PRODUCTION OF MONOCLONAL ANTIBODIES SPECIFIC FOR TWO DISTINCT STERIC PORTIONS OF THE GLYCOLIPID GANGLIO-*N*-TRIOSYLCERAMIDE (ASIALO GM_2)*

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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 GM_2)[1] (see structure in Table I). This carbohydrate structure was chosen because we previously demonstrated that asialo GM_2 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

* Supported by the National Institutes of Health grants GM23100 to Sen-Itiroh Hakomori and CA18074 to Robert C. Nowinski.

‡ Recipient of a fellowship from the Cancer Research Institute, Inc.

[1] *Abbreviations used in this paper:* asialo GM_2, ganglio-*n*-triosylceramide; BSA, bovine serum albumin; IPA, ^{125}I-protein A from *Staphylococcus aureus*; 4 MeUmP, 4-methyl umbelliferone phosphate; PBS, phosphate-buffered saline 140 mM NaCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 10 mM $NaPO_4$, pH 7.4; TLC, thin-layer chromatography.

 J. Exp. Med. © The Rockefeller University Press · 0022-1007/79/10/1008/12 $1.00
Volume 150 October 1979 1008–1019

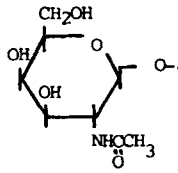
TABLE IB

Structural Analogues of Asialo GM_2

Glucosyl ceramide	Glcβ1→1Ceramide
Ceramide dihexoside	Galβ1→4Glcβ1→1Ceramide
Asialo GM2	GalNAcβ1→4Galβ1→4Glcβ1→1Ceramide
GM2 ganglioside	GalNAcβ1→4Galβ1→4Glcβ1→1Ceramide 3 ↑ 2 NANA
Asialo GM1	Galβ1→3GalNAcβ1→4Galβ1→4Glcβ1→1Ceramide
GM1 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

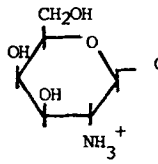
TABLE IB

Structural Analogues of Asialo GM_2



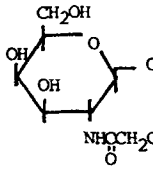
O-4 Galβ1→4Glcβ1→1Ceramide

Asialo GM_2



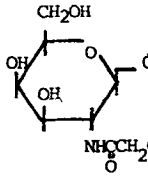
O-4 Galβ1→4Glcβ1→1Ceramide

Asialo GM_2-amine



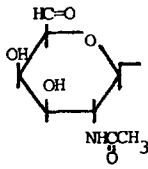
O-4 Galβ1→4Glcβ1→1Ceramide

Asialo GM_2 N-propionylated



O-4 Galβ1→4Glcβ1→1Ceramide

Asialo GM_2 N-butyrylated



O-4 Galβ1→4Glcβ1→1Ceramide

C6 aldehyde-asialo GM_2

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 GM_2 was produced by immunizing a New Zealand white rabbit with an emulsion containing asialo GM_2, 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 GM_2 from guinea pig erythrocytes; glucosyl ceramide, ceramide dihexoside, ceramide trihexoside, and globoside from human erythrocytes; GM_2 ganglioside from the brain of a Tay-Sachs patient; and GM_1 ganglioside from bovine brain.

Lacto-*N*-triosylceramide was prepared from purified sialyl paragloboside 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 GM_1 was prepared by mild acid hydrolysis of GM_1 ganglioside.

