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Effects of IgM Anti-Galnac-GD1a Monoclonal Antibody on Neuromuscular Transmission and Calcium Channel Binding in the Rat Neuromuscular Junction

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Abstract

Guillain-Barré syndrome with antibodies against ganglioside N-acetylgalactosaminyl GD1a (GalNAc-GD1a) is characterized by a rapid onset of predominantly distal pure motor neuropathy. However, the pathogenic role of anti-GalNAc-GD1a antibody and calcium channels in neuromuscular junctions (NMJs) remains unclear. We investigated the effects of IgM anti-GalNAc-GD1a monoclonal antibody (IgM anti-GalNAc-GD1a mAb) on spontaneous muscle action potentials (SMAP) in a rat spinal cord–muscle co-culture system and the localization of IgM anti-GalNAc-GD1a mAb and calcium channel binding in the rat hemi-diaphragm. Immunohistochemistry of the rat hemi-diaphragm showed that IgM anti-GalNAc-GD1a mAb binding overlapped with anti-neurofilament 200 antibody and α-bungarotoxin staining, demonstrating that IgM anti-GalNAc-GD1a mAb was localized at the motor nerve terminal. Moreover, IgM anti-GalNAc-GD1a mAb binding overlapped with anti-Cav2.1 antibody in the nerve terminal. We suggest that the inhibitory effect of IgM anti-GalNAc-GD1a mAb on SMAP is related to the GalNAc-GD1a epitope on P/Q-type calcium channels in motor nerve terminals at NMJs.

Keywords: Guillain-Barré syndrome; IgM anti-GalNAc-GD1a antibody; Spontaneous muscle action potential; calcium channel;Neuromuscular junctions; Hemi-diaphragm

Introduction

Interest in the pathological role of anti-ganglioside antibodies has been stimulated by the identification of monoclonal IgM antibodies reacting with specific gangliosides in patients with multifocal motor neuropathy [1-3]. These anti-ganglioside antibodies may play an important role in the pathogenesis of Guillain-Barré syndrome (GBS), as its clinical manifestation is associated with the detection of anti-ganglioside antibodies [4-5]. One such antibody, N-acetylgalactosaminyl GD1a (GalNAc-GD 1a), is frequently found in the sera of patients with acute motor axonal neuropathy (AMAN) and acute inflammatory demyelinating polyneuropathy (AIDP) [6]. Several different immune neuropathies have been described in patients with polyclonal antibodies, either immunoglobulin G (IgG) or immunoglobulin M (IgM), directed against GalNAc-GD1a. IgG anti-GalNAc-GD1a antibody is frequently associated with a purely motor variant of GBS, which is referred to as acute motor axonal neuropathy [7-9]. On the other hand, GBS with IgM anti-GalNAc-GD1a monoclonal antibody (IgM anti-GalNAc-GD1a mAb) is associated with cranial nerve involvement and sensory disturbance [10].

Several studies have reported that anti-ganglioside antibodies cause changes in calcium channels at neuromuscular junctions (NMJs) [11-12]. Moreover, L-type voltage-dependent calcium channels (VDCCs) current is functionally stimulated after exposure to IgM monoclonal antibodies against GM2, suggesting a close relation between GM2 gangliosides and L-type VDCCs downregulation [13]. We previously reported that IgM anti-GQ1b mAb decreases spontaneous muscle action potentials in spinal-muscle co-culture systems and VDCC current in cerebellum granule cells [14]. In fact, lipid microdomains, which contain gangliosides, are involved in clustering P/Q-type VDCCs and organizing the presynaptic membrane sites of synaptic exocytosis. Moreover, we reported that IgG anti-GalNAc-GD1a antibodies purified from a rabbit immunized with GalNAc-GD1a inhibit VDCC currents in nerve growth factor-differentiated PC12 pheochromocytoma cells [15].

In the present study, we investigated the effects of IgM anti-GalNAc-GD1a mAb on spontaneous muscle action potentials using a rat spinal-muscle co-culture system. Immunohistochemistry with IgM anti-GalNAc-GD1a mAb and calcium channel antibodies was used to show colocalization of GalNAc-GD1a and calcium channels in NMJs of the rat hemi-diaphragm.

Materials and Methods

Monoclonal IgM anti-GalNAc-GD1a antibody

Mouse monoclonal IgM anti-GalNAc-GD1a antibody was purchased from Seikagakukogyo (Tokyo, Japan) and was stored at 4°C until use.

