by entry of Ca^{2+} via voltage-gated Ca channels at the presynaptic membrane, the possibility exists that antibodies to gangliosides such as GM1 or GalNAc-GD1a block spontaneous muscle action potentials at the neuromuscular junction through impairment of the voltage-gated Ca channel.

In *ex vivo* experiments using mouse hemidiaphragmas, anti-GM1 or anti-GD1a monoclonal antibodies induce a decrease in presynaptic transmitter release in a complement-independent manner through antibody–antigen interaction in the presynaptic membrane of motor nerves, probably because depolarization-induced calcium influx is inhibited [41]. Furthermore, in *in vitro* experiments using cultured olfactory bulb neurons that express P/Q-type Ca channels, these monoclonal antibodies reduced depolarization-induced calcium influx, which was complement-independent [41]. These observations indicate that the complement independent functional blockade of motor nerve terminals by antibodies to GM1 or GD1a can explain limb weakness in AMAN. Considering that the blood–nerve barrier is absent and gangliosides are abundant at presynaptic membranes in the neuro-muscular junction, the presynaptic membranes are likely to be susceptible to antiganglioside antibody attack [42].

Thus, the functional blockade of voltage-gated Ca channels at the presynaptic membrane can be the alternative pathophysio-logy in GBS. Neuromuscular transmission failure, however, has never been confirmed by clinical electrophysiological tests in GBS patients with antibodies to GM1, GD1a or GalNAc-GD1a.

Ganglioside complexes as target antigens in GBS & its variants

There is no question as to the importance of routine measurement for serum antiganglioside antibodies in GBS and its variants. The measurement has generally been done using ELISA system. To exactly detect serum antibodies, we use purified single antigens as test antigens and must avoid contamination of the antigens. Antiganglioside antibody negative has meant so far that the sera have no antibodies to single ganglioside antigens.

Recently, we detected in some GBS and FS sera IgG antibodies to a ganglioside complex (GSC) consisting of two different gangliosides [43,44]. IgG antibodies to the GD1a–GD1b complex (GD1a/GD1b) were identified in eight out of 100 patients with GBS, and their sera showed sharp and strong immunostaining in the overlapping portion of GD1a and GD1b on a thin-layer chromatographic plate (Figure 2) [43]. The anti-GSC antibody-positive sera have little or no reactivity with each constituent ganglioside, but a strong one with a mixture of the two gangliosides in a well of an ELISA plate. This indicates that novel glycoepitopes are formed in the GSC and may function as target molecules in antibody-mediated events. The antibody activity to GD1a/GD1b became maximal when the mixture consisted of an equal amount of GD1a and GD1b. Thus, we cannot state now that sera are antiganglioside antibody negative before antibodies to various GSCs are examined. In a larger population of GBS, 39 out of 234 patients (17%) had IgG antibodies to GSCs consisting of two of the four major ganglio-series gangliosides, GM1, GD1a, GD1b and GT1b . Patients with anti-GD1a/GD1b or anti-GD1b/GT1b antibodies are significantly predisposed to severe disability [45]. Most of anti-GD1a/GD1b- or anti-GD1b/GT1b-positive sera react with GM1/GD1a and GM1/GT1b, indicating that these sera are more multivalent than the antibodies reacting only with GM1/GD1a or GM1/GT1b, or with single ganglioside antigen.

In FS, characterized by a clinical triad of ophthalmoplegia, ataxia and areflexia, a ganglioside GQ1b is considered to be a prime antigen [46,47]. We detected IgG antibodies to GSCs containing GQ1b or GT1a, such as GQ1b/GM1 and GQ1b/GD1a, in half of FS patients [44,48]. FS-associated antibodies are probably subdivided into three types based on antibody specificity: GQ1b-specific, GQ1b/GM1-reactive and GQ1b/GD1a-reactive [44]. Such antibody specificity appears to be regulated by conformation of terminal residues containing sialic acids (Figure 3). That is, anti-GQ1b/GM1-reactive sera react with a combination of (Galβ1–3GalNAc) and (NeuAcα2–8 NeuAcα2–3Galβ1–3GalNAc) in the terminal residues of ganglio-*N*-tetraose structures, and anti-GQ1b/GD1a-reactive sera react with a combination of (NeuAcα2–3Galβ1–3GalNAc) and (NeuAcα2–8 NeuAcα2–3Galβ1–3GalNAc) in the terminal residues [44,48]. A proportion of GBS patients also have IgG antibodies to GSCs containing GQ1b or GT1a, and such anti-GSC antibodies are highly associated with development of ophthalmoplegia in GBS [48]. Both FS patients with and without the anti-GSC antibodies demonstrated the clinical triad, suggesting

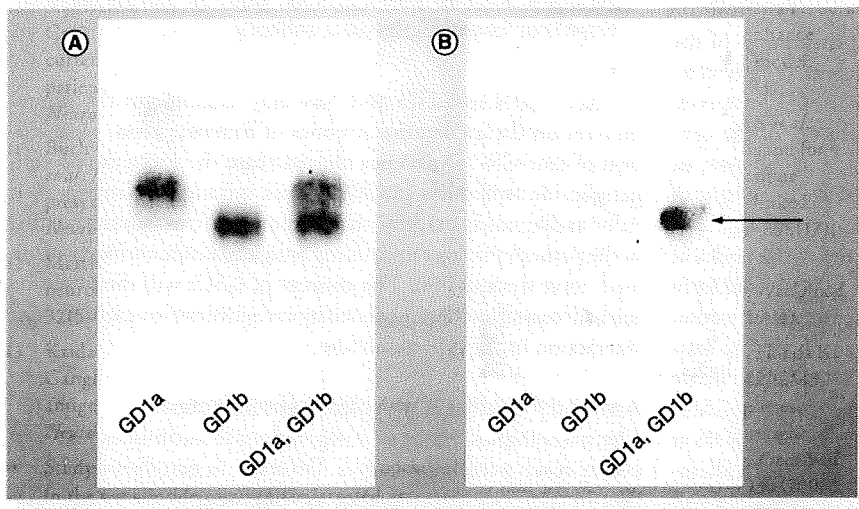

Figure 2. Thin layer chromatography (TLC) study. (A) TLC bands are visualized with orcinol reagent. **(B)** TLC immunostaining study reveals that the overlapping portion between GD1a and GD1b is strongly stained (arrow). Serum is diluted to 1:100.

the possibility that same glycoepitopes are recognized by GQ1b-specific, GQ1b/GM1-reactive, or GQ1b/GD1a-reactive antibody, or that each target molecules are in the vicinity on the nerve membrane. In view of the clustering of gangliosides in the biological membrane, GSCs containing GQ1b appear to be preferential antigens in some cases (Figure 4). As a matter of course, GQ1b must be a key molecule in the immunobiology of FS, and anti-GQ1b IgG antibody remains in place as an excellent diagnostic marker of FS.

The mechanism of anti-GSC antibody-mediated nerve injury remains to be elucidated, although a complement-mediated mechanism is speculated as seen in *ex vivo* or *in vitro* experiments using anti-GQ1b antibody. Ganglioside complexes may be preferentially formed in clustered glycoepitopes in the microdomains, such as lipid rafts or glycosynapses in nerve cell membranes, and anti-GSC antibodies may directly cause dysfunction of nerve cells through binding of anti-GSC antibodies to GSCs in the microdomains. Furthermore, tight binding between such multi-valent anti-GSC antibodies and clustered glycoepitopes may correlate with a predisposition to a severe form of the disease. Alternatively, anti-GSC antibodies may promote the breakdown of the blood–nerve barrier by binding to clustered glycoepitopes in various ligands on the membranes of vascular endothelial cells [49,50]. It has been inferred that ligands of adhesion molecules, such as selectins, comprise diverse and complex glycoconjugates, called clustered saccharide patches, in which oligosaccharides are packed closely together to form rigid rod-like structures with multivalency and strict binding specificity [51,52]. The discovery of anti-GSC antibodies in GBS serum suggests that clustered oligosaccharides on the plasma membrane are actually involved in immune-mediated events. As described in the latest research, clustered glycoepitopes of GSCs in the cell membrane may function in a more effective manner than a solo glycoepitope of isolated ganglioside [53].

