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Ganglioside alterations in YAC-1 cells cultivated in serum-supplemented and serum-free growth medium

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Gangliosides of the 'G_{M1b}-pathway' (G_{M1b} and GalNAc-G_{M1b}) have been found to be highly expressed by the mouse T lymphoma YAC-1 grown in serum-supplemented medium, whereas G_{M2} and G_{M1} ('G_{M1a}-pathway') occurred only in low amounts [Müthing, J., Peter-Katalinić, J., Hanisch, F.-G., Neumann, U. (1991) *Glycoconjugate J* **8**:414–23]. Considerable differences in the ganglioside composition of YAC-1 cells grown in serum-supplemented and in well defined serum-free medium were observed. After transfer of the cells from serum-supplemented medium (RPMI 1640 with 10% fetal calf serum) to serum-free medium (RPMI 1640 with well defined supplements), G_{M1b} and GalNAc-G_{M1b} decreased and only low amounts of these gangliosides could be detected in serum-free growing cells. The expression of G_{M1a} was also diminished but not as strongly as that of G_{M1b} and GalNAc-G_{M1b}. These growth medium mediated ganglioside alterations were reversible, and the original ganglioside expression was achieved by readaptation of serum-free growing cells to the initial serum-supplemented medium. On the other hand, a 'new' ganglioside, supposed to represent GalNAc-G_{D1a} and not expressed by serum-supplemented growing cells, was induced during serum-free cultivation, and increased strongly after readaptation. These observations reveal that the ganglioside composition of *in vitro* cultivated cells can be modified by the extracellular environment due to different supplementation of the basal growth medium.

Keywords: YAC-1 T lymphoma, G_{M1b}-type gangliosides, antibodies, choleragenoid, overlay technique, cell cultivation, serum

Abbreviations: BSA, bovine serum albumin GSL(s), glycosphingolipid(s); HPTLC, high-performance thin-layer chromatography; LDL, low density lipoprotein; NeuAc, *N*-acetylneuraminic acid; NeuGc, *N*-glycoloylneuraminic acid. The designation of the following glycosphingolipids follows IUPAC–IUB recommendations. $GgOse_3Cer$ or gangliotriaosylceramide, GalNAcβ1-4Galβ1-4GlcCer; $GgOse_4Cer$ or gangliotetraosylceramide, Galβ1-3GalNAcβ1-4Galβ1-4GlcCer; $GgOse_5Cer$ or gangliopentaosylceramide, GalNAcβ1-4Galβ1-3GalNAcβ1-4Galβ1-4GlcCer; $GgOse_6Cer$ or gangliohexaosylceramide, Galβ1-3GalNAcβ1-4Galβ1-3GalNAcβ1-4Galβ1-4GlcCer or $GgOse_6Cer$; II^3NeuAc-$GgOse_3Cer$ or G_{M2}; II^3NeuAc-$GgOse_4Cer$ or G_{M1} or G_{M1a}; IV^3NeuAc-$GgOse_4Cer$ or G_{M1b}; IV^3NeuAc-$GgOse_5Cer$ or GalNAc-G_{M1b}; IV^3NeuAc-$GgOse_6Cer$ or Gal-GalNAc-G_{M1b}; IV^3NeuAc, II^3NeuAc-$GgOse_4Cer$ or G_{D1a}; $II^3(NeuAc)_2$-$GgOse_4Cer$ or G_{D1b}; IV^3NeuAc, III^6NeuAc-$GgOse_4Cer$ or $G_{D1\alpha}$; IV^3NeuAc, II^3NeuAc-$GgOse_5Cer$ or GalNAc-G_{D1a}.

Enzymes: *Vibrio cholerae* and *Arthrobacter ureafaciens* neuraminidase (EC 3.2.1.18).

Gangliosides are glycosphingolipids (GSLs) containing one or more sialic acid residues and are found in the plasma membrane of all vertebrate cells. They act, for instance, as markers for development and differentiation and as regulators for cell proliferation [1]. Specific gangliosides can be assigned to particular differentiation stages, e.g., of T lymphocytes [2] or macrophages [3, 4]. Furthermore, changes during cell aging [5] and cell density dependent changes of gangliosides of adherent growing cells [6, 7] have been described. Modifications in the GSL-expression of cultured cells due to the extracellular environment have been reported by several groups. Significant differences in the GSL composition were obtained by altered carbohydrate feeding [8], pH shift [9] and changes in the osmolarity of the medium [10], indicating that biosynthesis of cell membrane constituents is affected by growth medium components. In this study, we describe altered ganglioside expression of the mouse T lymphoma YAC-1 in serum-supplemented and serum-free growth medium [11].

The murine T cell lymphoma YAC-1 represents the most

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Table 1. RPMI 1640 basal medium used in the experiments.

