Acta Biochimica Polonica

Vol. 45 No. 2/1998

343-349

QUARTERLY

Comparative study of sialidase activity and G_{M3} content in B16 melanoma variants with different metastatic potential*

Masashi Sawada[1, 2], Setsuko Moriya[1], Ryuzaburo Shineha[2], Susumu Satomi [2] and Taeko Miyagi[1]✉

[1]*Division of Biochemistry, Research Institute, Miyagi Prefectural Cancer Center, Natori, Miyagi 981-1293, and* [2]*2nd Department of Surgery, Tohoku University, School of Medicine, Sendai, Miyagi 980-8577, Japan*

Key words: sialidase, B16 melanoma, gangliosides, sialyltransferase, sialic acids

We previously demonstrated that transfection of a sialidase cDNA into B16-BL6 cells, a highly metastatic and invasive cell line derived from the mouse B16 melanoma, resulted in a marked suppression of metastasis accompanied by decreased cellular content of the G_{M3} that is one of the target molecules of the sialidase expressed (Tokuyama *et al.*, 1997 *Int. J. Cancer*, 73, 410–415). To obtain further insight into the involvement of sialidase in metastasis, we made a comparison of the levels of sialidase activity and G_{M3} content between B16 melanoma cell lines with low (B16-F1) and high (B16-F10 and -BL6) metastatic potential. The cells exhibited sialidase activity towards 4-methylumbelliferyl *N*-acetylneuraminic acid (4MU-Neu5Ac) and gangliosides at acidic pH in the particulate fractions, but not in the cytosol. The activity toward 4MU-NeuAc was significantly lower in highly metastatic cells. The activity toward gangliosides, on the other hand, varied independently of metastatic potential: B16-F10 cells with a high potential for experimental metastasis showed the lowest level and B16-BL6 cells having high invasiveness had rather a higher level of ganglioside sialidase along with a much greater G_{M3} synthase activity than the other two cell lines. Flow cytometric analysis with anti-G_{M3} antibody revealed that highly metastatic cell lines were higher in the binding affinity as compared to B16-F1 cells, B16-BL6 cells containing twice as much cellular G_{M3} as B16-F1 cells on thin-layer chromatography.

*Paper presented at the International Symposium on Structure, Metabolism and Functions of Glycosphingolipids and Sphingolipids, held in Warsaw, Poland, in September, 1997.

✉To whom correspondence should be addressed: Taeko Miyagi, The Division of Biochemistry, Research Institute, Miyagi Prefectural Cancer Center, Medeshima-Shiode, Natori-shi, Miyagi, Japan 981-1293.

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; 4MU-Neu4Ac, 4-methylumbelliferyl *N*-acetylneuraminic acid; mAb, monoclonal antibody; MAM, *Maackia amurens* mutagen; PNA, pea nut agglutinin; RCA, *Ricinus communis* agglutinin; SSA, *Sambucus amurens* agglutinin; WGA, wheat germ agglutinin.

Alterations in sialylation have been found to be closely associated with metastatic potential [1–5]. To elucidate how such aberrant sialylation occurs in cancer cells and why such changes affect metastasis, we have been studying sialidase expression in metastatic cells. In our previous studies murine tissues were found to contain at least four types of sialidase differing in subcellular location and in enzymatic and immunological properties [6–8]. They are located mainly in the lysosomal matrix, lysosomal membranes, cytosol and plasma membranes. Metastatic potential was found to be inversely correlated with lysosomal matrix-type sialidase activity in transformed rat 3Y1 cell lines [9]. We also presented evidence suggesting an important role for sialidase in highly metastatic and invasive B16 melanoma variants (B16-BL6 cell lines), which exhibited decreased pulmonary metastasis on transfection of a cytosolic sialidase cDNA, with an associated decrease in G_{M3} content [10]. These results stimulated us to further investigation of sialidases and their target molecules in metastatic cells.

The present work was undertaken to determine whether phenomena such as G_{M3} reduction brought about by the sialidase gene transfection described above might be also observed in B16 melanoma cells with low metastatic potential. Although B16 melanoma cells have been utilized as a good tool for metastatic studies, no information is available regarding their endogenous sialidase expression. We therefore assessed the levels of sialidase and sialyltransferase activities and ganglioside components using B16 melanoma variants with different metastatic ability. The results obtained are essentially consistent with our previous findings.

