*Glycoconjugate Journal* (1996) **13:** 461–469

# Microscale analysis of glycosphingolipids by TLC blotting/secondary ion mass spectrometry: a novel blood group A-active glycosphingolipid and changes in glycosphingolipid expression in rat mammary tumour cells with different metastatic potentials

T. KASAMA[1], Y. HISANO[2], M. NAKAJIMA[3], S. HANDA[2] and T. TAKI[2]*

[1]*Laboratory for Biomedical Analysis, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku Tokyo 113, Japan*
[2]*Department of Biochemistry, Faculty of Medicine, Tokyo Medical and Dental University, Bunkyo-ku Tokyo 113, Japan*
[3]*Institute of Molecular Cellular Biology, Tokyo University, Bunkyo-ku Tokyo 113, Japan*

Received 7 July 1995, revised 30 November 1995

The glycosphingolipid compositions of rat mammary tumour cell lines with different metastatic potentials for the lung [a parental tumour cell line (MTC) and its subclones MTLn2 (a non metastatic subclone) and MTLn3 (a subclone with high metastatic potential to the lung)] were studied using a newly developed TLC blotting/secondary ion mass spectrometry system and crude glycosphingolipids obtained from 0.5–1 $\times 10^7$ cells of each cell line. $G_{M3}$ and $G_{M2}$ were the major components of the MTC cell line, but they were very minor components in the MTLn2 and MTLn3 cell lines, $G_{D1a}$ being the major ganglioside. HexNAc-fucosyl-$G_{M1a}$ was found in the MTLn2 cells by the TLC blotting/SIMS method, and the terminal sugar linkage was shown to be a blood group A-type structure by immunostaining. These findings suggest that the ganglioside is a novel type of blood group A-active ganglioside, GalNAc$\alpha$1-3(Fuc$\alpha$1-2)$G_{M1a}$. No blood group A-active lipid was present in MTLn3 cells, whereas Hex-$G_{M1a}$ and neutral glycosphingolipids with more than 5 sugar residues were.

*Keywords*: TLC blotting/SIMS, blood group A-active ganglioside, glycosphingolipids, metastasis, mammary tumour cell lines

*Abbreviations*: TLC, thin-layer chromatography; HPTLC plate, high performance thin-layer chromatography-plate; PVDF, polyvinylidene difluoride; SIMS, secondary ion mass spectrometry; GC-MS, gas chromatography-mass spectrometry; C16:0, hexadecanoic acid; C18:0, octadecanoic acid; C22:0, docosanoic acid; C24:0, tetracosanoic acid; d18:1, 2-amino-4-octadecene-1,3-diol; Hex, hexose; HexNAc, *N*-acetylhexosamine; Gal, galactose; Glc, glucose; GalNAc, *N*-acetylgalactosamine; Lac, lactose; NeuAc, *N*-acetylneuraminic acid; Cer, ceramide; Glob, globoside; iGlob, isogloboside; GlcCer, glucosylceramide; LacCer, lactosylceramide; $Gb_3$Cer, Gal$\alpha$1-4Gal$\beta$1-4Glc$\beta$1-1Cer; $Gb_4$Cer (Glob), GalNAc$\beta$1-3Gal$\alpha$1-4Gal$\beta$1-4Glc$\beta$1-1Cer; $iGb_3$Cer, Gal$\alpha$1-3Gal$\beta$1-4Glc$\beta$1-1Cer; $iGb_4$Cer (iGlob), GalNAc$\beta$1-3Gal$\alpha$1-3Gal$\beta$1-4Glc$\beta$1-1Cer; Ganglio-series gangliosides are named according to Svennerholm [1].

## Introduction

Glycosphingolipids in the cell membrane function in cell-cell adhesion through carbohydrate-carbohydrate interaction [2], the regulation of cell adhesion [3], and adhesion events mediated by selectin family molecules [4]. Their carbohydrate sequences change in association with oncogenic transformation [5–7] and cell differentiation [8–10]. These findings suggest that glycosphingolipids expressed on tumour cell membranes are involved in

*To whom correspondence should be addressed.

the metastatic process, e.g. in adhesion to the target tissue. To clarify the changes in glycosphingolipid composition that occur during the metastatic mutation of tumour cells, we studied the glycosphingolipid composition of a rat mammary tumour cell line (MTC), originally derived from an 13762 NF mammary adenocarcinoma, and its subclones MTLn2 and MTLn3. These cell lines were cloned from metastatic colonies in lung tissue. MTLn2 has low metastatic potential and MTLn3 high metastatic potential for the lung [11].

We used a newly developed TLC blotting/secondary ion mass spectrometry (TLC blotting/SIMS) system [12] for the glycosphingolipid analysis. Glycosphingolipids separated on an HPTLC plate were transferred to a PVDF membrane sheet and analysed directly by secondary ion mass spectrometry (SIMS). The major glycosphingolipids transferred to the membrane were subjected directly to methylation analysis using gas chromatography mass spectrometry (GC-MS). We here report changes in glycosphingolipid expression in association with metastatic potential and discuss the advantage of this TLC blotting procedure for the microscale analysis of glycosphingolipids.

## Materials and methods

### *Cells*

Cell lines derived from a 13762 NF rat mammary adenocarcinoma growing at the local site of implantation (MTC) and from its spontaneous lung metastasis, MTLn2 and MTLn3, were used [11]. The MTLn2 and MTLn3 cell lines were cloned from lung metastatic colonies. Cells were grown in a humidified incubator (5% $CO_2$-95% air) at 37 °C on 100 mm tissue culture plates containing RPMI 1640 supplemented with 10% FCS. Trypsin (0.05%) in phosphate-buffered saline (PBS) containing 0.53 mM EDTA was used to passage the cells.

### *Glycosphingolipid preparation*

Approximately $4 \times 10^8$ cells of MTC, $8 \times 10^8$ cells of MTLn2, and $8 \times 10^8$ cells of MTLn3 were collected for glycosphingolipid extraction. The cells were suspended in 1 ml of distilled water, to which 50 ml of a mixture of chloroform:methanol (2:1, by vol) was added. Lipids were extracted by stirring the mixture at 4 °C for 12 h, then filtering it through a glass wool-packed Pasteur pipette and evaporating the filtrate to dryness. Total glycosphingolipids were obtained by the method of Saito and Hakomori [13].

