Review Article

Detection of anti-ganglioside antibodies in Guillain-Barré syndrome

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Abstract: Gangliosides are a class of glycosphingolipid molecules that are highly enriched in cellular membranes of the nervous system. The gangliosides associated with autoimmune diseases of the nervous system are mainly GM1, GD1a, GalNAc-GD1a, GM1b, GD3, CD1b, GT1a, and GQ1b. Multiple antibodies recognizing gangliosides are associated with some acute or chronic peripheral neuropathies, especially Guillain-Barré syndrome (GBS) and its clinical variants. Antibodies binding to gangliosides can activate complement system and recruit macrophages on the axolemma at the nodes of Ranvier of motor fibers, which are found in the course of GBS, causing axonal degeneration and reversible conduction block or conduction failure. Testing of anti-gangliosides autoantibodies is helpful for diagnosis of autoimmune peripheral neuropathies or support the diagnosis of the subtypes. These anti-gangliosides antibodies are usually detected by several qualitative or quantitative methods, particularly enzyme-linked immunosorbent assay (ELISA) and immunodot assays, which have been commercialized or established in-house worldwide. Herein, we introduce the methods and clinical applications of these assays in the diagnosis of autoimmune peripheral neuropathies. Anti-gangliosides antibodies are diagnostic markers of GBS subtypes. We use GBS as an example to explain the role of anti-gangliosides antibodies in the pathogenesis and diagnostic classification of neuropathies.

Keywords: Anti-gangliosides antibodies; Guillain-Barré syndrome (GBS); detection methods; clinical applications

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Introduction

Gangliosides, mainly located in the outer layer of the bilayer structure of the neuronal cell membrane, play important roles in cellular growth and differentiation, signal transduction, and immune reactions (1,2). Primary gangliosides associated with nervous system autoimmune diseases are GM1, GD1a, GalNAc-GD1a, GM1b, GD3, CD1b, GT1a, and GQ1b (3-6). Anti-ganglioside antibodies binding to the corresponding gangliosides, activate the complement system and lead to neural damage, including axonal degeneration and loss of myelin (4). IgG and complement deposits on the axolemma at the nodes of Ranvier of motor fibers are found early in the course of the disease. Clinical manifestations are variable and electrophysiological features are axonal degeneration and block conduction (4,7). The functions of gangliosides in auto-immune reactions may depend on their carbohydrate and ceramide structures (3): the hydrophobic ceramide tail of gangliosides is embedded in the lipid bi-layer of the plasma membrane, usually in the cholesterol-enriched microdomains, while the extracellular hydrophilic

Table 1 Target antigens of anti-ganglioside antibodies testing based on clinical features of GBS

Classification	Clinical features	Presence of anti-ganglioside antibodies
MFS	Ophthalmoplegia, ataxia and flexia/hyporeflexia	Anti-GQ1b, anti-GT1a (14,15)
BBE	Hypersomnolence and ophthalmoplegia and ataxia without limb weakness	AntiGQ1b, antiGD1b (16)
AIDP	Paresthesia, limb weakness	Anti-GM1, anti-GD1a (17)
AMAN	Weakness without paresthesia	Anti-GD1a, anti-GM1 (18,19)
PCB	Bulbar, cervical and upper limbs	Anti-GT1a, anti-GQ1b (20)
AMSAN	Weakness accompanied by paresthesia	Anti-GM1, anti-GM1b, anti-GD1a (5,21)

AMAN, acute motor axonal neuropathy; GBS, Guillain-Barré syndrome; AIDP, acute inflammatory demyelinating polyradiculoneuropathy; MFS, Miller Fisher syndrome; PCB, pharyngeal-cervical-brachial variant; AMSAN, acute motor and sensory axonal neuropathy; BBE, Bickerstaff brainstem encephalitis.