Component	*mg l⁻¹*	*Component*	*mg l⁻¹*	*Component*	*mg l⁻¹*
L-Arginine	200	L-Proline	20	Pyridoxine-HCl	1.0
L-Asparagine	50	L-Serine	30	Riboflavin	0.2
L-Aspartic acid	20	L-Threonine	20	Thiamine-HCl	1.0
L-Cystine-2HCl	65.15	L-Tryptophan	5	Vitamin B12	0.005
L-Glutamic acid	20	L-Tyrosine	28.83		
L-Glutamine	300	L-Valine	20	$Ca(NO_3)_2 4H_2O$	100
Glycine	10			KCl	400
L-Histidine	15	Biotin	0.2	$MgSO_4$	48.84
L-Hydroxyproline	20	Pantothenic acid	0.25	NaCl	6000
L-Isoleucine	50	Choline chloride	3.0	Na_2HPO_4	800
L-Leucine	50	Folic acid	1.0		
L-Lysine-HCl	40	Inositol	35	D-Glucose	2000
L-Methionine	15	Nicotinamide	1.0	Glutathione	1.0
L-Phenylalanine	15	*p*-Aminobenzoic acid	1.0	Phenol red	5

used target cell line for mouse natural killer cell mediated lysis. The involvement of gangliosides in the recognition process of natural killer cells and the target lymphoma was clearly demonstrated by Bergelson *et al.* [12]. Therefore, individual gangliosides of this important cell line have been structurally characterized recently and gangliosides of the 'G_{M1b}-pathway' (G_{M1b} and GalNAc-G_{M1b}) were found to be highly expressed by YAC-1 cells [13]. Many investigations dealing with GSL metabolism of *in vitro* cultivated mammalian cells have been performed in serum-supplemented media. To avoid effects of uncharacterized bioactive molecules in serum, recently developed serum-free culture media [14, 15] are very useful for the analysis of GSL expression of *in vitro* cultivated cells in well defined media, providing only the essential growth promoting compounds. A serum-free growth medium for the cultivation of a wide spectrum of mammalian cells has been developed by Jäger *et al.* [16]. Besides many cell lines of different origin, the murine T cell lymphoma YAC-1, regularly propagated in serum-supplemented medium, could be adapted to this medium [16]. The aim of this work was to elucidate altered ganglioside expression of this cell line after serum-free adaptation and readaptation to the original serum-supplemented medium.

Materials and methods

Cells and culture conditions

The T cell lymphoma YAC-1 [13] obtained from the American Type Culture Collection (ATCC, MD, USA; unknown number of passages) was propagated in a humidified atmosphere of 5% CO_2 in air at 37 °C, seeded with 2×10^5 cells per ml and harvested at a final concentration of 1.5×10^6 cells per ml. Cell numbers were determined by trypan blue exclusion with a hemocytometer. After centrifugation, the cells were washed twice with phosphate-buffered saline and finally suspended in chloroform–methanol, 2:1 by vol, for storage at −20 °C.

Media

RPMI 1640 medium (Gibco, 074-01800 P, supplemented with 65 mg l⁻¹ penicillin G, 100 mg l⁻¹ streptomycin, 20 mM $NaHCO_3$, 10 mM HEPES) served as the basal medium (see Table 1). The cells were initially propagated in medium 1: basal medium supplemented with 10% (by vol) heat inactivated fetal calf serum (Boehringer, Mannheim). The serum-free growth medium has been developed by Jäger *et al.* [16]. RPMI 1640 basal medium was supplemented with fatty acid free bovine serum albumin (BSA, Serva, 11924; 500 mg l⁻¹) oleic acid (Sigma, 0-3879; 4 mg l⁻¹) complex and BSA (500 mg l⁻¹) linoleic acid (Sigma, L-1376; 4 mg l⁻¹) complex, iron saturated human transferrin (Behring, OTRE 04/05; 10 mg l⁻¹) and bovine insulin (Sigma, I-5500; 10 mg l⁻¹). Further supplements used were bovine plasma low density lipoprotein (LDL, 10 mg l⁻¹) [16]), dioleic phosphatidylcholine (Sigma, P-1013; 0.5 mg l⁻¹) and dilinoleic phosphatidylcholine (Sigma, P-7649; 0.5 mg l⁻¹). Medium 2 was completed with all the above supplements, whereas the cell cultivation in medium 3 was carried out without the last three listed supplements. The components of the three media are summarized in Table 2. The preparation of the supplements for the serum-free media has been described in detail by Jäger *et al.* [16]. To adapt cells to medium 2 (serum-free), they were harvested from serum-supplemented medium 1 by low speed centrifugation, suspended in medium 2 and propagated for several passages before they were metabolically labeled with radioactive hexoses (see below). Adaptation from medium 2 to medium 3 and readaptation

Table 2. Composition of supplemented RPMI 1640 basal media.

Supplements	*Medium 1 serum-suppl.*	*Medium 2 serum-free*	*Medium 3 serum-free*
Fetal calf serum (10% by vol)	+	–	–
BSA-oleic acid ($500\ mg\ l^{-1}$–$4\ mg\ l^{-1}$)	–	+	+
BSA-linoleic acid ($500\ mg\ l^{-1}$–$4\ mg\ l^{-1}$)	–	+	+
Human transferrin ($10\ mg\ l^{-1}$)	–	+	+
Bovine insulin ($10\ mg\ l^{-1}$)	–	+	+
Bovine plasma LDL ($10\ mg\ l^{-1}$)	–	+	–
Dioleic phosphatidyl-choline ($0.5\ mg\ l^{-1}$)	–	+	–
Dilinoleic phosphatidyl-choline ($0.5\ mg\ l^{-1}$)	–	+	–

from medium 3 to serum-supplemented medium 1 were performed in the same way. Metabolic labelling was performed in all cases after several passages in the changed medium.