Spinal cord–muscle co-culture

Pregnant Wistar rats were housed individually under automatically controlled environmental conditions and a 12 h light/dark cycle with free access to food and water. Experiments were carried out in accordance with the guidelines for animal care of Showa Pharmaceutical University, as well as the guidelines for animal use published by the National Institutes of Health.

The spinal cord–muscle co-culture was performed according to the method of Taguchi et al. [16]. Muscle was excised from pre-natal day 17 fetal rats and cut into small pieces in Tyrode's solution containing 100 μg/mL streptomycin and 100 μg/mL penicillin. The pieces were then incubated at 37°C for 20 min in Ca^{2+}- and Mg^{2+}-free Tyrode's solution containing 1 mg/mL collagenase. For the innervation experiments, explants of whole transverse slices of fetal spinal cord, including the dorsal root ganglia, from pre-natal day 17 fetal rats were placed on the bottom of a collagen-coated 35-mm Petri dish. Individual muscle cells dissociated by trituration were placed on the slices of spinal cord and cultured. Muscle cells and spinal cord were co-cultured in 67% Dulbecco's modified Eagle's medium (DMEM; GIBCO BRL, Carlsbad, CA, USA), 23% medium 199 (GIBCO BRL), and 10% fetal calf serum (Roche, Basel, Switzerland) supplemented with 25 ng/mL fibroblast growth factor (Sigma, St. Louis, MO, USA) and 20 μg/mL insulin (GIBCO BRL). The muscle-spinal cord co-cultures were kept in a CO_2 incubator (5% CO_2, 95% O_2, at 37°C). In this experiment, cultured cells were maintained for up to 1 week in the same medium.

Measurement of spontaneous muscle action potentials

Spontaneous muscle action potentials were measured according to the method of Taguchi et al. [11]. After one week of co-culture, the innervated muscle specimens were placed in an experimental chamber (35-mm petri dish) on the stage of an inverted microscope. The experimental chamber (volume: 1 mL) was continuously perfused with medium (67% DMEM and 23% medium 199) at a rate of 1-2 mL/min, with continuous bubbling using 5% CO_2/95% O_2. Glass microelectrodes filled with 3 M KCl with a tip resistance of 20-40 MΩ were used to record spontaneous muscle action potentials. Electrical activity was recorded by each intra-cellular electrode, which was connected to a microelectrode amplifier, and displayed on an oscilloscope. Data were transferred to and stored in a computer. The action potentials were low-pass filtered at 1 kHz. All experiments were performed at 33±1°C.

Immunohistochemistry

A Wistar rat was anesthetized deeply and perfused transcardially with 0.01 M phosphate-buffered saline (PBS), pH 7.4. The hemi-diaphragm was excised, snap frozen in liquid nitrogen, and cut using a cryostat at -25°C. Longitudinal and cross-sections (10 μm) were air dried on albumin-coated glass slides and fixed with cold acetone for 3 min. Immunohistochemistry was performed using the triple fluorescence labeling technique to determine the co-localization of GalNAc-GD1a and calcium channels in the rat hemi-diaphragm. Hemi-diaphragm sections were incubated with 10% normal goat serum (NGS, Funakoshi. Co., Tokyo, Japan) in Block Ace (Dainipponseiyaku Co., Osaka, Japan) solution for 30 min at room temperature to block non-specific binding. After blocking, hemi-diaphragm sections were incubated for 5 h at 4°C with IgM anti-GalNAc-GD1a mAb (diluted 1:300 with 10% NGS and 10% Block Ace in 0.01 M PBS). To detect IgM anti-GalNAc-GD1a antibody, hemi-diaphragm sections were then incubated for 1 h at 4°C with goat anti-mouse IgM conjugated to fluorescein isothiocyanate (FITC) (1:20; SIGMA). Then, hemi-diaphragm sections were incubated with one of the following primary antibodies: rabbit anti-neurofilament 200 kDa antibody (NF200) (1:1000; Chemicon, Inc., Temecula, CA, USA), rabbit anti-S100 protein antibody-1 (S100)(1:100; Neomarkers, Kalamazoo, MI, USA), rabbit anti-syntaxin 1antibody (Syn) (1:100 and 1:1000; SIGMA), rabbit anti-Cav2.1 (α1A) antibody, rabbit anti-Cav2.2 (α1B) antibody, and rabbit anti-Cav1.2 (α1C) antibody (1:1000 each; Alomone Labs, Jerusalem, Israel) for 5 h at 4°C. To detect the primary antibody, hemi-diaphragm sections were incubated for 1 h at 4°C with goat anti-rabbit IgG conjugated to Cy5 (1:100; Chemicon International Inc.) and tetramethylrhodamine α-bungarotoxin (α-BuTx; 1:300; Invitrogen, Carlsbad, CA, USA). Each reaction was terminated via a few washings with PBS. The immunostained sections were mounted on slides with Aqua Poly/Mount (Polyscience, Inc., Warrington, PA, USA), covered with microslips, and observed with an Olympus laser-scanning confocal microscope (FLUOVIEW BW50, Olympus, Tokyo, Japan) at wavelengths of 488 nm, 647 nm and 568 nm.

