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Involvement of the Acyl Chain of Ceramide in Carbohydrate Recognition by an Anti-Glycolipid Monoclonal Antibody: the Case of an Anti-Melanoma Antibody, M2590, to G_{M3}-Ganglioside

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The effect of the chain length of the fatty acid residue of the ceramide moiety of ganglioside G_{M3} on the binding ability of monoclonal antibody M2590, which is specific for the carbohydrate structure of G_{M3}-ganglioside, was examined by means of a direct binding assay on thin layer chromatography plates (TLC immunostaining) and a quantitative enzyme-linked immunosorbent assay (ELISA). Derivatives of G_{M3} with a long fatty acid chain reacted with the M2590 antibody, but those with a short fatty acid chain showed no reaction in either assay system. These results suggested that the acyl fatty acid moiety of the ganglioside played an important role in the formation or maintenance of the antigenic structure of the carbohydrate moiety of the ganglioside.

Glycosphingolipids are ubiquitous membrane components of animal cells and are believed to be involved in cell-cell and cell-extracellular matrix interactions, as well as cellular development, differentiation and growth [1]. In recent years, many tumor-specific monoclonal antibodies have been shown to be directed toward the carbohydrate moieties of glycosphingolipids [2]. These facts suggest that carbohydrates at the cell surface play important roles in tumorigenicity.

Glycosphingolipids consist of ceramide moieties (fatty acid linked to a lipophilic sphingosine), to which oligosaccharide chains are attached. Although the majority of glycosphingolipids are assumed to be located in the external leaflet of the lipid bi-layer of a cell membrane [3, 4], the conformation and organization of the carbohydrate moieties of glycosphingolipids at the cell surface remain unclear. The recent advances in antigenic analysis with carbohydrate-directed monoclonal antibodies have led to the discovery that

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the antigenic presentation of the carbohydrate moieties of glycosphingolipids on the cell surface is restricted by their ceramide structures. For example, variation in the reactivities of different cells with the specific antibody to a certain glycolipid was associated with a change in its ceramide composition and not in the amount of it in the cell membrane [5].

Some monoclonal antibodies directed toward glycosphingolipids react preferentially with cells which predominantly contain antigenic glycolipid species with long chain fatty acids [6] or hydroxy fatty acids [7]. From these studies, the question arises whether or not the antigenicity of the carbohydrate moiety of a glycosphingolipid is affected directly by its ceramide structure, without the involvement of other cellular membrane components or auxiliary lipids. In the present study, we examined the effect of the chain length of a fatty acid residue in the ceramide moiety of G_{M3} on its reactivity with melanoma specific monoclonal antibody M2590, which is directed toward a terminal NeuAcα2-3Galβ1-4Glc (or GlcNAc), by means of a direct binding assay on thin layer chromatographic plates (TLC immunostaining) and an enzyme-linked immunosorbent assay (ELISA).

Materials and Methods

Materials

G_{M3} (II^3NeuAc-LacCer) was extracted from dog erythrocytes and purified by DEAE-Sephadex chromatography [8], followed by high performance liquid chromatography (HPLC) on an Iatrobeads 6RS8010 column (Iatron Laboratories, Kanda, Tokyo, Japan) [9]. 9-Fluorenylmethylchloroformate (Fmoc-Cl) was obtained from Sigma Chemical Co., St. Louis, MO, USA. Pre-coated thin-layer silica gel plates (Polygram SilG) were obtained from Macherey-Nagel, W. Germany. All other chemicals were of the highest purity available.

Analytical Assay

Quantitative determination of gangliosides was carried out by TLC densitometry [10] with a TLC Scanner CS-9000 (Shimadzu, Kyoto, Japan).

Monoclonal Antibody

The monoclonal antibody, M2590, was prepared as previously described [11, 12], and the culture supernatant, containing 100 ng/ml of IgM, was used throughout this work.

