細胞と比較し、コンドロイチン硫酸の総量が増加した。次に、ChGn-1 過剰発現細胞におけるコンドロイチン硫酸鎖の増加が、本数の増加と鎖長の伸長のどちらに起因するのかを明らかにするため、細胞から抽出・精製したグリコサミノグリカン画分をそれぞれゲルろ過クロマトグラフィーにより分画し、各画分に含まれるコンドロイチン硫酸の二糖総量を求めることにより鎖長解析を行った。その結果、親株の L 細胞および sog9 細胞のコンドロイチン硫酸鎖長と比較すると、ChGn-1 を過剰発現してもその鎖長にほとんど変化がなかった。従って、ChGn-1 を過剰発現することによるコンドロイチン硫酸合成量はコンドロイチン硫酸鎖の本数が増加していることに起因することが明らかとなった。これらの結果より、C4ST-1 が欠損している sog9 細胞において、ChGn-1 を過剰発現するによりコンドロイチン硫酸鎖量の増加が見られたことより、C4ST-1 以外の因子と共同し、コンドロイチン硫酸鎖の本数を制御していることが示唆された。そこで次に、GalNAc-結合領域に効率よく硫酸基を転移した C4ST-2 を L 細胞で過剰発現およびノックダウンした細胞を構築し、細胞が産生したコンドロイチン硫酸鎖の総量および鎖長を解析した。その結果、C4ST-2 の発現量とコンドロイチン硫酸鎖の本数および総量が相関していることが明らかとなった。（1）。

D. 考察

今回、C4ST-2 もコンドロイチン硫酸鎖の本数の制御に関与していることが明らかになった。したがって、ニューロパチーの症例の中には、ChGn-1 ばかりでなく C4ST-2 が変異することによりコンドロイチン硫酸鎖の本数が減少している症例も存在することが示唆された。そのため、C4ST-2 の変異によりコンドロイチン硫酸プロテオグリカンの合成異常が存在すると、minor trauma にさらされやすい末梢神経の障害からの修復や再生が十分にできなくなる可能性が考えられる。さらに、C4ST-2 は ChGn-1 と共同してコンドロイチン硫酸鎖の合成を制御していることも判明した。そのため、引き続きコンドロイチン硫酸の合成を制御していることが明らかとなった C4ST-2 や ChGn-1 のニューロパチー症例における検討、および最近作成された ChGn-1 のノックアウトマウスを用いたコンドロイチン硫酸プロテオグリカンの機能の解析を行うことで、ニューロパチーの病態におけるプロテオグリカンの役割が明らかになるものと期待される。

E. 結論

ニューロパチーとの関連が疑われているコンドロイチン硫酸鎖の本数の減少には、ChGn-1 ばかりでなく C4ST-2 も関与しており、その二つの酵素は共同して作用していることが明らかになった。

F. 健康危険情報

なし

G. 研究発表

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H. 知的財産権の出願・登録状況

1. 特許取得:なし
2. 実用新案登録:なし

研究成果の刊行に関する一覧表

書籍：該当なし

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ORIGINAL ARTICLE

Chondroitin beta-1,4-*N*-acetylgalactosaminyltransferase-1 missense mutations are associated with neuropathies

Kazumasa Saigoh[1], Tomomi Izumikawa[2], Toshiyasu Koike[2], Jun Shimizu[3], Hiroshi Kitagawa[2] and Susumu Kusunoki[1]

Chondroitin sulfate proteoglycans (CSPGs) in the peripheral nervous system likely participate as regulatory molecules in the process of axonal degeneration and regeneration. We investigated the *chondroitin beta1,4-N-acetylgalactosaminyltransferase-1* (*ChGn-1*) gene in 114 patients affected with neuropathies including Guillain-Barré syndrome, chronic inflammatory demyelinating polyneuropathy, hereditary motor and sensory neuropathy (HMSN) and unknown etiology. The controls were 196 patients with other neurological diseases. We found novel missense mutations in two patients with neuropathy (Bell's palsy, unknown HMSN) in exons 5 (H234R) and 10 (M509R), respectively. None of the patients with other neurological diseases had either of these mutations. We then synthesized the two soluble forms of ChGn-1, containing each of the above mutations. Each of the soluble mutants was expressed in COS-1 cells and the mutant proteins were purified. The purified mutant proteins were used for western blotting analysis using an anti-ChGn-1 antibody and evaluated for glycosyltransferase activities. Although the expression of the ChGn-1 mutant proteins was confirmed by western blotting, they exhibited no *N*-acetylgalactosamineT-II activities. It is possible that these mutations are associated with the pathogenetic mechanisms of the peripheral neuropathies.
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Keywords: ChGn-1; chondroitin GalNAcT-I; chondroitin GalNAcT-II; mutation; neuropathy

INTRODUCTION

Peripheral neuropathies are often caused by genetic factors. In particular, hereditary motor and sensory neuropathy (HMSN) or Charcot-Marie-Tooth disease is known to be associated with a number of causative genes.[1,2] HMSN is a heterogeneous group of degenerative peripheral nerve disorders, which altogether constitute the most common inherited neurological disease, with an incidence of 1 in 2500.[3]

Glycoconjugates, such as glycoproteins, proteoglycans and gangliosides, are important constituents of both the central and peripheral nervous systems. However, no association between human peripheral neuropathies and glycosyltransferases, which are involved in the synthesis of carbohydrate chains of glycoproteins, proteoglycans, gangliosides and so on, has been reported to date. Recently, it has been shown that beta1,4-*N*-acetylgalactosaminyltransferase (also called GM2/GD2 synthetase)-deficient mice are affected by sensory-dominant neuropathies.[4]

Chondroitin sulfate proteoglycans (CSPGs) have been shown to be present in the matrix of the nervous system. Several species of molecules are known such as neurocan, versican, phosphacan and so on. They show developmental and post-traumatic changes both spatially and temporally. CSPGs are known to act as growth inhibitory molecules.[5] On the other hand, some of the CSPGs promote neurite outgrowth.[6] CSPGs are likely to act not only as a chemical barrier but also as regulatory molecules for nerve regeneration.[7] Chondroitin beta1,4-*N*-acetylgalactosaminyltransferase-1 (ChGn-1) is involved in an important step for the synthesis of CSPGs.[8] Chondroitin sulfate chains consist of repeating disaccharide units of *N*-acetylgalactosamine (GalNAc) and glucuronic acid, which are sulfated at either the C6 or C4 position of GalNAc. The integrity of the chondroitin sulfate chain is maintained by elongation (biosynthesis) of the chain, which is catalyzed by ChGn-1, ChGn-2 and sulfotransferases.[9] Recently, it has been reported that a broad spectrum of skeletal dysplasias result from mutations causing undersulfation of chondroitin sulfate chains in humans and in mice.[10–14] In contrast, no association between CSPGs and human peripheral neuropathies has been reported to date.

In this study, we found novel missense mutations that resulted in a profound decrease of enzymatic activities in two patients with neuropathies.

MATERIALS AND METHODS

Subjects and patient populations

We recruited 310 patients with neurological disorders. We investigated 114 patients with neuropathy (40 with Guillain-Barré syndrome, 40 with chronic inflammatory demyelinating polyneuropathy, 5 with hereditary motor sensory neuropathy and 29 with unknown-etiology) and 196 disease control subjects. This study was approved by the internal review board of Kinki University

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School of Medicine. All patients provided written informed consent before participation in the study. Genomic DNA extraction and genotyping were performed using standard protocols.

Sequence analysis

Genomic DNA was extracted from whole blood using the QiaAmp Mini DNA kit (Qiagen, Tokyo, Japan). PCR amplicons generated with oligonucleotide primers were digested from *ChGn-1* gene on the basis of GenBank sequence. We sequenced all exons and their boundaries, using the ABI Prism 3700 DNA analyzer (Applied Biosystems, Foster City, CA, USA).

