Glycoconjugate Journal (1991) **8**:414–423

Structural studies of gangliosides from the YAC-1 mouse lymphoma cell line by immunological detection and fast atom bombardment mass spectrometry

JOHANNES MÜTHING[1]*, JASNA PETER-KATALINIĆ[2], FRANZ-GEORG HANISCH[3] and ULRICH NEUMANN[4]

[1] *Institut für Zellkulturtechnik, Universität Bielefeld, Germany*
[2] *Institut für Physiologische Chemie der Universität, Bonn, Germany*
[3] *Abt. Immunobiologie der Universitätsklinik, Köln, Germany*
[4] *Klinik für Geflügel der Tierärztlichen Hochschule, Hannover, Germany*

Received 18 February, revised 14 June 1991

YAC-1 cells were propagated in bioreactors in 1 l and 7.5 l volumes. The cells were metabolically labelled with D-[1-^{14}C]galactose and D-[1-^{14}C]glucosamine. The ganglioside fraction, purified by DEAE-Sepharose and silica gel column chromatography, showed on thin layer chromatography four major bands with mobilities between G_{M1} and G_{D1a}. Gangliosides, obtained by further purification steps including high performance liquid chromatography on silica gel 60 columns with a gradient system of isopropanol:hexane:water, and preparative high performance TLC were characterized by (1) immunostaining of corresponding asialogangliosides obtained by mild acid hydrolysis and neuraminidase treatment and (2) fast atom bombardment mass spectrometry of native and permethylated samples and methylation analysis of G_{M1b} ganglioside. As well as small amounts of G_{M2} and G_{M1}, the major gangliosides found in the complex mixture were G_{M1b} and GalNAc-G_{M1b}. The structural heterogeneity of these gangliosides was cased by (a) substitution of the ceramide moiety by fatty acids of different chain length and degree of unsaturation ($C_{16:0}$, $C_{24:0}$, $C_{24:1}$) and (b) N-substitution of the sialic acid moieties with either acetyl or glycolyl groups. Disialogangliosides were detected only in low amounts and will be the subject of further investigation. A polyclonal chicken antiserum was raised against IVNeuAc-$GgOse_5Cer$. The antiserum was highly specific for gangliosides (IVNeuAc and IVNeuGc) and asialogangliosides with a $GgOse_5Cer$ backbone. No cross-reaction with G_{M1b} or $GgOse_4Cer$ was observed.

Keywords: gangliosides, YAC-1 T lymphoma, antibodies, FAB MS

Abbreviations: FAB-MS, fast atom bombardment mass spectrometry; GSL(s), glycosphingolipid(s); HPLC, high performance liquid chromatography, HPTLC, high performance thin layer chromatography; NK, natural killer; SIM, selective ion monitoring; TIC, total ion current. NeuAc, *N*-acetylneuraminic acid; NeuGc, *N*-glycolylneuraminic acid. The designation of the following glycosphingolipids follows the IUB-IUPAC recommendations. $GgOse_3Cer$ or gangliotriaosylceramide or asialo G_{M2}, GalNAcβ1-4Galβ1-4GlcCer; $GgOse_4Cer$ or gangliotetraosylceramide or asialo G_{M1}, Galβ1-3GalNAcβ1-4Galβ1-4GlcCer; $GgOse_5Cer$ or gangliopentaosylceramide, GalNAcβ1-4Galβ1-3GalNAcβ1-4Galβ1-4GlcCer; II^{3}NeuAc-$GgOse_4Cer$ or G_{M1}; IV^{3}NeuAc-$GgOse_4Cer$ or G_{M1b}; IV^{3}NeuAc-$GgOse_5Cer$ or GalNAc-G_{M1b}; IV^{3}NeuAc, II^{3}NeuAc-$GgOse_4Cer$ or G_{D1a}; II^3(NeuAc)$_2$-$GgOse_4Cer$ or G_{D1b}; IV^3(NeuAc)$_2$-$GgOse_4Cer$ or G_{D1c}; IV^{3}NeuAc,III^{6}NeuAc-$GgOse_4Cer$ or $G_{D1\alpha}$; IV^{3}NeuAc,II^3(NeuAc)$_2$-$GgOse_4Cer$ or G_{T1b}; *Vibrio cholerae* and *Arthrobacter ureafaciens* neuraminidase (EC 3.2.1.18).

Glycosphingolipids (GSLs) are ubiquitous components of the outer surface of animal cell membranes [1]. Structures and functions of GSLs have been widely reviewed [2, 3]. In recent years reports have appeared on the structural characterization of GSLs, in particular, gangliosides of immunocompetent cells and target cells. The neutral GSLs and gangliosides of immune cells have been analysed by others and by us with the aim of detecting GSLs which might be specifically expressed by thymocytes [4], natural killer (NK) cells [5], macrophages [6, 7], T lymphocytes [8] and related subpopulations [9, 10] as well as by lymphoid tumour cell lines [11–13].

* To whom correspondence should be addressed.

0282-0080/91 $03.00+.12

The T cell lymphoma YAC-1, a Moloney virus-induced lymphoma of A/Sn origin, is known to be highly sensitive to NK cell mediated lysis and is the most commonly used target cell for mouse NK cells. YAC-1 lymphoma variants with different NK sensitivity have been shown by Yogeeswaran *et al.* [14] to express different ganglioside patterns, and the neutral GSL $GgOse_3Cer$ (asialo G_{M2}) has been discussed as the putative 'NK target structure'. Certain higher ganglioside homologues (than G_{M2} [14]) of the original YAC-1 cell line and several subclones showed a positive correlation with NK sensitivity, indicating the involvement of gangliosides in the recognizing process of NK cells and the targets, the YAC-1 cell line and its subclones. These results were confirmed in a recent report by Bergelson *et al.* [15], pointing out that target cell-associated gangliosides may function as target structures for NK cells. Furthermore, it is known that gangliosides shed by YAC-1 cells have inhibitory activity on mitogen- and antigen-induced lymphoproliferation [16]. The failure of a structural characterization of the gangliosides from this important cell line led us to a detailed structural analysis of YAC-1 gangliosides [17]. We could demonstrate that the gangliosides of the 'G_{M1b}-pathway' IVNeuAc/NeuGc-$GgOse_4Cer$ (G_{M1b}) and IVNeuAc/NeuGc-$GgOse_5Cer$ (GalNAc-G_{M1b}) were the main gangliosides expressed by YAC-1 cells.

Materials and methods

Animals

Female CBA/J inbred mice 6–8 weeks of age were purchased from Gl. Bomholtgard Ltd (Ry, Denmark).

Cells and culture conditions; metabolic labelling of cells

The cultivation of Concanavalin A (Con A) stimulated T lymphocytes from CBA/J spleens was carried out exactly as previously described [8].

