Immunosuppressive Activity of Chemically Synthesized Gangliosides†

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ABSTRACT: New chemical synthetic methods have permitted the synthesis of a spectrum of glycosphingolipid molecular species, some of which are not naturally occurring. Here we have studied a number of chemically synthesized gangliosides for immunosuppressive activity, using a human *in vitro* specific antigen (tetanus toxoid)-induced assay of the cellular immune response. Chemically synthesized G_{M3} and G_{M4} had the same high degree of immunosuppressive activity as did natural G_{M3} and G_{M4} gangliosides, verifying that inhibition is intrinsic to the ganglioside molecules and not caused by other molecules sometimes found in natural preparations (e.g., proteins). Studies of modified molecular species of G_{M3} and G_{M4}, also prepared by chemical synthesis, have shown the influence of certain structural details upon the immunosuppressive activity of gangliosides: (i) the inverse relationship between fatty acyl chain length and immunosuppressive activity is extended to even shorter chain lengths, with the synthetic gangliosides d18:1-C2:0-G_{M3} and d18:1-C14:0-G_{M3} being more immunosuppressive than d18:1-C18:0-G_{M3} and d18:1-C24:0-G_{M3}; (ii) hydroxylation of the fatty acyl group decreases immunosuppressive activity; (iii) substitution of an *S*-glycosidic bond for an *O*-glycosidic bond in the sialic acid ketosidic linkage in G_{M4} does not alter its activity; and (iv) modifications of the sialic acid group variably influence immunosuppressive activity, since KDN-G_{M3} and -G_{M4} ganglioside analogues, which contain a 3-deoxy-D-*glycero*-D-*galacto*-2-nonulopyranosonic acid in place of *N*-acetylneuraminic acid, retain activity, while other modifications such as 8-epi-G_{M3}, and to a lesser extent 9-deoxy-G_{M3}, reduce immunosuppressive activity. In conclusion, gangliosides produced by total chemical synthesis have the immunosuppressive properties of the natural molecules and are useful in probing which elements of ganglioside structure are critical to their biological activity.

Increasing interest in the potential biological activity of gangliosides has resulted in a close examination of the relation of structure to biological activity (Lengle et al., 1979; Hakomori, 1981; Sharom et al., 1990; Zeller & Marchase, 1992; Ladisch et al., 1992, 1994), as well as critical evaluations of the possibility that contaminating molecules might be responsible for the immunosuppressive activity of gangliosides that had been isolated from natural sources (Krishnaraj & Kemp, 1982; Ladisch et al., 1983; Fidelio et al., 1991). Although substantial advances have been made in the purification of gangliosides from natural sources, absolute purity and the isolation of substantial quantities of material remain somewhat elusive goals. The development of new chemical synthetic methods for the total synthesis of glycosphingolipids (Hasegawa & Kiso, 1992) has opened a new perspective on the biological studies of gangliosides, since chemical synthesis can yield not only large quantities of homogeneous molecular species but also ganglioside analogues.

In contrast to extensive studies of potential recognition sites on synthetic molecules (e.g., sialyl Le^x), and their relation to the natural ganglioside species involved in recognition processes (Tyrrell et al., 1991; Brandley et al., 1993), the functional activities of chemically synthesized gangliosides, i.e., how they may act upon living cells, have not yet been systematically studied. Here we demonstrate that chemically synthesized gangliosides, of identical structure to certain naturally occurring species, have similar degrees of immunosuppressive activity. The biological activity of these synthetic molecules and their analogues confirms that the originally observed immunosuppressive activity of naturally occurring gangliosides is intrinsic to these molecules and not due to potential contaminants in natural preparations (such as proteins or other lipids). Furthermore, the present studies of chemically synthesized gangliosides reveal that immunosuppressive activity is influenced by certain previously unstudied structural determinants.

MATERIALS AND METHODS

Purification of Natural Gangliosides. Total gangliosides were purified from normal human brain by a sequence of steps, including extraction of the cells with chloroform/methanol (1:1), partition of the total lipid extract in diisopropyl ether/1-butanol, and Sephadex G-50 gel filtration of the ganglioside-containing aqueous phase (Ladisch & Gillard, 1985). The individual gangliosides G_{M3} and G_{M4} used in

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this study were then separated and purified by HPLC (Gazzotti et al., 1985). Gangliosides were quantified by resorcinol assay (Svennerholm, 1957) and analyzed by high-performance thin-layer chromatography (HPTLC).[1] The developing solvent system was chloroform/methanol/0.2% $CaCl_2 \cdot 2H_2O$ (60:40:9, by volume), and the gangliosides were visualized by resorcinol staining (Ledeen & Yu, 1982). The structures of these natural gangliosides were confirmed (Ladisch et al., 1992) using negative ion fast atom bombardment mass spectrometry and collisionally activated dissociation tandem mass spectrometry (Domon & Costello, 1988).