Collagenase and chloroform/methanol treatments

The rat hemi-diaphragm was excised as described earlier and treated with: (1) 1% collagenase (Funakoshi Co.) in PBS for 20 min at 37°C to dissociate the muscle into intact single muscle fibers; and (2) chloroform-methanol (1:1) for 20 min at room temperature to dissociate the glycolipids and the nerve fibers.

Statistical analysis

All data are expressed as the mean ± S.E.M. A paired t-test was used to compare the effects of IgM anti-GalNAc-GD1a mAb. p values less than 0.05 were regarded as significant.

Results

Effects of IgM anti-GalNAc-GD1a mAb on neuromuscular transmission

After 5-7 days of co-culture, asynchronous contraction of several individual muscle fibers was observed at the newly developed NMJs. Spontaneous action potentials within innervated muscle cells were recorded, with a frequency of 12.6 ± 6.9 per 5 sec and an amplitude of 69.9 ± 7.6 mV (n=7). Figure 1A shows the effects of IgM anti-GalNAc-GD1a mAb on spontaneous muscle action potentials at the NMJs. IgM anti-GalNAc-GD1a mAb (1:100) rapidly reduced the frequency of spontaneous muscle action potentials at the NMJs within 3 min. After 3 min, the spontaneous muscle action potentials at the NMJs remained decreased. Spontaneous muscle action potentials were recovered by washing away IgM anti-GalNAc-GD1a mAb from the bath solution (Figure 1B). IgM anti-GalNAc-GD1a mAb (1:100) significantly decreased spontaneous muscle action potentials at the NMJs (n = 4, 69.9 ± 8.5%, $p<0.01$) (Figure 1C).

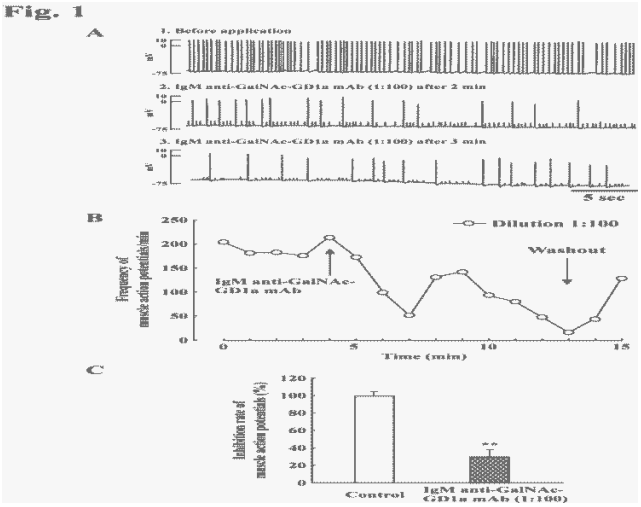

Figure 1: Effects of IgM anti-GalNAc-GD1a mAb on spontaneous muscle action potentials in spinal cord–muscle co-cultured cells. (A) Blockade of spontaneous muscle action potentials by IgM anti-GalNAc-GD1a mAb (1:100 dilution, 10 µL) in spinal cord–muscle co-cultured cells. This blockade was fully reversed after 4 min by washing with the co-culture solution. (B) Time course of the frequency of spontaneous muscle action potentials after addition of IgM anti-GalNAc-GD1a mAb (1:100 dilution, 10 µL). IgM anti-GalNAc-GD1a mAb decreased the spontaneous muscle action potentials in the spinal cord–muscle co-cultured cells. After washing out the IgM anti-GalNAc-GD1a mAb, recovery from inhibition was observed. The number of spontaneous muscle action potentials was measured every 5 sec. (C) Inhibition rate of the spontaneous muscle action potentials following addition of IgM anti-GalNAc-GD1a mAb (1:100 dilution). Data are the mean ± SEM. n = 4 for IgM anti-GalNAc-GD1a mAb (1:100 dilution, 10 µL). **P < 0.01 versus control.

