Ceramide structure predicts tumor ganglioside immunosuppressive activity

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ABSTRACT Molecular determinants of biological activity of gangliosides are generally believed to be carbohydrate in nature. However, our studies of immunomodulation by highly purified naturally occurring tumor gangliosides provide another perspective: while the immunosuppressive activity of gangliosides requires the intact molecule (both carbohydrate and ceramide moieties), ceramide structure strikingly influences ganglioside immunosuppressive activity. Molecular species of human neuroblastoma G_{D2} ganglioside in which the ceramide contains a shorter fatty acyl chain (C16:0, C18:0) were 6- to 10-fold more active than those with a longer fatty acyl chain (C22:0/C24:1, C24:0). These findings were confirmed in studies of ceramide species of human leukemia sialosylparagloboside and murine lymphoma GalNAcG$_{M1b}$. Gangliosides that contain shorter-chain fatty acids (and are most immunosuppressive) are known to be preferentially shed by tumor cells. Therefore, the results suggest that the tumor cell is optimized to protect itself from host immune destruction by selective shedding of highly active ceramide species of gangliosides.

Gangliosides, a class of biologically active cell surface molecules, are expressed in particularly high concentrations on the plasma membranes of tumor cells, from which they are actively shed (1–3) into the cellular microenvironment (4–7). Their chemical structure consists of a carbohydrate portion (one or more sialic acids linked to an oligosaccharide) attached to a lipid composed of a long-chain base and a fatty acid (ceramide). The known properties of gangliosides include antigenic specificity (8), immunosuppressive activity (9), and roles in cell recognition (10), adhesion (11), and signal transduction (12). The importance of the ceramide portion, which anchors the ganglioside molecule in the cell membrane, is suggested by recent studies showing that some glycosphingolipid metabolic products, such as lysogangliosides (13–15), sphingosine (15–17), and ceramide (18), may modulate intracellular signal transduction as second messengers.

Here we show that the molecular structure of the ceramide portion of naturally occurring gangliosides has a previously unrecognized functional importance. A simple difference in ceramide structure of the intact ganglioside—the length of the fatty acyl chain—dramatically influences ganglioside immunosuppressive activity. Gangliosides with shorter fatty acyl chains are far more potent in inhibiting the in vitro human lymphoproliferative response to a soluble antigen than are those containing longer fatty acyl chains. Importantly, these ganglioside species having shorter fatty acid chains (and thereby more potent immunosuppressive activity) are exactly the same species that are preferentially shed into the tumor microenvironment by tumor cells (19, 20).

MATERIALS AND METHODS

Ganglioside Purification. Total gangliosides were purified from normal human brain and from human and murine tumor cells by a sequence of steps including extraction of the cells with chloroform/methanol, 1:1 (vol/vol), partition of the total lipid extract in diisopropyl ether/1-butanol (21), and Sephadex G-50 gel filtration of the ganglioside-containing aqueous phase.

HPLC. Individual gangliosides were separated and purified by HPLC methods of Gazzotti et al. (22, 23). Briefly, 600–800 nmol of lipid-bound sialic acid of total gangliosides was dissolved in 100 μl of water and chromatographed using the Perkin-Elmer HPLC system, on a LiChrosorb-NH2 column (length, 250 mm; i.d., 4 mm, Merck). The solvent system for normal-phase HPLC includes acetonitrile/5 mM Sorensen's phosphate buffer [83:17 (vol/vol), pH 5.6; solvent A] and acetonitrile/20 mM Sorensen's phosphate buffer (1:1, pH 5.6; solvent B). The gradient elution program was as follows: 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, at a flow rate of 1 ml/min. Reversed-phase HPLC chromatographic separation of total G_{D2} {N-acetylgalactosaminyl(β1-4)[N-acetylneuraminosyl-(α2-8)N-acetylneuraminosyl(α2-3)]galactosyl(β1-4)glucosyl-ceramide} ganglioside (10 nmol in 25 μl of water) was carried out using a LiChrosorb RP-8 column (length, 250 mm; i.d., 4 mm; Merck). The solvent system was acetonitrile/5 mM sodium phosphate, pH 7.0, maintained at 55:45 for 10 min, increased linearly to 60:40 over 20 min, and then increased linearly to 65:35 over the next 20 min. The flow rate was 0.52 ml/min. The elution profile was monitored by flow-through detection at 215 nm for normal-phase HPLC and at 195 nm for reversed-phase HPLC. The ceramide species of sialosylparagloboside (SPG) and GalNAcG$_{M1b}$ {N-acetylgalactosaminyl-(β1-4)[N-acetylneuraminosyl(α2-3)]galactosyl(β1-3)N-acetylgalactosaminyl(β1-4)galactosyl(β1-4)glucosylceramide} were separated and purified in the same manner (24).

