Glycoconjugate Journal 16, 39–43 (1999)

Antigenicity of the carbohydrate moiety of ganglioside GM3 having 3-*O*-acetyl ceramide

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To elucidate the effect of a modification of ceramide on antigenicity of the carbohydrate of ganglioside, the reactivity of *O*-acetyl GM3 having 3-*O*-acetyl ceramide, which has been characterized as a glioma-related ganglioside, with monoclonal antibody M2590 was examined in comparison to that of non-acetylated GM3, by means of quantitative enzyme-linked immunosorbent assay, TLC-immunostaining and liposome immune lysis assay. In all these assay systems, *O*-acetyl GM3 showed less activity than GM3 as follows: GM3 was detected till 0.1 nmol in TLC-immunostaining, whereas *O*-acetyl GM3 could not be detected even at 0.25 nmol; the GM3 reaction was approximately twofold that of *O*-acetyl GM3 at each diluted point in the enzyme-linked immunosorbent assay; and 20% of the liposomes containing GM3 were lysed at 6 mol%, while liposomes containing *O*-acetyl GM3 did not lyse at that concentration. The lesser antigenicity of the sugar moiety of *O*-acetyl GM3 could be ascribed to the presence of an acetyl group in the ceramide at the 3-position of sphingosine.

Keywords: **ganglioside, acetyl ceramide, antigenicity, GM3.**

Abbreviations: **Ac, acetyl; Cer, ceramide; CMW, chloroform-methanol-water; NeuAc, *N*-acetyl neuraminic acid; NeuGc, *N*-glycolyl neuraminic acid; GM3 (NeuAc), GM3 having NeuAc; GM3 (NeuGc), GM3 having NeuGc; DPPC, dipalmitoyl phosphatidylcholine; DCP, dicetyl phosphate; ELISA, enzyme-linked immunosorbent assay; LILA, liposome immune lysis assay; TLC, thin-layer chromatography. Nomenclature and abbreviations of glycosphingolipids follow the system of Svennerholm (*J. Neurochem* [1963] 10, 613–23) and those recommended by the IUPAC-IUB Nomenclature Commission (Lipids [1977] 12, 455–68).**

Introduction

The sugar moiety of glycosphingolipid acts as a membranous antigen on cells, and induces an intercellular interaction [1]. In contrast, ceramide (Cer) of the glycosphingolipid has been regarded to be only an anchor molecule in the cell membrane. However, differences of the Cer moiety in the glycolipids, particularly the chain length of the fatty acids, have been reported to affect the antigenicity of the sugar moiety [2–5]. The Cer itself has been found to have apoptotic activity as a second messenger [6,7].

3-*O*-Acetyl (Ac) GM3 was isolated by us for the first time from transplanted rat glioma tissue as a glioma-related ganglioside and the structure was determined to contain 3-*O*-Ac Cer and *N*-Ac neuraminic acid (NeuAc) (3-*O*-Ac GM3 (NeuAc)) [8]. This modified GM3 has been chemically derived from non-acetylated GM3 through selective insertion of the *O*-Ac group into the C-3 hydroxyl group of the Cer, and the antigenicity of the sugar moiety toward melanoma-specific monoclonal antibody M2590 has been examined [9]. In the present paper, the antigenicity of the sugar moiety of the 3-*O*-Ac GM3 (NeuAc) toward M2590, which recognized a carbohydrate structure in GM3 (NeuAc) [10] was extensively studied in comparison to that of non-acetylated GM3 (NeuAc) by three different techniques, and the effect of modification of the Cer moiety on the antigenicity was estimated.

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Materials and methods

Chemicals DEAE-Sephadex, A-25 and Sephadex LH-20 were obtained from Pharmacia-LKB (Uppsala, Sweden); silica beads (Iatrobeads) from Iatron Laboratories (Tokyo); silica-gel on polyester thin-layer chromatography (TLC)-plates and 3′-sialyl lactose, from Sigma-Aldrich (MO); cholesterol from Nakarai Biotesque (Kyoto); monoclonal anti-GM3 (NeuAc) antibody M2590 from Biotest Res. (Tokyo) and horseradish peroxidase (HRP)-labeled

goat antibody (whole serum) against mouse IgM from Jackson Immuno Res. Lab. (PA). Other standard glycolipids were prepared in this laboratory, and all other reagents were of analytical grade.

