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Identification of a human neuroectodermal tumor antigen (OFA-I-2) as ganglioside GD2

(monospecific human antitumor antibody/membrane glycolipid/sialic acid)

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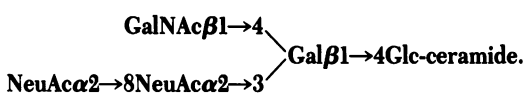
ABSTRACT **Two monospecific human antibodies (anti-OFA-I-1 and anti-OFA-I-2) produced *in vitro* by lymphoblast cell lines originating from melanoma patients have been shown previously to recognize cell surface antigens (OFA-I-1 and OFA-I-2) on human tumors and fetal brain: OFA-I-1 is expressed on a variety of human tumors, while OFA-I-2 has been detected only on tumors of neuroectodermal origin. Evidence presented in this report suggests that the two antigens expressed by a cultured human melanoma cell line (M14) are chemically distinct and that OFA-I-2 is a cell surface glycolipid, ganglioside GD2:**



The fact that some cancer patients produce antibodies that react with antigens on tumor cells is well documented (1–5). This report describes the identification of the chemical nature of a tumor antigen, previously detected by Irie *et al.* by using serum antibodies from melanoma patients (6, 7). The antigen was present on cells of melanomas and other human tumors and in fetal human brain and thus was designated OFA-I for oncofetal antigen (immunogenic) (8). Naturally occurring anti-OFA-I antibodies are cytotoxic to cells that express the antigen (9). Melanoma patients (stages 2 and 3) who have high levels of circulating anti-OFA-I antibodies have a significantly improved survival when compared with patients who lack the antibody (10, 11). Recently, lymphocytes obtained from melanoma patients were transformed by Epstein–Barr virus, yielding two continuous cell lines that produced monospecific antibodies (12, 13). On immunologic grounds, the antibodies from the two lymphoblast cell lines exhibited tissue reactivities similar to the anti-OFA-I antibodies detected previously in serum. However, the two cell lines produced antibody directed against two different antigens designated OFA-I-1 and OFA-I-2. By tissue screening, OFA-I-1, like the previously described OFA-I (8), was found on a variety of histologic types of human cancer cells. OFA-I-2 was detected only on tumors of neuroectodermal origin including melanoma, glioma, and neuroblastoma. In this report, we present evidence that OFA-I-1 and OFA-I-2 are chemically distinguishable, that OFA-I-2 in cultured melanoma cells is a ganglioside, that the anti-OFA-I-2 antibody has a high specificity for the ganglioside GD2 [gangliosides are named according to the nomenclature of Svennerholm (14)], and that the biochemical basis for the expression of OFA-I-2 may be an alteration in ganglioside biosynthesis.

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MATERIALS AND METHODS

Antigen Sources. Two established human melanoma cell lines, UCLA-SO-M14 (M14) and UCLA-SO-M15 (M15), were grown in a chemically defined medium (13). As a source of soluble crude antigen, spent culture medium was centrifuged at 1,000 × *g* for 10 min to remove cell debris and the medium was concentrated 100-fold on an Amicon filter (M_r cutoff, 10,000).

Anti-OFA-I Sources. Monospecific anti-OFA-I-1 and anti-OFA-I-2 antibodies secreted in the spent tissue culture media of human lymphoblastoid cell lines (L55 and L72, respectively) were used as the source of antibody to detect both soluble OFA-I-1 and OFA-I-2 and antigens on membranes of intact M14 cells (12, 13). The antibodies were purified free of bovine immunoglobulin and albumin present in the fetal bovine serum used to supplement the culture media of the cell lines.

Immunologic Studies. The OFA-I antigens on tumor cells were identified by an immune adherence assay as described (6). In this procedure, rosettes are formed when M14 cells are exposed to antibody, guinea pig complement, and human (type O+) erythrocytes. Soluble antigen was assayed by inhibition of immune adherence (6).