Recently, a GSC consisting of GM1 and GalNAc-GD1a (GM1/GalNAc-GD1a) has been reported as a target antigen for pure motor variant of GBS [54]. Electrophysiological findings of the anti-GM1/GalNAc-GD1a-positive patients featured early conduction block at intermediate nerve segments of motor nerves. Serial nerve conduction studies show rapid recovery of the conduction block and no findings indicative of remyelination or axonal degeneration. Hence, the conduction block is thought to be a reversible conduction failure on the axolemma and originates from impairment of axonal membrane properties at the nodes of Ranvier [17,54]. GM1 and GalNAc-GD1a may aggregate and form GM1/GalNAc-GD1a in the motor axonal membrane at nodes, since GM1-like epitopes and GalNAc-GD1a locate on the axolemma at nodes of the motor nerves [26,27]. In view of the dense cluster of of Nav in the axolemma at the nodes, the anti-GM1/GalNAc-GD1a antibody is likely to bind GM1/GalNAc-GD1a at the nodes and cause reversible conduction block through alteration of the regulatory function of Nav. Further investigation will clarify whether anti-GM1/GalNAc-GD1a antibody induces conduction block through complement activation, direct breakdown of Nav function, or both.

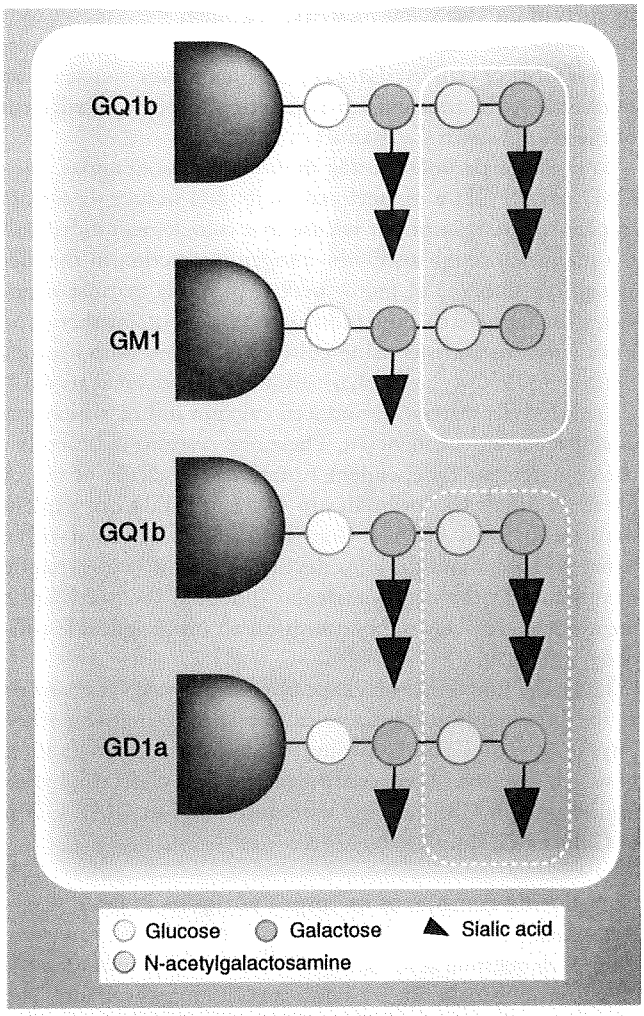

Figure 3. Carbohydrate structures of GQ1b, GM1, and GD1a. The rectangle with solid lines indicates a putative antigenic epitope for anti-GQ1b/GM1 antibody, and the one with dotted lines for anti-GQ1b/GD1a antibody.

GM1, GalNAc-GD1a and Nav may assemble in the microdomain on the axolemma at nodes of Ranvier. Thus, examination of anti-GSC antibodies may increase the spectrum of anti-ganglioside antibodies in GBS and its variants, enhancing their value as diagnostic markers and promoting further understanding of the pathophysiology underlying antiganglioside antibody-mediated nerve dysfunction. The concept of GSCs will shed light on microdomain function mediated by carbohydrate-to-carbohydrate interaction in the cell membrane.

Antibody avidity & glycolipid environment

The pathological effect of antiganglioside antibodies does not evenly reach peripheral nerves. Diversity in ganglioside expression in the PNS can influence development of the symptomatology of GBS with antiganglioside antibodies [6,55], and GBS is subdivided into some clinical phenotypes, each of which is probably associated with specific antiganglioside antibodies [55].

The IgG anti-GQ1b antibody is well known to be an excellent diagnostic marker of FS and have a pathogenetic potential for development of FS [11,46,47]. On the other hand, there are multiple reports showing close association of IgG anti-GD1b antibody with ataxia in GBS and experiments using rabbits sensitized with GD1b also indicate that IgG GD1b-specific antibodies can induce ataxic neuropathy [56,57]. Only half of GBS patients with IgG anti-GD1b antibody, however, demonstrated ataxia in GBS in a larger population of subjects [58]. Considering that different gangliosides can form clusters in living cell membranes [59] and clustered gangliosides such as GSCs are actually involved in antibody-mediated events [43,44], anti-GD1b antibodies may bind to a part of epitopes in the GSCs containing GD1b and differential specificity of the anti-GD1b antibodies may account for the clinical diversity. This hypothesis prompted us to investigate the activities of the anti-GD1b IgG-positive sera against GSCs containing GD1b [60]. We compared antibody activities to GSCs containing GD1b with use of anti-GD1b antibody-positive sera from GBS patients with or without ataxia, demonstrating that anti-GD1b activities to GD1b in sera from patients with ataxia were significantly inhibited by the addition of gangliosides with two or more sialic acids to GD1b. The addition of GD1a to GD1b completely inhibited the binding activity of anti-GD1b antibody to GD1b, suggesting that target epitopes of GD1b can be masked or modified by colocalization of GD1a. These findings indicate that IgG antibodies highly specific for GD1b induce ataxia in GBS and that colocalization of another ganglioside and GD1b may influence the accessibility of the anti-GD1b antibodies (Figure 5) [60]. Finally, the sugar chain of gangliosides may *cis*-interact in the microdomains of the biological membrane and modify the conformation of the glycoepitopes. Such complex glycolipid environments in the cell membrane may affect accessibility and avidity of antiganglioside antibodies against target gangliosides. Regarding IgG anti-GM1 antibodies highly involved in development of pure motor GBS, a recent study using GalNAcT$^{-/-}$ and GD3 synthase-deficient (GD3s$^{-/-}$) mice demonstrated also that the local glycolipid environment in the cell membrane is critical for the exertion of the pathogenetic effect of antiganglioside antibodies [61]. The binding ability of the pathogenetic anti-GM1 antibody to GM1-like epitopes is dependent upon what gangliosides neighbor upon GM1 in the cell membrane and whether GM1-like epitopes are unmasked. Such study has drawn the conclusion that ganglioside interaction may either enable or inhibit antibody binding to the neuronal membrane or be neutral [61]. Thus, to understand the pathogenetic role of antiganglioside antibodies, we should bear in mind that the neuropathic action of antiganglioside antibodies is dependent not only upon the fine specificity of individual antibodies but also upon the conformational diversity of glycoepitopes in glycolipid environments in the biological membrane. GSC antigens can be useful tools for assay of antiganglioside antibody activities against the conformational diversity of the clustered gangliosides in the cell membrane.