Metabolic labelling of cells

YAC-1 cells were labeled for 40 h with $1\ \mu Ci\ ml^{-1}$ ($3.7 \times 10^4\ Bq\ ml^{-1}$) D-[1-^{14}C]glucosamine hydrochloride ($58.7\ Ci\ mol^{-1}$ or $2.17 \times 10^{12}\ Bq\ mol^{-1}$) and $1\ \mu Ci\ ml^{-1}$ ($3.7 \times 10^4\ Bq\ ml^{-1}$) D-[1-^{14}C]galactose ($58\ Ci\ mol^{-1}$ or $2.15 \times 10^{12}\ Bq\ ml^{-1}$; Amersham Buchler, Braunschweig, Germany) as previously described [17]. Labeled cells were harvested and stored as described above.

Ganglioside isolation from YAC-1 cells

^{14}C-Labeled gangliosides were isolated as described for mouse T-lymphocytes by Müthing *et al.* [17] and were further purified after the anion exchange chromatography step on a small Iatrobeads column (6RS-8060, Macherey-Nagel, Düren, Germany) as described by Ueno *et al.* [18]. Radioactivity was determined in a Packard Tri-Carb 1900 CA liquid scintillation spectrometer (Packard Inst. Co) in aliquots from fractions after evaporation of organic solvents.

Purification of gangliosides from fetal calf serum

Gangliosides composition from the total lipid extract of fetal calf serum was checked according to Ladisch and Gillard [19]. 10 ml frozen serum were lyophilized, resuspended in 20 ml chloroform–methanol, 1:1 by vol, sonicated for 5 min and insoluble material was removed by centrifugation in a conical centrifuge tube ($750 \times$ **g**, 5 min). The total lipid extract was evaporated under N_2, followed by oil pump vacuum, and dispersed in 10 ml of diisopropyl ether–1-butanol, 60:40 by vol, by several minutes of vortexing and sonication. Next, 5 ml 50 mM aqueous NaCl was added and the sample was mixed and sonicated again as described. The two phases were separated by centrifugation ($750 \times$ **g**, 5 min). After removal of the upper organic phase (containing the neutral lipids and phospholipids) the lower aqueous phase (containing the gangliosides) was reextracted with 10 ml fresh organic solvent mixture. The lower aqueous phase was lyophilized and desalted with a Sep-Pak™ C_{18} cartridge (Millipore, Germany) according to Williams and McCluer [20].

Reference gangliosides

Human brain gangliosides were purchased from Supelco (Bellefonte, PA, USA). Reference gangliosides from serum-supplemented grown YAC-1 cells were isolated as published recently [13] and are listed in Table 3. The isolation and characterization of gangliosides from concanavalin A stimulated murine T lymphocytes has been described [17].

Thin-layer chromatography

High-performance thin-layer chromatography plates (HPTLC plates, size 10 cm × 10 cm, thickness 0.24 mm, E. Merck, Darmstadt, Germany) were used. Gangliosides were separated in chloroform–methanol–water, 120:85:20 by vol, containing 2 mM $CaCl_2$. ^{14}C-Labeled gangliosides were located by autoradiography on Hyperfilm™-^{3}H (Amersham Buchler, Braunschweig, Germany).

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

Terminally sialylated gangliosides with the $GgOse_4Cer$ backbone (G_{M1b}, $G_{D1\alpha}$) were detected as described [21]. Briefly, gangliosides were chromatographed on HPTLC plates, and the silica gel was fixed with polyisobutyl-methacrylate (Plexigum P28, Röhm, Darmstadt, Germany). The plates were incubated with $5\ mU\ ml^{-1}$ *V. cholerae* neuraminidase (EC 3.2.1.18, Behring, Marburg, Germany) for 2 h at room temperature in 0.05 M sodium acetate, 9 mM $CaCl_2$, pH 5.5. Desialylated gangliosides were analysed with a polyclonal anti-$GgOse_4Cer$ antiserum which has been raised in a rabbit according to the method of Kasai *et al.* [22]. The polyclonal anti-$GgOse_4Cer$ and the goat anti-rabbit IgG antiserum (alkaline phosphatase conjugated, Medac, Hamburg, Germany) were diluted 1:2000, respectively, in phosphate buffered saline containing 1% bovine serum albumin (solution A) and incubated for 1 h at room temperature. The washing steps and the detection of alkaline phosphatase activity with 5-bromo-4-chloro-3-indolyl phosphate were done exactly as described [21].

Table 3. Main gangliosides from serum-supplemented grown YAC-1 cells (data from Müthing *et al.* [13]).