Ganglioside Quantification, Structural Characterization, and Radiolabeling. Gangliosides were quantified by resorcinol assay (25) and analyzed by high performance thin-layer chromatography (HPTLC). The developing solvent system was chloroform/methanol/0.2% CaCl2·2H2O, 60:40:9 (vol/vol), and the gangliosides were visualized by resorcinol staining. Ganglioside structures were confirmed by negative-ion fast atom bombardment mass spectrometry and collisionally activated dissociation tandem mass spectrometry. For binding studies (9), the individual molecular species of G_{D2} ganglioside were radiolabeled with tritiated borohydride (26) and purified.

Lymphocyte Proliferation Assay. An assay of antigen-induced human lymphoproliferation has been used to measure the immunosuppressive effects of purified gangliosides (9). Normal human peripheral blood mononuclear leukocytes (PBMCs) were isolated by Ficoll/Hypaque density gradient centrifugation (27) from whole blood collected in preservative-free heparin (50 units/ml). The cells were washed three times and resuspended in complete HB104 medium. Autologous human plasma was added to a final concentration of 0.5%. Normal human PBMCs were cultured in 96-well (A/2) tissue culture clusters (Costar 3696).

Gangliosides purified by HPLC were suspended in medium by brief sonication before addition to the cell cultures. Ganglioside solution was added at 10 μl per well, followed by addition of the PBMCs (25 μl, 2 × 10^6 cells per ml of complete medium). After a 3-h preincubation at 37°C, 10 μl of the previously determined optimal concentration of the stimulant of lymphoproliferation, tetanus toxoid [3.5 Lf (limes flocculating) doses/ml, Massachusetts Department of Health, Boston] was added. Basal medium (10 μl per well) alone was added to unstimulated control cultures. The complete cultures were incubated at 37°C in 95% air/5% CO_2 for 6 days (9). As has been documented under these conditions (9), purified gangliosides are not toxic to the cells. At the end of the culture period, 0.5 μCi of [^{3}H]thymidine in 50 μl of medium was added to each well (1 Ci = 37 GBq). The cultures were incubated for an additional 4.5 h and harvested onto glass fiber filters. Cellular [^{3}H]thymidine uptake was quantitated by β-scintillation counting. Mean net [^{3}H]thymidine uptake in stimulated cultures was determined by subtracting the mean cpm of unstimulated cultures. Percent inhibition was calculated by comparing the mean net [^{3}H]thymidine uptake of cultures containing gangliosides with that of cultures without gangliosides.

RESULTS AND DISCUSSION

Relative Immunosuppressive Activity of Tumor-Derived and Normal Brain Gangliosides. Comparison of a series of highly purified normal human brain gangliosides showed that those with a terminal sialic acid linked to a compact neutral oligosaccharide had the highest immunosuppressive activity (9). Because of a particular interest in tumor gangliosides, we also studied the analogous tumor-derived ganglioside molecules. We assumed that any specific ganglioside, whether isolated from a human tumor or from normal human brain, should have the same immunosuppressive activity. Surprisingly, we found that naturally occurring human and other vertebrate tumor gangliosides are frequently more immunosuppressive than are the corresponding normal human brain gangliosides. For example, G_{D2} isolated from a human neuroblastoma was significantly more active than G_{D2} from normal human brain tissue (Fig. 1). Since the tumor-derived G_{D2} and the brain-derived G_{D2}, which were characterized by HPTLC, mass spectrometry, and immunostaining with an anti-G_{D2} monoclonal antibody, had identical carbohydrate structures, the explanation for this difference was not intuitively obvious. The only possible difference between these two gangliosides on a molecular basis was in their ceramide structure. This led us to postulate that ceramide structure, a relatively unstudied portion of the ganglioside molecule, is critical in determining ganglioside biological activity.

