

A Mouse IgG₁ Monoclonal Antibody Specific for *N*-Glycolyl GM3 Ganglioside Recognized Breast and Melanoma Tumors

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ABSTRACT

14F7 murine monoclonal antibody (MAb) is an IgG1 immunoglobulin that is generated by immunizing Balb/c mice with GM3(NeuGc) ganglioside hydrophobically conjugated with human very-low-density lipoproteins and in the presence of Freund's adjuvants. 14F7 MAb binds specifically to GM3(NeuGc), whereas neither *N*-glycolyl or *N*-acetyl gangliosides, nor a sulfated glycolipid, are recognized as assessed by enzyme-linked immunosorbent assay or immunostaining on thin layer chromatograms. Immunohistochemical studies in fresh tumor tissues showed that 14F7 MAb strongly recognized in antigen expressed in human breast and melanoma tumors.

INTRODUCTION

GANGLIOSIDES are sialic acid-containing glycosphingolipids that are present in the plasma membranes of vertebrates.⁽¹⁾ Some of these molecules have been reported as tumor-associated antigens or tumor markers,⁽²⁾ leading to the use of anti-ganglioside monoclonal antibodies (MAbs) in the diagnosis and therapy of cancer.^(3,4) *N*-acetyl (NeuAc) and *N*-glycolyl (NeuGc) neuraminic acids are the most common types of sialic acids found in animals.⁽⁵⁾ In general, the NeuGc residue is not expressed in human and chicken normal tissues, but is widely present in other vertebrate.^(6,7) In contrast, it has been reported that Anti-NeuGc-containing ganglioside antibodies^(8,9) recognize some human tumors and cancerous cell lines. These studies have been carried out using polyclonal or monoclonal antibodies of chicken,⁽¹⁰⁾ human,⁽¹¹⁾ or murine⁽¹²⁾ origin, but in all these tumors minimal levels of NeuGc were reported. On the other hand, recently, we found increased levels of GM3(NeuGc) ganglioside in human breast cancer,⁽¹³⁾ a finding that certainly makes this molecule an attractive target for cancer therapy.

Several MAbs recognizing NeuGc-containing gangliosides showing different binding specificity have been obtained, while some of them react with an epitope shared by more than one of these gangliosides, as GMR8 or GMR3,⁽¹⁴⁾ YK3,⁽¹⁵⁾ and P3,^(16,17) other MAbs are highly specific against a particular ganglioside, as Y-2-HD1,⁽¹⁸⁾ MK-2-34,⁽¹⁹⁾ or SHS1.⁽²⁰⁾ A common characteristic of all these MAbs is that they are IgM antibodies.

Here, we describe for the first time the generation and characterization of an IgG1 highly specific anti-GM3(NeuGc) MAb that has been designated as 14F7. This MAb was generated by immunization of mice with a vaccine formulation containing GM3(NeuGc) hydrophobically conjugated with human very-low-density lipoproteins (VLDL).⁽²¹⁾ Noteworthy is the fact that a preliminary immunohistochemical study with 14F7 MAb showed a strong recognition of breast and melanoma tumor tissues.

MATERIALS AND METHODS

Animals

Six- to 8-week-old female Balb/c mice were purchased from CENPALAB, Havana, Cuba.

Glycolipids

GM3(NeuAc) and GM3(NeuGc) were obtained from dog and horse erythrocytes using a modification of the Folch method.⁽²²⁾ GM1, GM2, GD1a, GD1b, and GT1b were purified from bovine brain,⁽²³⁾ and GD3 was purchased from Sigma. GM2(NeuGc) was obtained from the liver of Balb/c mice,⁽²⁴⁾ and (NeuGc-NeuGc)GD3, from bear erythrocytes, was kindly supplied by Y. Hashimoto and S. Suzuki (Tokyo Metropolitan Institute of Medical Science, Japan). S. Sonnino (Milan University, Italy) and J. Portoukalian (Faculty of Medicine Lyon-

Sud, France) kindly supplied ISO₃-Gal-Cer and IV³NeuGc α -nLc₄Cer, respectively.