Chemical Synthesis of Gangliosides. The synthesis and structural analyses of the glycosphingolipids, which differ either in carbohydrate structure or in ceramide structure from the naturally occurring ganglioside molecules, have been published. In each case, the structures and purity of the chemically synthesized glycosphingolipids were confirmed by chemical analysis, IR spectrometry, and ^1H NMR spectrometry, as cited here. G_{M3} homologues varying in the ceramide moiety include species with fatty acyl groups containing 14−24 carbons (Murase et al., 1989) or even shorter chain lengths and α- or β-hydroxylation (Hasegawa et al., 1990a). Gangliosides varying in the carbohydrate moiety include G_{M3} analogues with modified sialic acids, 9-deoxy-*N*-acetylneuraminic acid (Hasegawa et al., 1992a) and 8-epi-*N*-acetylneuraminic acid (Hasegawa et al., 1992b), as well as KDN-G_{M3} and -G_{M4} analogues that contain 3-deoxy-D-*glycero*-D-*galacto*-2-nonulopyranosonic acid (KDN) in place of *N*-acetylneuraminic acid (Terada et al., 1993). S-G_{M4} and S-GlcG_{M4}, in which the *O*-glycosidic bond is replaced by an *S*-glycosidic bond in the sialic acid ketosidic linkage (and glucose replaced galactose as indicated), were also synthesized (Hasegawa et al., 1990b).

Lymphocyte Proliferation Assay. An assay of the human cellular immune response, lymphoproliferation stimulated by a specific antigen, tetanus toxoid (Ladisch et al., 1992), has been used to measure the immunosuppressive effects of purified gangliosides. Briefly, normal human peripheral blood mononuclear leukocytes were isolated from Ficoll-hypaque density gradient centrifugation (Boyum, 1968) 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 peripheral blood mononuclear leukocytes were cultured in 96-well (A/2) tissue culture clusters (Costar No. 3696).

[1] Abbreviations: HPTLC, high-performance thin-layer chromatography; KDN, 3-deoxy-D-*glycero*-D-*galacto*-2-nonulopyranosonic acid; all gangliosides used in this study contain sphingosine (d18:1) and either saturated fatty acyl groups (C2:0, C14:0, C18:0, and C24:0: the number after C indicates the carbon number) or a hydroxylated fatty acyl group (αh- or βh-); G_{M3}, *N*-acetylneuraminosyl-α(2,3)-galactosyl-β(1,4)-glucosylceramide; G_{M4}, *N*-acetylneuraminosyl-α(2,3)-galactosylceramide; 9-deoxy-G_{M3}, 9-deoxy-*N*-acetylneuraminosyl-α(2,3)-galactosyl-β(1,4)-glucosylceramide; 8-epi-G_{M3}, 8-epi-*N*-acetylneuraminosyl-α(2,3)-galactosyl-β(1,4)-glucosylceramide; α(2,3)-KDN-G_{M3}, 3-deoxy-D-*glycero*-D-*galacto*-2-nonulopyranosyl-α(2,3)-galactosyl-β(1,4)-glucosylceramide; α(2,6)-KDN-G_{M3}, 3-deoxy-D-*glycero*-D-*galacto*-2-nonulopyranosyl-α(2,6)-galactosyl-β(1,4)-glucosylceramide; α(2,3)-KDN-G_{M4}, 3-deoxy-D-*glycero*-D-*galacto*-2-nonulopyranosyl-α(2,3)-galactosylceramide; α(2,6)-KDN-G_{M4}, 3-deoxy-D-*glycero*-D-*galacto*-2-nonulopyranosyl-α(2,6)-galactosylceramide; S-G_{M4}, *S*-(*N*-acetylneuraminosyl)-α(2,6)-galactosylceramide; S-GlcG_{M4}, *S*-(*N*-acetylneuraminosyl)-α(2,6)-glucosylceramide; Lf, limes flocculating.