Materials

UDP-[^{3}H]GalNAc (10 Ci mmol^{-1}) and unlabeled UDP-GalNAc were purchased from NEN Life Science Products (Waltham, MA, USA) and Sigma (Tokyo, Japan), respectively. Chondroitin was purchased from Seikagaku (Tokyo, Japan).

Construction of a Soluble Form of ChGn-1

The complementary DNA fragment of a truncated form of *ChGn-1*, lacking the first 41 N-terminal amino acids, was amplified by reverse transcription-PCR with total RNA derived from G361 human melanoma cells (ATCC CRL-1424) as a template using a 5′-primer (5′-GCTCTAGACAGCTGGCACTGCCCAGG-3′) containing an in-frame *Xba*I site and a 3′-primer (5′-CGGGATCCCATCTCT GACCCATCAGTCC-3′) containing a *Bam*HI site located 58 bp downstream of the stop codon.

Site-directed Mutagenesis

A two-stage PCR mutagenesis method was used to construct the ChGn-1 H234R or ChGn-1 M509R mutants. Two separate PCR reactions were performed to generate two overlapping gene fragments using the soluble form of *ChGn-1* complementary DNA as a template. In the first PCR, the sense 5′-primer described above and either of the antisense internal mutagenic primers listed below were used: H234R 5′-GTTTGAATTCGCGTTTGTGGTCCCC-3′ or M509R 5′-CCTGAACACCAGCCTGCCCAGCTGGCCG-3′ (the mutated nucleotides are underlined). In the second round of PCR, the respective sense internal mutagenic primers (complementary to the antisense internal mutagenic primer) and the antisense 3′-primer described above were used.

Expression of soluble forms of the ChGn-1 and ChGn-1 mutants and enzyme assays

The expression plasmids (6.0 μg each) were transfected into COS-1 cells on 100-mm plates using FuGENE 6 (Roche Molecular Biochemicals, Tokyo, Japan) according to the manufacturer's instructions. At 2 days after transfection, 1 ml of the culture medium was collected and incubated with 10 μl of HIS-Select Cobalt Affinity beads (Sigma) for 1 h at 4 °C. The beads recovered by centrifugation were washed and then resuspended in the assay buffer described below, and GalNAcT transferase activity was assessed using polymer chondroitin (167 μg) as an acceptor. Reaction mixtures were incubated at 37 °C for 1 h, and radiolabeled products were then separated from UDP-[^{3}H]GalNAc by gel filtration using a syringe column, as described previously.[9]

Western blot analysis

After 2 days of culture, the culture medium was collected and incubated with 10 μl of HIS-Select Cobalt Affinity beads (Sigma) for 1 h at 4 °C. The beads recovered by centrifugation were washed with phosphate-buffered saline, resolved on 7.5% SDS-polyacrylamide gels, and proteins were transferred to a polyvinylidene difluoride membrane. The membrane was incubated for 1 h with an anti-ChGn-1 mouse antibody (Transgenic, Kobe, Japan). The antibody was diluted 1:1000 with 25 mM Tris-buffered saline. The bound antibody was detected with anti-mouse IgG conjugated with horseradish peroxidase and enhanced chemiluminescence.

RESULTS

Mutation analysis of patient DNA

In mutation analysis of the *ChGn-1* gene, we found two novel heterozygous missense mutations in patients with neuropathies, H234R (1355 A >G) in exon 5 from one patient and M509R (2180 T >G) in exon 10 from the other. Those mutations were not observed in 196 unrelated disease control DNA samples.

The patient with the H234R missense mutation was a 38-year-old man. He had been affected by acquired idiopathic generalized anhidrosis since his childhood. He had no apparent family history. He was also affected by hemi-facial palsy since the age of 34. The hemi-facial palsy developed similar to an episode of acute idiopathic facial palsy, so called Bell's palsy, but was irreversible.

The patient with the M509R mutation was a 25-year-old man. He had motor and sensory neuropathy without any apparent family history. He had experienced intermittent postural tremor since he was in elementary school. Nerve conduction studies revealed an absence of sensory nerve action potentials in the median, ulnar and sural nerves. The compound muscle action potentials were reduced (left median nerve: 2.88 mV (control: >4 mV), left ulnar nerve: 0.85 mV (>3 mV) and left tibial nerve: 0.19 mV (>7 mV)). Motor nerve conduction velocities were also decreased (left median nerve: 14.7 m s^{-1} (>45 m s^{-1}), left ulnar nerve: 13.6 m s^{-1} (>45 m/s) and left tibial nerve: 9.34 m s^{-1} (>40 m s^{-1})). He had been diagnosed with HMSN of unknown type.

Sequence analysis on PMP22 and MPZ, and PMP22 duplication and deletion study using fluorescence *in situ* hybridization method showed no abnormal findings in either patient.

Expression and glycosyltransferase activities of soluble forms of the ChGn-1 H234R and M509R mutants

To clarify whether these mutations of *ChGn-1* influence glycosyltransferase activities, we constructed soluble forms of the two ChGn-1

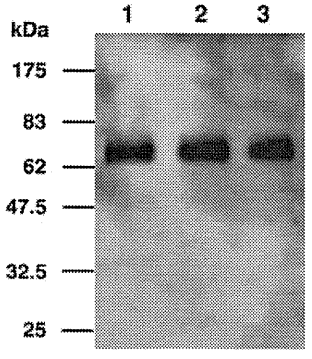

Figure 1 Western blot analysis of ChGn-1 H234R and ChGn-1 M509R. A soluble form of ChGn-1, ChGn-1 H234R or ChGn-1 M509R was expressed as a fusion protein tagged with 6× His in COS-1 cells as described in 'Materials and methods.' The recombinant proteins secreted in the medium were purified and then separated by SDS-PAGE, and the expression of each His-tagged protein was examined using an anti-ChGn-1 antibody. *Lane 1*, ChGn-1-His; *lane 2*, ChGn-1 M509R-His; *lane 3*, ChGn-1-H234R-His.

Table 1 The GalNAcT-II activity of the fusion proteins secreted into the culture medium by transfected COS-1 cells

Protein	*GalNAcT-II activity (pmol ml^{-1} medium per h)*[a]
ChGn-1	1.2
ChGn-1 mutant H234R	ND[b]
ChGn-1 mutant M509R	ND[b]

Abbreviation: ChGn-1, chondroitin beta-1,4-*N*-acetylgalactosaminyltransferase-1.
Values represent the average of three independent experiments.
[a]Polymer chondroitin was used as an acceptor substrate.
[b]ND, not detected (<0.01 pmol ml^{-1} medium per h).

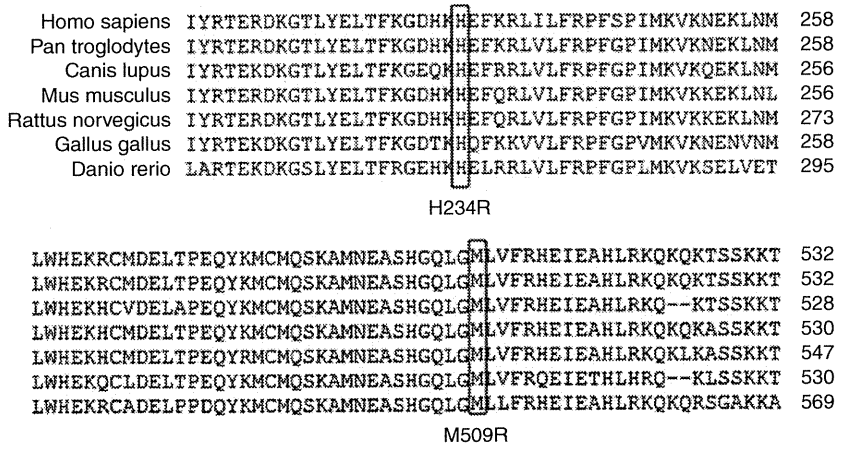

Figure 2 Amino acid sequence alignment of *ChGn-1* gene. H234R and M509R positions are highly conserved across species. Sequence include human (homo sapience; NP_060841.3), chimpanzee (Pan troglodytes; XP_519635.2), dog (Canis lupus; XP_539946.2), mouse (Mus musculus; NP_766341.3), rat (Rattus norvegicus; XP_224757.4), chicken (Gallus gallus; XP_420453.2) and zebra fish (Danio rerio; XP_001333479.2). Sequence were aligned using the NCBI homologene web site (http: //www.ncbi.nlm.nih.gov/ homologene).