Generation of Mabs

Balb/c mice were immunized intramuscularly four times, at 2-weeks interval, with 200 μ g of GM3(NeuGc) coupled hydrophobically with human VLDL and emulsified for the first injection in complete Freund's adjuvant and incomplete Freund's adjuvant in the subsequent doses. Three days after the last boost, the animals were sacrificed and the spleen cells were harvested and fused with mouse myeloma cell line P3X63Ag653, using polyethylenglycol 3,000–3,600 (Sigma, St. Louis, MO). The antibodies produced by the hybridoma were tested by ELISA for their binding to GM3(NeuGc) and GM3(NeuAc), as described below. The selected hybridomas were cloned twice by the limiting dilution method.

Enzyme-linked immunosorbent assay (ELISA)

ELISA experiments using gangliosides were carried out as previously described.⁽²⁵⁾ Briefly, gangliosides (200 ng/well) in 50 μ L of methanol were dried in 96-well flexible polyvinyl chloride-activated microtiter plates (ICN-Flow Laboratories, Oxfordshire, U.K.) for 90 min at 37°C. Then, the plates were blocked with 1% bovine serum albumin (BSA) in 0.05 M Tris HCl buffer pH 7.8 for 30 min at 37°C. Serum samples or hybridoma supernatants were incubated for 2 h at 37°C. After washing with phosphate-buffered saline (PBS), the second antibody, consisting of alkaline phosphatase conjugated goat anti mouse IgM+IgG (Jackson Immunoresearch Laboratories, Inc, West Grove, PA) diluted 1:5000, was added and incubated for 1 h at 37°C. Afterwards, the plates were washed again and a substrate solution of 1 mg/mL of p-nitrophenylphosphate (Sigma) in diethanolamin buffer pH 9.8 was added. After 30 min, the absorbance was measured at 405 nm with an ELISA reader (Organon Teknika, Salzburg, Austria).

Isotype and subclass determination

Mab isotype was determined by ELISA using biotinylated goat anti-mouse IgM (μ chain specific) and goat anti-mouse IgG (δ chain specific); (Jackson Immunoresearch Laboratories), and, for subclass analysis, biotinylated MAbs specific for mouse IgG1, IgG2a, IgG2b, and IgG3, (PharMingen, San Diego, CA) were used.

Thin layer chromatography

HPTLC-aluminium sheets (Merck, Darmstadt, Germany) were used for the glycolipids fractionation. The solvent system used for chromatographic development chromatography was chloroform/methanol/0.25% KCL and 2.5 M NH₃ (5:4:1) (v:v). TLC plates were stained either with orcinol (neutral glycolipids) or resorcinol reagent⁽²⁶⁾ (gangliosides). Additionally, sulfated glycolipid was visualized by the Azure A method.⁽²⁷⁾

Enzyme immunostaining on HPTLC plates

Immunostaining on HPTLC plates was performed as previously reported.⁽²⁸⁾ Briefly, after chromatography of glycolipids, the plates were soaked for 75 s on hexane containing 0.1% poly-

isobutylmethacrylate (Aldrich Chemical Company Inc., Milwaukee, WI). After drying, the plates were incubated with PBS containing 1% BSA for 30 min. Then, plates were incubated with 14F7 Mab solution for 1 h at room temperature. After washing with PBS, the plates were incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (Jackson Immunoresearch Laboratories) for 1 h at room temperature. The plates were washed again and incubated with the substrate solution consisting of 40 μ g/mL o-phenyldiamin (Sigma) in 80 mM citrate phosphate buffer, pH 5.0, containing 0.12% H₂O₂. Dipping the plates in PBS stopped the reaction.

