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Monoclonal anti-GD3 antibodies selectively inhibit the proliferation of human malignant glioma cells *in vitro*

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The frequently occurring alteration of ganglioside expression in tumor cells has been implicated to play a role in the uncontrolled growth of these cells; antibodies to such gangliosides might affect tumor cell growth. We have studied the effect of IgM monoclonal antibodies to two glioma-associated gangliosides, GD3 and GM2, on cell proliferation of four human glioma cell lines and one renal tumor cell line. Of the two anti-ganglioside antibodies tested, only the anti-GD3 antibody resulted in a significant ($p < 0.005$) inhibition of cell proliferation as measured by thymidine incorporation and Brd-U labeling, after 24 h incubation. The effect was not dependent on any serum factor and no increased cell death was observed. All cell lines contained higher or similar amounts of GM2 than GD3, and both antigens were shown to be expressed on the cell surface and accessible to antibodies. The selective effect of anti-GD3 antibodies as contrasted to the inactivity of anti-GM2 antibodies suggests a possible role for ganglioside GD3 in tumor cell proliferation.

Keywords: **carbohydrate antigens, gangliosides, glioma, monoclonal antibodies, proliferation**

Abbreviations: **BCA, bicinchoninic acid; BCIP, 5-bromo-4-chloro-3′-indolylphosphate; Brd-U, 5-Bromo-2′-deoxy-Uridine; EGF, epidermal growth factor; FCS, fetal calf serum; HPTLC, high performance thin layer chromatography; MAbs, monoclonal antibodies; PDGF, platelet derived growth factor; PVP, polyvinylpyrrolidone; TCA, trichloro-acetic acid; TGF-α, transforming growth factor alpha.**

Gangliosides have been designated according to CBN recommendations [57] and to the coding system of Svennerholm [58].

Introduction

It is well documented that many tumor cells exhibit an altered cell surface phenotype with regard to glycolipids, particularly gangliosides (glycolipids containing sialic acid) [1]. This phenomenon has been studied in human gliomas, which have been shown to have a ganglioside composition that differs from normal adult human brain cells [2–4]. Some of these glioma-associated gangliosides were also found to be expressed during human brain development [5]. *In vivo* expressed gangliosides were also found in glioma cells grown *in vitro*, although their relative expression was changed [6]. As gangliosides in general have been found to be expressed mainly on the cell surface, they have been considered to be suitable targets for antibodies or other ligands, and thus utilized in therapy.

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There are accumulating data to support the premise that gangliosides are involved in cell growth and adhesion [7]. Therefore, an altered expression of gangliosides might be part of the mechanism behind the uncontrolled growth and migration of tumor cells. Several studies give support to the hypothesis that monoclonal antibodies (MAbs) against tumor-associated gangliosides might inhibit tumor cell growth. MAbs reacting with ganglioside GM2 were shown to induce necrosis in *in vitro*-spheroid cultures of a GM2-rich human glioma cell line [8]; anti-GD3 MAbs were demonstrated to inhibit growth of human melanoma cells both *in vivo* [9,10] and *in vitro* [11]; and human MAbs against GD2 were shown to suppress growth of human melanoma xenografts in nude mice [12]. MAbs against melanoma gangliosides have also been used in clinical trials with encouraging results: murine MAbs against ganglioside GD2 activated human complement and had an antitumor effect on neuroblastoma and malignant melanomas *in vivo* [13].

The mechanisms underlying ganglioside involvement in growth regulation and the inhibitory effects of anti-ganglioside

antibodies have not been fully elucidated. It has been shown that the growth stimulation induced by epidermal growth factor (EGF), platelet derived growth factor (PDGF) and transforming growth factor-α (TGF-α) [14–16] might be inhibited by exogenously added gangliosides [7,17]. These findings led to the suggestion that the role of gangliosides in the membrane might be interaction with various receptors. More recent studies have focused on the interaction of endogenous gangliosides and signal transduction. Kasahara et al. [18] showed an association of Src-family tyrosine Lyn with ganglioside GD3 in rat brain in caveolae-like domains in the cell membranes. The assembly of glycolipids with signal transducer molecules was reviewed by Hakomori et al. [19]. If a particular ganglioside is involved in the activation of a receptor or some other steps in the signaling pathway, an antibody directed against this ganglioside might interfere with the signal and possibly result in an inhibition of growth stimulation.

As described above, we have previously found that anti-GM2 antibodies induced necrosis in three-dimensional cultures of D54MG human malignant glioma cells. Another ganglioside over-expressed in human gliomas as well as in many other tumors which might be a target for monoclonal antibodies is ganglioside GD3 [2,20–26]. The purpose of this study was to investigate if anti-GD3 monoclonal antibodies might have an inhibitory effect on the proliferation of human glioma cells grown in monolayer culture and, if so to compare that effect with that of the anti-GM2 MAb which induced necrosis in spheroids of glioma cells. We have therefore measured the effect of mouse anti-GD3 and anti-GM2 MAbs on glioma cell proliferation by a variety of techniques chosen to measure DNA synthesis and cell growth rate.