Ganglioside fraction	*No.*	*Major fatty acid*	*Symbol*	*Structure*
I	1	$C_{24:0,24:1}$	G_{M1b} (NeuAc)	IVNeuAc-GgOse$_4$Cer[a]
II	2	$C_{16:0}$	G_{M1b} (NeuAc)	IVNeuAc-GgOse$_4$Cer[a]
	3	$C_{24:0,24:1}$	G_{M1b} (NeuGc)	IVNeuGc-GgOse$_4$Cer[a]
	4	$C_{24:0,24:1}$	GalNAc-G_{M1b} (NeuAc)	IVNeuAc-GgOse$_5$Cer[b]
III	5	$C_{16:0}$	G_{M1b} (NeuGc)	IVNeuGc-GgOse$_4$Cer[a]
	6	$C_{16:0}$	GalNAc-G_{M1b} (NeuAc)	IVNeuAc-GgOse$_5$Cer[b]
	7	$C_{24:0,24:1}$	GalNAc-G_{M1b} (NeuGc)	IVNeuGc-GgOse$_5$Cer[b]
IV	8	$C_{16:0}$	GalNAc-G_{M1b} (NeuGc)	IVNeuGc-GgOse$_5$Cer[b]

[a] G_{M1b}-type gangliosides, detectable by immunostain with the anti-GgOse$_4$Cer antibody after *Vibrio cholerae* neuraminidase treatment [21].
[b] GalNAc-G_{M1b}-type gangliosides, detectable by immunostaining of GgOse$_5$Cer with the cross-reacting anti-GgOse$_3$Cer antibody after *Arthrobacter ureafaciens* neuraminidase treatment [26].

Detection of extended ganglio-series gangliosides with terminal GalNAcβ1-4Gal sequence (GalNAc-G_{M1b}) on HPTLC plates

For the analysis of gangliosides with the GgOse$_5$Cer core, plates were incubated with 50 mU ml^{-1} *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.* [23]. The monoclonal anti-GgOse$_3$Cer antibody 2D4 (TIB 185, American Type Culture Collection, Bethesda, MD, USA [24]) which crossreacts with GgOse$_5$Cer [17, 25] was used for the enzyme-immunostaining procedure, which has been published recently by Müthing and Ziehr [26].

HPTLC-immunostaining assay with cholera toxin B subunit (choleragenoid)

The HPTLC-binding method using cholera toxin for specific detection of G_{M1} has been developed by Magnani *et al.* [27] and also been approved to be practicable for the detection of the G_{M1b} elongation product Gal-GalNAc-G_{M1b} as recently published by Nakamura *et al.* [28]. We modified the described methods as follows. The silica gel fixed HPTLC plate was incubated with cholera toxin B subunit (Sigma, No. C-7771, Munich, Germany) with a final concentration of 250 ng ml^{-1} in solution A (see above) for 2 h at room temperature. Goat anti-cholera toxin B subunit (choleragenoid) antiserum (Calbiochem, No. 227040, Frankfurt, Germany) and alkaline phosphatase conjugated rabbit anti-goat IgG (Dianova, Hamburg, Germany) antibody (1:1000 dilution in solution A) were used for the immunostaining procedure (1 h at room temperature). The washing steps and the detection of alkaline phosphatase activity on the plate were done as described [21, 26].

To reveal the presence of other members of the gangliotetraose family, i.e., G_{D1a}, G_{D1b}, G_{T1b} and G_{Q1b} neuraminidase is employed to convert these gangliosides to G_{M1a} prior to treatment with cholera toxin B subunit. *V. cholerae* neuraminidase (and other neuraminidases from bacterial and animal sources) hydrolyse the mentioned gangliosides to G_{M1a} but fail to hydrolyse the latter [29]. Therefore, silica gel fixed plates were incubated with 50 mU ml^{-1} *V. cholerae* neuraminidase for 18 h at 37 °C in 0.05 M sodium acetate, 9 mM $CaCl_2$, pH 5.5. The subsequent immunostaining assay with cholera toxin B subunit was performed as described above. This technique was developed originally by Wu and Ledeen [30].

Results

Cultivation of YAC-1 cells in serum-supplemented and serum-free medium

RPMI 1640 medium represents a well suitable basal medium for the cultivation of lymphocytes and lymphocyte-derived cell lines. The common components of the basal medium used in our experiments are listed in Table 1. To propagate the T cell lymphoma YAC-1 supplementation of the basal medium with 10% fetal calf serum (by vol) was essential, whereas the application of newborn calf serum led to increasing doubling times and cell death after several passages. Therefore, initial cultivation of YAC-1 cells was carried out in fetal calf serum-supplemented medium 1 (see Table 2). The cells were seeded and harvested as described in the Materials and methods section with an average of 90% viable cells and doubling times of about 15 h. YAC-1 cells could be adapted easily to the serum-free medium 2 (see Table 2) and identical doubling times compared with serum-supplemented growth were achieved. After several passages metabolical labelling of the cells was performed as described in the Materials and methods section. Next, serum-free adapted cells were transferred to serum-free medium 3 lacking LDL, dioleic phosphatidylcholine and dilinoleic phosphatidylcholine compared with medium 2 (see Table 2). Successful growth with doubling times

comparable with the cultivations in medium 1 and 2 was obtained after several passages and metabolical labelling of the cells was done as described. Finally, serum-free growing cells were readapted to the serum-supplemented medium 1 (see Table 2) without any differences of growth parameters compared with the original cultivation.