### *Thin-layer chromatography (TLC)*

Glycosphingolipids were separated by TLC on precoated HPTLC plates (HPTLC plate silica gel 60, E. Merck, Germany). The solvent systems were Solvent A, chloroform:methanol:0.2% $CaCl_2$ (60:35:8, by vol), Solvent B, chloroform:methanol:0.2% $CaCl_2$ (55:45:10, by vol). Gangliosides were made visible by spraying the plate with resorcinol/HCl reagent. Neutral glycosphingolipids were made visible by spraying the plates with the orcinol/$H_2SO_4$ reagent.

### *TLC blotting*

Glycosphingolipids (unknowns and standards) that separated on the HPTLC plate were transferred to a PVDF membrane by TLC blotting [14, 15]. The developed HPTLC plate was immersed in a blotting solvent mixture of isopropanol:0.2% $CaCl_2$:methanol (40:20:7, by vol) for 20 s, after which it was placed on a glass plate and covered with a PVDF membrane (Clear Blot Membrane-P, ATTO Co. Ltd, Japan) and a glass micro fibre filter (glass microfibre filters GF/A, Whatman, International Ltd, England). The whole was pressed for 30 s with a 180 °C iron, after which the PVDF membrane was separated from the plate and dried.

### *TLC blotting/SIMS*

Glycosphingolipids developed on the HPTLC plate were sprayed with primuline reagent and detected under ultraviolet light (365 nm). The visible bands were marked with a coloured drawing pencil. The glycosphingolipids then were blotted on a PVDF membrane as described above, the coloured markings on the plate being transferred with the glycosphingolipids. Each marked glycosphingolipid was punched out and placed on a SIMS target tip of a mass spectrometer with 1 µl of triethanolamine as the matrix. Negative secondary ion mass spectrometry (SIMS) spectra were obtained with a TSQ 70 triple quadrupole mass spectrometer (Finnigan MAT, USA). The glycosphingolipid on the membrane was bombarded with a $Cs^+$ beam accelerated at 20 kV. The ion multiplier was 1.5 kV, and the conversion dynode 20 kV.

### *Densitometric determination of glycosphingolipids*

Glycosphingolipids were subjected to TLC, and the neutral glycosphingolipids and gangliosides detected as described above. Densitometric glycosphingolipid determinations were done with a Densitograph AE 6920-M (ATTO Co. Ltd, Japan) using neutral glycosphingolipids and $G_{M3}$ ganglioside standards.

### *Methylation analysis of glycosphingolipid*

The glycosphingolipids that separated were made visible by spraying the HPTLC plate with primuline reagent. The major neutral glycosphingolipid bands were marked using a coloured drawing pencil then transferred to a PVDF membrane as described above. The marked area on the membrane then was excised and immersed in distilled water to remove the primuline reagent. The excised

membrane was transferred to a small tube and methylated by the method of Mansson *et al.* [16]. Methylated glycosphingolipids released into the methylation solvent were extracted with chloroform then hydrolysed. The methylated carbohydrates then were converted to partially methylated alditol acetates as described elsewhere [17]. The alditol acetates were analysed by GC-MS using a Varian 3400 gas chromatograph interfaced to a TSQ70 triple quadrupole mass spectrometer (Finnigan MAT, USA). The gas chromatography column was a fused silica capillary column DB-5 (0.25 mm i.d. × 30 m, J & W Scientific, USA). The temperature was programmed from 140 °C to 250 °C at the rate of 4 °C per min.

*TLC immunostaining*

TLC immunostaining was done as described elsewhere [18]. Anti-blood group A antibody (rabbit IgG) was used as the first antibody, and peroxidase conjugated anti-rabbit IgG antibody (sheep) (Cappel product, purchased from Cosumo Biochem. Co. Ltd, Japan) as the second antibody. A Konica Immunostain Kit (Konica Co. Inc., Japan) was used as the staining reagent.

## Results and discussion

*TLC profiles of glycosphingolipids in rat mammary tumour cell lines with different metastatic potentials*

TLC profiles of the total glycosphingolipids from rat mammary tumour cell lines with different metastatic potentials for the lung are shown in Fig. 1. MTC has two major resorcinol positive bands that correspond to $G_{M3}$ and $G_{M2}$, also a minor band that moves slightly faster than the standard $G_{D1a}$. Only faint bands corresponding to $G_{M3}$ and $G_{M2}$ are present in the MTLn2 and MTLn3 cells, the double bands near the standard $G_{D1a}$ band being the major gangliosides. In addition, there are several bands between standard $G_{M1a}$ and $G_{D1a}$ in these subcloned cell lines. In MTLn3, orcinol-positive double bands are present just above the standard $G_{M3}$ ganglioside, identified as isogloboside (below).

*Structural analysis of the glycosphingolipids by TLC blotting/SIMS*

Glycosphingolipid fractions of all three cell lines were subjected to TLC blotting and then used for the SIMS analysis. The glycosphingolipid pattern of MTC cells was relatively simple compared with the patterns of MTLn2 and MTLn3 cells (Fig. 1). The total glycosphingolipids obtained from $5 \times 10^6$ or $1 \times 10^7$ MTLn2 and MTLn3 cells were applied to an HPTLC plate and made visible with ultraviolet light after being sprayed with primuline reagent (Fig. 2a). These bands were marked with a coloured drawing pencil and transferred to a PVDF membrane (Fig. 2b). Thirteen bands were present in the MTLn2 cells and 19 in the MTLn3 cells. Eight bands were found for the MTC glycosphingolipids (data not shown). The solid circles on the marked bands show the areas punched out for SIMS analysis.