MATERIALS AND METHODS

Materials. 4-Methylumbelliferyl *N*-acetylneuraminic acid (4MU-NeuAc) was purchased from Nakarai (Kyoto, Japan). Bovine mixed gangliosides (type II) were from Sigma (St. Louis, MO, U.S.A.). CMP-[^{14}C]NeuAc was obtained from New England Nuclear (Boston, MA) and diluted with cold CMP-NeuAc from Sigma to give a final specific activity of 2.5 Ci/mol. The sources of gangliosides and glycoproteins used as acceptors for sialyltransferase assays were described elsewhere [11]. The lectins of *Sambucus siebaldiana* agglutinin (SSA), *Maackia amurens* mutagen (MAM), wheat germ agglutinin (WGA), pea nut agglutinin (PNA) and *Ricinus communis* agglutinin (RCA) were products of Honen (Tokyo, Japan). Anti-G_{M3} mAb (M2590) was from Snow Brand (Tokyo, Japan).

Cell lines. B16-F1 (low incidence of lung colonization), B16-F10 (high incidence of lung colonization) and B16-BL6 (high incidence of lung colonization and invasion) derived from the B16 murine melanoma, the origins and properties of which were described by Poste *et al.* [12] and Fidler [13], were kindly provided by Dr. S. Taniguchi (Shinshu University, School of Medicine). They were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. Cells in the exponential growth phase within four passages were used for this investigation.

Sialidase assay. Cells were washed with phosphate-buffered saline (PBS, pH 7.2) and sonicated in ice bath in 9 vol. of ice-cold 0.25 M sucrose containing 1 mM EDTA and 0.2 mM phenylmethylsulfonyl fluoride for 10 s at the minimum setting (Sonifier 250; Branson, Danbury). The mixture was centrifuged at 600 × ***g*** for 10 min and then the supernatant was again spun at 105000 × ***g*** for 60 min at 4°C. The resultant supernatant was used as the cytosolic fraction, and the pellet suspended in 9 vol. of sucrose solution as the particulate fraction, both being assayed for sialidase activity under optimal conditions. The assay mixture contained 60 nmol of the substrate 4-MU-NeuAc, 5 μmol of sodium acetate (pH 4.7 or 5.5), 100 μg of bovine serum albumin (BSA) and aliquots of the enzyme fractions (20–60 μg protein) in a final volume of

0.1 ml. After incubation at 37°C for 1–2 h, the reaction was terminated by the addition of 0.25 M glycine-NaOH (pH 10.4). 4-Methylumbelliferone released was determined fluorometrically as described previously [7]. With gangliosides as the substrate, the assay mixture was composed of bovine mixed gangliosides (100 nml as bound sialic acid), 5 μmol of sodium acetate (pH 4.7 or 5.5), 100 μg of BSA, 100 μg of Triton X-100 and enzyme fraction in 0.1 ml. After incubation for 1–2 h, the sialic acid released was determined by fluorometric high-performance liquid chromatography with 1,2-diamino-4,5-methylene dioxybenzene [14]. One unit of sialidase was defined as the amount of enzyme which catalyzed the release of 1 nmol of sialic acid/h.

Sialyltransferase assay. Sialyltransferase activities were determined under the optimal conditions described below. When asialo-(AS-) glycoproteins were the acceptors, the assay mixture contained 0.04 μmol of CMP-[^{14}C]NeuAc, 50–100 μmol of acceptor (expressed in terms of acceptor site), 5 μmol of sodium cacodylate (pH 6.3), 100 μg of Triton X-100 and enzyme (20–60 μg) in 0.1 ml. After incubation at 37°C for 1–2 h, protein-bound radioactivity was determined as described previously [11]. With glycolipid as acceptor, the assay mixture was the same as for glycoprotein acceptors except that 20–50 μmol of acceptor was used. The reaction product was measured by counting the radioactivity incorporated into the corresponding area comigrating with a standard in a thin-layer plate [11].

Flow cytometric analysis. Cells (10^6) were stained on ice for 30 min with FITC-labeled lectins (20 μg/ml). For immuno-fluorescence the cells were incubated for 1 h with anti-G_{M3} mAb (M2590). After washing twice with PBS containing 0.5% BSA, the cells were stained with FITC-conjugated goat anti-mouse IgG $F(ab')_2$ for 30 min on ice. They were then washed, suspended in 0.5 ml of cold PBS and analyzed using a FACS (Becton Dickinson, San Jose, CA, U.S.A.).