Immunohistochemical technique

Normal adult tissues were obtained at autopsy, from trauma victims, within a few hours after death. Tumor samples were taken at surgery, frozen in liquid nitrogen, and stored at -70°C. Immunostaining of cryostat sections was performed by the avidin-biotin-peroxidase complex method as previously described.⁽²⁹⁾ Staining of all tissues was performed with the 3,3 di-Amino Bencidine (Sigma) and H₂O₂ peroxidase substrate, except for melanoma tissues, which were stained with the 3-amino-9-ethyl-carbazole (Sigma) and H₂O₂ peroxidase substrates.

RESULTS

Specificity of Mab 14F7 against different glycolipids

One mouse showing the highest IgG antibody titer against GM3(NeuGc) was selected for the fusion experiment. A total of 1536 culture supernatants from growing hybridomas were screened by ELISA against GM3(NeuGc) and GM3(NeuAc), and only 5.5% of the supernatants contained specific antibody.

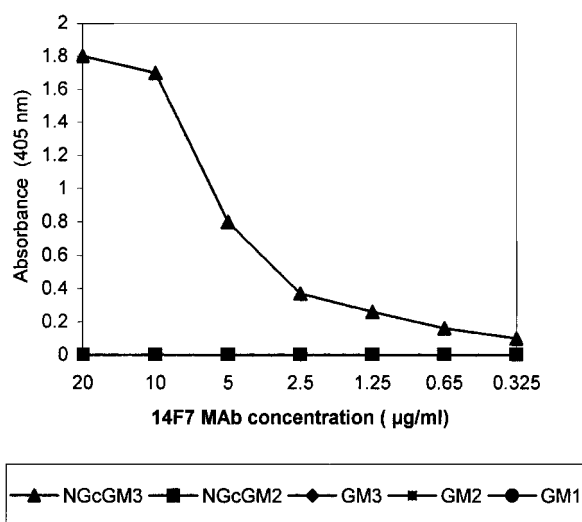


FIG. 1. Specificity of 14F7 Mab against different purified gangliosides by ELISA. Each well of a 96-well plate was coated with 200 ng of gangliosides of 50 μ L in methanol. Reactivity was determined with serial dilutions of the purified antibody.

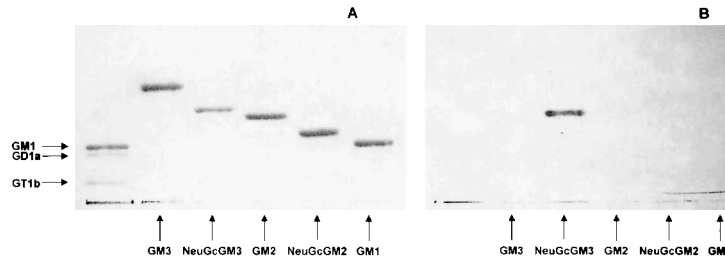


FIG. 2. TLC immunostaining of standard monosialogangliosides with 14F7 MAb. Standard monosialogangliosides were developed in chloroform: methanol: 0.25% KCL in 2.5 M ammonium solution (A,B). Resorcinol staining (A); and immunostaining with 14F7 MAb (B).

ies to GM3(NeuGc) ganglioside and one hybridoma designated as 14F7 was selected and cloned. The MAb produced by this hybridoma belongs to the IgG1 subclass.

Specificity test of 14F7 MAb against several gangliosides, performed by ELISA, showed strong and exclusive reactivity with GM3(NeuGc). No reactivity was observed with the other monosialogangliosides tested (GM3, GM2, GM1 and GM2(NeuGc)); (Fig. 1). Antibody binding to different purified glycolipids separated on HPTLC plates were also tested (Fig. 2). 14F7 MAb reacted with GM3(NeuGc) ganglioside but not with other *N*-glycolylated gangliosides tested, such as GM2(NeuGc), GD3(NeuGc-NeuGc), and IV³NeuGc α -nLc₄Cer. Reactivity of 14F7 MAb was not observed with all the NeuAc-containing gangliosides and with the sulfated glycolipid tested. The reactivity of 14F7 MAb against glycolipids was summarized in Table 1.

