Glycoconjugate Journal (1996) **13:** 385–389

Structural characterization and *in vivo* immunosuppressive activity of neuroblastoma G_{D2}

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Received 28 July 1995, revised 13 October 1995

Shedding of neuroblastoma gangliosides is positively correlated with tumour progression in patients with neuroblastoma. In assessing the biological activity of these ganglioside molecules, we recently found that total human neuroblastoma gangliosides inhibit cellular immune responses. Here, we have studied the major neuroblastoma ganglioside, G_{D2}. G_{D2} was purified by high performance liquid chromatography and structurally characterized by mass spectrometry. Immunoregulatory effects of G_{D2} *in vivo* were then determined in an established murine model. G_{D2} significantly downregulated the local cellular immune response to an allogeneic cell challenge; the usual increase in mass of the lymph node draining the injection site was reduced by 88%, from 1.52 to 0.19 mg (control versus G_{D2}-treated mice; $p < 0.01$). In parallel, lymphocyte recovery from each node was also reduced from 2.4 to 1.2×10^6 cells, and lymphocyte DNA synthesis was reduced to half of the control level. These results show that certain shed tumour gangliosides, such as G_{D2}, function as intercellular signalling molecules, downregulate the cellular immune response, and may thereby enhance tumour formation and progression.

Keywords: ganglioside G_{D2}, immunosuppression *in vivo*, mass spectrometry

Introduction

Tumour formation and progression are two complex processes which remain poorly understood. It is clear, however, that the interaction between tumour cells and the host is critical to the tumour formation [1]. For example, host immunological reactions against a tumour enhance the immunological destruction of the tumour by the host. However, the existence and progression of tumours *in vivo* speak against the effectiveness of this process, even though this phenomenon, the destruction of tumour cells by host leukocytes, is easily demonstrable *in vitro*. Significant efforts made to understand the lack of effectiveness of the cellular immune response in eliminating tumour cells *in vivo* have been largely unsuccessful. One possibility, and the hypothesis that underlies our study, is that ganglioside molecules shed from the surface of tumour cells strikingly modulate (downregulate) host antitumour immune responses [2, 3].

We have focused our study on human neuroblastoma gangliosides, and have shown that rapid progression of a tumour and a lower survival rate are related to higher circulating (shed) G_{D2} levels measured at the time of diagnosis [4]. This potential relationship between ganglioside shedding and tumour progression is supported by studies on immunoregulatory effects of gangliosides. Tumour gangliosides isolated from different tumour sources, such as neuroblastoma [5, 6], lymphoma [2], leukaemia [7] and melanoma [8], all strikingly inhibit cellular immune responses *in vitro*. Gangliosides isolated from normal tissues have also been shown to have a variety of immunoregulatory functions [9–15].

However, an important gap in this work is the demonstration of inhibition of the cellular immune response *in vivo*. This problem has been addressed by the development of an animal model in which the

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inhibitory effect of a small quantity of tumour ganglioside molecules can be tested. Using this animal model, we recently found that total gangliosides isolated from human neuroblastoma cells inhibit the cellular immune response *in vivo*. With this finding, we have now highly purified a major ganglioside of human neuroblastoma, G_{D2}, and have determined its immunoregulatory effects. This study demonstrates that neuroblastoma ganglioside G_{D2} significantly downregulates the murine allogeneic cellular immune response *in vivo*.

Materials and methods

LAN-5 neuroblastoma cell culture

LAN-5 human neuroblastoma cells were grown in adherent monolayer cultures in 75 cm^2 flasks in Waymouth's MB 752/1 medium supplemented with 2 mM L-glutamine (Gibco Laboratories, USA) and 10% heat-inactivated fetal bovine serum (Hyclone Laboratories, USA), and cultured in a humidified 5% CO_2:95% air atmosphere. The cells were harvested by trypsin treatment. The cell suspension was centrifuged at $300 \times \mathbf{g}$ for 10 min, and the cell pellet was washed once with phosphate buffered saline and then was frozen at –70 °C until being processed for ganglioside isolation [16, 17].

Ganglioside purification, quantification and high performance thin-layer chromatography (HPTLC)

The total lipid extract of the cells was obtained by chloroform:methanol extraction. Gangliosides were purified by diisopropyl ether/1-butanol partition [18] followed by Sephadex G-50 gel filtration. The purified gangliosides were quantified by the modified resorcinol method [19] as nmol lipid-bound sialic acid.

