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A Monoclonal Antibody Directed to *N*-Acetylneuraminosyl-α2→6-galactosyl Residue in Gangliosides and Glycoproteins*

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A hybridoma cell line producing a monoclonal antibody directed to *N*-acetylneuraminosyl-α2→6-galactosyl residue has been established. The antibody is IgG2b and reacts only with lacto-series gangliosides as well as with glycoproteins having an *N*-acetylneuraminosyl-α2→6-galactosyl residue, but does not react with gangliosides or glycoproteins having an *N*-acetylneuraminosyl-α2→3- or -α2→4-galactosyl residue. The antibody is useful for detecting the specific carbohydrate chain having this terminal structure by immunostaining of glycolipids separated on thin layer chromatography or glycoproteins separated on gel electrophoresis after blotting on nitrocellulose sheet. A remarkable accumulation of a few gangliosides having this terminal structure has been detected by this monoclonal antibody in some human cancer.

Monoclonal antibodies directed to defined carbohydrate structures have been isolated and have proven to be useful probes in determination of the carbohydrate profile at the cell surface and expedient reagents in determination of carbohydrate structures (1–9). This approach has been conveniently applied for analysis of antigen profiles, in combination with the immunostaining of glycolipids separated on thin layer chromatography (10) and glycoproteins separated on gel electrophoresis followed by blotting on nitrocellulose sheet (11).

Recently, a new type of lacto-series ganglioside having a sialosyl-α2→6-galactosyl terminus was isolated and characterized from human erythrocytes (12) and human meconium (13). Two gangliosides having this terminal structure, sialosyl-lacto-*neo*tetraosylceramide and sialosyl-lacto-*nor*hexaosylceramide, were isolated and characterized as minor components of human erythrocytes (12). The former ganglioside was found to be the major component of human meconium (13). Glycoproteins having the carbohydrate chain with the terminal sialosyl-α2→6-galactosyl residue have been found to be widely distributed (see for a review Ref. 14), particularly in blood plasma glycoproteins (*e.g.* Refs. 15–17). The present paper describes establishment of a hybridoma secreting the IgG2b antibody, which reacts specifically with the sialosyl-α2→6-galactosyl structure in gangliosides as well as in glycoproteins, and the application of this antibody to detect this structure at nanogram order.

MATERIALS AND METHODS[1]

Mouse IgM and IgG subclasses were obtained from commercial suppliers (Bionetics Laboratory Products, Kensington, MD 20795) as previously described (1). Glycolipids from human erythrocytes and human adenocarcinoma tissue were extracted with isopropanol-hexane-water (55:25:20, v/v/v) (9) and partitioned according to Folch-Pi et al. (18). The ganglioside fraction was prepared by DEAE-Sephadex according to the method of Yu and Ledeen (19). The gangliosides eluted from the column were dialyzed in a Spectropore dialysis tube (Spectrum Medical Industries, 60916 Terminal Annex, Los Angeles, CA 90054) and lyophilized. The gangliosides were purified on high performance low pressure, as well as high pressure, liquid chromatography on porous silica gel columns (Iatrobeads 6RS8060 and 6RS8010) (Varian model 502) as described previously (9,20). Gradient elution was performed with an isopropanol-hexane-water system originally described by Watanabe and Arao (21). Sialosylα2-3 and sialosylα2-6 lacto-*neo*tetraosylceramide were prepared from human erythrocytes and from human adenocarcinoma. Sialosylα2-3 and sialosylα2-6 lacto-*nor*hexaosylceramide were prepared from human erythrocytes (12).