Sialidase Treatment of Cells. M14 cells (1×10^6) were suspended in 250 μl of Tris·HCl/saline buffer, pH 7.4, containing 0.6 unit of *Clostridium perfringens* sialidase (type X; Sigma) at 37°C for 2 hr. Control cells were incubated in buffer alone. After reaction, the cells were suspended in 15 ml of cold (4°C) barbital-buffered saline, collected by centrifugation, and washed two more times in the same manner. The cells were then suspended in 1 ml of barbital-buffered saline for use in the immune adherence assay.

Analysis of Gangliosides. Purified human brain ganglioside standards (GM1, GD1a, GD1b, GT1b) and neutral glycosphingolipids were provided by Lars Svennerholm (University of Goteborg, Sweden). GD3 was a gift from John Magnani and Victor Ginsburg (National Institutes of Health, Bethesda, MD). TLC was done on plates precoated with silica gel 60 (0.25-mm layer thickness; Merck, Darmstadt, Federal Republic of Germany). Solvent systems used for chromatography were chloroform/methanol/0.22% $CaCl_2$ (55:45:10) (solvent 1), 1-propanol/0.25% KCl (3:1) (solvent 2), and chloroform/methanol/2.5 M NH_3 in water (60:32:7) (solvent 3). TLC of neutral glycolipids was carried out in chloroform/methanol/water (65:25:4) (solvent 4). Gangliosides were visualized with the resorcinol stain (15) and neutral glycolipids, by the orcinol stain (16). Sialic acid contents were determined after hydrolysis in 50 mM H_2SO_4 at 80°C for 1 hr by the thiobarbituric acid assay (17).

Abbreviations: Gangliosides are named according to the nomenclature of Svennerholm (14); for example, the structure of GT1b is NeuAcα2→3Galβ1→3GalNAcβ1→4(NeuAcα2→8NeuAcα2→3)Galβ1→4Glc-ceramide. Other ganglioside structures are given in Fig. 3. OFA-I, oncofetal antigen (immunogenic).

Extraction of M14 Cell Glycolipids and Purification of M14 Gangliosides. The concentrated culture medium containing soluble OFA-I antigens was partitioned in chloroform/methanol/water (1.0:2.0:1.4) as described by Fredman and Svennerholm (18). The lower phase was extracted once with 0.01 M KCl as described (18). After partitioning, the combined ganglioside-enriched upper phases were evaporated, dialyzed against distilled water for 72 hr, and lyophilized. The lower phase was concentrated by evaporation only. Glycolipids were stored in chloroform/methanol/water (60:30:4.5) at −8°C. Immediately prior to immunologic studies, aliquots of the glycolipids were taken to dryness under N_2 and the residues were suspended in phosphate-buffered saline (0.15 M NaCl/0.01 M sodium phosphate, pH 7.4). Further purification of M14 cell gangliosides was done by preparative TLC in solvent 1. Gangliosides were eluted from the silica by extraction into chloroform/methanol (1:2) overnight at room temperature.

Preparation of GM2 and GD2. These gangliosides were prepared from GM1 and GD1b, respectively, by removing terminal galactose residues with bovine testes β-galactosidase (19), a gift of George W. Jourdian (University of Michigan). Reaction mixtures (1.0 ml) contained GM1 (290 nmol) or GD1b (280 nmol) and 1 mg of delipidized bovine serum albumin (Sigma) in 0.01 M potassium acetate, pH 5.0/1% sodium taurocholate (Sigma) containing 0.3 or 0.4 unit of bovine testes β-galactosidase for digestion of GM1 and GD1b, respectively. The reaction mixtures were incubated at 37°C. As judged by TLC in solvent 1, the reaction was >95% complete after 48 hr for conversion of GM1 to GM2 and in 29 hr for conversion of GD1b to GD2. The reaction mixtures were lyophilized and the gangliosides were desalted on a column containing 1.0 g of Sephadex G-25 and eluted as described by Wells and Dittmer (20). Final purification was carried out by preparative TLC as described above for purification of M14 cell gangliosides.