Gangliosides A large amount of GM3 (NeuAc) was obtained after replacement of the *N*-glycolyl (Gc) residue at the sialic acid moiety of GM3 (NeuGc) from equine erythrocytes [11,12] with an *N*-Ac group through the de-*N*-glycolyl GM3 derivative, as reported previously [9]. 3-*O*-Ac GM3 (NeuAc) and lyso GM3 (NeuAc) were chemically synthesized from GM3 (NeuGc) as described earlier [9,13]. The amount of the ganglioside was measured according to the method of Yu and Ledeen [14] for the sialic acid by gas-liquid chromatography.

TLC-immunostaining The immunostaining of the glycolipid on the TLC-plate was performed according to the method of Higashi *et al.* [15] with a slight modification. The indicated amount of the glycolipid was applied and developed on a TLC-plate with chloroform-methanol-water (CMW), 60:40:9 (by vol). The plate was dried, and dipped in an *n*-hexane solution containing 0.1% (w/v) polyisobutylmetacrylate for 1 min, followed by drying in a desiccator *in vacuo.* The dried plate was incubated in Blockace solution (UK-B25, Dainihonseiyaku, Tokyo) for 2 h at room temperature, and was incubated again in diluted M2590 (1,000-fold) solution in phosphate-buffered saline (PBS) for 2 h. The plate was washed with PBS three times, and incubated in an HRP-linked goat anti-mouse IgM antibody (5,000-fold diluted with PBS) for 2 h at room temperature. After washing the plate with PBS, a solution of Chemiluminescence Reagent Kit (Renaissance, Dupont NEN, Boston) was added to the plate, followed by exposure to X-ray film (Fuji, Tokyo) for 30 sec and visualization of the spot by development.

Enzyme-linked immunosorbent assay The enzyme-linked immunosorbent assay (ELISA) for glycolipid was carried out according to the method of Tai *et al.* [16]. Briefly, 50 µl of a solution containing various concentrations of ganglioside in ethanol was placed in the well of a polystyrene microtiter plate (Immuron I, Dynatech, Tokyo) and allowed to stand to dryness overnight at room temperature. The glycolipid-fixed well was incubated with diluted Blockace solution (4-fold with PBS, 200 µl) for 1 h at room temperature, followed by removal of the solution; the well was further incubated after addition of diluted M2590 (1,000-fold with PBS, 100 µl) for 1 h. When 3′-sialyl lactose was employed as an inhibitor, GM3 (NeuAc) was fixed on the wells at 0.5 nmol and the inhibitor was added from 0 to 5 nmol, followed by reaction with diluted antibody in a manner similar to that reported above. The well was washed with PBS three times, incubated with diluted HRP-labeled goat antibody against mouse IgM (5,000-fold with PBS, 100 µl) for 1 h, and then washed again with PBS. The well was incubated with *O*-phenylene diamine solution (400 µg/ml, 100 µl) in 0.1 M citrate-phosphate buffer, pH 5.5 for 15 min, the reaction was terminated by addition of 4 N HCl (100 µl) and the oxidized diamine produced in the well of the microtiter plate was measured by photometer (Immunomini, NJ-2300, Inter Med. Tokyo) at a wavelength of 490 nm. The mean value for binding activity between antigen and antibody of triplicate determinations was used after subtraction of the blank-value, which was measured in the absence of antigen.

Liposome immune lysis assay The liposome immune lysis assay (LILA) (dependant on complement) was carried out according to the method of Nores *et al.* [17], with some modification. Briefly, 500 nmol of both cholesterol and dipalmitoyl phosphatidyl choline (Calbiochem, CA), 20 nmol of dicetyl phosphate (Sigma, MO) and variable amounts of *O*-Ac GM3 or GM3 were dried and dispersed in 0.1 ml of a solution containing 0.1 M 4-methyl umberiferyl phosphate (Wako Pure Chemicals, Tokyo). The resultant liposomes were thoroughly dialyzed against a solution of 75 mM KCl and 75 mM NaCl to remove the untrapped fluorescence marker. Aliquots (100 µl) of the liposome suspension (diluted 100-fold with PBS) were incubated with 1 µg per ml of M2590 antibody and guinea pig complement (Wako Pure Chemicals, Tokyo) in the presence (10 nmol) or absence of 3′-sialyl lactose. The liberation of 4-methyl umberiferone from the liposomes was measured with a spectrofluorophotometer (RE-510, Shimadzu) after incubation with alkaline phosphatase (*E. coli,* type III, Sigma) for 30 min at room temperature. Each assay was performed in triplicate and the mean value was used.

