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Significant inhibition of hybridoma cells by exogenous application of ganglioside G_{M3}, a possible modulator of cell growth *in vitro*

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Abstract

Gangliosides of the mouse-rat hybridoma cell line 187.1, which secretes an antibody against $\varkappa$-light chain of mouse IgG, were isolated and structurally characterized by biochemical and immunological methods (overlay technique), and fast atom bombardment-mass spectrometry. Exclusively G_{M3}, substituted with $C_{24:1}$ and $C_{16:0}$ fatty acid and $C_{18:1}$ sphingosine, was found in this B cell derived cell line. A G_{M3}(NeuGc) to G_{M3}(NeuAc) ratio (80 to 20), was characteristic for 187.1 cells, and absolute G_{M3} amounts of about 0.3 mg 10^{-9} viable cells were determined. Exogenous application of G_{M3}, which has been isolated from large cell preparations, to 187.1 cells showed growth inhibition in a concentration dependent manner. Using the MTT-assay and the [^{3}H]thymidine incorporation assay, the cells exhibited a strong reduction in metabolic and proliferative activity, respectively, after exposure of cells to G_{M3}. G_{M3} was applied in concentrations between 3μM and 30μM, giving evidence for strong inhibitory effects at 30μM G_{M3} and less but significant suppression after application of G_{M3} concentrations lower than 20μM. No cellular response was observed at the lowest concentration (3μM) used in this study. Hybridoma cells as well as other cell types like fibroblasts, muscle cells and endothelial cells, are in general characterized by high expression of the G_{M3} ganglioside, which is known to act as a modulator of cellular growth in monolayer cultures of adherent cells. Since gangliosides are released to the culture medium by cell lysis, i.e. cell death, and/or by active membrane shedding, the results obtained in this study suggest a growth regulatory role of G_{M3} in high density hybridoma cell cultures.

Abbreviations: DMB–1,2-diamino-4,5-methylenedioxybenzene; FAB-MS – fast atom bombardment-mass spectrometry; GSL(s) – glycosphingolipid(s); HPLC – high performance liquid chromatography; HPTLC – high performance thin layer chromatography; MTT – 3,(4,5 dimethylthiazol-2-yl)2,5 diphenyl tetrazolium bromide; NeuAc – N-acetylneuraminic acid; NeuGc – N-glycolylneuraminic acid; PBS – phosphate buffered saline. The designation of the following glycosphingolipids follows the IUPAC-IUB recommendations (1977) and the nomenclature of Svennerholm (1963). Lactosylceramide or LacCer, Galß1-4Glcß1-1Cer; gangliotriaosylceramide or $GgOse_3Cer$; GalNAcß1-4Galß1-4Glcß1-1 Cer; gangliotetraosylceramide or $GgOse_4Cer$, Galß1-3GalNAcß1-4Galß1-4Glcß1-1Cer; G_{M3}(NeuAc), II^{3}NeuAc-LacCer; G_{M3}(NeuGc), II^{3}NeuGc-LacCer; G_{M2}(NeuGc), II^{3}NeuGc-$GgOse_3Cer$; G_{M1} or G_{M1a}, II^{3}NeuAc-$GgOse_4Cer$; G_{M1b}, IV^{3}NeuAc-$GgOse_4Cer$.

Introduction

Gangliosides are a diverse class of complex glycosphingolipids (GSLs)[1] and primarily located in the external membrane of animal cells (Thompson and Tillack, 1985). GSLs consist of two structural elements: a lipophilic membrane anchor, the ceramide portion, which is formed by a long chain base and a fatty acid, and a hydrophilic carbohydrate moiety, which protrudes from the cell surface (Hakomori, 1981). The structures of gangliosides, which are characterized by the presence of one or more sialic acid units in

the oligosaccharide moiety, as well as their functions have been widely reviewed (Hakomori, 1984; Stults *et al.*, 1989; Zeller and Marchase, 1992). The parent sialic acids are N-acetylneuraminic acid (NeuAc) and N-glycolylneuraminic acid (NeuGc), which both play crucial roles in various biological events (Schauer, 1988).

Murine leukocytes in general express complex ganglioside patterns. In mouse macrophages (Yohe *et al.*, 1991) and T-Lymphocytes (Müthing *et al.*, 1987; Müthing *et al.*, 1989) ganglio-series gangliosides of the G_{M1b}-pathway are predominant, whereas murine B lymphocytes are characterized by the presence of G_{M1a}-type gangliosides (Pörtner *et al.*, 1993). In contrast to their primary counterparts, B cell lymphomas (= myelomas) and hybridomas, which are generated by fusion of B lymphocytes with myeloma cells, express a simple ganglioside pattern. Our group has recently classified G_{M3}(NeuGc) and G_{M3}(NeuAc) as the main gangliosides in these cell lines (Müthing *et al.*, 1994).

The hybridoma cell line 187.1, which produces a monoclonal rat antibody against mouse IgG antibodies, has been studied intensively and a variety of metabolic data as well as growth parameters have been derived from this cell line (Büntemeyer *et al.*, 1992). Furthermore, 187.1 cells were also found to express the ganglioside G_{M3} exclusively (Brandt *et al.*, 1993a). G_{M3} has been reported by several authors as an important modulator of cell growth in monolayer cultures of adherently growing cells (Bremer *et al.*, 1986; Igarashi *et al.*, 1989; Hakomori, 1990; Rösner *et al.*, 1990). The focus of this work was to investigate the growth modulatory activity of ganglioside G_{M3} in hybridoma cell cultures, using the well characterized mouse-rat hybridoma 187.1 as a model cell line. Gangliosides are known to be shed by lymphoma cells (Ladisch *et al.*, 1983). They are also released in considerable amounts into the culture medium due to lysis of dead cells, especially at high cell densities. In this study the response of 187.1 cells to exogenous G_{M3} was investigated and the growth modulatory function of G_{M3} is discussed. Preliminary results have been published (Brandt *et al.*, 1993b).