Putative targets for AIDP

Acute inflammatory demyelinating polyneuropathy is the most prevalent form of GBS in Western countries, the frequency of which is approximately 90% of total GBS [62]. In addition to cellular immune response associated with activated helper T cells, humoral immune response involved with surface antigens of Schwann cells has been speculated [7,62]. Some glycolipids such as GD1b, LM1 or galactocerebroside have been proposed as target antigens of AIDP [58,63–67]. Experimental allergic neuritis (EAN) induced by inoculation of peripheral myelin protein such as P0 or P2 has been considered as an animal model of AIDP. The predominant pathology of the EAN shows infiltration of T cells and macrophages and demyelination, similar to the histopathology of AIDP [7,68]. Indeed, sera from patients with AIDP often show antibodies to various peripheral nerve myelin, but it is not clear how the antibodies are involved with thr pathophysiology of AIDP [69–71]. The mechanism of conduction failure and demyelination in AIDP has also not been well understood. The disruption of Nav channel clusters at nodes is observed in spinal roots of EAN rats immunized with peripheral myelin, although the mechanism of the disruption was not elucidated [72]. A recent study by Lonigro and Devaux shed light on the disruption of Nav channel clusters at nodes

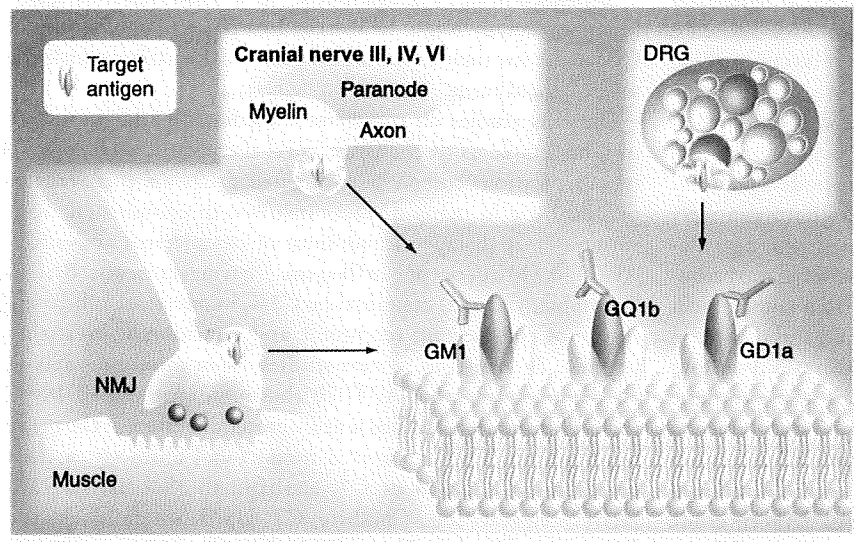

Figure 4. Putative target antigens for autoantibodies in Fisher syndrome. The lower right illustration shows a closeup of the antigenic molecules in the biological membrane. The target antigens are thought to be localized on presynaptic axolemma, perisynaptic Schwann cells, paranodal myelin in oculomotor, trochlear, and abducens nerves, and a subset of large dorsal root ganglion cells. Small balls at neuromuscular junction indicate acetylcholine released from presynaptic axonal membrane.

Table 1. Novel therapeutics for Guillain–Barré syndrome and its variants in preclinical development.

Agent	Nature & activity	Trials in animal	Ref.
APT070 (Mirococept)	Truncated sCR1 with a membrane-localizing peptide Inhibition of C3/C5 convertase	Mouse model of anti-GQ1b-positive neuropathy	[16]
Eculizumab	Humanized monoclonal antibody against C5 Blocking of the formation of C5a and C5b-9	Mouse model of anti-GQ1b-positive neuropathy	[80]
rEV576	Recombinant form of a complement inhibitory protein derived from the saliva of the soft tick Specific prevention of the conversion of C5 into C5a and C5b	Rat model of myasthenia gravis Mouse model of anti-GQ1b-positive neuropathy	[83,84]
Nafamostat mesilate	Synthetic serine protease inhibitor Inhibition of C1r and C1s, leading to inhibition of C3 fragment deposition	Rabbit model of AMAN	[85]
Flecainide	Sodium channel (Nav 1.5) blocking agent Axonal protection; reduction of axonal degeneration	Rat model of AIDP	[86]
sCR1	Complement regulatory protein Protection of nerves from early axonal degeneration after injury	Rat model of nerve crush injury	[89]

AIDP: Acute inflammatory demyelinating polyneuropathy; AMAN: Acute motor axonal neuropathy; sCR1: Soluble complement receptor 1.

and demyelination in EAN, a model of AIDP [73]. In EAN rats induced by inoculation of crude peripheral myelin, disruption of Nav channel clusters at nodes were found in parallel with the clinical signs, with dispersion of Kv1 channels at nodes and paranodes [73]. Neurofascin 186, a neuronal protein exposed on the surface of axon, and gliomedin, a myelin protein exposed on Schwann cell microvilli, were broken down prior to Nav channel dispersion and demyelination. The early breakdown of neurofascin and gliomedin, which are involved with aggregation of Nav channels at nodes, was followed by the node alteration. Interestingly, antibodies to neurofascin and gliomedin were found in sera from the EAN rats, and associated with degradation of axo–glial units and node alteration. A series of the pathological events at the node were independent from complement deposition, suggesting that antineurofascin and antigliomedin antibodies directly induce the node disorganization without complement activation. EAN rats immunized with synthetic P2 peptide showed little nodal changes and no antibodies to neurofascin and gliomedin with pathology of inflammatory demyelination, indicating that mechanism in EAN induced by immunization of P2 is different from that of EAN by crude peripheral myelin [73]. Thus, the pathophysiology of AIDP are heterogeneous, and humoral factors such as antibodies to neurofascin and gliomedin may also play a crucial role in the pathogenesis of a part of AIDP. From the aspect of treatment, it will be beneficial to know whether the predominant mechanism in a patient with AIDP is humoral or a cellular immune response, dependent upon complement activation or independent. Clinical, electrophysiological and immunobiological studies with a large population of AIDP patients will be required to select more effective treatments or to develop a new regimen.

New therapeutic strategy for GBS & FS

Specific immunomodulatory therapies such as IVIg and PE have hitherto been established in GBS [74]. Plasmapheresis, such as immunoadsorption with tryptophanyl ligands or double membrane filtration, is often utilized as an alternative to PE [75], although a large randomized trial for the efficacy has not yet been executed. Some clinical trials have demonstrated ineffectiveness of corticosteroid in GBS, which might be associated with inhibitory effects of corticosteroids against macrophage repair processes in demyelination [74], and intravenous methylprednisolone (IVMP) therapy executed together with IVIg had no significant benefit for improvement of disability compared with IVIg alone [76]. IVIg plus IVMP may be worthy of reconsideration, because IVIg plus IVMP yielded a better improvement of disability than IVIg alone ($p = 0.06$) [76]. Furthermore, adjustment for age and degree of disability at entry revealed superiority of a combination of IVIg and IVMP ($p = 0.03$) [76]. Such combination therapy might be worthy in a particular subgroup of GBS patients, such as ventilated patients or patients with the severe axonal form.

Ongoing studies on complement-mediated pathophysiology in GBS enable us to challenge novel therapeutic strategies [11] (Table 1). Various complement-targeted drugs are prepared for practical use in many diseases involved in complement activation [77]. A therapeutic inhibitor of complement activation, APT070 (Mirococept, Inflazyme Pharmaceuticals, British Columbia, Canada) contains the C3/C5 convertase inhibiting region of complement receptor 1 and a membrane-localizing peptide. *In vitro* and *in vivo* studies using mouse models of FS demonstrated that APT070 thoroughly prevented MAC (C5b-9) formation and had a neuroprotective effect at the nerve terminal [16]. The humanized monoclonal antibody against complement component C5, eculizumab (Soliris; Alexion Pharmaceuticals Inc., Cheshire, CT, USA), which blocks the formation of human C5a and C5b-9, is the first complement-specific drug authorized by the US FDA, and now applied to treatment of paroxysmal nocturnal hemoglobinuria [78,79]. As with Mirococept, eculizumab also showed neuroprotective potency at motor nerve terminals in a novel mouse model of FS [80]. Intraperitoneal administration of monoclonal anti-GQ1b

Table 2. The target sites of antiganglioside antibodies.