H234R and M509R mutants. To examine the expression and activity of the mutant proteins, each of the soluble mutants was expressed in COS-1 cells and the culture medium was purified with HIS-Select Cobalt Affinity beads. The purified mutant proteins were used for western blotting analysis, and glycosyltransferase activities were assessed using chondroitin as an acceptor. When the soluble form of the ChGn-1 H234R and M509R mutants were expressed in COS-1 cells, proteins, ~70 kDa in size were secreted as shown by western blotting using an anti-ChGn-1 antibody (Figure 1). Although the ChGn-1 H234R and M509R mutant proteins were expressed (Figure 1, lanes 2 and 3), these mutant proteins showed no GalNAcT-II activities (Table 1).

We analyzed amino acid sequence alignment of *ChGn-1* gene. The novel coding mutations identified in this study changed the charge of the amino acid sequence. And the both mutation sites of *ChGn-1* genes are highly conserved across species from zebrafish to humans (Figure 2). The mutation of H234R and M509R would be possible to form a new salt-bridge and contribute to the formation of the protein turn around the positive-charged residue. Moreover, these two mutations were not represented in the single-nucleotide polymorphism database (http://www.ncbi.nlm.nih.gov/projects/SNP/).

DISCUSSION

In this study, we found two novel mutations in *ChGn-1* gene, both of which were associated with a profound decrease of their enzyme activity, in two patients with neuropathies of unknown etiology.

Proteoglycans are considered to be involved in the development of the nervous system. Defects in the production of CSPGs therefore may be associated with developmental errors in both the central and peripheral nervous systems. Complete loss of chondroitin polymerization has been studied in nematodes, indicating that condroitin is required for embryonic cytokinesis and cell division.[15,16]

In addition, CSPGs are known to have an important role in nerve injury. CSPG is known to be a major component of glial scarring. It is considered to be a major obstacle for recovery of the adult nervous system after injury, especially in light of its well-known activity in limiting axonal growth. *In vitro*, many CSPG family members have been reported to inhibit neurite outgrowth. However, the inhibitory activity of CSPGs does not necessarily mean that they are simple barrier molecules that contribute to nerve regeneration. They may avoid excessive fiber regeneration and inappropriate reinnervation.[17] In addition, in some situations, CSPG has been shown to strongly promote neurite outgrowth.[18] Thus, CSPG has a pivotal role in the repair of the injured spinal cord and in the recovery of motor function during the acute phase after injury.[17]

The peripheral nervous system is not as completely protected against injuries as the central nervous system. It should therefore be more vulnerable to minor trauma, such as compressions or bruises. Defect in the glycosyltransferases of the CSPGs, such as ChGn-1, may be associated with the pathogenesis of the peripheral neuropathies by disturbing the recovery from the minor trauma.

As the mutations we identified in this study are heterozygous mutations, the amount of CSPGs may not decrease profoundly in either case. We therefore consider that those mutations may not usually cause any obvious clinical disturbances. However, these mutations may reduce the effectiveness of the reparation of the peripheral nervous system because of poor productivity of CSPGs at the time of the emergency. It may be associated with the development of the irreversible hemi-facial palsy in the patient with the H234R mutation, whereas Bell's palsy is reversible in most of the cases. Another possible mechanism is toxic or dominant-negative effect. For example, as for superoxide dismutase mutation for familial amyotrophic lateral sclerosis, the pathogenetic mechanism is not from the loss of enzymatic activities.[19] This possibility should be investigated in future.

An association between the abnormality in the CSPG genes and skeletal diseases has been reported.[12] However, to the best of our knowledge, the association with neurological disorders has never been reported. The present investigation did not prove that the heterozygous *ChGn-1* mutations cause neuropathy in two patients. However, the possible association between the mutations and pathogenesis of neuropathy may exist. Studies on larger number of patients and complete family studies are necessary.

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Anti-GM1/GD1a complex antibodies in GBS sera specifically recognize the hybrid dimer GM1–GD1a

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It is now emerging the new concept that the antibodies from some patients with Guillain–Barré syndrome (GBS) recognize an antigenic epitope formed by two different gangliosides, a ganglioside complex (GSC). We prepared the dimeric GM1–GD1a hybrid ganglioside derivative that contains two structurally different oligosaccharide chains to mimic the GSC. We use this compound to analyze sera from GBS patients by high-performance thin-layer chromatography immunostaining and enzyme-linked immunosorbent assay. We also synthesized the dimeric GM1–GM1 and GD1a–GD1a compounds that were used in control experiments together with natural gangliosides. The hybrid dimeric GM1–GD1a was specifically recognized by human sera from GBS patients that developed anti-oligosaccharide antibodies specific for grouped complex oligosaccharides, confirming the information that GBS patients developed antibodies against a GSC. High-resolution ^{1}H-^{13}C heteronuclear single-quantum coherence-nuclear overhauser effect spectroscopy nuclear magnetic resonance experiments showed an interaction between the IV Gal-H1 of GM1 and the IV Gal-H2 of GD1a suggesting that the two oligosaccharide chains of the dimeric ganglioside form a single epitope recognized by a single-antibody domain. The availability of a method capable to prepare several hybrid gangliosides, and the availability of simple analytical approaches, opens new perspectives for the understanding and the therapy of several neuropathies.

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Introduction

High titers of antibodies that recognize carbohydrate epitopes shared by mammalian and bacterial cell membranes can be found in human sera following bacterial infection and in the course of such neuropathies, as Guillain–Barré syndrome (GBS; Kusunoki et al. 2008), promoted by them, by targeting the nodes of Ranvier or motor nerve terminals (Willison and Yuki 2002; Van Doorn et al. 2008).

Gangliosides are widely used in thin-layer chromatography (TLC) immunostaining and in enzyme-linked immunosorbent assays (ELISAs) to recognize serum anti-carbohydrate antibodies. The specificity of anti-oligosaccharide antibodies is variable. Some sera recognize several oligosaccharides, linked to lipids and/or proteins, with different complexity, whereas others seem highly specific (Kaida, Kamakura, et al. 2008). This not necessarily represents the antibody–antigen interaction properties in vivo as proved by the non-constant capability of anti-oligosaccharide antibodies to develop neuropathies when injected in animals (Paparounas et al. 1999). Glycolipids, and particularly gangliosides, are not randomly distributed within the membrane but rather form domains (Sonnino et al. 2006) that participate and modulate signal transduction processes. Oligosaccharides of membrane glycoconjugates, and particularly the oligosaccharides of glycolipids, are cryptic structures. Glycoproteins protrude from the plasma membrane into the extracellular environment, but neighboring proteins can prevent external interactions. The oligosaccharide chain of glycolipids protrude from the plasma membrane to a maximum of only 20–25 Å (Sonnino et al. 1994) and from many years are considered very cryptic for targeting by antibodies, lectins and enzymes (Prince 1992; Greenshields et al. 2009).

A new concept that is emerging in recent times suggests that a specific combination of oligosaccharide structure, of their position and clustering can form a distinct epitope for serum anti-oligosaccharide antibodies that not necessarily show binding properties for the individual oligosaccharides or part of them (Kaida et al. 2004; Kaida and Kusunoki 2010). This needs the availability at the same time of specific anti-oligosaccharide antibodies and of a specific plasma membrane organization, for the developing of a neuropathology.