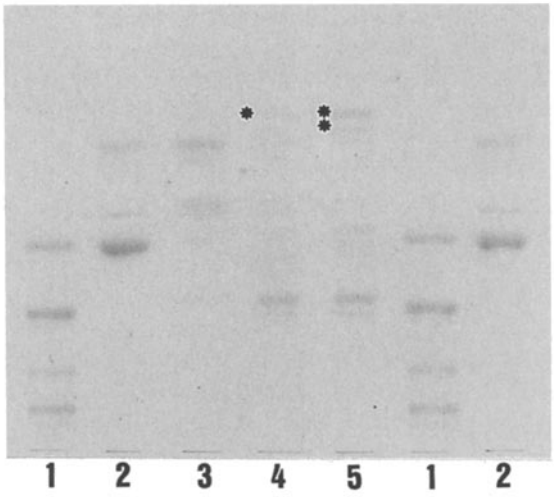


**Figure 1.** TLC profile of the glycosphingolipids from three rat mammary tumour cell lines. Lane 1: ganglioside mixture; from top to bottom, $G_{M1a}$, $G_{D1a}$, $G_{D1b}$, and $G_{T1b}$. Lane 2: standard gangliosides; from top to bottom, $G_{M3}$, $G_{M2}$, and $G_{M1a}$. Lanes 3, 4, and 5: the respective glycosphingolipids of MTC, MTLn2, and MTLn3. Glycosphingolipids were developed with solvent B. Gangliosides were made visible by spraying them with the resorcinol reagent. Glycosphingolipids were made visible with the orcinol reagent. * shows bands made visible only with the orcinol reagent.

*MTC cell*

Typical SIMS spectra of the glycosphingolipids in the three cell lines are shown in Figs 3, 4, and 6. (All references to band numbers are based on Fig. 2). In the SIMS spectra the major double bands of MTC cells have a mobility similar to that of the standard $G_{M3}$ ganglioside (Fig. 3). The SIMS spectrum in Fig. 3a clearly shows that the upper band is $G_{M3}$ ganglioside with a C24:0 fatty acid and d18:1 long chain base in its ceramide moiety. The SIMS spectrum of the lower band (Fig. 3b) is consistent with that of $G_{M3}$ with a ceramide moiety that has C16:0 and d18:1. These fragmentation patterns are strong evidence that the sugar sequence is NeuAc-Hex-Hex-Cer.

*MTLn2 cell*

The characteristic SIMS spectra of glycosphingolipids from MTLn2 cells are shown in Fig. 4. The spectra of bands 3 and 4 (not shown) indicate that the sugar sequences of these glycosphingolipids are HexNAc-Hex-Hex-Hex-. The difference in their mobilities on TLC is assumed to be due to the difference in their fatty acid constituents; C24:0 in the upper and C16:0 in the lower band. Two mol of 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylga-

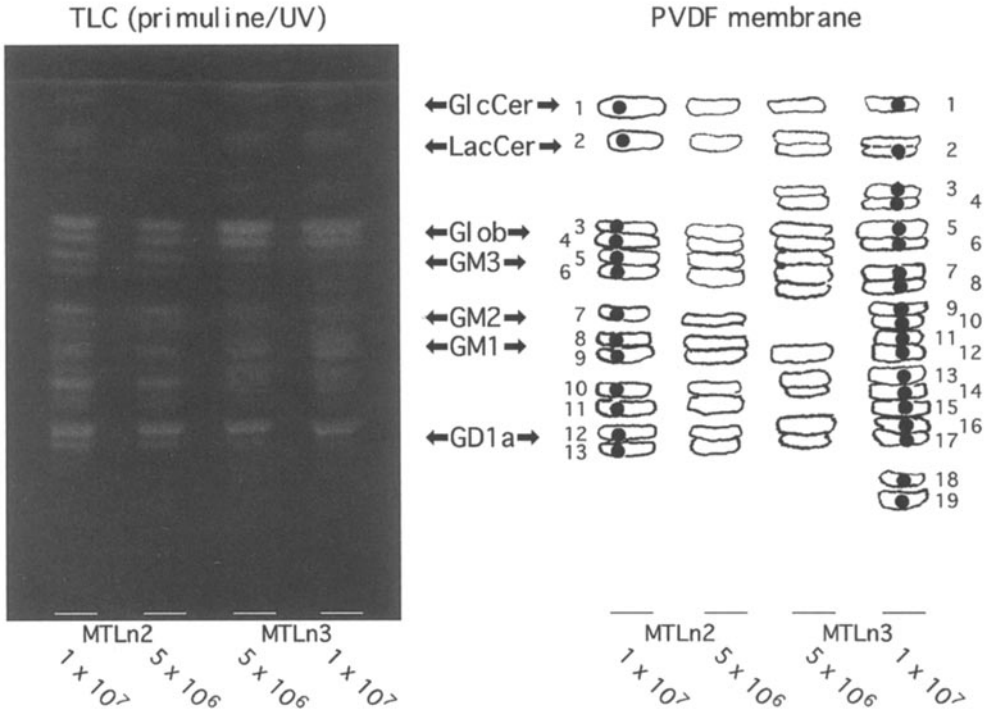


**Figure 2.** TLC profile of glycosphingolipids from MTLn2 and MTLn3 cells. (A) Glycosphingolipids developed with solvent B and viewed under ultraviolet light after being sprayed with primuline reagent. Glycosphingolipids from $1 \times 10^7$ (lane 1) and $5 \times 10^6$ (lane 2) cells of MTLn2. Glycosphingolipids from $5 \times 10^6$ (lane 3) and $1 \times 10^7$ (lane 4) cells of MTLn3. (B) Photocopy of the PVDF membrane after TLC blotting/SIMS. The thin outlines are prints of the coloured pencil markings on the HPTLC plate. The solid circles in panel B show the punched out areas used for the SIMS analysis.

lactitol, 1 mol of 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylglucitol, and *N*-acetyl-1,5-di-*O*-acetyl-3,4,6-tri-*O*-methylgalactosaminitol were found in the sugar linkage analysis of both bands by methylation. These results indicate that these glycosphingolipids are isoglobosides. Bands 5 and 6 were identified as $G_{M3}$ from their SIMS spectra (Fig. 4a