Materials and Methods

Chemicals

^{3}H-thymidine was purchased from Amersham Life Science, Buckinghamshire, England. Glass microfiber filters (Ø4.7 cm) were obtained from Whatman International Ltd, Maidstone, England, UK. The liquid scintillation fluid, Ultima Gold, was from Packard Instrument Company, Meriden, CT, USA. High performance thin layer chromatography (HPTLC) plates (0.25 mm silica gel on pre-coated plastic sheets) were purchased from Macherey-Nagel, Düren, Germany. Fractogel TSK DEAE-650 (M) was obtained from Merck, Darmstadt, Germany. The substrate 5-bromo-4-chloro-3′-indolylphosphate (BCIP) was obtained from Sigma Chemical Co, St. Louis, USA, and the bicinchoninic acid (BCA) reagent was from Pierce, Rockford, Illinois, USA. The synthetic serum, Ultroser, was from Réactifs IBF, LKB, Pointet Girard, France. Dako mounting medium was from DAKO PATTS AB, Carpinteria, USA. Proliferation kit WST-1 and 5-Bromo-2′-deoxy-uridine (Brd-U) labeling and detection kit were purchased from Boehringer Mannheim Scandinavia AB, Bromma, Sweden. Trypan blue (0.5% solution in 0.85% saline) was from Flow Laboratories, Virginia, USA.

Glycolipid standards

Ganglioside GM2 was isolated from the brain of a patient who had died from Tay-Sachs disease [27]. Other gangliosides and neutral glycolipids used as standards were all isolated from human sources and all glycolipid standards were characterized by fast atom bombardment-mass spectrometry as previously described [28,29].

Antibodies

The anti-ganglioside-MAb producing hybridoma cells were generated by fusion of spleen cells, from immunized mice and myeloma cells [30,31]. The monoclonal antibodies were purified from the supernatant of the hybridoma culture by anti-IgM Sepharose (Sigma chemical Co., St. Louis, MO) column chromatography; purification was performed at Duke University Medical Center, Durham, North Carolina, USA [32]. These antibodies, including the control IgM MOPC104E-antibody (a mineral oil-induced plasmacytoma for which the binding epitope is unknown, [33]), were demonstrated to contain only IgM and κ light chains by SDS-gel electrophoresis, and were kindly provided by Dr. D. D. Bigner. The alkaline phosphatase-conjugated goat anti-mouse IgG + IgM (H + L) antibody and Texas red-conjugated anti-mouse IgM antibody were purchased from Jackson Immunoresearch Laboratories Inc., West Grove, PA, USA. The defined binding epitopes of the mouse monoclonal antibodies have been previously published; DMAb-1 (anti-GM2) is specific for GalNAcβ1-4(NeuAcα2-3)Galβ1-4Glc [30], DMAb-8 (anti-GD3) for NeuAcα2-8NeuAcα2-3Galβ1-4-Glc [32]. The anti-GM2 antibodies have shown lipid specificity [30], thus cross-reactivity with the carbohydrate epitope expressed elsewhere is unlikely and it appears to be the same for anti-GD3 as well. During the experiments different batches of the anti-ganglioside-MAbs were tested and they gave similar results.

Cell lines

The human glioma cell lines TP483 [34], D54MG [35,36], U118 [37,38], U138 and the renal cell line SMKT-R2 [39] were grown in Ø1.55 cm wells (NUNC) in complete medium (Iscoves medium supplemented with 10% fetal calf serum (FCS), 1% sodium pyruvate, 1% L-glutamine and antibiotics (streptomycin/penicillin 1%)) at 37°C in 6–8% CO_2. They were harvested by trypsinization (0.25 g/L in Versene) for three minutes at 37°C. Thereafter, media containing 10% FCS was added, and centrifugation at $500 \times g$ for 5 min at room temperature was performed.

Effect of MAbs on thymidine incorporation

The cells were cultured to confluency (i.e. approximately 10^5 cells/cm^2) in Ø1.55 cm culture wells (NUNC) and then harvested as described above. The cells were suspended in complete medium to a final concentration of 2×10^4 cells/mL

and 2 mL were seeded per well (i.e. approximately 2×10^4 cells/cm^2) and cultured at 37°C for 4 h. Thereafter, the medium was changed to medium (2 mL) containing MAbs (anti-GD3, anti-GM2 or MOPC104E) and ^{3}H-thymidine (to a final concentration of 2 μCi/mL). The antibody concentration was measured by the BCA-method [40]. The cells were then incubated for another 24 h (total 28 h) and harvested (non-confluent conditions). Trichloro-acetic acid (TCA) (1 mL of a 10% aqueous solution) was added and mixed well with the cell pellet. After 30 min at +4°C, the precipitate was filtered through a glass microfiber filter. The filter was washed twice with 10% TCA, dried and the radioactivity measured in a β-counter.