The complexity of the natural membranes makes difficult to proof this using simple experimental models.

To overcome this, we prepared the dimeric GM1–GD1a hybrid ganglioside that contains two structural different oligosaccharide chains. We found that the serum IgG reactive with the GM1/GD1a complex actually recognize the GM1–GD1a hybrid dimer.

Results

The preparation of ganglioside dimers GM1–GM1, GD1a–GD1a and GM1–GD1a was carried out starting from lysogangliosides. Lysoganglioside preparations were described in great details (Neuenhofer et al. 1985; Sonnino et al. 1992; Mauri et al. 2004, Valiente et al. 2001) in the past and they are currently available in our laboratory.

The lysogangliosides are connected with adipic acid to form the dimer compounds. Figures 1 and 2 show schemes for the preparation of ganglioside dimers and for the hybrid dimer GM1–GD1a. The yield of preparation was good and determined to be 70% for dimers GM1–GM1 and GD1a–GD1a and ~50% for the hybrid dimer GM1–GD1a. TLC of the dimers (Figure 3) shows homogeneity over 98% for the three compounds that due to the high sugar content displayed a retention time lower than that of natural gangliosides. As expected, the chromatographic behavior of the hybrid dimer GM1–GD1a was intermediate between those of dimers GM1–GM1 and GD1a–GD1a.

The three dimers were characterized by mass spectrometry (MS) and nuclear magnetic resonance (NMR). By NMR, we also performed experiments aimed to have information on the three-dimensional structure of the hybrid dimer GM1–GD1a.

The synthesis of the three dimers started from lysogangliosides prepared from natural compounds extracted from calf brains. These gangliosides are a mixture of C18- and C20-sphingosine. Combining the lysoderivatives, each compound homogeneous in the oligosaccharide chains results heterogeneous in the lipid moiety, displaying the three combinations of sphingosine C18/C18, C18/C20 and C20/C20. MS confirmed the calculated molecular mass for the three compounds. The electro-spray ionization (ESI)-MS spectra show the main charged ions $[M-2H]^{2-}$ at 1334, 1348 and 1362, $[M-3H]^{3-}$ at 986, 995 and 1004 and $[M-4H]^{4-}$ at

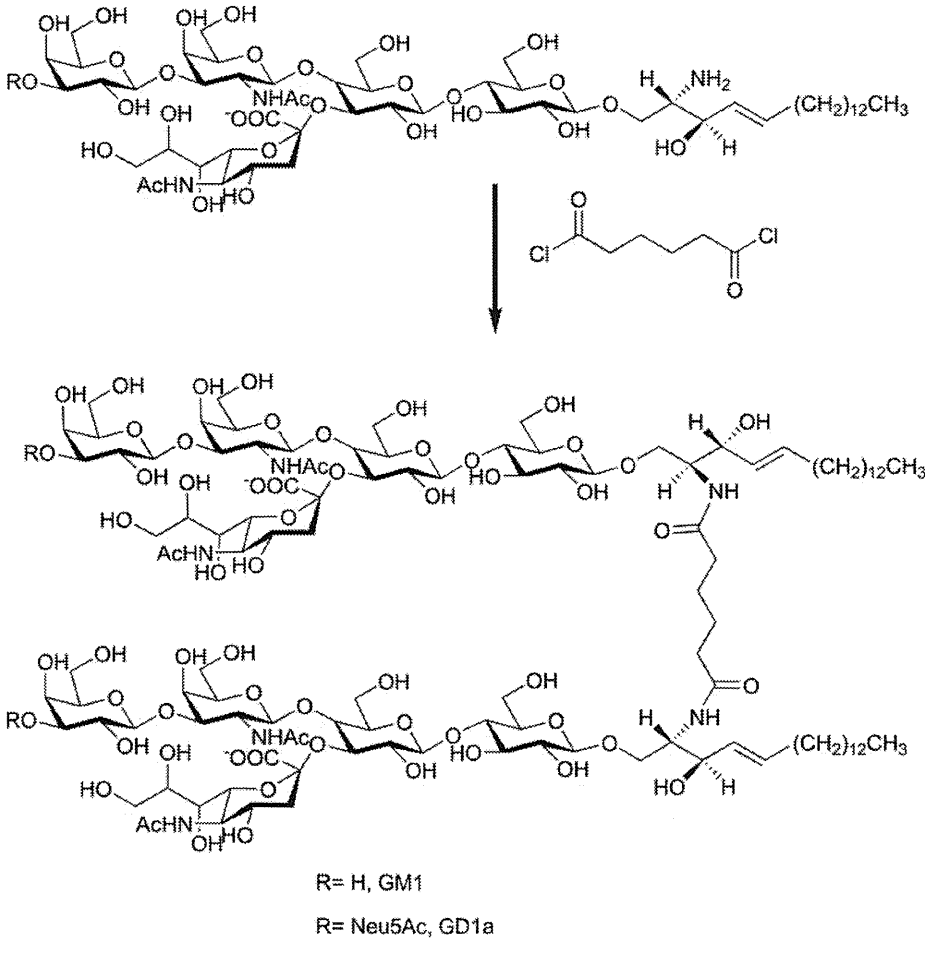

Fig. 1. Scheme of the preparation of ganglioside dimers GM1–GM1 and GD1a–GD1a.

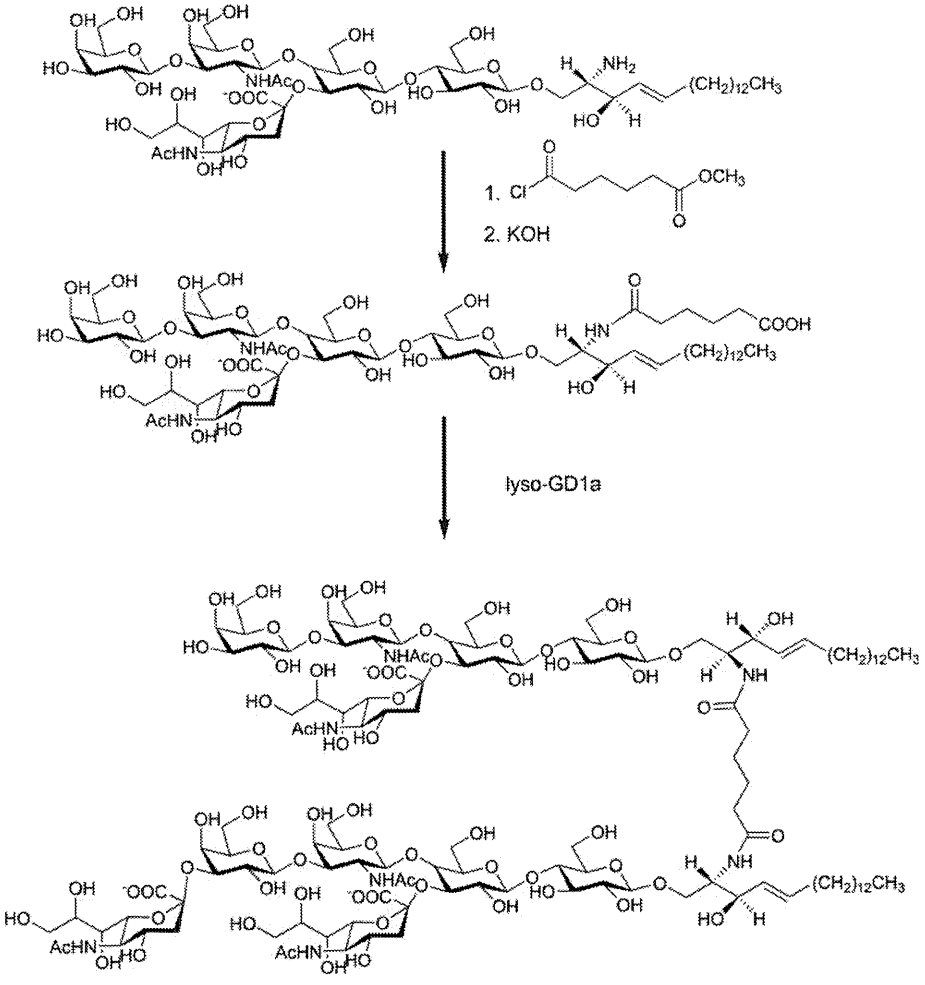

Fig. 2. Scheme of the preparation of ganglioside hybrid dimer GM1–GD1a.