Results

Gangliosides The chemical structures of GM3 (NeuAc) and 3-*O*-Ac GM3 (NeuAc) are presented in Figure 1. The purity of the GM3 (NeuAc) as well as 3-*O*-Ac GM3 (NeuAc) was determined by TLC-analysis and proton nuclear magnetic resonance spectroscopy to be over 99%.

TLC-immunostaining Various amounts of the gangliosides, GM3 (NeuAc) and 3-*O*-Ac GM3 (NeuAc) were immunostained on TLC using M2590. As demonstrated in Figure 2, GM3 (NeuAc) was visible at 0.1 nmol, whereas 3-*O*-Ac GM3 (NeuAc) was not detected at less than 0.25 nmol. This indicated the weaker reactivity of the 3-*O*-Ac GM3 (NeuAc) toward M2590 on the TLC-plate. LysoGM3 was not suitable for the TLC-immunostaining because it was removed from the plate by washing with aqueous solution.

Enzyme-linked immunosorbent assay The reactivity of the two gangliosides toward M2590 was examined by ELISA, in which the gangliosides used as antigens and the antibody M2590 were independently diluted. The GM3 (NeuAc) reacted with higher sensitivity than 3-*O*-Ac GM3 (NeuAc) in

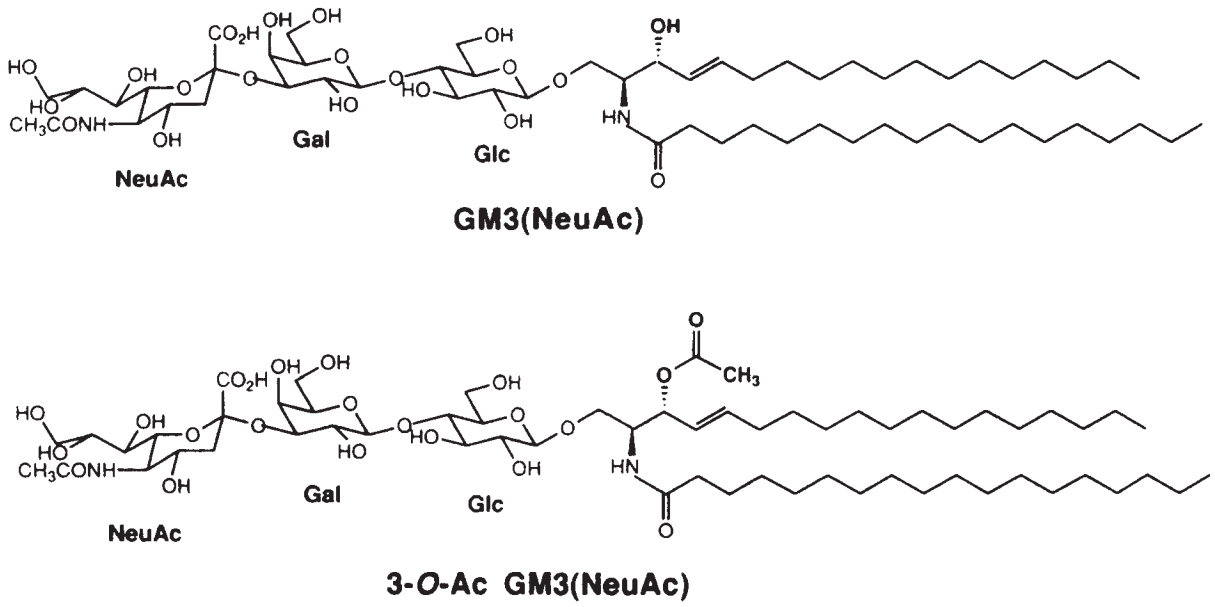

Figure 1. Structures of GM3 (NeuAc) and 3-*O*-Ac GM3 (NeuAc).

both cases, as shown in Figure 3. The GM3 (NeuAc) was revealed to be approximately twice as sensitive as 3-*O*-Ac GM3 (NeuAc) at each dilution of antigen or antibody. In the presence of 3′-sialyl lactose at variable concentrations, the reactivities of GM3 (NeuAc) and 3-O-Ac GM3 (NeuAc) toward the antibody were both inhibited, with a maximum of 40%, as compared to the value obtained in the absence of the inhibitor (data not shown).