Materials and methods

Cells and culture conditions

The hybridoma 187.1 was obtained from the American Type Culture Collection (ATCC, Bethesda, MD, USA, HB58). The clone was produced by fusing cells of the mouse myeloma X63Ag8.653 line with spleen cells from Sprague-Dawley rats, immunized with purified mouse IgG ($IgG_{2b},\varkappa$) (Yelton *et al.*, 1981). The hybridoma secretes an IgG_1 rat monoclonal antibody against the κ-light chain of mouse antibodies.

The cells were cultivated in serum-free standard medium DMEM/Ham's F12 (1/1) (Gibco BRL, Eggenstein, Germany) supplemented with 10 mg l^{-1} iron saturated human transferrin (Behring, OTR 04/05, Marburg, Germany), 10 mg l^{-1} bovine insulin (Sigma, I5500, Deisenhofen, Germany) and 1 ml l^{-1} Pentex ExCyteI (Bayer Diagnostics, München, Germany) (Jäger *et al.*, 1988). The cells were cultivated in a humidified atmosphere of 10% CO_2 in air at 37 °C and routinely passaged in concentrations from 2 x 10^5 cells ml^{-1} (seeding) up to 1 x 10^6 cells ml^{-1}. Large amounts of cells were propagated in 21 volume bioreactors as described by Büntemeyer *et al.*, (1992).

Isolation of gangliosides

Glycosphingolipids were extracted from hybridoma 187.1 cells with chloroform/methanol (2/1), (1/1), (1/2), each by volume (Merck, Darmstadt, Germany). Gangliosides were isolated according to standard procedures (Ledeen and Yu, 1982) as demonstrated in the flow sheet (Figure 1). Briefly, the GSL containing extract was rotary evaporated, resuspended in high purity Milli-Q-water (Millipore, Inc., Bedford, MA, USA) and dialyzed against Milli-Q-water. Neutral GSLs and negatively charged gangliosides were separated by anion exchange chromatography on a DEAE-Sepharose CL-6B column (Pharmacia Fine Chemicals, Freiburg, Germany) in the acetate form as described by Müthing *et al.* (1991). Neutral GSLs passed the column (eluate A) and bound gangliosides were eluted with 0.45 M ammonium acetate in methanol (see Figure 1). After evaporation and desalting by dialysis, the ganglioside fraction was incubated 1 h at 37 °C in 1 M NaOH to hydrolyse contaminating phospholipids, followed by neutralization with acetic acid and desalting by dialysis. Then the gangliosides were further purified by Iatrobeads 6RS-8060 chromatography (Macherey & Nagel, Düren, Germany) according to Ueno *et al.* (1978). Fatty acids and other impurities were removed with chloroform/methanol (85/15, by vol.) and gangliosides were eluted with chloroform/methanol (1/2, by vol.). Finally, gangliosides were taken up in chloroform/methanol (2/1, by vol.) and stored at –20 °C.

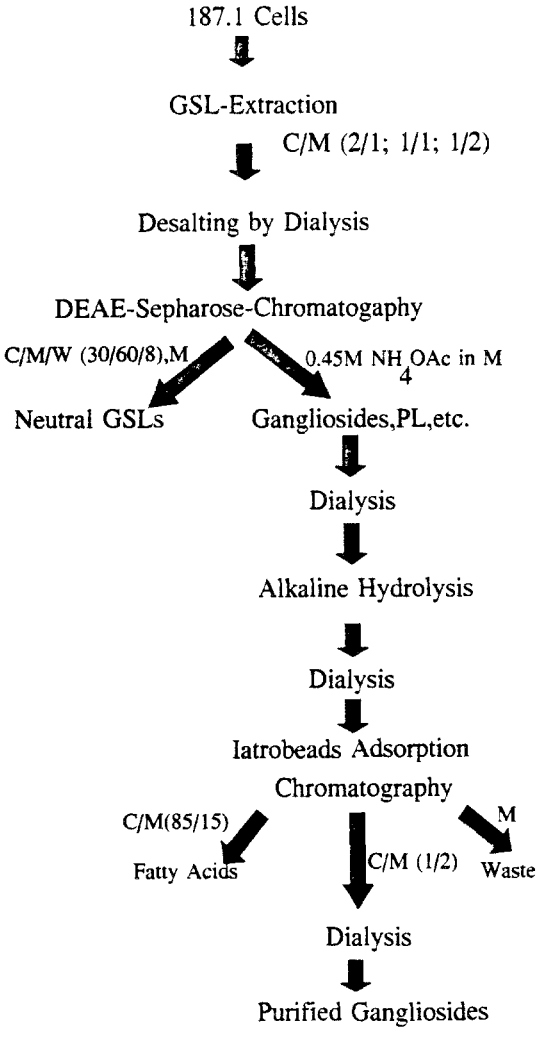

Fig. 1. Flow scheme of the ganglioside isolation from hybridoma 187.1 cells. C = chloroform, M = methanol, W = water, PL = phospholipids.

High performance thin layer chromatography

Gangliosides were separated on glass backed silica gel 60 precoated high performance thin layer chromatography plates (HPTLC plates, size 10 cm x 10 cm, thickness 0.24 mm, Merck, Darmstadt, Germany) in chloroform/methanol/2.5 M NH_4OH, (120/85/20, each by vol.) containing 2 mM $CaCl_2$, and visualized by spraying the plate with resorcinol according to Svennerholm (1957).

Lipid bound sialic acid was estimated by densitometry. Resorcinol stained ganglioside chromatograms were scanned with a densitometer (Desaga, CD 60 scanner, Heidelberg, Germany). Intensities of the bands were measured in reflectance mode at 580 nm with a light beam slit of 0.1 mm x 2mm.

HPTLC immunostaining (overlay technique)

Specific chicken anti-G_{M3}(NeuAc), anti-G_{M3}(NeuGc) and anti-lactosylceramide antibodies were produced as recently published (Müthing *et al.*, 1994; Müthing and Neumann, 1993).