The C6 position of terminal GalNAc of asialo GM_2 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 GM_2 was removed by heating at 80°C for 5 h in the presence of hydrazine hydrate. The de-*N*-acetylated asialo GM_2 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 GM_2. 10 μg 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 GM_2 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 N_2 stream in the presence of excess toluene. Re-*N*-acetylated asialo GM_2 comigrated with native asialo GM_2 on TLC in chloroform:methanol:water (60:35:8, vol/vol) with R_f of 0.43; *N*-propionylated asialo GM_2 had an R_f of 0.48, and *N*-butyrylated had an R_f of 0.51. The methyl ester of GM_2 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 GM_2 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 μg asialo GM_2 on day 0, 10 μg on day 4, 15 μg on day 7, and 20 μg on day 12. After a rest period of at least 2 wk the mice received a final booster injection (20 μg asialo GM_2) 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 GM_2 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 GM_2 (20 μg/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 μl 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 ^{125}I-protein A from *Staphylococcus aureus* (IPA) in 50 μl 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 GM_2 is the major glycolipid of guinea pig erythrocyte membranes (27), and these cells are readily agglutinated by anti-asialo GM_2. 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 multicompartment liposomes prepared with a mole ratio of dimyristoyl phosphatidyl-choline: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 GM_2 Hybridomas. The first fusion of the NS-I/1 myeloma with spleen cells from three BALB/c mice immunized against asialo GM_2 yielded 72 culture wells from which one anti-asialo GM_2-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 GM_2 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 GM_2 antibodies were initially compared by hemagglutination of guinea pig erythrocytes. The highest hemagglutination titer in the sera of mice immunized against asialo GM_2 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 GM_2 Hybridomas

	Clone 2D4	Clone D11G10
Immunoglobulin:		
Heavy chain type	μ	$\gamma 3$
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 GM_2 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 GM_2 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. GM_2 ganglioside, which differs from asialo GM_2 only by the presence of sialic acid attached to C3 of the galactose residue, also did not inhibit. To rule out the possibility that GM_2 was not recognized simply because of the negative charge of the sialic acid carboxyl group, the GM_2 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 GM_1 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 GM_2 were tested (Table I B). Removal of the acetyl group of the terminal GalNAc by hydrazinolysis produced a free amino asialo GM_2 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 GM_2 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 GM_2 to an aldehyde group by galactose oxidase. Although this structure inhibited agglutination by the IgG3 to the same extent as

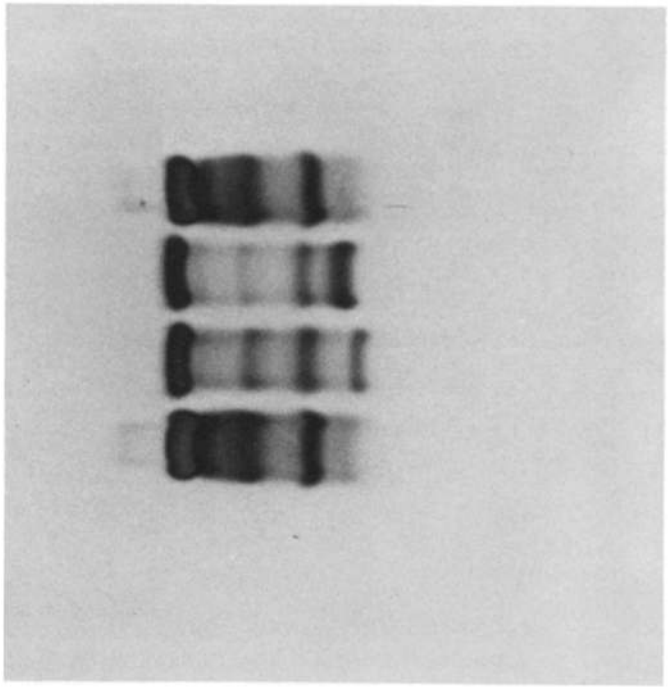


FIG. 1. Electrophoretic separation of proteins in the ascites fluid of mice bearing anti-asialo GM2-secreting hybridomas. Electrophoresis on cellulose acetate membranes was performed with (from top to bottom): a. normal mouse serum; b. ascites fluid from IgM secreting hybridoma; c. ascites fluid from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

native asialo GM_2, it was not recognized by the IgM antibody in this assay. Therefore, the terminal GalNAc of asialo GM_2 including C6 portion was identified as a major immunodeterminant for the IgM antibody.