812, 819 and 826 for GM1–GM1, GM1–GD1a and GD1a–GD1a, respectively. Figure 4 shows the mass spectra of the hybrid dimer GM1–GD1a. Less abundant signals corresponding to differently charged ions were also in the spectra.

The hybrid dimer GM1–GD1a was characterized by high-resolution NMR experiments. Table I reports the proton NMR assignments and Figure 5 a portion of the ^{1}H-^{13}C HSQC (heteronuclear single-quantum coherence)-NOESY (nuclear overhauser effect spectroscopy) spectrum. Amphiphilic compounds, like gangliosides and ganglioside/phospholipid mixtures, form big aggregates not suitable for NMR analyses in water solution. To mimic a membrane organization, experiments in water were carried out inserting the hybrid dimer GM1–GD1a into DPC aggregates. These aggregates have been shown to be small and to display high mobility in solution; this allowed to obtain high-resolution spectra (Poppe et al. 1994).

Figure 5 shows a correlation between the IV Gal-H1 of the monosialyl oligosaccharide chain and the IV Gal-H2 of the disialyl oligosaccharide chain. This suggests a possibility that the two oligosaccharide chains should be locked in a quite rigid conformation forming a single epitope.

GD1a has an additional sialic acid with respect to GM1 and it is very well separated by high-performance TLC (HPTLC). Figure 3 shows the results of TLC immunostaining with GBS serum. Both gangliosides were not recognized by the serum from a patient with GBS in the HPTLC-immunostaining procedure. Nevertheless, if GD1a is applied on the plate above GM1 to have a less separation from GM1, an immunostained spot with chromatographic behavior intermediate between GM1 and GD1a was observed. We know that the chromatographic resolution is not absolute and that each ganglioside is represented on the TLC plate by a large spot due to heterogeneity of the lipid moiety. Between the two spots of GM1 and GD1a, we expect to have a mixture of the more hydrophilic species of GM1 (short-chain fatty acids) and less hydrophilic species of GD1a (long-chain fatty acids). Of course, the serum antibodies recognized the GM1/GD1a mixture in a dot-spot assay.

The three dimers GM1–GM1, GD1a–GD1a and GM1–GD1a are separated each other by HPTLC. The dimeric hybrid GM1–GD1a was very well recognized by the GBS serum (Figure 3). As a control, we used dimeric GM1 and dimeric GD1a. No reactivity was observed with the patient

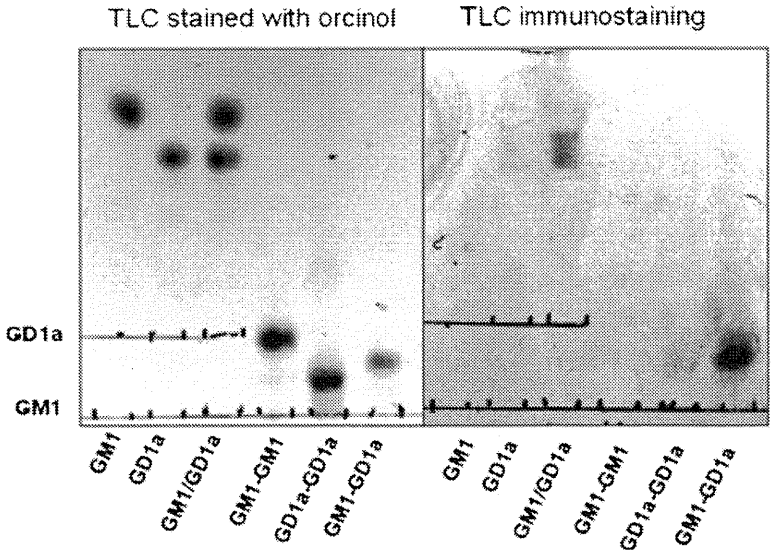

Fig. 3. HPTLC and HPTLC immunostaining of gangliosides and ganglioside dimers. Two micrograms of GM1, GD1a, a mixture of GM1 and GD1a, GM1–GM1 dimer, GD1a–GD1a dimer and GM1–GD1a hybrid dimer was applied to a TLC plate, developed with a solvent system chloroform:methanol:0.2% $CaCl_2$-$2H_2O$ (50:45:10, v/v). The left panel was stained by the orcinol reagent and the right panel was immunostained by a patient serum IgG (diluted 1:100) specifically reactive with a mixture of GM1 and GD1a. Anti-human IgG Fc, diluted to 1:200 with 1% BSA in PBS, was used as a secondary antibody. Each horizontal line indicates the start line for GM1 and GD1a. The start line for each dimer is same as that for GM1.

serum, excluding any possible artifact deriving by the presence of two general oligosaccharide chains. This strongly suggests that both the GM1 and GD1a chains are necessary for a strong interaction and to maintain the stability of the antibody–antigen complex.

Twelve sera that had been confirmed to be IgG positive for a mixture of GM1 and GD1a according to the criteria reported by Kaida et al. (2004) were used for the ELISA using the three dimers as an antigen (Table II). The 12 sera showed no reactivity against GM1 and no or minor reactivity against GD1a, suggesting that both the oligosaccharide chain must be present for antibody–antigen complex formation. Among the sera, 10 showed strong antibody activity to the GM1–GD1a hybrid dimer, whereas the two sera #3 and 11 did not. Sera #3 and 11, from patients that had ophthalmoplegia, showed a strong cross-reactivity against GD1b, GQ1b and GT1a. Within the sera that recognize the hybrid dimer GM1–GD1a, sera #4, 5 and 6 reacted with GD1b, but not with GQ1b or GT1a.

Discussion

Several data (Sonnino et al. 2006; Prinetti et al. 2009; Sonnino and Prinetti 2010; van Zanten et al. 2010) have been reported on the segregation of gangliosides in membranes and on the role of gangliosides in stabilizing lipid membrane domains known as lipid rafts. The existence of lipid rafts subdomains has been also reported (Lingwood and Simons 2010). This is probably one of the reasons that make the oligosaccharide chain cryptic reducing the possibility of an oligosaccharide–serum antibody interaction. Sometimes, this occurs and it is followed by a process of serious neuropathy. Sometimes, this interaction, in in vitro assays, requires an epitope obtained mixing two structurally different gangliosides. Anti-ganglioside complex (GSC) antibodies, in vivo, may recognize a conformational epitope formed by two different gangliosides on the lipid rafts.

Antibodies against a GM1/GD1a complex were detected in 39 of 234 GBS sera (Kaida et al. 2007). A subset of anti-GM1/GD1a-positive patients also had the antibodies against GD1a/GD1b and/or GD1b/GT1b complexes, which are significantly associated with severe GBS (Kaida et al. 2007). The antibodies against the GM1/GalNAc-GD1a complex were associated with pure motor GBS or acute motor conduction block neuropathy (Kaida, Sonoo, et al. 2008). The antibodies against GSCs containing GQ1b or GT1a are present in the Fisher syndrome and related disorders (Kanzaki et al. 2008). In contrast, the binding activities of the antibodies highly specific to GD1b, associated with sensory ataxic neuropathy, are decreased by the addition of such gangliosides as GD1a to GD1b antigen due to the interaction between the two gangliosides and resultant conformational change (Kaida, Kamakura, et al. 2008). A similar inhibitory effect of neighboring gangliosides on the antibody binding has also been reported by the other research groups (Greenshields et al. 2009; Nobile-Orazio et al. 2010). Therefore, the analyses of the clustered gangliosides on the plasma membrane are more and more important for clarifying the pathogenetic mechanisms of autoimmune neuropathies.