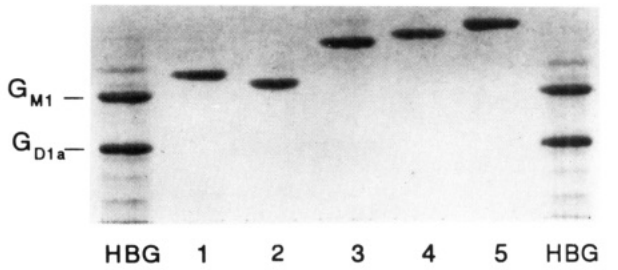

FIGURE 1: High-performance thin-layer chromatographic analysis of chemically synthesized gangliosides. Lanes 1−5 contain 2 nmol, respectively, of the following molecular species: lyso-G_{M3}, d18:1-C2:0-G_{M3}, d18:1-C14:0-G_{M3}, d18:1-C18:0-G_{M3}, and d18:1-C24:0-G_{M3}. HBG, human brain gangliosides (3 nmol). Stain: resorcinol−HCl.

Gangliosides and their analogues were suspended in medium by brief sonication before addition to the cell cultures. Ganglioside solution was added at 10 μL/well, followed by the addition of the peripheral blood mononuclear leukocytes (PBMC, 25 μL, 2×10^6 cells/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/mL, Massachusetts Department of Health, Boston, MA), was added. Ten microliters of basal medium alone was added to unstimulated control cultures. The complete cultures were incubated at 37 °C in 95% air/5% CO_2 for 6 days (Ladisch et al., 1992). As has been previously documented, gangliosides are not toxic to the cells under these conditions (Lengle et al., 1979; Whisler & Yates, 1980; Ladisch et al., 1992). At the end of the culture period, 0.5 μCi of [^{3}H]thymidine in 50 μL of medium was added to each well. The cultures were incubated for an additional 4.5 h and harvested onto glass fiber filter paper. Cellular [^{3}H]thymidine uptake was quantified 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

Characterization of Chemically Synthesized Gangliosides. Molecules with modifications in either the ceramide portion or the sialic acid moiety have been synthesized. Shown in Figure 1 is the HPTLC of a representative series of chemically synthesized glycosphingolipid species. In this case they are subspecies of G_{M3} ganglioside, each containing a fatty acyl group of different chain length varying from 2 to 24 carbons or having no fatty acyl group (lyso-G_{M3}). Each purified individual molecule has been analyzed for structure by mass spectrometry and HPTLC. As is evident by HPTLC, the final product of the chemical synthetic process is a series of homogeneous subspecies of G_{M3}. Of interest, the relative HPTLC mobilities of these individual ganglioside species generally vary with fatty acyl chain length, as do the mobilities of naturally occurring ganglioside species. Gangliosides with longer fatty acyl groups, such as d18:1-C24:0-G_{M3}, are less polar and migrate more rapidly in the solvent system used than do molecular species with very short fatty acyl groups, such as d18:1-C2:0-G_{M3}. Lyso-G_{M3} migrates slightly faster than does d18:1-C2:0-G_{M3}.