In some experiments FCS was exchanged for the same percentage (10%) of heat inactivated FCS serum (FCS pre-heated to 56°C in a water-bath for 45 min) or synthetic serum (2% Ultroser). In each experiment, quadruplicate samples for each set-up were analyzed.

Cell count

The cells were cultured as described under '*Effect of MAbs on thymidine incorporation*' with or without addition of thymidine. The cells were cultured with antibodies for 24 h as described above (total 28 h). After harvest of cells and culture medium, all cells were centrifuged to a pellet, resuspended in medium and diluted 1 : 1 with trypan blue. Live and dead cell counts were determined in a Bürker chamber by two independent readers.

Proliferation assayed with WST-1 kit

The cells were cultured at the same concentration (2×10^4 cells/cm^2) as described above for the thymidine incorporation analyses, but in 96-well plates (NUNC), 100 μL/well, for 4 h. The medium was changed to medium containing antibodies according to the above-described concentrations (see *Effect of MAbs on thymidine incorporation*). The cells were then cultured for another 24 h (total 28 h) before the WST-1 reagent was added to the cell culture according to the manufacturer's directions. The plate was scanned in an ELISA-reader, Milenia Kinetic analyzer, at 450 nm with 650 nm as reference, after one to four hours. The absorption of the different media was compared with the absorption of the control (no antibody in the medium) and decreased/increased proliferation was estimated as decreased/increased absorption compared to the control. The cells were also cultured 24, 48 and 72 h with antibody present before analysis.

The proliferation assay WST-1 is a tetrazolium salt (slightly red) that is cleaved to formazan (dark red) by cellular enzymes. An increase in the number of viable cells results in an increase in the overall activity of mitochondrial dehydrogenase in the sample. This augmentation in enzyme activity leads to an increase in the amount of formazan dye formed, which directly correlates to the number of metabolically active cells in the culture. The formazan dye produced by metabolically active cells is quantified with a scanning multiwell spectrophotometer (ELISA reader) by measuring the absorbance of the dye solution [41].

5-Bromo-2′-deoxy-uridine labeling and detection kit (Brd-U kit)

The cells were cultured at the same concentration (2×10^4 cells/cm^2) as described above for the thymidine incorporation and WST-1 analyses, in 96-well plates (NUNC), 100 μL/well, for 4 h. The medium was changed to medium containing antibodies according to the above-described concentrations (see *Effect of MAbs on thymidine incorporation*). The cells were then cultured for another 24 h (total 28 h) before the Brd-U labeling solution was added to the cell culture according to the manufacturer's directions. After 4 h the cells were fixed with 70% ethanol in HCl (final concentration 0.5 M) at −20°C for 30 min. Thereafter nucleases were added for 30 min at 37°C, anti-Brd-U antibody was then added at 37°C for 30 min. Lastly the peroxidase substrate ABTS were added and incubated at room temperature for 20 to 60 min before the plate was scanned in an ELISA-reader, Milenia Kinetic analyzer, at 405 nm with 490 nm as reference. The Brd-U is incorporated into the cells DNA and detected by immunofluorescence [42,43], using an anti-Brd-U antibody [44] and ELISA measurement [45].

Ganglioside analyses

The cells were cultured to confluency in 25 cm^2 bottles, harvested and suspended as described above (2×10^4 cells/cm^2), cultured for 28 h (non-confluent conditions) or for 72 h (confluent conditions) in 25 cm^2 bottles and harvested by trypsinization as described above. The cell pellet was homogenized in distilled water. An aliquot was used for protein determination by the BCA-method [40]. Gangliosides were extracted and separated according to previously described methods [46–48] with the exception that Fractogel was used as the ion exchange chromatography resin. Quantitative determination was performed by HPTLC-ELISA as previously described [47], except that all incubations were performed at room temperature. The gangliosides were quantified by densiometric scanning at 620 nm with a CAMAG TLC Scanner. Purified gangliosides were used as standards (see glycolipid standards).

Metabolic labeling of ganglioside

The cells were cultured to confluency in 25 cm^2 bottles, harvested and suspended as described above (2×10^4 cells/cm^2), cultured for 28 h (non-confluent conditions) in 25 cm^2 bottles with ^{3}H-galactose (2 μCi/mL) or ^{14}C-serin (0.5 μCi/mL) present in the media and harvested by trypsinization as described above. The gangliosides were extracted and separated as described above (*Ganglioside analyses*). The labeled gangliosides were separated by high

performance thin layer chromatography (HPTLC) using C/M/0.25% aqueous KCl (5:4:1, by volume) as developing solvent. Individual gangliosides were detected by autoradiography (the film was exposed at −80°C for 3 days). The quantity of each ganglioside band was determined by scanning with Bioscan, System 200 Imaging Scanner. Ganglioside- and glycolipid standards were chromatographed at the end of the plate and that part was cut off after the chromatography. The standards were visualized by spraying with orcinol [49].