Target sites	Antiganglioside antibodies (target gangliosides)	Complement-mediated action	Ref.
Motor nerve			
Axonal membrane at nodes of Ranvier	GM1	Yes	[25,26,31]
	GalNAc-GD1a	?	[27]
Presynaptic axonal membrane	GM1	No	[41]
	GD1a	Yes	[41]
		No	[90]
	GalNAc–GD1a	No	[38]
	GQ1b	Yes	[11–13,16]
Perisynaptic Schwann cell	Disialosyl epitope (e.g., GQ1b, GD1b)	?	[15]
Paranodal myelin	GD1b	?	[63]
Paranodal myelin in oculomotor, trochlear, abducens nerves	GQ1b	?	[47]
Dorsal root ganglion			
Subset of large cells	GD1b	? (apoptosis)	[56,87]
	GQ1b	?	[93]

antibody and normal human serum as a complement source induced respiratory paralysis and destruction of nerve terminals in the mouse diaphragm through complement activation. Intravenous eculizumab injection prevented the respiratory paralysis and the complement-mediated nerve injury, which were confirmed immunohistochemically, electrophysiologically and functionally [80]. These results will provide promising therapeutic strategies, but we need take into account some problems. First, it appears to be impossible to administer eculizumab before or at the same time anti-GQ1b antibody attacks peripheral nerves in order to maximize the efficacy. The late administration of eculizumab may fail to inhibit nerve injury that has already begun to progress through complement activation. Second, normal human serum is required as a source of complement in the mouse model of FS. The reason why mouse complement system is not activated by mouse monoclonal anti-GQ1b antibody has not yet been elucidated [11,80]. Considering that it has already been administered to patients with paroxysmal nocturnal hemoglobinuria, a clinical trial of eculizumab will be planned also in GBS and FS patients in near future.

rEV576 is a recombinant form of a complement inhibitory protein identified from the saliva of the soft tick *Ornithodoros moubata*, and inhibits both the classic and the alternative pathways through specific prevention of the conversion of mouse and human C5 into C5a and C5b [81]. Recent reports have described that rEV576 has therapeutic efficacy in animal models of myasthenia gravis in which the terminal stage of complement pathway has been demonstrated to play a key role in pathophysiology [82,83]. In an *in vitro* mouse model of FS using monoclonal anti-GQ1b antibody, rEV576 completely prevented the formation of MAC and protected motor nerve terminals from antibody-mediated nerve injury [84]. Electrophysiological and functional assays revealed the dramatic efficacy of rEV576. This novel agent raises hopes for better-than-expected improvements in the treatment of a subset of GBS and FS in which complement activation plays a pathogenetic role. rEV576 that specifically blocks the C5 activation step is a more attractive agent in that C3b opsonization of pathogens and immune complex solubilization are unaffected by its complement-inhibitory ability.

In a rabbit model of AMAN with anti-GM1 antibodies, a complement inhibitory agent has been shown to inhibit complement deposition and complement-mediated disruption of Nav at the nodes of Ranvier in peripheral motor nerves [85]. A synthetic serine protease inhibitor, Nafamostat mesilate (6-amidino-2-naphtyl-p-guanidinobenzoate dimethanesulfonate), which has been used for acute pancreatitis and disseminated intravenous coagulation, has the complement inhibitory effect and prevents sodium channel disruption in the rabbit model of AMAN [85]. Nafamostat mesilate has often been used for plasmapheresis instead of heparin in Japan, and administered for patients with GBS when they experience plasmapheresis. Such combination of Nafamostat mesilate and plasmapheresis might be more beneficial than plasmapheresis alone. This agent may be a potent candidate for complement inhibitory therapy in light of its affordable price and easy administration.

A sodium channel-blocking agent, Flecainide, which works by blocking the voltage-gated sodium channel Nav1.5 in the heart, has been reported to have an effect on axonal protection in EAN rats sensitized with bovine peripheral myelin [86]. Flecainide ameliorates the neurological deficits, electrophysiological findings indicating demyelination and axonal loss, and axonal damage in tibial nerve fibers in the rat model of AIDP, although the mechanism of its axonal protection has been obscure [86]. This agent may improve the outcome of GBS and its variants, especially AIDP, by protecting the axonal damage.

Expert commentary

Growing evidence supports the importance of complement activation in the pathophysiology of GBS and FS and the neuroprotective effect of complement inhibitory agents, and encourage us to challenge clinical trials of the agents, although some issues of actual use remain to be solved. As is the case of IVIg and PE, its use in the early stage of the disorders should be more beneficial and critical. In the clinical scene, however, complement system has already been activated and nerve injury has progressed to some extent when patients with GBS and FS visit hospitals a few days after the onset of the neurological symptoms. It is unknown whether such late administration of the complement inhibitory agents

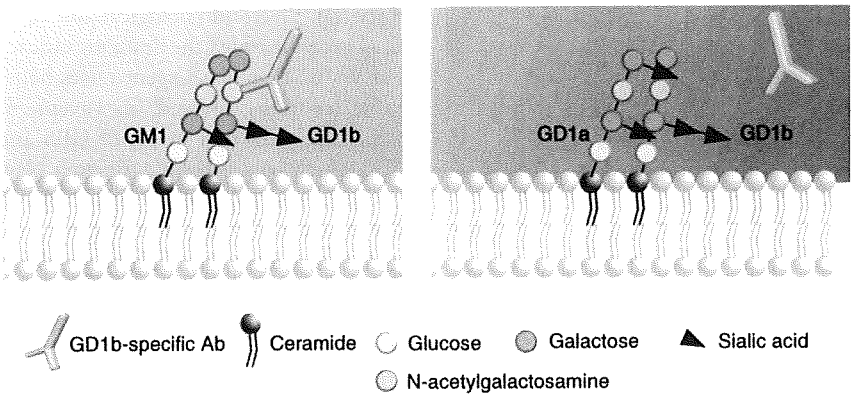

Figure 5. Putative antigen–antibody interaction between GD1b-specific antibody and ganglioside complexes containing GD1b in the nerve cell membrane. In **(A)**, GD1b and GM1 co-localize and *cis*-interact together in the membrane. The GD1b-specific antibody can easily access antigenic epitopes of GD1b in the GD1b-GM1 complex. In **(B)** membrane, GD1b and GD1a co-localize and *cis*-interact together in the membrane. The GD1b-specific antibody can hardly access the antigenic epitopes of GD1b in the GD1b-GD1a complex.

improves the disability and the outcome significantly. Considering that IVIg and PE are more effective if conducted within 2 weeks after the onset of the diseases, the complement inhibitory agents appear to have a potency to limit nerve injury until pathogenetic autoantibodies disappear from the patient's serum. Furthermore, in addition to cost–effectiveness, when or how to administer the complement inhibitory agents should be carefully determined in the practical use, because GBS is a monophasic disorder different from chronic relapsing complement-mediated diseases for which complement inhibitory agents are considered.

The pathophysiology of GBS appears to be heterogeneous. A recent study using a rabbit model of anti-GD1b antibody-associated sensory ataxic neuropathy demonstrated that an apoptotic mechanism is predominant in the pathophysiology of the rabbit model [87]. This suggests that the activation of apoptotic cascade plays a key role in development of anti-GD1b-positive GBS with ataxia, and the complement inhibition may exert little efficacy of nerve protection in the disorder. The efficacy of the complement inhibition has been shown exclusively in a rabbit model of AMAN or a mouse model of FS in which target antigens such as GM1 or GQ1b are shown to be located on the axonal membrane of motor nerves [11,85]. Cellular immune response to myelin antigens, including macrophage activation, can be an alternative mechanism in AIDP, in addition to the complement activation and the formation of the MAC in peripheral nerves [62]. Complement inhibition may be more effective in AIDP in which complement-mediated pathophysiology is predominant. The therapeutic strategy of GBS and its variants should depend on the individual immunobiological mechanism; whether the activation of complement system is predominant or not as well as whether the disorders are electrophysiologically AIDP or AMAN.

Besides blockade of antibody-mediated complement activation, complement inhibition has the therapeutic potential to rescue axons from Wallerian degeneration in impaired nerves. Acute peripheral nerve injury can activate the classical pathway of the complement system and induce the formation of the MAC, which is essential for the early events of axonal degradation during Wallerian degeneration [88]. Moreover, complement inhibition can suppress the recruitment and activation of macrophages [88]. Complement inhibition by soluble complement receptor 1, an inhibitor of all complement pathways, can shield peripheral nerves from early axonal loss [89]. These observations indicate that complement inhibition has a therapeutic potency to directly preclude axonal damage and indirectly inhibit macrophage accumulation in impaired nerves after acute nerve injury, leading to an improved outcome of GBS. Thus, we can expect the protective effect of the complement inhibitory agents against primary or secondary axonal damage in autoimmune neuropathy, although it may be supplemental.

As with of GBS patients demonstrating rapid recovery after immunomodulatory therapies, reversible conduction failure with little pathological changes of nerve structure may cause limb weakness. Antibody-mediated dysfunction of Nav in the axonal membrane at the nodes of Ranvier is probably a cause of the functional block without pathological changes. Interaction between gangliosides and Nav at nodes of Ranvier may contribute to development of such antibody-mediated functional block, which is one of the issues to be solved.