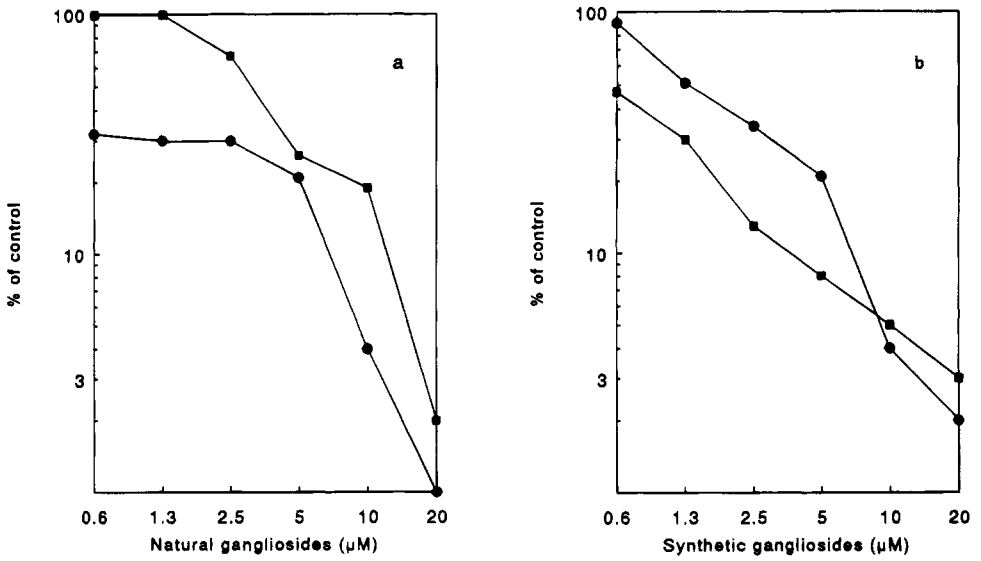

FIGURE 2: Comparison of the immunosuppressive activities of chemically synthesized and natural gangliosides. An assay of antigen (tetanus toxoid)-induced human lymphoproliferation was used to measure the immunosuppressive effects of natural (panel a) or synthetic (panel b) gangliosides G_{M3} (■) and G_{M4} (●). The % inhibition of cellular proliferation by ganglioside-treated cultures was calculated by comparing the mean net [^{3}H]thymidine uptake of triplicate ganglioside-treated cultures with control cultures. Control stimulation = (7.1 ± 1.8) × 10^3 cpm (mean ± SEM).

Chemically Synthesized Gangliosides of Identical Structure to Those of Natural Origin Are Immunosuppressive. Substantial information exists on the immunosuppressive activity of G_{M3} and G_{M4} gangliosides isolated from natural sources (Ladish et al., 1992). The present experiments were undertaken to assess the relative immunosuppressive activity of gangliosides that were chemically synthesized but had the same molecular structure as those that were isolated from natural sources, such as tissue samples.

In these experiments, we measured the immunosuppressive activities of d18:1-C18:0-G_{M3} and d18:1-C18:0-G_{M4}, prepared by total chemical synthesis, and compared the activities with those of G_{M3} and G_{M4} isolated from normal human brain. Shown in Figure 2a are the immunosuppressive activities of the naturally occurring G_{M3} and G_{M4}. These two gangliosides are highly immunosuppressive, having 50% inhibitory concentrations (ID_{50}) of 3.0 and <0.6 μM, respectively, consistent with our previous demonstration of their highest activity, relative to all other brain-derived gangliosides (Ladisch et al., 1992). As shown in Figure 2b, synthetic gangliosides G_{M3} and G_{M4} have levels of activity similar to those of the natural G_{M3} and G_{M4}, with ID_{50}'s of <0.6 and 1.4 μM, respectively. It has been previously shown that the inhibition by gangliosides is not due to a direct toxic effect on the responding cell population or altered lectin binding and that this inhibition is reversible (Lengle et al., 1979; Whisler & Yates, 1980; Ladisch et al., 1992). In the present study, when normal human peripheral blood mononuclear leukocytes were exposed to 20 μM synthetic d18:1-C18:0-G_{M3} (which caused 97% inhibition of the lymphoproliferative response), there was no toxic effect observed at the end of the 6 day culture period (1.06 × 10^5 vs 0.82 × 10^5 viable cells/well, control vs G_{M3}-exposed cells, respectively). These results, which demonstrate the similarity of the activities of naturally occurring and chemically synthesized ganglioside molecular species, provide the first evidence for the functional activity of chemically synthesized gangliosides in assays of cellular immunity. Importantly, they exclude the possibility that the activity of the gangliosides isolated from natural sources may have been due to contaminating proteins, since there is no protein present in the chemical synthetic procedures.

Role of Ceramide Structure in Determining the Immunosuppressive Activity of Chemically Synthesized Gangliosides. Previously, we showed a significant relationship between the fatty acyl chain length of naturally occurring gangliosides and their immunosuppressive activity: shorter fatty acyl chain lengths were associated with greater immunosuppressive activity of the corresponding gangliosides (Ladisch et al., 1994). Here we have studied a number of synthetic molecules of the carbohydrate structure of G_{M3} ganglioside and of varying fatty acyl chain lengths. In the first experiment, the activities of two synthetic gangliosides, d18:1-C2:0-G_{M3} and lyso-G_{M3}, were compared. The influence of a fatty acyl group was evidenced by the higher degree of immunosuppressive activity of G_{M3} containing a two-carbon fatty acyl group in its ceramide structure (ID_{50} = 0.2 μM), compared to that of lyso-G_{M3} (ID_{50} = 3.0 μM) (Figure 3). Next, as shown in Figure 4, we compared the relative activities of a number of species of G_{M3} (d18:1-C2:0, d18:1-C14:0, d18:1-C18:0, d18:1-C24:0). These studies demonstrated increasing immunosuppressive activity with decreasing fatty acyl chain length and confirm previous findings obtained using naturally occurring gangliosides, in which all of three tumor-derived gangliosides studied (G_{D2}, GalNAcG_{M1b}, and sialosylparagloboside), which had a shorter fatty acyl group (e.g., C16:0 or C18:0), were consistently more active than the corresponding gangliosides that contained a longer fatty acyl group (C22:0 or C24:1/C24:0) (Ladisch et al., 1994).