YAC-1, a T cell lymphoma, induced by inoculation of the Moloney virus into newborn A/Sn mice [18], was a kind gift from B. Schwinzer (Medical High School Hannover, Germany). The cell line is available through the American Type Culture Collection (ATCC, Bethesda, MD, USA) designated as TIB 160. The cells were cultured in RPMI 1640 medium, supplemented with 10% (by vol) heat-inactivated foetal calf serum (Boehringer, Germany), in a humidified atmosphere of 7.5% CO_2 in air at 37°C. Larger amounts of cells were propagated in bioreactors in 1 l and 7.5 l volumes [19] by repeated batch cultivation.

ConA stimulated T lymphocytes and YAC-1 cells were labelled with D[1-^{14}C]glucosamine hydrochloride and D-[1-^{14}C]galactose as previously described [8]. After centrifugation at 225 × g for 10 min, the cells were washed twice with Dulbecco's phosphate-buffered saline (D-PBS). Finally, the cells were suspended in chloroform:methanol, 2:1 by vol, and stored at −20°C.

Isolation of YAC-1 gangliosides

1.0×10^{11} YAC-1 cells were mixed with 1.2×10^8 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 [20], and applied to 100 ml DEAE-Sepharose CL-6B (Pharmacia Fine Chemicals, Germany) in the acetate form. The gangliosides were eluted from the column with 0.15 M ammonium acetate in methanol. Alternatively, DEAE-Sepharose bound gangliosides were eluted with an acetate gradient system as previously described [8]. After evaporation and desalting by Sep-Pak™C_{18} cartridges (Millipore, Germany [21]), gangliosides were further purified by Iatrobeads 6RS-8060 chromatography (Iatron Laboratories, Inc., Japan [22]) and high performance liquid chromatography (HPLC, details see below). The final purification step was performed by high performance thin layer chromatography (HPTLC, see below).

Reference gangliosides of mouse and human brain

Gangliosides of CBA/J brains were isolated and purified by standard procedures [20–23]. A mixture of reference gangliosides from human brain was purchased from Supelco, Inc. (Bellefonte, PA, USA).

Preparation of asialogangliosides

Gangliosides (10 μg) were hydrolysed with 1 ml 1 M acetic acid for 45 min at 100°C. After neutralization by adding 1.67 ml 0.6 M NaOH in methanol, the sample was partitioned according to the method of Folch *et al.* [23]. The lower phase, containing the asialogangliosides, was washed twice with Folch's theoretical upper phase and dried in a stream of N_2.

High performance liquid chromatography (HPLC)

HPLC was carried out on a LiChrosorb Si 60 500-16 column with an average particle size of 5 μm (Merck, Germany) by use of a Varian 5060 liquid chromatograph equipped with a Jasco Uvidec 100-III variable wavelength detector (Japan Spectroscopic Co., Tokyo, Japan). Underivatized gangliosides were separated by preparative HPLC using a method originally described by Watanabe and Arao [24] and modified as follows: flow rate 1.5 ml min^{-1}; linear gradient from 55% 2-propanol, 42% hexane, 3% water to 55% 2-propanol, 25% hexane, 20% water (600 min). The UV response was monitored at 215 nm. Fractions were collected every 6 min (9 ml volumes). Radioactivity was determined in a Packard Minaxi Tri-Carb 4000 liquid scintillation spectrometer (Packard Inst. Co., Downers Grove, IL, USA)

in aliquots of each fraction after evaporation of the organic solvents. The final purification was done by preparative HPTLC.

Analytical and preparative thin layer chromatography

High performance thin layer chromatography plates (HPTLC plates, size 10 cm × 10 cm, thickness 0.24 mm, Merck, Germany) were used for analytical and preparative purposes. Two solvent systems were used for the separation of gangliosides (all ratios are by vol): I, chloroform:methanol:water, 120:85:20, containing 2 mM $CaCl_2$; II, chloroform:methanol:2.5 N NH_4OH, 120:70:16. Neutral GSLs were separated in III, chloroform:methanol:water, 120:70:17, containing 2 mM $CaCl_2$. Gangliosides were visualized by resorcinol [25]. Radioactive GSLs were located by autoradiography on Hyperfilm™-^{3}H (Amersham Buchler, Germany). Preparative amounts of GSLs were applied to the HPTLC plates with an automatic sample applier Linomat IV (Camag, Germany). Zones containing GSL were scraped off, and the silica gel was extracted as described in a preceding paper [8].

Detection of gangliosides of the G_{M1b}-type on HPTLC plates by immunostaining after neuraminidase treatment

The method for the detection of terminally sialylated gangliosides with the $GgOse_4Cer$ backbone (G_{M1b}, $G_{D1\alpha}$) has been recently described by Müthing and Mühlradt [26].

For the analysis of gangliosides with the $GgOse_5Cer$ core, plates were incubated with 50 mU/ml *A. ureafaciens* neuraminidase (Boehringer, Mannheim, Germany) for 24 h at 30°C in the presence of 0.5 mg ml^{-1} sodium taurodeoxycholate in 0.1 M sodium acetate (pH 4.8) as described by Hirabayashi *et al.* [27]. The monoclonal anti-$GgOse_3Cer$ antibody 2D4 (TIB 185, American Type Culture Collection, Bethesda, MD, USA [29]) which cross-reacts with $GgOse_5Cer$ [8, 26, 28] was used for the enzyme immunostaining procedure, which has been published recently by Müthing and Ziehr [30].

Preparation of antisera against YAC-1 gangliosides

Specific pathogen free chickens (VALO, Lohmann, Cuxhaven, Germany) were hatched and thereafter raised in isolators under filtered negative air pressure. At the age of 12 weeks the animals were immunized according to the method of Kasai *et al.* [31]. Two chickens were injected each with approximately 100 µg of the gangliosides 6 and 8, adsorbed to 1 mg of methylated bovine serum albumin (Serva, Germany), respectively (see Table 2). The preparations were emulsified with equal parts of complete Freund's adjuvant (Difco, Detroit, MI, USA) in a final volume of 1 ml and administered at multiple intramuscular sites. Preimmune sera were collected just before immunization. After 4 weeks, the chickens were boostered and exsanguinated 14 days later.

Immunostaining of gangliosides with chicken anti-ganglioside antisera on HPTLC plates

Gangliosides were chromatographed on HPTLC plates. The silica gel was fixed and the immunostaining procedure was carried out as described previously [26]. Chicken anti-ganglioside antisera were diluted 1:200 and alkaline phosphatase labelled anti-chicken IgG (Dianova, Hamburg, Germany) used at 1:1000 dilution.

Mass spectrometric analysis of purified gangliosides

Fast atom bombardment mass spectrometry (FAB-MS) was performed on a ZAB HF mass spectrometer (VG Analytical, Manchester, UK) essentially as described earlier [32]. The native gangliosides were dissolved in methanol:2N hydrochloric acid, 2:1 by vol, and analysed by negative ion FAB-MS in thioglycerol as a matrix. The permethylation was carried out as described by Ciucanu and Kerek [33]. The permethylated gangliosides were separated from reagents by exhaustive extraction with chloroform and chromatographed on a 1 cm × 20 cm Sephadex LH-20 column using methanol:chloroform, 1:1 by vol, as eluent. The permethylated ganglioside samples were analysed by positive ion FAB-MS in thioglycerol as a matrix. The atom gun was operated at 9 kV.