Metabolically labeled gangliosides of YAC-1 cells grown in serum-supplemented and serum-free media

Previous investigations have shown the considerable heterogeneity of gangliosides from YAC-1 cells which have been produced in commonly used fetal calf serum supplemented medium [13]. The basic core structures of the major YAC-1 monosialogangliosides were identified as $GgOse_4Cer$ and $GgOse_5Cer$ sialylated in position IV with *N*-acetyl- and *N*-glycoloylneuraminic acid. Additionally, heterogeneity was found in the ceramide portions of the gangliosides substituted with $C_{16:0}$- and $C_{24:0,\,24:1}$-fatty acids. Overall, the main monosialoganglosides from YAC-1 comprise four different types of G_{M1b} and GalNAc-G_{M1b}, and these are listed in Table 3 (fractions I to IV). An autoradiograph of [^{14}C]glucosamine and [^{14}C]galactose labeled gangliosides of YAC-1 cells cultivated in serum-supplemented medium is given in Fig. 1 (lane a). The monosialoganglioside fractions are marked with roman numerals (see Table 3, fractions I to IV), and the individual gangliosides are designated with arabic numbers (also Table 3, gangliosides 1 to 8). It should be noted that up to three different individual gangliosides chromatograph as one band, e.g. in fraction II (gangliosides 2, 3 and 4, see Table 3): G_{M1b}(NeuAc) substituted with $C_{16:0}$-fatty acid and G_{M1b}(NeuGc) and GalNAc-G_{M1b}NeuAc), both carrying $C_{24:0,\,24:1}$-fatty acids. Fraction III also comprises three different individual gangliosides 5, 6 and 7 listed in Table 3. Besides these gangliosides of the 'G_{M1b}-pathway', gangliosides of the 'G_{M1a}-pathway' have been also detected

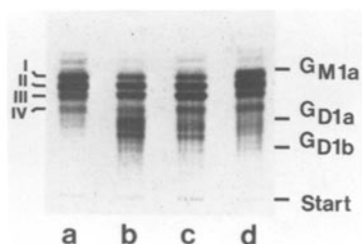

Figure 1. Autoradiography of gangliosides from metabolically labeled YAC-1 cells cultivated in serum-supplemented and serum-free media. ^{14}C-Galactose and ^{14}C-glucosamine labeled gangliosides (3000 counts min^{-1}) from YAC-1 cells propagated in medium 1 (10% FCS, lane a), medium 2 (serum-free, lane b), medium 3 (serum-free, lane c) and medium 1 (cells readapted to 10% FCS, lane d) were chromatographed. Exposure time was 168 h. The components of the three media used are listed in Table 2. The positions of human brain gangliosides G_{M1}, G_{D1a} and G_{D1b} are marked at the margin. Gangliosides of fractions I, II, III and IV are enumerated in Table 3.

in YAC-1 cells [13]. Since G_{M2} and G_{M1a} are expressed only in small amounts and to avoid confusion these minor gangliosides have been omitted from Table 3.

The transfer of cells grown in serum-supplemented medium to serum-free conditions caused evident alterations of the metabolically labeled gangliosides as demonstrated in Fig. 1 (lane a and b). The transfer of serum-free growing cells from medium 2 to medium 3 (=medium 2 without LDL, dioleic- and dilinoleic phosphatidylcholine) revealed obvious changes in the G_{D1a} area (Fig. 1, lane b and c). The readaptation of these cells to the original medium 1 (serum-supplemented) raised almost the same labelling pattern (Fig. 1, lane d) as the pattern of the cells originally grown in serum-supplemented medium (Fig. 1, lane a).

To characterize the alterations in the ganglioside expression on the level of the individual gangliosides, a comparison of the GSL-patterns is insufficient, since 'one band' on the HPTLC-plate does not represent 'one ganglioside' (see Table 3). To analyse GSL changes during the different periods of cultivation on the one hand in the 'monosialoganglioside range' (fractions I to IV, see Fig. 1 and Table 3) and on the other hand in the 'disialoganglioside range' (beyond fraction IV, see Fig. 1) the well working overlay technique was used as demonstrated in the following sections.

G_{M1b}-expression of serum-supplemented and serum-free grown cells

Three G_{M1b}-bands are detectable in the ganglioside fraction of serum-supplemented grown cells (Fig. 2, lane a, fractions I, II and III, see Table 3): G_{M1b} (NeuAc) with C_{24}-fatty acid

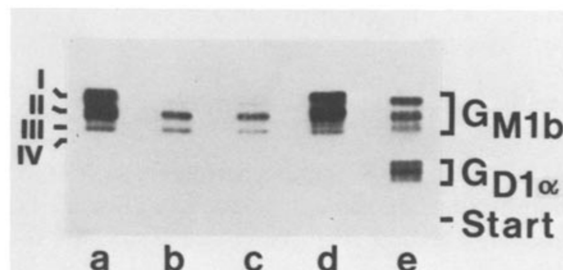

Figure 2. Detection of G_{M1b}-type gangliosides in the whole ganglioside fraction of YAC-1 cells cultivated in serum-supplemented and serum-free media. Gangliosides of 2.5×10^6 YAC-1 cells propagated in medium 1 (10% FCS, lane a), medium 2 (serum-free, lane b), medium 3 (serum-free, lane c) and medium 1 (cells readapted to 10% FCS, lane d) were chromatographed. Gangliosides of 5×10^6 concanavalin A stimulated murine T lymphocytes were co-chromatographed (positive control, lane e). After chromatography the silica gel was fixed (see the Materials and methods section) and the plate was overlayed with *V. cholerae* neuraminidase (5 mU ml^{-1}, 2 h at room temperature). $GgOse_4Cer$ bands were detected by immunostaining with specific anti-$GgOse_4Cer$ antibodies. Gangliosides of fractions I, II, III and IV are enumerated in Table 3.