A rabbit antiserum specific for asialo GM_2 was also tested for its ability to bind analogs of asialo GM_2 (Table III). Agglutination of guinea pig erythrocytes by the rabbit IgG fraction, unlike IgG3 antibody, was not inhibited by asialo GM_2 C6 aldehyde, and it also failed to recognize *N*-butyrylated asialo GM_2. The fact that asialo GM_2 *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 GM_2 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 GM_2 *N*-butyrylated and asialo GM_2 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 GM_2 but reacted with asialo GM_2 and with the three re-*N*-acylated structures to the same degree. There was a weak but detectable reaction of the IgM with asialo GM_2 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 GM_2 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-*N*-acetylated	1.6	6.3	1.6	12.5
Asialo GM_2-*N*-propionylated	1.6	3.1	3.2	12.5‡
Asialo GM_2-*N*-butyrylated	0.8	>100	6.4‡	>100
Asialo GM_2-C6 aldehyde	>100	6.2	12.5‡	>100
Glucosylceramide Ceramide dihexoside GM_2, GM_2 methyl ester GM_1, asialo GM_1, globoside Ceramide trihexoside Lacto-*N*-triosylceramide	>100	>100	ND	ND

ND, not determined.

* Minimum dose of glycolipid in μg/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 GM_2 did fix complement in agreement with the description of another anti-carbohydrate IgG3 (32). The IgG3 anti-asialo GM_2 culture supernate concentrated 30-fold had a complement fixation titer of only 1:8. Nevertheless, this antibody reacted to an equal extent with asialo GM_2, 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 GM_2.

Lysis of multicompartment 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. 3 A). The IgM reacted with asialo GM_2 and the three re-*N*-acylated structures to the same extent. Reaction with liposomes containing asialo GM_2 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 GM_2, the C6-aldehyde structure and the re-*N*-acetylated and *N*-propionylated derivatives (Fig. 3 B), but failed to react with the asialo GM_2 *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, GM_2 ganglioside, GM_2 methyl ester, GM_1 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

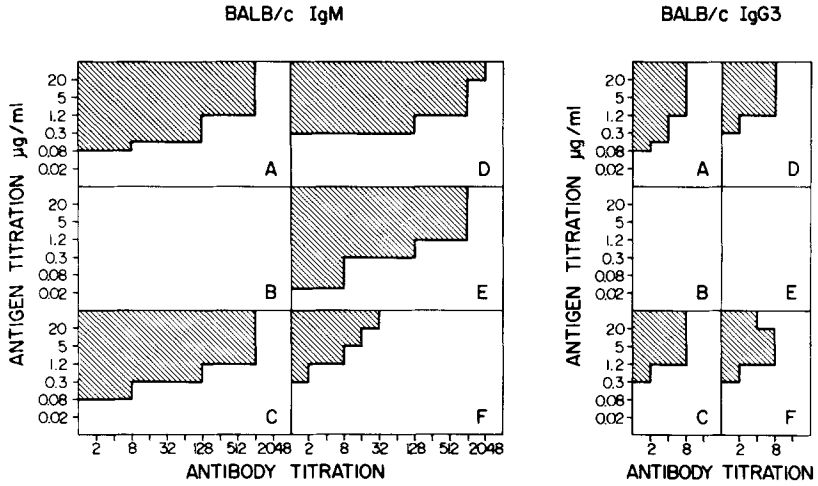


FIG. 2. Complement fixation patterns produced by anti-asialo GM2 hybridoma antibodies of the IgM class (left) and IgG3 subclass (right) and various glycolipid derivatives. Abscissa, reciprocal of the antibody dilution; in both cases the antibody source was culture medium from the hybridoma cells which 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 GM_2. Using structural analogs of asialo GM_2, 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 GM_2 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 GalNAcβ1→4Gal structure without sialosyl attachment at the penultimate Gal, as neither globoside nor GM_2 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-iso*octaosyl structure (33, 34). The determinants recognized by four out of six monoclonal anti-i antibodies are located on various domains of the lacto-*N-nor*hexaosyl structure, which is a linear chain with two repeating Galβ1→4GlcNAc residues (33–35). These data provide additional examples that specific domains within the same carbohydrate chain can be recognized by different monoclonal antibodies.