Another aspect of fatty acyl structure that we could investigate using chemically synthesized gangliosides is the effect of hydroxylation of the fatty acyl groups. Two homologues of d18:1-C14:0-G_{M3}, which contain a hydroxyl group in either the α- or β-position of the fatty acyl chain, were synthesized. Both d18:1-αhC14:0-G_{M3} and d18:1-βhC14:0-G_{M3} were much less active than the parent mol-

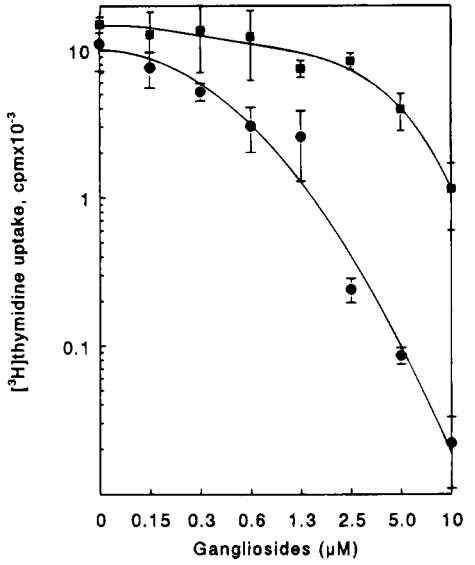

FIGURE 3: Inhibition of the human cellular immune response by d18:1-C2:0-G_{M3} and lyso-G_{M3}. Chemically synthesized gangliosides d18:1-C2:0-G_{M3} (●) and lyso-G_{M3} (■) were assessed for immunosuppressive activity in the tetanus toxoid-induced human lymphoproliferation assay over a range of ganglioside concentrations (0−10 μM), as in Figure 2. Each point represents the mean ± SEM of triplicate cultures from the same donor. Control stimulation = (11.5 ± 3.0) × 10^3 cpm. The ID_{50} for d18:1-C2:0 was 0.2 μM and that for lyso-G_{M3} was 3 μM.

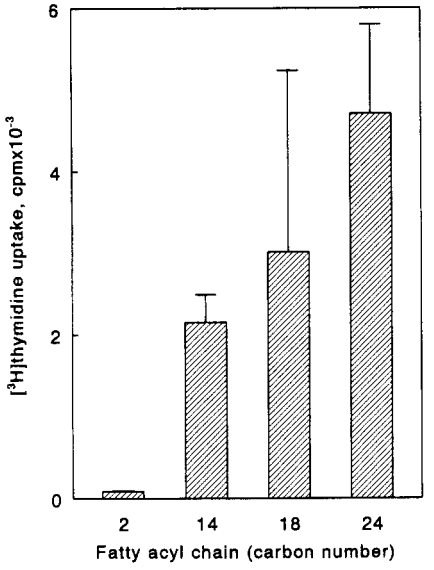

FIGURE 4: Effects of fatty acyl chain length on immunosuppressive activity of chemically synthesized gangliosides. The immunosuppressive activity of G_{M3} gangliosides, of ceramide structure d18:1-C*n*:0 (*n* = carbon number of the fatty acyl chain as indicated in the figure), is compared. Each bar represents the mean ± SEM [³H]thymidine uptake of cultures exposed to 5 μM of the indicated G_{M3} species. Control stimulation = (11.5 ± 3.0) × 10^3 cpm.

ecule, d18:1-C14:0-G_{M3} (Table 1). For example, whereas the ID_{50} of d18:1-C14:O-G_{M3} upon the antigen-induced lymphoproliferative response of human leukocytes to tetanus toxoid was approximately 2.5 μM, neither d18:1-αhC14:0-G_{M3} nor d18:1-βhC14:0-G_{M3} was inhibitory at the 2.5 μM concentration. These results show that hydroxylation of the fatty acyl group reduces ganglioside immunosuppressive activity.