Ceramide Structural Diversity of Human Neuroblastoma Gangliosides. To determine how ceramide structure affects immunosuppressive activity of gangliosides, we first isolated G_{D2} from LAN-5 human neuroblastoma cells (Fig. 2). By using recently developed techniques (28), we isolated and purified G_{D2} to homogeneity with respect to both carbohydrate and ceramide structure. Although G_{D2} is generally considered a single molecular species, we found that actually
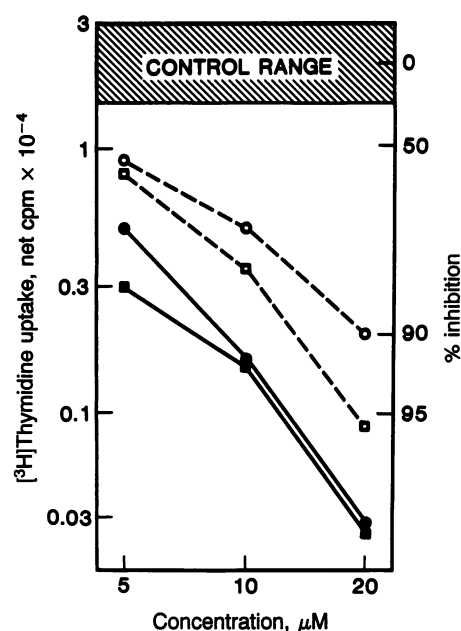

FIG. 1. Inhibition of the human cellular immune response by normal human brain and tumor-derived gangliosides *in vitro.* The effects of gangliosides G_{D3} (○) and G_{D2} (□) isolated from normal human brain are compared to those of gangliosides with identical carbohydrate structure, G_{D3} (●) and G_{D2} (■), isolated from human neuroblastoma tissue. An assay of antigen-induced human lymphoproliferation was used to measure the immunosuppressive effects of purified gangliosides (9). The range of the proliferative response of multiple control cultures, not exposed to gangliosides, is shown. The data represent the mean of three experiments; the SEM was <10% of the mean.

it is not: G_{D2} could be separated into four major and many minor peaks by reversed-phase HPLC, each peak representing a different molecular species (Fig. 3). Structural characterization by mass spectrometry revealed that every one of these species has a different ceramide structure linked to the same carbohydrate structure of G_{D2}. Within the ceramide, although the long-chain base structure was constant (sphingosine, d18:1), substantial differences occurred in the fatty acids that varied in the length of the fatty acyl chains from 16 to 24 carbon atoms. These results demonstrated a marked diversity of ceramide structure of tumor-derived G_{D2} ganglioside.

Immunosuppressive Activity of Neuroblastoma Ganglioside G_{D2} Molecular Species. The effect of the ceramide structure of subspecies of human neuroblastoma-derived G_{D2} on the cellular immune response [measured as modulation of the *in vitro* human lymphoproliferative response to a soluble antigen, tetanus toxoid (9)] was then determined. Strikingly,
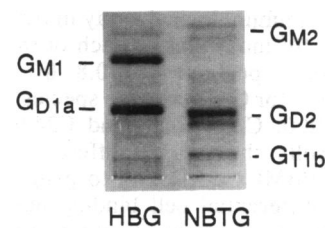

FIG. 2. HPTLC analysis of human neuroblastoma gangliosides (NBTG). Major gangliosides in normal human brain (HBG) revealed by HPTLC are G_{M1} and G_{D1a} as indicated. In the LAN-5 neuroblastoma cells, G_{D2} (56%), G_{M2} (15%), and G_{T1b} (11%) are the main ganglioside components. Note that in contrast to single bands formed by each brain ganglioside, each tumor ganglioside appears as a doublet.
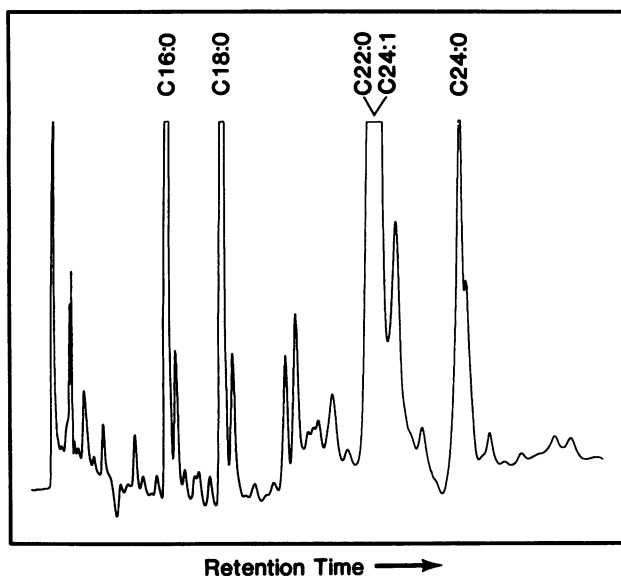
FIG. 3. HPLC separation of human neuroblastoma gangliosides. Ganglioside G_{D2} was isolated by normal-phase HPLC from the total neuroblastoma gangliosides shown in Fig. 2. This G_{D2} was further resolved into multiple individual ceramide species by reversed-phase HPLC (23). Four major peaks were isolated, and their ceramide structures were determined by negative-ion fast atom bombardment mass spectrometry and collisionally activated dissociation tandem mass spectrometry (29). All species are G_{D2} with the same carbohydrate structure and long-chain base (sphingosine, $d18:1$). They differed only in the fatty acid compositions, as shown in the figure. Their relative proportion, by HPLC estimation, was C16:0 (13%), C18:0 (12%), C22:0/C24:1 (41%), and C24:0 (14%).