Synthesis of Lyso-G_{M3}

Lyso-G_{M3} (II^3NeuAc-Lac-sphingosine) was prepared as described previously [13]. Briefly, ganglioside G_{M3} was deacylated with 1 M methanolic potassium hydroxide and then its sphingoid amino group was protected with 9-fluorenylmethylchloroformate. The free amino group of the sialic acid residue was then reacetylated with acetic anhydride. The *N*-9-fluorenylmethoxycarbonyl derivative of lyso-G_{M3} (*N*-Fmoc-lyso-G_{M3}) was purified by HPLC as described above. Lyso-G_{M3} was obtained from *N*-Fmoc-lyso-G_{M3} by treatment with liquid ammonia, the overall yield being 18%.

Synthesis of G_{M3} Derivatives

The *N*-acylation of lyso-G_{M3} was performed with acetic, butyric, hexanoic, decanoic, tetradecanoic or octadecanoic anhydride by the method of Suzuki *et al.* [14]. The *N*-acetylated derivatives were purified with Sep-Pak silica cartridges (Waters Associates, Milford, MA, USA).

Mass Spectrometry

FAB mass spectrometry was performed with a JEOL HX-110 mass spectrometer equipped with a JMA-DA 5000 computer system (JEOL Ltd., Japan) according to the methods previously described [15, 16].

TLC Immunostaining

The enzyme immunostaining of gangliosides on TLC plates was performed according to the method of Magnani *et al.* [17] as modified by Higashi *et al.* [18] with a slight modification. Briefly, appropriate amounts of derivatives were spotted on TLC plates, which were developed with a solvent system of $CHCl_3/CH_3OH/2$ M NH_4OH, 60/35/8 by vol. Each chromatogram was soaked in 0.5% polyisobutylmethacrylate in ether and then dried. The plastic-coated chromatograms was then soaked for 1 h at room temperature in solution A [phosphate-buffered saline (PBS; 10 mM sodium phosphate buffer, pH 7.4, 150 m M NaCl) containing 1% bovine serum albumin and 1% polyvinylpyrrolidone]. Monoclonal antibody M2590 was applied to the chromatogram, which was then kept at room temperature with shaking for 2 h. Then the chromatogram was soaked in solution A for 15 min and re-incubated with horseradish peroxidase-conjugated goat anti-mouse immunoglobulin (Cappel Laboratories, Cochranville, PA, USA) diluted 1:400 with solution A for 1 h. After several successive washings with PBS, the chromatogram was exposed to the substrate solution (0.6 mg/ml of 4-chloro-1-naphthol, 0.01% H_2O_2, 200 mM NaCl in Tris-HCl, pH 7.4). The stained chromatogram was dried and then subjected to densitometry with a TLC Scanner for quantitative determination of the antibody reactivity. For evaluation of the effect of coating with polyisobutylmethacrylate on the retention of G_{M3} derivatives on TLC plates, chromatograms, on which appropriate amounts of the derivatives had been spotted, developed and dried as described above, were processed with PBS instead of the reagents used for the TLC immunostaining described above, and then the spots on each plate were visualized with resorcinol-HCl reagent [19]. The quantity of each derivative was determined with a TLC Scanner.

Enzyme-linked Immunosorbent Assay (ELISA)

The antigen activity of each G_{M3} derivative was determined by evaluating the inhibition of the reaction between the M2590 antibody and G_{M3} immobilized on a microtiter plate, by ELISA as described by Higashi *et al.* [20]. The inhibitory activity was calculated from the absorbance of each well, as follows:

Inhibition (%) = (A-B)/A x 100

where A is A_{500} of wells without an inhibitor, and B A_{500} of wells with an inhibitor.