The immunostaining procedure was carried out according to Magnani *et al.* (1982) with some modifications as described by Müthing and Mühlradt (1988). The plates were overlayed with anti-G_{M3} antibodies, diluted 1:1000 in phosphate buffered saline (PBS) supplemented with 1% bovine serum albumin. Rabbit anti-chicken IgG (Dianova, Hamburg, Germany), labelled with alkaline phosphatase and diluted 1:1000 in the same buffer, was used as secondary antibody. Bound antibodies were visualized with 0.05% (w/v)5-bromo-4-chloro-3-indolylphosphate (Biomol, Hamburg, Germany) (Bethke *et al.*, 1986).

G_{M3} ganglioside was alternatively detected by immunostaining with anti-lactosylceramide antibody on the HPTLC plate after *V. cholerae* neuraminidase treatment according to the procedure described by Müthing and Mühlradt (1988).

Reference gangliosides

Mouse liver gangliosides from female CBA/J mice, composed of small amounts of G_{M3}(NeuAc), G_{M3}(NeuGc) and G_{M1}(NeuGc) and G_{M2}(NeuGc) as the major component (Hashimoto *et al.*, 1983; Suzuki *et al.*, 1986), were isolated by standard procedures as described above (Ledeen and Yu, 1982). G_{M1}(NeuAc) was obtained from Dr. Pallmann GmbH (München, Germany).

Determination of sialic acids

Ganglioside derived NeuAc and NeuGc were identified and quantified as their fluorescent derivatives by HPLC essentially as described by Hara *et al.* (1987). Sialic acids were released from gangliosides with 25 mM H_2SO_4 (aq.) (2.5 h, 80 °C) and converted with 1,2-diamino-4,5-methylenedioxybenzene (DMB, Sigma, Deisenhofen, Germany) into their fluorescent derivatives. Reference NeuAc was from Biomol (Hamburg, Germany) and NeuGc from Sigma. HPLC was carried out with a Kontron D450 system consisting of two type 420 HPLC pumps, a type 460 autosampler,

an SFM 25 fluorometer and an MT 450 data system (Büntemeyer *et al.*, 1991). Isocratic HPLC was performed on a reversed phase column (Supersphere RP18, 125 x 8 x 4, particle size 4μm, Bischoff, Leonberg, Germany). Sialic acid derivatives were separated by isocratic elution with methanol/acetonitrile/water (9/7/110 by vol.) and a flow rate of 1.2 ml min^{-1}. The fluorometric detection was operated at an excitation wavelength of 373 nm and an emission wavelength of 448 nm.

Mass spectrometric analysis of G_{M3}

The structure of the isolated G_{M3} was analyzed as its permethylated derivative by positive ion mode fast atom bombardment – mass spectrometry (FAB-MS) as previously reported by Peter-Katalinić and Egge (1990). The permethylation was carried out according to Ciucanu and Kerek (1984). G_{M3} derivatives were separated from reagents by exhaustive extraction with chloroform followed by chromatography on a 1 cm x 23 cm Sephadex LH-20 column (Pharmacia Fine Chemicals) with chloroform/methanol (1/1, by vol.) as eluent. Final purification was performed by chromatography on Iatrobeads (column 0.5 cm x 5 cm) by elution with chloroform, chloroform/methanol (98/2), (95/5), (90/10) and chloroform/methanol/water (75/25/4), each, by vol. Permethylated G_{M3} eluted with chloroform/methanol (90/10, by vol.).

FAB-MS was performed on a ZAB-HF mass spectrometer (VG Analytical, Manchester, UK). The mass spectra were acquired as single scans in the upscan mode on an AMD DP10 data system fitted with SAM II (KWS) hardware and SUSY software (AMD Intectra, Beckeln, Germany).

MTT-assay

Indirect measurement of cell viability was achieved by conversion of 3,(4,5 dimethylthiazol-2-yl)2,5 diphenyl tetrazolium bromide (MTT, Sigma) into coloured formazan by cells according to Hansen *et al.* (1989). 5 mg ml^{-1} MTT were dissolved in sterile PBS and stored at +4 °C in the dark for no longer than one month. Cells were desintegrated with 20% (w/v) sodium dodecylsulphate in N,N dimethylformamide/water (1/1, by vol.), which was adjusted to pH 4.7 with 1N HCl (aq.) (=lysis buffer).

10 μl of MTT in PBS were added to 100μl cell suspensions in 96 well microtiter plates (Nunc, Wiesbaden, Germany) and incubated for 3 h at 37 °C. Then 100μl of the lysis buffer was added and after overnight incubation at 37 °C the concentration of blue formazan was determined spectrophotometrically in a microplate autoreader (model EL311, Bio-Tek Instruments, Fernwald, Germany) at 570 nm.

[^{3}H]thymidine incorporation assay

The DNA synthesis rate of cells, which is routinely utilized as a measure for cell proliferation, was determined by incorporation of [^{3}H]-labelled thymidine into cell DNA (Andersson *et al.*, 1972). 12 h before harvest, cells were pulsed with 0.2 μCi (7.4 kBq) [^{3}H]thymidine (Amersham Buchler, Braunschweig, Germany) per well. 20μl of [^{3}H]thymidine in PBS (10μCi ml^{-1}; specific activity 2Ci $mmol^{-1}$ or 74 GBq $mmol^{-1}$) was added to 200μl cell suspensions in 96 well microtiter plates (Nunc). Cells were collected in a 12 channel cell harvester (Skatron, Transby, Norway) by pouring them onto glass fiber filters (Skatron, 1 μm pore diameter), washed successively with PBS, 10% trichloracetic acid, and 95% ethanol, and dried finally. The filters were placed into scintillation vials, and 3 ml of Aqualuma plus (Baker, Gross-Gerau, Germany) were added to each vial. The radioactivity of the precipitated DNA was determined 1 h later in a Tri-Carb 1900 CA liquid scintillation spectrometer (Packard Instruments Co., Downers Grove, IL, USA). The data were presented as counts per minute (cpm) per microtiter plate cultures.