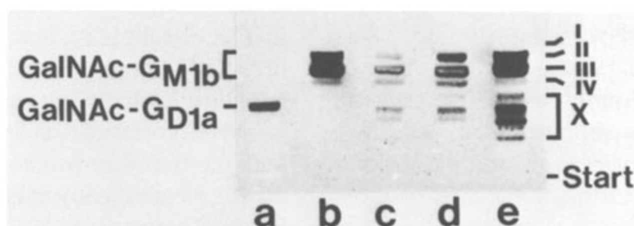

Figure 3. Detection of GalNAc-G_{M1b} in the whole ganglioside fraction of YAC-1 cells cultivated in serum-supplemented and serum-free media. 10 µg human brain gangliosides (lane a) were co-chromatographed with gangliosides of 1×10^7 YAC-1 cells propagated in medium 1 (10% FCS, lane b), medium 2 (serum-free, lane c), medium 3 (serum-free, lane d) and medium 1 (cells readapted to 10% FCS, lane e). After chromatography, silica gel fixation and *A. ureafaciens* neuraminidase treatment (50 mU ml^{-1}, 24 h at 30 °C), $GgOse_5Cer$ bands were detected with anti-$GgOse_3Cer$ antibody. Gangliosides of fractions I, II, III and IV are enumerated in Table 3.

(ganglioside 1 of fraction I), G_{M1b} (NeuAc) with C_{16}- and G_{M1b} (NeuGc) with C_{24}-fatty acid (gangliosides 2 and 3, respectively) co-chromatographing as one band in fraction II and G_{M1b} (NeuGc) with C_{16}-fatty acid (ganglioside 5 of fraction III) [13]. Stimulated mouse T lymphocytes produce identical G_{M1b}-type gangliosides [17] and served as references (see Fig. 2, lane e). $G_{D1\alpha}$, the main disialoganglioside of T lymphocytes, is not expressed by YAC-1 cells. An evident reduction of G_{M1b}-gangliosides could be observed in serum-free growing cells (medium 2, Fig. 2, lane b). Furthermore these cells are lacking G_{M1b} (NeuAc) with C_{24}-fatty acid (ganglioside 1). Band II is considerably reduced and band III seems not to be affected by serum-free conditions. This GSL-status was not changed by transfering the cells to serum-free medium 3 (Fig. 2, lane c). The cells raised the same G_{M1b}-pattern after readaptation to serum-supplemented medium 1 compared to the original GSL-status (see Fig. 2, lane d and a, respectively).

GalNAc-G_{M1b}-expression of serum-supplemented and serum-free grown cells

Three GalNAc-G_{M1b}-bands are detectable in YAC-1 cells grown in serum-supplemented medium (Fig. 3, lane b, fractions II, III and IV, see Table 3): GalNAc-G_{M1b} (NeuAc) with C_{24}-fatty acid (ganglioside 4 of fraction II), GalNAc-G_{M1b} (NeuAc) with C_{16}- and GalNAc-G_{M1b} (NeuGc) with C_{24}-fatty acid (gangliosides 6 and 7, respectively) co-chromatographing as one band in fraction III and GalNAc-G_{M1b} (NeuGc) with C_{16}-fatty acid (ganglioside 8 of fraction IV) [13]. In analogy to the G_{M1b}-decrease in serum-free growing cells, GalNAc-G_{M1b}-bands are diminished, too (Fig. 3, lane c). After cell transfer to medium 3, GalNAc-G_{M1b} positive bands increased modestly (Fig. 3, lane d), reaching the original strength after readaptation to serum-supplemented medium 1 (Fig. 3, lane e). A 'new' ganglioside, not found in serum-supplemented grown cells, is induced during serum-free conditions (Fig. 3, lane c). This (these) ganglioside(s) is/are strongly expressed after readaptation to serum-supplemented medium (Fig. 3, lane e, designated with 'X'). The two main bands of ganglioside X chromatograph similarly compared with GalNAc-G_{D1a}, a well characterized disialoganglioside of normal human brain [31], which can be detected with this assay (Fig. 3, lane a). The uptake of ganglioside X by the cells from serum can be excluded, since compound X was not found in the serum used in our experiments and expression was not observed in cells cultivated in serum-supplemented medium before medium change (Fig. 3, lane b).