Methylation analysis

The purified permethylated gangliosides were acetolysed, reduced and acetylated as previously described [34]. Total ion current (TIC) and selective ion monitoring (SIM) profiles were acquired.

Results

Metabolically labelled gangliosides from stimulated T lymphocytes and YAC-1 cells

Previous investigations [8] have shown the considerable heterogeneity of gangliosides from Con A stimulated splenic T lymphocytes. The main components found in T cells were G_{M1b} (NeuAc and NeuGc), IVNeuAc/Gc-$GgOse_5Cer$ and two disialogangliosides of the $G_{D1\alpha}$ type [8, 9, 26]. The reference gangliosides from T cells are listed in Table 1. Comparing metabolically labelled gangliosides from YAC-1 T lymphoma cells and stimulated T cells, we found high conformity in the monosialoganglioside range of both cell types (see Fig. 1, fractions I–IV).

Detection of G_{M1b}-type gangliosides in the whole ganglioside fraction of YAC-1 T lymphoma cells

The expression of terminally sialylated gangliosides with the $GgOse_4Cer$ backbone ($=G_{M1b}$) in YAC-1 cells was proved by specific desialylation of terminally sialylated $GgOse_4Cer$ with *V.cholerae* neuraminidase and immunostaining with anti-$GgOse_4Cer$ antibodies on HPTLC plates [26]. Three

Table 1. Structures of gangliosides from mouse T-lymphocytes.[a]

Ganglioside fraction	*Fatty acid component*	*Structure*
I	$C_{24:1}$	$IV^3NeuAc\text{-}GgOse_4Cer$[b]
II	$C_{16:0}$	$IV^3NeuAc\text{-}GgOse_4Cer$[b]
	$C_{24:0}$ $C_{24:1}$	$IVNeuGc\text{-}GgOse_4Cer$[b]
	$C_{24:0}$ $C_{24:1}$	$IVNeuAc\text{-}GgOse_5Cer$[c]
III	$C_{16:0}$	$IVNeuGc\text{-}GgOse_4Cer$[b]
	$C_{16:0}$	$IVNeuAc\text{-}GgOse_5Cer$[c]
	$C_{24:0}$	$IVNeuGc\text{-}GgOse_5Cer$[c]
IV	$C_{16:0}$	$IVNeuGc\text{-}GgOse_5Cer$[c]
V	$C_{24:0}$ $C_{24:1}$	$IVNeuAc,IIINeuAc\text{-}GgOse_4Cer$[b]
VI	$C_{16:0}$	$IVNeuAc,IIINeuAc\text{-}GgOse_4Cer$[b]

[a] Data drawn from Müthing *et al.* [8, 26] and Müthing and Ziehr [30].
[b] G_{M1b} type gangliosides, detectable by immunostain with the anti-$GgOse_4Cer$ antibody after *Vibrio cholerae* neuraminidase treatment [26].
[c] Gangliosides with the $GgOse_5Cer$ backbone, detectable by immunostain with the cross-reacting anti-$GgOse_3Cer$ antibody after *Arthrobacter ureafaciens* neuraminidase treatment [30].

G_{M1b}-bands are detectable in the whole ganglioside fraction of YAC-1 cells (Fig. 2, lane a, fractions I, II and III). These gangliosides chromatograph identically compared to the well characterized G_{M1b}-type gangliosides of T cells (Fig. 2, lane b, fractions I, II and III, see Table 1). Whereas two disialogangliosides of the $G_{D1\alpha}$ type with C_{24} and C_{16} fatty acids are principal components of T cells, YAC-1 cells are completely lacking these GSLs (see Fig. 2, fractions V and VI, Table 1).

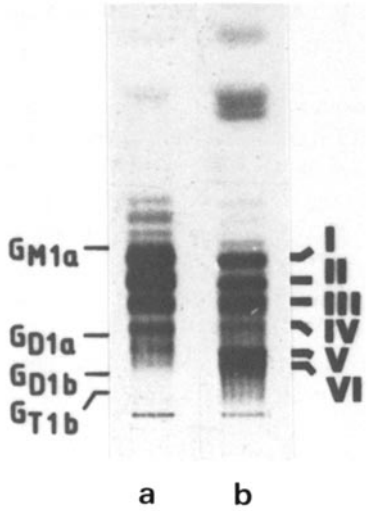


Figure 1. Autoradiography of gangliosides from metabolically labelled YAC-1 T lymphoma cells (lane a) and Con A-stimulated T cells (lane b). ^{14}C-Labelled gangliosides (3000 cpm) were applied and chromatographed in chloroform:methanol:water, 120:85:20 by vol, with 2 mM $CaCl_2$. Exposure time 190 h. The positions of human brain gangliosides are marked. T cell gangliosides of bands I to VI are enumerated in Table 1.

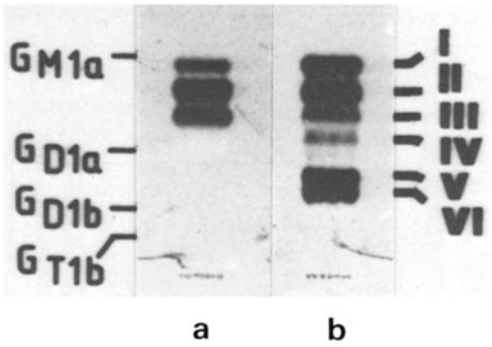


Figure 2. Detection of G_{M1b} type gangliosides in the whole ganglioside fraction of YAC-1 T lymphoma cells. Gangliosides of 1.8×10^6 YAC-1 cells (lane a) and 4.5×10^6 Con A stimulated T cells (lane b) were chromatographed as described in Fig. 1. After chromatography the silica gel was fixed (see the Materials and methods section) and the plate was overlayed with *V.cholerae* neuraminidase (2.5 mU ml^{-1}, 2 h at room temperature). $GgOse_4Cer$ bands were detected by immunostaining with specific antibodies. The positions of human brain gangliosides are marked. T cell gangliosides of bands I to VI are enumerated in Table 1.

Detection of GalNAc-G_{M1b} in the whole ganglioside fraction of YAC-1 cells

Prior neuraminidase treatment is also necessary for the binding of the monoclonal antibody 2D4 to gangliosides with the $GgOse_5Cer$ backbone. The cleavage of *N*-glycolylneuraminic acid from the $GgOse_5Cer$ core has been demonstrated to be a critical step using the neuraminidase from *A. ureafaciens* under standard conditions [9] but, after increase of enzyme concentration, incubation temperature and time, complete removal of NeuGc was achieved as described by Müthing and Ziehr [30]. This technique was then applied to the identification of all gangliosides with the $GgOse_5Cer$ backbone. Three GalNAc-G_{M1b} positive bands were detected in the whole ganglioside fraction of YAC-1 cells (not shown, published in [30]), chromatographing in fractions II, III and IV (cf. Figs 1, 2).

