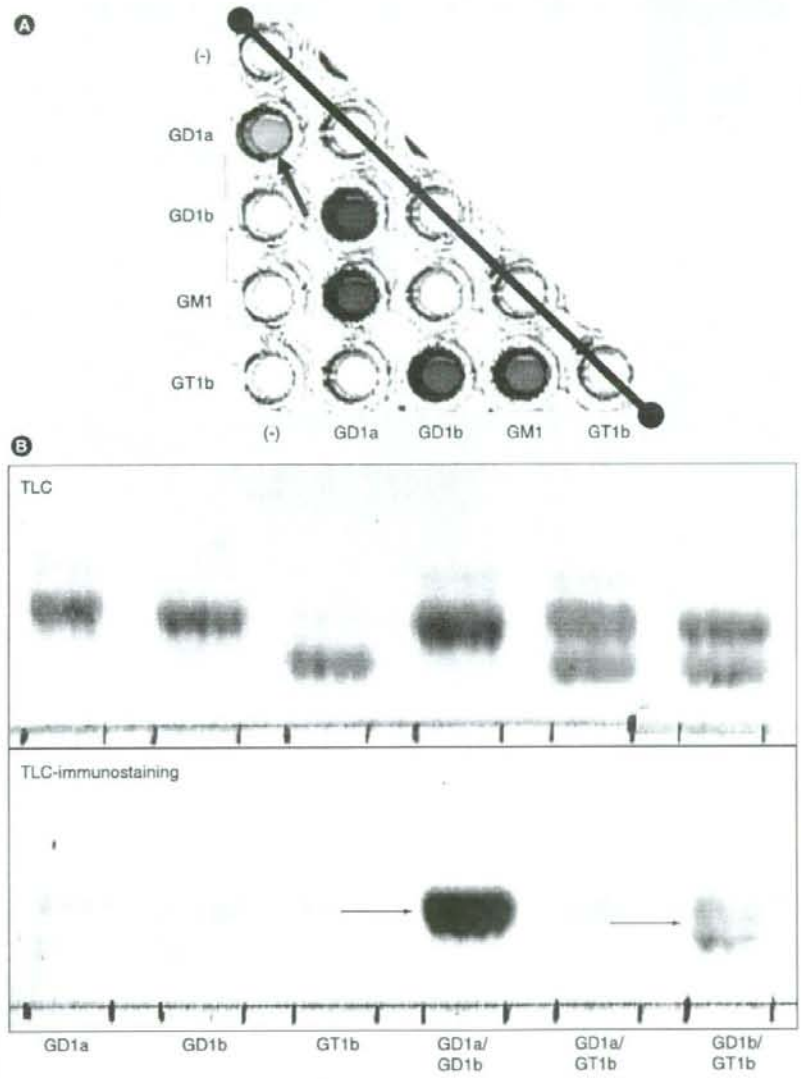
GBS and FS patients, and labeled these antibodies as anti-GSC antibodies [17,18]. We have demonstrated that anti-GSC antibodies might function in the development of GBS and FS [18,19], and the clinical and pathophysiological significance of anti-GSC antibodies in GBS and related disorders is described here.

Antibodies to ganglioside complexes in Guillain–Barré syndrome

To detect serum antiganglioside antibodies, single gangliosides have generally been used as the test antigens for ELISA in various laboratories, and purification of antigens has been considered to be important for identification of antiganglioside antibodies. Meanwhile, TLC-immunostaining can be useful for the detection of antibody activity to unidentified antigens when a crude fraction of bovine brain gangliosides is applied. We first observed positive staining just below GD1a in TLC-immunostaining using serum from a GBS patient with severe tetraplegia, whereas ELISA with the serum produced negative results on the test gangliosides (GM1, GM2, GM3, GD1a, GalNAc-GD1a, GD1b, GD3, GT1a, GT1b and GQ1b) [17]. However, when GD1a and GD1b were developed to overlap in the same lane (developing solvent, chloroform[C]/methanol[M]/0.2%$CaCl_2$–$2H_2O$ = 50/45/10, volume/volume), TLC-immunostaining studies demonstrated that the serum IgG reacted strongly with the overlapping portion of them. In another developing solvent (C/M/0.2%$CaCl_2$–$2H_2O$ = 30/65/10) that clearly separated the positions of GD1a and GD1b, the immunoreaction disappeared. In ELISA with GD1a, GD1b and a mixture of both, the serum IgG had a positive reaction only in a well coated with the mixture of GD1a and GD1b. The optimal mixture rate of GD1a and GD1b was approximately 1:1. These findings make it clear that the mixture of GD1a and GD1b induces formation of the GSC GD1a/GD1b with a novel glycoepitope entirely different from that of either GD1a or GD1b. We conducted further studies using other major gangliosides, GM1 and GT1b, in the human PNS, demonstrating that the anti-GD1a/GD1b-positive serum had strong activity against GM1/GD1a, GM1/GT1b and GD1b/GT1b, with no response to GM1, GD1b, GD1a and GT1b (Figure 1). Thus, the anti-GSC antibody does not bind to each constituent ganglioside, but binds to *de novo* epitopes formed in the GSCs. These findings may indicate that GSCs exist in the biological membrane and their clustered glycoepitopes are actually involved in immune-mediated events.