Immunohistochemistry

The cells were cultured on cover slips in Ø3 cm wells for 28 h in the same concentration as described above for the ganglioside analyses. They were washed with PBS (phosphate buffered saline with 0.145 M NaCl, 2.5 mM Na_2HPO_4, 7.5 mM NaH_2PO_4 and pH 7.4) and fixed with 4% paraformaldehyde in PBS for 20 min. After washing with PBS, the cells were permeabilized by incubation in 0.05% Tween20 in 1% polyvinylpyrrolidone (PVP) as blocking agent, various primary MAbs (anti-GM2, anti-GD3, MOPC104E) (20 μg/mL) diluted in 1% PVP (in PBS) were added and the cells were incubated for 60 min at room temperature. After extensive washing with PBS, Texas red-conjugated goat anti-mouse IgM (diluted 1 : 800) was added for 30 min (room temperature), and the cells were washed with PBS again. The cells were also incubated with primary antibody before fixation and without permeabilization, with the same incubation times and conditions. Both staining procedures were also made on cover slips with cells but without any primary antibodies added, as controls. The cover slips, with the cells, were mounted on slides with mounting medium (DAKO), dried, and stored at +4°C and in darkness until studied. The maximum storage time was 6 days.

Results

Immunohistochemistry

Both anti-GM2 and anti-GD3 MAbs stained permeabilized as well as unpermeabilized TP483 and D54MG cells (see Figure 1A–D). Approximately ten percent of unpermeabilized TP483 cells showed detectable staining with anti-GD3, as compared to about one percent of the D54MG cells. However, both these cell lines were 100% positive for anti-GD3 when permeabilized. The MOPC104E control antibody did not stain either of these two cell lines, independently of whether the cells were permeabilized or not (see Figure 1E–F). The anti-GM2 antibody stained 100% of the unpermeabilised cells and the staining was more intense than the staining with anti-GD3 antibody.

Ganglioside analyses

The amount of GD3 and GM2 ganglioside expressed by the five cell lines used is presented in Table 1. It is noteworthy that two of the cell lines contained markedly more GM2 than GD3. The amount of GM2 per mg protein was increased in confluent cells compared to non-confluent cells, while the GD3 concentration in all cell lines was similar and low. In confluent D54MG the GD3 concentration was below the detection limit

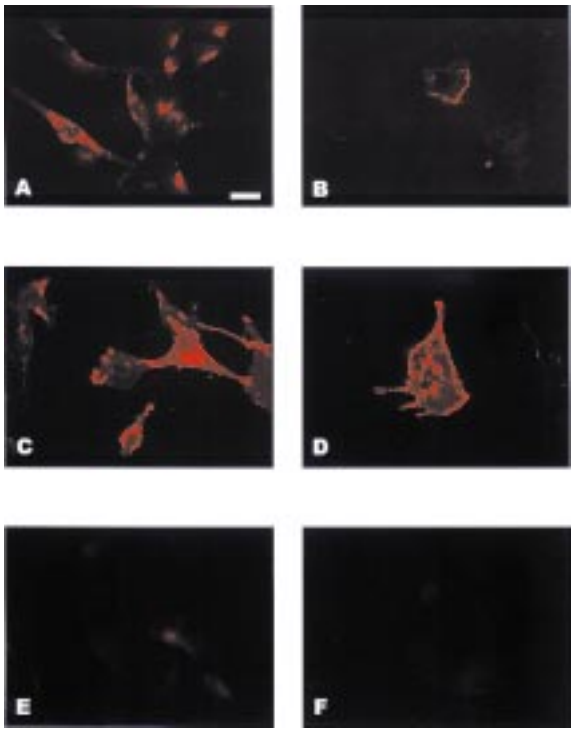

Figure 1. Immunofluorescence-staining of D54MG glioma cells with anti-GD3 MAb, anti-GM2 MAb and MOPC104E MAb. Cells were cultured and treated as described under Materials and Methods. In Figure 1A, 1C and 1E the cells were permeabilized before addition of antibody, but not in Figure 1B, 1D and 1F. A and B: anti-GD3 MAb; C and D: anti-GM2 MAb; E and F: MOPC104E MAb. Scale bar = 20 μm. The procedure is described in detail under Materials and Methods.

