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The ganglioside $G_{D1\alpha}$, $IV^3Neu5Ac$, $III^6Neu5Ac$-$GgOse_4Cer$, is a major disialoganglioside in the highly metastatic murine lymphoreticular tumour cell line MDAY-D2

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The aim of the present study was to investigate the ganglioside expression of the highly metastatic murine lymphoreticular tumour cell line MDAY-D2. Cells were propagated under controlled pH conditions and oxygen supply in bioreactors of 1 and 7.5 l volumes by repeated batch fermentation. Gangliosides were isolated from 2.7×10^{11} cells, purified by silica gel chromatography and separated into mono- and disialoganglioside fractions by preparative DEAE anion exchange high performance liquid chromatography. Individual gangliosides were obtained by preparative thin layer chromatography. Their structural features were established by immunostaining, fast atom bombardment and gas chromatography mass spectrometry. In addition to gangliosides of the G_{M1a}-pathway (G_{M2}, G_{M1a} and G_{D1a}) and G_{M1b} ($IV^3Neu5Ac$-$GgOse_4Cer$) and GalNAc-G_{M1b} of the G_{M1b}-pathway, the disialoganglioside $G_{D1\alpha}$ ($IV^3Neu5Ac$, $III^6Neu5Ac$-$GgOse_4Cer$) was found in equal amounts compared to G_{D1a} ($IV^3Neu5Ac$, $II^3Neu5Ac$-$GgOse_4Cer$). All gangliosides were substituted with $C_{24:0,24:1}$ and $C_{16:0}$ fatty acids, sphingosine and *N*-acetylneuraminic acid as the sole sialic acid.

Keywords: MDAY-D2, metastatic tumour cell line, $G_{D1\alpha}$, FAB-MS, GC-MS

Abbreviations: FAB-MS, fast atom bombardment-mass spectrometry; GC-MS, gas chromatography-mass spectrometry; GSL(s), glycosphingolipid(s); HPLC, high performance liquid chromatography; HPTLC, high performance thin layer chromatography; Neu5Ac, *N*-acetylneuraminic acid; Neu5Gc, *N*-glycoloylneuraminic acid [57]. The designation of the following glycosphingolipids follows the IUPAC-IUB recommendations [58] and the nomenclature of Svennerholm [59]. Gangliotriaosylceramide or $GgOse_3Cer$, GalNAcβ1-4Galβ1-4Glcβ1-1Cer; gangliotetraosylceramide or $GgOse_4Cer$, Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-1Cer; gangliopentaosylceramide or $GgOse_5Cer$, GalNAcβ1-4Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-1Cer; G_{M2}, $II^3Neu5Ac$-$GgOse_3Cer$; G_{M1a}, $II^3Neu5Ac$-$GgOse_4Cer$; G_{M1b}, $IV^3Neu5Ac$-$GgOse_4Cer$; GalNAc-G_{M1b}, $IV^3Neu5Ac$-$GgOse_5Cer$; G_{D1a}, $IV^3Neu5Ac$, $II^3Neu5Ac$-$GgOse_4Cer$; G_{D1b}, $II^3(Neu5Ac)_2$-$GgOse_4Cer$; $G_{D1\alpha}$ or G_{D1e}, $IV^3Neu5Ac$, $III^6Neu5Ac$-$GgOse_4Cer$; G_{D1c}, $IV^3(Neu5Ac)_2$-$GgOse_4Cer$; G_{T1b}, $IV^3Neu5Ac$, $II^3(Neu5Ac)_2$-$GgOse_4Cer$.

Introduction

Certain gangliosides, which are sialic acid-containing glycosphingolipids (GSLs), are known to be closely associated with cell growth, oncogenesis, differentiation and malignant transformation [1]. Aberrant glycosylation of GSLs in tumour cells has been well established [2], and tumourigenic cells which progress to a more aggressive, metastatic phenotype express elevated levels of sialylated GSLs and glycoproteins [3], although the cell biological significance is not known. Two general types of changes are either deletion of complex GSLs due to a block in synthesis or activation of normally unexpressed glycosyl- or sialyltransferases leading to neosynthesis of aberrant GSL structures. These common features of animal tumours are manifested phenotypically by the expression of tumour-associated carbohydrate antigens [4]. Qualitative and quantitative differences in sialylation and expression of GSLs in tumour cells having different metastatic potentials have been reported by several authors [5–7].

Murine leukocytic tumour cell lines were found to express G_{M1b}-type gangliosides. G_{M1b} and GalNAc-G_{M1b} have been detected in an MDAY-D2 clone [8], in YAC-1 lymphoma cells [9] and in macrophage-like WHI-3 cells [10]. The sialidase-susceptible disialoganglioside G_{D1c} has been

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isolated from AKR and WHT mouse thymomas [11, 12]. The unusual disialoganglioside $G_{D1\alpha}$ was found in murine lymphoma Eb and erythroleukaemia cells [6, 13] as well as in several rat ascites hepatomas (also named G_{D1e}) [14, 15]. G_{M1b} and $G_{D1\alpha}$ of metastatic mouse lymphosarcoma RAW117-H10 cells were recently shown to be crucial membrane compounds in adhesion to hepatic sinusoidal endothelial cells, which enables metastasis of tumour cells throughout the body [16]. Here we report on the ganglioside expression of the highly metastatic mouse lymphoreticular tumour cell line MDAY-D2. Cells were found to express gangliosides of the G_{M1a}- as well as of the G_{M1b}-pathway. $G_{D1\alpha}$ ($IV^3Neu5Ac$, $III^6Neu5Ac\text{-}GgOse_4Cer$) was characterized as one of the major gangliosides of MDAY-D2 cells.