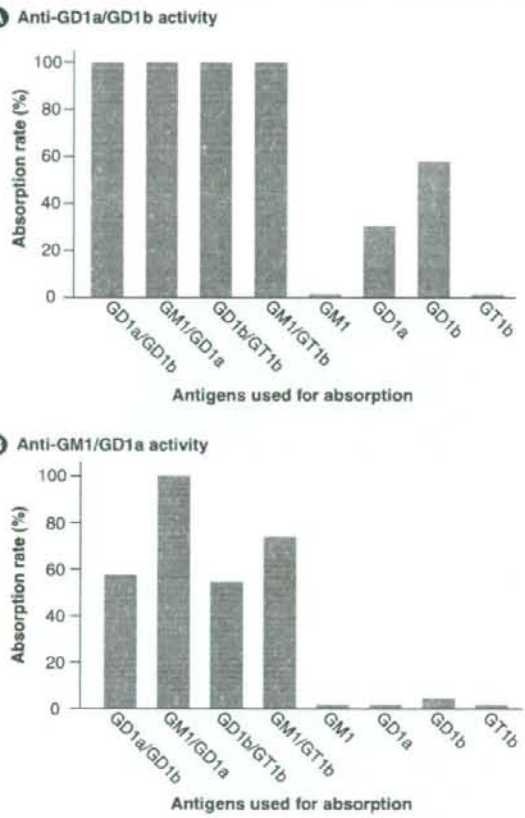
Next, we surveyed a large population of GBS patients on antibodies to ganglioside complexes consisting of two of the four major gangliosides (GM1, GD1a, GD1b and GT1b) [19]. An ELISA was performed in a grid manner with horizontal and vertical mixing lines (Figure 1A). The mixture rate of two gangliosides was 1:1 (weight/weight). Since anti-GSC antibodies often exhibited, to some extent, reactivity with constituent gangliosides of the GSCs, we decided that the detection criteria of anti-GSC antibodies (e.g., anti-GD1a/GD1b antibody); when optical density (OD) corresponding to the anti-GD1a/GD1b antibody was first, 0.2 higher than that corresponding to anti-GD1a or anti-GD1b antibody and second, was more than the sum of those of anti-GD1a and anti-GD1b antibodies, the sera were judged to be anti-GD1a/GD1b-positive. The cutoff value (0.2) for anti-GSC antibodies is decided arbitrarily. A total of 39 (17%) of 234 GBS patients studied had IgG antibodies to at least one of GSCs. All the 39 patients had anti-GM1/GD1a antibodies, 27 had anti-GM1/GT1b antibodies, 16 had anti-GD1a/GD1b antibodies and 13 had GD1b/GT1b antibodies. Clinical analysis indicated close association between presence of antibodies to GD1a/GD1b or GD1b/GT1b and severe disability requiring mechanical ventilation [18]. The rate of mechanical ventilation was 46% in anti-GD1b/GT1b-positive and 38% in anti-GD1a/GD1b-positive patients, significantly higher compared with 11% in anti-GSC-negative patients. Most of the anti-GD1a/GD1b or anti-GD1b/GT1b antibodies also reacted with GM1/GT1b as well as GM1/GD1a. Considering results of immunoabsorption study (Figure 2), it is likely that anti-GSC antibodies specifically react with clustered glycoepitopes common to these GSCs, rather than individually with each GSC. Interestingly, anti-GSC antibodies never bound to GD1a/GT1b and rarely to GM1/GD1b, implying that a particular combination of gangliosides is apt to form glycoepitopes closely involved in antibody-mediated response. When mixtures of three or four gangliosides were put in a well and used as antigens in ELISA, most anti-GSC antibodies showed a decrease or no change in their activity. For example, anti-GM1/GD1a antibodies, which also had activity to GD1a/GD1b showed less reactivity to a mixture of GM1,

Figure 1. Antiganglioside complex antibodies in a serum sample from a patient with Guillain–Barré syndrome.