Table 2 Target antigens of anti-glycolipid antibodies located in human peripheral nerves

Classification	Localization in PNS
GQ1b	The extramedullary part of the cranial nerve and the presynaptic membrane of the neuromuscular junction (23,24)
GD1b	Large neurons in DRG, paranodal myelin (25)
GM1	The axolemma at the nodes of Ranvier, the myelin of motor nerves, and dorsal root ganglia (26)
GD1a	The Ranvier node of motor neurons (27)
GT1a	Glossopharyngeal and vagus nerves (28)
GalNAc-GD1a	Axonal membrane of motor nerves at node and paranode, axolemma of small fibers in sural nerves (29)

PNS, peripheral nervous system; DRG, dorsal root ganglion.

oligosaccharide moiety is exposed to specific auto-antibodies, and its conformations can be varied to enhance or reduce the autoantibody binding affinity, depending on the binding requirements for a particular antibody (8,9).

Guillain-Barré syndrome (GBS) is a broad term used to describe a number of acute inflammatory immune-mediated syndromes consisting of sensory dysfunction, autonomic dysfunction, progressive weakness and pain (10). By the clinical symptoms and nerve conduction test, GBS is classified into several subtypes: acute inflammatory demyelinating polyradiculoneuropathy (AIDP), acute motor and sensory axonal neuropathy (AMSAN); pharyngeal-cervical-brachial variant (PCB); Miller Fisher syndrome (MFS) and Bickerstaff brainstem encephalitis (BBE); and pure motor GBS, which is subdivided into acute motor axonal neuropathy (AMAN) and acute motor demyelinating neuropathy (11,12). As a treatable rare disease, timely diagnosis and subtype-classification play crucial roles in GBS treatment (13). In this review, we discuss the different types of GBS and clinical features associated with anti-ganglioside antibodies (*Table 1*).

Correlation between GBS and anti-ganglioside antibodies

Premorbid infection with micro-organisms, such as *Campylobacter jejuni* (*C. jejuni*), is recognized as a main triggering event for GBS. Lipooligosaccharides (LOS) on the surface of both infectious micro-organisms and ganglioside lipopolysaccharides (LPS) induces cross-reactive humoral and cellular immune responses to nerve structures. Patients with GBS carry anti-ganglioside antibodies, resulting in autoimmune targeting of the peripheral nervous system (PNS), and leading to neural damage or functional impairment (22). Targeting gangliosides of different autoantibodies locate in different parts of the PNS (*Table 2*). Heterogeneity of ganglioside expression in the PNS may cause differential clinical manifestations of GBS and the GBS variants (30).

Gangliosides are required for axonal regeneration and stabilization of cytoskeletal structures. Therefore, ganglioside antibodies may inhibit nerve reparative processes (31). The antibodies binding to the exposed neuron membrane, such as Ranvier's neuromuscular junctions and lymph nodes, causing disordered ion channel regulation and axonal conduction disorders (9,31), and results in axonal degeneration (32). Several studies reported the role of complement activation in mediating axonal

injury in inflammatory neuropathy. Anti-GM1 and GD1a antibodies binding to gangliosides activate the complement system, and lead to the dysfunction of the PNS and conduction block (33,34).

Gangliosides have long been used intravenously as a neurotrophic drug in China. However, safety concerns for GBS following intravenous ganglioside treatment were aroused in Europe several decades ago (35). Ganglioside-associated GBS was reported to manifest more functional deficits and poorer outcomes after standard treatment with gangliosides in China (36). High titers of anti-GM1 and GT1a antibodies were found in some of the patients who developed GBS following intravenous ganglioside treatment. GM1 is the major immunogen (37). However, the etiology of GBS is still unclear. One explanation is that the low purity of gangliosides may alter the individual's susceptibility by molecular mimicry, and trigger GBS (36).