Sialosylα2-6 lacto-*neo*tetraosylceramide isolated from human cancer (metastatic liver tumor from colon) was used as immunogen for production of hybridoma antibodies. The structure of the glycolipid antigen was verified by methylation analysis. Balb/c mice were immunized with sialosylα2-6 lacto-*neo*tetraosylceramide, adsorbed to naked *Salmonella minnesota* by the procedure of Galanos et al. (22). Mice were immunized intravenously through tail veins. The first 5 injections contained 25 µg of glycolipid and 125 µg of the bacteria suspended in sterile saline solution, and the sixth injection contained 50 µg of glycolipid with 240 µg of bacteria. The first five injections were made on day 0, 4, 11, 16, and 25, and the final booster injection was made on day 137. Two days after the last injection, spleen was harvested and cell fusion was made according to the procedure of Köhler and Milstein using 38% polyethylene glycol and mouse myeloma NS/1 cells (23,24). Addition of thymocytes as feeder cells and selection of the hybridoma were performed as previously described (1,24). Cloning of the hybridoma and assay procedure of the antibody were the same as previously described (1), except that the glycolipids were coated onto 96-well Dynatech plates (Immunolon II) by evaporation technique. Briefly, 5 µg of glycolipids, 5 µg of lecithin, and 3 µg of cholesterol were dissolved in 1 ml of absolute ethanol. 20 µl of this mixture were placed in each well of the Dynatech Immunolon II plates and allowed to dry at room temperature. The plates were blocked with 0.25% gelatin in phosphate-buffered saline overnight. The reactivity of the glycolipids on the plates after blocking with gelatin was determined by the second antibody and I^{125}-labeled protein A (1). Immunostaining of gangliosides on HPTLC was performed according to a modified procedure (25) originally described by Magnani et al. (10).

Fibronectin was prepared from human and bovine plasma by affinity chromatography on a gelatin-Sepharose column (26). Glycophorin A was prepared from human erythrocyte membranes (27) and was donated by Dr. William Carter. Bovine submaxillary mucin was purchased from Sigma Chemical Co. (St. Louis, MO).

The reactivity of these glycoproteins with the antibody was determined by solid phase radioimmunoassay as previously described (1), and the protocol is described in the legend for Fig. 2. Glycoproteins separated on gel electrophoresis were immunostained after blotting on nitrocellulose sheet (11).

RESULTS

Production of the Hybridoma—Fusion of the NS/1 myeloma with spleen cells of mice immunized against sialosyl-α2→6-lacto-*neo*tetraosylceramide yielded about 3% positive clones by the first assay after the fusion. Two clones, IB2 and IB9, were isolated which appeared to have the same reactivity, specifically to sialosyl-α2→6-lacto-*neo*tetraosylceramide, but showed no reactivity to sialosyl-α2→3-lacto-*neo*tetraosylceramide. They were derived from the same well (IIIC8) of the original fusion plate and were derivatives of the same clone. Both hybridomas, IB2 and IB9, can be propagated in BALB/c mice as ascites form, producing high titer antibody. Antibodies from these hybridomas were identified as IgG2b class, and were successfully propagated as ascites form in "pristane"-treated BALB/c mice.

Specificity of the Antibody—Antibodies produced by both hybridomas, IB2 and IB9, showed identical specificity, and the supernatant fluid of the hybridomas showed the same specificity as ascites. The results described in this paper are those with the IB9 antibody. The reactivity of the antibody with various gangliosides and glycoproteins is shown in Figs. 1 and 2, respectively. It is clear that only lacto-series gangliosides containing the sialosyl-α2→6-galactosyl residue showed a specific reactivity. All other gangliosides failed to react with

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[1] "Materials and Methods" are presented in miniprint as prepared by the authors. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 83M-1087, cite the authors, and include a check or money order for $1.00 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