Materials and methods

Cells and culture conditions; large scale cell production; metabolic labelling of cells

MDAY-D2, a lymphoreticular tumour cell line of the murine DBA/2 strain [17, 18], was cultivated in RPMI 1640 medium containing 10% heat inactivated fetal calf serum, 2 mM glutamine, in a humidified atmosphere of 5% CO_2 in air at 37 °C. The cells were seeded with 1×10^5 cells per ml and harvested at a final concentration of 1.2×10^6 cells per ml. Larger amounts of cells were propagated in bioreactors of 1 and 7.5 l volumes [19] by repeated batch cultivation. MDAY-D2 cells were labelled with D-[1-^{14}C]glucosamine hydrochloride and D-[1-^{14}C]galactose as previously described for stimulated T cells [20]. Cell numbers were determined by trypan blue exclusion with a hemocytometer. After centrifugation the cells were washed twice with phosphate buffered saline (100 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.2) and finally suspended in chloroform:methanol, 2:1 by vol, and stored at −20 °C.

Isolation of MDAY-D2 gangliosides

2.7×10^{11} cells produced in bioreactors were mixed with 2.1×10^8 radioactively labelled cells, suspended in chloroform:methanol, 2:1 by vol, and sonicated for 5 min. After centrifugation, the sediment was subsequently extracted with chloroform:methanol, 1:1 by vol, and chloroform:methanol, 1:2 by vol. The combined extracts were evaporated, dissolved in water and dialysed against distilled water. The dialysate was freeze dried, the GSL extract taken up in chloroform:methanol:water, 30:60:8 by vol [21], and applied to 100 ml DEAE-Sepharose CL-6B (Pharmacia Fine Chemicals, Freiburg, Germany) in the acetate form. Whole gangliosides were eluted from the column with 0.15 M ammonium acetate in methanol. After desalting by dialysis, gangliosides were further purified on a column packed with 75 g Iatrobeads 6RS-8060 (Macherey-Nagel, Düren, Germany) [22].

High performance liquid chromatography (HPLC)

Whole gangliosides were fractionated by anion-exchange HPLC on a column of Fractogel DEAE 650-S (1.0 cm × 15 cm, Merck, Darmstadt, Germany). The HPLC equipment (Gilson Abimed, Langenfeld, Germany) consisted of three M303 pumps, a high pressure mixer M811, an M166 UV variable wavelength detector and a computer control module. The Fractogel column was rinsed with three column volumes of chloroform:methanol:water, 30:60:8 by vol, and an aliquot of the whole ganglioside fraction (about 10 mg gangliosides) was applied to the column in the same solvent via a 5 ml Rheodyne 7125 loop. After rinsing with three column volumes of chloroform:methanol:water, 30:60:8 by vol, and methanol, respectively, gangliosides were eluted with a programmed linear gradient up to 0.1 M of methanolic ammonium acetate. The flow rate was 0.5 ml min^{-1} and fractions were collected every 4 min (2 ml fractions). Radioactivity was determined in a Packard TRI CARB CA1900 liquid scintillation counter (Packard Inst. Co., Warrenville Road, IL, USA) in aliquots of each fraction after evaporation of the organic solvent.

Analytical and preparative thin layer chromatography

High performance thin layer chromatography plates (HPTLC plates, silica gel coated, size 10 cm × 10 cm, thickness 0.24 mm, Merck, Darmstadt, Germany) were used for analytical and preparative purposes. Gangliosides were separated in chloroform:methanol:water, 120:85:20 by vol, containing 2 mM $CaCl_2$, and visualized by resorcinol [23]. Lipid bound sialic acid was determined by densitometry. Resorcinol stained ganglioside chromatograms were scanned with the Desaga CD60 scanner (Desaga, Heidelberg, Germany) equipped with an IBM compatible personal computer and densitometric software. Intensities of bands were measured in reflectance mode at 580 nm with a light beam slit of 0.1 mm × 2 mm. Preparative GSL amounts were applied to HPTLC plates with an automatic sample applier Linomat IV (Camag, Muttenz, Switzerland) and visualized after chromatography with primulin (Aldrich-Chemie, Steinheim, Germany) 0.01% in acetone:H_2O, 8:2 by vol [24]. Zones containing GSLs were scraped off and the silica gel was transferred to small sintered glass columns. Gangliosides were freed from primulin by stepwise gradient elution of isopropanol:hexane:H_2O (from 55:40:5 to 55:25:20, each by vol) according to Levery *et al.* [25].

Determination of sialic acids

N-Acetyl- and *N*-glycoloylneuraminic acid of gangliosides were identified and quantified as their perbenzoylated derivatives by high performance liquid chromatography according to Karamanos *et al.* [26] with some modifications as published recently [27]. Two hundred µg of gangliosides were dissolved in 0.5 ml 25 mM aqueous HCl and heated for 2 h at 80 °C. After evaporation the hydrolysis

products were partitioned according to Folch *et al.* [28]. To the dried upper phase containing sialic acids, 0.25 ml of the benzoylation reagent (10% (w/v) benzoic anhydride and 5% (w/v) 4-dimethyl-aminopyridine in pyridine) was added and allowed to stand at room temperature for 12 h. The reaction was stopped by adding 0.25 ml of water and heating for 10 min at 80 °C. Pyridine was removed by evaporation and the benzoylation reagents by Folch partition. Twenty per cent aliquots of the lower phase were analysed by HPLC. Isocratic HPLC was performed using an RP_{18} column as the stationary phase and methanol: water, 8:2 by vol, as the mobile phase. The flow rate was 1.5 ml min^{-1} and elution was monitored at 230 nm (for further details see [27]).

Detection of G_{M1b}- and G_{M1a}-type gangliosides on HPTLC plates by immunostaining

Gangliosides G_{M1b} and $G_{D1\alpha}$ were detected on thin layer plates by immunostaining after neuraminidase treatment as described [29]. Briefly, silica gel fixed plates were incubated with 5 mU ml^{-1} *Vibrio cholerae* neuraminidase followed by incubation with a rabbit anti-$GgOse_4Cer$ antiserum. Alkaline phosphatase-conjugated second antibodies were used in all cases (see below) to visualize bound first antibodies by generating a blue dye from 5-bromo-4-chloro-3-indolyl-phosphate. GalNAc-G_{M1b} was identified with a highly specific chicken antiserum according to Müthing *et al.* [9].