Exogenous application of G_{M3} to microtiter plate cultures

Aliquots of purified ganglioside G_{M3} from 187.1 hybridoma cells (see above) were transferred to sterile screw capped glass tubes. The organic solvent was removed by gentle heating at 37 °C. The dried ganglioside probes were resuspended in sterile culture medium (see above), sonicated for 5min and then applied to microtiter plate cultures in final concentrations from 3μM up to 30μM at different times from culture initiation. In case of the MTT-assay (see above), the cell concentration was adjusted to 1.5 x 10^5 cells ml^{-1}, and 1.5 x 10^4 cells were seeded per well (=100μl cultures). G_{M3} was added immediately (t = 0 h) and 12 h after seeding to parallel microtiter plate cultures. Using the [^{3}H]thymidine incorporation assay, the cell concentration was adjusted to 1.3 x 10^5 cells ml^{-1}, and 2.6 x 10^4 cells were seeded per well (=100μl cultures). To show the influence of exogenously added G_{M3} to cells dur-

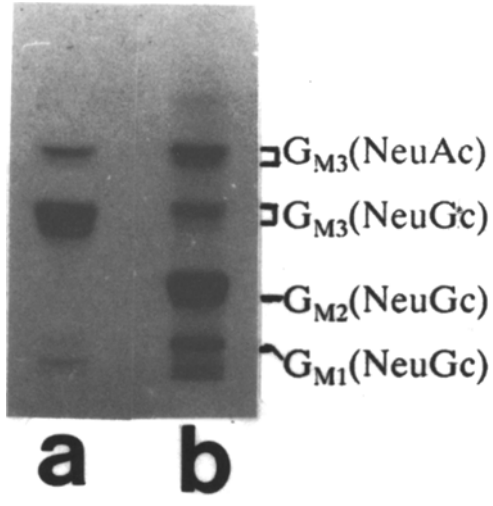

Fig. 2. Resorcinol stain of gangliosides from 187.1 cells on an HPTLC plate. a: 10μg gangliosides of hybridoma 187.1, b: 15μg of mouse liver gangliosides (reference).

ing various stages of initial growth, G_{M3} was applied immediately (t = 0 h), and 12 h as well as 24 h after initiation to parallel cultures.

Results

Gangliosides of hybridoma 187.1 cells

Glycosphingolipids were extracted from bulk quantities of cells obtained from bioreactors. Gangliosides were purified and contaminating phospholipids were removed as described above. Figure 1 shows the flow chart of the isolation procedure. 15 mg gangliosides were isolated from 5.4 x 10^{10} hybridoma 187.1 cells (93% viable cells), corresponding to 0.3 mg 10^{-9} viable cells. Gangliosides were structurally characterized by means of chemical as well as immunochemical methods (overlay technique) and FAB-MS (see below).

Exclusively G_{M3} bands were found in the ganglioside fraction. To elucidate differences in sialylation, i.e. substitution of the lactosylceramide backbone with NeuAc and/or NeuGc, ganglioside extracts were applied onto HPTLC plates and chromatographed in alkaline solvent (Nagai *et al.*, 1980). The resorcinol stain is shown in Figure 2 (lane a). As concluded from thin layer chromatography, the mouse-rat hybridoma showed characteristic high G_{M3}(NeuGc) and low G_{M3}(NeuAc) content, which has been found recently to be a typical attribute of a variety of hybridoma cell lines analysed until now in our group (Müthing *et al.*, 1994). The identity of both ganglioside species was verified by HPTLC immunostaining with specific chicken anti-G_{M3}(NeuAc) and anti-G_{M3}(NeuGc) antibodies as well as with an anti-lactosylceramide antibody after neuraminidase treatment (data not shown). The ganglioside pattern in Figure 2 (lane a) is characterized by expression of G_{M3}(NeuGc) and G_{M3}(NeuAc) in an 80/20 ratio, respectively, each species appearing as a double band on the HPTLC plate suggesting variability in the fatty acids of the ceramide portions. To facilitate the explanation of the structural heterogeneity of G_{M3} in its respective sialic acid and ceramide moiety, the chemical formula of G_{M3}, substituted with $C_{18:1}$ sphingosine, $C_{16:0}$ fatty acid and NeuGc is presented in Figure 3. NeuAc and NeuGc, released by mild acid treatment, were separated and quantified as their DMB-derivatives. The proportion of G_{M3} derived sialic acids is demonstrated in Fig. 4B compared to a reference mixture of NeuAc and NeuGc (Figure 4A). The ratio of NeuGc/NeuAc of about 80/20 coincided to the HPTLC data (see above).

FAB-mass spectrometry

The structural analysis for sequencing of gangliosides can be performed by negative and positive ion FAB-mass spectrometry on the native sample or by positive ion FAB-mass spectrometry after perderivatisation. The main purpose of derivatisation is to increase sensitivity and to enable the analysis of subnanomole amounts of analyte, even if several molecular species are present in the sample (Peter-Katalinić and Egge, 1990).

The structural characterization of the G_{M3}-fraction isolated from hybridoma 187.1 cells was carried out by combining biochemical, immunological and spectroscopic data. The HPTLC separation of the intact gangliosides and their corresponding lactosylceramides, obtained after neuraminidase treatment, was followed by immunostaining of the entire gangliosides and the asialogangliosides with specific anti-G_{M3} and anti-lactosylceramide antibodies as described above. The major constituents of the G_{M3} ganglioside sample, analyzed by positive ion FAB-MS after permethylation, were carrying the same oligosaccharide moiety, but differing in the fatty acid species, present