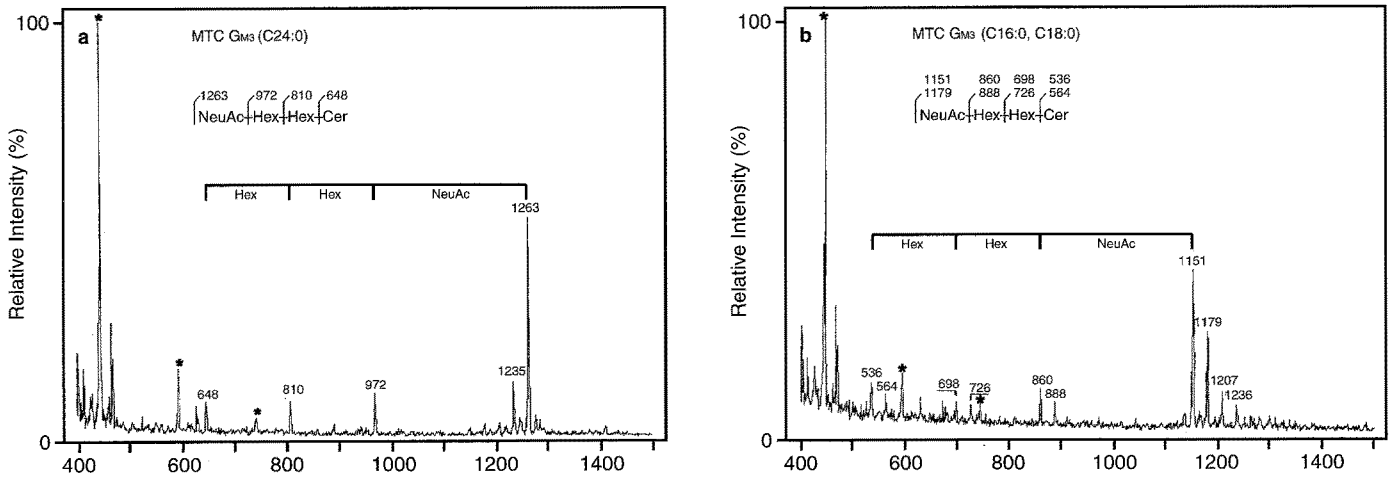


**Figure 3.** SIMS spectra of the major MTC cell glycosphingolipids that migrated similar to the migration of standard $G_{M3}$. (a) SIMS spectrum of the upper major glycosphingolipid band. The *m/z* 1235 and 1263 ions are the deprotonated molecules of $G_{M3}$ with, respectively, C22:0/d18:1 and C24:0/d18:1 in the ceramide moiety. (C22:0/d18:1 means the pairing of C22:0 fatty acid and a d18:0 long chain base.) The interpretation of the fragment ions is shown. (b) SIMS spectrum of the lower major glycosphingolipid band. The *m/z* 1151, 1179, 1207, and 1235 ions are the deprotonated molecules of $G_{M3}$ with, respectively, C16:0/d18:1, C18:0/d18:1, C20:0/d18:1, and C22:0/d18:1 in the ceramide moiety. The interpretation of the fragment ions is shown. The ions marked * are the cluster ions of the matrix.