(A) ELISA is performed in a grid way on a microtiterplate. Strong reactions are found in wells of GD1a/GD1b, GM1/GD1a, GM1/GT1b and GD1b/GT1b, with a weak reaction in a well of GD1a (arrow), but no reactions in wells of the single ganglioside antigens, GM1, GD1b or GT1b. Serum is diluted to 1:40. A diagonal solid line indicates blank wells (control wells). **(B)** TLC study. TLC bands are visualized with orcinol reagent in the upper panel. TLC immunostaining study demonstrates that overlapping portions between GD1a and GD1b and between GD1b and GT1b, are strongly stained (lower panel, arrow). Serum is diluted to 1:100. Horseradish peroxidase-conjugated anti-human IgG antibody is used as a secondary antibody. 3 µg of each ganglioside is applied to each lane on the TLC plate. The developing solvent comprised of chloroform, methanol, and 0.2% $CaCl_2.H_2O$ (volume/volume, 55/40/10).
TLC: Thin-layer chromatogram.

Figure 2. Immunoabsorption study on antiganglioside complex antibodies.



Patient's serum with antibody activities to GD1a/GD1b, GM1/GD1a, GD1b/GT1b and GM1/GT1b is used for this study. Each antiganglioside complex (GSC) antibody is absorbed in antigen-coated ELISA wells of a microtiter plate. The absorption rate of the anti-GSC antibody activity is calculated: absorption rate (%) = (1 – the corrected optical density [OD] after absorption in antigen-coated wells ÷ the corrected OD after absorption in antigen-uncoated wells) × 100. **(A)** IgG activity of anti-GD1a/GD1b antibody was absorbed completely by GSC antigens, but barely by single ganglioside antigens. **(B)** Similarly, IgG anti-GM1/GD1a antibody activity was absorbed to a greater extent by the GSC antigens than by the single antigens. IgG activities of anti-GD1b/GT1b and GM1/GT1b antibodies were also absorbed thoroughly by the complex antigens (data not shown). These findings imply that GSCs such as GD1a/GD1b, GM1/GD1a, GD1b/GT1b and GM1/GT1b have glycoepitopes with mutual conformation.

GD1a and GD1b than to a mixture of GM1 and GD1a or a mixture of GD1a and GD1b. In view of a specific combination of constituent gangliosides of GSCs, an epitope formed by a combination of [Galβ1–3GalNAc] and [NeuAcα2–3Galβ1–3GalNAc] in the terminal moieties of ganglio-*N*-tetraose structures is likely to be essential for antibody binding (Figure 3).

Further studies are required to elucidate the clinical significance and pathogenic role of anti-GSC antibodies. Clinical features of anti-GSC antibody-positive GBS also need to be confirmed in western countries, because the subjects in our studies were mostly Japanese. Why anti-GD1a/GD1b and GD1b/GT1b antibodies are associated with severe disabilities remains to be settled. Given that GSCs have more multivalent glycoepitopes than single gangliosides, the binding of anti-GSC antibodies to clustered glycoepitopes is considered to be extremely tight and can induce more severe nerve dysfunction, which may explain a predisposition to a severe form of GBS with the anti-GD1a/GD1b or anti-GD1b/GT1b antibody.

Antiganglioside complex antibodies in Fisher syndrome

As described above, the presence of IgG anti-GQ1b antibody has been an excellent diagnostic marker for FS [17]. In view of the pathophysiological similarity between GBS and FS, we extended an investigation of anti-GSC antibodies to FS patients. Aside from the four major gangliosides (GM1, GD1a, GD1b and GT1b), GQ1b and GT1a were used as test gangliosides. Of 12 FS patients studied, ten had IgG anti-GQ1b antibodies and seven patients had antibodies to GSCs, such as GQ1b/GM1, GQ1b/GD1b, GQ1b/GD1a, GQ1b/GT1b, GT1a/GM1, GT1a/GD1b and GT1a/GD1a, but not to the complexes without GQ1b and GT1a [18]. No FS patients had antibodies to GSCs consisting of two of the four major gangliosides, which may be a cleavage between FS and GBS.