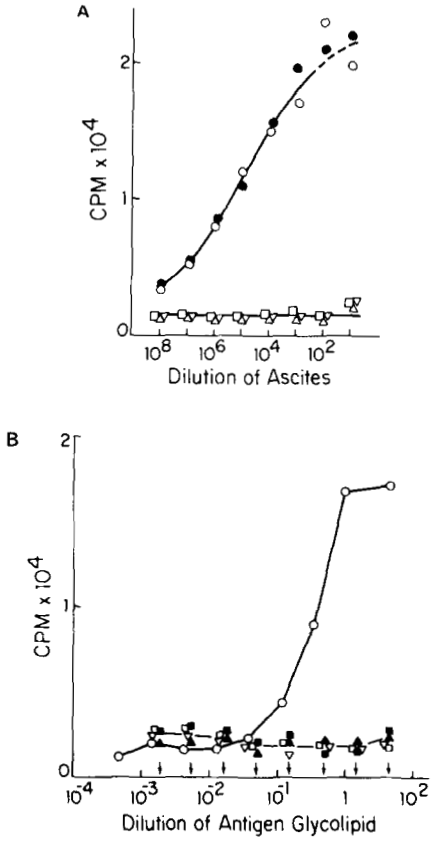

FIG. 1. **Reactivity of various gangliosides with the monoclonal antibody IB9 determined by solid phase radioimmunoassay.** *A* shows the reactivity of various gangliosides using 100 ng/well with lecithin and cholesterol (see text). The activity was determined with various dilutions of hybridoma ascites. ○, sialosyl-α2→6-lacto-*neo*tetraosylceramide; ●, sialosyl-α2→6-lacto-*nor*hexaosylceramide; △, sialosyl-α2→3-lacto-*neo*tetraosylceramide; □, G_{M3} ganglioside; ▽, G_{M1} ganglioside. *B* shows the reactivity of various gangliosides with different concentrations by solid phase radioimmunoassay. The original concentration (100 ng/well) was diluted and coated on the plate with cholesterol and lecithin and determined with 100× diluted ascites of IB9 hybridoma. ○, sialosyl-α2→6-lacto-*neo*-tetraosylceramide; □, lacto-*neo*tetraosylceramide; ▽, G_{M3} ganglioside; ■, G_{M1} ganglioside; ▲, cholesterol and lecithin without ganglioside.

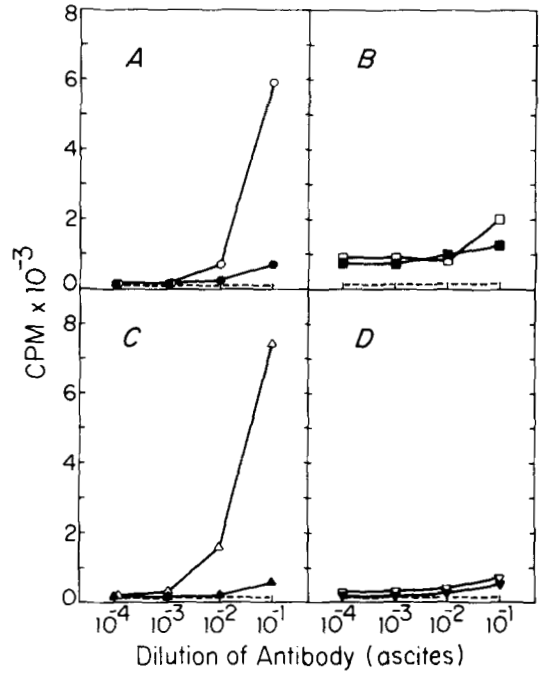

FIG. 2. **Reactivity of glycoproteins in solid phase radioimmunoassay with the monoclonal antibody IB9.** The glycoprotein solutions were incubated in 96-well Dynatech plates overnight and analyzed according to the procedure as previously described (1). The concentration of glycoprotein for primary coating on Dynatech plates was as follows: 0.1 mg/ml of human plasma fibronectin, bovine plasma fibronectin, and bovine submaxillary mucin; 0.25 mg/ml of human erythrocyte glycophorin. Bovine serum albumin (0.5 mg/ml) was used as a background coat. *A*, human plasma fibronectin; *B*, bovine plasma fibronectin; *C*, bovine submaxillary mucin; *D*, human erythrocyte glycophorin. ○, □, △, and ▽, intact molecules; ●, ■, ▲, and ▼, glycoproteins treated with *V. cholerae* sialidase; - - -, background value with bovine serum albumin.

the antibodies present in the supernatant or in ascites. Human plasma fibronectin was shown to have an *N*-acetylneuraminosyl-α2→6-galactosyl residue (17), and gave a positive reaction with this antibody (Fig. 2*A*). In contrast, the majority of the sialosyl residue in bovine plasma fibronectin is linked through the α2→4Gal structure and only a minority is linked through the α2→6Gal structure (25). This reflects a low reactivity of bovine fibronectin with this antibody (Fig. 2*B*). Bovine submaxillary mucin, which has the NeuAcα2→6GalNAc structure (14), showed a strong reactivity with this antibody (Fig. 2*C*). However, human erythrocyte glycophorin (27), which has a major oligosaccharide chain with the structure NeuAcα2→3Galβ1→3(NeuAcα2→6)GalNAc (28), did not show any appreciable reaction with this antibody (Fig. 2*D*), although glycophorin has one asparagine-linked complex-type oliogasaccharide containing the NeuAcα2→6Gal residue as a minor component (29). The reactivity of human plasma fibronectin and bovine submaxillary mucin to this antibody was abolished by treatment with *Vibrio cholerae* sialidase (see Fig. 2, *A* and *C*). The weak reactivity of bovine plasma fibronectin to this antibody was slightly reduced by sialidase treatment. These results clearly indicate that the antibody recognizes NeuAcα2→6Gal and NeuAcα2→6GalNAc at the terminus. The latter structure at the internal residue, such as found in glycophorin (28), was not reactive. Other types of sialosyl linkages in various glycoproteins, *i.e.* α2→3Gal, α2→4Gal, and α2→6GlcNAc, were not reactive to this antibody.