GM1 locates on the axolemma at the nodes of Ranvier, the myelin of motor nerves, and dorsal root ganglia (26,38). Anti-GM1 antibody is associated with a pure motor variant of GBS without sensory loss (14,39). AMAN subtype of GBS is characterized by primary axonal degeneration, which is rare (about 5% of all GBS cases) in North America and Europe, but is the most prevalent form of GBS in China and Japan (40,41). GD1a is mainly distributed in the Ranvier node of motor neurons, and the IgG form of anti-GD1a antibody is also closely associated with the AMAN (27). In Asia, including China, nearly half of the patients with GBS are eventually diagnosed with AMAN, of which approximately 60% of the patients are anti-GM1 and GD1a autoantibody positive (17,42). Anti-GM2 antibodies are positive in some cytomegalovirus-infected individuals, but association with the incidence of GBS is still controversial (43). N-acetylgalactosaminyl GD1a (GalNAc-GD1a) has been reported as the most potent target antigen in AMAN (41,44,45). GalNAc-GD1a exists in the nodes of Ranvier in motor nerves and locates on the membrane of motor nerves at node and paranode, and the axolemma of small fibers in sural nerves (29). Anti-GalNAc-GD1a IgG antibody may cause conduction failure of motor nerves by binding the axolemma at nodes or nerve terminals, which is closely associated with AMAN and pure motor GBS (46). Anti-GM1b IgG antibodies are closely associated with pure motor GBS. Some anti-GM1b-antibody positive GBS patients also had anti-GM1 and anti-GalNAc-GD1a antibodies (47). AIDP patients rarely have anti-GD3 antibody (48). However, the precise localizations of GD1a, GM1b, and GD3 in motor nerves has not yet been confirmed.

In MFS, the antibodies of *C. jejuni*, produced during prior infection, can partially cross-recognize the ganglioside GQ1b and GM1 and contribute to MFS pathogenesis. The dense distribution of GQ1b exists in paranodal myelin of oculomotor, trochlear and abducens nerves, and some large neurons in the dorsal root ganglion could also be immunostained by monoclonal anti-GQ1b antibody (49). The distribution of GQ1b is critical for the symptomatology of MFS. Anti-GQ1b antibodies bind to GQ1b at these locations, causing nerve damage or conduction block (23). Anti-GQ1b antibody is closely associated with extraocular muscle paralysis, ataxia, and a possible cause of decreased levels of consciousness in BBE patients. After plasma exchange in patients with MFS, the levels of serum anti-GQ1b IgG antibody decreased, and extraocular muscle paralysis and ataxia symptoms were alleviated (15,50). The cross-reactivity of anti-GQ1b antibody with GD1b is involved in the development of impaired deep sense in MFS (51).

Other anti-ganglioside antibodies, for example GT1a, are densely expressed in human glossopharyngeal and vagus nerves, and PCB patients often have antiGT1a IgG antibodies (28). AntiGQ1b antibodies were detected in 65% of ataxic GBS, and 18% of GBS with acute sensory ataxic neuropathy. AntiGD1b antibodies without GQ1b cross-reactivity were found in 14% of ataxic GBS, and 35% of GBS with acute sensory ataxic neuropathy (52).

Anti-gangliosides antibodies are used as diagnostic markers of some subtypes and to support the diagnosis of other subtypes. For example, the existence of antiGQ1b antibodies in 83% of MFS patients and 68% of BBE patients demonstrated that these disorders belong to the same disease spectrum (53,54). Some GBS patients with limb weakness and retained deep tendon reflexes are more likely to have anti-GM1 or anti-GM1a antibodies, neurophysiological features consistent with AMAN, compared with patients with decreased reflex. The presence of antiGT1a or antiGQ1b IgG antibodies further supports the clinical diagnosis of PCB weakness or one of its incomplete forms. The presence of antiGQ1b or antiGD1b antibodies can further confirm the clinical diagnosis of these MFS subtypes (52,55). The detection rate of anti-GD1b antibody was 35% of the patients with acute sensory ataxic neuropathy (the detection rate was 14% in ataxia GBS) (56,57). Although antiganglioside antibody testing can be informative for classifying GBS, the classification system does not stipulate the results of antiganglioside antibody testing.