Tissue specificity of 14F7 MAb

A preliminary immunohistochemical study was performed with fresh tissue sections of human benign and malignant tumors (Table 2). 14F7 MAb reacted with all ductal infiltrating breast carcinoma 18/18 and melanoma 10/10 tissues tested. Positive cells showed a cytoplasmatic staining. None of the samples from lung carcinoma of different histological types and nervous system tumors analyzed were stained with this MAb.

However, some benign lesions of the breast (such as breast fibrocystic and breast fibroadenoma) showed a positive staining to extracellular secretion. Figure 3 shows the recognition by 14F7 MAb of a breast carcinoma, while Figure 4 shows the strong staining with 14F7 of a melanoma tumor and not of the skin tissue. The results of the immunohistochemical analysis of adult fresh normal tissues are shown in Table 3. 14F7 MAb stained the extracellular secretion of breast glands and mucus cells from small intestine and large intestine.

DISCUSSION

Some MAbs specific against NeuGc-containing gangliosides have been reported previously. Three murine MAbs—Y-2-HD-1,⁽¹⁸⁾ MK2-34,⁽¹⁹⁾ and GMR14⁽³⁰⁾—were reported to react specifically with GM2(NeuGc). On the other hand, one murine and one human MAbs were generated by Ozawa et al.⁽¹⁴⁾ and Furokawa et al.⁽¹¹⁾ respectively, which recognized NeuGc α 2 \rightarrow 3Gal sequences internal position. Also, they have obtained murine and human MAbs that recognize NeuGc-containing disialogangliosides but these MAbs did not react with NeuGc-containing monosialyl derivatives. YK3 MAb was described to identify the NeuGc α 2 \rightarrow 8NeuGc2 \rightarrow 3Gal β 1 structure in different disialo gangliosides⁽¹⁵⁾ and SHS1 is a very specific MAb

TABLE 1. STRUCTURE OF GLYCOLIPIDS USED IN THIS STUDY

Abbreviation	Structure	Binding TLC _i
LacCer	Gal β 1-4GlcCer	—
IV ³ NeuAc α -Gc ₃ Cer	GalNeuAc β 4-Gal β 1-4GlcCer	—
IV ³ NeuAc α -Gc ₄ Cer	Gal β 3-GalNeuAc β 4-Gal β 1-4GlcCer	—
GM3	NeuAc α 2-3 Gal β 1-4GlcCer	—
GM2	GalNAc β 1-4(NeuAc α 2-3) Gal β 1-4GlcCer	—
GM1a	Gal β 1-3 GalNAc β 1-4(NeuAc α 2-3) Gal β 1-4GlcCer	—
GD3	NeuAc α 2-8NeuAc α 2-3Gal β 1-4GlcCer	+++
GD2	Gal β 1-4(NeuAc α 2-8NeuAc α 2-3)Gal β 1-4GlcCer	—
GD1a	NeuAc α 2-3 Gal β 1-3 GalNAc β 1-4(NeuAc α 2-3) Gal β 1-4GlcCer	—
GD1b	Gal β 1-3GalNAc β 1-4(NeuAc α 2-8Neuac α 2-3)Gal β 1-4GlcCer	—
GT1b	NeuAc α 2-3Gal β 1-3GalNAc β 1-4(NeuAc α 2-8Neuac α 2-3)Gal β 1-4GlcCer	—
GM3(NeuGc)	NeuGc α 2-3 Gal β 1-4GlcCer	—
GM2(NeuGc)	GalNAc β 1-4(NeuGc α 2-3) Gal β 1-4GlcCer	—
GD3(NeuGc-NeuGc)	NeuGc α 2-8NeuGc α 2-3Gal β 1-4GlcCer	—
IV ³ NeuGc α -nLc ₄ Cer	NeuGc α 2-3Gal β 1-4GlcNeuAc β 1-33Gal β 1-4GlcCer	—
I SO ₃ -GalCer	HSO ₃ -3GalCer	—