The HPTLC-binding method using cholera toxin B subunit for specific detection of G_{M1a} was developed by Magnani *et al.* [30]. A modification of this procedure was used as published recently by Müthing *et al.* [31]. Neuraminidase is employed to convert G_{D1a} to G_{M1a} which was identified by the same procedure. This technique was originally developed by Wu and Ledeen [32]. The monoclonal anti-$GgOse_3Cer$ antibody 2D4 (TIB 185, American Type Culture Collection, Bethesda, MD, USA) [33] was used to detect G_{M2} after neuraminidase treatment [34].

Mass spectrometric analysis of gangliosides

Fast atom bombardment mass spectrometry (FAB-MS) was carried out on a ZAB-HF mass spectrometer (VG Analytical, Manchester, Great Britain) essentially as described earlier [35]. The native samples of gangliosides were dissolved in chloroform:methanol (1:1 by vol) and desorbed from the matrix thioglycerol (EGA Chemie, Steinheim, Germany) using xenon as bombarding gas. The mass spectra were acquired as single scans in the upscan mode on an AMD DP10 data system fitted with SAMII (KWS) hardware and SUSY software (AMD Intectra, Beckeln, Germany). The mass values found were obtained after calibration with cesium iodide and do not represent nominal, but physical numbers.

The permethylation was carried out as described by Ciucanu and Kerek [36]. Permethylated gangliosides were separated from reagents by exhaustive extraction with chloroform and chromatographed on a 1 cm × 23 cm Sephadex LH-20 column (Pharmacia Fine Chemicals, Freiburg, Germany) with chloroform:methanol (1:1 by vol) as eluent. Final purification was performed on a small Iatrobeads column (0.5 cm × 5 cm, Iatrobeads 6RS-8060, Macherey-Nagel, Düren, Germany) by elution with chloroform, chloroform:methanol (98:2 by vol), (95:5 by vol), (95:10 by vol) and chloroform:methanol:water (75:25:4 by vol).

Methylation analysis

Purified gangliosides were permethylated, hydrolysed and the resulting monosaccharide derivatives were reduced with NaB^3H_4 followed by acetylation according to Levery and Hakomori [37]. The derivatives were analysed by gas chromatography-mass spectrometry (GC-MS) essentially as described earlier [38]. Briefly, separation of the partially methylated alditol acetates was achieved on a capillary column (15 m) wall coated with SE54, heated from 100 °C to 300 °C at 10 °C per min. Detection of the derivatives was performed by single-ion monitoring using dwell times of 0.02 s on an MSD 5970.

Reference gangliosides

Gangliosides of CBA/J brains were isolated and purified by standard procedures [20, 21, 39]. A mixture of reference gangliosides from human brain was purchased from Supelco, Inc. (Bellefonte, PA, USA). The isolation and characterization of gangliosides from the murine T lymphoma YAC-1 have been described in [9].

Results

Whole gangliosides from MDAY-D2 cells

2.7×10^{11} MDAY-D2 cells were produced in bioreactors in 1 and 7.5 l volumes by repeated batch cultivation. GSL extraction gave 144 mg gangliosides. A thin layer chromatogram of the whole ganglioside mixture is shown in Fig. 1. Monosialo- and disialogangliosides were designated with roman numerals from (-II) to III and from IV to VII, respectively. Densitometric scanning revealed major bands 0 and I (25% and 18%, respectively) followed by the two disialogangliosides IV and VI and the monosialoganglioside II representing 14%, 13% and 11%, respectively, of the whole ganglioside fraction. Compounds -I, -II, V and VII each contributed from 3–6% of the whole GSL mixture. Less than 1% (about 0.7%) of compound II was detected.

Sialic acid profile

Sialic acids were released by mild acid treatment of whole gangliosides and their perbenzoylated derivatives were separated by isocratic HPLC [26]. Ganglioside derived sialic acids from the T lymphoma YAC-1 served as the standard mixture of Neu5Ac and Neu5Gc [9]. The elution profile of YAC-1 reveals the presence of Neu5Ac/Neu5Gc in

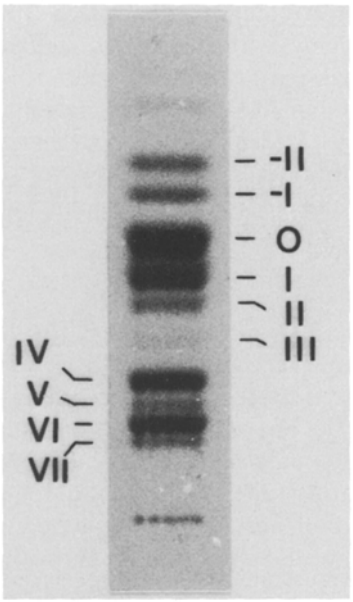

Figure 1. Thin layer chromatogram of MDAY-D2 gangliosides. 10 µg gangliosides were chromatographed in chloroform:methanol:H_2O (120:85:20 by vol) with 2 mM $CaCl_2$ and visualized by spraying the plate with resorcinol-HCl reagent. Monosialogangliosides and disialogangliosides are marked with roman numerals from (-II) to III and from IV to VII, respectively, at the margin.

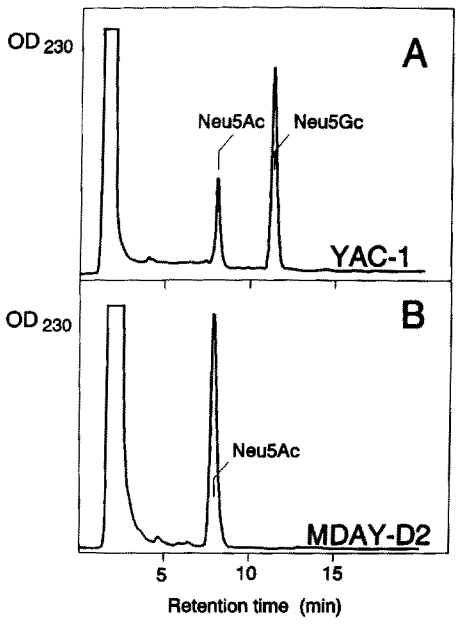

Figure 2. HPLC elution profiles of perbenzoylated neuraminic acids from YAC-1 (A) and MDAY-D2 (B) gangliosides. Neuraminic acids were isolated from 200 µg gangliosides and a 20% aliquot was applied on to the column, respectively.