Thin-layer chromatography. Cells (10^7) harvested at subconfluency were washed with PBS and lyophilized. Glycolipids were extracted successively with 5 ml chloroform/methanol (C/M, 1:1, v/v), 2.5 ml C/M (2:1,v/v) and 2.5 ml C/M (1:2, v/v), and then evaporated to dryness. After desalting by dialysis, the glycolipids were again lyophilized, dissolved in a small volume of C/M (2:1, v/v) and 1/10 amounts were chromatographed on HPTLC plates (Baker, Phillipsburg, NJ, U.S.A.) in C/M/0.5% $CaCl_2$ (60:40:9, by vol.). Glycolipids were visualized with the orcinol-H_2SO_4 and resorcinol-HCl reagents, and quantified by densitometric scanning (Image Master; Pharmacia, Uppsala, Sweden).

RESULTS AND DISCUSSION

In the present study three B16 melanoma variants with different metastatic potentials were selected for determination of the levels of sialidase activities using 4MU-NeuAc or mixed gangliosides as substrates. The cells

Table 1. Sialidase activities in three metastatic variants of B16 melanoma

Substrate	4MU-NeuAc		Gangliosides
	Homogenate	Particulate	Homogenate
	(units/mg protein)		
B16-F1	62.5 ± 3.3	136.5 ± 24.2	1.64 ± 0.16
B16-F10	34.9 ± 6.3	72.9 ± 14.9	0.94 ± 0.19
B16-BL6	54.1 ± 1.6	91.8 ± 13.9	1.89 ± 0.17

Values are means ± S.D. of four different batches of cells.

showed sialidase activities towards 4MU-NeuAc and gangliosides at acidic pH in the particulate but not in the cytosolic fractions. As shown in Table 1, highly metastatic B16-F10 and B16-BL6 cells exhibited lower sialidase activity towards 4MU-NeuAc at acidic pH in either homogenates or particulate fractions than did the B16-F1 cells with low metastatic potential. The sialidase assayed under these conditions is a lysosomal-type sialidase, as demonstrated in previous studies [6]. The observed inverse relationship between its activity and the metastatic potential was similar to that we previously reported for transformed rat 3Y1 cell lines [9]. When mixed gangliosides were applied as the substrate in the presence of Triton X-100, the sialidase activity of B16-F10 was significantly lower than those of the other cells and only half the B16-BL6 value, although the absolute levels in these cells were very low so that a sensitive assay method using 1,2-diamino-4,5-methylene dioxybenzene was needed for their accurate estimation. Since the Triton X-100 activatable sialidase activity has been suggested to be localized in the plasma membrane [15, 16], the responsible enzyme probably was the plasma membrane form, with a less close relation to metastatic ability of the cells.

We next examined by flow cytometry with various FITC-conjugated lectins the question whether sialic acid containing molecules on the cell surface might differ among these cells (Fig. 1a). The lectins used here were SSA and MAM which preferentially recognize $\alpha 2 \rightarrow 6$ and $\alpha 2 \rightarrow 3$ sialyl linkages, respectively, and WGA showing affinity for clustered sialyloligosaccharide groups as well as for terminal N-acetyl-glucosamine. While the cells hardly reacted with SSA lectin, all of them demonstrated binding affinity for MAM and WGA lectins. B16-BL6 cells were found to react less with WGA than the other two cell lines but no differences in the binding to MAM were apparent. Similarly no variation in binding to RCA and PNA lectins was noted. When the