Detection methods of anti-ganglioside antibodies

A major obstacle in the detection of anti-ganglioside antibodies is that gangliosides are different from classic antigens. First, Low molecular weight may have steric hindrance to impede antibody binding (38). Second, gangliosides, unlike proteins, are difficult to be separated and displayed using well-developed methods that rely on molecular weight or charges, such as polyacrylamide gel electrophoresis, so anti-ganglioside antibodies are hard to be detected using western blot. Third, there is no reliable cell overexpression method for gangliosides, so the anti-ganglioside antibody cannot be detected by cell-based assay, which has been widely used in anti-membrane protein antibodies testing (58,59). Fourth, similar skeletons of some gangliosides can cause cross-reaction (51). Anti-ganglioside antibodies are detected in serum using enzyme linked immune sorbent assay (ELISA), immunodot assays, thin-layer chromatography (TLC) overlay, agglutination tests, and flow cytometry (56,57,60,61). Conventionally, in house or commercially available ELISAs and nitrocellulose/polyvinylidene fluoride (PVDF) dot blots are used, in which purified gangliosides as the adhered antigens are probed with sera from patients with neuropathies. In this review, we mainly focus on current and emerging detection methods of anti-gangliosides antibodies.

Enzyme-linked immunosorbent assays (ELISAs)

ELISAs are immunoassays with the sensitivity of enzyme reactions that are based on the specific binding of antibodies to antigens to a solid phase-bound substance. These antigens can be glycolipids, including gangliosides (62). Serum samples were serially diluted commencing at 1:100. The antibody titer is the highest serum dilution that was defined by a basic optical density (OD) at 490 nm. Serum is considered positive when the titer was 1:500 or more (63). Specific binding is detected using the reactants, which will generate a colored reaction product. Antibodies from a particular species react with the antigen, which binds to the solid phase and the bound antibodies are detected by the addition of horseradish peroxidase (HRP)-labeled antibody. ELISA combines the specificity of antibodies with the sensitivity of enzyme reactions. In one study, the specificity and sensitivity of an ELISA were reported to be 97% and 32% respectively (64). The main problem of ELISA method for detecting anti-ganglioside antibodies is the high unspecific binding of unknown antibodies of samples (called background) in all ELISA plate wells. The background of high unspecific binding affects the judgment of the results, making the ELISA prone to false positives. It is laborious to find the maximum dilution by gradient dilution.

Immunodot assays

Immunodot assay is a rapid and efficient assay for detecting antibodies against gangliosides (65). PVDF membranes are cut into appropriate sizes (squares, strips or other shape), and coated with purified gangliosides. Individual gangliosides are dissolved with organic solvents. The qualitative assay of anti-ganglioside antibody was optimized based on positive/negative sample testing results. Sera samples are typically diluted 1/50–1/200. The secondary antibody, alkaline phosphatase (AP)-conjugated anti-human IgG and IgM is incubated for recognizing human serum antibodies, then subjected to a color reaction by an enzyme-reactive substrate to form a spot-like coloration observable by the naked eye. If the coated region has a brown speckled color, that means there are antibodies specific to given gangliosides antigen in the samples (*Figure 1*). Positive results: the antigen-coated area presents a clearly distinguishable circle or approximately circular shape, colored light blue-gray or dark blue-black, with a darker color than the blank control. Negative result: the color of the antigen-coated area is shallower or equivalent to the blank control area, or that of the antigen-coated area is slightly darker than the blank control, but there is no clear boundary. Immunodot assays can detect multiple antibodies simultaneously. However, the inspection of testing results is somewhat subjective. For example, for a weak positive or suspected positive result, different people may give different judgment results. Therefore, the interpretation of the results generally requires 2–3 experienced personnel to make judgments. When there are differences in the results, other detection methods are needed to determine the results. It is reported that the sensitivity of immunoblotting method was 56% and specificity was 100% (64).