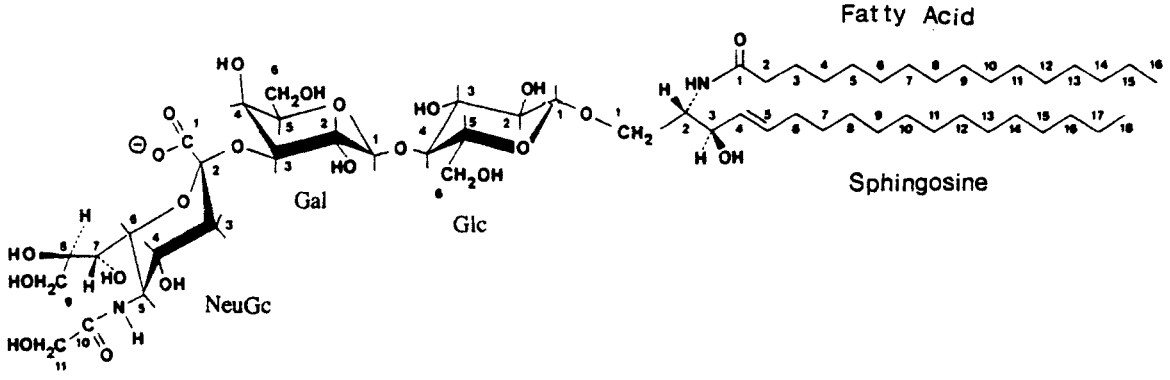

Fig. 3. Structure of G_{M3}(NeuGc). For example, ceramide composed of $C_{18:1}$ sphingosine and $C_{16:0}$ fatty acid is shown in this chemical formula.

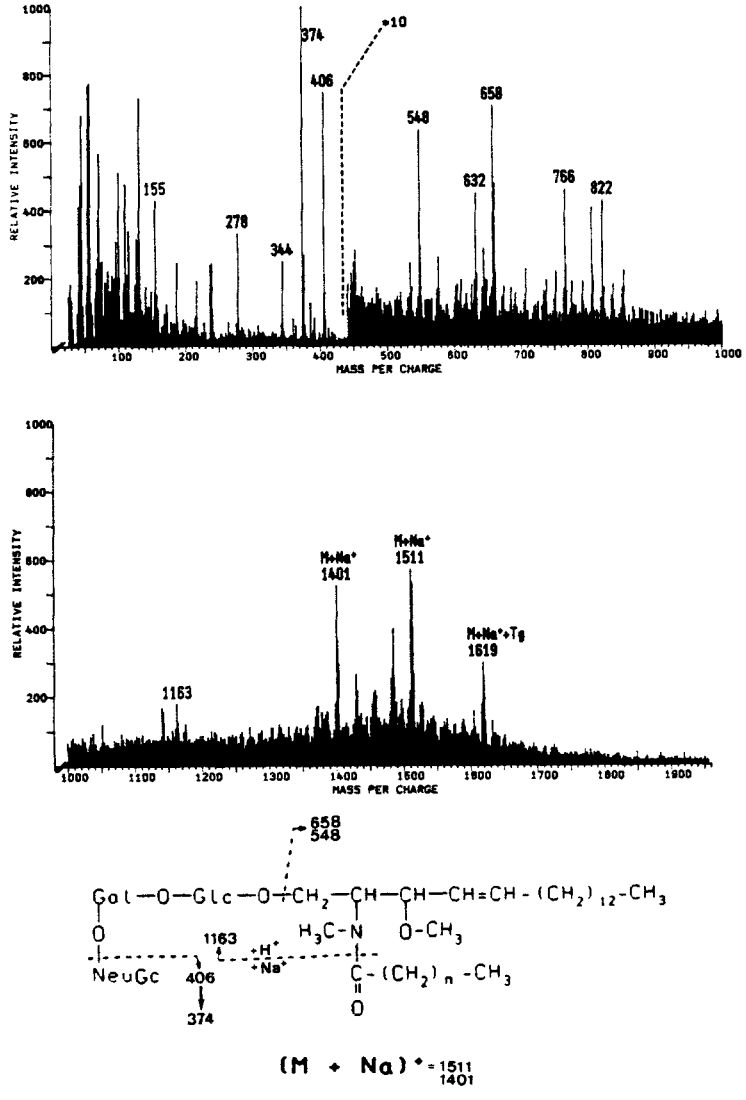

Fig. 5. Positive ion FAB-MS of the hybridoma-derived G_{M3} ganglioside sample after permethylation and the fragmentation scheme of its two major constituents (n = 14 and 22:1 respectively).

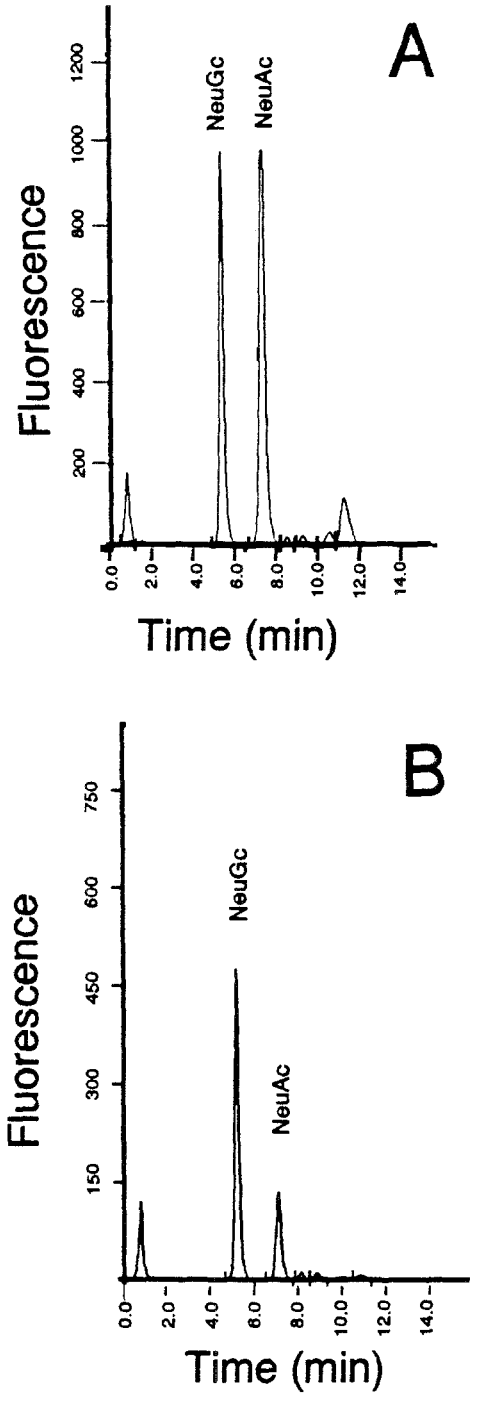

Fig. 4. HPLC elution profile of fluorescent DMB derivatives of G_{M3} released sialic acids from hybridoma 187.1 cells. A: NeuAc and NeuGc standards, B: hybridoma 187.1 cells.