Role of Carbohydrate Structure in Determining the Immunosuppressive Activity of Chemically Synthesized Gangliosides. A series of ganglioside G_{M3} and G_{M4} analogues were synthesized in which the sialic acid was either modified or replaced by a KDN structure (3-deoxy-D-*glycero*-D-*galacto*-2-nonulopyranosonic acid). The purpose was to investigate the role of the integrity of the sialic acid in determining the immunosuppressive activity of gangliosides. These modified molecules are shown in Figure 5.

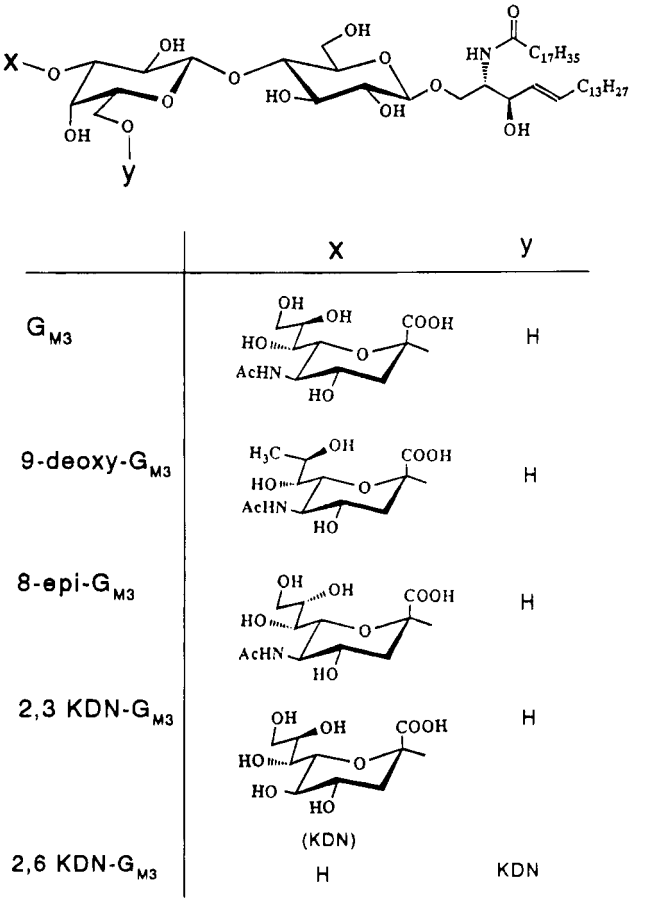

FIGURE 5: Structural modifications of G_{M3}. Changes in carbohydrate structure include a modified sialic acid and a 3-deoxy-D-*glycero*-D-*galacto*-2-nonulopyranosonic acid (KDN).

Table 1: Influence of Fatty Acid Hydroxylation on Ganglioside Immunosuppressive Activity

	[³H]thymidine uptake (cpm × 10^{-3})		inhibition (%)	
	2.5 μM[a]	5.0 μM	2.5 μM	5.0 μM
control	9.8	9.7		
G_{M3}				
d18:1-C14:0	4.6	0.8	53	92
d18:1-αhC14:0	9.1	3.2	7	67
d18:1-βhC14:0	8.2	3.4	16	65

[a] Final concentration of gangliosides in culture. An assay of antigen-induced human lymphoproliferation was used to measure the immunosuppressive effects of the gangliosides. The data represent the means of three separate cultures from the same donor. The SEM was routinely ≤10%.

G_{M3} analogues with modified sialic acid varied in their effects on ganglioside immunosuppressive activity. One modified molecule, 9-deoxy-G_{M3}, in which a hydroxyl group (OH) at the C_9-position of the sialic acid is replaced with a hydrogen, evidenced the reduced immunosuppressive activity compared with G_{M3} with an unmodified sialic acid. Another species, 8-epi-G_{M3}, a stereoisomer of G_{M3} at the C_8-position

Table 2: Immunosuppressive Activity of Gangliosides and Their Analogues

	[^{3}H]thymidine uptake[a] (cpm × 10^{-3})	
	G_{M3}[b]	G_{M4}
control	23.9	27.1
ganglioside modification	<0.1 (99)[c]	2.2 (92)
9-deoxy	10.1 (58)	*d*
8-epi	17.1 (28)	*d*
α(2,3)-KDN	1.2 (95)	0.2 (99)
α(2,6)-KDN	0.1 (99)	0.8 (97)

[a] The data represent the mean [^{3}H]thymidine uptake of three separate cultures from the same donor. The SEM was routinely ≤10%. [b] Final concentration of gangliosides in cell culture was 10 μM. All gangliosides and their analogues contain d18:1-C18:0-ceramide. [c] % inhibition is in parentheses. [d] Not tested.