RESULTS

OFA-I-1 and OFA-I-2 Are Chemically Distinct and OFA-I-2 Is a Ganglioside. To obtain information about the chemical nature of the human tumor antigen OFA-I-2, spent concentrated culture medium from M14 cells was treated with trypsin and a variety of purified glycosidases from *Streptococcus pneumoniae* (21). Only the *S. pneumoniae* sialidase totally abolished antigenicity of OFA-I-2, suggesting that sialic acid was essential for its expression (data not shown). The role of sialic acid in OFA-I-1 and OFA-I-2 antigenicity was further investigated by incubation of M14 cells with *C. perfringens* sialidase immediately prior to testing for rosette formation with anti-OFA-I-1 and anti-

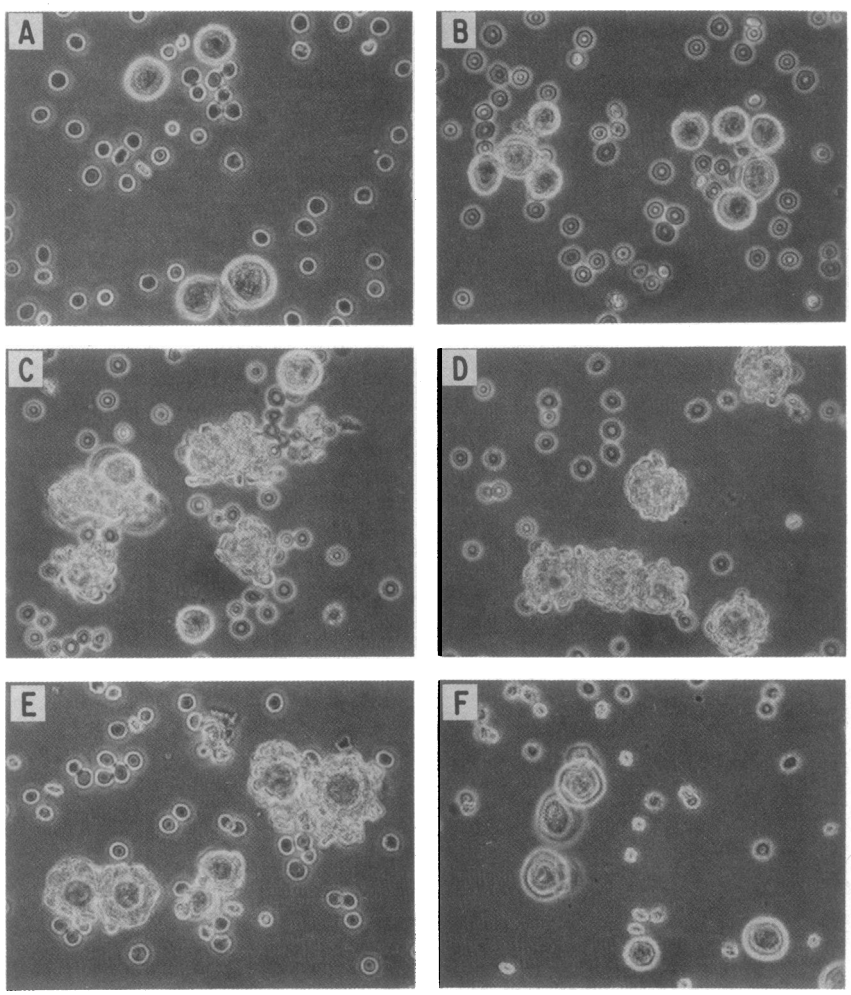


FIG. 1. Differential sensitivity of OFA-I-1 and OFA-I-2 antigens on M14 melanoma cells to treatment by sialidase. Cells were incubated with or without *C. perfringens* sialidase in a Tris·HCl/saline buffer. The presence of OFA-I-1 and OFA-I-2 antigens on the cell surface was monitored by the immune adherence assay using monospecific antibodies to OFA-I-1 and OFA-I-2. In this assay, cells containing antigen–antibody complexes on their cell surface form rosettes with indicator human erythrocytes in the presence of complement. (*A*) Native M14 cells with no antibody. (*B*) Sialidase-treated cells with no antibody. (*C*) Native cells with anti-OFA-I-1. (*D*) Sialidase-treated cells with anti-OFA-I-1. (*E*) Native cells with anti-OFA-I-2. (*F*) Sialidase-treated cells with anti-OFA-I-2.