in the ceramide residue (Figure 5 and fragmentation scheme). This was documented by the molecular $[M + Na^+]^+$ ions at m/z = 1401 ($C_{16:0}$) and 1511 ($C_{24:1}$) and by the ceramide ions at m/z = 548 ($C_{16:0}$) and 658 ($C_{24:1}$). Both species gave the same fragment $[M\text{-acyl}^+ + H^+ + Na^+]^+$ ion, diagnostic for the structure of the long chain base, in this case the $C_{18:1}$ sphingosine species, represented by the ion at m/z = 1163 (Figure 5 and fragmentation schme). Besides the two amide-linked major fatty acid, palmitic ($C_{16:0}$) and nervonic acid ($C_{24:1}$), minor amounts of $C_{18:0}$, $C_{22:0}$ and $C_{24:0}$ were also present, as documented by the molecular $[M + Na^+]^+$ ions at m/z = 1429, 1485 and 1513. In the carbohydrate moiety the terminal NeuGc was represented by the ions at m/z = 406 and 374. The less abundant ions at m/z = 376 and 344 are characteristic for the NeuAc sialic acid. NeuAc carrying trisaccharides, bound to the same ceramide species as those with NeuGc, were present only in low amounts of about 20%, indicated by G_{M3} molecular $[M + Na^+]^+$ ions at m/z = 1371 ($C_{16:0}$) and 1481 ($C_{24:1}$).

Exogenous application of G_{M3} to 187.1 cell cultures

Purified ganglioside G_{M3}, composed of about 80% G_{M3}(NeuGc) and 20% G_{M3}(NeuAc), was prepared from large scale propagated 187.1 cells and used in the following studies. Addition of G_{M3} to the serum-free growing cells resulted in a growth inhibition as determined by the MTT-assay and [^{3}H]thymidine incorporation. In the following parts of this article the cellular response due to exposure to low and high concentrations of exogenously applied G_{M3} (3μM and 30μM, respectively) will be discussed. G_{M3} preparations not only from 187.1 cells but also from various hybridoma cells expressing exclusively this ganglioside (Müthing *et al.*, 1994) were tested and in all cases G_{M3} was found to be the biological active compound. To exclude unspecific ganglioside effects, the monosialoganglioside G_{M1} was used as reference in control cultures in concentrations up to 50μM. No growth modulatory action of G_{M1} was observed.

G_{M3} induced reduction in cell viability determined by the MTT-assay

G_{M3} added to cultured 187.1 cells generated reduced metabolic activity. This cellular response is concentration dependent as measured by the conversion of yellow MTT to blue formazan (Hansen *et al.*, 1989). Cells were incubated in final concentrations between 3μM and 30μM G_{M3} in microtiter cultures and their metabolic activity was compared to controls without gangliosides. As exemplified in Figure 6 for 3μM G_{M3},

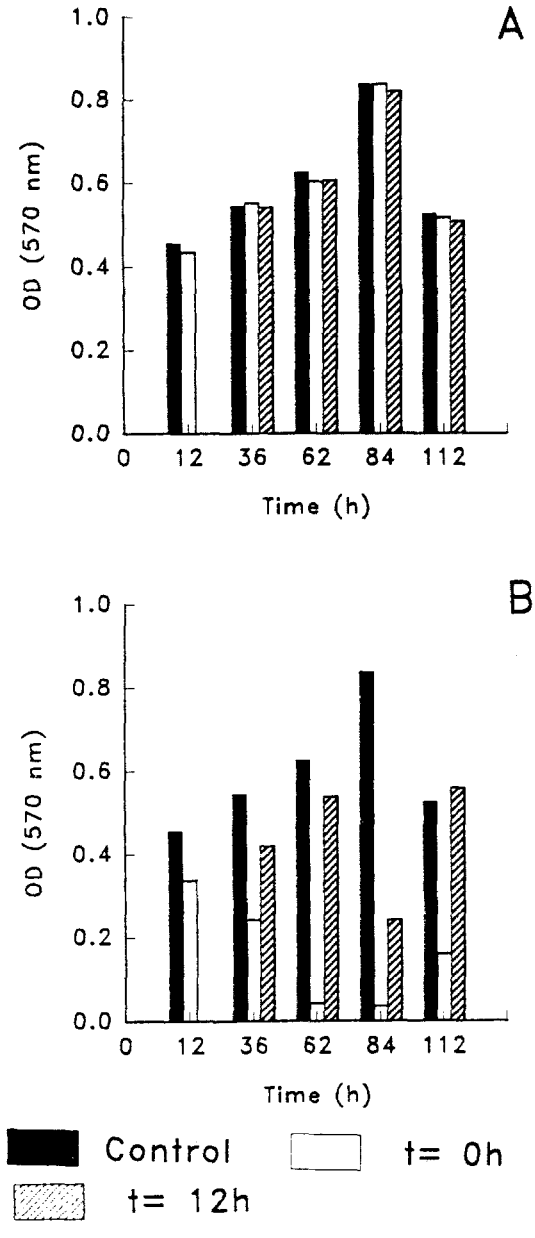

Fig. 6. Dose dependent reduction in cell viability obtained by exogenous application of G_{M3} and determined by the MTT-assay. 3μM G_{M3} (A) and 30μM G_{M3} (B) were added to microtiter plate cultures. 5 x 10^4 cells per well (= 1.5 x 10^5 cells ml^{-1}) were seeded and G_{M3} was applied immediately (t = 0 h) and 12 h after cell seeding. Cultures without G_{M3} served as control. Quintuple MTT-tests were performed in time intervalls as indicated and standard deviations < ± 10% were revealed.