Table 3: Influence of Sulfur Substitution in the Sialic Acid Ketosidic Linkage on Ganglioside Immunosuppressive Activity

	[^{3}H]thymidine uptake (cpm × 10^{-3})		inhibition (%)	
	5.0 μM[a]	10 μM	5.0 μM	10 μM
control	27.1	27.1		
G_{M4}		2.2		92
S-G_{M4}	1.1	0.9	95	97
S-GlcG_{M4}[b]	2.4	1.7	91	94

[a] Final concentration of gangliosides in cell culture. Each ganglioside has the ceramide structure, d18:1-C18:0. The data represent the means of three separate cultures from the same donor. The SEM was routinely ≤10%. [b] S-GlcG_{M4} contains glucose in place of the usual galactose.

of sialic acid, was almost devoid of immunosuppressive activity compared with native G_{M3} (Table 2).

Two KDN-G_{M3} analogues, α(2,3)-KDN-G_{M3} and α(2,6)-KDN-G_{M3}, were synthesized. α(2,3)-KDN-G_{M3} contains a 3-deoxy-D-*glycero*-D-*galacto*-2-nonulopyranosonic acid in place of an *N*-acetylneuraminic acid. The overall structural difference between G_{M3} and α(2,3)-KDN-G_{M3} is that, in KDN-G_{M3}, the acetamido group (CH_3CONH) is replaced by a hydroxyl group (OH) at the C_5-position of the sialic acid. In α(2,6)-KDN-G_{M3}, an additional change is that the ketosidic linkage is α(2,6) rather than α(2,3). As shown in Table 2, these modifications in the sialic acid structure of G_{M3} gangliosides had little effect on immunosuppressive activity; both molecules caused ≥95% inhibition when added to the leukocyte cultures in a 10 μM concentration. The same two KDN structural analogues of G_{M4} were also synthesized. Once again, immunosuppressive activity was completely preserved (≥97% inhibition of lymphoproliferation at a 10 μM concentration) despite the substitution of a KDN for a sialic acid (Table 2). Thus, the immunosuppressive activities of G_{M3} and G_{M4} were maintained even though the sialic acid structure and the glycosidic linkage had been altered.

Finally, sulfur-containing ganglioside G_{M4} analogues were also synthesized. In these molecules, the oxygen atom in the sialic acid ketosidic linkages is replaced by a sulfur atom, i.e., an *O*-glycosidic bond is replaced by an *S*-glycosidic bond. In S-GlcG_{M4}, the monosaccharide was glucose instead of the usual galactose. Substitution of sulfur for oxygen (and glucose for galactose) in these molecules did not significantly affect ganglioside immunosuppressive activity, and in fact, the activity was even slightly greater than that of native G_{M4} (Table 3).

DISCUSSION

Ganglioside molecules have been recognized to have a number of important biological activities. These include, for example, a role in defining the antigenic specificity of many cell types (Hakomori, 1984) and roles in cell recognition (Feizi, 1985), adhesion (Cheresh et al., 1986), and signal transduction (Bremer et al., 1986). Gangliosides also inhibit cellular immune responses, including the lymphoproliferative response induced by specific antigens as well as by allogenic cells (Whisler & Yates, 1980). The successful chemical synthesis of a range of glycosphingolipid structures should make a significant contribution to advancing the field of glycobiology by permitting a thorough study of the structure–activity relationship affecting these cellular immune responses. As a first step, the present study provides a first demonstration of functional activity (immunosuppression *in vitro*) of chemically synthesized ganglioside molecules: synthetic gangliosides of identical structure to the naturally occurring gangliosides G_{M3} and G_{M4} have very similar immunosuppressive activities. Traces of contaminating molecules (such as proteins) are potentially present in ganglioside preparations from natural sources, and immunosuppression by gangliosides has been suspected to be due to these contaminating molecules (Krishnaraj & Kemp, 1982; Fidelio et al., 1991). On the other hand, studies with murine lymphoma gangliosides suggested that protein contamination was not the cause of the immunosuppressive activity, since the higher the purity of the lymphoma gangliosides, the greater their immunosuppressive activity (Ladisch et al., 1983). The demonstration that gangliosides produced by chemical synthesis are active definitively excludes the potential trivial cause of ganglioside immunosuppressive activity being the presence of contaminating molecules. The present study also provides a strong rationale for using synthetic gangliosides (available in larger quantities) in studies of ganglioside biological activities and their mechanisms.