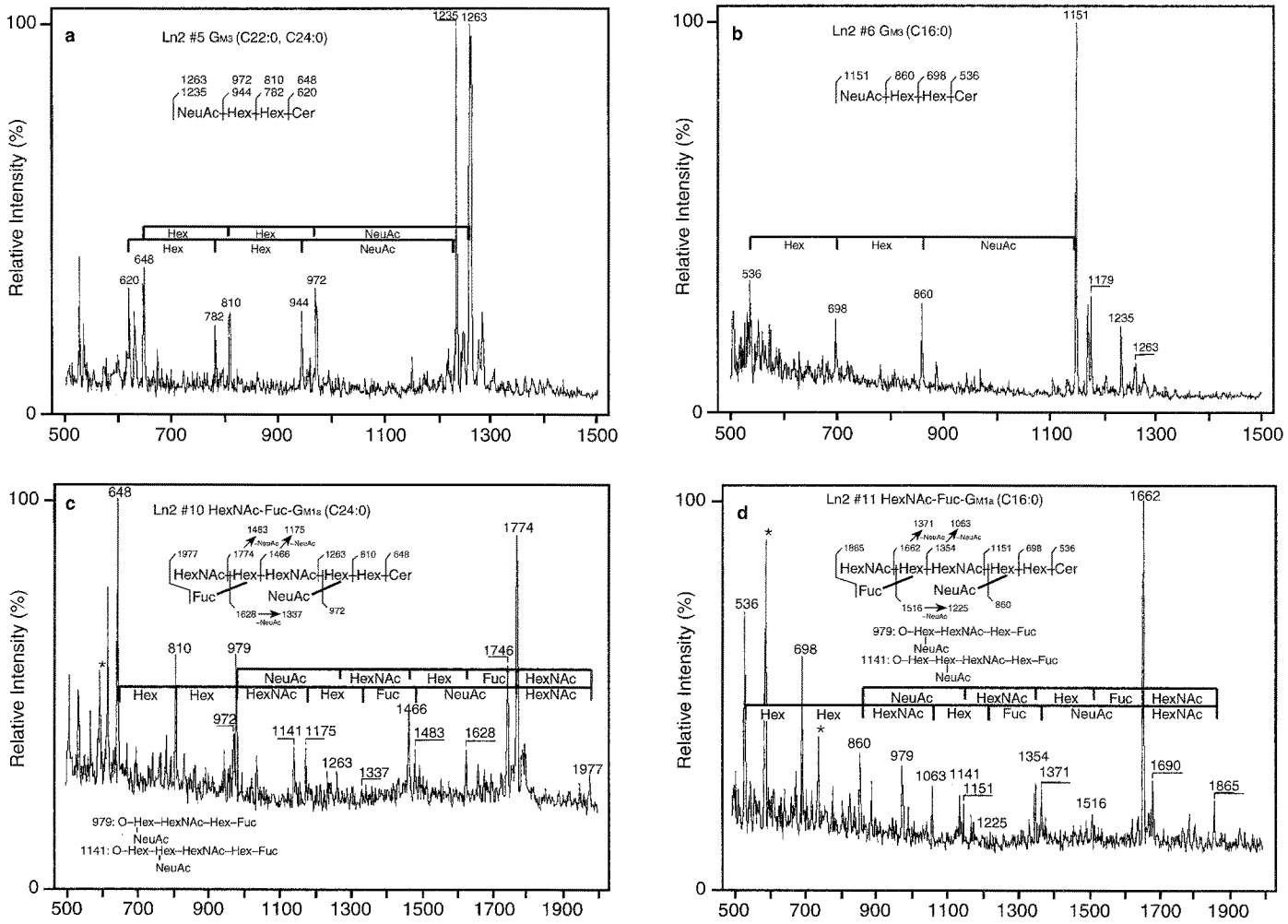


**Figure 4.** Characteristic SIMS spectra of the glycosphingolipids in MTLn2 cells. (a) SIMS spectrum of MTLn2 cell band 5. The *m/z* 1235 and 1263 ions are deprotonated molecules of $G_{M3}$ with, respectively, C22:0/d18:1 and C24:0/d18:1 in the ceramide moiety. (C22:0/d18:1 means the pairing of C22:0 fatty acid and a d18:0 long chain base.) The interpretation of the fragment ions is shown. (b) SIMS spectrum of MTLn2 cell band 6. The *m/z* 1151, 1179, 1235, and 1263 ions are deprotonated molecules of $G_{M3}$ with, respectively, C16:0/d18:1, C18:0/d18:1, C22:0/d18:1, and C24:0/d18:1 in the ceramide moiety. The interpretation of the fragment ions is shown. (c) SIMS spectrum of MTLn2 cell band 10. The *m/z* 1977 ion is the deprotonated molecule of GalNAc-fucosyl-$G_{M1}$ with C24:0/d18:1 in its ceramide moiety. The *m/z* 1746 ion corresponds to the fragment ion-released hexasamine residue of the deprotonated molecule of GalNAc-fucosyl-$G_{M1}$ with C22:0/d18:1 in its ceramide moiety. The interpretation of the fragment ions is shown. (d) SIMS spectrum of MTLn2 cell band 11. The *m/z* 1865 ions is the deprotonated molecule of GalNAc-fucosyl-$G_{M1}$ with C16:0/d18:1 in its ceramide moiety. The interpretation of the fragment ions is shown. Ions marked * are the cluster ions of the matrix.

and 4b). They have different fatty acids in their ceramide moieties that are similar to the difference in bands 3 and 4. The fragmentation patterns of the spectra are the same as those of the $G_{M3}$ from MTC cells (Fig. 3a and 3b). The SIMS spectra of bands 7 and 8 (not shown) indicate that these two glycosphingolipids are $G_{M2}$ ganglioside and have different fatty acid constituents in their ceramide moieties; C24:0 in band 7 and C16:0 in band 8. As shown in Fig. 1, only faint bands corresponding to $G_{M3}$ and $G_{M2}$ are detectable for the glycolipids of MTLn2 and MTLn3 cells; whereas, TLC blotting/SIMS clearly shows their presence and molecular species. The combination of visualization with primuline and SIMS analysis of the glycosphingolipid on a PVDF membrane therefore makes it possible to determine very small amounts of glycosphingolipids. The spectrum of band 9 (not shown) corresponds to $G_{M1a}$.

Glycosphingolipid bands 10 and 11 are shown in Fig. 4c and d. The fragmentation profiles are the same. The major peak at *m/z* 1774 (Fig. 4c) and 1662 (Fig. 4d) respectively correspond to fucosyl-$G_{M1}$ with C24:0 and C16:0 in the ceramide moieties. The signals at *m/z* 1978 (Fig. 4c) and 1866 (Fig. 4d), however, suggest that HexNAc may be attached to fucosylated $G_{M1a}$. We therefore investigated the presence of the HexNAc residue at the nonreducing end of fucosyl $G_{M1a}$ using

immunostaining with anti-blood group A antibody. The results (Fig. 5) support this assignment and indicate that the epitope structure of these glycosphingolipids is the same as that of blood group A. The ions of *m/z* 979 and 1141 are fragment ions that correspond to the oligosaccharide moieties of the glycosphingolipids. On the basis of these findings, the ganglioside structure is proposed to be a novel type of blood group A-active ganglioside with the structure, GalNAc$\alpha$1-3(Fuc$\alpha$1-2)Gal$\beta$1-3GalNAc$\beta$1-4(NeuAc$\alpha$2-3)Gal$\beta$1-4Glc$\beta$1-1Cer. This demonstrates that TLC blotting/SIMS analysis of very small amounts of ganglioside provides useful information for structural determinations. The amounts of glycosphingolipid bands 10 and 11 applied to the HPTLC plate were less than 0.5 μg, and about one-fourth of the band (excised area vs total band area) was used for the SIMS analysis (Fig. 2b). SIMS spectra of glycosphingolipid bands 12 and 13 (not shown) show that these are $G_{D1a}$ with different fatty acid constituents in the ceramide moiety. The deprotonated molecule of a disialoganglioside generally is detected as a monosodium salt of the lipid in negative SIMS. In our study, the deprotonated molecules of bands 12 and 13 were shown to be monosodium salt forms.

*MTLn3 cell*

Characteristic SIMS spectra of the glycosphingolipids from MTLn3 cells are shown in Fig. 6. The spectrum of band 1 (not shown) indicates that the glycosphingolipids are a monohexosylceramide (probably GlcCer) with

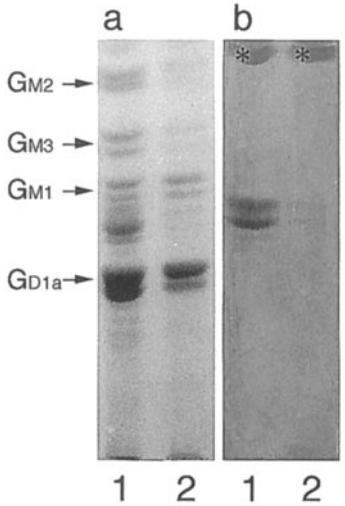


**Figure 5.** Immunostaining of MTLn2 glycosphingolipids with anti-blood group A antibody. Glycosphingolipids from MTLn2 (lane 1) and MTLn3 (lane 2) cells were separated on an HPTLC plate. (a) TLC of the MTLn2 glycosphingolipids. Bands were made visible by spraying the plate with the resorcinol reagent. (b) Glycosphingolipid immunostaining with anti-blood group A antibody. Solvent B was used for the developing. * shows nonspecific bands. Arrows indicate the migration positions of the standard gangliosides.