TABLE 2. RECOGNITION PATTERN OF 14F7 MAB WITH DIFFERENT HUMAN TUMORS

Localization	Positive cases/total
Breast	
Fibrocystic disease	5/9 ^a
Fibroadenoma	5/5 ^a
Infiltrating ductal carcinoma	18/18
Skin	
Epidermoid carcinoma	0/9
Melanoma	10/10
Lung	
Non small cell lung cancer	0/20
Small cell lung carcinoma	0/10
Central nervous system	
Oligodendroglioma grade II	0/3
Meningioma	0/3
Multiple glioblastoma	0/1
Meningeal sarcoma	0/2
Immune system	
B cell lymphoma (lymph node)	0/5
T cell lymphoma (lymph node)	0/3
Sézary syndrome	0/2

^aPositive staining of extracellular secretion.

to i-active ganglioside (NeuGc). One common characteristic of all these MABs is that they are IgM antibodies.

To our knowledge, this is the first description of a murine IgG1 MAB specific for *N*-glycolyl neuraminic acid containing gangliosides. The binding specificity of 14F7 MAB was strictly restricted to *N*-glycolyl function, since this MAB was able to discriminate between NeuGc and NeuAc-variants of GM3 ganglioside. On the other hand, the antibody was completely unreactive with GM2(NeuGc) having NeuGc α 2 \rightarrow 3Gal structure in an internal position, indicating that the addition of one *N*-acetyl-galactosamine to the galactose residue affected MAB

binding. The finding that 14F7 did not react with GD3 (NeuGc-NeuGc) containing NeuGc α 2 \rightarrow 8NeuGc2 \rightarrow 3Gal β 1 terminal structure suggests that MAB binding is dependent not only on the external position of the *N*-glycolyl neuraminic acid group but also on the sialic acid linkage. These evidence together with the fact that 14F7 MAB did not bind to IV3NeuGc α nLc4-Cer, differing from GM3 (NeuGc) in that the terminal NeuGc α 2 \rightarrow 3Gal-structure is linked to GlcNAc, suggest that the epitope recognized by 14F7 comprises the tri-saccharide structure NeuGc α 2 \rightarrow 3Gal β 1 \rightarrow 4Glc-. Ganglioside recognition pattern of 14F7 MAB is different from the previously reported for other murine and human MABs, which bind not only to GM3 (NeuGc) but also to several gangliosides having NeuGc α 2 \rightarrow 3Gal-terminal structure.^(11,14,16)

Positive evidences of NeuGc-containing gangliosides in human tumors are still controversial between different groups. However, it is generally agreed that normal human tissues only express NeuAc-gangliosides. Some articles have described *N*-glycolylated neuraminic acid in human tumors stained with a murine monoclonal antibody⁽²⁰⁾ or chicken polyclonal antibodies.⁽¹⁰⁾ Additionally, there are some reports where NeuGc-containing ganglioside composition has been determined by biochemical analysis in human tumors^(31,32) and cell lines.⁽⁸⁾

We used a preliminary immunohistochemical study with 14F7 in normal and tumor sections to clarify if human tumors express NeuGc antigen or not. Previous evidences of NeuGc antigen have been shown in breast cancer. Breast cancer cells were detected by membrane immunofluorescence⁽³³⁾ with anti-Hanganutziu Deicher (HD) antibodies, where the NeuGc is the immunogenic residue of the HD antigen. Furthermore, recently our group reported, for the first time, the chemical quantification of NeuGc-containing gangliosides in breast cancer. On the other hand, immunohistochemical analysis in these tissues with 3E1.2 MAB, which is specific to the mucin determinant expressed and secreted in breast tumor *N*-glycolyl sialyl Tn glycoprotein,⁽³⁴⁾ showed a positive staining in more than 90% of