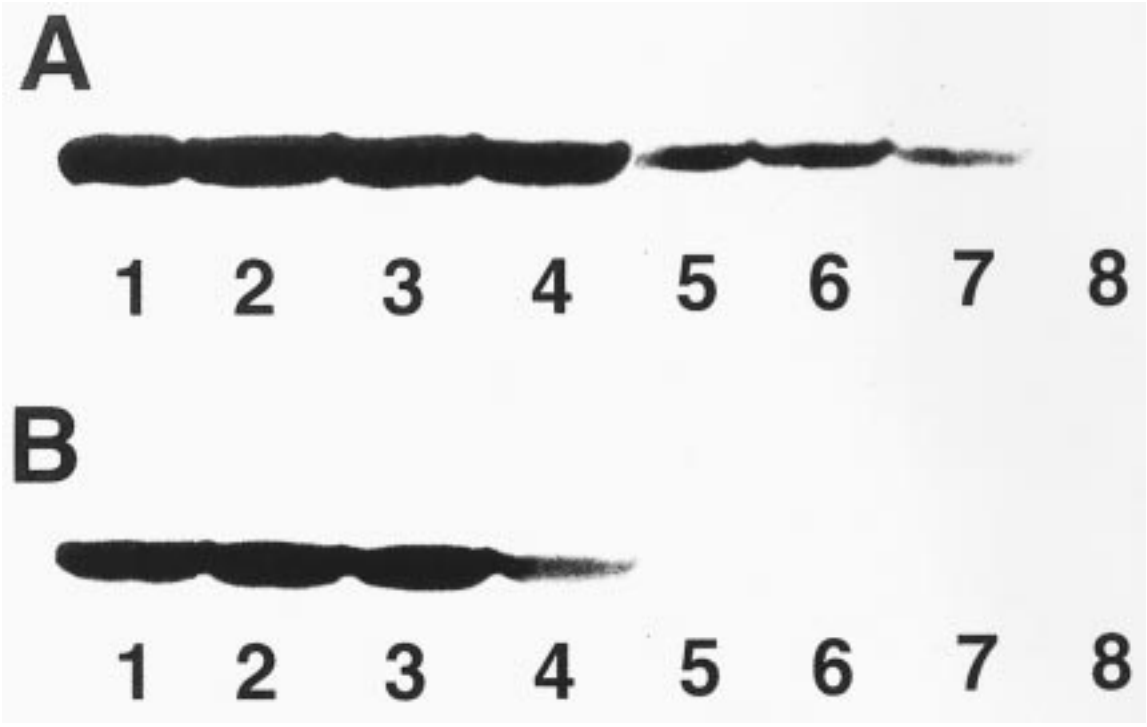

Figure 2. TLC-immunostaining of GM3 (NeuAc) and 3-*O*-Ac GM3 (NeuAc) with M2590. Panels A and B indicate chromatograms of GM3 (NeuAc) and 3-*O*-Ac GM3 (NeuAc), respectively. Through the panels, the gangliosides applied to the lanes were: 1, 2.5 nmol; 2, 1.5 nmol; 3, 1.0 nmol; 4, 0.5 nmol; 5, 0.25 nmol; 6, 0.15 nmol; 7, 0.1 nmol and 8, 0.05 nmol.