a 1:3 ratio (Fig. 2A), whereas Neu5Gc is absent in MDAY-D2 cells as demonstrated in Fig. 2B. Only traces of Neu5Gc (<0.2%) could be detected by application of

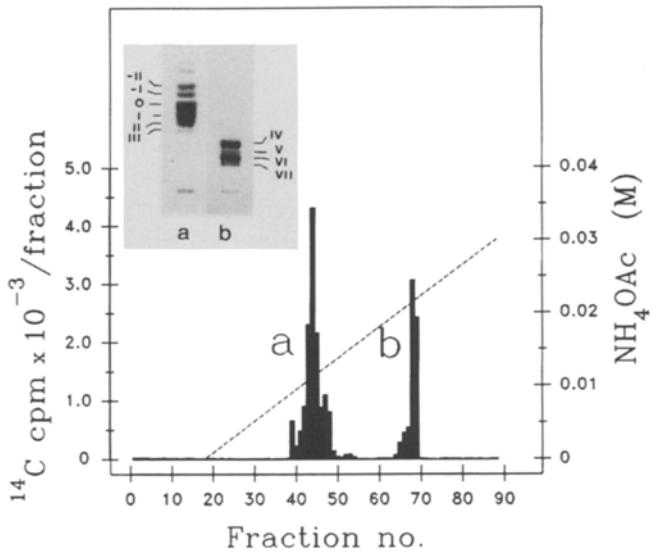

Figure 3. Anion-exchange HPLC elution profile of MDAY-D2 gangliosides. 600 000 cpm (about 10 mg) of ^{14}C-labelled gangliosides were applied on to a Fractogel DEAE column and eluted with a linear gradient of methanolic ammonium acetate. Two ml fractions were collected every 4 min and the radioactivity of 3.3% aliquots (=67 µl) was determined in a liquid scintillation counter. a, Monosialogangliosides; b = disialogangliosides.

extremely high amounts of sialic acids to the column (not shown).

Anion exchange HPLC of MDAY-D2 gangliosides

Whole gangliosides were separated into mono- and disialogangliosides by HPLC on DEAE Fractogel as demonstrated in Fig. 3. The radioactivity profile revealed 65% monosialo- and 35% disialogangliosides, respectively. Gravimetrical estimation of several preparative separations of whole gangliosides gave 105 mg mono- and 39 mg disialogangliosides (73% and 27%, respectively). Individual GSLs were isolated and purified by preparative HPTLC (see below).

Detection of GalNAc-G_{M1b} in the MDAY-D2 monosialoganglioside fraction

A highly specific chicken anti GalNAc-G_{M1b} antibody was used to trace out this GSL by *in situ* immunostaining on the HPTLC plate. Fig. 4A shows the overlay assay indicating two immunostained bands in the MDAY-D2 monosialoganglioside fraction (lane c, numbers II and III). None of the other resorcinol positive bands (Fig. 4B) of human and mouse brain (lanes a and b) as well as MDAY-D2 disialogangliosides (lane d) showed any cross-reaction with this specific antibody.

Structural characterization of MDAY-D2 gangliosides

The structural mapping of individual gangliosides isolated by preparative HPTLC was carried out by combining immunological and spectroscopic data as described

Table 1. Monosialogangliosides from MDAY-D2 cells.[a].

Ganglioside fraction	*No.*	*$[M\text{-}1]^-$ of native gangliosides*	*$[M+H]^+/[M+Na]^+$ of permethylated gangliosides*	*Major fatty acid component*	*Symbol*	*Structure*
–II	1	1466	ND	24:0	G_{M2}	IINeu5Ac-GgOse$_3$Cer
		1464		24:1		
–I	2	1354	ND	16:0	G_{M2}	IINeu5Ac-GgOse$_3$Cer
0	3	1628	ND	24:0	G_{M1a}	IINeu5Ac-GgOse$_4$Cer
		1626		24:1		
I	4	1516	ND	16:0	G_{M1a}	IINeu5Ac-GgOse$_4$Cer
	5	1628	1910/1932	24:0	G_{M1b}	IVNeu5Ac-GgOse$_4$Cer
		1626	1908/1930	24:1		
II	6	1516	1798/1820	16:0	G_{M1b}	IVNeu5Ac-GgOse$_4$Cer
	7	1831	ND	24:0	GalNAc-G_{M1b}	IVNeu5Ac-GgOse$_5$Cer
		1829		24:1		
III	8	1719	ND	16:0	GalNAc-G_{M1b}	IVNeu5Ac-GgOse$_5$Cer

[a] ND = not determined.

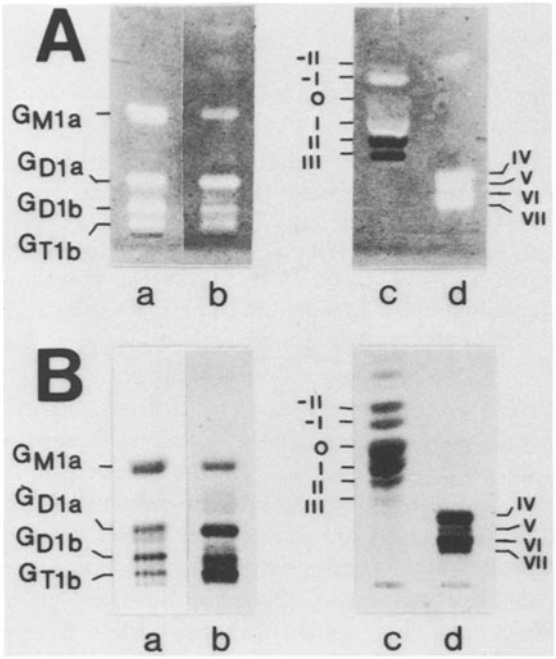

Figure 4. Detection of GalNAc-G_{M1b} in MDAY-D2 monosialogangliosides. (A) Immunostain and (B) resorcinol stain of different ganglioside mixtures: 10 µg gangliosides each from human brain (lane a), mouse brain (lane b) and monosialogangliosides (lane c) and disialogangliosides (lane d) from MDAY-D2 were chromatographed as described in Fig. 1. Immunostaining was performed with a chicken anti-GalNAc-G_{M1b} antiserum. Monosialogangliosides and disialogangliosides from MDAY-D2 are marked with roman numerals from (-II) to III and from IV to VII, respectively, and the positions of human and mouse brain gangliosides are marked at the margin.