Determination of Gangliosides and Glycoprotein Profiles Having NeuAcα2→6Gal Residue—Ganglio-series and lacto-series gangliosides having the NeuAcα2→6Gal residue were separated on high performance thin layer chromatography and immunostained as shown in Fig. 3. None of the ganglio-series gangliosides were stained (Fig. 3, *Lane 5*). A doublet corresponding to sialosyl-α2→6-lacto-*neo*tetraosylceramide (*band h*) and a band corresponding to a sialosyl-α2→6-lacto-*nor*hexaosylceramide (*band i*) were stained (*Lane 6*). In addition, a slower migrating band was detected which has not been characterized (*Lane 6*). This component could be contamination present in the reference sample of G_9[2] ganglioside (30). A ganglioside fraction isolated from two cases of human cancer (Fig. 3, *Lanes 3* and *4*) and their immunostaining pattern (*Lanes 7* and *8*) are also shown. G_{M3} was the major component in both cases (*Lanes 3 and 4*); however, a glycolipid showing a doublet corresponding to sialosyl-α2→6-lacto-*neo*-tetraosylceramide (a doublet in *Lane 3* corresponding to *band h* in *Lane 2*) was found as the second major component in one

[2] The abbreviations used are: G_9, Fucα1→2Galβ1→4GlcNAcβ1→6[NeuAcα2→3Galβ1→4GlcNAcβ1→3]Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→1Cer (other assignments for ganglio-series gangliosides are according to Svennerholm (33)); HPTLC, high performance thin layer chromatography.