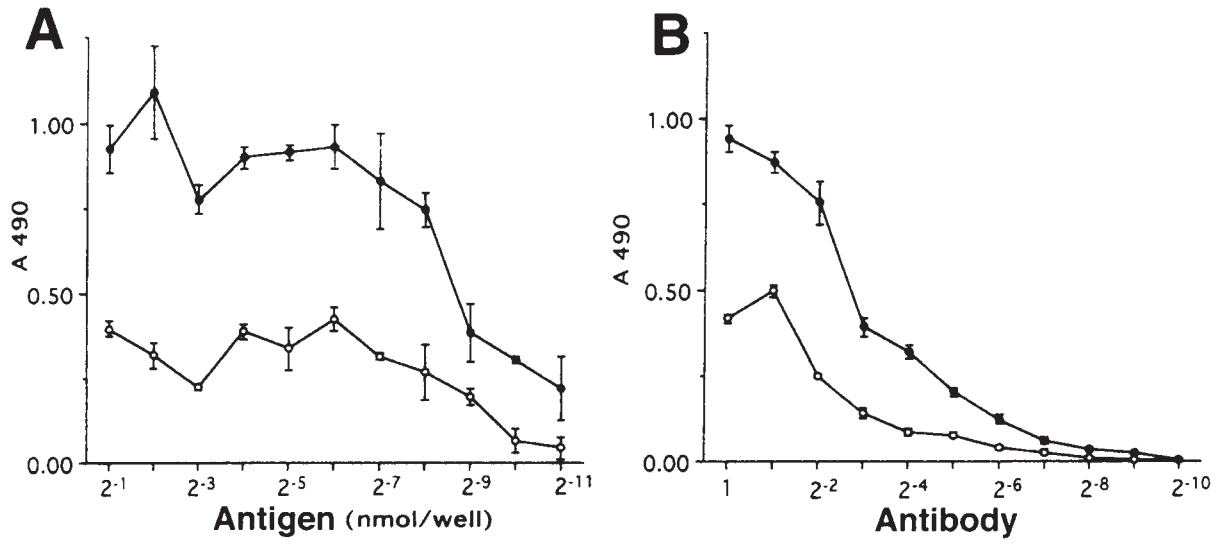

Figure 3. Reactivity of M2590 with GM3 (NeuAc) (closed circles) and 3-*O*-Ac GM3 (NeuAc) (open circles) in enzyme-linked immunosorbent assay. In panel A, wells were coated with both gangliosides at indicated concentrations and incubated with 1 μg/ml of M2590. In panel B, wells were coated with both gangliosides (0.5 nmol/well) and incubated with different dilutions of M2590 (undiluted solution at 1 μg/ml). Each vertical bar represents the standard error.

Liposome immune lysis assay The results of the LILA using two gangliosides and M2590 are summarized in Figure 4. About 20% of the liposomes containing GM3 (NeuAc) were lysed by the antibody and complement at the concentration of 6 mol%, whereas those containing 3-*O*-Ac GM3 (NeuAc) were not lysed at the same concentration. Similarly, at a ganglioside concentration of 8 mol% in the respective liposomes, 45% of GM3-liposomes were lysed, while only 25% of 3-*O*-Ac GM3-liposomes were lysed ($p<0.01, n=5$). These results indicated that the threshold of the lysis of the liposomes containing GM3 (NeuAc) was lower than that of those containing 3-*O*-Ac GM3 (NeuAc). Furthermore, the maximal degree of lysis of the liposomes was slightly higher (50%) for GM3-liposomes than for 3-*O*-Ac GM3-liposomes (45%) ($p<0.1, n=5$). In the presence of 3′-sialyl lactose, these lysis curves were both shifted to a concentration higher than 12 mol% (data not shown). Lysis of the liposomes containing mixtures of these gangliosides with ratios of 65:35, 50:50 or 35:65 was similar to GM3-liposomes as shown in Figure 4. Thus the results obtained from three different techniques indicated that 3-*O*-Ac GM3 (NeuAc) was less reactive toward M2590 than GM3 (NeuAc).

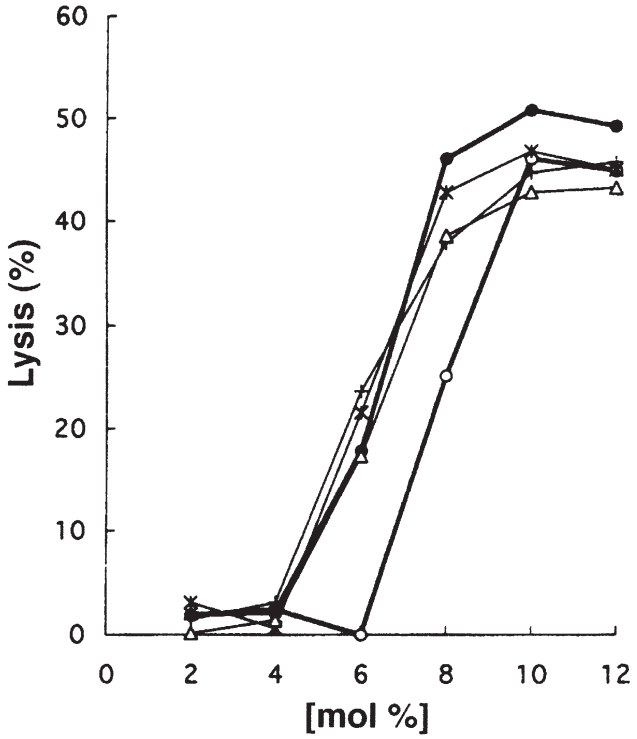

Figure 4. Reactivity of M2590 with GM3 (NeuAc) and 3-*O*-Ac GM3 (NeuAc) and their mixture in the liposome immune lysis assay. The liposomes were prepared with the indicated amounts of gangliosides and other lipids and liberation of umberiferyl phosphate by M2590 and complement from the liposomes was measured after hydrolysis with alkaline phosphatase. Each value represents the mean of triplicate determinations. ●, GM3 (NeuAc); ❍, 3-*O*-Ac GM3 (NeuAc); +, mixture of 3-*O*-Ac GM3 (NeuAc) and GM3 (NeuAc) at 65:35; X, a mixture at 50:50; Δ, a mixture at 35:65.