Isolation of G_{D2} ganglioside by high performance liquid chromatography

G_{D2} {*N*-acetylgalactosaminylβ(1-4)[sialylα(2-8) sialyl(α2-3)]galactosylβ(1-4)glucosylceramide} was isolated by normal phase HPLC from total LAN-5 cellular gangliosides [20]. Briefly, total gangliosides (800 nmol lipid-bound sialic acid) were dissolved in 100 μl water and chromatographed using the Perkin-Elmer HPLC system, on a LiChrosorb-NH2 column (250 mm length, 4 mm ID, Merck, Germany). Solvent A was acetonitrile-5 mM Sorensen's phosphate buffer (83:17), pH 5.6, and solvent B was acetonitrile-20 mM Sorensen's phosphate buffer (1:1), pH 5.6. The following gradient elution programme at a flow rate of 6.25 ml min^{-1} was used: a linear gradient from 100% solvent A to solvent A:solvent B (66:34) over 58 min, and then a linear gradient to solvent A:solvent B (36:64) over 20 min. The elution profile was monitored by flow-through detection at 215 nm.

Structural characterization of gangliosides by mass spectrometry

The carbohydrate structure of ganglioside G_{D2} was characterized by negative-ion fast atom bombardment mass spectrometry, without prior derivatization [21]. Approximatley 1 μl of ganglioside solution in methanol (0.1–2.0 nmol μl^{-1}) was mixed with 2 μl of triethanolamine (matrix) on the fast atom bombardment probe tip. Ions were formed by bombardment with a 6 keV beam of xenon atoms in a JEOL HX-110 double focusing mass spectrometer. The accelerating voltage was 10 kV and the resolution was 3000.

Assessment of murine allogeneic immune responses in vivo

The inhibitory effect of ganglioside G_{D2} on the allogeneic immune response *in vivo* was assessed in a murine model [22]. In this model, mice are challenged by subcutaneous injection of allogeneic cells in the footpad, and the immune response in the lymph node draining the injection site is quantified 4 days later. Normally a brisk cellular immune response is noted at this point, observed as increases in popliteal lymph node mass, lymphocyte number, and specific proliferative response. C3H ($H\text{-}2^K$) and BALB/c ($H\text{-}2^d$), murine virus-free strains (Charles River, Wilmington, USA), at 7–12 weeks of age were used in the study. BALB/c splenocytes (stimulator cells) were γ-irradiated for 8 min with 2880 rads (137 Cesium, Nordion Int. Inc., Canada) and immediately injected subcutaneously into the left hind footpad of C3H mice (2.5×10^6 per 30 μl) as previously described [23]. When ganglioside G_{D2} was injected together with these allogeneic cells, G_{D2} was first resuspended in 0.9% NaCl and then mixed with the cell suspension. A positive control in these studies was the use of cyclosporin A (Sandoz Pharmaceutical Corp., USA), a known immunosuppressive agent. The cyclosporin A-treated mice received allogeneic cells in the footpad and 4 daily intraperitoneal (i.p.) injections (24 mg kg^{-1} day^{-1}) of cyclosporin A. Primed C3H mice were killed by cervical dislocation on day 4 and the popliteal lymph nodes draining the left and right footpads were aseptically removed, weighed, and processed to obtain a single cell suspension. After being washed in RPMI-1640 medium (Biowhittaker, USA) containing 0.1% 2-mercaptoethanol, the cells were counted and their viability determined by trypan blue exclusion.

To measure the proliferative response of these lymph node cells caused by their *in vivo* exposure to the allogeneic cells, 2×10^5 lymph node cells in 100 μl were cultured for 18 h in complete medium with 0.5 μCi [^{3}H]thymidine immediately after their recovery as from the lymph node. At the end of the 18 h, the cells were harvested and [^{3}H]thymidine uptake quantified by β-scintillation counting [12].

Statistical analysis

The difference between a control group and treatment group was determined at the significance level of $p = 0.05$, using Student's t test (one-tailed, unpaired).

Results and discussion

Structural characterization of G_{D2}

G_{D2} is the major ganglioside of LAN-5 human neuroblastoma cells (56%). Other major gangliosides are G_{M2} (15%) and G_{T1b} (11%). Lesser quantities of G_{D3}, G_{D1a}, and some other minor components are also present [16]. By normal phase HPLC, these total LAN-5 neuroblastoma gangliosides were separated into three major peaks, with retention times of 14.28, 36.03, and 59.10 min (Fig. 1). By HPTLC analysis, these three peaks were identified to correspond to G_{M2}, G_{D2}, and G_{T1b}, respectively (Fig. 2).