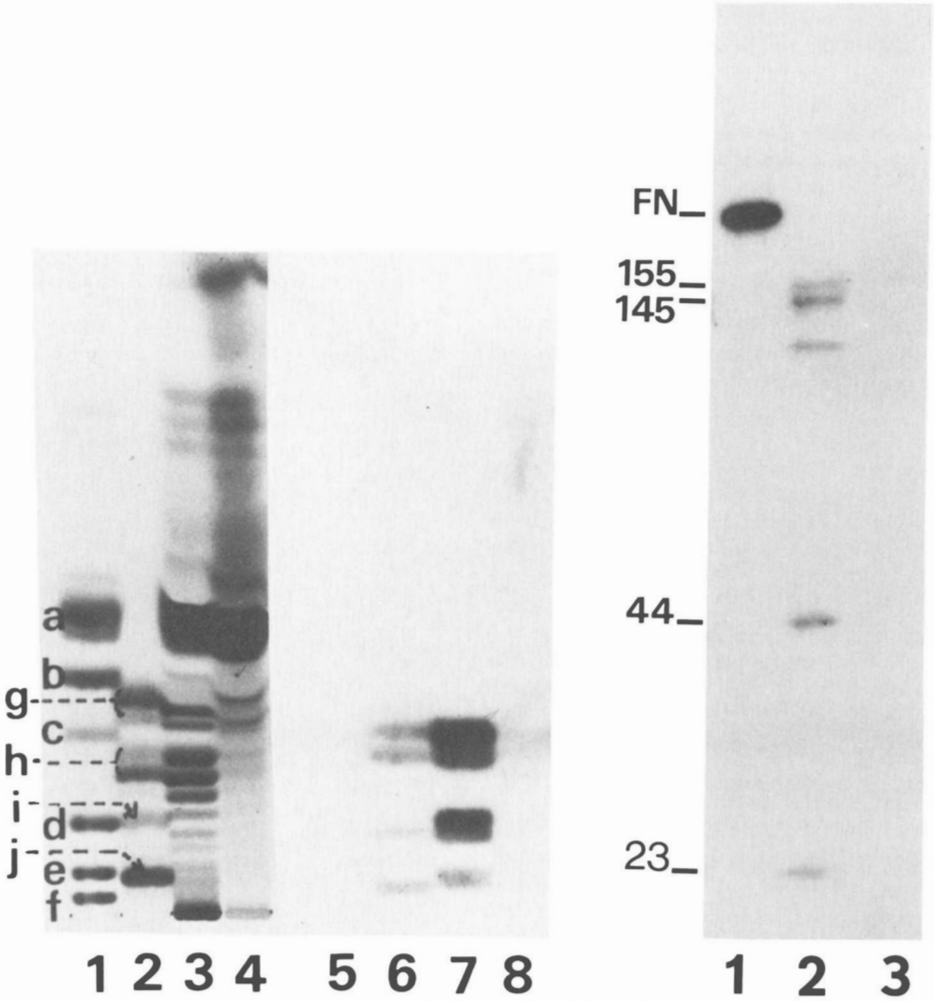

FIG. 3 (*left*). **Immunostaining pattern of various gangliosides with monoclonal antibody IB9.** The various gangliosides were separated on high performance thin layer chromotography plate (J. T. Baker Chemical Co., Phillipsburg, NJ) in a solvent mixture of choloroform/methanol/water (60:35:8, v/v/v). *Lanes 1–4* were revealed by orcinol/sulfuric acid reaction. *Lanes 5–8* are an autoradiogram of the chromatogram developed simultaneously and immunostained with monoclonal antibody IB9. *Lane 1,* a mixture of standard brain gangliosides. *Bands a–f* are, respectively, G_{M3}, G_{M2}, G_{M1}, G_{D1a} G_{D1b}, and G_{T1b} gangliosides. Designation of these brain gangliosides is according to Svennerholm (33). *Lane 2* is a mixture of standard lacto-series gangliosides isolated and previously characterized (12). A doublet *band g* represents sialosyl-α2→3-lacto-*neo*tetraosylceramide (the two bands represent different ceramides); a doublet *band h* represents sialosyl-α2→6-lacto-*neo*tetraosylceramide (the two bands represent different ceramides); *band i,* sialosyl-α2→6 lacto-*nor*hexaosylceramide; *band j,* G_9 ganglioside (a branched fucoganglioside with ceramide decasaccharide structure (30). *Lane 3,* ganglioside fraction of hepatocarcinoma (diagnosed as adenocarcinoma). *Lane 4,* ganglioside fraction of primary lung carcinoma metastatic to liver.

FIG. 4 (*right*). **Immunostaining of fibronectins separated on gel electrophoresis followed by blotting on nitrocellulose sheet.** *Lane 1,* human plasma fibronectin; *Lane 2,* thermolysin digest of human plasma fibronectin (31); *Lane 3,* desialylated human plasma fibronectin by hydrolysis in 1% acetic acid. *Left margin* shows identification of the fragments. *FN,* intact fibronectin.

case (*Lane 3*). This component was immunostained intensely by the antibody (*Lane 7*). In addition, slower migrating bands, including the one corresponding to sialosyl-α2→6-lacto-*nor*-hexaosylceramide and another with slower mobility, were detected as being stained by the antibody (*Lane 7*). This type of large accumulation of gangliosides having the NeuAcα2→6Gal residue was not observed in the other case of human cancer (lung cancer metastatic to liver) shown in *Lanes 4* and *8.* The major gangliosides chemically detectable in this case were G_{M3} and sialosyl-α2→3-lacto-*neo*tetraosylceramide (*Lane 4*), which were not immunostained by this antibody (*Lane 8*). Only a small quantity of sialosyl-α2→6-lacto-*neo*-tetraosylceramide was detected, which was weakly immunostained by this antibody (*Lane 8*).

As an example of glycoproteins having the NeuAcα2→6Gal residue, human plasma fibronectin, its thermolysin digest, and desialylated products were separated on gel electrophoresis and immunostained after blotting on nitrocellulose sheet. An intense band was immunostained for intact fibronectin (Fig. 4, *Lane 1*), and a doublet with M_r = 140,000–150,000, 105,000, 44,000, and 23,000 was immunostained after thermolysin digestion (*Lane 2*) (31). An intense fibronectin stain was completely lost after the sialosyl residue of fibronectin was eliminated (*Lane 3*).