these individual ceramide subspecies isolated from G_{D2} had markedly different immunosuppressive activity (Fig. 4). G_{D2} molecules containing fatty acids of 16 and 18 carbon atoms were >6 times more active than gangliosides with fatty acids of 22 and 24 carbon atoms. This very dramatic difference in effectiveness of immunosuppression can also be seen in the ID_{50}. The ID_{50} value for G_{D2} with the shortest fatty acid (16 carbons) was 0.4 μM or 10-fold lower than that of the G_{D2} molecule with the longest fatty acid (24 carbons, >5.0 μM). These overall results define an inverse relationship between the degree of activity and the length of the fatty acyl chains in G_{D2} ceramide subspecies and suggest that ganglioside immunosuppressive activity is enhanced when the fatty acyl chain is among the naturally occurring shorter fatty acid species.

We considered the possibility that striking differences in immunosuppressive activity related to ganglioside ceramide structure could be due to differential binding of these molecules to PBMCs. We therefore quantified the binding of 5 μM ^{3}H-labeled G_{D2} subspecies to PBMCs under the culture conditions of the immunological assay in three experiments. On the order of 10^7 molecules of each of the G_{D2} ceramide species were bound per cell (1.2, 0.8, 0.7, and 0.5 $\times$ 10^7 molecules per cell, for G_{D2} ceramide species containing fatty acids C16:0, C18:0, C22:0/24:1, and C24:0, respectively). Thus, these results show some differences in binding of gangliosides to PBMCs according to ganglioside ceramide structure. This differential cell binding may contribute to differences in immunosuppressive activity but cannot explain the observed 6- to 10-fold differences in activity.

Immunosuppressive Activity of Human Leukemia and Murine Lymphoma Gangliosides. Additional experiments were performed to ascertain whether the clear relationship we defined among G_{D2} species reflects a general phenomenon. That is, does ceramide structure itself significantly influence ganglioside immunosuppressive activity independent of the
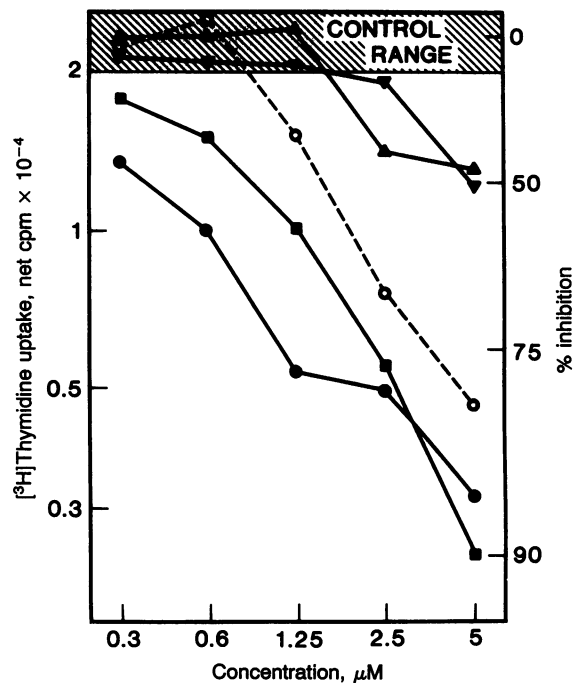