We successfully prepared the hybrid dimer GM1–GD1a. In addition, we also prepared the GM1–GM1 and GD1a–GD1a dimers that were very useful in biological assays as controls. The synthesis was carried out from lysogangliosides and did

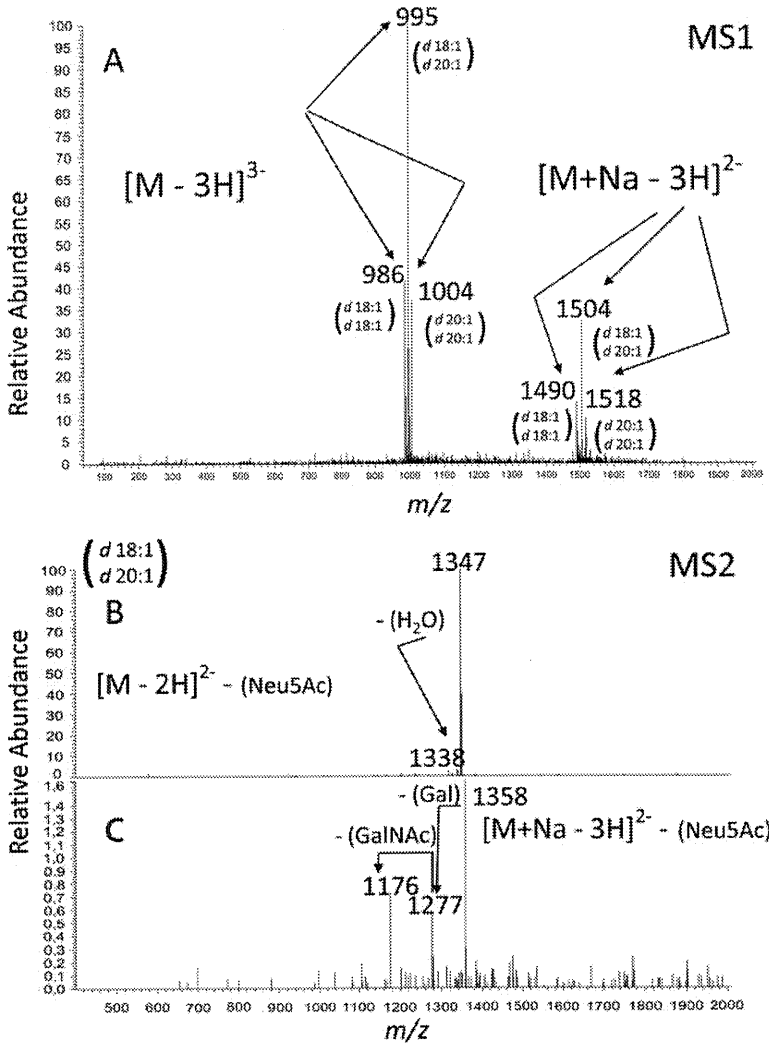

Fig. 4. Negative-ion ESI/MS spectra of ganglioside hybrid dimer GM1–GD1a. (**A**) MS1 spectrum of the double-charged ions and triple-charged ions, resulting from the ionization of the carboxylic group of sialic acid, from the three different possible combinations between lysogangliosides containing 18:1 and 20:1 sphingosine. (**B**) MS2 spectrum from ion at 995; the loss of an Neu5Ac residue originated bi-charged ions. (**C**) MS2 spectrum from ion at 1504.

not present specific difficulties and the final yields were from 50 to 70%. The availability of procedures for the preparation of several lysogangliosides, such as lyso-GM3, lyso-GM1, lyso-GD1a and lyso-GD1b, opens the possibility to prepare several ganglioside hybrid dimers.

Most of the antibodies specifically reactive with a mixture of GM1 and GD1a recognized the GM1–GD1a hybrid dimer, suggesting that, at least in some cases, the hybrid dimer actually mimics the GM1/GD1a complex, a novel antigen formed by the carbohydrate structures of GM1 and GD1a gangliosides.

However, the two sera #3 and 11 (Table I) did not recognize the GM1–GD1a dimer. Both of the patients 3 and 11 had ophthalmoplegia and anti-GQ1b and anti-GT1a IgG antibody activities in the sera. It indicates that a difference exists between the antigenic epitope of the GM1/GD1a complex and that of the GM1–GD1a dimer and that some of the IgG antibodies reactive with both GQ1b and GT1a could cross-react to the GM1/GD1a complex but not to the GM1–GD1a dimer. These results suggest that the position and the features of protein domain(s) interacting with the epitope(s) are different, also if all our sera recognized the GM1/GD1a complex.

Many further NMR experiments will be necessary to have the complete secondary structure of the two chains of the hybrid dimer GM1–GD1a. This is due to the complexity of the spectra, to some pick overlapping and to the fact that some interactions occur via water bridges (Brocca et al. 1998). Nevertheless, while chemical-shift assignments suggest that the conformation reported for the chains in the natural gangliosides should be maintained in the hybrid dimer GM1–GD1a, a defined interaction between the two external galactoses could be observed (Figure 5). Thus, the covalent linkages of the two glucoses with the lipid moiety and the hydrogen bond interactions at the end of the two chains

Table I. Chemical shifts of hybrid dimer GM1–GD1a in DPC aggregates in D_2O at 30°C

GM1 part				GD1a part				Ceramide part		
		^1H (ppm)	^{13}C (ppm)			^1H (ppm)	^{13}C (ppm)		^1H (ppm)	^{13}C (ppm)
Glc (I)	1	4.40	103.01	Glc (I)	1	4.40	103.01	1a	3.69	69.55
	2	3.29	72.79		2	3.29	72.79	1b	4.13	69.61
	3	3.60	74.33		3	3.60	74.33	2	3.87	53.45
	4	3.56	79.05		4	3.56	79.05	3	4.01	71.10
	5	3.54	74.92		5	3.54	74.92	4	5.37	130.23
	6	3.75, 3.93	60.31		6	3.75, 3.93	60.31	5	5.66	133.25
Gal (II)	1	4.48	102.84	Gal (II)	1	4.48	102.84	6	1.93	32.76
	2	3.32	70.09		2	3.32	70.09	7		
	3	4.10	74.47		3	4.10	74.47	8		
	4	4.08	77.36		4	4.08	77.36	9		
	5	3.71	74.32		5	3.71	74.32	10		
	6	3.7–3.8	60.5–61.3		6	3.7–3.8	60.5–61.3	11	1.2–1.3	29.0–30.3
GalNAc (III)	1	4.74	102.66	GalNAc (III)	1	4.74	102.66	12		
	2	4.00	51.17		2	4.00	51.17	13		
	3	3.76	80.65		3	3.76	80.65	14		
	4	4.12	67.98		4	4.12	67.98	15		
	5	3.68	74.50		5	3.68	74.50	16		
	6	3.7–3.8	60.5–61.3		6	3.7–3.8	60.5–61.3	17	1.20	22.50
	7				7		174.73	CH_3	0.77	13.52
	8	1.96	22.75		8	1.96	22.75			
Gal (IV)	1	4.49	104.90	Gal (IV)	1	4.56	104.66			
	2	3.48	70.80		2	3.49	69.26			
	3	3.58	72.73		3	4.04	75.63	Linker part		
	4	3.87	68.75		4	3.91	67.59		^1H (ppm)	^{13}C (ppm)
	5	3.64	74.93		5	3.63	74.77	1	2.11, 2.21	35.65
	6	3.7–3.8	60.5–61.3		6	3.7–3.8	60.5–61.3	2	1.49, 1.54	25.20
NeuAc (A)	1			NeuAc (A)	1		180.60			
	2				2		101.52			
	3a	1.89	37.13		3a	1.89	37.13			
	3e	2.62	37.14		3e	2.62	37.14			
	4	3.74	68.52		4	3.74	68.86			
	5	3.77	51.75		5	3.77	51.75			
	6	3.45	73.23		6	3.45	73.23			
	7	3.55	68.24		7	3.55	68.24			
	8	3.71	72.36		8	3.71	72.36			
	9a	3.59	62.97		9a	3.59	62.97			
	9b	3.83	62.97		9b	3.83	62.97			
	10				10		175.07			
	11	1.99	22.21		11	1.99	22.21			
				NeuAc (B)	1		174.17			
					2		99.86			
					3a	1.76	39.74			
					3e	2.71	39.78			
					4	3.64	68.53			
					5	3.79	51.82			
					6	3.58	72.96			
					7	3.55	68.24			
					8	3.85	71.95			
					9a	3.59	62.74			
					9b	3.83	62.73			
					10		175.07			
					11	1.99	22.21			