Since the discovery of antiganglioside antibodies in sera from GBS or FS patients, clinical and immunobiological studies on the pathophysiology of GBS has greatly progressed. Close association of anti-GQ1b antibodies with ophthalmoplegia is principally founded on the specific localization of GQ1b on the paranodal myelin in human oculomotor, trochlear and abducens nerves [47]. The antibodies highly specific to GD1b contribute to ataxia in GBS and induce experimental ataxic neuropathy in rabbits immunized with GD1b [60], associated with specific location of GD1b in subsets of large dorsal root ganglion cells [56,57]. Thus, antiganglioside antibody-mediated nerve dysfunction is fundamentally regulated by antibody specificity and specific distribution of target gangliosides in the PNS (Table 2) [6,55]. Recent studies, meanwhile, indicate that some specific conditions of glycoepitopes in the cell membrane of peripheral nerves are required for induction of pathogenetic action of antiganglioside antibodies. First, as mentioned previously, complex glycolipid environments in the cell membrane may affect accessibility and avidity of antiganglioside antibodies against target gangliosides [60,61]. Screening for antibodies to GSCs is essential for elucidating the pathogenetic role of antiganglioside antibodies in the development of GBS and its variants. Analysis of the reactivity of antiganglioside antibodies against GSC antigens is also important. Second, the large amount of targeted ganglioside in specific loci of peripheral nerves makes the loci predisposed to antibody-mediated injury. Anti-GD1a-mediated injury was found

in GD3-synthase knockout mice that overexpressed GD1a but not found in normal mice, suggesting that the high level of expression of GD1a at neuromuscular junctions is required for development of anti-GD1a-mediated disorders [90]. Third, the steric microstructure of gangliosides can influence the binding ability of antiganglioside antibodies. In immunohistochemical experiments using GD1a derivatives which sialic-acid residues were chemically modified, anti-GD1a monoclonal antibodies that preferentially stained motor axons specifically reacted with such GD1a derivatives as GD1a-1-ethyl methyl, GD1a-1-alcohol and GD1a-1-metylester, different from reaction of another anti-GD1a monoclonal antibodies that stained both motor and sensory axons [91]. Anti-GD1a antibodies from AMAN patients revealed the similar reaction to the motor-specific anti-GD1a monoclonal antibodies. Both the fine specificity and ganglioside exposure in the nerves are probably significant contributions to target recognition by antiganglioside antibodies [91].

Various functional molecules that are located on nodes or paranodes and involved in conduction property of motor nerves may be identified in the future. Thrombin receptor PAR-1 (G-protein-coupled protease-activated receptor) is predominantly localized on noncompacted Schwann cell microvilli at the nodes of Ranvier, and interaction of thrombin with the PAR-1 is likely to produce a reversible conduction block in peripheral nerves [92]. Antiganglioside antibody–antigen interaction at nodes may alter the function of molecules such as PAR-1, inducing conduction failure at nodes and muscle weakness in GBS.

Five-year view

Many autoimmune, inflammatory, and infectious diseases have been demonstrated to be attributable to excessive complement activity. Complement-targeted therapeutics is emerging as a hopeful strategy and a salvor to such refractory complement-mediated diseases [77]. There are much data indicating that complement activation underlies development of GBS and its variants. In addition to authorized complement-specific drugs, such as eculizumab, various drug candidates that are in clinical trials and preclinical development will be used to verify their efficacy in *in vitro*, *ex vivo* or *in vivo* models of autoimmune neuropathy, and in the near future will be applied to clinical trials for GBS and its variants. Combination therapy such as complement inhibitory agents and IVIg or PE may be challenged in these trials. To adequately apply novel drugs to patients with GBS and its variants, it is important to understand the mechanisms underlying the disorders in individual cases. Complement-dependent pathophysiology is probably a key role in the development of GBS, and complement-independent mechanisms such as the functional blockade of voltage-gated Ca channel, the apoptotic mechanism, or antineurofascin antibody-mediated disorganization of nodes is not negligible. Precise identification of target epitopes and analysis of their conformation will lead to the development of various efficacious remedies such as anti-idiotypic antibody neutralizing antiganglioside or antineurofascin antibodies. In the near future, indication of novel drugs, the timing of administration, and a various combinations of established treatments and novel drugs should be examined before clinical trials. Combination therapies such as IVIg and a complement inhibitory agent, in practice, will be more feasible than single administration of the novel agent.

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Key issues

- In a rabbit or mouse model of Guillain–Barré syndrome (GBS) or Fisher syndrome (FS), complement activation through the classical pathway is essential for development of the disorders.
- In anti-GM1-positive acute motor axonal neuropathy, motor conduction failure is probably a result of antibody-mediated disruption of voltage-gated Na channel clusters at the nodes of Ranvier.
- IgG antibodies to GM1, GD1a or GalNAc-GD1a can induce complement-independent blockade of voltage-gated Ca channel at the presynaptic membrane, explaining a part of the paralysis.
- Ganglioside complexes (GSCs) consisting of two different gangliosides work as target antigens in a proportion of GBS and a half of FS patients.
- Anti-GSC antibodies in GBS sera are real examples indicating that clustered oligosaccharides on the plasma membrane are actually involved in immune-mediated events. Screening for anti-GSC antibodies will broaden the spectrum of antiganglioside antibodies, enhancing their value as diagnostic markers.
- Antiganglioside antibody-mediated nerve dysfunction is fundamentally regulated by antibody specificity and specific distribution of target gangliosides in the PNS.
- The exertion of the pathogenetic effect of antiganglioside antibodies depends upon local glycolipid environment in the cell membrane.
- In acute inflammatory demyelinating polyneuropathy, neurofascin 186 and gliomedin are candidates for target molecules, associated with complement-independent node disorganization.
- In experimental conditions of GBS and its variants, complement inhibitory agents exert a neuroprotective effect by inhibiting activation of the classical pathway.

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Short communication

Antibodies to ganglioside complexes consisting of asialo-GM1 and GQ1b or GT1a in Fisher and Guillain–Barré syndromes

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ABSTRACT

To determine the epitopes of ganglioside complexes (GSCs) containing GQ1b or GT1a, we investigated their reactivity to GSCs consisting of asialo-GM1 (GA1) and GQ1b or GT1a using IgG anti-GQ1b- or anti-GT1a-positive sera. Nine anti-GQ1b-positive sera had higher activity to GA1/GQ1b than to GQ1b, only five of which reacted with GM1/GQ1b and GD1b/GQ1b. Five of 14 sera positive for GA1/GT1a and GM1/GT1a were negative for GA1/GQ1b and GM1/GQ1b. Sialic acids attached to the internal galactose of gangliotetraose can influence the reactivity of anti-GSC antibodies. Screening for antibodies to GSCs containing GA1 is useful for elucidation of the antibody-mediated pathophysiology.

1. Introduction

Fisher syndrome (FS), a variant of Guillain–Barré syndrome (GBS), presents with external ophthalmoplegia, ataxia and areflexia (Fisher, 1956). Anti-GQ1b antibody may play a pathogenetic role in the development of FS, GBS with ophthalmoplegia, and Bickerstaff's brainstem encephalitis (Chiba et al., 1992, 1993; Odaka et al., 2003), and FS patients often have antibodies to ganglioside complexes (GSCs) containing GQ1b (Kaida et al., 2006; Kanzaki et al., 2008). The anti-GSC antibodies are classified into two groups based on their reactivity to GSCs, GM1/GQ1b or GD1a/GQ1b, and the reactivity of these antibodies appear to depend upon the number of sialic acids in terminal residues in the GSCs (Kaida et al., 2006; Kanzaki et al., 2008).

Aisialo-GM1 (GA1) has terminal residues with a gangliotetraose structure, as for GM1 or GD1b (Fig. 1A). GA1 is contained in the human peripheral nerve tissue (Ogawa-Goto and Abe, 1998) but its location in the peripheral nerve and the pathogenetic role of the anti-GA1 antibody in GBS have not been defined, although an ex vivo study showed that anti-GA1 antibody had a presynaptic blocking effect at neuromuscular junctions (NMJs) through inhibition of voltage-gated Ca^{2+} channels (Taguchi et al., 2004). Some anti-GM1 antibodies in GBS sera cross-react with GA1 and probably bind to the terminal N-acetylgalactosamine-galactose moiety (Koga et al., 2001). The terminal residues of GA1/GQ1b are similar to those of GM1/GQ1b or GD1b/GQ1b (Fig. 1A), and anti-GM1/GQ1b or anti-GD1b/GQ1b antibody may cross-react with GA1/GQ1b. The same holds true for GA1/GT1a. Recently, we have found anti-GA1/GQ1b and anti-GA1/GT1a antibodies in sera from FS patients that did not react with GM1/GQ1b and GD1b/GQ1b, implying that factors other than the number of sialic acids in terminal residues of GSCs can influence the antigen-antibody interaction. To examine this hypothesis, we explored the specificity of antibodies to GA1/GQ1b and GA1/GT1a in sera from FS or GBS patients.