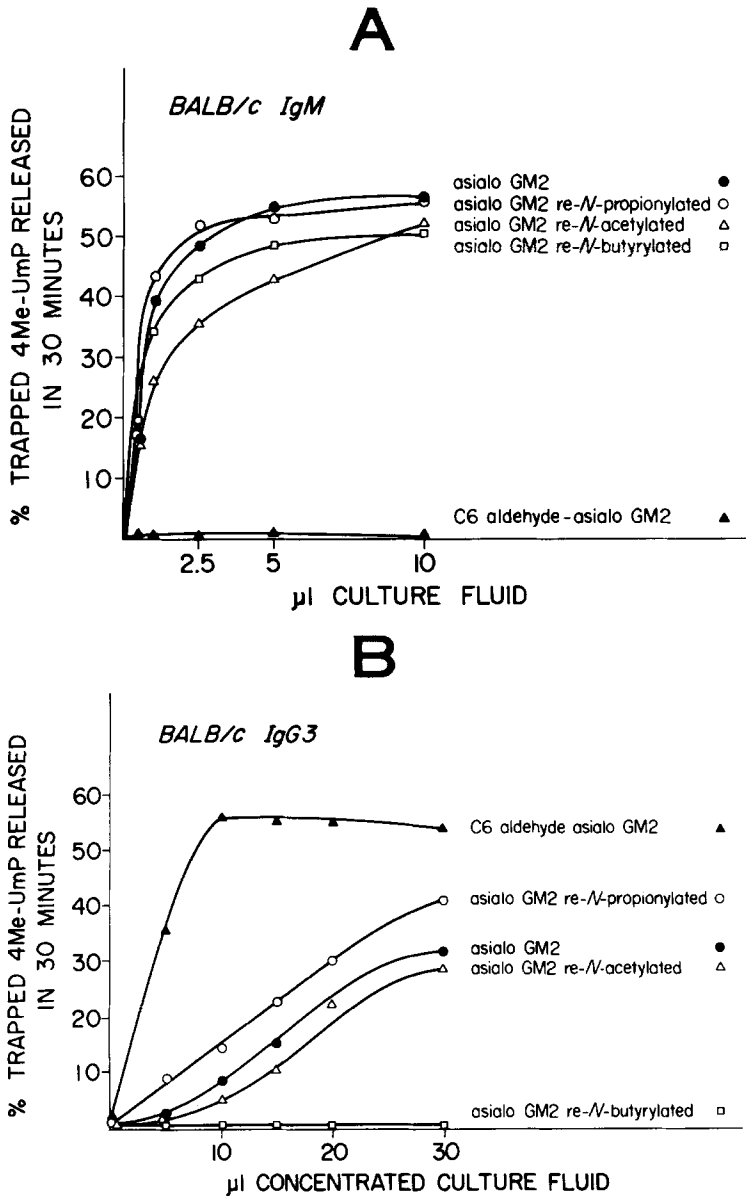


FIG. 3. Effect of anti-asialo GM2 hybridoma antibodies on 4Me-UmP release from glycolipid liposomes. A, IgM antibody; B, IgG3 antibody. See Materials and Methods for assay details.

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.

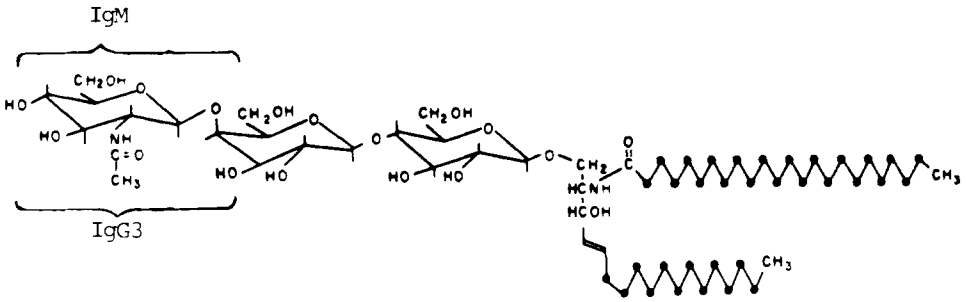


FIG. 4. Proposed structural specificities for BALB/c monoclonal anti-asialo GM_2 antibodies.