Structural characterization of YAC-1 gangliosides

The structural mapping of the monosialganglioside fraction isolated from the YAC-1 ganglioside mixture was carried out by combining immunological and spectroscopic data. The HPTLC separation of the intact gangliosides and their corresponding asialogangliosides obtained after acid and neuraminidase treatment was followed by immunostaining of the asialogangliosides with specific antibodies as described.

FAB-MS

The direct evidence about structural parameters of all native gangliosides was obtained by negative ion FAB-MS as described previously [8, 32]. The mapping data are summarized in Table 2.

Table 2. Main monosialogangliosides form YAC-1 cells.[a]

Ganglioside fraction	*No.*	[M − 1] *of native gangliosides*	$[M+H]^+/[M+Na]^+$ *of permethylated gangliosides*	*Major fatty acid component*	*Symbol*	*Structure*	*References*
I	**1**	1628	1910/1932	24:0	G_{M1b}	IVNeuAcGgOse$_4$Cer[b]	8, 11, 35,
		1626	1908/1930	24:1			36, 38
II	**2**	1516	n.d.	16:0	G_{M1b}	IVNeuAc-GgOse$_4$Cer[b]	
	3	1644	1940/1962	24:0	G_{M1b}	IVNeuGc-GgOse$_4$Cer[b]	8, 35, 37
		1642	1938/1960	24:1			
	4	1831	n.d.	24:0	GalNAc-G_{M1b}	IVNeuAc-GgOse$_5$Cer[c]	
		1829		24:1			
III	**5**	1532	n.d.	16:0	G_{M1b}	IVNeuGc-GgOse$_4$Cer[b]	8, 37
	6	1719	2043/2065	16:0	GalNAc-G_{M1b}	IVNeuAc-GgOse$_5$Cer[c]	8, 35, 38
	7	1847	n.d.	24:0	GalNAc-G_{M1b}	IVNeuGc-GgOse$_5$Cer[c]	8, 37
		1845		24:1			
IV	**8**	1735	2073/2095	16:0	GalNAc-G_{M1b}	IVNeuGc-GgOse$_5$Cer[c]	8, 35, 37

[a] n.d. = not determined.
[b] G_{M1b} type gangliosides, detectable by immunostain with the anti-GgOse$_4$Cer antibody after *Vibrio cholerae* neuraminidase treatment [26].
[c] GalNAc-G_{M1b} type gangliosides, detectable by immunostaining with the cross-reacting anti-GgOse$_3$Cer antibody after *Arthrobacter ureafaciens* neuraminidase treatment [30].

These results revealed that the structural variations of monosialogangliosides arise from the variation in the carbohydrate backbone chain length and the types of sialic acid and fatty acid in the ceramide portion present. The HPTLC separation of the ganglioside species with the same carbohydrate core and ceramide portions, but different sialic acids, like gangliosides **6** and **8** of the YAC-1 fractions III and IV (see Table 2) can, however, be achieved (Fig. 3). The gangliosides IVNeuAc-GgOse$_4$Cer (**1**), IVNeuGc-GgOse$_4$Cer (**3**), IVNeuAc-GgOse$_5$Cer (**6**), and IVNeuGc-GgOse$_5$Cer (**8**) were postulated to be structurally homogeneous in their respective carbohydrate and lipid portion, based on data from negative ion FAB-MS concerning the molecular ions $[M-1]^-$ as well as sequence relevant fragments [36] (Table 2). The sole structural difference between the gangliosides **1** and **3** and the gangliosides **6** and **8** was located in sialic acid moiety (Δ (amu) = 16 for NeuAc versus NeuGc).

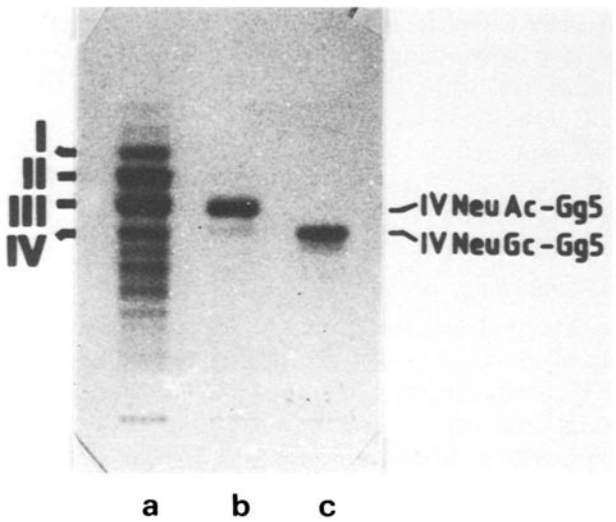
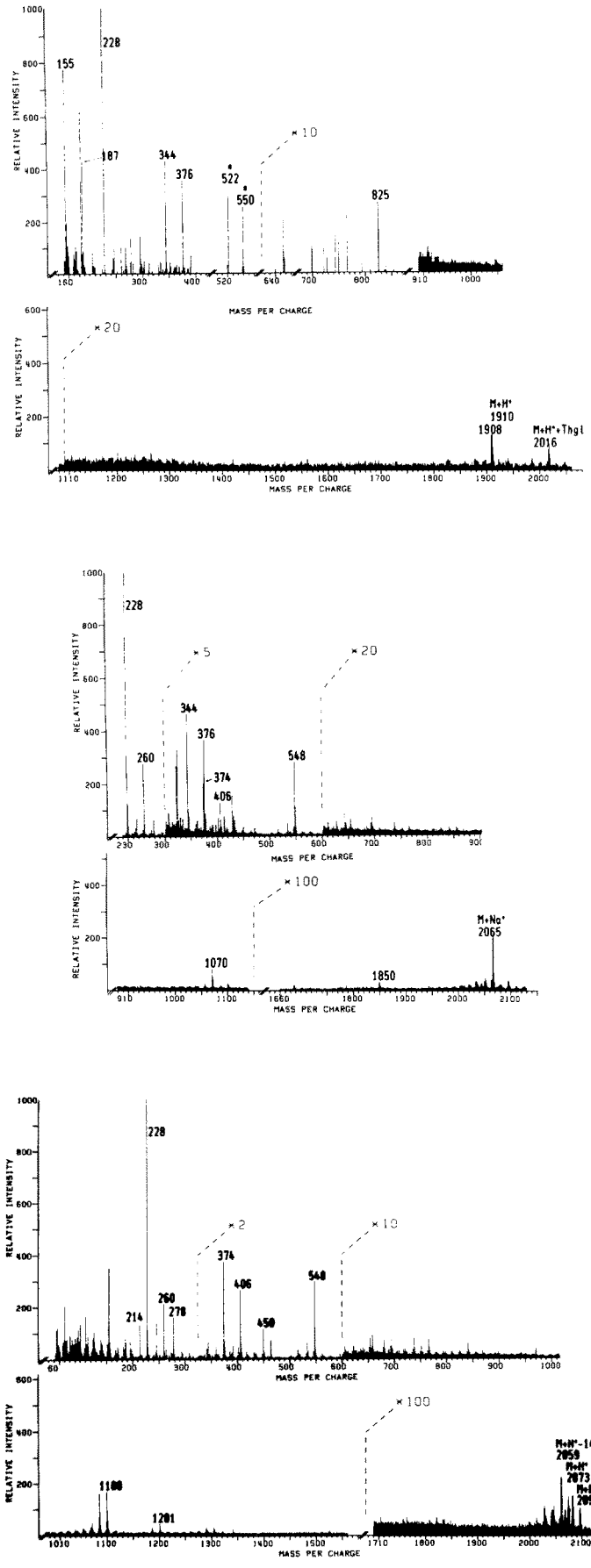