OFA-I-2 in the immune adherence assay. Phase-contrast photomicrographs of the immune adherence assays for both antibodies on native and sialidase-treated M14 cells are shown in Fig. 1. Since M14 cells contain both OFA-I-1 and OFA-I-2 (13), both antibodies formed rosettes with native cells (Fig. 1 *C* and *E*). However, although anti-OFA-I-1 could still bind to surface antigen and form rosettes on sialidase-treated M14 cells (Fig. 1*D*), anti-OFA-I-2 could not (Fig. 1*F*). These results confirmed that sialic acid is an essential antigenic determinant of OFA-I-2 and provided evidence that OFA-I-1 and OFA-I-2 were chemically distinct.

Cell surface sialic acids occur on both glycoproteins and glycolipids. To determine whether sialyloligosaccharides present on glycoproteins could mimic the antigenic activity of OFA-I-2, a variety of soluble glycoproteins (human glycophorin, bovine prothrombin, bovine fetuin, ovine submaxillary mucin, and human α_1-acid glycoprotein) were tested for antigenic activity by the immune-adherence-inhibition assay. None exhibited OFA-I-2 antigenicity (data not shown). To investigate the possibility that OFA-I-2 was a ganglioside (a glycolipid containing sialic acid), concentrated culture medium from M14 cells, which contains both OFA-I-1 and OFA-I-2 (13), was partitioned in chloroform/methanol/water into a ganglioside-enriched upper phase and a lower phase containing mainly neutral glycolipids and phospholipids (18). This procedure minimizes the extraction of amphipathic glycoproteins reported to contaminate ganglioside preparations (22, 23). Both fractions were tested for the presence of OFA-I-1 and OFA-I-2. The results (not shown) showed that OFA-I-2 was extracted into the upper phase while OFA-I-1 was detected in both the upper and lower phases. Thus, it appeared likely that OFA-I-2 was a ganglioside. Although the identity of OFA-I-1 is not considered further in this report, preliminary evidence suggests that it is a glycolipid.

Characterization of OFA-I-2 as a Disialylganglioside. The upper-phase ganglioside fraction obtained from the culture fluids of M14 and M15 melanoma cell lines, which, respectively, express and do not express OFA-I-2, were compared by TLC (Fig. 2). Major differences were observed. Although the gangliosides from M15 cells had prominent bands corresponding to the major naturally occurring brain gangliosides (GM1, GD1a, GD1b, and GT1b), the M14 cells lacked these gangliosides. Moreover, gangliosides from the M15 cells appeared to migrate as single bands while those in M14 cells appeared as doublets.

Antigenic activity of the M14 gangliosides was also assessed from a duplicate sample in an adjacent lane before staining with resorcinol. As detected by inhibition of anti-OFA-I-2-mediated immune adherence, all antigenic activity (OFA-I-2) comigrated with the major staining doublet (Fig. 2). Virtually identical results were obtained in two solvent systems (nos. 1 and 2). Both bands in the doublet were verified to exhibit antigenic activity after purification and separation by preparative TLC.

The sialic acid composition of the purified OFA-I-2 ganglioside was investigated by graded sialidase treatment and analysis of the products by TLC. The results showed that each separated band of the doublet was converted to a faster migrating band with no evidence of any other kinetic intermediates. This is shown in Fig. 3, where the lower bands (lanes A, B, and C) and the upper bands (lanes E, F, and G) were each treated with a high dose and a low dose of *Vibrio cholera* sialidase. By comparison with the total M14 gangliosides (lane D), the two bands of the antigenic doublet (centered at ganglioside standard GD2) were converted to the corresponding upper and lower bands of another M14 ganglioside doublet centered around ganglioside standard GM2. Complete removal of sialic acid from the purified OFA-I-2 doublet by mild formic acid hydrolysis (1 M