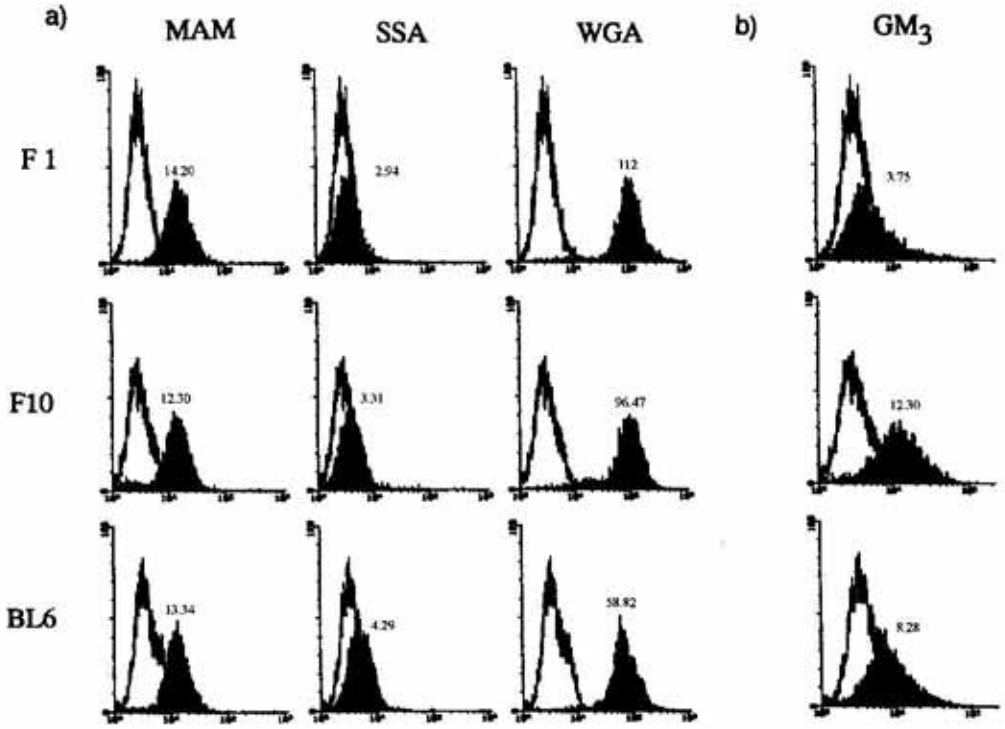

Figure 1. Flow cytometry analysis of three metastatic variants of B16 melanoma.

The numbers indicate the fluorescent intensity of the cells (black). Positivity was evaluated using FITC-conjugated isotype-matched controls (open white). For abbreviations of lectins, see "Materials and Methods"

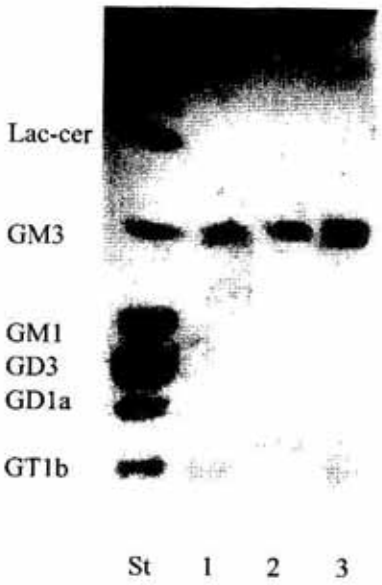

Figure 2. Thin-layer chromatography pattern of glycolipids extracted from the B16 melanoma variants (10^7 cells).

The glycolipids were analyzed on an HPTLC plate and visualized with resorcinol reagents. St, Standards; 1, B16-F1 cells; 2, B16-F10 cells; 3, B16-BL6 cells.

anti-G_{M3} antibody was used for flow cytometric analysis, B16-F10 and B16-BL6 cells, respectively, showed the reactivity 3- and 2-fold that of B16-F1 cells, which indicates that content of the G_{M3} accessible to the antibody was higher in highly metastatic cells (Fig.1b). To determine cellular G_{M3} levels, glycolipid extracts from the same number of the cells (10^7) were subjected to thin-layer chromatography. As shown in Fig. 2, G_{M3} was the major ganglioside detected by resorcinol staining in these cells. This level in B16-BL6 was the highest, with those for B16-F1 and B16-F10 cells being 65% and 57%, respectively, of the B16-BL6 value as quantified by densitometric analysis.

The activity of sialyltransferases was also compared using glycolipids and glycoproteins as acceptors, as shown in Fig. 3. The activity of B16-BL6 towards asialo-fetuin and -orosomucoid were by 50% and 70%, respectively, higher than in the case of B16-F1, while little variation was observed in the activities with asialo-submaxillary mucins. This indicates a specific increase in sialic acid transfer to N-linked glycoproteins in B16-BL6 cells. They also showed a marked increase in G_{M3} synthesis as compared with the other two cell lines.