Table 1. Ganglioside analyses. Ganglioside content measured by HPTLC-ELISA on extracted glycolipids from human cell lines. The term 'undetectable' stands for a value lower than 0.0008 nmol NeuAc. ∗, nmol NeuAc/mg protein

Cell line	*Confluent/ nonconfluent*	*GM2**	*GD3**
TP483	Non confluent	0.16	0.06
TP483	Confluent	0.37	0.05
D54MG	Non confluent	1.34	0.06
D54MG	Confluent	2.23	undetectable
U118	Non confluent	0.05	0.04
U138	Non confluent	0.06	0.06
SMKT-R2	Non confluent	0.05	0.007
SMKT-R2	Confluent	0.13	0.004

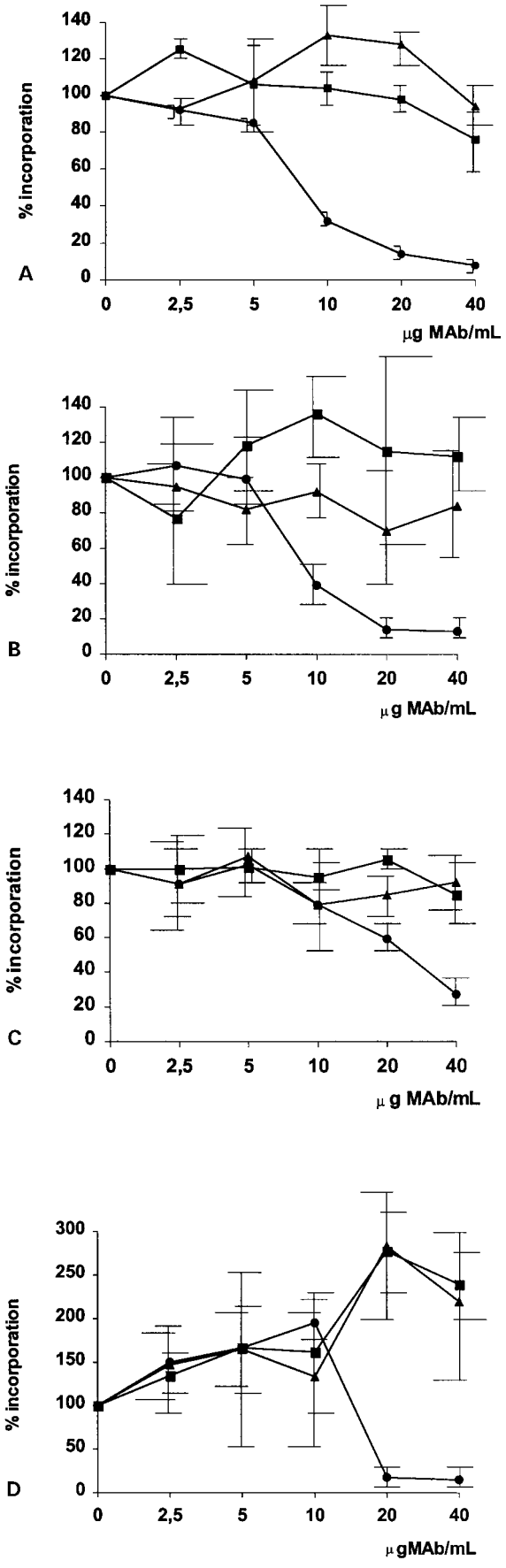

Figure 2. The effect of various concentrations of monoclonal antibodies to GD3, GM2 and MOPC104E on ^{3}H-thymidine incorporation by four human glioma cell lines. The inhibition was calculated as the percentage (^{3}H-) thymidine incorporation as compared to the control, i.e. no antibody addition. The results are the mean values of four individual samples within a single experiment. Anti-GD3 antibody (●), anti-GM2 antibody (■) and the MOPC antibody (▲). A: TP483 cells; B: D54MG cells; C: U118 cells and D: U138 cells. The procedure is described in detail under Materials and Methods.

(i.e. 0.0008 nmol NeuAc). The SMKT-R2 cell line contained approximately 10 fold lower amounts of ganglioside GD3 as compared to the glioma cell lines (Table 1). It should be noted that all cell lines were analyzed at a non-confluent stage.

Radioactive labeling of ganglioside synthesis

The radioactive labeling of the gangliosides was performed on TP483 and D54MG glioma cells and showed a pattern similar to the total content of the two gangliosides in the cells. The rate of synthesis of GD3 thus was lower than that of GM2.