FIG. 4. Ceramide structure predicts immunosuppressive activity of human neuroblastoma G_{D2} ganglioside molecules. The four major neuroblastoma G_{D2} peaks isolated by reversed-phase HPLC as shown in Fig. 3 had the carbohydrate structure of G_{D2} and the same long-chain base (sphingosine, $d18:1$) and differed only in fatty acid structure. These molecules were tested for immunosuppressive activity as described in Fig. 1. Activity of the individual species is shown. The fatty acids were C24:0 (▲), C22:0/C24:1 (▼), C18:0 (■), and C16:0 (●). Highly purified total G_{D2} (○), which is a mixture of the ceramide species, had intermediate activity. From the results, the interpolated ID_{50} (ganglioside concentration causing 50% inhibition of the lymphocyte proliferative response) is 1.6 μM for the unseparated total G_{D2}. The ceramide species had ID_{50} values of 0.4 μM ($d18:1$-C16:0), 0.8 μM ($d18:1$-C18:0), 4.9 μM ($d18:1$-C22:0/$d18:1$-C24:1), and >5.0 μM ($d18:1$-C24:0).

carbohydrate structure of the ganglioside molecules? Or, are these findings restricted to G_{D2}? To answer this question, two other gangliosides, SPG and GalNAcG_{M1b} were isolated from two other tumors, a human leukemia and a murine lymphoma, respectively. They were separated and characterized in the same way as were the G_{D2} gangliosides. Two major ceramide species of each ganglioside were identified, and again they differed only in the fatty acyl chain (length 16 and 24 carbons). When SPG and GalNAcG_{M1b} were tested for immunosuppressive activity, in each case the ganglioside containing a short fatty acyl chain (16 carbons) was much more immunosuppressive than the same ganglioside containing a 24-carbon fatty acyl chain (Fig. 5). Thus, independent of the origin of the gangliosides (human or mouse; neuroblastoma, lymphoma, or leukemia), greater immunosuppressive activity is associated with gangliosides containing shorter-chain fatty acids. The present work, therefore, clearly defines the important concept that the ceramide structure of the ganglioside molecule dramatically influences ganglioside immunosuppressive activity.

How do these findings relate to what is already known about ceramide? Ceramide (18, 30–32) and certain lipid molecules, either structurally or metabolically related to ceramide [e.g., sphingosine 1-phosphate (16, 17) and sphingolipid breakdown products such as lysogangliosides (13–15)], may have important functional roles, such as in membrane signal transduction. Other synthetic lipid molecules, including D-$threo$-1-phenyl-2-decanoylamino-3-morpholino-1-propanol, an analogue of ceramide, block the synthesis of
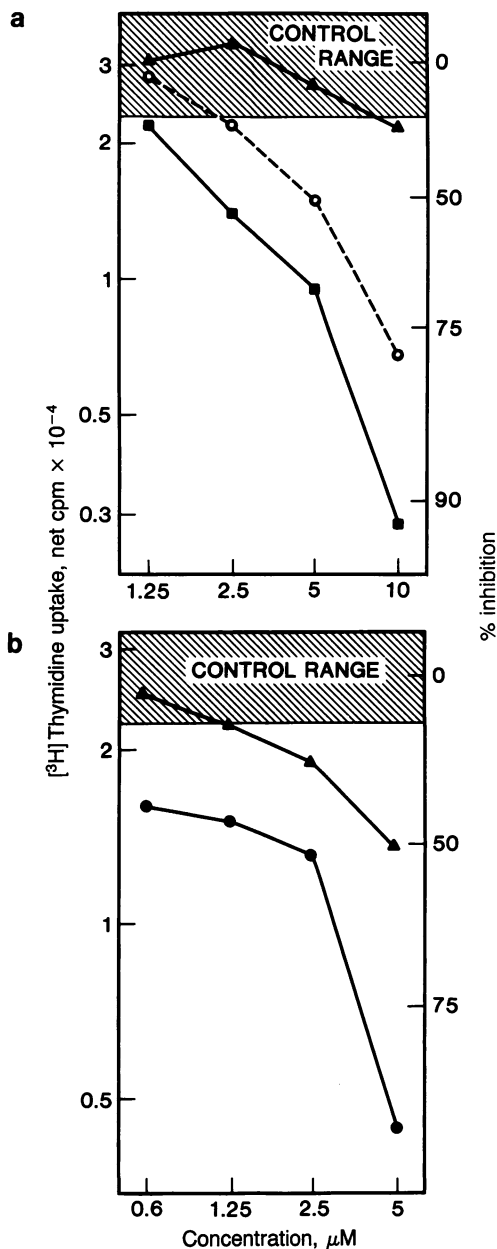
FIG. 5. Ceramide structure and immunosuppressive activity of human leukemia and mouse lymphoma gangliosides. The individual ceramide subspecies isolated from human leukemia and murine lymphoma gangliosides were assessed for immunosuppressive activity. (a) Human leukemia-derived SPG of ceramide structure d18:1-C24:1 (▲) and d18:1-C16:0 (■). Highly purified total SPG (○), from which the ceramide species were isolated, had intermediate activity. The ID$_{50}$ value for total purified SPG is 4.8 μM. The ceramide species had ID$_{50}$ values of 2.1 μM (d18:1-C16:0) and >>10 μM (d18:1-C24:1). (b) Murine lymphoma-derived GalNAcG$_{M1b}$ of ceramide structure d18:1-C16:0 (●) or d18:1-C24:1 (▲). The ID$_{50}$ value for the GalNAcG$_{M1b}$ ceramide species is 2.2 μM (d18:1-C16:0) and 5.0 μM (d18:1-C24:1).