should arrange the eight neutral sugars in a quite fixed and rigid conformation. This suggests that the GM1–GD1a oligosaccharide system behave as a single epitope recognized by a single-antibody domain. This domain is present in 10 of the 12 sera analyzed. The remaining two sera #3 and 11 should have two distinct domains, one for the GM1 oligosaccharide and one for the GD1a oligosaccharide. Thus, these two sera are capable to recognize a mixture of GM1 and GD1a but not the single epitope formed by the two interacting chains.

The availability of hybrid gangliosides, containing two or more oligosaccharide chains, mimicking the cluster of oligosaccharide chains occurring on cell membranes, provides a new tool to analyze anti-oligosaccharide and correlated them with the clinical features of the pathology. In addition to this, their use to generate monoclonal antibodies could be useful to develop animal models. It should open new perspectives for understanding the pathogenesis and developing a novel therapy of GBS.

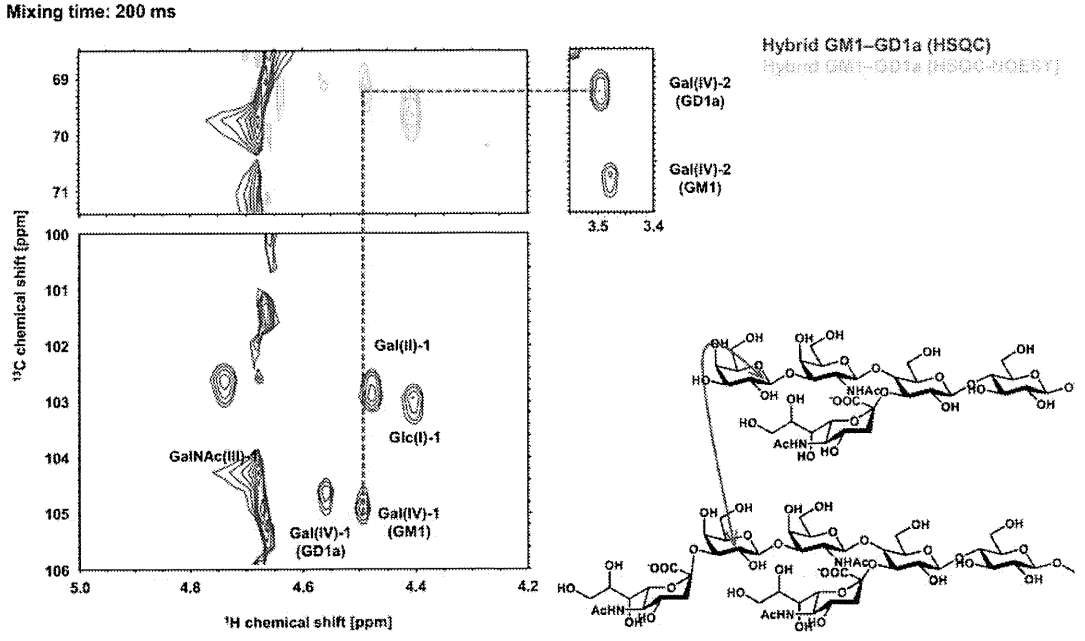

Fig. 5. Portion of the ^{1}H-^{13}C HSQC-NOESY spectrum of ganglioside hybrid dimer GM1–GD1a mixed with DPC-d_{38} in 10 mM phosphate buffer (pH 7.0) D_2O solution at 2.0 mM final concentration. Mixing time was 200 ms.

Table II. OD of the ELISA

Patient number	GM1	GD1a	GM1/ GD1a mixture	GM1–GM1 dimer	GD1a–GD1a dimer	GM1–GD1a hybrid dimer	IgG antibodies to other gangliosides
1	<0.1	<0.1	0.516	<0.1	<0.1	0.799	No
2	<0.1	0.154	0.52	0.134	0.104	0.489	No
3	<0.1	0.145	0.65	<0.1	<0.1	<0.1	GD1b, GQ1b, GT1a
4	<0.1	<0.1	0.664	<0.1	<0.1	0.583	GD1b
5	<0.1	<0.1	0.49	0.111	<0.1	0.742	GD1b
6	<0.1	0.135	0.519	<0.1	<0.1	0.548	GD1b
7	<0.1	0.199	0.497	<0.1	0.131	0.482	No
8	<0.1	0.111	0.671	<0.1	0.151	0.678	No
9	<0.1	<0.1	0.605	<0.1	<0.1	0.34	No
10	<0.1	<0.1	0.594	<0.1	0.127	0.68	No
11	<0.1	<0.1	0.382	<0.1	<0.1	<0.1	GD1b, GQ1b, GT1a
12	<0.1	0.218	0.774	<0.1	0.101	0.751	No

Antigens: GM1, GD1a, GM1 and GD1a mixture, GM1–GM1 dimer, GD1a–GD1a dimer and GM1–GD1a hybrid dimer.

Materials and methods

Chemicals

LiChroprep RP18 for column chromatography (particle size, 40–63 μm), silica gel 100 (particle size, 63–200 μm) and 60 (particle size, 15–40 μm), Amberlite® IR-120 H^+ form (particle size 300–900 μm) and high-performance silica gel-precoated thin-layer plates (HPTLC, Kieselgel 60) were obtained from Merck (Darmstadt, Germany). All the chemicals were of the highest purity available. The solvents were distilled before use and deionized water was freshly distilled in a glass apparatus. Dialysis tubes 12.000–14.000 Da were from Medicell International Ltd (London).

Gangliosides GM1 and GD1a were extracted from calf brain, purified to homogeneity and characterized by NMR and MS. Lyso-GM1 and lyso-GM1 were prepared from the corresponding natural gangliosides (Neuenhofer et al. 1985; Sonnino et al. 1992).

Methods

Preparation of GM1 and of GD1a dimers. To a solution of 0.15 M adipic acid in CH_2Cl_2/toluene 2:1 by vol., five equivalents of thionyl chloride were added drop wise at −10° C (Figure 1). After standing for 10 min at the same temperature, the reaction mixture was heated at 50°C for 3 h under reflux. The exceeding of thionyl chloride was removed by evaporation at reduced pressure and the product used without further purification. Adipoyl chloride and tributylamine were added to a solution of lyso-GM1 or lyso-GD1a in anhydrous CH_2Cl_2/CH_3OH 1:2 by vol. (50 μmol/mL) to a final molar ratio of 0.5:2:1. After vigorous stirring for 2 h at room temperature, the reaction mixture was dried and the residue purified by flash chromatography with silica gel 60 column equilibrated and eluted with $CHCl_3/CH_3OH/H_2O$ 60:35:5, by volume. The yield of reaction, after purification, was 70%.