previously [9]. G_{M1b}-type gangliosides were detected by immunostaining after neuraminidase treatment and GalNAc-G_{M1b} was identified as described above. As an example, the resorcinol stained monosialogangliosides No. 5 of fraction I, No. 6 of fraction II, No. 7 of fraction II and No. 8 of fraction III after HPTLC separation are given in Fig. 5A (numbers related to Table 1). Immunostaining with anti-GgOse$_4$Cer antibody after neuraminidase treatment of gangliosides Nos. 5 and 6 indicates the G_{M1b}-structure (Fig. 5B, lanes b and c) whereas gangliosides Nos. 7 and 8 were reactive to the anti-GalNAc-G_{M1b} antibody (Fig. 5B, lanes d and e). Two disialogangliosides of fractions VI and VII in the whole ganglioside fraction were immunostained with the anti-GgOse$_4$Cer antibody after neuraminidase treatment (Fig. 5B, lane a) indicating the presence of two disialogangliosides with an unusual sialylation compared with G_{D1a} or G_{D1b} (see below).

FAB-MS of MDAY-D2 monosialogangliosides

The monosialogangliosides G_{M2}, G_{M1a}, G_{M1b} and GalNAc-G_{M1b} of fractions (–II)–III were analysed as native samples by immunochemical methods, by negative ion FAB-MS and in part by positive ion FAB-MS of their permethylated derivatives after the separation steps described above. High enrichment, but not the complete separation, of discrete structures was accomplished, in particular with respect to the composition of the ceramide portions. The identification of sugar sequence was done by analysing diagnostic ions as previously described and complete assignment of single structures was possible by matching the ions present in the spectra [35] (Table 1).

FAB-MS and GC-MS of MDAY-D2 disialogangliosides

The resorcinol stain of individual disialogangliosides from MDAY-D2 is shown in Fig. 6. The structural features of disialogangliosides IV–VII were established by immunochemical and spectroscopic methods. After treatment with *V. cholerae* neuraminidase positive immunostaining reactions were obtained from IV and V against cholera toxin

Table 2. Diagnostic molecular and fragment ions (m/z)[a] for identification of G_{D1a} and $G_{D1\alpha}$ gangliosides in negative ion FAB-MS.

Ganglioside fraction[b]	$[M-1]^-$	Cer^-	$Cer\text{-}Hex^-$	$Cer\text{-}Hex\text{-}Hex^-$	$Neu5Ac\text{-}Hex\text{-}HexNAc^-$
IV	1919/1917	648/646	810/808	–	673
V	1807	536	698	–	673
VI	1919/1917	648/646	810/808	972/970	–
VII	1807	536	698	860	–

[a] Nominal masses.
[b] According to Fig. 6.

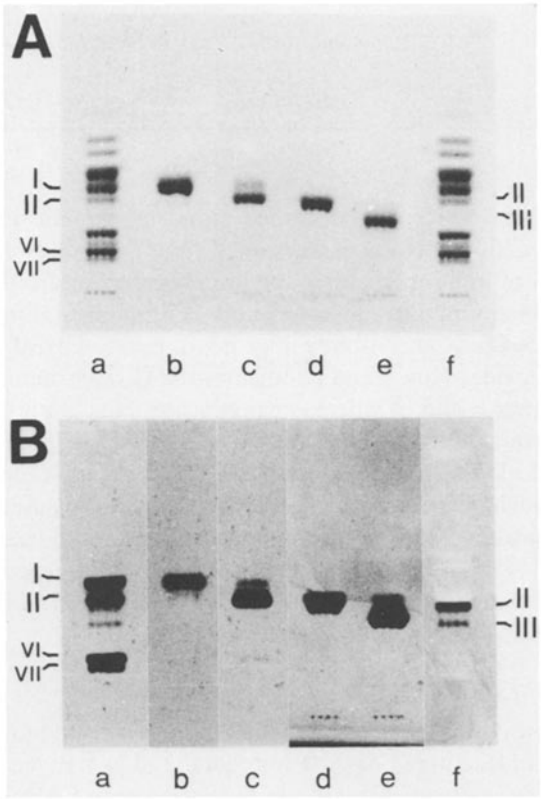

Figure 5. Resorcinol stain (A) and immunostain (B) of individual monosialogangliosides from MDAY-D2. 10 µg of whole gangliosides from MDAY-D2 (lane a and f) and 2 µg, respectively, of G_{M1b} (C_{24}-fatty acid; =No. 5 from fraction I; lane b), G_{M1b} (C_{16}-fatty acid; =No. 6 from fraction II; lane c), GalNAc-G_{M1b} (C_{24}-fatty acid; =No. 7 from fraction II; lane d) and GalNAc-G_{M1b} (C_{16}-fatty acid; =No. 8, fraction III; lane e) were chromatographed as described in Fig. 1. For specific immunological detection of G_{M1b} and $G_{D1\alpha}$, lanes a, b and c were overlaid with *Vibrio cholerae* neuraminidase and $GgOse_4Cer$ bands were detected with specific antibodies. GalNAc-G_{M1b} was identified with a chicken anti-GalNAc-G_{M1b} antiserum (lanes d, e and f). The monosialogangliosides are enumerated in Table 1.

