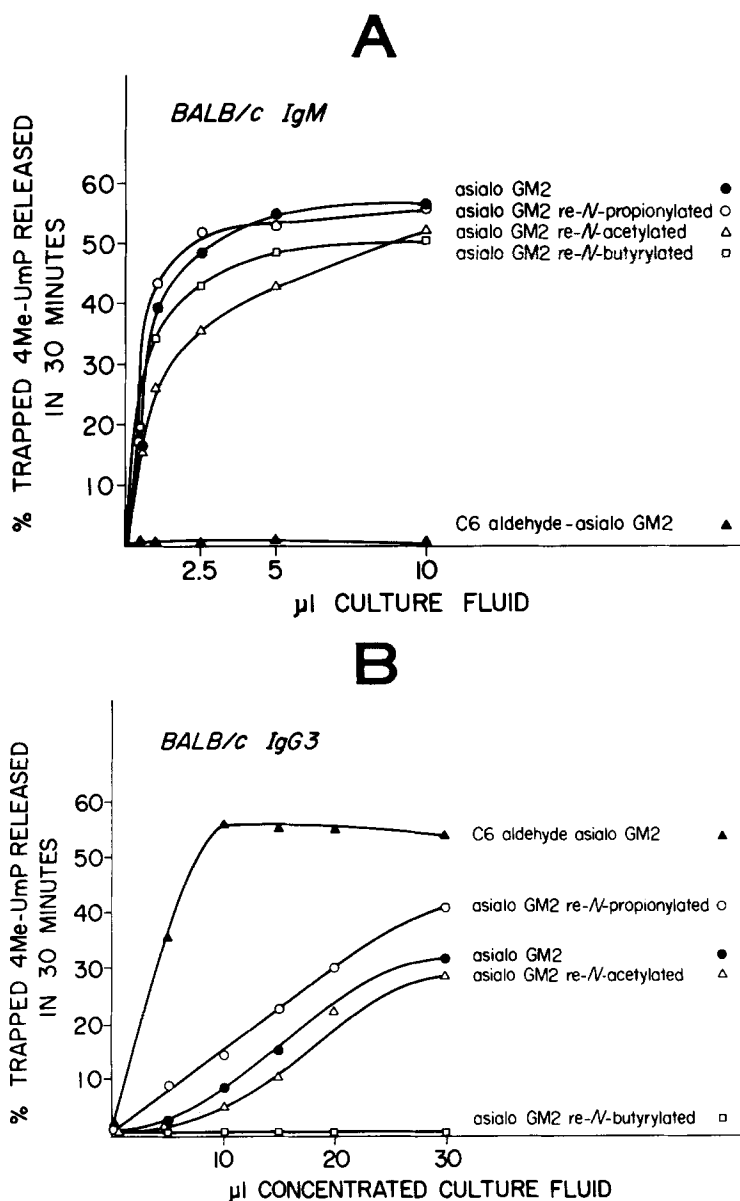


FIG. 3. Effect of anti-asialo GM2 hybridoma antibodies on 4Me-UmP release from glycolipid liposomes. A, IgM antibody; B, IgG3 antibody. See Materials and Methods for assay details.

Polyclonal anti-carbohydrate antibodies have also been shown to possess strict specificity. For example Laine et al. (8) reported that affinity purified rabbit antibodies specific for *N*-glycolylhematoside reacted only weakly with *N*-acetylhematoside. Nevertheless, the results of the present paper demonstrate that monoclonal anti-carbohydrate antibodies prepared by the hybridoma technique are clearly the reagents of choice in terms of both the quantity and specificity of antibody that can be produced.

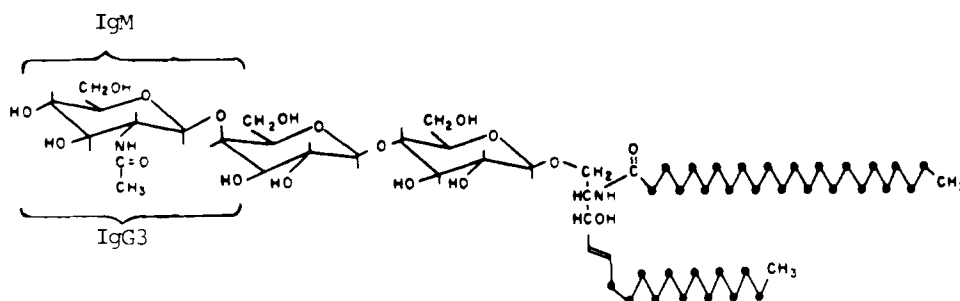


Fig. 4. Proposed structural specificities for BALB/c monoclonal anti-asialo GM₂ antibodies.

Perlmutter et al. (30) have recently reported that murine IgG responses against several bacterial carbohydrate antigens are largely restricted to the rare IgG3 subclass. They proposed the existence of a subclass-specific regulatory mechanism in the mouse activated by such antigens, which may exist in other species as well. Interestingly, our anti-asialo GM₂ IgG is also of the IgG3 subclass. The immunogen used in these studies was asialo GM₂ noncovalently adsorbed to an acid-treated mutant of *Salmonella minnesota* (24). Apparently such a complex is sufficient to activate on IgG3 response.

In conclusion, we have described the production of two anti-asialo GM₂ antibodies which interact with distinct portions of the carbohydrate chain. Using reagents with such strict specificity it may be possible to probe cell surface membrane architecture at a new level. For example, it is conceivable that access of exogenous reagents such as antibodies to one portion of a carbohydrate chain might be restricted by neighboring molecules, whereas other regions of the same chain would be exposed; such situations may be detectable using sets of highly specific monoclonal antibodies.

Summary

Two hybrid cell lines were prepared by the fusion of mouse myeloma cells with the spleen cells of BALB/c mice that had been immunized with the glycolipid ganglio-*N*-triosylceramide (asialo GM₂). The specificity of the monoclonal antibodies produced by these hybridomas, one an IgM and the other an IgG3, has been defined by hemagglutination inhibition, complement fixation, and lysis of glycolipid liposomes by antibody and complement.

A major determinant recognized by the IgM antibody is the nonreducing terminal *N*-acetylgalactosamine including the C6 primary hydroxyl group, but excluding the C2-acetamido group of *N*-acetylgalactosamine, because oxidation with galactose oxidase produced a structure showing only minimal cross-reaction with the IgM but replacement of the *N*-acetyl group with an *N*-*n*-butyryl group produced a glycolipid that reacts with IgM antibody to the same extent as with the unmodified glycolipid. A major determinant recognized by the IgG3 antibody is the terminal *N*-acetylgalactosamine including the C2-acetamido group, but excluding the C6 primary hydroxyl group of *N*-acetylgalactosamine, because replacement of the *N*-acetyl group with an *N*-*n*-butyryl group produced a glycolipid that did not react with the IgG3 antibody; in striking contrast the IgG3 antibody reacted with the C6-oxidized glycolipid as well as with the native glycolipid. Neither antibody reacted significantly with any other natural glycolipids tested including several that are structurally related to asialo GM₂ such as ganglioside GM₂, ganglio-*N*-tetraosylceramide (asialo GM₁), or ceramide

dihexoside. These results indicated that in addition to the fine structure specificity described above both antibodies recognize the nonreducing terminal GalNAc β 1 \rightarrow 4Gal structure.

The strict antigenic specificity of these monoclonal anti-glycolipid antibodies indicates their great potential as specific probes for cell surface studies.

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