cells were not effected by exposure to low G_{M3} concentration (Figure 6A), whereas a distinct decrease in metabolic activity was obtained by exposure to 30μM G_{M3} (Fig. 6B). By adding 30μM G_{M3} from the beginning (t = 0 h) of the incubation period, the level of dehydrogenases activities decreased to 74%, 44%, 7%, 4% and 30% after 12 h, 36 h, 62 h, 84 h and 112 h of G_{M3} exposure, respectively, compared to control cultures without G_{M3}. After 112 h some recovery of the cells could be observed (see Figure 6B). Increasing cell densities reduced the inhibitory effects, i.e. G_{M3} added 12 h after culture initiation showed less reduction in cell viability than immediately applied G_{M3} (t = 0 h). Less but also significant suppression of metabolic activity was gained by application of G_{M3} in concentrations from 10μM to 20μM (data not shown).

G_{M3} induced inhibition of cell growth determined by [^{3}H]thymidine incorporation

Dose dependent inhibition of cell growth by adding G_{M3} to 187.1 microwell cultures was detected by radioactive DNA labelling. The incorporation of [^{3}H]thymidine as measure for growth was not effected by addition of 3μM G_{M3} into the culture medium compared to reference microwell cultures without G_{M3} (Figure 7A). Again, a strong decrease of the proliferation rate was detected when the cells were incubated with 30μM G_{M3} (Figure 7B). Immediate addition of G_{M3} (t = 0 h) reduced DNA label to 65%, 54% and 60% after 36 h, 48 h and 64 h of G_{M3} exposure, respectively, compared to control cultures without G_{M3}. Inhibition of [^{3}H]thymidine incorporation occurred retard of G_{M3} application, i.e. 12 h and 24 h cultures gave the same DNA label compared to G_{M3} free cultures (Figure 7B). Inhibitory effects were abolished by increasing cell densities i.e. G_{M3} added 12 h and 24 h after starting the cell cultures showed less reduction in proliferation than G_{M3} added just from culture initiation (see Figure 7B). Inhibition by G_{M3}, added 12 h and 24 h after cell seeding, was overcome at cultivation time t=60 h (see Figure 7B) indicating recovery of the cells.

Discussion

Growth modulatory function of G_{M3}

G_{M3} is a common and ubiquitous component of vertebrate cells and represents the major ganglioside in mammalian cells of mesodermal origin. Several lines of evidence that G_{M3} regulates cell proliferation (for review see Hakomori, 1990; and references therein) and induces cell differentiation (Saito, 1989) as

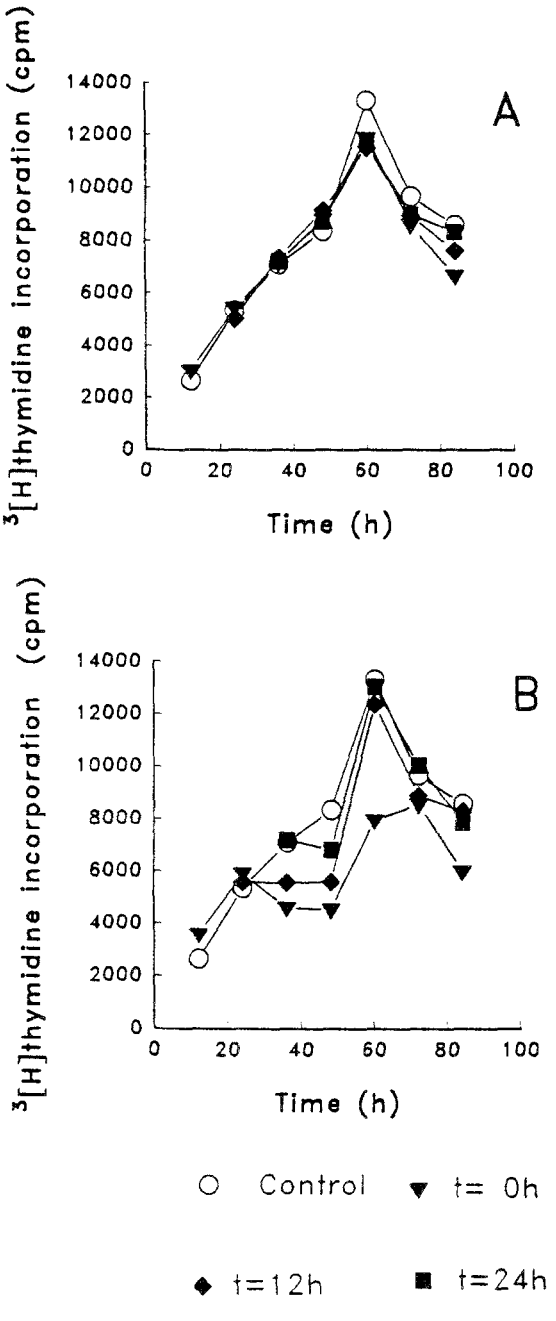

Fig. 7. Dose dependent inhibition of cell growth obtained by exogenous application of G_{M3} and determined by [^{3}H]thymidine incorporation. 3μM G_{M3} (A) and 30μM G_{M3} (B) were added to microtiter plate cultures. 2.6 x 10^4 cells per well (= 1.3 x 10^5 cells ml^{-1}) were seeded and G_{M3} was added immediately (t = 0 h), 12 h and 24 h after cell seeding. 12 h before harvest the cells were pulsed with 2μCi ml^{-1} [^{3}H]thymidine. Each point represents the mean value of quintuple cultures with standard deviations < ± 10%.

well as immunosuppression (Prokazova *et al.*, 1988; Dyatlovitskaya *et al.*, 1991) have accumulated during the past decade. Growth modulatory actions of G_{M3} exogenously applied to cell cultures have been reported by several groups (also reviewed by Zeller and Marchase, 1992) as well as possible involvement in contact inhibited cell growth (Rösner *et al.*, 1990). GSLs exogenously added to cultured cells become membrane associated in at least three ways (Radsak *et al.*, 1982): one mode of attachment susceptible to serum albumin and another by adsorption to trypsin-labile structures, which were shown to consist of ganglioside micelles attached to the cell surface (Schwarzmann *et al.*, 1983). The remaining albumin and trypsin-resistant third portion suggested a location in the plasma membrane.