glycosphingolipids and affect cell growth, cell adhesion, and mitogen-stimulated lymphocyte proliferative responses (33–35). Finally, ceramide, as a structural component of the intact glycosphingolipid molecule, affects ganglioside–monoclonal antibody binding affinity (36). It also influences the relative antigenicity of glycolipids (37, 38) probably by affecting exposure of the carbohydrate epitope in the cell plasma membrane.

It is known that the ceramides from certain normal ganglioside molecules (e.g., bovine brain gangliosides) have no immunosuppressive activity when cleaved from the carbohydrate portion of the molecule (39). To confirm whether this is true for ceramides found in tumor gangliosides, we assessed the immunosuppressive activity of ceramides containing C18 (Matreya, Inc) or C24/C18 (type III, Sigma) fatty acyl groups under the same conditions as intact gangliosides were studied. Neither of these ceramides was active (ID$_{50}$ > 30 μM). Thus, the immunosuppressive activity of tumor-associated gangliosides cannot be reproduced by the ceramide alone. One can conclude that while the immunosuppressive activity requires the intact ganglioside consisting of both carbohydrate and ceramide moieties, the molecular structure of the ceramide predicts degree of activity.

Gangliosides interfere with the cellular immune response by at least several mechanisms. These include reversible inhibition of adherent leukocyte antigen processing/presentation (40), direct binding to cytokines such as interleukin 2 (41, 42), and downregulation of CD4 expression (43). Programmed cell death, or apoptosis, may also be influenced by gangliosides and related molecules. Most recently, C$_2$-ceramide has been shown to cause apoptosis (44) and globotriaosylceramide may be a receptor for a signal molecule that induces apoptosis (45), whereas ganglioside G$_{M1}$ protects against tumor necrosis factor-induced apoptosis (46). The lack of toxicity of gangliosides to PBMCs (4, 9, 47), reversible inhibition (40), and the protective effects of G$_{M1}$ (46) all argue against gangliosides acting by causing apoptosis. Clearly, however, the molecular mechanisms of immunosuppression by tumor gangliosides remain to be established.

The present findings provide another perspective on ganglioside function and ceramide structure. Tumor gangliosides clearly have a dynamic effect on the tumor cell microenvironment and are a critical factor in tumor formation and progression (48). By the continuous (20) shedding of these biologically active cell surface molecules, the tumor cell creates a microenvironment containing a high concentration of tumor-derived gangliosides that may downregulate the host antitumor response by inhibiting the function of leukocytes that infiltrate tumors, thereby facilitating tumor progression. The accumulated evidence supports this hypothesis. *In vitro*, gangliosides inhibit several steps in the cellular immune response, such as antigen processing/presentation (40), lymphocyte proliferation (4, 47, 49–51), and cytotoxic effector function (52–54). *In vivo*, tumor formation by otherwise poorly tumorigenic cells can be enhanced by the addition of gangliosides in experimental model systems (48, 55). In humans, substantial shedding was chemically detected in the circulation of patients with neuroblastoma (6) and tumor progression is highly linked statistically to the circulating concentration of shed tumor gangliosides (56). And gangliosides with short fatty acyl chains, which we identified to be highly immunosuppressive, are also those molecules that are selectively shed by tumors such as neuroblastoma (20), leukemia and lymphoma (57, 58), melanoma (7, 19), and sarcoma (59). The combined findings strongly suggest that the tumor cell is optimized to protect itself *in vivo* by selective shedding of certain highly active ganglioside species. Finally, since gangliosides inhibit antigen-induced proliferation of PBMCs without toxicity to unstimulated resting cells (9), certain highly active ganglioside structures may, under other circumstances, paradoxically have therapeutic potential as immunosuppressive molecules.

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