Figure 3. Resorcinol stain of IVNeuAc- and IVNeuGc-GgOse$_5$Cer isolated from YAC-1 cells. 5 µg YAC-1 gangliosides (lane a) and approximately 0.5 µg each IVNeuAc-GgOse$_5$Cer, $C_{16:0}$ fatty acid (lane b, ganglioside **6** of fraction III) and IVNeuGc-GgOse$_5$Cer, $C_{16:0}$ fatty acid (lane c, ganglioside **8** of fraction IV) were chromatographed as described in Fig. 1. Gangliosides were detected with resorcinol. YAC-1 gangliosides of bands I to IV are enumerated in Table 2. Gg5 = GgOse$_5$Cer.

The gangliosides **1**, **3**, **6** and **8** were further characterized by positive ion FAB-MS on their permethylated derivatives. In positive ion FAB-MS of permethylated IVNeuAc-GgOse$_4$Cer (**1**) the molecular ion $[M+H]^+$ for NeuAcHex NAcHex$_3$Cer$_{24:0/24:1}$ appeared at m/z = 1910/1908. The ion at m/z = 825 was indicative for the trisaccharide fragment ion $[NeuAcHexHexNAc]^+$ (Fig. 4(a), Fig. 5). The ceramide ions were not detected. The GC/MS analysis of partially methylated alditol acetates derived from the ganglioside **1** revealed in its TIC profile the presence of 3- and 4-substituted galactose, as expected for G_{M1b} type ganglioside (R_F = 8.69 and 8.51 min). 3-Substituted *N*-acetylgalactosamine at R_F = 11.75 min was characterized by ions at m/z = 158, 230, 274 and 318 obtained by SIM acquisition. The permethylated compound **3** would be expected to show in its FAB mass spectrum the specific terminal trisaccharide ion for $[NeuGcHexHexNAc]^+$ at m/z = 855 as reported for its homologue with fatty acid 16:0 [38].

The FAB-MS spectra of compounds **6** and **8** showed a



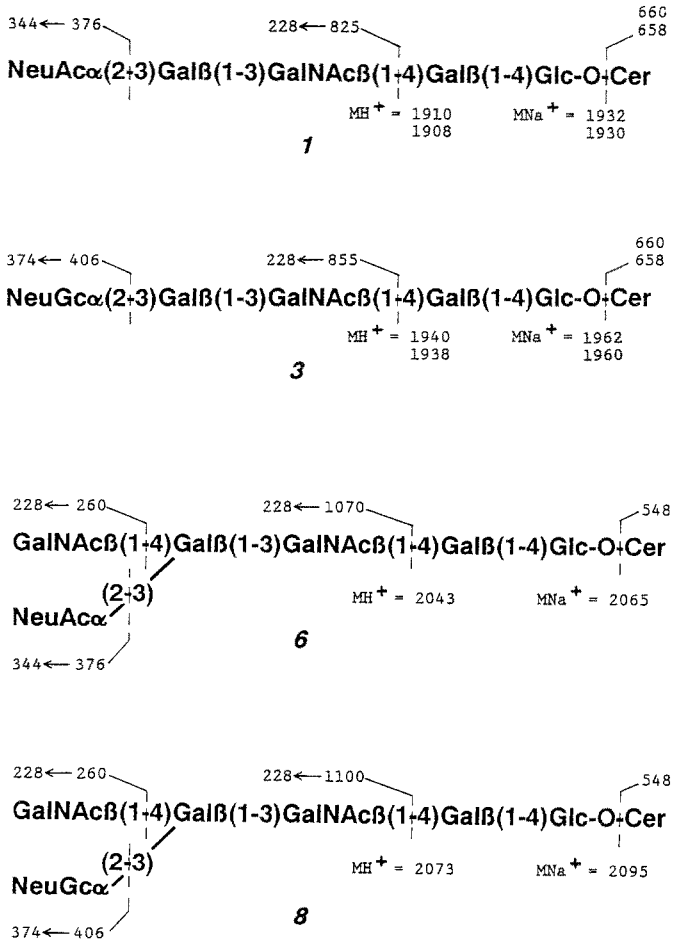


Figure 5. Fragmentation patterns of permethylated gangliosides **1**, **3**, **6** and **8** isolated from YAC-1 cells (see Table 2).

high degree of homology in the carbohydrate and lipid part. The molecular ions were shifted by 30 amu due to the occurrence of NeuAc and NeuGc (Fig. 4b, c). The molecular ion $[M + Na]^+$ of ganglioside **6** appeared at $m/z = 2065$ (Fig. 4(b)), accounting for $NeuAcHexNAc_2Hex_3Cer_{16:0}$. The terminal $[HexNAc]^+$ was represented by the ion at $m/z = 260$ and the corresponding terminal tetrasaccharide $[HexNAc[NeuAc]HexHexNAc]^+$ by $m/z = 1070$. Both ions produce the same daughter ion at $m/z = 228$ after eliminating the substituents bound to position 3 of the HexNAc. The ceramide ion was at $m/z = 548$. A minor ganglioside component $NeuGcHexHexNAcHex_2Cer$ (16:0) would account for the presence of the $[NeuGc]^+$ ion at

Figure 4. Positive ion FAB-MS of permethylated YAC-1 gangliosides IVNeuAc-$GgOse_4Cer$ with C_{24} fatty acid (ganglioside **1** of fraction I) (a), IVNeuAc-$GgOse_5Cer$ (ganglioside **6** of fraction III) (b) and IVNeuGc-$GgOse_5Cer$ (ganglioside **8** of fraction IV) (c) with C_{16} fatty acid, respectively. The numbered YAC-1 gangliosides are listed in Table 2. In FAB-MS depicted in part (b) the matrix background has been subtracted. * = impurities from solvents.