2. Materials and methods

2.1. Subjects

Sera were collected in the acute phase before treatment at various hospitals in Japan between August 2006 and September 2007. Diagnosis was based on questionnaires sent by attendant physicians. The samples were sent to our laboratory for screening for antibodies to GM1, GM2, GM3, GD1a, GD1b, GT1a, GT1b, GA1, galactocerebroside and GQ1b, using enzyme-linked immunosorbent assay (ELISA) (Kusunoki et al., 1994). From these samples, we selected 20 sera (GQ1b group) positive only for anti-GQ1b IgG antibody and 5 sera (GT1a group) positive only for anti-GT1a IgG antibody. This selection was performed to attenuate the impact of cross-reactivity between anti-GQ1b and anti-GT1a antibodies.

2.2. ELISA for antibody to GSCs

Reactivity to GSCs containing GQ1b or GT1a was investigated by ELISA using 100 ng of each glycolipid (Kaida et al., 2006; Kanzaki

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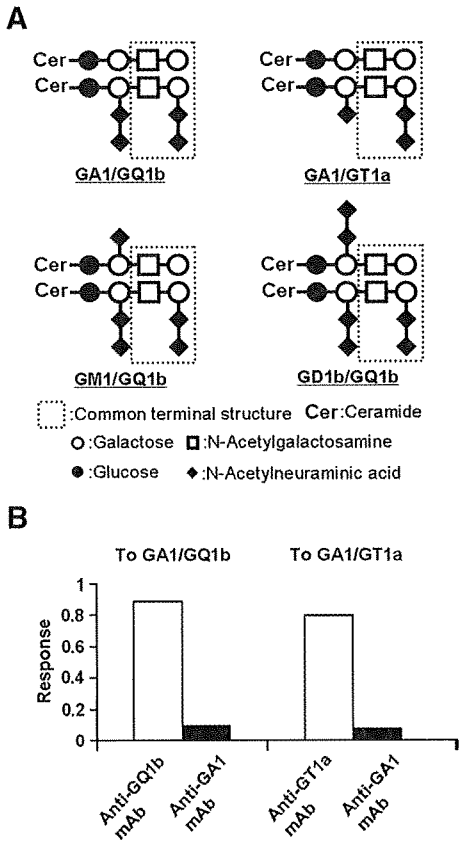

Fig. 1. (A) Pattern diagrams of four glycolipid complexes containing GQ1b and GT1a. GA1/GQ1b, GM1/GQ1b, GD1b/GQ1b and GA1/GT1a have a common terminal structure, which is shown by a dotted box. (B) In the left graph, the white bar shows the response (0.88) of monoclonal anti-GQ1b antibody to GA1/GQ1b, and the black bar shows the response (0.11) of monoclonal anti-GA1 antibody to GA1/GQ1b. In the right graph, the white bar shows the response (0.80) of monoclonal anti-GT1a antibody to GA1/GT1a, and the black bar shows the response (0.08) of monoclonal anti-GA1 antibody to GA1/GT1a. Note the strong inhibition of anti-GA1 antibody activity to GSCs such as GA1/GQ1b and GA1/GT1a.

et al., 2008). The other component of the GSCs was GA1, GM1, GD1a or GD1b. GQ1b and GT1a were purchased from HyTest (Joukahaisenkatu, Turku, Finland) and other glycolipids were purchased from Sigma-Aldrich (St. Louis, MO, USA). The purity of these antigens was confirmed by thin-layer chromatography as mentioned elsewhere (Kusunoki et al., 1994). Peroxidase-conjugated anti-human IgG antibody (MP Biomedicals, Solon, OH, USA) was used as the secondary antibody. The optical density (OD) was determined at 490 nm and corrected by subtracting the average OD of uncoated control wells. The sera were judged to be positive for one glycolipid antibody when the corrected OD was >0.1. Anti-GQ1b-positive sera for which the corrected OD of the anti-GA1/GQ1b antibody was 0.2 higher than the corrected OD of the antibodies to GQ1b or GA1 were considered anti-GA1/GQ1b antibody-positive. The same criterion was applied for the other anti-GSC antibodies. ELISA was performed in duplicate and mean ODs were calculated (Kaida et al., 2004, 2006).

The reactivity of monoclonal IgM anti-GA1, anti-GQ1b and anti-GT1a antibodies (Seikagaku Biobusiness Corp., Tokyo, Japan) to GA1/GQ1b and GA1/GT1a was also evaluated to verify whether epitopes of GA1, GQ1b or GT1a are preserved in these GSCs. Peroxidase-labeled anti-mouse IgM antibody (Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA) was used as the secondary antibody. The response of each monoclonal antibody against GA1/GQ1b or GA1/GT1a was calculated as follows:

Response of monoclonal anti−GA1 antibody (GA1−mab)
= (corrected OD of GA1−mab to GA1/GQ1b or GA1/GT1a)
÷(corrected OD of GA1−mab to GA1)

Response of monoclonal anti−GQ1b antibody (GQ1b−mab)
= (corrected OD of GQ1b−mab to GA1/GQ1b)
÷(corrected OD of GQ1b−mab to GQ1b)

Response of monoclonal anti-GT1a antibody (GT1a-mab) was calculated in the same way as GQ1b-mab.

3. Results

3.1. ELISA with patient's samples

A summary of patients is shown in Table 1. Nine of the 20 GQ1b-group sera (45%) were GA1/GQ1b-positive and 5 of these 9 sera (patients 2, 3, 4, 6 and 17) were negative for GM1/GQ1b and GD1b/GQ1b, but positive for GA1/GT1a and GM1/GT1a (Table 1). Fourteen GQ1b-group sera had higher reactivity with GA1/GT1a or GM1/GT1a, and 5 of these 14 (patients 7, 8, 11, 13 and 19) reacted only with GSCs containing GT1a. One GT1a-group serum (patient 21) was specific to GA1/GQ1b and GA1/GT1a, and another GT1a-group serum (patient 23) reacted with not only GM1/GT1a but also GM1/GQ1b. Anti-GD1a/GT1a antibody was not detected in any samples.

Table 1
Diagnosis and anti-GSC antibodies in patients in the GQ1b and GT1a groups.

Patient no.	Diagnosis	cOD for	Anti-GA1/GQ1b	Anti-GM1/GQ1b	Anti-GD1a/GQ1b	Anti-GD1b/GQ1b	Anti-GA1/GT1a	Anti-GM1/GT1a	Anti-GD1b/GT1a
GQ1b group		GQ1b							
1	FS	0.14	++	++	−	−	++	++	−
2	FS	0.26	++	−	−	−	+++	++	−
3	FS	0.12	++	−	−	−	+	++	++
4	FS	0.27	++	−	−	−	+	++	−
5	FS	0.13	+	+	−	−	+	+	−
6	FS	0.13	+	−	−	−	++	++	++
7	FS	0.20	−	−	−	−	++	++	+
8	FS	0.43	−	−	−	−	+	+	−
9	FS	0.11	−	−	−	−	−	−	−
10	FS	0.17	−	−	−	−	−	−	−
11	FS	0.56	−	−	−	−	++	++	+
12	FS	0.26	−	−	−	−	−	−	−
13	FS	0.15	−	−	−	−	++	++	++
14	FS	0.17	−	−	−	−	−	−	−
15	FS	0.35	−	−	−	−	−	−	−
16	GBS-op	0.30	++	++	−	++	++	+++	++
17	GBS	0.28	++	−	−	−	++	++	++
18	GBS-op	0.23	++	++	−	++	++	++	++
19	GBS	0.49	−	−	−	−	++	++	++
20	BBE	0.17	−	−	−	−	−	−	−
GT1a group		GT1a							
21	FS	0.34	+++	−	−	−	++	−	−
22	FS	0.18	−	−	−	−	−	−	−
23	GBS	0.11	+++	+++	−	++	++	+++	+
24	GBS	0.63	−	+	+	−	−	−	−
25	BBE	0.19	−	−	−	−	−	−	−

cOD: corrected optical density; FS: Fisher syndrome; GBS-op: Guillain–Barré syndrome with ophthalmoplegia; BBE: Bickerstaff's brainstem encephalitis. GD1a/GT1a was negative in all cases. Patients 17 and 19 were diagnosed with GBS without cranial nerve palsy. Corrected OD for anti-GSC after subtraction of corrected OD of anti-GQ1b or anti-GT1a. −; <0.2, +; ≥0.2, ++; ≥0.3, +++; ≥0.8, ++++; ≥1.3.