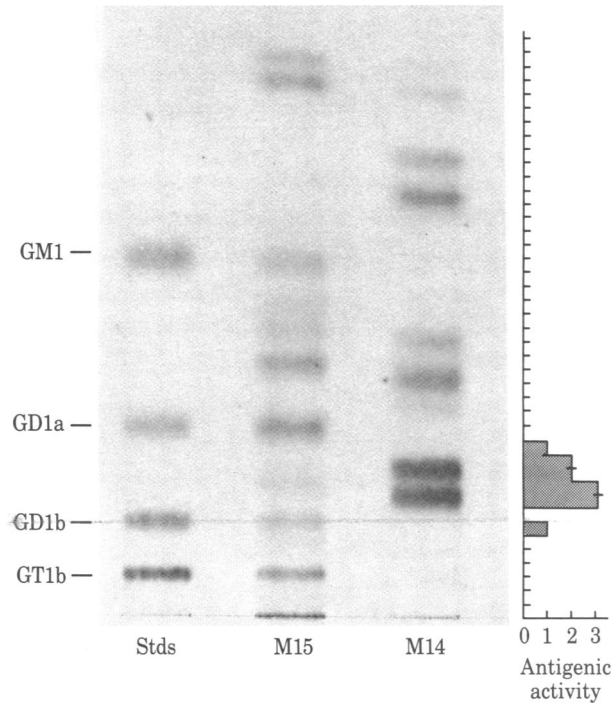


FIG. 2. Identification of a ganglioside from human melanoma M14 cells as OFA-I-2. Gangliosides were extracted from the concentrated cell culture fluid of the M14 and M15 melanoma cell lines. Selected ganglioside standards (Stds), gangliosides from M15 cells, which do not contain OFA-I-2, and gangliosides from M14 cells were resolved by TLC in solvent 1. One of two lanes containing duplicate samples of M14 cell gangliosides was sectioned and assessed for OFA-I-2 by inhibition of immune adherence. Ganglioside bands in the remaining lanes were then visualized by resorcinol staining (15). A semiquantitative representation of the immunologic data is shown on the right: 0, no inhibition detected; 1, 25% inhibition; 2, 50% inhibition; 3, complete inhibition of the immune adherence assay.

formic acid at 100°C for 30 min) converted the ganglioside to a major neutral glycolipid that corresponded on TLC in solvent 4 with authentic gangliotriosylceramide (GalNAcβ1→4Galβ1→4Glc-ceramide; data not shown). Because *V. cholera* sialidase is unable to cleave the sialic acid from a branched galactose (24), these results are consistent with both antigenic bands being the disialylganglioside GD2 [GalNAcβ1→4(NeuAcα2→8NeuAcα2→3)Galβ1→4Glc-ceramide] with sialidase treatment yielding the partially desialylated derivative GM2 [GalNAcβ1→4(NeuAcα2→3)Galβ1→4Glc-ceramide]. Although the chromatographic results obtained might also be obtained if OFA-I-2 were an amphipathic sialoglycoprotein that comigrated on TLC with GD2, this seems unlikely.

The structural heterogeneity giving rise to the OFA-I-2 doublet has not yet been identified. Neither band appeared to contain fucose as evidenced by their insensitivity to digestion by α-fucosidase (Boehringer Mannheim) and their more rapid migration relative to GM1 on TLC in an alkaline solvent (solvent 3) compared with neutral solvents (solvents 1 and 2) (25). Only *N*-acetylneuraminic acid was detected in the OFA-I-2 doublet by TLC (26) of the sialic acids released by mild acid hydrolysis (pH 2 at 80°C for 1 hr). At present, the chromatographic doublet is presumed to be due to the ceramide moiety, but this remains to be established.