Detection of G_{M1a} in serum-supplemented and serum-free grown cells with cholera toxin B subunit (choleragenoid)

Besides the main monosialogangliosides of the G_{M1b}-type produced by YAC-1 cells, minor amounts of G_{M1a} (indicating the 'underlying' G_{M1a}-pathway) can be made visible by cholera toxin, which is known to have a high affinity for G_{M1a} [32]. As shown in Fig. 4, two strong G_{M1a}-positive bands can be identified in all YAC-1

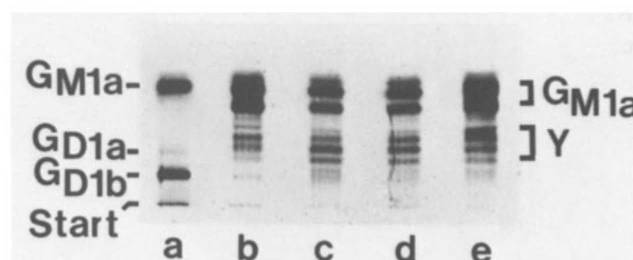

Figure 4. HPTLC-immunostaining assay with cholera toxin B subunit of whole ganglioside fractions from YAC-1 cells cultivated in serum-supplemented and serum-free media. 1 µg human brain gangliosides (lane a) were co-chromatographed with gangliosides of 2.5×10^6 YAC-1 cells propagated in medium 1 (10% FCS, lane b), medium 2 (serum-free, lane c), medium 3 (serum-free, lane d) and medium 1 (cells readapted to 10% FCS, lane e). After chromatography and silica gel fixation the immunostaining procedure was performed as described in the Materials and methods section.

ganglioside fractions. A significant decrease could be observed in serum-free medium propagated cells (Fig. 4, lane c and d), reaching the same G_{M1a}-pattern after readaptation to serum-supplemented medium (Fig. 4, lane e). Furthermore, several cholera toxin positive bands with slower mobilities than G_{M1a} are immunostained, marked with 'Y' in Fig. 4. It is suggested that these gangliosides represent Gal-GalNAc-G_{M1b}-type gangliosides. This is in accordance with data of Nakamura *et al.* [28], who have shown the choleragenoid-binding activity to the ganglioside Gal-GalNAc-G_{M1b}, isolated from mouse spleen. This ganglioside with $GgOse_6Cer$ core, substituted with NeuGc in positive IV of the oligosaccharide, contains the terminal tetrasaccharide structure identical with that of G_{M1a} [28]. Since choleragenoid shows binding activity to both NeuAc- and NeuGc-substituted G_{M1a} [33], no exact structural information concerning the sialylation are available from the immunostained G_{M1a}- and supposed Gal-GalNAc-G_{M1b}-bands in Fig. 4. However, it should be noted, that G_{M1a} and Gal-GalNAc-G_{M1b}, each representatives of a different biosynthetic pathway, are susceptible to this specific immunobinding assay.

Comparing Fig. 4 (G_{M1a}-stain) and Fig. 3 (G_{M1b}-stain), G_{M1a} seems to be synthesized as much as G_{M1b}, from the intensities of the immunostained bands, e.g., in serum-supplemented grown YAC-1 cells (see Fig. 4, lane b and Fig. 3, lane a). This sophism is due to the high sensitivity of the choleragenoid-assay detecting 0.01 ng G_{M1a} on the HPTLC-plate [34] whereas a minimum of about 30 ng of G_{M1b} is needed for specific detection by the overlay technique [21]. Therefore, G_{M1a}-type gangliosides are indeed minor GSLs compared with G_{M1b}-type gangliosides in serum supplemented grown YAC-1 cells [13].

The structure of the immunostained ganglioside in the human brain ganglioside mixture (containing G_{M1a} as a positive control) migrating identically to G_{D1b}, remains unclear (see Fig. 4, lane a). Binding of choleragenoid to the disialoganglioside G_{D1b} of the gangliotetraose family has been reported by several authors using the described overlay technique [30, 34, 35]. Since low cross-reactions of G_{D1a} and G_{D1b} with cholera toxin B subunit are of the same order of magnitude [36, 37] and the affinity of choleragenoid for G_{D1b} is several 1000-fold less than for G_{M1a} [36, 38], G_{D1b} might be excluded from being the main binding ganglioside of the immunostained band. Therefore, a cross-reacting disialoganglioside, e.g., Gal-GalNAc-G_{D1a}, with identical 'G_{M1a}-terminus' which might co-chromatograph with G_{D1b}, would explain contradictory explanations.

Gangliosides from fetal calf serum

It is obvious from our data that cells grown in serum supplemented medium show higher ganglioside expression than serum-free propagated cells. Uptake of gangliosides by *in vitro* cultivated cells from their environment is a well described phenomenon [39, 40]. Therefore, fetal calf serum was checked for the presence of relevant gangliosides of the G_{M1a}- and the G_{M1b}-pathway. For microscale purification, serum gangliosides were extracted by three-component solvent partition according to Ladisch and Gillard [19]. Aliquots of ganglioside containing lower phase were applied to HPTLC-plates and checked by the overlay technique as described above.

The G_{M1b} content of serum was analysed by application of lower phase aliquots of 167 µl serum to HPTLC plates. These aliquots correspond to maximum ganglioside amounts which could be taken up from the environment by 2.5×10^6 cells (see Fig. 2, lanes a and d) harvested at final cell densities of 1.5×10^6 cells per ml grown in 10% (by vol) serum-supplemented medium. The presence of GalNAc-G_{M1b} (and supposed GalNAc-G_{D1a}) was checked by application of aliquots from 667 µl serum corresponding to 1×10^7 cells in Fig. 3. None of these mentioned gangliosides was found in fetal calf serum (not shown), and so their uptake from surrounding medium can be excluded.