To summarize the present data, the differences in the properties observed between cells with high and low metastatic potentials were as follows: (1) lysosomal-type sialidase activities were decreased in highly metastatic cells, in agreement with studies on transformed rat 3Y1 cells [9] and mouse colon adenocarcinoma cell lines (unpublished), and (2) the levels of cell surface G_{M3} accessible to anti-G_{M3}, on the other hand, were increased in the highly metastatic cells, although this was not paralleled by changes in total cellular G_{M3}. The highly metastatic B16-F10 and B16-BL6 cells were distinct from each other in terms of their ganglioside sialidase activity and cellular G_{M3} content. B16-F10 cells possessed more cell surface G_{M3} accessible to the antibody than B16-BL6 cells, despite a reverse order for total cellular G_{M3}. Although the molecular basis for this difference is unclear, it is possibly related to the fact that B16-BL6 cells are more invasive than B16-F10 cells. The differences in G_{M3} levels between the two highly metastatic cell lines are presumably due to the variation evident on the activities of ganglioside sialidase and G_{M3} synthase. The higher binding affinity of B16-F10 cells to the anti-G_{M3} may account for its reduced Triton X-100 activatable ganglioside sialidase that is thought to be responsible for hydrolysing surface G_{M3} in the plasma membrane. In contrast, less binding of B16-BL6 cells to the antibody, relative to B16-F10, may be the result of increased ganglioside sialidase, and increased G_{M3} synthase activity, on the other hand, would cause a higher cellular G_{M3} level, as analyzed by HPTLC.

The present finding that reduced lysosomal-type sialidase activity occurs together with increased G_{M3} content in highly metastatic B16 melanoma cells suggests that this may be a general characteristic of highly metastatic cells, at least of murine origin. It is also in

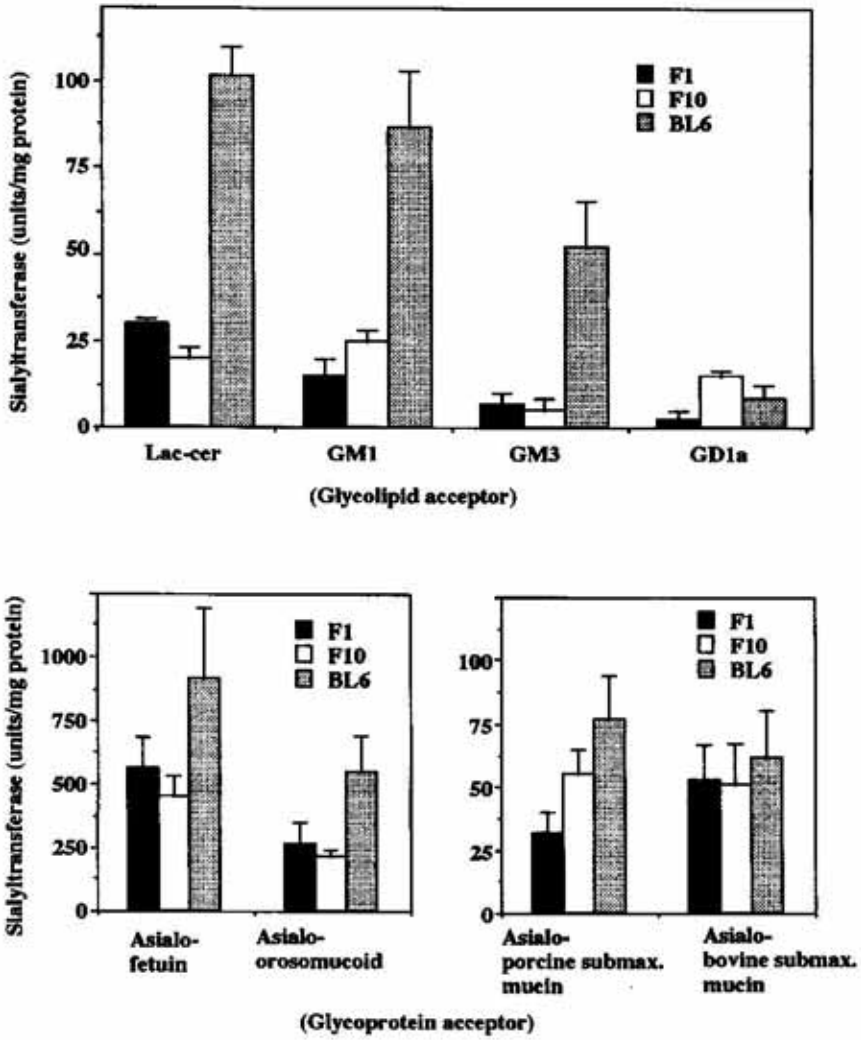

Figure 3. Sialyltransferase activities in three metastatic variants of B16 melanoma.