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 GM_2 IgG is also of the IgG3 subclass. The immunogen used in these studies was asialo GM_2 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 GM_2 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 GM_2). 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 GM_2 such as ganglioside GM_2, ganglio-*N*-tetraosylceramide (asialo GM_1), or ceramide

dihexoside. These results indicated that in addition to the fine structure specificity described above both antibodies recognize the nonreducing terminal GalNAcβ1→4Gal structure.

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

We thank Dr. C. Milstein (Molecular Research Council, Cambridge) for providing the BALB/c myeloma cell line MOPC 21 NSI/1.

Received for publication 5 July 1979.

References

1. Marcus, D. M., and G. A. Schwarting. 1976. Immunochemical properties of glycolipids and phospholipids. *Adv. Immunol.* **23:**203.
2. Hakomori, S., and A. Kobata. 1974. Blood group antigens. *In* The Antigens. M. Sela, editor. Academic Press, Inc., New York. II:79.
3. Hakomori, S., and W. W. Young, Jr. 1978. Tumor-associated glycolipid antigens and modified blood group antigens. *Scand. J. Immunol.* **6:**97.
4. Hakomori, S. 1975. Structures and organization of cell surface glycolipids and glycoproteins, dependency on cell growth and malignant transformation. *Biochim. Biophys. Acta.* **417:**55.
5. Fishman, P. H., and R. O. Brady. 1976. Biosynthesis and function of gangliosides. *Science (Wash. D. C.).* **194:**906.
6. Koscielak, J., S. Hakomori, and R. W. Jeanloz. 1968. Glycolipid antigen and its antibody. *Immunochemistry.* **5:**441.
7. Marcus, D. M., and R. Janis. 1970. Localization of glycosphingolipids in human tissues by immunofluorescence. *J. Immunol.* **104:**1530.
8. Laine, R. A., G. Yogeeswaren, and S. Hakomori. 1974. Glycosphingolipids covalently linked to agarose gel or glass beads. Use of the compounds for purification of antibodies directed against globoside and hematoside. *J. Biol. Chem.* **249:**4460.
9. Zopf, D. A., and V. Ginsburg. 1975. Preparation of precipitating antigens by coupling oligosaccharide to polylysine. *Arch. Biochem. Biophys.* **167:**305.
10. D. A. Zopf, A. Ginsburg, and V. Ginsburg. 1975. Goat antibody directed against a human Le^b blood group hapten, lacto-N-difucohexaose I. *J. Immunol.* **115:**1525.
11. Wolf, B. A., and P. W. Robbins. 1974. Cell mitotic cycle synthesis of NIL hamster glycolipids including the Forssman antigen. *J. Cell Biol.* **61:**676.
12. Lingwood, C., and S. Hakomori. 1977. Selective inhibition of cell growth and associated changes in glycolipid metabolism induced by monovalent antibody to glycolipids. *Exp. Cell Res.* **108:**385.
13. Hakomori, S., C. Teather, and H. D. Andrews. 1968. Organizational difference of cell surface hematoside in normal and virally transformed cells. *Biochem. Biophys. Res. Commun.* **33:**563.
14. Hakomori, S., and S. Kijimoto. 1972. Forssman reactivity and cell contacts in cultured hamster cells. *Nature (Lond.).* **239:**87.
15. Lingwood, C. A., A. Ng, and S. Hakomori. 1978. Monovalent antibodies directed to transformation-sensitive membrane components inhibit the process of oncogenic transformation. *Proc. Natl. Acad. Sci. U. S. A.* **75:**6049.
16. Kohler, G., and C. Milstein. 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature (Lond.).* **256:**495.
17. Kohler, G., and C. Milstein. 1976. Derivation of specific antibody-producing tissue culture and tumor lines by cell fusion. *Eur. J. Immunol.* **6:**511.