Detection of gangliotetraose-type gangliosides of the G_{M1a}-pathway in fetal calf serum was performed by combined neuraminidase and choleragenoid immunostaining assay according to Wu and Ledeen [30]. G_{M1a} of human brain gangliosides (reference mixture containing G_{M1a}, G_{D1a}, G_{D1b}, G_{T1b} and G_{Q1b}) was immunostained by choleragenoid without (Fig. 5A, lane a) and the latter gangliosides from the reference mixture with (Fig. 5A, lane b) prior neuraminidase treatment of the plates. Neuraminidase converts G_{D1a}, G_{D1b}, G_{T1b} and G_{Q1b} to

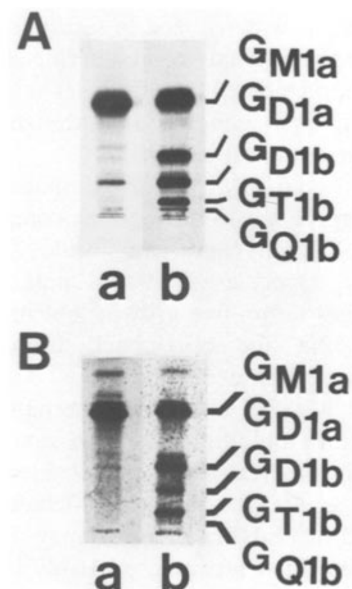

Figure 5. Detection of gangliotetraose-type gangliosides in fetal calf serum by combined neuraminidase and choleragenoid immunostaining assay. 25 ng human brain gangliosides (A) and ganglioside aliquots corresponding to 167 µl fetal calf serum (B) were chromatographed and immunostained with choleragenoid (lanes a). Neuraminidase treatment prior to immunostain (lanes b) was done as described in the Materials and methods section.

G_{M1a} as demonstrated with 25 ng human brain gangliosides (Fig. 5A, lane b), indicating the extreme sensitivity of this specific overlay assay [27, 30, 34]. Almost identical patterns were obtained from serum aliquots (Fig. 5B, lanes a and b), revealing the presence of G_{D1a}, G_{D1b} and G_{T1b} in addition to G_{M1a} in nanogram range in serum aliquots. Therefore, upper G_{M1a} bands of serum-supplemented grown cells in Fig. 4 (lane b and e) may be a serum artefact due to uptake by growing cells. However, G_{D1a}, G_{D1b} and G_{T1b}, representatives of the G_{M1a}-pathway, were not detected in serum-supplemented and serum-free grown cells (not shown).

Discussion

In this study we demonstrated that the addition of different kinds of high molecular supplements to the basal medium affected the content and metabolism of gangliosides in cells. Ganglioside alterations during the different cultivation periods were obvious comparing metabolically labeled GSLs. Using the overlay technique which has been introduced to GSL-research by Magnani *et al.* [41] ganglioside changes could be specified. G_{M1b}-type gangliosides, highly expressed in serum-supplemented grown cells, were reduced manifold in serum-free cultured cells. The low G_{M1b} expression was maintained during cultivation in the second serum-free medium, lacking LDL and phosphatidyl cholines. After readaptation of cells to serum-supplemented medium, the original G_{M1b}-status was achieved. By analogy, GalNAc-G_{M1b}, the elongation product of G_{M1b}, was reduced in serum-free growing cells, too, reaching the original expression rate after readaptation to serum-supplemented medium. The proposed Gal-GalNAc-G_{M1b}-type gangliosides (designated with 'Y') showed insignificant alterations.

The expression of G_{M1a} was also diminished in serum-free growing cells but not as strongly as compared with G_{M1b} and GalNAc-G_{M1b}. A 'new' ganglioside 'X' proposed to be GalNAc-G_{D1a} (speculative at this point of research) is induced during serum-free growth and increases manifold after transfering the cells back from serum-free to serum-supplemented medium.

At present it is not clear what mechanism leads to the activation and/or inhibition of hexosyl- and sialyltransferases involved in the biosynthesis of gangliosides in cultured cells. From our results it can be concluded that specific hexosyl- and/or sialyltransferases may be stimulated in serum-supplemented medium, probably by serum factors, and suppressed in serum-free medium lacking stimulating compounds.

Changes in the GSL-expression of *in vitro* cultured cells by altered environments have been reported by several researchers. Source of carbohydrate [8], osmolarity [10] and pH of medium [9] can modify GSL expression. Furthermore, ganglioside changes of human melanoma during *in vitro* growth (i.e., tissue culture cell lines) and *in vivo* growth (i.e., biopsied tumor and nude mice) were caused by culture environment as shown by Tsuchida *et al.* [42].

According to these results, we have demonstrated modified ganglioside expression of *in vitro* cultured cells grown in serum-supplemented and serum-free medium. It should be kept in mind that most cell technologists use xenogenic sera (e.g., calf serum for propagation of human or murine cells) which may contain stimulatory and/or inhibitory factors, especially for lymphocyte-derived cell lines. For future research it would be of interest to identify serum factors which are triggering activities of enzymes involved in ganglioside biosynthesis.

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