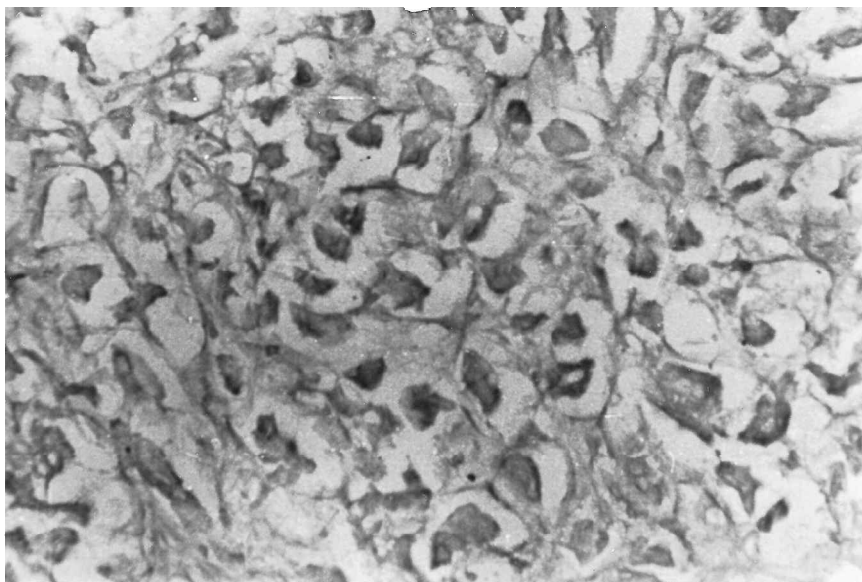


FIG. 3. Intense immunoperoxidase staining of infiltrating ductal breast carcinoma showing membrane and cytoplasmic recognition with 14F7 MAB (\times 500).

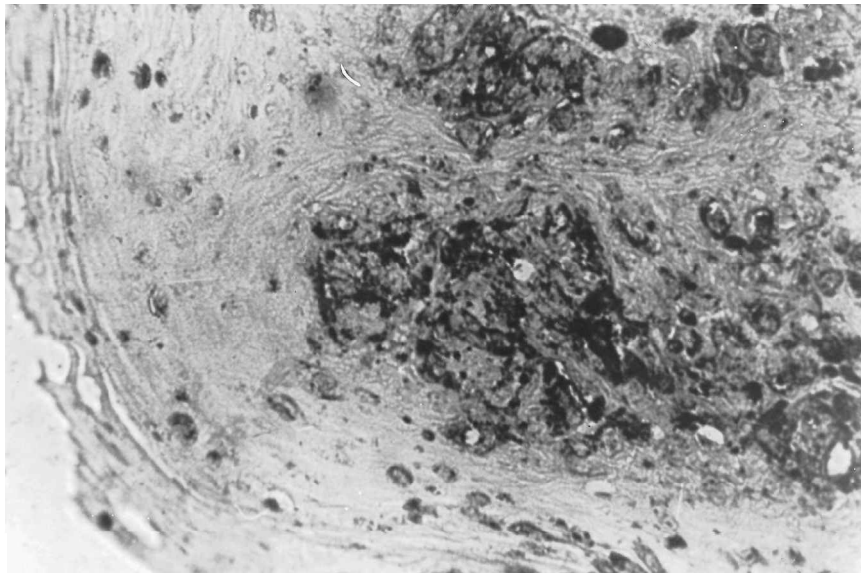


FIG. 4. Strong immunoperoxidase reactivity with 14F7 MAb in melanoma tumors and not with normal skin tissue ($\times 250$).

the breast tumor samples. Another MAb (P3), reported by our group, binds to several NeuGc containing gangliosides and also reacts with sulfated glycolipids showing a positive staining of the breast tumors and lymph node metastases samples. However, 14F7, which is a more specific anti-GM3(NeuGc) ganglioside MAb, is also capable of showing a strong cytoplasmic and membrane staining in breast malignant tumor cells (Ductal infiltrating carcinoma). This finding allows us to preclude that the structure recognized in breast cancer tissues could be the oligosaccharide core of GM3(NeuGc) ganglioside in glyco-lipids, glycoproteins, or mimics of this antigen. On the other hand, benign disease or normal tissues showed positive staining only in the extracellular secretions.