Localization of monoclonal IgM anti-GalNAc-GD1a mAb in rat hemi-diaphragm sections

In the rat hemi-diaphragm, immunostaining with IgM anti-GalNAc-GD1a mAb (green) overlapped with that of α-BuTx (red), a marker of postsynaptic acetylcholine receptor (AChR) clusters, and partially with NF200, a neuronal marker (blue) (Figure 2A). IgM anti-GalNAc-GD1a mAb staining was also present inside the AChR clusters. Longitudinal sections of the NMJs demonstrated that IgM anti-GalNAc-GD1a mAb corresponded to the axons stained by NF200 (blue) (Figure 2A). However, these longitudinal sections demonstrated that IgM anti-GalNAc-GD1a mAb staining was not overlapped with a Schwann cell (glial cell) marker, S100 (blue) (Figure 2B). Also, cross-sections demonstrated that the IgM anti-GalNAc-GD1a mAb signal was not corresponded to a presynaptic nerve marker, syntaxin antibody (blue) (Figure 2C). Therefore, GalNAc-GD1a was localized to the motor nerve terminal within the NMJs.

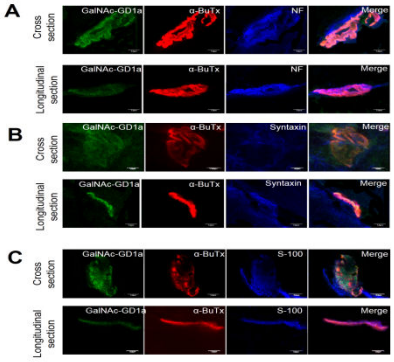

Figure 2: Immunostaining with IgM anti-GalNAc-GD1a mAb in cross-sectional and longitudinal sections of rat hemi-diaphragms. (A) Staining with IgM anti-GalNAc-GD1a mAb (green) overlapped with the postsynaptic acetylcholine receptor cluster marker, α-bungarotoxin (α-BuTx, red), and anti-neurofilament 200 kDa antibody (NF200; blue) at the nerve terminal. (B) Staining with IgM anti-GalNAc-GD1a mAb (green) was not overlapped with the Schwann cell (glial cell) marker, anti-S100 protein antibody-1 (S100; blue) (C) Staining with IgM anti-GalNAc-GD1a mAb (green) was not overlapped with anti-syntaxin antibody (Syn; blue). The bars represent 5 µm.

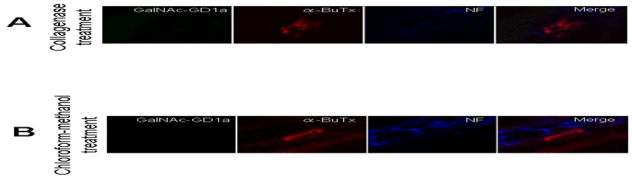

Figure 3: Immunostaining with IgM anti-GalNAc-GD1a mAb in cross-sections of rat hemi-diaphragm after collagenase and chloroform/methanol treatment. (A) Following collagenase treatment, which dissociates the motor nerve, IgM anti-GalNAc-GD1a mAb (green) and anti-neurofilament 200 kDa antibody (NF200; blue) did not stain these sections. However, the postsynaptic acetylcholine receptor cluster marker, α-bungarotoxin (α-BuTx), did stain these sections (red). (B) Following chloroform/methanol treatment, which dissociates glycolipids and nerve fibers, IgM anti-GalNAc-GD1a mAb did not stain these sections, but α-BuTx (red) and NF200 (blue) did. The bars represent 5 µm.