The carbohydrate structure of the ganglioside with retention time of 36.03 min was characterized by mass spectrometry. Figure 3 shows the negative-ion fast atom bombardment mass spectrum of this ganglioside. In the region near m/z at 1755.4, there are multiple peaks, corresponding to the molecular ions $[M–H]^-$ of G_{D2}. In addition to these molecular ions, there are four sets of peaks in the mass spectrum, which resulted from the loss of *N*-acetylneuraminic acid (NeuAc), a second NeuAc, *N*-acetylgalactosamine (GalNAc), and glucose. For example, the molecular ion $[M–H]^-$ at m/z 1755.4 (one molecular species of G_{D2}) yielded the peak at m/z 1464.5 upon losing one *N*-acetylneuraminic acid group, as well as the peak at m/z 1173.6 from the loss of two *N*-acetylneuraminic acid groups. The loss of two *N*-acetylneuraminic acid residues and a *N*-acetylgalactosamine group gave the peak at m/z 970. It is likely that the

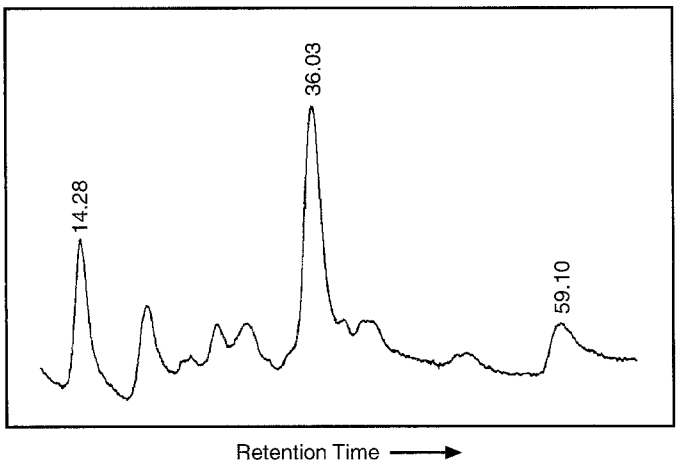

Figure 1. Normal phase HPLC separation of LAN-5 neuroblastoma gangliosides. Three major peaks with retention times of 14.28, 36.03 and 59.10 min (corresponding to G_{M2}, G_{D2} and G_{T1b}, respectively) were separated.

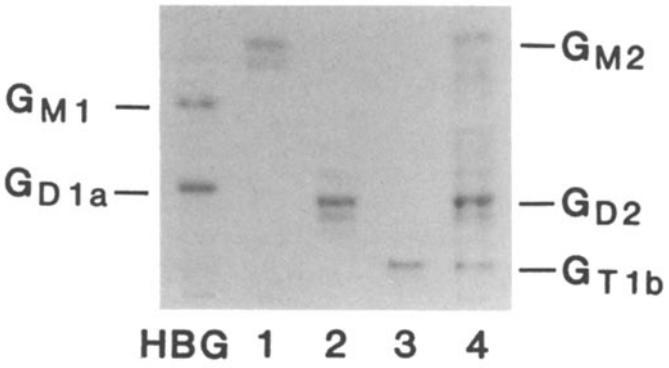

Figure 2. HPTLC analysis of human neuroblastoma gangliosides. Lane 1, 2, 3 correspond to the fractions 14.28 min (G_{M2}), 36.03 min (G_{D2}) and 59.10 min (G_{T1b}) isolated by normal phase HPLC. Lane 4, total gangliosides of LAN-5 neuroblastoma cells. HBG, human brain gangliosides G_{M1} and G_{D1a}. Gangliosides were stained with resorcinol-HCl.

elimination of a galactose group, together with two *N*-acetylneuraminic acid groups and one *N*-acetylgalactosamine group yielded an ion at m/z 808.3. The corresponding $[CerO]^-$ ion was also detected at m/z 646 in the mass spectrum, although it was of low abundance. Similar results were obtained for other G_{D2} molecular ions. G_{D2} was also characterized by enzymatic analysis. When G_{D2} was treated with neuraminidase at pH 5.0 (in the absence of detergent), it formed G_{M2} upon the removal of a sialic acid group, as identified by HPTLC (not shown). The results are consistent with a carbohydrate structure of G_{D2}:GalNAc-(NeuAc-NeuAc-)Gal-Glc-Cer.

The multiple peaks in the molecular ion region suggest heterogeneity of the ceramide structure of G_{D2}. This has been confirmed by the isolation of the ceramide subspecies of G_{D2} of human neuroblastoma LAN-5 cells by reversed-phase HPLC. The major ceramide structures were characterized by negative-ion fast atom bombardment collisionally activated dissociation tandem mass spectrometry [21] as d18:1-C16:0, d18:1-C18:0, d18:1-C22:0/d18:1-C24:1 and d18:1-C24:0 [6]. They all contain sphingosine as the long chain base while the fatty acyl chain varies from C16 to C24.