If cell growth is indeed regulated by the quantity and/or kind of GSL in the plasma membrane of cultured cells, such growth might be altered by increasing or decreasing the levels of the specific GSL in the plasma membrane. In fact, this was convincingly demonstrated in early experiments by the addition of GSLs to the culture medium. The first reports concerning the growth modulatory function of GSLs administered to *in vitro* grown cells (Laine and Hakomori, 1973; Keenan *et al.*, 1975) implicated the prolongation of the prereplicate G_1 phase of the cell cycle (resting state), rendering reduced growth rate and diminished saturation cell density. A specific functional role of the ganglioside G_{M3} has been reported later by several authors. Inhibition of mouse 3T3 fibroblasts by exogenously administered G_{M3} was reported to be more obvious in chemically defined serum-free than in serum-supplemented medium (Bremer *et al.*, 1984). Their results indicated a modulation of growth factor receptor function by affecting the degree of tyrosine phosphorylation and altering the affinity of growth factor receptor. This approach was extended to the epidermal growth factor receptor of human epidermal carcinoma cell lines, that had been cultured in medium containing 50μM G_{M3} to effect cell growth inhibition (Bremer *et al.*, 1986). The molecular basis for these effects is not yet fully understood, but their results suggest that membrane lipids, especially G_{M3}, can modulate receptor phosphorylation *in vitro* as well as *in situ*.

Intracellular transport of GSLs

In general, two major pathways of intracellular GSL flow can be distinguished. First, there is a transport of synthesized GSLs from the Golgi complex to the

plasma membrane, and second, there is a GSL flow *vice versa* from the plasma membrane to intracellular organelles during endocytosis. GSLs within the membrane structures associated with both routes will end up in lysosomes or the plasma membrane following endo- or exocytosis, respectively. As mentioned above, exogenous gangliosides can insert into the plasma membrane of cultured cells and mix with the pool of endogenous GSLs (Schwarzmann *et al.*, 1983). Following the metabolic fate and the intracellular transport of GSLs, experiments performed by Schwarzmann *et al.* (1987) clearly demonstrated that exogenous gangliosides, once inserted into the outer leaflet of the plasma membrane of cultured cells, are subject to endocytosis via coated pits and, besides being transported to lysosomes, are also delivered to the Golgi apparatus. The amount of gangliosides transported to the Golgi complex is, however, rather small compared to that ending up in lysosomes (Schwarzmann and Sandhoff, 1990).

Ganglioside G_{M3} sialidases

If cell surface glycoconjugates such as G_{M3} are localized in the membrane, where they are able to inhibit the growth factor-mediated cell growth, a mechanism must exist to relieve the inhibition, otherwise cells could not progress towards DNA synthesis and would presumably be blocked at some point in the cell cycle. There are several different sialidases that might account for the turnover of G_{M3}, lysosomal and plasma membrane bound ganglioside G_{M3} sialidase (Schneider-Jakob and Cantz, 1991). The presence of sialidase activity in the conditioned medium of cultured human fibroblasts and the rapid turnover of the sialic acid residue of G_{M3} has been reported by Usuki *et al.* (1988a). An extracellular sialidase was shown to be involved in the regulation of growth, and inhibition of this activity was accompanied by decreased cell growth (Usuki *et al.*, 1988b). Studies of turnover rates for gangliosides in brain revealed half-lives varying from a few days to several weeks (reviewed by Ledeen, 1989). These lengthy half-lives imply that GSLs might be protected from catabolic degradation and recycle many times between the endosomes and the plasma membrane prior to being finally delivered to lysosomes. Further investigations are necessary to unambiguously establish the intracellular traffic routes of GSLs and the exact sites of biosynthesis to get more detailed insights into the metabolic pathway of gangliosides.

Conclusion

In our experiments reduced cell viability and growth inhibition was determined in hybridoma cultures after exogenous application of cell identical G_{M3}. The metabolic breakdown, determined by the MTT-assay, occurred immediately after G_{M3} addition, whereas the DNA synthesis declined 24 h later. Cell recovery was observed after defined time spans, indicating "arresting" of cells, probably in the replicative G_1 phase of the cell cyclus, and not cell killing. G_{M3}, suggested by several authors to play a crucial role as a potential modulator of cell growth, represents the dominant ganglioside found in a wide spectrum of different hybridomas, and consequently the described effects might not be restricted to the 187.1 hybridoma cell line used in this study. In general, hybridomas are used as a tool for large scale production of monoclonal antibodies in bioreactors or hollow fiber cartridges. Low molecular weight inhibitors, e.g. lactate or ammonium, can be easily removed from high cell density bioreactors by perfusion or dialysis. In such systems, glycolipids as well as other amphipathic membrane lipids, which might be delivered to the culture medium due to cell lysis (cell death) and/or membrane shedding, do not escape from the reactor vessel, due to their capability to form large micelles and/or vesicles. By the fact that these "exogenous" compounds can be reincorporated into the plasma membrane, cellular G_{M3} becomes one of the potent candidates to regulate growth in high density hybridoma cultures. This "cannibalism" of own cellular compounds might also prevent considerable accumulation in the surrounding medium. Moreover, additive inhibitory effects raised by other cell-released components seem more feasible than a single action of one extracellular kind of molecule. In artificial (experimentally) high cell density cultures a wide range of cell-derived constituents, which "normally" do not influence viability and cell growth, might change to negative effectors.

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