Immunohistological staining of the hemi-diaphragm with monoclonal IgM anti-GalNAc-GD1a mAb after collagenase and chloroform/methanol treatments

To demonstrate that IgM anti-GalNAc-GD1a mAb localized to motor nerve terminals, we dissociated the motor nerve from the hemi-diaphragm with collagenase treatment. Collagenase treatment prevented IgM anti-GalNAc-GD1a mAb (green) and NF200 (blue)

staining in the hemi-diaphragm (Figure 3A). However, α-BuTx (red) staining was selectively retained in the hemi-diaphragm following collagenase treatment (Figure 3A). Pretreatment with chloroform: methanol (1:1) to dissociate the glycolipids and the nerve fibers eliminated IgM anti-GalNAc-GD1a mAb staining in the hemi-diaphragm, but NF200 (blue) and α-BuTx (red) staining were preserved (Figure 3B). IgM anti-GalNAc-GD1a mAb also appeared to be localized in the motor nerve terminals of the hemi-diaphragm.

Immunohistological staining using monoclonal IgM anti-GalNAc-GD1a mAb and calcium channel antibodies in hemi-diaphragm sections

Next, we examined the localization of IgM anti-GalNAc-GD1a mAb and different calcium channels in the nerve terminal. The rat hemi-diaphragm stained positive with anti-Cav2.1 (α1A) antibody (P/Q-type calcium channel) (blue), and the staining overlapped partially with IgM anti-GalNAc-GD1a mAb staining (green) and α-BuTx staining (red) (Figure 4A). However, anti-Cav2.2 (α1B) antibody (N-type calcium channel) (Figure 4B) and anti-Cav1.2 (α1C) antibody (L-type calcium channel) (Figure 4C) staining (blue) was not overlapped with IgM anti-GalNAc-GD1a mAb staining. Thus, IgM anti-GalNAc-GD1a mAb was localized at the motor nerve terminal, and staining corresponded to that of P/Q-type calcium channels at the motor nerve terminal.

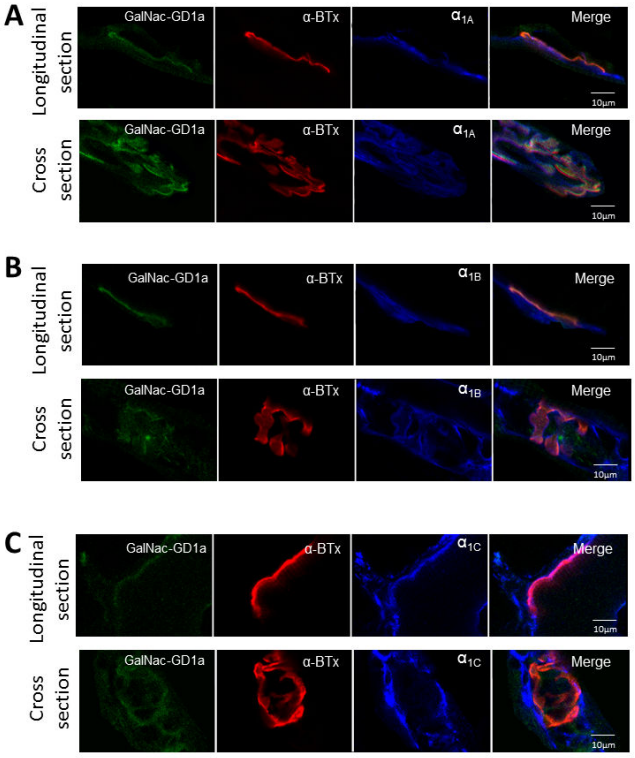

Figure 4: Immunostaining with IgM anti-GalNAc-GD1a mAb and calcium channel antibody in cross-sections and longitudinal sections of rat hemi-diaphragms. (A) Staining with IgM anti-GalNAc-GD1a mAb (green) overlapped with the postsynaptic acetylcholine receptor cluster marker α-bungarotoxin (α-BuTx, red) and anti-Cav2.1 (α1A) antibody (P/Q-type calcium channel; blue) at the nerve terminal. (B) Staining with IgM anti-GalNAc-GD1a mAb (green) was not overlapped with anti-Cav2.2 (α1B) antibody (N-type calcium channel; blue). (C) Staining with IgM anti-GalNAc-GD1a mAb (green) was not overlapped with anti-Cav1.2 (α1C) antibody (L-type calcium channel; blue). The bars represent 5 μm.