Inhibitory effects of G_{D2} on murine immune responses in vivo

To determine the inhibitory effects of G_{D2} on cellular immune responses *in vivo*, we used the murine model developed by Kroczek *et al.* [22]. This model measures the class II-restricted allogeneic immune response in the popliteal lymph node draining the injection site of allogeneic cells. Four days after subcutaneous injection of γ-irradiated allogeneic splenocytes (BALB/c) in the left hind footpad of C3H mice (2.5×10^6 per 30 μl), the popliteal lymph nodes draining the left (stimulated) and

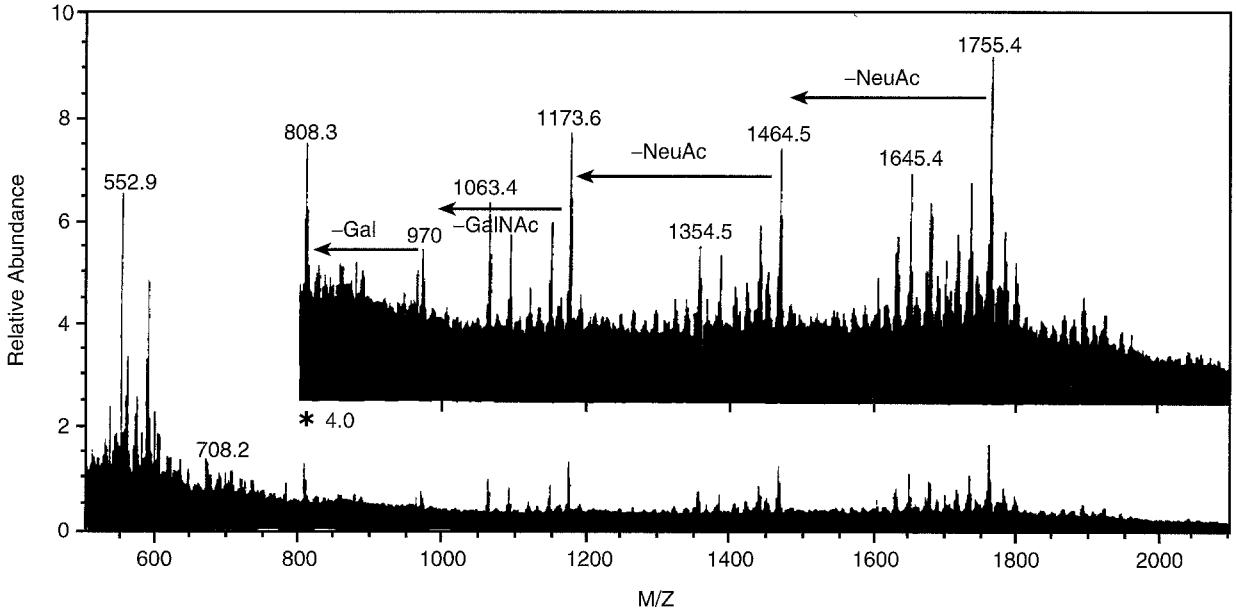

Figure 3. The negative-ion fast atom bombardment mass spectrum of ganglioside G_{D2}. The peak at *m/z* 1755.4 represents one of the multiple molecular ions $[M-H]^-$ of ganglioside G_{D2}. Four additional fragmental ions for this molecular species were observed in this mass spectrum, which resulted from the loss of -NeuAc, -NeuAc, -GalNAc, -Gal. The ceramide fragment ion corresponding to this molecular species was also detected at *m/z* 646, although it was of low abundance.

the right footpads (unstimulated) were removed, the lymph node mass was measured, and the increase in lymph node mass, lymphocyte number and lymphocyte DNA synthesis were assessed. The injection of allogeneic cells into the footpad of mice normally causes a two-fold increase of popliteal lymph node mass when compared with the lymph node mass of naive (untreated) mice (e.g. from 1.34 to 2.99 mg), and a six-fold increase in both lymphocyte number (from 6.4×10^5 to 41.8×10^5 cells per node) and [^{3}H]thymidine uptake (from 217 to 1376 cpm per 2×10^5 cells), as shown in our previous study [23]. Human neuroblastoma ganglioside G_{D2} coinjected with the stimulating allogeneic cells significantly suppressed this *in vivo* immune response (Table 1): the increase in the lymph node mass was reduced by 88% (0.19 versus 1.52 mg for the control, $p < 0.01$). Likewise, the increase in lymphocyte number was inhibited by 50% (1.2×10^6 versus 2.4×10^6 cells per node in the control). The *in vitro* spontaneous proliferation of the lymphocytes recovered *in vivo*, measured as [^{3}H]thymidine uptake, was also reduced, from 2496 to 1353 cpm per 2×10^5 cells. Ten nmol G_{D2} has a similar immunosuppressive activity to that of the total neuroblastoma gangliosides [23]. This inhibitory effect of G_{D2} on the murine allogeneic immune response *in vivo* is quite significant, since it is of a similar order of magnitude as that of systematically administered cyclosporin A (24 mg kg^{-1} day^{-1} i.p. $\times$ 4 days), which is a well-known immunosuppressive molecule [24] and exten-