Effects on exogenous thymidine incorporation

In the first series of experiments, various concentrations (2.5, 5, 10, 20 and 40 μg MAb/mL) of anti-GD3 (DMAb-8), anti-GM2 (DMAb-1) and IgM control (MOPC104E) antibodies were applied to the cultures and thymidine incorporation was determined (Figure 2). The anti-GD3 antibodies, but neither the anti-GM2 nor the MOPC104E antibodies, induced a marked reduction of thymidine incorporation. Although the dose-dependent inhibitory effect started at different anti-GD3 concentrations for various cell lines, it was found that at a concentration of 20 μg MAb/mL the thymidine incorporation in all four cell lines was down to 10–30% of the controls (Figure 2). The control is a mean value of the control samples of one cell line at each experiment, and is accounted for as 100% proliferation. All further experiments were thus performed with 20 μg of MAb/mL. As shown in Figure 3A–B, neither the anti-GM2 nor the MOPC antibody showed, in any experiment, an inhibitory effect on thymidine incorporation into any of the cell lines. The anti-GM2 antibody in fact increased the incorporation in one cell line, U138, but the same effect was seen with the control antibody. Anti-GD3 MAb, on the other hand, reduced the thymidine incorporation by $81 \pm 15.9\%$ in TP483 cells (Figure 3A), $91 \pm 17.7\%$ in D54MG cells (Figure 3A), $71 \pm 24.6\%$ in U118 cells (Figure 3B), $91 \pm 8.6\%$ in U138 cells (Figure 3B) and $56 \pm 20\%$ in SMKT-R2 cells (Figure 3B), as compared to the controls without any antibody. Different batches of the anti-GD3 antibody gave similar inhibitory effects. The glioma cell lines U118 and U138 and the renal tumor cell line SMKT-R2 were included to confirm a general effect of GD3 antibodies on thymidine incorporation into tumor cells and were not used in further experiments.

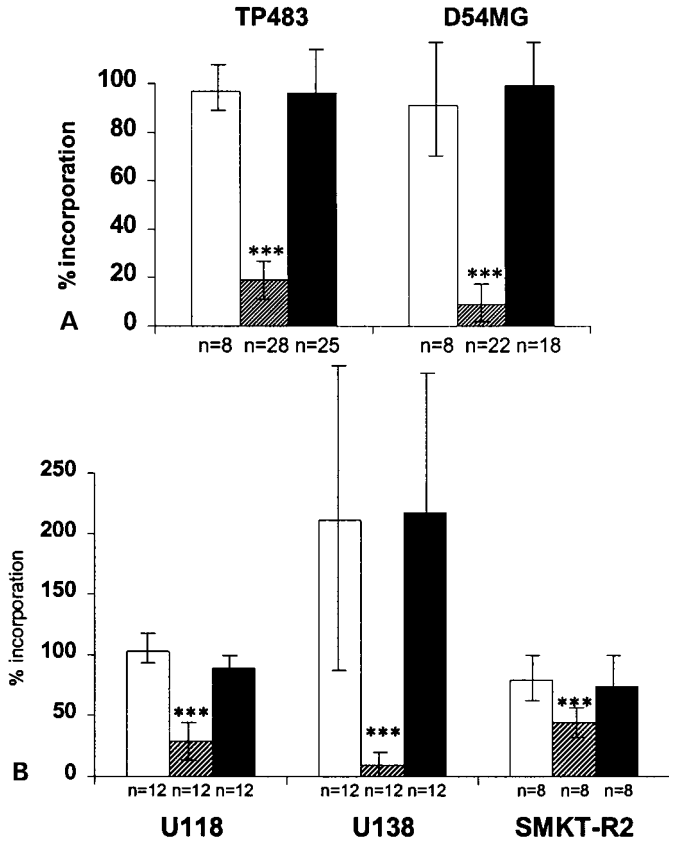

Figure 3. The effect of a fixed amount of monoclonal antibodies to GM2 and GD3 on ^{3}H-thymidine incorporation by TP483 and D54MG glioma cell lines (A) or U118, U138 and SMKT-R2 cell lines (B). Inhibition was calculated by dividing the value of the samples by the mean value of ^{3}H-thymidine incorporation in the control wells, i.e. without any antibody (four to five wells per assay, at two to six assays). The different antibodies, anti-GM2 (white pile), anti-GD3 (gray pile) and MOPC (black pile), were all used at a concentration of 20 μg/mL. The procedure is described in detail under Materials and Methods.

No light microscopically detectable difference in the morphology or significant cell death was observed. Each experiment was performed with four to five wells for each antibody. When the FCS used in the culture media was heat inactivated or replaced by synthetic serum, an identical inhibitory effect of anti-GD3 MAb on the TP483 and D54MG cell lines was maintained (Figure 4).

Proliferation assayed with the Brd-U kit

A significant effect on the cell proliferation of the anti-GD3 antibody was seen on the glioma cells, TP483 and D54MG (Figure 5). The effect corresponded well to that found with thymidine incorporation.

Cell counts

Cells were counted independently by two people (two 'A' squares each of every sample, n = 8) in a Bürker chamber. The number of cells were counted as a mean value of the squares of

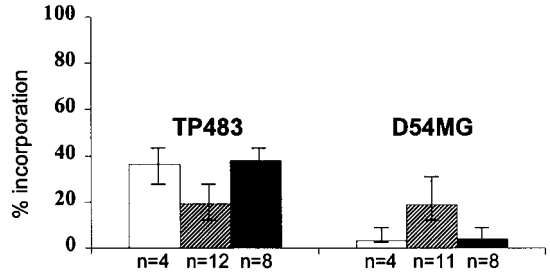

Figure 4. Influence of culture media on the effects of anti-GD3 monoclonal antibodies on ^{3}H-thymidine incorporation by TP483 and D54MG glioma cell-lines. Inhibition was calculated by dividing the value of the samples by the mean value of ^{3}H-thymidine incorporation in the control wells, i.e. without any antibody (three to four wells per assay, one to three assays). In this experiment, different culture medium supplements were tested, 2% synthetic serum (Ultroser) (white pile), 10% fetal calf serum (FCS) (gray pile) and heat inactivated FCS (H.I.) (black pile), and on the human glioma cell lines TP483 and D54MG. The concentration of anti-GD3 monoclonal antibody was 20 μg/mL. The procedure is described in detail under Materials and Methods.