different fatty acid constituents in the ceramide moiety. The spectrum of band 2 (not shown) corresponds to a dihexosylceramide (probably LacCer) with a C16:0 fatty acid. Bands 3 and 4 were identified by their SIMS spectra (not shown) as trihexosylceramides with different fatty acid constituents in their ceramide moieties. These neutral glycosphingolipids were only faintly visible when orcinol reagent was used (Fig. 1a), further evidence of the very sensitive detection and identification of the glycosphingolipids provided by the present method. Bands 5 and 6 were identified as isoglobosides from their SIMS spectra (not shown) and by methylation. The glycosphingolipid of band 7 had a fragmentation profile (not shown) similar to that of $G_{M3}$ with the C24:0 fatty acid. The spectrum of band 8, shown in Fig. 6a, suggests that this band is composed of pentaglycosylceramide (Hex-Hex-HexNAc-Hex-Hex-Cer) with the C24:0 fatty acid and $G_{M3}$ with the C16:0 fatty acid in the ceramide moiety. The signal intensity of the deprotonated molecule *m/z* 1499 is similar to that of $G_{M3}$, indicative that the amount of pentaglycosylceramide is similar to that of $G_{M3}$. Bands 10 and 11 are identified as $G_{M2}$ with different fatty acid constituents (data not shown). The SIMS spectrum of band 12 (not shown) shows that the glycosphingolipid is $G_{M1a}$ with the C24:0 fatty acid, and the spectrum in Fig. 6b shows that band 13 contains $G_{M1a}$ with the C16:0 and Hex-$G_{M1}$ with the C24:0 fatty acid. The possible assignment is indicated in the spectrum. The SIMS spectrum in Fig. 6c suggests that band 14 is a mixture of Hex-$G_{M1a}$ with the C16:0 fatty acid and heptaglycosylceramide with the C24:0 fatty acid. Bands 16 and 17 correspond to $G_{D1a}$ with C24:0 and C16:0 fatty acid, respectively, as the major component of the ceramide moiety (data not shown).

These findings for the total glycosphingolipid fractions suggest the presence of neutral glycosphingolipids that have longer sugar chains than isogloboside. We therefore tried to isolate the neutral glycosphingolipids with long sugar chains. Each glycosphingolipid fraction first was separated into a neutral and an acidic fraction using a DEAE Sephadex A-25 column then subjected to TLC. The profiles are shown in Fig. 7. The amounts of the glycosphingolipids used for TLC in Fig. 7 were about five times the amount in Fig. 1. In all three cell lines, there are three orcinol-positive bands that move more slowly on the HPTLC plate than the major isogloboside (Fig. 7a). They appear to be the glycosphingolipids detected in the SIMS spectra together with the gangliosides. These neutral glycosphingolipids and gangliosides were used for TLC blotting/SIMS. The SIMS spectra obtained showed the presence of the predicted glycosphingolipids and confirmed their sugar sequences (data not shown).

We have compared the glycosphingolipid compositions of MTC (a rat parental mammary tumour cell line), MTLn2 (a non metastatic subclone) and MTLn3