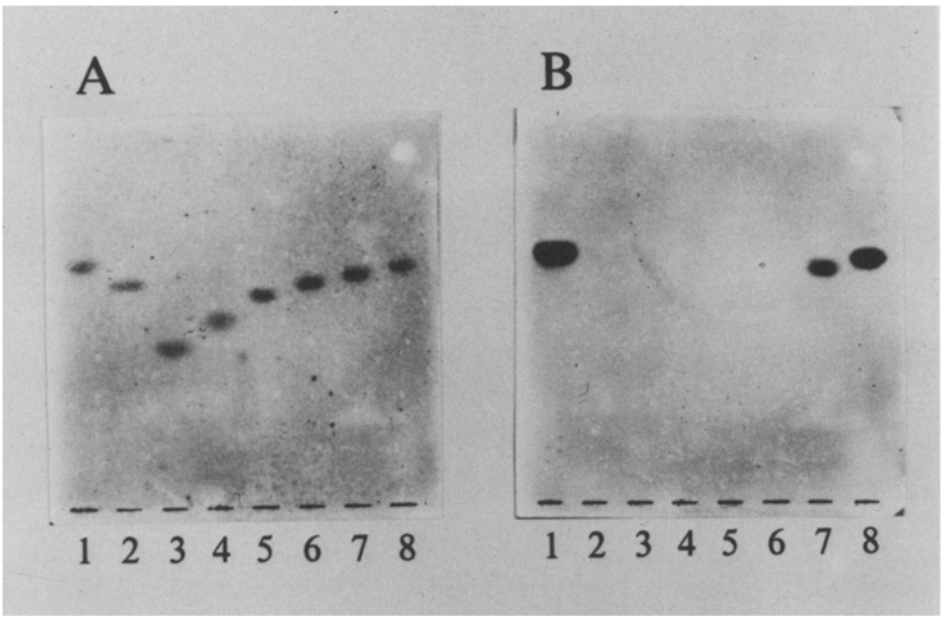


Figure 1. The thin layer chromatographic pattern of G_{M3} derivatives and their TLC immunostaining profile with the M2590 monoclonal antibody.
500 pmol of each derivative were spotted on the TLC plate, which was developed with chloroform/methanol/2 M NH_4OH, 60/35/8 by vol, as described in the Materials and Methods section.
Lane 1, G_{M3} (NeuAc) from dog erythrocytes; lane 2, *N*-Fmoc-lyso-G_{M3}; lane 3, lyso-G_{M3}; lane 4, *N*-acetyl-G_{M3}; lane 5, *N*-hexanoyl-G_{M3}; lane 6, *N*-decanoyl-G_{M3}; lane 7, *N*-tetradecanoyl-G_{M3}; lane 8, *N*-octadecanoyl-G_{M3}. The plate in panel A was visualized with resorcinol-HCl reagent. The plate in panel B was immunostained with the M2590 antibody.

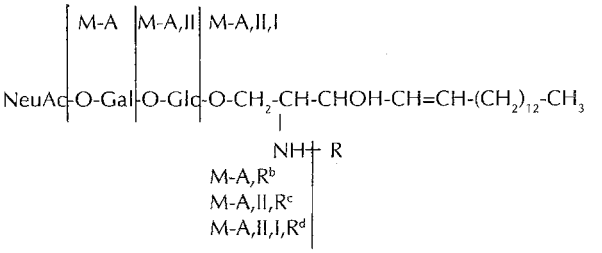
Results

Characterization of G_{M3} Derivatives

Fig. 1 (Panel A) shows the behaviour of G_{M3} derivatives on TLC. G_{M3}, *N*-Fmoc-lyso-G_{M3}, lyso-G_{M3} and synthetic *N*-acyl-G_{M3} derivatives were obtained as clearly separated single bands, respectively, suggesting that these derivatives were each mainly composed of a single type of sphingoid.

The structures of the G_{M3} derivatives were confirmed by FAB mass spectrometry. The negative FAB mass spectrum of lyso-G_{M3}, with triethanolamine as the matrix, showed an $[M-H]^-$ ion at m/z 913, and fragment ions at m/z 622 and 460; while lyso-G_{M3} sphingosine showed a fragment ion at m/z 298, indicating that it exclusively contained a C_{18} long chain base, though it was obscured by the isotope peak of the intense matrix dimer ion (m/z 297; $[(triethanolamine)_2\text{-}H]^-$). Similarly, $[M-H]^-$ ions and fragment ions corresponding to each of *N*-acylated derivatives were observed, as shown in Table 1.

Table 1. Mass (m/z) of relevant fragments produced on negative ion FAB mass spectrometry of G_{M3} and synthetic derivatives.