$m/z = 406 \rightarrow 374$, beside the corresponding molecular ion $[M + Na]^+$ at $m/z = 1850$, both of low intensity. IVNeuGc-$GgOse_5Cer$ (**8**) was identified on the basis of the molecular ion MH^+ at $m/z = 2073$, with palmitic acid in the ceramide part and specific ions for terminal $[HexNAc]^+$ at $m/z = 260$ and $[HexNAc[NeuGc]HexHexNAc]^+$ at $m/z = 1100$ ($\rightarrow$228). As well as the ceramide ion at $m/z = 548$, a sphingosine characteristic ion appeared at $m/z = 278$ giving indirect information about the fatty acid substitution in the lipid part. The ion at $m/z = 1201$ could possibly arise from the unusual cleavage of the glycosidic linkage at the HexNAc unit derived from the ceramide end of the molecule. The undermethylation, probably due to the steric crowding in the outer galactose area, gave rise to the heterogeneity in the tetrasaccharide ion as well as in the molecular ion area, as documented by sequences differing in 14 mass units.

IINeuAc-$GgOse_3Cer$ and IINeuGc-$GgOse_3Cer$ (G_{M2}), each substituted with $C_{24:0,24:1}$ and $C_{16:0}$ fatty acids were identified by FAB-MS (data not shown) and found in low amounts among YAC-1 gangliosides, chromatographing beyond band I (see Fig. 1, lane a). G_{M1} occurred in trace amounts and was detected with a modification of the highly sensitive cholera toxin-immunostaining procedure as described by Nakamura *et al.* [37] (data not shown) indicating the low expression of the G_{M1} pathway as well as the predominant G_{M1b} pathway of YAC-1 cells. $G_{D1\alpha}$, which has been found in normal murine T lymphocytes [9] (see Fig. 2, lane b, fractions V and VI, and Table 1), was not detectable in YAC-1 cells.

Preparation of antibodies against YAC-1 gangliosides IVNeuAc- and IVNeuGc-$GgOse_5Cer$

Two chickens were immunized with IVNeuAc- and IVNeuGc-$GgOse_5Cer$, both with $C_{16:0}$ fatty acid (see Fig. 3 and Table 2, gangliosides **6** and **8**), respectively, as described above. Both antisera were tested for their specific recognition of gangliosides from different origin: human brain, mouse brain, murine T cells and YAC-1 cells. Figure 6A shows the resorcinol stain of the gangliosides and Fig. 6B the overlay assay of the same gangliosides, using the antiserum against IVNeuAc-$GgOse_5Cer$ (ganglioside **6**). Figure 6B shows three immunostained bands in the T cell ganglioside fraction (lane c, fractions II, III and IV, see Table 1) and three positive bands in the YAC-1 ganglioside fractions (lane d, whole mixture, and lane e, monosialogangliosides, fractions II, III and IV, see Table 2), representing IVNeuAc- and IVNeuGc-$GgOse_5Cer$: IVNeuAc-$GgOse_5Cer$ (C_{16}-fatty acid, ganglioside **6**, see Table 2) and IVNeuGc-$GgOse_5Cer$ (C_{24}-fatty acid, ganglioside **7**, see Table 2) which cochromatograph as one band between IVNeuAc-$GgOse_5$ Cer with C_{24} fatty acid (weak upper band, fraction II, ganglioside 4) and IVNeuGc-$GgOse_5Cer$ with C_{16} fatty acid (lower one of the three bands, fraction IV, ganglioside **8**) as shown for purified T cell gangliosides [8] (see Table 1, fractions II, III and IV). Alternatively, identical immunostained bands were achieved by the combined method of *A. ureafaciens* neuraminidase treatment followed by immunodetection of the desialylated gangliosides with the monoclonal antibody 2D4 (not shown, see [30]).

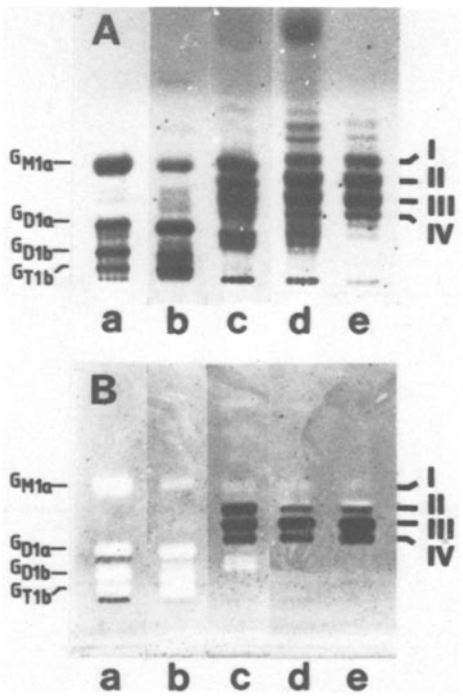


Figure 6. Test of a chicken anti-IVNeuAc-$GgOse_5Cer$ antiserum for cross-reactions with gangliosides from different sources. A, Resorcinol stain of different ganglioside mixtures: gangliosides from human brain (lane a, 10 µg), mouse brain (lane b, 10 µg), Con A-stimulated T cells (lane c, 15 µg), YAC-1 (lane d, 15 µg) and monosialogangliosides from YAC-1 (lane e, 10 µg) were chromatographed as described in Fig. 1. The positions of human brain gangliosides are marked. YAC-1 gangliosides of bands I to IV are enumerated in Table 2. B, Immunostain of the same ganglioside mixtures used in A with a chicken anti-IVNeuAc-$GgOse_5Cer$ antiserum. Gangliosides were applied to the HPTLC plate as in A but, in the case of lanes c, d and e, only 1/5 in comparison to the resorcinol stain were used to prevent overloading of the plate, corresponding to 8.7×10^6 T cells (lane c) and 4.8×10^6 YAC-1 cells (lane d and e, respectively). The assay was performed as described in the Materials and methods section. The positions of human brain gangliosides are marked. YAC-1 gangliosides of bands I to IV are enumerated in Table 2.

A weak cross-reactivity of the polyclonal antiserum is observed with the human brain ganglioside fraction (Fig. 6B, lane a, below G_{D1a}), probably displaying the disialopentahexosylceramide IVNeuAc,IINeuAc-$GgOse_5Cer$ previously isolated from normal human brain by Svennerholm *et al.* [39]. A pre-immuneserum taken from the animal before immunization did not react with any of the gangliosides and neutral GSLs available for testing.