DISCUSSION

Profiles of cell surface carbohydrates have been probed and defined by lectins (32). This approach has been strengthened and partially replaced by monoclonal antibodies directed to defined carbohydrate structures. Another potentially important use of anti-carbohydrate monoclonal antibodies is their application in structural analysis of carbohydrates. The im-

munostaining procedure recently developed on thin layer chromatography (10) and blotting of electrophoretic gels on nitrocellulose sheets (11) is highly sensitive and requires only nanogram quantities of material. Immunostaining of complex carbohydrates by multiple monoclonals in combination with enzymatic hydrolysis will be potentially useful to elucidate the structure of nanogram quantities of carbohydrates, if the necessary number of well defined monclonal antibodies are available. Efforts to gain increasing numbers of well defined monoclonal anti-carbohydrate antibodies are essential to reach such a goal.

The antibody described in this paper defines the terminal residue NeuAcα2→6Gal in glycolipids and glycoproteins and the NeuAcα2→6GalNAc residue in glycoproteins. The antibody does not react with the internal sialosylα2→6GalNAc residue (28) linked to polypeptides since glycophorin, which contains this structure, was not reactive.

The structure NeuAcα2→6Gal (or GalNAc) is known to be widely distributed in a variety of glycoproteins as the terminus of the short *O*-linked oligosaccharide in mucin-type glycoproteins, *i.e.* NeuAcα2→6GalNAcα1→*O*-Ser (Thr), and as the terminus in the side chain of a complex type asparagine-linked structure, *i.e.* NeuAcα2→6Galβ1→4GlcNAcβ1→2Manα1→R (see for a review Ref. 14), although such a structure has been confirmed only relatively recently based on methylation analysis (15–17, 25). Determination of such structures in glycoproteins requires isolation of oligosaccharides or glycopeptides after degradation, and methylation analysis before and after desialylation, and needs at least 0–200 μg of glycopeptides. If glycoproteins contain 3–5% carbohydrates (like fibronectin), a minimum quantity to obtain the information on sialosyl linkage would be at least 2–3 mg of glycoprotein. The results shown in Figs. 3 and 4 required only 100 ng of the glycoprotein. Since neither a ganlioside having *N*-glycolylneuraminosyl-α2→6-galactosyl residue nor a glycoprotein exclusively containing this structure have been isolated, we have had no chance to test the reactivity of the IB9 antibody to this structure.

On the other hand, the NeuAcα2→6Gal structure is rarely found in sialosyl glycolipids (gangliosides), and the structure constitutes the terminus of the type 2 *N*-acetyllactosaminyl chain, *i.e.* NeuAcα2→6Galβ1→4GlcNAcβ1→3Galβ1→R. Gangliosides with this structure have been isolated and characterized only recently (12, 13). Two types of gangliosides have been isolated and characterized from human erythrocytes (12); one is sialosyl-α2→6-lacto-*neo*tetraosylceramide (G_4) and the other is sialosyl-α2→6-lacto-*nor*hexaosylceramide (G_7). The quantity of these gangliosides in human erythrocytes was very low (G_4 and G_7 comprised 1.9 and 1.4% of the total gangliosides of human erythrocytes, respectively) (12). Normal colonic mucosal tissue and normal liver contain similar quantities of these gangliosides. Interestingly, the quantity of gangliosides having this terminal structure was found to be much higher in some human cancers, and sialosyl-α2→6-lacto-*neo*tetraosylceramide is a major ganglioside of some human cancers. The ganglioside composition of only two cases of human cancer are presented in this paper, as shown in Fig. 3. One accumulated sialosyl-α2→6 gangliosides and the other did not. However, we have observed many other cases, and the results of these studies will be published elsewhere. Since sialosyl-α2→6-lacto-*neo*tetraosylceramide is the major ganglioside in meconium (13), but represents only a minor component in various normal tissues, the remarkable accumulation of this ganglioside in human cancer may reflect an oncofetal expression of this ganglioside. The antibody is useful in determining the profile of gangliosides and sialosyl structures in tissues and cells.

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