3.2. ELISA with monoclonal antibody

The responses of monoclonal IgM anti-GA1, -GQ1b, and -GT1a antibodies to GSCs are shown in Fig. 1B. The response of the anti-GA1 antibody to GA1/GQ1b was much lower than that of the anti-GQ1b antibody to GA1/GQ1b. Similarly, the activity of the anti-GA1 antibody was strongly inhibited by GA1/GT1a.

4. Discussion

GSC consisting of two gangliosides may express new epitopes that differ from its constituent gangliosides (Kaida et al., 2004, 2006). A combination of [Galβ1-3GalNAc] and [NeuAcα2-8NeuAcα2-3Galβ1-3GalNAc] in the terminal residues of gangliotetraose structures may be important as an epitope for the anti-GM1/GQ1b antibody (Kaida et al., 2006; Kanzaki et al., 2008). GA1/GQ1b has an above combination in the terminal residue, as for GM1/GQ1b or GD1b/GQ1b. GA1/GT1a, GM1/GT1a and GD1b/GT1a also share this structure. However, in the present study GQ1b-group sera often showed a different response to GA1/GQ1b and GA1/GT1a (Table 1). Some sera in the GQ1b group that were positive for GA1/GT1a and GM1/GT1a (patients 7, 8, 11, 13 and 19) had no reactivity to GA1/GQ1b or GM1/GQ1b. These results suggest that sialic acids attached to internal galactose residues in the gangliotetraose structure may also be essential for antibody binding to such GSCs. Interestingly, an IgG antibody specific to GA1 fixed on an ELISA plate can be absorbed by soluble GD1a in glycolipid-detergent mixtures (Lopez et al., 2006), with speculation that the different flexibility of the glycolipid chain between the solid phase and the soluble micellar phase produced this phenomenon and that access of the antibody was regulated by the three-dimensional structure. Similarly, the specificity of anti-GSC antibody may depend upon the steric structure of targeted GSCs, which can be influenced not only by sialic acids in the terminal residues but also by those attached to an internal galactose. Conformational analyses of glycoepitopes in GSCs are required for precise identification of target antigens.

The presynaptic blocking effect of anti-GA1 antibody (Taguchi et al., 2004) and the abnormal presynaptic transmission at NMJs observed in FS patients with anti-GQ1b antibody (Lo et al., 2006) raise the possibility that GA1 and GQ1b coexist in the presynaptic membrane of motor nerve terminals. GA1/GQ1b-positive sera always showed similar strong activities to GA1/GT1a or GM1/GT1a compared with those to GA1/GQ1b. Eleven GQ1b-positive sera (patients 7–15, 19, 20) were GA1/GQ1b-negative, and 5 of the 11 showed specificity for GA1/GT1a and GM1/GT1a. Therefore, glycoepitopes of GA1/GT1a or GM1/GT1a, as well as those of GA1/GQ1b, may be preferentially targeted.

On another front, it appears that the epitope for the monoclonal anti-GA1 monoclonal antibody was masked in GA1/GQ1b, whereas that for the monoclonal anti-GQ1b antibody was still expressed. Therefore, even if GA1 and GQ1b actually form complexes in motor nerve terminals, the anti-GQ1b antibody can access GQ1b epitopes in GA1/GQ1b but the anti-GA1 antibody cannot access GA1 epitopes in GA1/GQ1b. Therefore, prudent interpretation of immunohistochemical results using monoclonal anti-ganglioside antibodies is required in the determination of target glycoepitopes in biological membranes (Greenshields et al., 2009).

In our series, the frequency of anti-GM1/GQ1b antibody was low compared with previous reports (Kaida et al., 2006; Kanzaki et al., 2008). This may depend on the selection bias in subjects such as GQ1b- or GT1a-positive patients. Larger numbers and more clinical information for patients are required to clarify the clinical association of anti-GA1/GQ1b and anti-GA1/GT1a antibodies.

The concept of "anti-GQ1b IgG antibody syndrome" has been advanced to explain the shared pathophysiology among FS, GBS with ophthalmoplegia, and Bickerstaff's brainstem encephalitis (Odaka et al., 2001). Investigation of antibodies to various GSCs containing GQ1b or GT1a may solve this nosological issue and more precisely elucidate the mechanism of anti-ganglioside antibody-associated nerve dysfunction. We recently found a serum from an FS patient that reacted with GA1/GQ1b but not with GA1, GQ1b or GT1a (data not shown). GA1 has not been considered to be an important antigen in GBS, but screening for antibodies to GSCs containing GA1 may be meaningful for diagnosis and elucidation of the antibody-mediated pathophysiology of the disorders.

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Suppression of Experimental Autoimmune Encephalomyelitis by Ghrelin[1]

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Ghrelin is a recently identified gastric hormone that displays strong growth hormone-releasing activity mediated by the growth hormone secretagogue receptor. While this unique endogenous peptide participates in the regulation of energy homeostasis, increases food intake, and decreases energy expenditure, its ability to inhibit the production of proinflammatory cytokines in vitro indicates its role in the regulation of inflammatory process in vivo. Here we examine the effect of exogenous ghrelin on the development of experimental autoimmune encephalomyelitis (EAE), a representative model of multiple sclerosis. In the C57BL/6 mouse model of EAE induced by sensitization to myelin oligodendrocyte glycoprotein 35–55 peptide, we found that alternate-day s.c. injections of ghrelin (5 μg/kg/day) from day 1 to 35 significantly reduced the clinical severity of EAE. The suppression of EAE was accompanied by reduced mRNA levels of proinflammatory cytokines such as TNF-α, IL-1β, and IL-6 in the spinal cord cellular infiltrates and microglia from ghrelin-treated mice at the peak of disease, suggesting the role of ghrelin as an antiinflammatory hormone. Consistently, ghrelin significantly suppressed the production of proinflammatory cytokines in LPS-stimulated microglia in vitro. These results shed light on the new role of ghrelin in the regulation of inflammation with possible implications for management of human diseases. *The Journal of Immunology,* **2009, 183: 2859–2866.**

Small synthetic compounds, referred to as growth hormone (GH)[3] secretagogues (GHS), have been known to stimulate GH release, working through a G protein-coupled receptor called GHS receptor (GHS-R) (1–3). It is now established that a new endogenous peptide, ghrelin, discovered in rat gastric extracts, is an endogenous ligand for GHS-R and is involved in the regulation of GH release. Ghrelin is a 28-aa polypeptide with an essential *n*-octanoyl modification on serine at position 3 (4). Although ghrelin is predominantly secreted from mucosal endocrine cells of stomach, it is widely distributed in various organs, including lymphoid tissues (5, 6). Furthermore, it is measurable in the systemic circulation, indicating its hormonal nature (7).

Ghrelin does not only stimulate GH release, but it also increases food intake, regulates energy homeostasis, and decreases energy expenditure by lowering the catabolism of fat (4, 8, 9). Because of its orexigenic and adipogenic character, ghrelin may be potentially useful for the treatment of anorexia and cachexia (10, 11). Although the precise mechanisms remain to be clarified, the orexigenic activities of ghrelin may be mediated by another feeding regulatory hormone neuropeptide Y (NPY) via stimulation of Y1 and Y5 receptors (12). Furthermore, the antagonistic effect of ghrelin on leptin-induced decrease of food intake seems to be mediated by ghrelin-induced release of NPY and subsequent stimulation of the Y1 receptor (13).