Anti-OFA-I-2 Antibody Has a High Specificity for Ganglioside GD2. Since the OFA-I-2 antigen from M14 cells appeared to be a ganglioside, the specificity of the anti-OFA-I-2 antibody toward a variety of purified gangliosides of known structure was

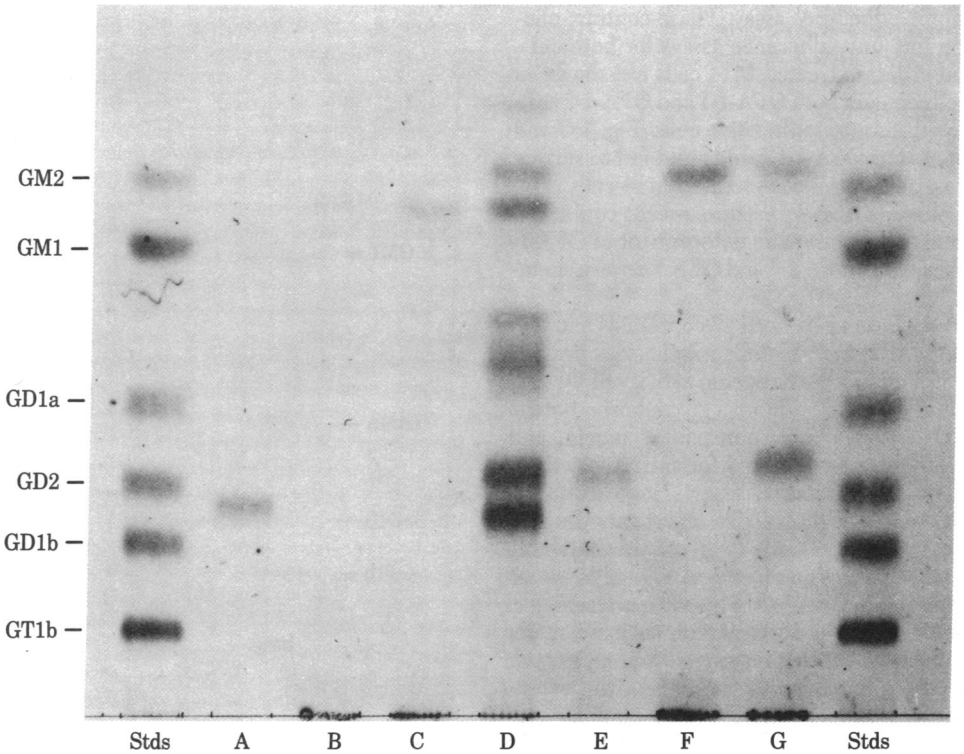


FIG. 3. Graded sialidase treatment of OFA-I-2 ganglioside. The ganglioside doublet recognized as an antigen by anti-OFA-I-2 was isolated by preparative TLC into two single bands. Samples of each band containing approximately 20 nmol of sialic acid were incubated with and without *V. cholera* sialidase (GIBCO) for 2 hr at 37°C in 55 μl of 0.01 M sodium cacodylate (pH 6.5). Samples were then evaporated under N_2, suspended in 10 μl of chloroform/methanol/water (60:30:4.5), subjected to TLC in solvent 1, and stained with resorcinol (15). Results obtained for the lower band are shown in lanes A–C and those for the upper band are shown in lanes E–G. Samples were incubated with no sialidase (lanes A and E) or with 5 units (lanes B and F) or 0.5 units (lanes C and G) of *V. cholera* sialidase. Ganglioside standards (Stds) and total M14 cell gangliosides (lane D) are shown for comparison.

investigated. Purified gangliosides were compared at equal molar concentrations with the purified OFA-I-2 ganglioside for their ability to bind anti-OFA-I-2 antibody by the immune-adherence-inhibition assay. The data show that, of the series of gangliosides tested, only GD2 and the OFA-I-2 ganglioside had antigenic activity (Fig. 4; the carbohydrate structure of each ganglioside is shown symbolically). An exquisite specificity was exhibited by the anti-OFA-I-2 antibody. Gangliosides that differ by the addition of one galactose (GD1b) or the loss of one sialic acid (GM2) or *N*-acetylgalactosamine (GD3) residue were completely devoid of antigenicity.