	R	M-H	M-A	M-A,II	M-A,II,I
G_{M3}	$COC_{23}H_{45}$	1261	971	808	646
N-Fmoc-lyso-G_{M3}	$COOCH_2C_{13}H_9$	1135	622[b]	460[c]	298[a,d]
Lyso-G_{M3}	H	913	622	460	298[a]
N-acetyl-G_{M3}	$COCH_3$	955	664	502	340
N-butyryl-G_{M3}	COC_3H_7	983	692	530	368
N-hexanoyl-G_{M3}	COC_5H_{11}	1011	720	558	396
N-decanoyl-G_{M3}	COC_9H_{19}	1067	776	614	452
N-tetradecanoyl-G_{M3}	$COC_{13}H_{27}$	1123	832	670	508
N-octadecanoyl-G_{M3}	$COC_{17}H_{35}$	1179	888	726	564

[a] Obscured by the triethanolamine matrix dimer ion at m/z.297.
[b] $(M-A,R)^-$
[c] $(M-A,II,R)^-$
[d] $(M-A,II,I,R)^-$

Fig. 2 shows typical examples of negative ion FAB mass spectra of *N*-acetyl- and *N*-octadecanoyl-G_{M3} derivatives with triethanolamine matrix. The conversion through *N*-acylation was obviously complete for these derivatives.

TLC Immunostaining and ELISA

In order to establish a TLC immunostaining system for ganglioside derivatives bearing short chain lengths of fatty acid residues in their ceramide moieties, we modified the method of Higashi *et al.* [18] according to the procedure of Magnani *et al.* [17], i.e., we coated the chromatograms with plastic for retention of the derivatives on the TLC plates during the TLC immunostaining process. Lyso-G_{M3} and derivatives bearing short fatty acid chains in their ceramide moieties were washed off the TLC plates without the plastic coating, whereas with the coating these derivatives with low affinity to TLC plates were retained in comparable amounts to native G_{M3} (Fig. 3). There were sufficient amounts for TLC immunostaining, because even a few percent of a retained G_{M3} were quantitatively recognized by the M2590 antibody (as little as 5 pmol of G_{M3}, data not shown).