Although FS is a clinically and serologically well defined, relatively homogeneous syndrome, specificities of antiganglioside antibodies were not homogeneous, even in such small samples. According to the ELISA results for anti-GSC antibodies, there are at least three different specificities in FS-associated antibodies:

- Monospecific to GQ1b (no anti-GSC antibodies)
- GQ1b/GM1-reactive
- GQ1b/GD1a-reactive (Table 1)

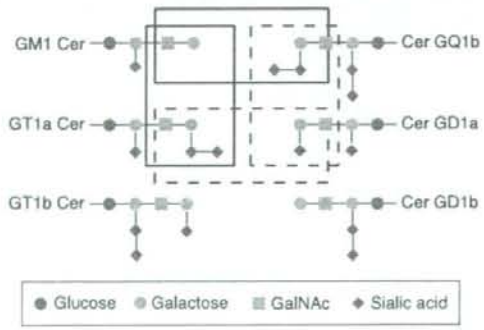
Immunoabsorption studies indicated that anti-GSC antibodies in FS bind to clustered glycoepitopes common to several GSCs. For

example, anti-GQ1b/GM1 antibodies also reacted with GT1a/GM1 or GT1a/GD1b. Considering the combination of constituent gangliosides in GSCs, a combination of [Galβ1–3GalNAc] and [NeuAcα2–8NeuAcα2–3Galβ1–3GalNAc] in the terminal residues of ganglio-*N*-tetraose structures is likely to be an important antigenic epitope for anti-GQ1b/GM1-reactive antibodies (Figure 3). Conversely, a combination of [NeuAcα2–3Galβ1–3GalNAc] and [NeuAcα2–8NeuAcα2–3Galβ1–3GalNAc] in the terminal residues may be an antigenic epitope for anti-GQ1b/GD1a-reactive antibodies (Figure 3). Further investigation in a larger population of patients is required to address issues related to clinical significance of antibodies to GSCs containing GQ1b or GT1a.

There have been many data indicating that the IgG anti-GQ1b antibody plays a crucial role in the development of FS [15,16,20–25]. Most of them have been obtained from immunohistochemical, *ex vivo*, or *in vitro* studies using anti-GQ1b monoclonal antibodies. In view of clustered glycoepitopes of glycolipids and glycoproteins on the plasma membrane, GQ1b is likely to form GSCs together with other gangliosides in the membrane, rather than to be isolated. Binding ability or binding sites may be different between anti-GQ1b monoclonal antibodies and antibodies to GSCs containing GQ1b, or between GQ1b and GSCs containing GQ1b. Similar investigation with antibodies to GSCs containing GQ1b can promote further understanding of pathogenetic mechanism of FS and is useful for elucidation of clinical diversity seen in anti-GQ1b antibody-associated disorders, such as GBS with ophthalmoplegia, Bickerstaff's brainstem encephalitis or acute ophthalmoparesis [26].

Specific immunoadsorption therapy to remove antibodies can palliate disabilities of patients with GBS or FS [27,28]. The synthetic disialylgalactose immunoaffinity columns with the minimal epitopes of GQ1b and GT1a may be useful for eliminating anti-GQ1b antibodies from sera [29]. Our data, however, suggest that an immunoabsorption column with the epitopes of GSCs, such as GQ1b/GM1 and GQ1b/GD1a, is required in a half of FS patients to achieve optimal effects.

Figure 3. Carbohydrate structures of GQ1b, GT1a, GD1b, GM1, GT1b and GD1a.



Squares with solid lines indicate a combination of [Galβ1–3GalNAc] and [NeuAcα2–8NeuAcα2–3Galβ1–3GalNAc] in the terminal residues of ganglio-*N*-tetraose structures, which is a putative antigenic epitope for anti-GQ1b/GM1-reactive antibodies. Squares with dotted lines indicate a combination of [NeuAcα2–3Galβ1–3GalNAc] and [NeuAcα2–8NeuAcα2–3Galβ1–3GalNAc] in the terminal residues, which is a putative antigenic epitope for anti-GQ1b/GD1a-reactive antibodies.
Cer: Ceramide.