The fact that the anti-IVNeuAc-$GgOse_5Cer$ antiserum

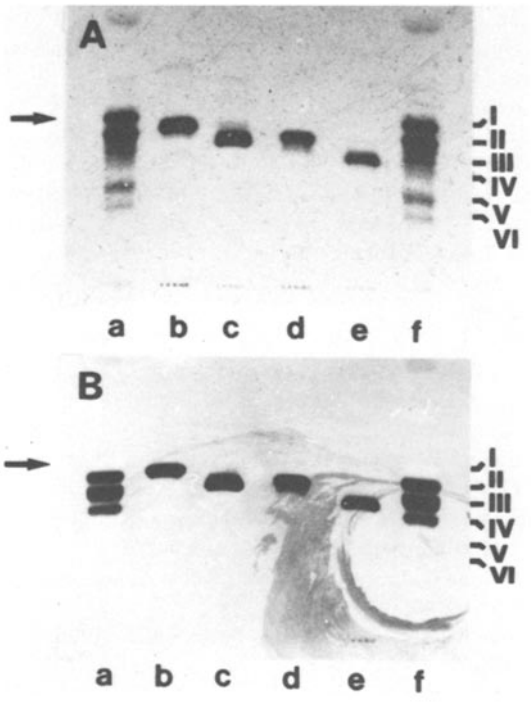


Figure 7. Test of a chicken anti-IVNeuAc-GgOse$_5$Cer antiserum for recognition of IVNeuAc- and IVNeuGc-GgOse$_5$Cer. A, Autoradiography of metabolically labelled gangliosides from ConA-stimulated T cells. 1000 cpm of ^{14}C-labelled ConA T cell gangliosides (lane a and f) and single gangliosides, each 250 cpm, IVNeuAc-GgOse$_5$Cer, C_{24} fatty acid (lane b), IVNeuAc-GgOse$_5$ Cer, C_{16} fatty acid (lane c), IVNeuGc-GgOse$_5$Cer, C_{24} fatty acid (lane d) and IVNeuGc-GgOse$_5$Cer, C_{16} fatty acid (lane e) were applied and chromatographed as described in Fig. 1. Exposure time 360 h. T cell gangliosides of bands I to VI are listed in Table 1. B, Immunostain of the same HPTLC plate as in A with a chicken anti-IVNeuAc-GgOse$_5$Cer antiserum. The assay was performed as described in the Materials and methods section. The autoradiography was taken from the same plate after the immunostain. T cell gangliosides of bands I to VI are enumerated in Table 1.

recognizes IVNeuAc- and IVNeuGc-GgOse$_5$Cer alike is demonstrated in Fig. 7: The four gangliosides IVNeuAc- and IVNeuGc-GgOse$_5$Cer and C_{24} and C_{16} fatty acid, respectively, isolated from the fractions II, III and IV of Con A stimulated T cells (Fig. 7A, see Table 1) could not be distinguished by the polyclonal antiserum (Fig. 7B). No crossreaction with G_{M1b} (NeuAc and NeuGc) or with GgOse$_4$Cer could be detected, but the antiserum strongly reacted with GgOse$_5$Cer from T cell asialogangliosides or human brain asialogangliosides (data not shown).

Similar results were obtained with a second antiserum raised by immunizing a chicken with IVNeuGc-GgOse$_5$Cer (ganglioside **8**). This antiserum showed additional weak cross-reactivity with GgOse$_4$Cer (from murine T cells and human brain). No reaction of this antiserum was observed with G_{M1b} (NeuAc and NeuGc), too. An attempt to raise antisera against G_{M1b} (NeuAc and NeuGc; see Table 2, gangliosides **1** and **3**) failed.

Discussion

G_{M1b} and GalNAc-G_{M1b}, previously characterized as T cell specific antigens [8], were the main gangliosides found in the complex GSL mixture of YAC-1 cells, revealing the predominance of the 'G_{M1b}-pathway' in this T-lymphoma (see Table 2). The heterogeneity in the gangliosides was caused by (a) substitution of the ceramide moiety by fatty acids of different chain length ($C_{16:0}$, $C_{24:0}$, $C_{24:1}$) and *N*-substitution of the sialic acids with either an acetyl or a glycolyl group. G_{M2} and G_{M1}, as the representatives of the 'G_{M1} pathway', were found only in low amounts. Interestingly, $G_{D1\alpha}$, previously demonstrated in normal murine T lymphocytes [9], the murine lymphoma Eb [11] and in murine Friend erythroleukemia cells [13] as well as G_{D1c}, a related disialoganglioside of the G_{M1b} pathway described in a mouse thymoma cell line [12] were not expressed by YAC-1 cells. Currently available data indicate that GalNAc-G_{M1b} seems to be restricted in mice to the T cell lineage [8, 9]. The antibody raised against IVNeuAc/NeuGc-GgOse$_5$Cer offers a good tool to screen other normal lymphoid cells, e.g., B lymphocytes, macrophages, etc., as well as related tumour lines [38] for the expression of GalNAc-G_{M1b}. The restriction to murine thymus and spleen [35, 37] can now be simply tested analysing GSL extracts from diverse organs with this antibody using the overlay technique as described in this paper.

The YAC-1 lymphoma represents the most commonly used target cell line for mouse NK cells. The original cell line and several YAC-1 lymphoma variants with different NK sensitivity have been shown by Yogeeswaran *et al.* [14] to produce different ganglioside patterns. Certain higher ganglioside homologues (than G_{M2}) with mobilities between G_{M1} and G_{D1a} showed a positive correlation with NK sensitivity. The failure of detailed structural characterization of these YAC-1 and subclones gangliosides [14] did not allow further conclusions about the participation of certain gangliosides in NK-cell-mediated lysis. The expression of G_{M1b} and GalNAc-G_{M1b} on YAC-1 subclones with different NK sensitivities [14, 40] could now be estimated by the described overlay technique. This approach would help to clarify the role of 'G_{M1b}-type' gangliosides in NK cell–target interactions. The occurrence of G_{M1b} and GalNAc-G_{M1b} among gangliosides of ConA stimulated T cells, which are not targets of NK cells, seems to rule out these structures as NK cell targets on YAC-1 cells. But it should be pointed out that GSL occurrence may not just reflect availability of GSL as target structures. However, both gangliosides are more abundant on YAC-1 cells (about factor 2 compared to T cells) as estimated by immunological detection (Figs 6B, lanes c and d) and it remains to be

determined whether NK cells bind to G_{M1b} and/or GalNAc-G_{M1b}. The involvement of gangliosides in the recognition process of NK cells and the target lymphoma YAC-1 was clearly demonstrated by Bergelson *et al.* [15]. The lysis of YAC cells by mouse splenocytes was most strongly increased by incubation of the target cells with ganglio-series gangliosides G_{D3} and G_{M3} (both substituted with NeuGc) prior to their exposure to NK effectors. These effects depended on the oligosaccharide structures of the gangliosides [15] and it was convincingly suggested that cell-associated gangliosides function as target structures recognized by NK cells. This hypothesis was supported by data of Ando *et al.* [41] indicating that G_{M2} is a strong candidate as a target in NK-cell-mediated lysis. Related to our data, it should be mentioned that 'naturally' occurring terminally sialylated G_{M1b} and GalNAc-G_{M1b} of YAC-1 cells correspond to G_{M3} [15] and G_{M2} [41] due to their terminal oligosaccharide sequences, respectively, but the role of 'G_{M1b}-type' gangliosides as NK target structures remains speculative without further biological data at this stage of research.