Preparation of hybrid GM1–GD1a dimer. To a solution of 0.15 M mono-methyl adipate in CH_2Cl_2/toluene 2:1 by vol., five equivalents of thionyl chloride were added drop wise at −10°C (Figure 2). After standing for 10 min at the same

temperature, the reaction mixture was heated at 50°C for 3 h under reflux. The exceeding of thionyl chloride was removed by evaporation at reduced pressure and the product used without further purification.

Methyladipoyl chloride and tributylamine were added to a solution of lyso-GM1 in anhydrous CH_2Cl_2/CH_3OH 1/2 (50 μmol/mL) to a final molar ratio of 1:2:1. After vigorous stirring at room temperature for 2 h, the reaction mixture was dried and the residue was dissolved in 0.5 M KOH in CH_3OH. After standing at room temperature for 18 h, the solution was neutralized and the solvent was removed. The residue was dissolved in water and the salts removed by dialysis. After lyophilization, the GM1 adipate was obtained as a white powder and used without further purification. The reaction mixture was analyzed by HPTLC, using the solvent systems $CHCl_3/CH_3OH$/0.2% $CaCl_2$ 50:42:11 by volume.

Dicyclohexylcarbodiimide and *N*-hydroxysuccinimide, both at a final concentration of 0.02 M, were added to a 0.017 M solution of GM1 adipate, in dry dimethylformamide. After stirring for 30 min at room temperature, the lyso-GD1a was added to give a final concentration of 0.02 M. The reaction was allowed to proceed at 60°C under vigorous stirring for 1 h. The reaction mixture was dried and the residue purified by flash chromatography with silica gel 60 column equilibrated and eluted with $CHCl_3/CH_3OH/H_2O$, 60:35:5 by vol. The yield of reaction, after purification, was 70%.

High-performance TLC. Ganglioside, ganglioside derivatives, ganglioside dimers and the reaction mixtures were analyzed by HPTLC using the solvent systems $CHCl_3/CH_3OH$/0.2% $CaCl_2$, 50:42:11 by vol., $CHCl_3/CH_3OH$/0.2% $CaCl_2$/50 mM KCl, 50:50:4:8 by vol. or $CHCl_3/CH_3OH/(CH_3)_2CHOH$/50 mM KCl, 30:35:15:13 by vol., according to the polarity of compounds.

Gangliosides, ganglioside derivatives and dimers were visualized on the HPTLC plates by treatment with anisaldehyde and *p*-dimethylaminobenzaldehyde spray reagents followed by heating at 130°C; amine-containing compounds were visualized by treatment with 20% methanolic ninhydrin followed by heating at 80°C.

For HPTLC immunostaining, 2 μg of gangliosides GM1 and GD1a of dimers GM1–GM1, GD1a–GD1a and GM1–GD1a were applied to the TLC plate and developed with the solvent of chloroform/methanol/aqueous 0.2% $CaCl_2$ 50:45:10, by vol. The line of application for GD1a was 1 cm above that for GM1 and the dimers. Sera positive for the IgG anti-GM1/GD1a complex antibody were overlaid for the TLC plate at a dilution of 1:100 (Kaida et al. 2004). Peroxidase-conjugated goat anti-human IgG Fc antibody (diluted 1:200, ICN Biomedicals Inc., Aurora, OH) was the secondary antibody. Immunoreactants were made visible with phosphate-buffered saline (PBS) containing 0.01% H_2O_2 and 50 mg/dL 3,3′-diaminobenzidine tetrahydrochloride.

Enzyme-linked immunosorbent assay. Sera from 12 GBS patients that displayed the IgG antibody against a mixture of GM1 and GD1a were examined for their reactivity against the hybrid dimer GM1–GD1a. ELISA was performed using purified gangliosides GM1 and GD1a, a mixture of GM1 and GD1a, the dimers GM1–GM1, GD1a–GD1a and GM1–GD1a. Each microtiter well was coated with 0.2 μg of each antigen. Serum diluted 1:40 with 1% bovine serum albumin (BSA) in PBS was added to wells, followed by the procedure as described previously (Kaida et al. 2004). Peroxidase-conjugated goat anti-human IgG Fc antibody (diluted 1:200, ICN Biomedicals Inc.) was the secondary antibody. A color reaction was obtained by incubation with 200 μL of orthophenylenediamine dihydrochloride (40 mg/dL of phosphate–citrate buffer, pH 5.0) at room temperature for 2 min. The reaction was stopped by the addition of 8 N H_2SO_4, after which the optical density (OD) at 492 nm was read with an ELISA reader. OD values were corrected by subtracting the OD of an uncoated well that had been processed similarly (Table II). When the corrected OD was >0.1, the serum was considered positive. ELISAs were repeated twice in the same way, and the mean OD of the two experiments was calculated. Antibody activities against other gangliosides, such as GM2, GM3, GD1b, GD3, GalNAc-GD1a, GT1a, GT1b and GQ1b, also were examined as described elsewhere (Kaida et al. 2004).

Mass spectrometry. ESI-MS was carried out in a negative mode on a ThermoQuest Finnigan LCQdeca mass spectrometer equipped with an electrospray ion source and an Xcalibur™ data system. Samples were dissolved in methanol at a concentration of 20–200 ng/μL prior to direct injection into the electrospray ionization ion-trap mass spectrometer (MS). Ions were monitored as MS1 or MS2 product ions. Ionization was performed under the following conditions: spray voltage, 4 kV; sheath gas flow rate, 50 arbitrary units; capillary temperature, 260°C; capillary voltage, −42 V. The scanning range was *m/z* 200–1600, and fragmentor voltage for collision induced dissociation was 25–90%.

Nuclear magnetic resonance. Two-dimensional NMR experiments (HSQC, heteronuclear 2 bond correlation, heteronuclear multiple bond correlation, chemical-shift correlated spectroscopy, HSQC-total correlation spectroscopy, HSQC-NOESY and NOESY) were performed with JEOL ECA-920 or JEOL ECA-600 spectrometers at 30°C. The pulse delay time of 2 s was used. All NMR spectra were processed and analyzed using Delta (JEOL) and Sparky (T. D. Goddard and D. G. Kneller: SPARKY 3, University of California, San Francisco, CA). Experiments were carried out on the hybrid dimeric GM1–GD1a inserted in a micelle of dodecylphosphocholine (DPC)-d_{38} in 10 mM phosphate buffer (pH 7.0) D_2O solution. The hybrid dimer GM1–GD1a/DPC molar ratio was 1:52 and the final hybrid dimer GM1–GD1a concentration was 2.0 mM.

Quantitative determinations. Gangliosides, ganglioside derivatives and ganglioside dimers were quantified by their sialic acid content using the HCl–resorcinol method and pure *N*-acetylneuraminic acid as a reference standard (Svennerholm 1957).

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Conflict of interest

None declared.

Abbreviations

BSA, bovine serum albumin; DPC, dodecylphosphocholine; ELISA, enzyme-linked immunosorbent assay; ESI, electrospray ionization; GBS, Guillain–Barré syndrome; GD1a, $IV^3NeuAc,II^3NeuAc\text{-}Gg_4Cer$; GD1b, $(II^3NeuAc)_2\text{-}Gg_4Cer$; GM1, $II^3NeuAc\text{-}Gg_4Cer$; GQ1b, $(IV^3NeuAc)_2,(II^3NeuAc)_2\text{-}Gg_4Cer$; GT1a, $(IV^3NeuAc)_2,II^3NeuAc\text{-}Gg_4Cer$; GSC, ganglioside complex; HPTLC, high-performance thin-layer chromatography; HSQC, heteronuclear single-quantum coherence; MS, mass spectrometry; NMR, nuclear magnetic resonance; NOESY, nuclear overhauser effect spectroscopy; OD, optical density; PBS, phosphate-buffered saline; TLC, thin-layer chromatography.

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