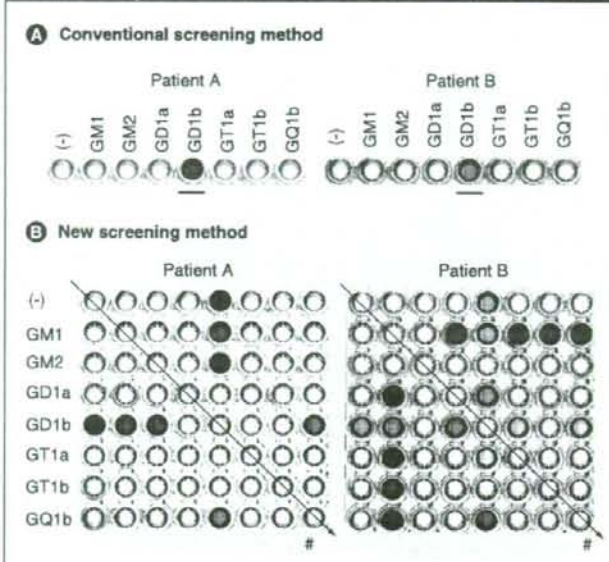
Antibody reactivity & affinity to ganglioside complexes

In view of clustering of gangliosides in the biological membrane, reactivity of antiganglioside antibodies to ganglioside antigens in the membrane may be different from reactivity to single antigens on the ELISA and TLC plates. We recently experienced the case that diversity of antiganglioside antibody reactivity against GSCs influenced clinical features in GBS. Representative data are shown in Figure 4.

According to a conventional ELISA study using single ganglioside antigens (Figure 4A), Patient A and B had only IgG anti-GD1b antibodies that appeared to be monospecific to GD1b. IgG anti-GD1b antibody is thought to be closely associated with ataxia in GBS [30–35]. Ataxia developed in Patient A as anticipated, but not in Patient B. Next, the patients' sera on antibodies to GSCs was investigated. To our surprise, as shown in Figure 4B, antibody activity to GSCs was individually different. A serum sample from Patient B exhibited stronger activity to GSCs, such as GM1/GD1a, GM1/GT1a, GM1/GT1b and GM1/GQ1b than to GD1b, considered not to be GD1b-specific. These results suggest that prime target antigens are different between serum samples from Patients A and B. Such difference may cause diversity of symptomatology in GBS.

As described above, investigation of anti-GSC antibodies is required to understand association between antiganglioside antibodies and clinical features in GBS. Moreover, it may expedite elucidation of a pathogenic role of antiganglioside antibodies. It is a challenge for the future to

Figure 4. Comparison of ELISA results between a conventional screening method with single gangliosides and a new screening method with ganglioside complexes.



(A) Results of a conventional screening for IgG antibodies to single ganglioside antigens. Serum samples from both patients reacted only with GD1b and appeared to have monospecific anti-GD1b antibodies (underlined wells). **(B)** Results of a screening for IgG antibodies to ganglioside complexes. Control wells (blank wells) on the plate are indicated by the oblique arrows. Antibody activity to ganglioside complexes is judged based on a criterion stated in the text. A serum sample from Patient A exhibited antibody activity only to GD1b, but not to any ganglioside complexes, including GD1b/GM1 and GD1b/GM2. On the other hand, a serum sample from Patient B exhibited intense activity to various ganglioside complexes. In Patient A's serum, anti-GD1b activity disappeared in wells containing GD1b/GD1a, GD1b/GT1a and GD1b/GT1b, probably because GD1b undergoes conformational changes in a mixture of gangliosides such as GD1a, GT1a or GT1b.

unveil how anti-GSC antibodies interfere with nerve function through its binding to clustered glycoepitopes in the biological membrane.