The physiological importance of YAC-1 gangliosides to the immune response of stimulated lymphocytes has been described by Ladisch *et al.* [16]. Gangliosides shed by YAC-1 cells caused inhibition of the proliferative response of mitogen- and antigen-stimulated lymphocytes. Furthermore, it was supposed by the authors that YAC-1 gangliosides shed *in vivo* may serve to protect tumor cells from host immune destruction.

In summary, the participation of gangliosides in the interactions of immunocompetent and target cells has been well documented by several authors. The structural investigations of GSLs by HPTLC only have been shown to be insufficient for final conclusions. More detailed structural characterizations are necessary to prove the hypotheses about the involvement of certain tumour GSLs in the process of self–nonself discrimination by cells of the immune system.

Acknowledgements

This study was supported in part by grants from the Gesellschaft für Biotechnologische Forschung mbH (GBF) for promotion of the Deutsche Sammlung von Mikroorganismen. We thank Prof. J. Lehmann and G. W. Piel for help with cultivating YAC-1 cells in bioreactors. Financial help by the Fonds der Chemischen Industrie is gratefully acknowledged. We express our warmest thanks to Prof. P. F. Mühlradt (GBF, Braunschweig, Germany) and Prof. H. Egge (University of Bonn, Germany) in whose laboratories parts of this work have been carried out. We are furthermore grateful to Dr. B. Kniep for HPLC analysis and to N. Rösel for excellent technical assistance. We thank Dr. H. Ziehr for critical reading of the manuscript.

References

1. Thompson TE, Tillack TW (1985) *Ann Rev Biophys Biophys Chem* **14**:361–86.
2. Hakomori S (1986) *Chem Phys Lipids* **42**:209–33.
3. Hakomori S (1984) In *The Cell Membrane* (Habes E, ed.) pp. 181–201. New York: Plenum.
4. Schwarting GA, Gajewski A (1983) *J Biol Chem* **258**:5893–8.
5. Kasai M, Iwamori M, Nagai Y, Okumura K, Tada T (1980) *Eur J Immunol* **10**:175–80.
6. Mercurio AM, Schwarting GA, Robbins PW (1984) *J Exp Med* **160**:1114–25.
7. Bethke U, Kniep B, Mühlradt PF (1987) *J Immunol* **138**:4329–35.
8. Müthing J, Egge H, Kniep B, Mühlradt PF (1987) *Eur J Biochem* **163**:407–16.
9. Müthing J, Schwinzer B, Peter-Katalinić J, Egge H, Mühlradt PF (1989) *Biochemistry* **28**:2923–9.
10. Kniep B, Hünig TR, Fitch FW, Heuer J, Kölsch E, Mühlradt PF (1983) *Biochemistry* **22**:251–5.
11. Murayama K, Levery SB, Schirrmacher V, Hakomori S (1986) *Cancer Res* **46**:1395–402.
12. Bartoszewicz Z, Koscielak J, Pacuszka T (1986) *Carbohydr Res* **151**:77–88.
13. Rokukawa C, Nakamura K, Handa S (1988) *J Biochem (Tokyo)* **103**:36–42.
14. Yogeeswaran G, Gronberg A, Hansson M, Dalianis T, Kiessling R, Welsh RM (1981) *Int J Cancer* **28**:517–26.
15. Bergelson LD, Dyatlovitskaya EV, Klyuchareva TE, Kryukova EV, Lemenovskaya AF, Matveeva VA, Sinitsyna EV (1989) *Eur J Immunol* **19**:1979–83.
16. Ladisch S, Gillard B, Wong C, Ulsh L (1983) *Cancer Res* **43**:3808–13.
17. Müthing J, Peter-Katalinić J, Egge H, Neumann U, Kniep B, Loyter A, Mühlradt PF (1989) Xth Int. Symposium on Glycoconjugates, Jerusalem, Israel.
18. Klein E, Klein G (1964) *J Natl Cancer Inst* **32**:547–68.
19. Lehmann J, Vorlop J, Büntemeyer H (1988) In *Animal Cell Biotechnology 3* (Spier RE, Griffiths JB, eds) pp. 221–37. New York: Academic Press.
20. Momoi T, Ando S, Nagai Y (1976) *Biochim Biophys Acta* **441**:488–97.
21. Williams MA, McCluer RH (1980) *J Neurochem* **35**:266–9.
22. Ueno K, Ando S, Yu RK (1978) *J Lipid Res* **19**:863–71.
23. Folch J, Lees M, Sloane Stanley GH (1957) *J Biol Chem* **226**:497–509.
24. Watanabe K, Arao Y (1981) *J Lipid Res* **22**:1020–4.
25. Svennerholm L (1957) *Biochim Biophys Acta* **24**:604–11.
26. Müthing J, Mühlradt PF (1988) *Anal Biochem* **173**:10–7.
27. Hirabayashi Y, Koketsu K, Higashi H, Suzuki Y, Matsumoto M, Sugimoto M, Ogawa T (1986) *Biochim Biophys Acta* **876**:178–82.
28. Bethke U, Müthing J, Conradt P, Mühlradt PF (1986) *J Immunol Methods* **89**:111–6.
29. Young WW, MacDonald EMS, Nowinski RC, Hakomori S (1979) *J Exp Med* **150**:1008–19.
30. Müthing J, Ziehr H (1990) *Biomed Chromatogr* **4**:70–2.
31. Kasai N, Naiki M, Ariga T, Hirabayashi Y, Yu RK (1985) *Biochem Biophys Res Commun* **129**:334–41.
32. Egge H, Peter-Katalinić J (1987) *Mass Spectrom Rev* **6**:331–93.

33. Ciucanu I, Kerek F (1984) *Carbohydr Res* **131**:209–17.
34. Hanisch FG, Uhlenbruck G, Peter-Katalinić J, Egge H (1988) *Carbohydr Res* **178**:29–47.
35. Nakamura K, Hashimoto Y, Suzuki M, Suzuki A, Yamakawa T (1984) *J Biochem (Tokyo)* **96**:949–57.
36. Pohlentz G, Klein D, Schmitz D, Schwarzmann G, Peter-Katalinić J, Sandhoff K (1988) *Biol Chem Hoppe-Seyler* **369**:55–63.
37. Nakamura K, Suzuki M, Inagaki F, Yamakawa T, Suzuki A (1987) *J Biochem (Tokyo)* **101**:825–35.
38. Laferté S, Fukuda MN, Fukada M, Dell A, Dennis JW (1987) *Cancer Res* **47**:150–9.
39. Svennerholm L, Mansson JE, Li YT (1973) *J Biol Chem* **248**:740–2.
40. Young WW, Durdik JM, Urdal D, Hakomori SI, Henney CS (1981) *J Immunol* **126**:1–6.
41. Ando I, Hoon DSB, Suzuki Y, Saxton RE, Golub SH, Irie RF (1987) *Int J Cancer* **40**:12–7.