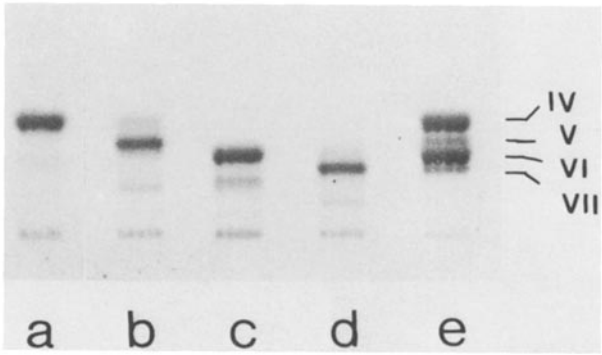

Figure 6. Resorcinol stain of individual disialogangliosides from MDAY-D2. Disialogangliosides (10 µg, lane e) and 2 µg, respectively of G_{D1a} (C_{24}-fatty acid; =No. 9, fraction IV), G_{D1a} (C_{16}-fatty acid; =No. 10, fraction V), $G_{D1\alpha}$ (C_{24}-fatty acid; =No. 11, fraction VI) and G_{D1a} (C_{16}-fatty acid; =No. 12, fraction VII) were chromatographed as described in Fig. 1. Disialogangliosides are designated according to Tables 2–5.

B subunit and from VI and VII against anti-$GgOse_4Cer$ antibody, respectively. Both native IV and V, analysed by negative ion FAB-MS, were identified by their molecular and fragment ions to belong to the G_{D1a} ganglioside-type, differing only in chain length of fatty acids in their respective ceramide portions ($C_{24:0,24:1}$ for IV and $C_{16:0}$ for V). Both VI and VII were postulated to belong to the $G_{D1\alpha}$ species as deduced from the ions at m/z = 972/970 and 860 ($Cer\text{-}Hex\text{-}Hex^-$) containing $C_{24:0,24:1}$ and $C_{16:0}$ fatty acids in their respective ceramide moieties (Table 2). This structural assignment was confirmed by positive ion FAB-MS analysis of permethylated gangliosides IV to VII (Table 3). The spectra of permethylated disialogangliosides V and VII are shown in Fig. 7A and B, respectively.

The patterns of monosaccharide substitution in disialogangliosides was investigated additionally by gas chromatography-mass fragmentography of partially methylated alditol acetates as represented in Fig. 8 (compounds IV and VI) by relevant ion traces and summarized qualitatively in Table 4. G_{D1a} in fractions IV and V was identified by the formation of 2,6-Me_2-Gal (peak 4 in ion traces a and c of Fig. 8 registered at m/z 118 or 305, respectively) representing a 3,4-di-*O*-substituted Gal residue and by the formation of 2,4,6-Me_3-GalNAcMe (peak 5 in ion trace b of Fig. 8 at m/z 159) representing a 3-mono-

Table 3. Diagnostic molecular and fragment ions $(m/z)^a$ for identification of G_{D1a} and $G_{D1\alpha}$ gangliosides in positive ion FAB-MS.

Ganglioside fraction[b]	$[M+H]^+$	$[M+Na]^+$	Cer^+	*Neu5Ac-Hex-HexNAc*$^+$	*Neu5Ac-Hex-(Neu5Ac)-HexNAc*$^+$
IV	2271/2269	2293/2291	660/658	825	–
V	2159	2181	548	825	–
VI	2271/2269	2269/2291	660/658	–	1186
VII	2159	2181	548	–	1186

[a] Nominal masses.
[b] According to Fig. 6.

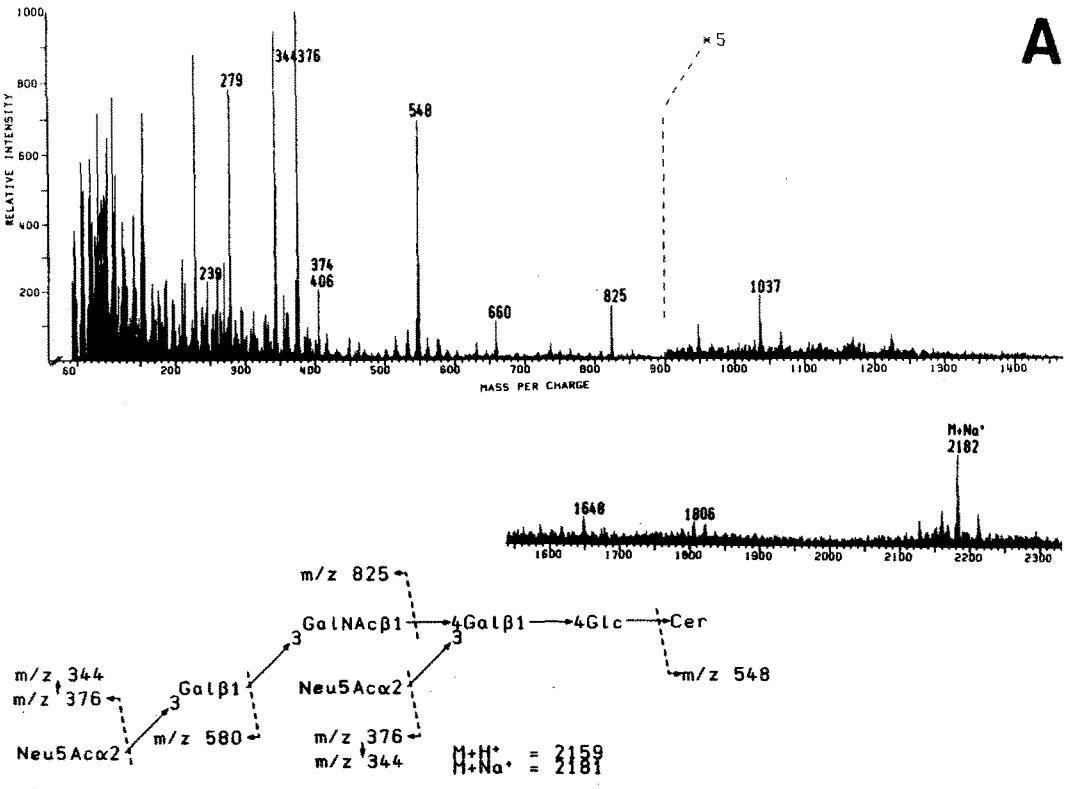

Figure 7. Positive ion FAB-MS of permethylated MDAY-D2 disialoganglioside fraction V (A).