A significant advantage of chemical synthesis, in addition to the ability to obtain much larger quantities of individual molecules, is the ability to obtain novel molecules. For example, previously only gangliosides of ceramide structures d18:1-C16:0 to d18:1-C24:0 had been isolated and studied for immunosuppressive activity. Chemical synthetic methods now allow us to extend these studies of ceramide structure beyond those of natural gangliosides. Thereby, it may be possible to identify molecular species of even higher activity than those that are naturally occurring.

The present study permits us to refine the overall view of a core structure imparting immunosuppressive activity to gangliosides. First, we have previously proposed that the highly immunosuppressive core structure of gangliosides consists of a compact oligosaccharide with a terminal sialic acid (Ladisch et al., 1992), coupled to a ceramide containing a relatively short fatty acyl group (Ladisch et al., 1994). This conclusion is confirmed by the finding of immunosuppressive activities of chemically synthesized G_{M3} and G_{M4} that are very similar to those of natural G_{M3} and G_{M4} species, which we previously showed to have the highest immunosuppressive activity among all the natural gangliosides studied (Ladisch et al., 1992).

Studies with a series of slightly modified structures were valuable in further establishing the nature of the core structure that imparts immunosuppressive activity to this class of glycosphingolipid molecules. With respect to the ceramide structure of these molecules, the conclusion that shorter fatty acyl chain-containing gangliosides were more active than longer fatty acyl chain-containing analogues of identical carbohydrate structure is also supported and extended by the present studies. Ganglioside G_{M3} species such as d18:1-C2:0 and d18:1-C14:0, not previously available for study, were demonstrated to have higher degrees of activity than do the G_{M3} species d18:1-C18:0 and d18:1-C24:0, which were obtained both by chemical synthesis and by isolation from a natural source. A third aspect of structure−activity relationships elucidated by these studies of chemically synthesized gangliosides concerns hydroxylation of the fatty acyl group. We studied the effect of hydroxylation in the α- or β-position of the fatty acyl group of d18:1-C14:0-G_{M3}, and hydroxylation of the fatty acyl chain in either position decreases ganglioside immunosuppressive activity. Thus, these studies confirm and extend the previous conclusions regarding fatty acyl chain structure and its effect on ganglioside immunosuppressive activity obtained by studying naturally occurring tumor gangliosides (Ladisch et al., 1994).

Finally, with respect to carbohydrate structure, the present results, together with previous findings, demonstrate the importance of sialic acid for immunosuppressive activity. An unsubstituted carboxylic group is important for activity, since lactones are less active than the corresponding native ganglioside (e.g., reduction of activity by lactonization of G_{D1b}; Ladisch et al., 1992). However, a fully intact *sialic* acid structure is *not* required for the highest immunosuppressive activity of a given glycolipid structure, since both α(2,3)-KDN and α(2,6)-KDN ganglioside species (G_{M3} and G_{M4}) are as active as the native forms. These findings also suggest that the acetamido group of the sialic acid may not be critical to activity. This is of potential importance with respect to the immunosuppressive activity of gangliosides *in vivo*, since alterations in linkage and sialic acid structure may render the molecules less susceptible to catabolism by sialidases *in vivo*, and thereby might increase the duration of the *in vivo* activities of these molecules. Clearly, however, additional studies are needed to definitively establish the relationship between the immunosuppressive activity and the carbohydrate structure of chemically synthesized gangliosides.

In summary, the results obtained regarding the immunosuppressive activity of synthetic gangliosides allow us to propose the following refined view of an optimal immunosuppressive ganglioside structure: the carbohydrate structure is compact and contains a terminal sialic acid in which the carboxyl group is in its free state (not a lactone), but in which the acetamido group of sialic acid is not essential; the ceramide contains a fatty acyl group that is preferably short and is not hydroxylated. Further modification of ganglioside structures, by chemical synthesis, will very likely lead to additional understanding of the core structure, which imparts the most potent immunosuppressive activity to a ganglioside. It is already clear, however, that synthetic gangliosides that have immunosuppressive properties will be useful, as are naturally occurring species, in probing ganglioside biological activities and their mechanisms *in vivo*.

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