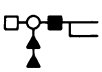
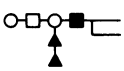
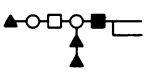
DISCUSSION

Evidence presented here suggests that OFA-I-2 (13), a membrane antigen associated with human tumors of neuroectodermal origin, is the ganglioside GD2 [GalNAcβ1→4(NeuAcα2→8NeuAcα2→3)Galβ1→4Glc-ceramide]. This conclusion is based on both the strict specificity of the human monospecific anti-OFA-I-2 antibody for binding authentic GD2 (Fig. 4) and the chromatographic characterization of the purified OFA-I-2 antigen before and after graded sialidase treatment (Figs. 2 and 3). These results further show that the OFA-I-1 and OFA-I-2 antigens are chemically distinct, as suggested earlier based on immunological evidence (13).

Other tumor-associated antigens detected with mouse monoclonal antibodies have recently been identified as gangliosides (27–30). Magnani *et al.* (27, 28) showed a colon carcinoma antigen to be an unusual fucosylated ganglioside [NeuAcα2→3Galβ1→3(Fucα1→4)GlcNAcβ1→3Galβ1→4Glc-ceramide]. Pukel *et al.* (29) found that a mouse monoclonal antibody produced against cultured human melanoma cells specifically binds to ganglioside GD3 (NeuAcα2→8NeuAcα2→3Galβ1→4Glc-ceramide). Ganglioside GD3 has been found in all melanoma tumors and cell lines examined (29, 31) and is presumably present in the M14 cell gangliosides migrating as a doublet between GM1 and GD1 (Fig. 3). To date, antibodies to these gangliosides have not been identified in cancer patients.

Ganglioside GD2 is found both on the surface of tumor cells and in fetal brain (unpublished data). However, it is also a biosynthetic intermediate of the abundant brain ganglioside GD1b and GT1b (32, 33) and is found as a minor ganglioside (1% to 2%) in normal adult brain (34). Yet GD2 appears to be immunogenic in man as evidenced by the production of the anti-OFA-I-2 antibody by a human lymphoblast cell line (13).

Alterations in glycosphingolipid biosynthesis are well known to be associated with transformation of cells (reviewed in refs. 35 and 36). In this regard, M14 melanoma cells, which express OFA-I-2, appear to contain large amounts of gangliosides GD2 and GM2, which contain the neutral trisaccharide core GalNAcβ1→4Galβ1→4Glc, and are totally deficient in the common gangliosides (GM1, GD1a, GD1b, GT1b), which contain the neutral tetrasaccharide core Galβ1→3GalNAcβ1→4Galβ1→4Glc. In addition, human melanomas have been reported to be rich in GM3 and GD3 and to have lesser amounts of GM2 and GD2 (29, 31). A biosynthetic alteration that would account for the observed ganglioside composition of M14 cells is a deficiency of the galactosyltransferase that converts GM2 to GM1 and GD2 to GD1b. The loss of this galactosyltransferase has been reported on transformation of BALB/c 3T3 cells and hepatocytes with Kirsten sarcoma virus, x-irradiation, and chemical carcinogens (37, 38). In each case, the predominant gan-

Ganglioside	Structure	Antigen Titer
OFA-I_2 Ganglioside		128
GM3		0
GD3		0
GM2		0
GD2		256
GM1		0
GD1a		0
GD1b		0
GT1b		0

Key: SA=▲, Gal=○, GalNAc=□, Glc=■, Ceramide=

FIG. 4. Antigenic specificity of the anti-OFA-I-2 antibody. Gangliosides (6 nmol) were serially diluted in phosphate-buffered saline and tested for inhibition of anti-OFA-I-2 binding to M14 cells in the immune adherence assay. Antigen titer is given as the reciprocal of the dilution that gave 50% inhibition of rosette formation (0, <2) (6).

glioside produced was GM2 instead of GM1 and GD1a in the nontransformed cells. Thus, the alteration in ganglioside metabolism for M14 melanoma cells may parallel that described in earlier reports, with the important additional consequence that the predominant ganglioside produced, GD2, is immunogenic in man.

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