Other detection methods

Serum from some GBS patients is bound to single gangliosides or to a mixture of ganglioside complexes (GSCs), including anti-GD1a:GD1b antibodies, anti-LM1-complex antibodies, anti-GM1:GalNAc-GD1a

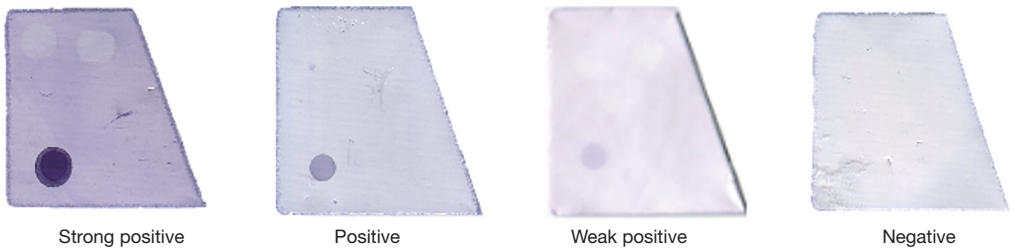

Figure 1 The test results of anti-ganglioside antibodies explained. Here, the C1Q antibody is used as an example of strong positive, positive, weak positive and negative.

antibodies, anti-GQ1b-complex antibodies, and other anti-glycolipid-complex antibodies (66). Furthermore, a small portion of sera binds only to the GSCs but not the individual gangliosides. Traditional antibody detection assays react with single ganglioside species that do not seem to greatly increase the diagnostic sensitivity of antibody testing. In 2012, the combinatorial glycol array method, a novel technique, was developed to test combinations of gangliosides and other glycolipids that significantly increase the sensitivity of serological testing (67). In this method, ganglioside solutions are diluted with methanol and GSCs are made by mixing equal volumes of different glycolipids. Antigens are then spotted onto PVDF membranes and each spot is separated from other spots by 2 mm. The PDVF membranes are then blocked with 2% bovine serum albumin (BSA) for 1 h at room temperature. Serum is diluted (1:100) with 1% BSA incubated on PVDF membranes for 2 h at 4 ℃. And then washed three times with phosphate-buffered saline (PBS), the corresponding secondary antibody is diluted (1:1,000) and applied for 1 h, washed with PBS, and PVDF membranes are washed with distilled water for 5 min. The PVDF membranes are dried at room temperature in the dark. Reactivities to GSCs are expressed in fluorescence intensities detected using Image Quent TL software. Intensities greater than the mean plus 3 standard deviations for healthy controls are considered positive. In addition to a single ganglioside species, the glycosyl array method also tests antibodies against several glycolipid complexes that can identify antibodies in 53% of GBS patients, while only 13% were identified by ELISA (68). The detection of anti-GSC antibodies may prove useful in identifying clinical and pathological subtypes. The LM1:GA1-complex antibodies associated with AMAN, absence of sensory impairment and reversible conduction failure, anti-GalNAc-GD1a, anti-GM1:GalNAc-GD1a, anti-GM1b:GA1, and anti-LM1:GA1 were associated with pure motor GBS (68).

Comparison among different detection methods and kits

Several commercial immunological assay kits are available worldwide for detecting anti-ganglioside antibodies such as immunodot assays (Euroimmun), line-immuno assay (GA Generic Assays), and ELISA (Buhlman). In one study, according to the clinical criteria of children with GBS, the specificity and sensitivity of immunoblotting were better than ELISA: the sensitivities of ELISA and immunoblotting methods were 32% and 56%, and the specificities were 97% and 100%, respectively (64). But the sensitivity and specificity are different for anti-ganglioside antibody detection in different commercial products (65). In developing detection methods for anti-ganglioside antibodies, different techniques are not necessarily expected to be fully concordant with each other, or superior to each other, as there is no recognized optimal assay for detecting these antibodies (69).