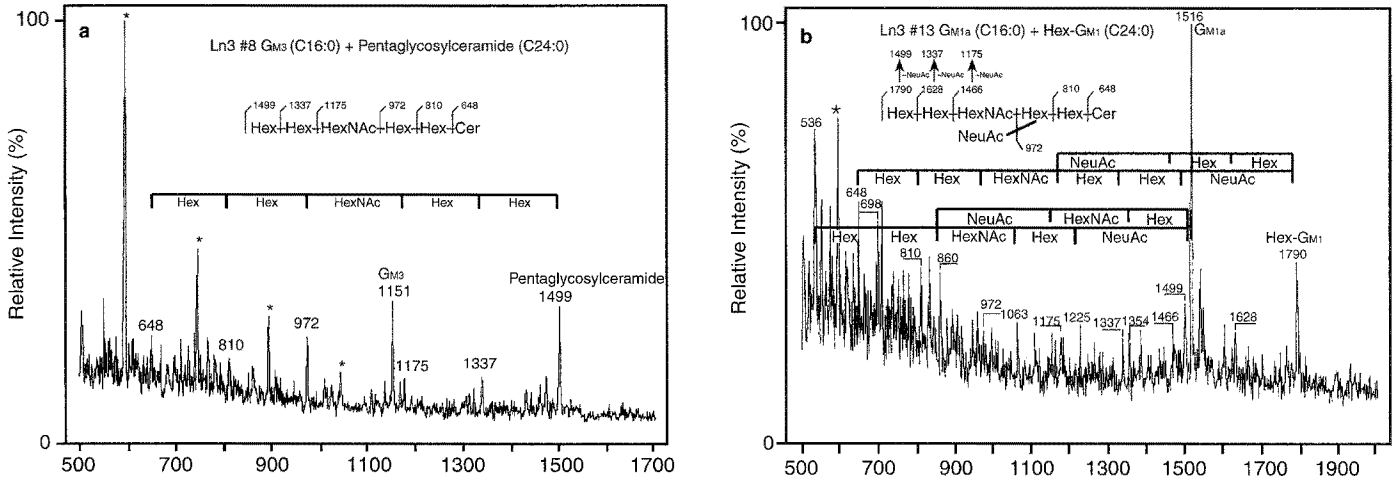


**Figure 6.** Characteristic SIMS spectra of the MTLn3 glycosphingolipids. (a) SIMS spectrum of MTLn3 cell band 8. The *m/z* 1499 ion is the deprotonated molecule of pentaglycosylceramide with C24:0/d18:1 in its ceramide moiety. (C24:0/d18:1 means the pairing of C24:0 fatty acid and a d18:0 long chain base.) The *m/z* 1151 ion is the deprotonated molecule of $G_{M3}$ with C16:0/d18:1 in its ceramide moiety. The interpretation of the fragment ions of pentaglycosylceramide is shown. (b) SIMS spectrum of MTLn3 cell band 13. The *m/z* 1790 ion is the deprotonated molecule of Hex-$G_{M1}$ with C24:0/d18:1 in its ceramide moiety. The *m/z* 1516 ion is the deprotonated molecule of $G_{M1}$ with C16:0/d18:1 in its ceramide moiety. The interpretation of the fragment ions of Hex-$G_{M1}$ is shown. (c) SIMS spectrum of MTLn3 cell band 14. The *m/z* 1678 ion is the deprotonated molecule of Hex-$G_{M1}$ with C16:0/d18:1 in its ceramide moiety. The *m/z* 1864 ion is the deprotonated molecule of heptaglycosylceramide with C24:0/d18:1 in its ceramide moiety. The interpretations of the fragment ions of Hex-$G_{M1}$ and the ion of heptaglycosylceramide are shown. Ions marked * are the cluster ions of the matrix.

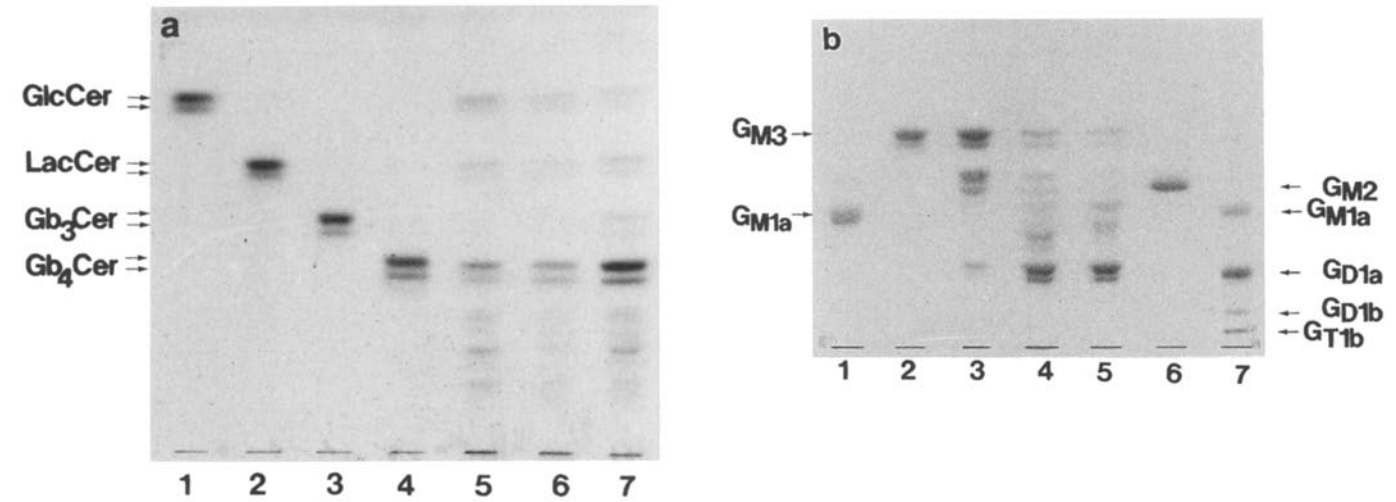


**Figure 7.** TLC profiles of neutral and acidic glycosphingolipids from the three cell lines. (a) Neutral glycosphingolipid fractions from all three cell lines. Lane 1, GlcCer; lane 2, LacCer; lane 3, $Gb_3Cer$; Lane 4, $Gb_4Cer$ (Glob); lanes 5, 6, and 7 are the respective neutral glycosphingolipids from the MTC, MTLn2, and MTLn3 cells. Bands were made visible by spraying the plate with the orcinol reagent. Solvent A was the developer. (b) TLC of the acidic glycosphingolipids from the three cell lines. Lane 1, $G_{M1a}$; lane 2, $G_{M3}$; lanes 3, 4 and 5 are the respective acidic glycosphingolipids from the MTC, MTLn2, and MTLn3 cells; lane 6, $G_{M2}$; and lane 7, the ganglioside mixture. Bands were made visible by spraying the plate with the resorcinol reagent. Solvent B was the developer.

(a subclone with high metastatic potential for the lung) using our newly developed TLC blotting/SIMS method. The major gangliosides in the parental cell line, $G_{M3}$ and $G_{M2}$, were markedly low in the two subclones, in which $G_{D1a}$ was the major ganglioside. There is a distinct difference, however, in the ganglioside compositions of MTLn2 and MTLn3. In the MTLn2 cells we found a new type of ganglioside which we characterized as a blood group A-active glycolipid with a $G_{M1}$ backbone structure. In contrast, we found no blood group A ganglioside in the subclone with high metastatic potential for the lung, but did find Hex-$G_{M1}$. The appearance of Hex-$G_{M1}$ in the MTLn3 cell line seems to be correlated with its metastatic properties for the lung. Moreover, its presence in the metastatic tumour cell line suggests there is a membrane component in the lung that has affinity for this ganglioside. Using our novel analytical method, we could characterize certain candidate molecules that are involved in special functions, such as the metastatic property. The method as described here offers a very powerful and useful means of elucidating which glycosphingolipids are involved in cell functions. The major results of our study are presented in Table 1.