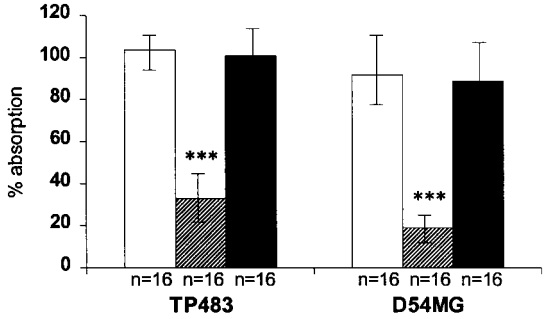

Figure 5. Proliferation analyses by proliferation kit Brd-U for 24 h incubation of TP483 and D54 glioma cells. Inhibition was calculated by dividing the value of the samples by the mean value of absorption of the control wells, i.e. without any antibody. The different antibodies, anti-GM2 (white pile), anti-GD3 (gray pile) and MOPC (black pile), were all used at a concentration of 20 μg/mL. The procedure is described in detail under Materials and Methods.

the individual reader's counts of the samples. No differences were seen between the number of cells cultured with the antibody (anti-GD3), mean value 7000 cells/cm^2 of D54MG and 11 300 cells/cm^2 of TP483, and those cultured without antibody present (control), mean value 8000 cells/cm^2 of D54MG and 10 300 cells/cm^2 of TP483. Nor was there any significant difference in the % of dead cells (D54MG, 5%; D54MG + anti-GD3, 7%; TP483, 4%; and TP483 + anti-GD3, 5%) (all averages of two separate counts).

Proliferation assay, WST-1

Neither of the two cell lines (TP483 and D54MG) showed a significantly decreased or increased proliferation in the presence of any of the antibodies, measured with this proliferation assay. Cell line D54MG showed a proliferation of 90% ± 29 (n = 89) with anti-GD3 present, 86% ± 25

(n = 115) with anti-GM2 present respectively 10l% ± 15 (n = 115) with MOPC104E present, as compared to controls (100% proliferation). The TP483 cell line gave similar results; 93% ± 20 (n = 103) with anti-GD3 present, 83% ± 22 (n = 127) with anti-GM2 present, and 85% ± 22 (n = 132) with MOPC104E present, compared to controls (100%). The results were the same even if thymidine was present during the incubation. When the cells were cultured for 48 and 72 h a slightly increasing effect of the antibody can be seen (Figure 6).

Statistical analyses

All statistical calculations were done with the Student's t-test, two-sided distribution and two sample unequal variance (*** = $p < 0.005$).

Discussion

This study has shown for the first time that anti-GD3, but not anti-GM2 monoclonal antibodies may directly inhibit proliferation of human glioma cells, despite the higher extracellular expression of ganglioside GM2. In addition, the GD3 concentration was relatively low; in no case >0.1 nmol NeuAc/mg protein. This supports the hypothesis that different gangliosides, GD3 and GM2, possibly play different roles in the tumor cells, and among those gangliosides only GD3 seemed to be associated with cell proliferation.

The aim of the study was to investigate whether anti-ganglioside antibodies, in particular anti-GD3, had any effect on human glioma cell proliferation and/or cell viability as such knowledge supports further exploration of the possibility to utilize anti-ganglioside antibodies in human glioma treatment strategies. Accessibility and local concentration of antigens are of great importance for immunotherapy strategies.

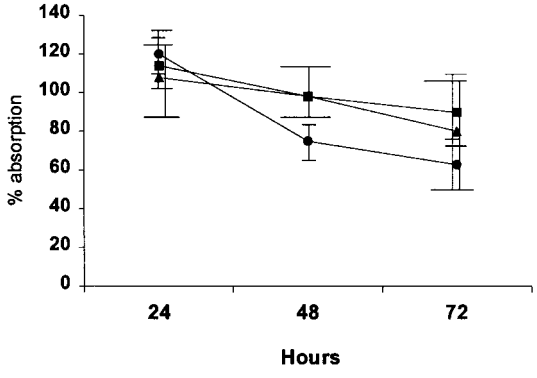

Figure 6. Proliferation analyses by proliferation kit WST-1 for 24, 48 and 72 h incubations of D54 glioma cells. The cells were cultured and treated as described under Materials and Methods. Inhibition was calculated by dividing the value of the samples (n = 24) by the mean value of absorption of the control wells, i.e. without any antibody. Anti-GD3 antibody (●), anti-GM2 antibody (■) and the MOPC antibody (▲).