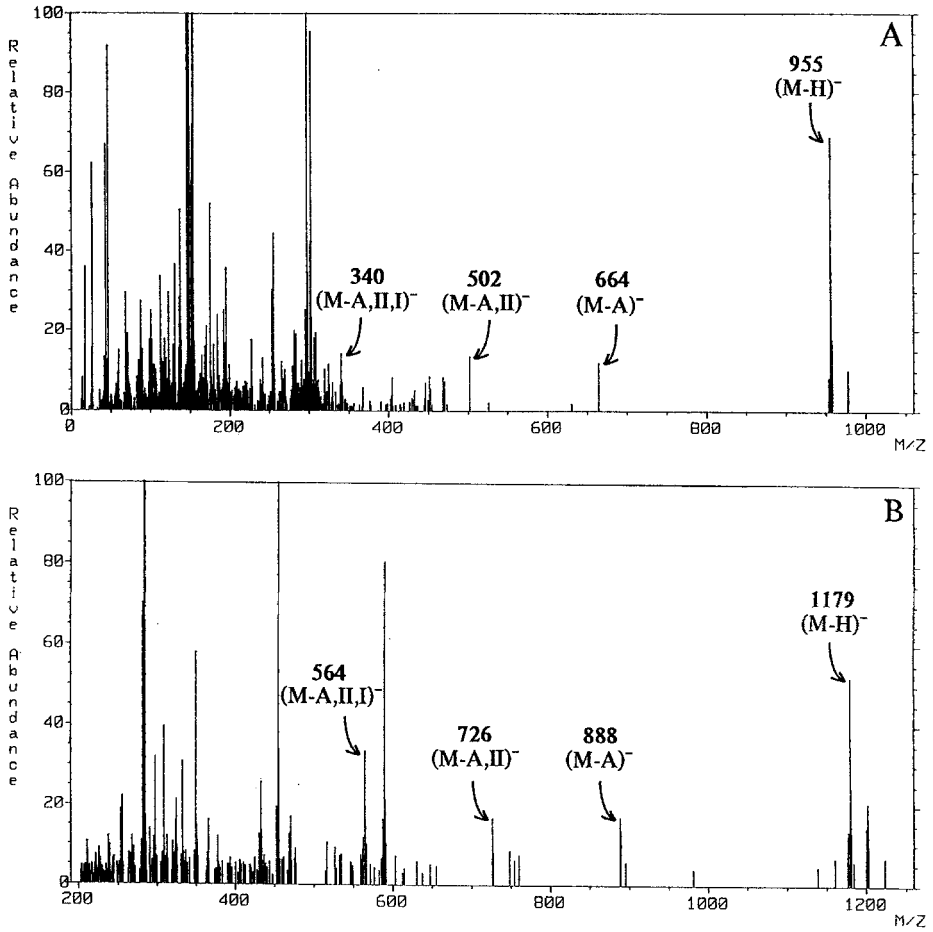


Figure 2. Negative ion FAB mass spectra of G_{M3} derivatives with triethanolamine as the matrix. A, *N*-acetyl-G_{M3}. B, *N*-octadecanoyl-G_{M3}.

Fig. 1 (panel B) shows that only native G_{M3} bearing *cis*-15-tetracosenoic acid (24:1) as major fatty acid species, synthetic *N*-tetradecanoyl- and *N*-octadecanoyl-G_{M3} were stained, whereas G_{M3} derivatives with 10 carbon atoms or less were not stained by the M2590 antibody under our experimental conditions. Even taking the effect of washing-out into consideration, G_{M3} derivatives with short acyl chains were still concluded to be non-reactive. The reactivity of the M2590 antibody with G_{M3} derivatives is summarized in Table 2.

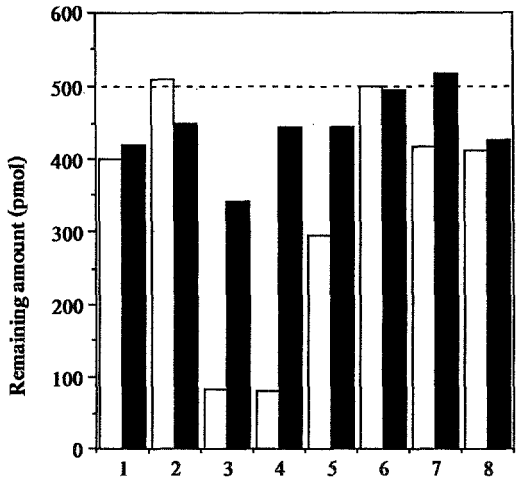


Figure 3. Effect of coating with plastic on the retention of G_{M3} derivatives on TLC plates. TLC chromatograms, on which G_{M3} derivatives (500 pmol each) had been spotted and developed under the conditions given in the Materials and Methods section were coated (closed columns) or not coated (open columns) with polyisobutylmethacrylate. After successive washings with PBS, the remaining glycolipids were visualized with resorcinol reagent and quantified by TLC densitometry. Column 1, G_{M3} from dog erythrocytes; column 2, *N*-Fmoc-lyso-G_{M3}; column 3, lyso-G_{M3}; column 4, *N*-acetyl-G_{M3}; column 5, *N*-hexanoyl-G_{M3}; column 6, *N*-decanoyl-G_{M3}; column 7, *N*-tetradecanoyl-G_{M3}; column 8, *N*-octadecanoyl-G_{M3}.

Decreases in the antigenic reactivities of G_{M3} derivatives with the M2590 antibody due to shortening of the chain lengths of their fatty acid residues were also observed even at five times higher concentration (500 ng/ml) of monoclonal antibody (data not shown). To confirm the antigenic reactivities of G_{M3} derivatives to M2590, the inhibition experiment was carried out by ELISA (Fig. 4). Antigenic activity toward M2590 appeared in *N*-hexanoyl G_{M3} and increased with further elongation of the acyl chain length.

Discussion

A large variety of tumor-associated glycolipid antigens has been discovered by monoclonal antibodies, which were prepared with tumor cells or tissues as antigens. The melanoma specific monoclonal antibody, M2590, was prepared by immunization of syngeneic C57BL/6 mice with the mouse B16 melanoma cells [11, 12]. Surprisingly, M2590 only reacted with melanoma cells from some species, although ganglioside G_{M3}, with an antigenic epitope in its sugar moiety, was found to be widely distributed in essentially all types of animal cells [21, 22]. One explanation for the melanoma specificity of M2590 demonstrated by Nores *et al.* [22] is that the reactivity of antibody M2590 depended greatly on the density of G_{M3}

Table 2. TLC Immunostaining reactivity of monoclonal antibody M2590 with G_{M3} and synthetic derivatives.