Mechanism of production of antiganglioside complex antibodies

In GBS and related disorders, antiganglioside antibodies are thought to be induced by lipooligosaccharides (LOS) of pathogens, causing antecedent infection through a molecular mimicry mechanism [36,37]. It has been well established that LOS of *Campylobacter jejuni* isolated from GBS patients induces IgG anti-GM1 antibody in a rabbit model of motor axonal GBS [38]. A similar mechanism can be speculated in production of anti-GSC antibodies. GBS patients with anti-GSC antibodies often have antecedent *C. jejuni* infection [19], and LOS of *C. jejuni* isolated from GBS and its variants frequently express structures mimicking GM1, GD1a, GD3 or GD1c [39]. A recent analysis on molecular structure of *C. jejuni* LOS has provided results supporting molecular mimicry between the LOS and GSCs targeted by serum antibodies from GBS patients [40]. In the analysis, the LOS fraction of *C. jejuni* strains isolated from GBS patients who had antibodies to GSCs such as GM1/GD1a, GD1a/GD1b, GD1a/GQ1b or GD3/GQ1b was used. Results of an inhibition ELISA showed that each anti-GSC antibody cross-reacted with LOS from the autologous *C. jejuni* strains, indirectly demonstrating that the LOS contained GSC-like structures. However, ganglioside-like structures expressed in some LOS of *C. jejuni* strains were not consistent with ones expected from serum anti-GSC antibodies. For example, strains isolated from GBS patients with anti-GQ1b/GD1a antibodies expressed a homogeneous LOS with only a GD1c-like structure [40]. Further studies are required to explain the cross-reaction between the anti-GQ1b/GD1a anti-bodies and GD1c-like moieties.

Future perspective

To identify specific antibodies associated with GBS and related disorders, purified single ganglioside antigens have generally been used and no response to the purified ganglioside antigens has been considered to be 'antibody-negative'. However, a series of studies on anti-GSC antibodies has indicated that a part of the 'antibody-negative' sera have anti-GSC antibodies, forcing us to reconsider how to identify antiganglioside antibodies. Moreover, the concept of GSC may influence future investigation on cell adhesion processes and signal transduction in which carbohydrate epitopes can play an essential role. Here, we will describe the clinical and the biochemical aspects of future studies on GSC.

Clinical aspect

From a clinical aspect, examination of anti-GSC antibodies provides a great benefit. For example, it may improve the detection rate of antiganglioside antibodies and the accuracy of the diagnosis for GBS and related disorders, and allow us to more precisely predict prognosis and select therapeutic strategy. According to results of the examination, additional treatment, such as intravenous metylprednisolone or a novel therapeutic inhibitor of complement activation (Mirococept, Inflazyme Pharmaceuticals, British Columbia, Canada) [41] may be applied to GBS patients with anti-GD1a/GD1b or anti-GD1b/GT1b antibodies,

besides intravenous immunoglobulin therapy or plasma exchange. Considering that gangliosides form clusters in the biological membrane, it is important to evaluate affinity or avidity of anti-ganglioside antibodies to various ganglioside antigens. Although artificial conditions such as ELISA or TLC are different from the microenvironment of the biological membrane, investigation of antibody reactivity against GSCs may allow us to predict antibody affinity to putative antigens and precisely identify target molecules. Consequently, we will be able to clarify associations between anti-ganglioside antibodies and clinical features. The predominant mechanism of conduction failure in GBS has been considered to be destruction of axon or myelin through complement activation induced by antibody–antigen interactions, usually accompanied by pathological changes. However, considering that some GBS patients make prompt recoveries soon after specific treatment (e.g., intravenous immunoglobulin or plasma exchange), it is likely that functional block without pathological changes causes limb weakness in such patients. Through binding of serum antibodies to GSCs on lipid rafts in nerve cell membranes, membrane property or cell function may be modified, leading to the malfunction of membrane molecules such as the sodium channel and then to consequent functional block of motor nerves. In future, induction of animal models of anti-GSC antibody-positive neuropathy, as well as identification of GSC antigens in the PNS, is required to confirm pathogenic roles of anti-GSC antibodies. The results of immunohistochemical and pathophysiological studies using monoclonal antibodies to single gangliosides must be carefully interpreted, because real target molecules may be masked by other gangliosides or because their binding ability to target antigens may be influenced by clustered glycoepitopes in GSCs on the cell membrane.