Immunohistochemical experiments showed that melanoma tumors were stained by 14F7 MAb, but no reactivity was found with normal skin. The expression of NeuGc-containing gangliosides in melanoma cells has been contradictory. Hirabayashi et al.⁽³⁵⁾ reported the presence of different HD gangliosides in melanoma tumors in small amount by enzyme TLC immunostaining. Furthermore, Kawachi and Saida⁽³⁶⁾ described that the glycoprotein fractions of human melanoma tissues were recognized by chicken anti-HD (anti-NeuGc lactosylceramide) antibody by Western Blotting assay. Saida et al.⁽³⁷⁾ has concluded that only melanoma tumors were clearly positive after comparing the NeuGc ganglioside expression in melanoma tumors, melanocytic nevus, and melanocytes from normal skin. Furthermore, we have recently demonstrated that the 14F7 MAb behaves like P3 MAb, also recognizing melanoma tumors (personal communication).

Although different biochemistry studies have shown either the absence or low level expression of these gangliosides in melanoma, Doré et al.⁽³⁸⁾ and Nakarai et al.⁽³⁹⁾ reported two previous evidences about the HD antigen as a possible target for immunotherapy in melanoma patients. Doré et al.⁽³⁸⁾ demonstrated the best prognosis in patients with IgG anti-GM3(NeuGc) gangliosides, after the immunization with vaccinia virus human

melanoma oncolysate, while Nakarai et al.⁽³⁹⁾ reported anti-HD antibodies in the sera of melanoma patients with strong recognition of HD antigen. Additionally, higher levels of IgG and IgM anti-HD antibodies were found in patients who were disease-

TABLE 3. RECOGNITION PATTERN OF 14F7 MAB WITH DIFFERENT NORMAL ADULT HUMAN TISSUES

<i>Tissues</i>	<i>Positive cases/total</i>
Urogenital tract	
Kidney	0/7
Prostate	0/5
Ovary	0/5
Breast gland (secretion)	3/4
Endocrine system	
Adrenal gland	0/3
Immune system	
Tonsils	0/5
Spleen	0/3
Central nervous system	
Brain	
Neuron	0/5
Glias	0/5
Cerebellum	0/5
Skin	
Keratinocytes	0/7
Melanocytes	0/7
Respiratory tract	
Bronchial epithelium	0/7
Neumocytes	0/7
Gastrointestinal tract	
Esophagus	0/7
Stomach	0/7
Small intestine (mucus cell)	5/5
Large intestine (mucus cell)	7/7
Pancreas	0/7
Liver	0/7

free for more than 5 years after surgery in comparison with those who relapsed within 2 years. The immunohistochemistry study with 14F7 MAB showed strong staining of tumor cells (more than 90% of positive cells). This evidence together with the results of the P3 staining in melanoma tumors could be an additional support to the hypothesis about the role of *N*-glycosylated GM3 as target for cancer immunotherapy.

The heterophilic characteristics of HD antigen on glycolipids or glycoproteins as well as the nonexpression in normal tissues in human and chickens have been reported. Although some anti-HD antibodies have recognized colon tumors by immunohistochemistry, we have not yet tested 14F7 MAB in these tumors tissues. However, the staining of mucus cells but not secretion of normal small and large intestine was observed. Studies with other fresh human tumor samples from different localizations are in progress in our laboratory.

Our results indicate that the 14F7 MAB could be a possible candidate for diagnosis used as a therapeutic agent in melanoma and breast tumors.

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