Antigen	Fatty acid	Amount remaining on TLC plate[a] (pmol)	Relative reactivity (%)
G_{M3} (dog erythrocytes)	24:1[b]	420	100
N-Fmoc-lyso-G_{M3}	-	450	0
Lyso-G_{M3}	-	340	0
N-acetyl-G_{M3}	2:0	440	0
N-hexanoyl-G_{M3}	6:0	440	0
N-decanoyl-G_{M3}	10:0	490	0
N-tetradecanoyl-G_{M3}	14:0	520	30
N-octadecanoyl-G_{M3}	18:0	430	80

[a] 500 pmol of each antigen were spotted on a TLC plate, which was processed as described in the Materials and Methods section, and then the remaining amount was determined by TLC densitometry.
[b] Only major fatty acid but contained $C_{24:0}$ and $C_{22:0}$ as minor components.

exposed at the cell surface, and melanoma cells expressed a high density of G_{M3} at the cell surface. The loss of reactivity of shorter chain fatty acid-containing G_{M3} derivatives with M2590 reported here may be due to their inability to express a density recognizable by M2590. The conformational change occurring in the carbohydrate moiety of G_{M3} on shortening of its fatty acid moiety is another possible mechanism for the decrease or loss of reactivity with the M2590 antibody, which was clearly demonstrated in the present experiment. Namely, a particular conformation of the carbohydrate moiety of G_{M3}, which arises on interaction with the ceramide moiety, is recognized by the M2590 antibody, and this conformation changes on shortening of the fatty acid chain length, resulting in a loss of reactivity. A complete loss of the reactivity on TLC of G_{M3} having fatty acids up to C_{10}, which still retain a sufficient density, strongly supports the latter possibility.

Fredman *et al.* suggested the possibility that the fatty acid residues of the ceramide moiety influence the reactivity of an anti-sulfatide monoclonal antibody [23]. It is also anticipated that a certain membrane factor or component may modulate the ceramide structure, having an effect like the shortening of the fatty acid chain, resulting in changes of carbohydrate conformation and reactivity.

The results presented in this study suggest that melanoma specific monoclonal antibody M2590 recognizes a particular conformation or organization of the sugar structure of G_{M3}, which is possibly perturbed by intramolecular fatty acid residues. The importance of knowledge concerning the highly ordered structures of glycolipids is being increasingly recognized, particularly in terms of various physiological functions or of specific tumor-associated expression of glycolipids, and this case of the M2590 monoclonal antibody may facilitate the solving of such problems.

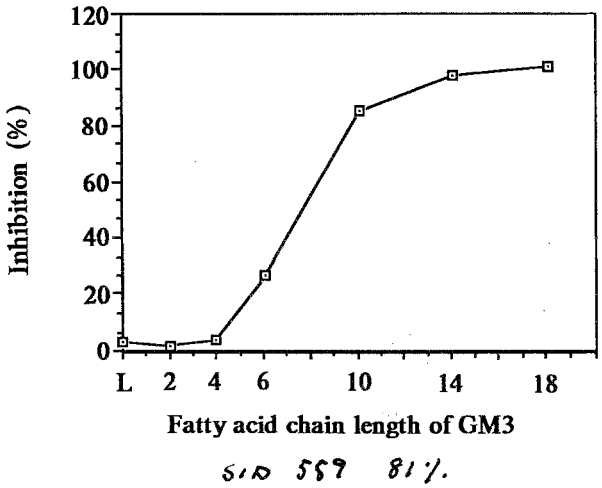


Figure 4. ELISA Reactivities of synthetic G_{M3} derivatives toward the M2590 monoclonal antibody. 100 μM of each G_{M3} derivative was used as an inhibitor. L, lyso-G_{M3}.

Acknowledgements

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