Activities are means ± S.D. of three batches of cells.

good agreement with the previous observation of suppression of metastasis in B16-BL6 cells overexpressing sialidase [10]. In this context, the results of transfection of sialidase genes of lysosomal and plasma membrane origins into metastatic cells would be very helpful for better understanding of the involvement of sialidases in metastasis.

REFERENCES

1. Yogeeswaran, G. & Salk, P. (1981) Metastatic potential is positively correlated with cell surface sialylation of cultured murine tumor cell lines. *Science* **212**, 1514–1516.

2. Tao, T.-W. & Burger, M.M. (1982) Lectin-resistant variants of mouse melanoma cells. I. Altered metastasizing capacity and tumorigenicity. *Int. J. Cancer* **29**, 425–430.

3. Fogel, M., Altevogt, P. & Schirrmacher, V. (1983) Metastatic potential severely altered by changes in tumor cell adhesiveness and cell-surface sialylation. *J. Exp. Med.* **157**, 371–376.

4. Collard, J.G., Schijven, J.F, Bikker, A., La Riviere, G., Bolscher, J.G.M. & Roos, E. (1986) Cell surface sialic acid and the invasive and metastatic potential of T-cell hybridomas. *Cancer Res.* **46**, 3521–3527.

5. Passaniti, A. & Hart, G.W. (1988) Cell surface sialylation and tumor metastasis. Metastatic potential of B16 melanoma variants correlated with their relative numbers of specific penultimate oligosaccharide structures. *J. Biol. Chem.* **263**, 7591–7603.

6. Miyagi, T. & Tsuiki, S. (1984) Rat-liver lysosomal sialidase. Solubilization, substrate specificity and comparison with the cytosolic sialidase. *Eur. J. Biochem.* **141**, 75–81.

7. Miyagi, T. & Tsuiki, S. (1985) Purification and characterization of cytosolic sialidase from rat liver. *J. Biol. Chem.* **260**, 6710–6716.

8. Miyagi, T., Sagawa, J., Konno, K., Handa, S. & Tsuiki, S. (1990) Biochemical and immunological studies on two distinct ganglioside-hydrolyzing sialidases from the particulate fraction of rat brain. *J. Biochem.* **107**, 787–793.

9. Miyagi, T., Sato, K., Hata, K. & Taniguchi, S. (1994) Metastatic potential of transformed rat 3Y1 cell lines is inversely correlated with lysosomal-type sialidase activity. *FEBS Lett.* **349**, 255–259.

10. Tokuyama, S., Moriya, S., Yasui, A., Miyazaki, J., Taniguchi, S. & Miyagi, T. (1997) Supression of pulmonary metastasis in mouse B16 melanoma cells. *Int. J. Cancer* **73**, 410–415.

11. Miyagi, T., Koseki, M. & Tsuiki, S. (1988) Comparative study of the levels of sialyltransferases responsible for the formation of sugar chains in glycoproteins and gangliosides in ray liver and hepatomas. *Jpn. J. Cancer Res.* **79**, 742–749.

12. Poste, G., Doll, J., Hart, I.R. & Fidler, I.J. (1980) *In vitro* selection of murine B16 melanoma variants with enhanced tissue invasive properties. *Cancer Res.* **40**, 1636–1644.

13. Fidler, I.J. (1973) Selection of successive tumor lines for metastasis. *Nature New Biol.* **242**, 148–149.

14. Hara, S., Yamaguchi, M., Takemori, Y. & Nakamura, M. (1986) Highly sensitive determination of *N*-acetyl- and *N*-glycolylneuraminic acids in human serum and urine and rat serum by reversed-phase liquid chromatography with fluorescence detection. *J. Chromatogr.* **377**, 111–119.

15. Lieser, M., Harms, E., Kern, H., Bach, G. & Cantz, M. (1989) Ganglioside G_{M3} sialidase activity in fibroblasts of normal individuals and of patients with sialidosis and mucolipidosis IV. *Biochem. J.* **260**, 69–74.

16. Kopitz, J., von Reitzenstein, C., Sinz, K. & Cantz, M. (1996) Selective ganglioside desialylation in the plasma membrane of human neuroblastoma cells. *Glycobiology* **6**, 367–376.