Discussion

We demonstrated that IgM anti-GalNAc-GD1a mAb inhibited spontaneous muscle action potentials at the NMJs. Antibodies from the serum of an AMAN patient with a high titer of antibodies to GalNAc-GD1a and antibodies from the serum of a rabbit immunized with GalNAc-GD1a blocked neuromuscular transmission in a rat muscle-spinal cord co-culture system [17]. These studies suggested that the blockade by the antibodies is a presynaptic effect at the motor nerve terminal. In another study, the serum of a patient with chronic demyelinating motor neuropathy with IgM monoclonal antibodies against GM2, GalNAc-GD1a, and GalNAc-GM1b was shown to block neuromuscular transmission by inhibiting neurotransmitter release at the NMJs. In addition, prior incubation with the P/Q-type calcium channel blocker ω-agatoxin IVA completely blocks the inhibitory

effect of the IgM monoclonal antibody against GM2, GalNAc-GD1a, and GalNAc-GM1b on neurotransmitter release [18]. Similarly, our results showed that IgM anti-GalNAc-GD1a mAb decreased the frequency of spontaneous muscle action potential at the NMJs. Thus, IgM anti-GalNAc-GD1a mAb may block neuromuscular transmission by suppressing P/Q-type calcium channel activity at the axon terminals of motor nerves.

In the present study, IgM anti-GalNAc-GD1a mAb staining overlapped with AChR clusters stained with α-BuTx and nerve terminal axons stained with NF200, suggesting that GalNAc-GD1a is localized to the motor nerve terminal of cross-sections and longitudinal sections of rat hemi-diaphragm. We previously reported that IgG anti-GalNAc-GD1a antibodies localize to motor axons, spanning the motor neurons from the spinal cord to muscle cells [17]. Thus, IgM anti-GalNAc-GD1a mAb staining corresponded to AChR clusters in nerve terminals. Following collagenase treatment, IgM anti-GalNAc-GD1a mAb and NF200 did not stain the axons. However, AChR clusters (which were stained with α-BuTx) were selectively stained in the rat hemi-diaphragm. Moreover, chloroform: methanol (1:1) treatment eliminated the IgM anti-GalNAc-GD1a mAb staining in the hemi-diaphragm, indicating that the IgM anti-GalNAc-GD1a mAb recognizes a glycolipid rather than a glycoprotein.

Several studies have demonstrated an interaction between gangliosides and calcium channels [19, 20]. Although the mechanism by which anti-GalNAc-GD1a antibody in the serum of GBS patients inhibits neurotransmitter release has not been fully clarified, Buchwald et al. [21] and Ortiz et al. [18] have proposed that the sera of GBS patients may block calcium channels located on axon terminals. Several studies suggest that calcium ion influx through voltage-gated calcium channels is the trigger for release of acetylcholine from motor neuron terminals at NMJs [22-24]. In addition, prior incubation with the P/Q-type calcium channel blockerω-agatoxin IVA completely blocks the inhibitory effect of the IgM monoclonal antibody against GM2, GalNAc-GD1a, and GalNAc-GM1b on neurotransmitter release [18]. The study also noted that anti-ganglioside antibodies block calcium influx via P/Q-type calcium channels [25]. In addition, we previously reported that anti-GalNAc-GD1a antibody appears to be related to the inhibitory effect of VDCC currents [15]. In this study, we found that IgM anti-GalNAc-GD1a mAb staining overlapped with P/Q-type but not N-type or L-type calcium channels, suggesting that GalNAc-GD1a is localized to the calcium channels at the motor nerve terminal of the rat hemi-diaphragm. A recent study showed rapid uptake of anti-ganglioside antibodies at the presynaptic motor nerve terminal compared to the axolemmal membrane at the node of Ranvier [26, 27]. The present data support the involvement of VDCC currents in the effects of anti-GalNAc-GD1a antibody on neurotransmitter release. However, GBS is associated with numerous antibodies to gangliosides in patients, including GM1, GM1b, GD1a, GD1b, and GQ1b. The question arises as to whether VDCC currents are also inhibited by elevation of other anti-ganglioside antibodies. Further research in this area is required.

Conclusion

Immunohistochemical techniques revealed that the epitope of GalNAc-GD1a is present on P/Q-type calcium channels in nerve terminals of MNJs. Thus, the anti-GalNAc-GD1a antibody may be one of the factors that cause muscle weakness in GBS patients by binding to calcium channels.

Acknowlegement

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