Immunostaining with the anti-GD3 MAb revealed a predominant intracellular localization, however a low but consistent level of membrane staining was apparent, acting as target for the antibody inducing the anti-proliferative effect. As a comparison, the effect of cholera toxin, which specifically binds to ganglioside GM1, is relevant. A biological effect is well known despite the observation that the amount of GM1 in target tissue, the mucosa of the intestinal cells, is only approximately 0.007 nmol GM1/mg protein [50].

The anti-GM2 antibody had no effect on thymidine incorporation, cell proliferation or cell death in any of the cell lines, but one of these cell lines, D54MG, with the highest concentration of GM2, has previously been shown to be affected by anti-GM2 antibodies. In that study, however, the cells were grown as spheroids and the antibody was not shown to affect proliferation but readily induced central necrosis [8]. The lack of effect cannot be ascribed to the accessibility of the target antigen; GM2 was demonstrated on the cell surface by immunohistochemistry. However, cell density might be one factor, cells in the center of the spheroids do not proliferate and it was noted in this study that the amount of GM2 was higher in confluent cells compared to non-confluent cells. Earlier studies have also indicated a correlation between confluency-dependent growth arrest and changes of ganglioside expression [51,52]. The role of GM2 might be associated with non-proliferating and densely packed cells, thus there was no effect observed in the proliferating cells studied.

The marked reduction in proliferation was shown with two commonly used assays, thymidine incorporation and Brd-U labeling. However, a third assay, the Wst-1 assay, did not reveal any effect of any of the antibodies unless the incubation period was prolonged from 24 to 72 h, thereafter a similar tendency was noticed. This result shows the importance of selecting more than one assay and that the sensitivity of Wst-1 assay is lower than that for the other two assays, and is not applicable in this type of experiment.

It is beyond the scope of this study to identify the mechanisms behind the anti-proliferative effect. However, the effect was also reproduced in serum-free medium and in heat-inactivated serum containing medium, excluding complement or other serum factors as mediators of the effect. There was no significant cell death, and the antibodies did not induce any notable detachment of cells. All three investigated antibodies were of the IgM type; therefore the effect of antibody subclass could not be addressed. These results indicate a selective effect on proliferation of the cell lines when treated with anti-GD3 antibody. It has been proposed before, as mentioned in the introduction, and also in a resent paper [53] that GD3 is involved in cell growth. We have now shown that antibody directed against GD3 inhibits proliferation of four glioma cell lines. There was also an effect on the proliferation of a renal tumor cell line, but to a lesser degree, which might be related to the much lower content of GD3 in the cells.

The *de novo* synthesis of gangliosides involves a series of enzymatic steps starting in the ER followed by glycosylation

in the Golgi. The gangliosides are then transported to the cell surface from where they are transported to the lysosome for complete degradation or partially degraded and recycled back to the Golgi for re-glycosylation (the Salvage pathway) [54]. During intracellular transport, the gangliosides are localized to vesicles. Thus, the antibody might react with the GD3 when expressed on the cell surface and there, or later during intracellular processes, induce its effect. A rapid turnover of GD3 might therefore explain why the anti-GD3 antibody had a significant effect despite the low concentration of GD3, but the metabolic labeling of the gangliosides did not however show any increased synthesis of GD3 compared to GM2. It should also be noted that the expression of GD3 in human glioma cells *in vivo* has been found to be much higher than in cells in culture [6,55]. Biochemical analyses have shown that the normal brain tissue contains ganglioside GD3 but in markedly lower concentration [25,26]. It is not yet known if the GD3 in normal tissue is expressed on the surface or not. For *in vivo* targeting one might have to consider lower concentrations of antibodies to selectively affect the tumor cells.

It should be noted that glycosphingolipids have engendered increasing interest as being enriched in caveolae-like domains, also named DIGs ([18], for short review [56]). Such domains contain, in addition to high glycosphingolipid concentrations, receptor proteins and related signaling kinases. Kasahara et al. [18] showed that anti-GD3 antibodies induced tyrosine phosphorylation in $p53/56^{lyn}$, which co-immunoprecipitates with GD3 in cerebellar cells. Hakomori et al. [19] postulated that the glycosphingolipids in these caveolae-like domains are involved in the binding of ligand to receptor protein or in the signaling transmission from the receptor protein into the cell. One might thus speculate that the effect observed in this study induced by anti-GD3 antibody might be localized to caveolae, thus requiring a smaller number, but focal density of surface GD3.

In summary, this study has shown a significant inhibition of tumor growth by anti-GD3 antibodies on tumor cells grown in *in vitro* monolayer cell cultures, which encourages further studies not only regarding how GD3 is involved but also to explore such antibodies in treatment of human gliomas.

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