Table 4. Partially methylated alditol acetates of disialogangliosides from MDAY-D2.

Partially methylated alditol acetates	*Ganglioside fraction*[a]			
	IV	*V*	*VI*	*VII*
2,3,6-Me_3-Glc	+	+	+	+
2,3,6-Me_3-Gal	–	–	+	+
2,4,6-Me_3-Gal	+	+	+	+
2,6-Me_2-Gal	+	+	–	–
4,6-Me_2-GalNAcMe	+	+	–	–
4-Me-GalNAcMe	–	–	+	+

[a] According to Table 2 and Table 3.

O-substituted GalNAc residue (see Table 4). In conjunction with data from FAB-MS (primary fragment ion at m/z 825 for the methylated compounds IV and V), supporting a Neu5Ac-Hex-HexNAc$^+$ sequence, these derivatives indicate that sialic acids of compounds IV and V are linked to the terminal and internal Gal residues. By contrast, $G_{D1\alpha}$ in fractions VI and VII is characterized by the absence of 2,6-Me_2-Gal and by the formation of 2,4-Me_2-GalNAcMe (peak 6 in ion traces e and f of Fig. 8 registered at m/z 159 or 275, respectively) representing a 3,6-di-*O*-substituted GalNAc residue (see Table 4). In accordance with this, the FAB-mass spectra of the permethylated compounds VI and VII exhibit sequence ions at m/z 1186 which correspond to the tetrasaccharide fragment Neu5Ac-Hex-(Neu5Ac)-

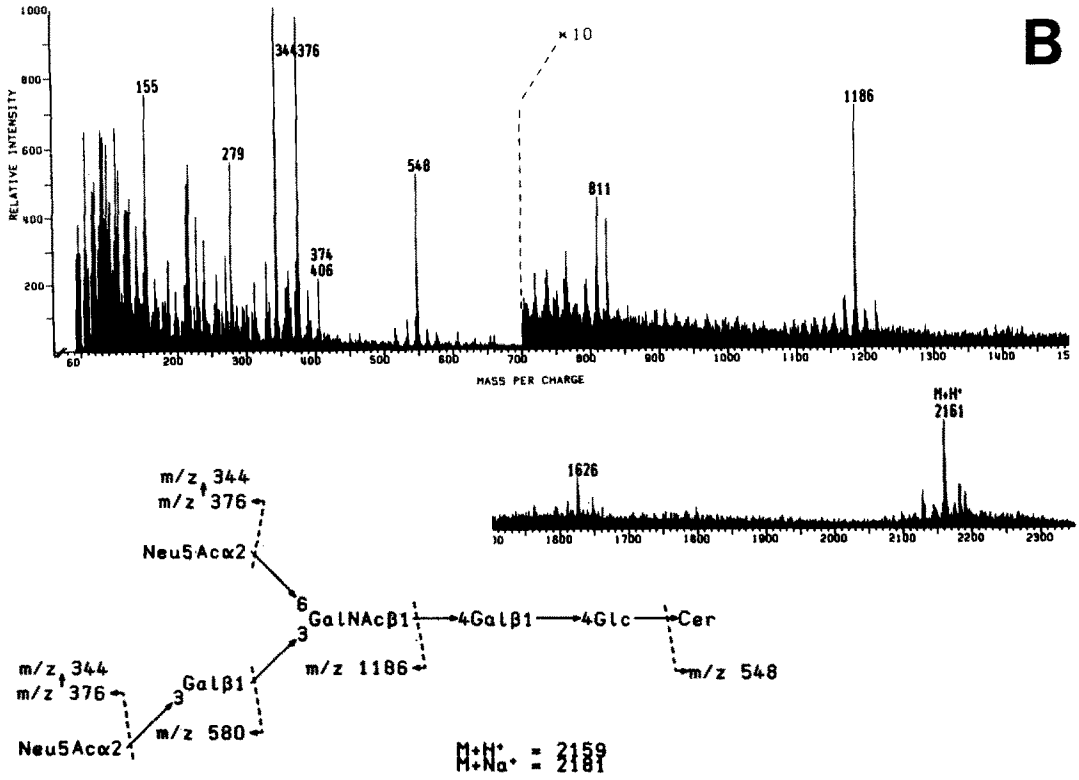

Figure 7. (*Continued*) and fraction VII (B).

$HexNAc^+$ (see Table 3 and Fig. 7B). The combined data support the assumption that the compounds VI and VII are sialylated at position 3 of the terminal Gal and at position 6 of the internal GalNAc residue. The structures of the disialogangliosides from MDAY-D2 are summarized in Table 5.

Discussion

To analyse unknown compounds in complex ganglioside mixtures, *in situ* immunostaining on HPTLC plates is the first approach to identification of G_{M1a}- or G_{M1b}-type gangliosides [32, 29]. Using this technique, an unusual disialoganglioside with chromatographic behaviour between G_{D1a} and G_{D1b} on thin layer chromatograms was detected in high amounts in the disialoganglioside fraction of MDAY-D2 cells. $G_{D1\alpha}$ ($IV^3Neu5Ac$, $III^6Neu5Ac$-$GgOse_4$-Cer) was identified by immunostaining with specific antibodies after sialidase degradation, FAB-MS of the native compound and the permethylated derivatives and GC-MS of partially methylated alditol acetates. Gangliosides of the G_{M1a}-pathway (G_{M2}, G_{M1a}, G_{D1a}) and the G_{M1b}-pathway (G_{M1b}, GalNAc-G_{M1b}) were found in MDAY-D2 cells as reported before by Laferté *et al.* [8], but contrary to our data, MDAY-D2 cells analysed by them did not express $G_{D1\alpha}$.

Table 5. Disialogangliosides from MDAY-D2 cells.