18. Rosenfelder, G., W. W. Young, Jr., and S. Hakomori. 1977. Association of the glycolipid pattern with antigenic alterations in mouse fibroblasts transformed by murine sarcoma virus. *Cancer Res.* **37:**1333.
19. Young, W. W., Jr., and S. Hakomori. 1979. Evidence that glycolipids can function as tumor-associated antigens. *Fed. Proc.* **38:**5247. (Abstr.)
20. Nowinski, R. C., M. E. Lostrom, M. R. Tam, M. R. Stone, and W. N. Burnette. 1979. The isolation of hybrid cell lines producing monoclonal antibodies against the p15(E) protein of ecotropic murine leukemia viruses. *Virology.* **93:**111.
21. Laine, R. A., K. Stellner, and S. Hakomori. 1974. Isolation and characterization of membrane glycosphingolipids. *Meth. Membrane Biol.* **2:**205.
22. Suzuki, Y., and K. Suzuki. 1972. Specific radioactive labeling of terminal N-acetylgalactosamine of glycosphingolipids by the galactose-oxidase sodium borohydride method. *J. Lipid Res.* **13:**687.
23. Udenfriend, S., S. Stein, P. Böhlen, W. Dairman, W. Leimgruber, and M. Weigele. 1972. Fluorescamine: a reagent for assay of amino acids, peptides, proteins, and primary amines in the picomole range. *Science (Wash. D. C.).* **178:**871.
24. Galanos, C., O. Lüderitz, and O. Westphal. 1971. Preparation and properties of antisera against the lipid-A component of bacterial lipopolysaccharides. *Eur. J. Biochem.* **24:**116.
25. Holmgren, J. 1973. Comparison of the tissue receptors for *Vibrio cholerae* and *Escherichia coli* enterotoxins by means of gangliosides and natural cholera toxoid. *Infect. Immun.* **8:**851.
26. Swanstrom, R., and P. R. Shank. 1978. X-ray intensifying screens greatly enhance the detection by autoradiography of the radioactive isotopes ^{32}P and ^{125}I. *Anal. Biochem.* **86:**184.
27. Seyama, Y., and T. Yamakawa. 1974. Chemical structure of glycolipid of guinea pig red blood cell membrane. *J. Biochem.* **75:**837.
28. Casey, H. L. 1965. Quantitative microcomplement fixation. *In* Public Health Monograph, No. 74, Dept. of Health, Education and Welfare, Washington, D.C. 1.
29. Six, H. R., W. W. Young, Jr., K. Uemura, and S. C. Kinsky. 1974. Effect of antibody-complement on multiple versus single compartment liposomes: application of a fluorometric assay for following changes in liposomal permeability. *Biochemistry.* **13:**4050.
30. Perlmutter, R., D. Hansburg, D. E. Briles, R. A. Nicolotti, and J. M. Davie. 1978. Subclass restriction of murine anti-carbohydrate antibodies. *J. Immunol.* **121:**566.
31. Grey, H. M., J. W. Hirst, and M. Cohn. 1971. A new mouse immunoglobulin: IgG3. *J. Exp. Med.* **133:**289.
32. Briles, D. E., and J. M. Davie. 1975. Detection of isoelectric focused antibody by autoradiography and hemolysis of antigen-coated erythrocytes. A comparison of methods. *J. Immunol. Methods.* **8:**363.
33. Watanabe, K., S. Hakomori, R. A. Childs, and T. Feizi. 1979. Characterization of a blood group I-active ganglioside. Structural requirements for I and i specificities. *J. Biol. Chem.* **254:**3221.
34. Feizi, T., R. A. Childs, K. Watanabe, and S. Hakomori. 1979. Three types of blood group I specificity among monoclonal anti-I autoantibodies revealed by analogues of a branched erythrocyte glycolipid. *J. Exp. Med.* **149:**975.
35. Niemann, H., K. Watanabe, and S. Hakomori. 1978. Blood group i and I activities of "lacto-N-*nor*hexaosylceramide" and its analogues: the structural requirements for i-specificities. *Biochem. Biophys. Res. Commun.* **81:**1286.