The ganglioside compositions of the three cell lines are given in Fig. 8. The analytical data shown is for 0.5–$1.0 \times 10^7$ cells. It is virtually impossible to obtain detailed information on individual glycosphingolipids from such a small amount of cultured cells when the ordinary silica gel column chromatography method for glycosphingolipid purification is used. Usually, silica

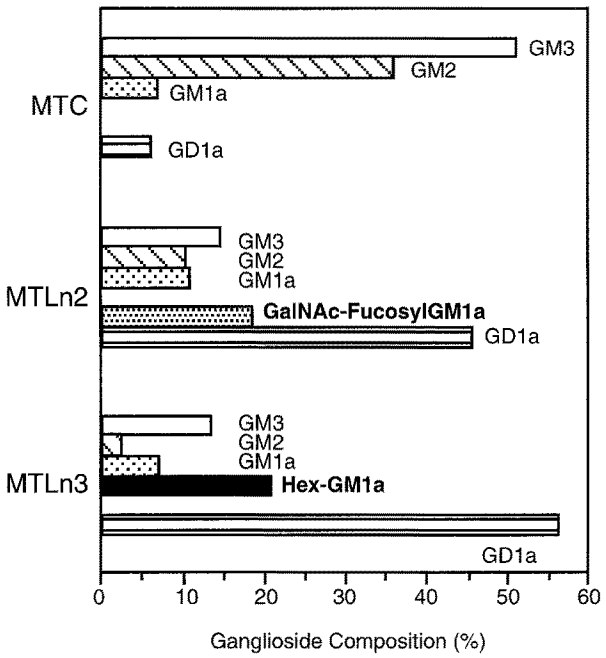


**Figure 8.** Ganglioside compositions of rat mammary tumour cell lines with different metastatic potentials.

**Table 1.** Glycosphingolipids in MTLn2 and MTLn3 cells identified by TLC blotting/SIMS.

| *MTLn2 cell* | | *MTLn3 cell* | |
|---|---|---|---|
| *Band no. in Fig. 2b* | *Sugar sequence (fatty acid)* | *Band no. in Fig. 2b* | *Sugar sequence (fatty acid)* |
| 1 | Hex-Cer (16:0 18:0 22:0 24:0) | 1 | Hex-Cer (16:0 18:0 22:0 24:0) |
| 2 | Hex-Hex-Cer (16:0 18:0 22:0 24:0) | 2 | Hex-Hex-Cer (16:0) |
| 3 | HexNAc-Hex-Hex-Hex-Cer(iGlob) (24:0) | 3 | Hex-Hex-Hex-Cer (24:0) |
| 4 | iGlob (16:0) | 4 | Hex-Hex-Hex-Cer (16:0) |
| 5 | GM3 (24:0 22:0) | 5 | HexNAc-Hex-Hex-Hex-Cer (iGlob) (24:0) |
| 6 | GM3 (16:0) | 6 | iGlob (16:0) |
| 7 | GM2 (14:0) | 7 | GM3 (24:0) |
| 8 | GM2 (16:0) | 8 | $Hex_2$-HexNAc-Hex-Hex-Cer (24:0) |
| 9 | GM1a (16:0 24:0) | 8 | GM3 (24:0) |
| 10 | GalNAc-fucosyl-GM1a (24:0) | 10 | GM2 (24:0) |
| 11 | GalNAc-fucosyl-GM1a (16:0) | 11 | GM2 (16:0) |
| 12 | GD1a (24:0) | 12 | GM1a (24:0) |
| 13 | GD1a (16:0) | 12 | $Hex_3$-HexNAc-Hex-Hex-Cer (24:0) |
| | | 13 | GM1a (16:0) |
| | | 13 | Hex-GM1 (24:0) |
| | | 14 | Hex-GM1 (16:0) |
| | | 14 | HexNAc-$Hex_3$-HexNAc-Hex-Hex-Cer (24:0) |
| | | 16 | GD1a (24:0) |
| | | 17 | GD1a (16:0) |
| | | 18 | No glycolipid |
| | | 19 | No glycolipid |

beads column chromatography must be repeated, entailing large amounts of toxic organic solvents and working time in order to purify the glycolipids, and the yield of purified glycosphingolipids obtained frequently has been too small to use for further chemical analysis. The TLC blotting/SIMS method makes it possible to analyze glycosphingolipids without prior purification, thereby overcoming the time-consuming difficulties of various purifying methods and making a structural analysis of glycosphingolipids. Our method is simple, rapid and reproducible with high resolution lipid separation on HPTLC plates. Moreover it reduces the time needed for analysis and the consumption of organic solvents. Use of this method should accelerate the development of glycosphingolipid research.

## Acknowledgement

This work was supported in part by Special Funds from the Science and Technology Agency of Japan.

## References

1. Svennerholm L (1963) *J Neurochem* **10**: 613–23.
2. Kojima N, Hakomori S (1989) *J Biol Chem* **264**: 20159–62.
3. Zheng M, Tsuruoka T, Tsuji T, Hakomori S (1992) *Biochem Biophys Res Commun* **186**: 1397–1402.
4. Phillips ML, Nudelman E, Gaeta FC, Perez M, Singhal AK, Hakomori S, Paulson JC (1990) *Science* **250**: 1130–32.
5. Hakomori S (1981) *Annu Rev Biochem* **50**: 733–64.
6. Hakomori S (1989) *Cancer Res* **52**: 257–331.
7. Taki T, Takamatsu M, Myoga A, Tanaka K, Ando S, Matsumoto M (1988) *J Biochem* **103**: 998–1003.
8. Taki T, Yamamoto K, Takamatsu M, Ishii K, Myoga A, Sekiguchi K, Ikeda I, Kurata K, Nakajima J, Handa S, Matsumoto M (1990) *Cancer Res* **50**: 1284–90.
9. Hakomori S, Kannagi R (1983) *J Natl Cancer Inst* **71**: 231–51.
10. Taki T, Kawamoto M, Seto H, Noro N, Masuda T, Kannagi R, Matsumoto M (1983) *J Biochem* **94**: 633–44.
11. Neri A, Welch DR, Kawaguchi T, Nicorson G (1983) *J Natl Cancer Inst* **68**: 507–17.
12. Taki T, Ishikawa D, Handa S, Kasama T (1995) *Anal Biochem* **225**: 24–27.
13. Saito T, Hakomori S (1971) *J Lipid Res* **12**: 257–59.
14. Taki T, Handa S, Ishikawa D (1994) *Anal Biochem* **221**: 312–16.
15. Taki T, Kasama T, Handa S, Ishikawa D (1994) *Anal Biochem* **223**: 232–38.
16. Mansson JE, Egge MME, Svennerholm L (1986) *FEBS Lett* **196**: 259–62.
17. Taki T, Rokukawa C, Kasama T, Kon K, Ando S, Abe T, Handa S (1992) *J Biol Chem* **267**: 11811–17.
18. Taki T, Rokukawa C, Kasama T, Handa S (1992) *Cancer Res* **52**: 4805–11.