Discussion

The antibody, M2590, employed herein was raised against melanoma B16 cells and recognizes a carbohydrate moiety with a NeuAcα2-3Galβ1-4Glc/GlcNAc structure [10]. The B16 cells were shown to have no 3-*O*-Ac GM3 (NeuAc) in a previous report [10] and by us (data not shown) indicat-

ing that M2590 was raised against the carbohydrate moiety of GM3 (NeuAc) on the cells. A monoclonal antibody recognizing the sugar moiety of a glycolipid has been found to be affected by the ceramide moiety, in particular by the chain length of the fatty acids [2–4]. With respect to the reactivity of M2590 toward GM3 (NeuAc), a shorter fatty acid chain in the Cer results in weakened reactivity in ELISA and TLC-immunostaining assays [3]. Furthermore, Nores *et al.* [17] showed that the reactivity of M2590 was expressed for the first time at a relatively high concentration of GM3 (NeuAc) in LILA, and the reactivity immediately reached a plateau above that concentration. This indicates that the recognition of the GM3 molecule in the liposomes does not occur in a monomeric state, but in a cluster state, and further that the high concentration results in a stereochemical structure of the sugar moiety of the GM3 (NeuAc) adequate for recognition by the antibody. In the present paper, comparison of the reactivity of M2590 toward GM3 (NeuAc) and 3-*O*-Ac GM3 (NeuAc) was done using ELISA, TLC-immunostaining and LILA. The results showed that the latter lipid had less reactivity than the former in all three assays. This inferior reactivity of 3-*O*-Ac GM3 (NeuAc) was in agreement with the above result using GM3 (NeuAc) having a shorter fatty acid chain [3]. The lesser reactivity might be due to a conformational alteration of the sugar epitope by modification (*O*-acetylation) of the hydroxyl group at the 3-position of the sphingosine of the parent GM3 (NeuAc). In other words, it is conceivable that the Cer moiety affected the immunological activity of the sugar moiety of the glycolipid. This can be further confirmed by investigation of the antigenicity of a GM3 derivative having a blocking group other than the *O*-Ac group. Furthermore, the reaction of M2590 toward GM3 (NeuAc) and *O*-Ac GM3 (NeuAc) was inhibited significantly by a large excess of 3′-sialyl lactose in ELISA and LILA, but not completely, at least, in the former assay. This suggested that the antibody might recognize the lipid moiety in addition to the sugar moiety. To estimate the recognition of the lipid moiety by the antibody, further studies were performed using GM3 species modified in the lipid moiety such as lysoGM3 (NeuAc) or C_2-GM3 (NeuAc) (synthesized from lysoGM3). These modified gangliosides, however, were not suitable for ELISA and LILA. Their unsuitability may be due either to their high solubility in aqueous solution or toxicity against liposomes.

In the LILA assay using GM3 (NeuAc) or 3-*O*-Ac GM3 (NeuAc) or a mixture of the two, it is of interest that the maximum lysis of the liposomes containing 3-*O*-Ac GM3 (NeuAc) or a mixture of both glycolipids was similar but lower than that containing GM3 (NeuAc) (Fig. 4). The threshold of the concentration for the lysis of the liposomes containing GM3 (NeuAc) was similar to that containing a mixture of both lipids, but different from that containing of 3-*O*-Ac GM3 (NeuAc), and the data were slightly different from our previous observations [9]. These differences could be ascribed to the different 3-*O*-Ac GM3 (NeuAc), as the previous lipid was isolated from rat glioma tissue and the present one was a modified lipid from equine erythrocytes, so they had different fatty acid components.

Acknowledgements

We are deeply indebted to Mr. Kim Barrymore for his help in the preparation of this article, Drs. Masahiko Chiba and Yoshihiro Maeda for their technical help in the preparation of glycolipids and the Hokkaido Ebetsu Meat Inspection Office for donation of equine blood. This work was supported in part by Grant-in-Aid for Scientific Research on Priority Areas No. 10142712 from the Ministry of Education, Science and Culture, Japan.

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Received 28 July 1998, revised 18 December 1998, accepted 22 December 1998