Table 1. Neuroblastoma G_{D2} inhibits murine allogeneic cellular immune responses.

Parameter	*Control*	*Cyclosporin A*	G_{D2}
Lymph node mass, mg[a]			
Unstimulated	0.90 ± 0.06	1.13 ± 0.10	1.23 ± 0.17
Stimulated	2.42 ± 0.37	1.30 ± 0.06	1.42 ± 0.18
Net increase	1.52 ± 0.41	0.16 ± 0.07	0.19 ± 0.16
Lymphocytes $\times 10^{-6}$ per node	2.4	0.3	1.2
[^{3}H]thymidine uptake, cpm[b]	2496 ± 70	120 ± 3	1353 ± 83

[a] γ-irradiated allogeneic splenocyte (BALB/c, 2.5×10^6 per 30 μl) were injected subcutaneously into the left hind footpad of C3H mice without (control) or with G_{D2} (10 nmol). Cyclosporin A was systematically administered i.p. (24 mg kg^{-1} day^{-1} $\times$ 4 days). On day 4, the popliteal lymph nodes draining the left (stimulated) and the right footpads (unstimulated) were removed, and the lymph node mass was measured. The data represent the mean ± SEM of five lymph nodes in each group. The difference in the net increase of popliteal nodes between control and G_{D2}-treated groups is statistically significant ($p < 0.01$).

[b] The spontaneous lymphoproliferation was measured by cellular [^{3}H]thymidine uptake at the cell density of 2×10^5 cells per well. The data represent the mean ± SEM of three cultures.

sively used clinically. These findings show that the major neuroblastoma ganglioside, G_{D2}, in a highly purified form, significantly inhibits the cellular immune response *in vivo*. This is the first demonstration of such an effect by a highly purified tumour ganglioside.

Gangliosides, G_{M3} and G_{M4}, isolated from normal human brain tissue also inhibit the murine allogeneic immune response (data not shown). In contrast, liposomes of cholesterol and lecithin (molar ratio 1:1) had no inhibitory effect on either lymph node mass or lymph node lymphocyte number [23], indicating that the observed immunosuppressive activity is specific for gangliosides, a conclusion which is consistent with the previous finding that G_{M3} ganglioside delays allograft (rat heart) rejection [25].

Ganglioside G_{D2} is a well-recognized marker for human neuroblastoma [26] to which several monoclonal antibodies have been generated [27–29]. G_{D2} is highly expressed in human neuroblastoma [30], and the significant shedding of G_{D2} by human neuroblastoma cells *in vitro* [16] as well as *in vivo* [31] has been documented. G_{D2} is easily chemically detectable at sometimes very high concentrations in the circulation of patients with neuroblastoma [32], where it is exclusively associated with serum lipoproteins [33]. Rapid progression of a tumour and a lower survival rate are related to higher circulating tumour-derived G_{D2} levels measured at the time of diagnosis [4], suggesting biological as well as marker properties of the molecules. The potent immunosuppressive properties of tumour gangliosides lead to the general hypothesis that gangliosides shed by tumour cells suppress the host antitumour immune response, in the microenvironment where host-tumour interactions occur.

The present study supports this hypothesis and extends our previous findings [23], by using the purified human neuroblastoma ganglioside G_{D2} to demonstrate that a highly purified tumour ganglioside downregulates the murine allogeneic immune response *in vivo*. This work lays the foundation for future studies to elucidate the mechanism by which tumour gangliosides have these inhibitory effects, and to develop strategies to eliminate these immunosuppressive ganglioside molecules or their effects *in vivo*. A tempting strategy is the potential modulation of ganglioside synthesis and shedding by a pharmacological agent, which would have the ultimate goal of achieving improved therapeutic approaches to neuroblastoma and other neuroectodermal tumours such as melanoma.

Acknowledgement

This work was supported in part by grants CA42361 and CA61010 from the National Cancer Institute, and by grant RR00480 from the NCRR, NIH, supporting the MSU-NIH mass spectrometry facility.

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