Development of anti-ganglioside antibodies detection in China

In China, the commercial kits, or in-house assays established in different clinical centers for antibody testing are usually ELISA and immunodot assays as elsewhere. Recently, immunodot assays have been further developed domestically. Several parameters may be adjusted from the current methods, such as ganglioside preparation, buffer composition, sample preparation, and time and temperature of incubation that may affect the detection sensitivity and specificity. The existence of ‘gray areas’ in immunology assays make the situation complicated. Identifying the color change in ELISA using an auto-measurement instrument should be less subjective than in immunodot with by eye observation. However, some reports had different conclusions (64,70). Since key parameters of the commercial kits are usually manufacturers’ proprietary information,

comparisons of sensitivity and specificity of the products is not adequate for evaluating the pros and cons of different technologies.

Our lab has compared different types of kits from different manufacturers, and set up an in-house immunodot assay to extensively study the parameters affecting the detection results (unpublished data). There are rational positive correlations between some parameters (for example, purity of antigen, affinity and specificity of secondary antibody, activity of enzyme) and the accuracy of testing. Changing the concentration of surfactant in diluent buffer or washing buffer, the washing duration, the dose of antigen, and the dilution ratio of samples will change the specificity and sensitivity to the opposite orientations. For example, reducing the concentration of the surfactant (such as from 0.5% to 0.2% in a certain testing system) will increase the titer value of positive sample dramatically, and the positive rate from 2% to 15%, even in a healthy patient group. It is reasonable to set up the assay using the following steps: first, set up the immunology assay with the reagents of the first class quantification, and with strict conditions: higher concentration of surfactant, longer washing duration, lower dose of antigen, etc., to ensure detection of the lowest positive rate (near zero) for the healthy group, and the positive signal from strong positive samples; second, relax the strict conditions to increase the detection titer value of the strong positive samples, while keeping the lowest positive rate from the healthy group, until the clinical requirements are met with clinical, randomized samples at a large scale.

A commercial kit from China was recently developed following the steps described above, with modified antigen solvents and dot technology to limit the antigen to quite a small region without diffusion, to keep the local antigen density. Using that kit, color spots with sharp margins will be obvious even when the weakest positive sample is tested, while the detection rate from the healthy group stays lower than 2% (anti-GM1) or goes to 0 (anti-GD1b and GQ1b). About 1,500 samples from suspected GBS patients in China were tested with this kit during the last several years, about 4/5 of IgG anti-GD1b positive patients were also IgG anti-GM1 positive; a few anti-GD1b positive patients were also anti-GQ1b positive (unpublished data). Those findings are somewhat different from previous reports: anti-GQ1b cross-reacts with GD1b and GT1a and *vice versa* (20). For the case of double-positivity, the reports mainly involved a large number of patients with GQ1b and GT1a double-positive, and it may be due to the cross-reaction between the antibodies and these structurally similar antigens (51). However, in our study, serum neutralization analysis was performed on GD1b and GM1 double positive serum. For example, serum samples were mixed with GM1 solution (50 μM) and incubated for 1 hour before detection of autoantibodies. Serum neutralization by the antigen GM1 did not affect the color development of anti-GD1b autoantibody and *vice versa*. These results show that the anti-GD1b and anti-GM1 double-positive sera are not caused by insufficient specificity of antibodies (the antibodies cross-react with GD1b and GM1), and it is more likely that both antibodies of anti-GD1b and anti-GM1 exist in the patient's sera.

Conclusions

Anti-ganglioside antibodies can be detected using several methods, including ELISA and immunodot assays. All methods have advantages and limitations. The evaluation for anti-ganglioside antibodies mainly includes two aspects. First, these methods differ substantially in their accuracy, including sensitivity and specificity. The accuracy values obtained are biased for different subgroups and are presumably higher than in actual practice in some reports. The specificity and sensitivity should be related to the disease spectrum. Second, several parameters (washing buffer, washing duration, the dose of antigen) are different in different detection methods. The immunodot assay has undergone many changes, making it more efficient and more convenient. Specific anti-ganglioside antibody detection can be used to define subgroups of GBS patients. Absolute sensitivities and specificities are unavailable. There is still a need for extensive standardization of all commercial anti-ganglioside assays.

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