Ganglioside fraction[a]	*Major fatty acid component*	*Symbol*	*Structure*
IV	24:0 24:1	G_{D1a}	$IV^3Neu5Ac$, $II^3Neu5Ac$-$GgOse_4Cer$
V	16:0	G_{D1a}	$IV^3Neu5Ac$, $II^3Neu5Ac$-$GgOse_4Cer$
VI	24:0 24:1	$G_{D1\alpha}$	$IV^3Neu5Ac$, $III^6Neu5Ac$-$GgOse_4Cer$
VII	16:0	$G_{D1\alpha}$	$IV^3Neu5Ac$, $III^6Neu5Ac$-$GgOse_4Cer$

[a] According to Tables 2–4.

The fact that cell lines with different metastatic potentials display differences in glycosylation has provided evidence that cell surface glycosylation may be related to metastatic potentials of cancer cells [6, 7]. The monosialoganglioside G_{M1b} and the disialoganglioside $G_{D1\alpha}$ were identified in less metastatic murine lymphoma Eb cells, but the high metastatic ESb cell line was characterized by the absence of these GSLs [6]. $G_{D1\alpha}$ was detected in trace amounts in mouse erythroleukaemia cells (Friend cells, 745A), but it was more strongly expressed in solid tumours than in Friend suspension cells [13]. Also several rat ascites hepatomas and Yoshida sarcoma [14, 15] were found to express $G_{D1\alpha}$ (designated as G_{D1c} in [14]), but from these data metastatic potential cannot be correlated in a simple manner with the presence or absence of this single

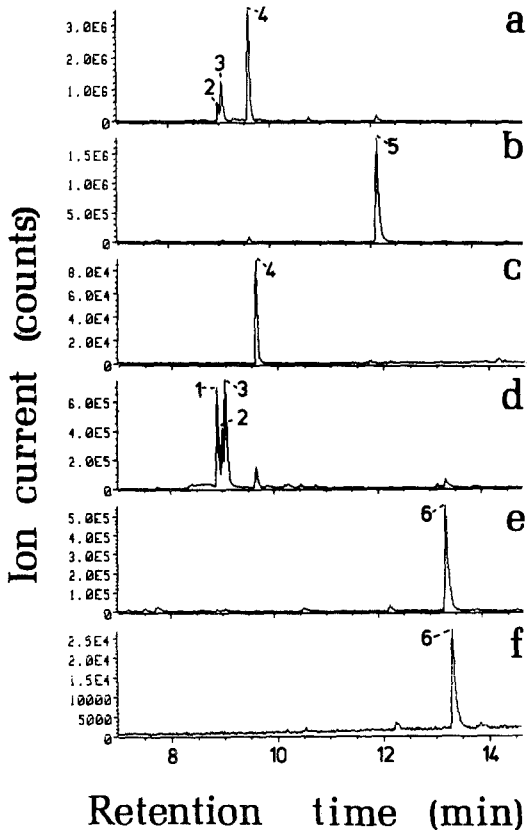

Figure 8. Gas–liquid chromatography-mass fragmentography of partially methylated alditol acetates obtained from the hydrolysis of permethylated MDAY-D2 disialoganglioside fractions IV and VI (see Table 4). Detection of the derivatives was performed by single ion monitoring at *m/z* 118 (a, d), *m/z* 159 (b, e), *m/z* 305 (c) and *m/z* 275 (f). Ion traces a–c correspond to ganglioside fraction IV, ion traces d–f to fraction VI. Peaks identified were as follows: (1) 2,3,6-Me_3-Gal; (2) 2,3,6-Me_3-Glc; (3) 2,4,6-Me_3-Gal; (4) 2,6-Me_2-Gal; (5) 4,6-Me_2-GalNAcMe; (6) 4-Me-GalNAcMe.

ganglioside. Interestingly, in a recent publication of Taki *et al.* [16] the involvement of G_{M1b} and $G_{D1\alpha}$, isolated from the highly metastatic murine lymphosarcoma RAW117-H10, in the adhesion process towards hepatic sinusoidal endothelial cells was clearly demonstrated.

However, these gangliosides are not restricted to tumour cells. The presence of gangliosides of the G_{M1b}-pathway in mouse was first described in thymus tissue [40, 41] and later in stimulated murine T lymphocytes [20, 42, 43] and T lymphocyte subpopulations [42, 44]. G_{M1b}-type gangliosides were also found in murine monocytes [45], but they are not present in mouse B lymphocytes as recently shown by Pörtner *et al.* [46]. From these results it is likely that gangliosides may play a role in immune cell circulation, e.g. localization to lymphoid organs (homing) and/or movement from the blood through the endothelium into the tissue (diapedesis). Extremely low concentrations of G_{M1b} and $G_{D1\alpha}$ were detected in normal brain and transient expression was observed in embryonic brain [47]. Therefore, the appearance of G_{M1b}-type gangliosides during embryogenesis and in tumor cells suggests an as yet unknown role for these antigens in early development as well as during tumourigenesis.

However, most of these data are based on GSL analysis of *in vitro* propagated cells. Although the synthesis of GSLs occurs through genetically programmed pathways [48, 49], there are additional pathways which are influenced by circumstantial conditions. Changes during cell ageing [50] and cell density dependent changes of gangliosides of adherent growing cells [51, 52] have been described. Modifications in the GSL-expression of cultured cells by the extracellular environment have been reported by several groups. Significant differences in the GSL composition were obtained by altered carbohydrate feeding [53], pH shift [54], changes in the osmolarity of the medium [55] and during cultivation in serum-supplemented and serum-free growth medium [31]. Moreover, ganglioside changes of human melanoma during *in vitro* growth (i.e. tissue culture cell line) compared with *in vivo* growth (i.e. biopsied tumour and nude mice) were caused by culture environment as shown by Tsuchida *et al.* [56].

Therefore, in this study care was taken to produce large amounts of cells in bioreactors under well defined and constant cultivation conditions. The oxygen supply was maintained at a constant level of 20% air saturation in the medium during fed-batch fermentation and the pH was adjusted to 7.0. Consequently, an important consideration in cell culture technology should be whenever possible to propagate cells *in vitro* under well defined conditions, e.g. in bioreactors, so that alterations in glycosylation, probably induced by changes in cultivation parameters such as oxygen concentration or pH, might be excluded. However, the effects of culture conditions on the GSL expression of MDAY-D2 cells have not as yet been carried out.

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