Biochemical aspect

The majority of cellular glycoconjugates exist in the cell surface to form a glycocalyx, and glycosyl epitopes in membrane microdomains involve cell adhesion and signal transduction events based on carbohydrate–carbohydrate or carbohydrate–protein interactions through molecular assemblies, termed glycosynapses [42]. Cell-adhesion processes are mediated by carbohydrate-binding proteins, such as selectins and Siglecs, and may be based on cis- or trans-carbohydrate–carbohydrate interactions [42,43]. Therefore, clustered epitopes of GSCs in the cell membrane may have a great influence on such cell adhesion processes. The carbohydrate–carbohydrate interaction, the strength of which is influenced by the degree of clustering, is considered to be weak *in vitro*. In the biological membrane, however, ligands of adhesion molecules such as selectins and Siglecs comprise diverse and complex glycoconjugates, where oligosaccharides are packed closely together to form rigid rodlike structures with multivalency and strict binding specificity [44]. GSCs with clustered sialic acid epitopes may affect the function of the sialic-acid recognizing proteins in the microdomain. Clustered glycoepitopes of GSCs in the cell membrane are likely to function in cell–cell recognition or immune-mediated events in a more effective manner than a solo glycoepitope of isolated ganglioside, which appears to be supported by the latest research exhibiting that ganglioside complex, GM2/GM3 coated on nanospheres more efficiently inhibited cell motility through blocking of cMet activation than GM2 or GM3 alone [45]. The concept of GSCs will provide new vistas to research on cell–cell interaction and shed light on microdomain function mediated by carbohydrate–carbohydrate interaction in the cell membrane [46].

Table 1. Antiganglioside complex antibodies in Fisher syndrome.

Patient number	GQ1b	GQ1b/ GM1	GQ1b/ GD1b	GT1a/ GM1	GT1a/ GD1b	GQ1b/ GD1a	GT1a/ GD1a	GQ1b/ GT1b
1–4	(+)							
5								
6	(+)	(+)		(+)	(+)			
7		(+)		(+)				
8	(+)	(+)		(+)	(+)			
9	(+)	(+)		(+)	(+)			
10	(+)	(+)	(+)	(+)	(+)			
11	(+)					(+)	(+)	(+)
12	(+)					(+)		(+)

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Executive summary

Introduction

- Gangliosides concentrate on the surfaces of neurons and are organized in clusters within membrane microdomains, termed lipid rafts or detergent-resistant membranes.
- Antiganglioside antibodies are present in the sera of approximately 60% of Guillain–Barré syndrome (GBS) patients.
- Heterogeneity of ganglioside expression in the peripheral nervous system influences symptomatology of GBS.
- Some GBS patients have antibodies to ganglioside complexes comprised of two different gangliosides.

Antibodies to ganglioside complexes in Guillain–Barré syndrome

- In the ELISA study on antiganglioside complex antibodies, the optimal mixture rate of two gangliosides is one to one.
- Antiganglioside complex antibodies are real examples indicating that the clustered glycoepitopes on the membrane are involved in immune-mediated events.
- Anti-GD1a/GD1b-positive serum had activity to GM1/GD1a, GM1/GT1b and GD1b/GT1b, suggesting that these antiganglioside complex antibodies specifically react with clustered glycoepitopes common to these ganglioside complexes, rather than with each ganglioside complex.
- Presence of antibodies to GD1a/GD1b or GD1b/GT1b is closely associated with severe disability requiring mechanical ventilation.

Antiganglioside complex antibodies in Fisher syndrome

- Approximately 60% of patients with Fisher syndrome (FS) have antibodies to ganglioside complexes containing GQ1b, but not to the complexes without GQ1b or GT1a.
- Based on antibody specificity, FS-associated antibodies are subdivided into at least three types; first, monospecific to GQ1b (no antiganglioside complex antibodies), second, GQ1b/GM1-reactive and third, GQ1b/GD1a-reactive.
- Investigation of antibodies to ganglioside complexes containing GQ1b may shed light on antibody-mediated pathophysiology in FS and explain clinical diversity in anti-GQ1b antibody-associated disorders.

Antibody reactivity & affinity to ganglioside complexes

- The various reactivity of antiganglioside antibodies against ganglioside complexes may generate diversity of clinical features in GBS.
- Antiganglioside complex antibodies should be investigated in order to clarify the close association of antiganglioside antibodies with clinical features in GBS.

Mechanism of production of antiganglioside complex antibodies

- Antiganglioside complex antibodies cross-react with lipooligosaccharides (LOS) from the autologous *Campylobacter jejuni* strains, indirectly demonstrating that the LOS contained a ganglioside complex mimic.

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