Ghrelin has been shown to exhibit antiinflammatory functions against T cells and macrophages in vitro (14–16). The potential activity of ghrelin as antiinflammatory reagent in vivo was shown in several animal models, including bowel disease (17), arthritis (16, 18), sepsis, and endotoxemia (16, 19, 20). Here we report that s.c. injections of ghrelin could significantly attenuate the clinical severity of the representative model of experimental autoimmune encephalomyelitis (EAE) induced in C57BL/6 (B6) mice by sensitization against myelin oligodendrocyte glycoprotein $(MOG)_{35-55}$ peptide. Furthermore, we demonstrate that in vivo treatment with ghrelin significantly suppressed the mRNA levels of the proinflammatory cytokines TNF-α, IL-1β, and IL-6 in microglia and infiltrating T cells derived from the spinal cords of ghrelin-treated mice. Finally, we confirm that LPS-stimulated microglia and monocytes produced lower amounts of proinflammatory cytokines when they were pretreated with ghrelin in vitro. In conclusion, the present study indicates the potential use of ghrelin as an antiinflammatory drug to control human CNS pathology.

Materials and Methods

Mice and reagents

We used female B6 mice (CLEA Japan) between 6 and 10 wk of age in specific pathogen-free conditions. Animal care and use were in accordance

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[3] Abbreviations used in this paper: GH, growth hormone; EAE, experimental autoimmune encephalomyelitis; GHS, growth hormone secretagogue; GHS-R, growth hormone secretagogue receptor; LN, lymph node; MOG, myelin oligodendrocyte glycoprotein; NPY, neuropeptide Y.

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Table I. *Amino acid sequence of mouse ghrelin and des-acyl ghrelin*

Peptide	Amino Acid Sequence[a]	Ser[3] acylation	Reference
Ghrelin	GSSFLSPEHQKAQQRKESKKPPAKLQPR	*n*-Octanoicacid	(4)
Des-acyl ghrelin	GSSFLSPEHQKAQQRKESKKPPAKLQPR		(7)

[a] The underlined letter S represents the third serine (Ser[3]).

with institutional guidelines. Animal experiments were approved by our institutional review committee. Rat MOG_{35-55} (amino acid sequence MEVGWYRSPFSRVVHLYRNGK) was synthesized at Toray Research Center (Tokyo, Japan). Ghrelin and des-acyl ghrelin (Table I) were synthesized as previously described (4, 7).

Immunization and clinical assessment of EAE

We immunized mice (n = 5–15 per group) s.c. in the tail base with 100 μg of MOG_{35-55}-peptide dissolved in 0.1 ml of PBS and 0.1 ml of CFA containing 1 mg of *M. tuberculosis* H37Ra (Difco). Shortly after immunization and 48 h later, the mice were injected i.p. with 200 ng of pertussis toxin (List Biological Laboratories). Clinical scores of EAE were daily assigned as follows: 0, normal; 1, weakness of the tail and/or paralysis of the distal half of the tail; 2, loss of tail tonicity; 3, partial hind limb paralysis; 4, complete hind limb paralysis; 5, forelimb paralysis or moribund; 6, death. The cumulative scores were calculated for individual mice by summing up the daily scores.

Administration of ghrelin and des-acyl ghrelin

For EAE treatment, we s.c. injected ghrelin and des-acyl ghrelin diluted in 0.9% saline. In the first series of experiments, mice were injected with ghrelin or des-acyl ghrelin at doses of 0.5, 5, or 50 μg/kg every other day for 35 days. Sham-treated animals were injected with 0.9% saline (standard protocol). In the next experiment, we injected the mice with 5 μg/kg ghrelin every day from day 1 to 10 (induction phase treatment) or from day 11 to 20 (effector phase treatment) and in-between with 0.9% saline. The controls were injected every day from day 1 to 20 with 0.9% saline (alternative protocol).

Assessment of histological EAE

To evaluate the histological manifestations of EAE, we treated mice with 5 μg/kg ghrelin or 0.9% saline following the standard protocol and sacrificed them on day 17 postimmunization. The spinal cords were removed and fixed in buffered formalin. They were embedded in paraffin, sectioned, and stained with H&E and Luxol fast blue for histopathological analysis.

Flow cytometry and isolation of mononuclear cells from the CNS

B6 mice were challenged for EAE, treated following the standard protocol with 5 μg/kg ghrelin or 0.9% saline and sacrificed on day 17 postimmunization. We removed spleen, lymph nodes (LN), and thymus as well as spinal cord from the ghrelin- and saline-treated mice for flow cytometer analysis. Single-cell suspensions were prepared according to standard methods. The spinal cord cell suspensions were centrifuged at 200 × g for 10 min and resuspended in 4 ml of 70% isotonic Percoll (Amersham Biosciences)/PBS and overlaid by equal volumes of 37% and 30% isotonic Percoll. The gradient was centrifuged at 500 × g for 15 min and the mononuclear cells were harvested from the 37%–70% interface, washed, and counted. The cells were stained for 5 min with anti-FcRγ III/II mAb (BD Pharmingen), washed, and labeled with the following mAbs for surface phenotype analysis: FITC-CD4 mAb, FITC-CD19 mAb, PE-CD8a mAb, PE-NK1.1 mAb, PE-CD25 mAb, allophycocyanin-FOXP3, and PerCP-Cy5.5-CD3e mAb (BD Pharmingen) and FITC-F4/80 mAb (Dainihon Seiyaku). The cytofluorometric analysis was performed using a FACSCalibur operated by CellQuest software (BD Biosciences).

Cytokine and cell proliferation assay

MOG_{35-55}-immunized B6 mice were treated s.c. with 5 μg/kg/day of ghrelin or 0.9% saline every day from day 1 to 10. The LN cells were collected on day 11 after immunization and suspended in our standard lymphocyte culture medium (RPMI 1640 supplemented with 5 × 10^{-5} M 2-ME, 2 mM L-glutamine, 100 U/ml penicillin/streptomycin) added with 1% syngeneic mouse serum. The cells were cultured in 96-well round-bottom plates at 1 × 10^6/well for 72 h in the presence of 100 μg/ml MOG_{35-55}. Levels of IFN-γ, IL-17, and IL-4 in the supernatant were determined by using a sandwich ELISA. Proliferative responses were measured using a Beta-1205 counter (Pharmacia) to detect the incorporation of [^{3}H]thymidine (1 μCi/well) for the final 16 h of culture.

Evaluation of encephalitogenic T cell induction in B6 mice treated with ghrelin

To evaluate whether in vivo ghrelin treatment may affect the induction of encephalitogenic T cells after immunization with MOG_{35-55}, we evaluated the ability of the lymphoid cells from ghrelin- or saline-treated mice to passively transfer EAE into naive recipients. Donor B6 mice were immunized with MOG_{35-55} and treated every day from day 1 to 10 with 5 μg/kg/day of ghrelin or 0.9% saline. We removed spleens and LN from the donor mice on day 11 and prepared lymphoid cell suspensions. The lymphoid cells were stimulated with MOG_{35-55} (33 μg/ml) in the standard medium added with FCS (10%) for 96 h and then we isolated the $CD4^+$ T cells for cell transfer by depletion of $CD8^+$, $CD19^+$, and $NK1.1^+$ cells. In brief, the MOG_{35-55}-stimulated total lymphoid cells were labeled with PE-CD8a mAb, PE-NK1.1 mAb, and PE-CD19 mAb (BD Pharmingen) for 30 min, washed, and incubated with anti-PE microbeads (Miltenyi Biotec) for 15 min. Using autoMACS (Miltenyi Biotec), we isolated $CD4^+$ T cells ($CD8^-$, $CD19^-$, and $NK1.1^-$ fraction) as a pass-through and suspended the cells in PBS. We injected 1.0 × 10^7 of the cells into the peritoneal cavity of syngeneic recipient mice that had been X-irradiated (550 rad) shortly before. We also injected 200 ng of pertussis toxin i.p. on the same day and 48 h later.

Reverse transcription and real-time PCR

To analyze the mechanism of ghrelin effects in vivo, we extracted total RNA from spinal cord, spleen, thymus, and LN samples using the RNeasy Mini Kit (Qiagen). The RNA was subjected to reverse transcription with the Advantage RT-for-PCR kit (BD Biosciences). Real-time PCR was conducted in the LightCycler quantitative PCR system (Roche Molecular Biochemicals) by using the LightCycler-FastStart DNS Master SYBR Green I kit (Roche Molecular Biochemicals). We followed the manufacturer's specification using 4 mM $MgCl_2$ and 1 pM primers. The primers used are as follows: TNF-α, CTGTGAAGGGAATGGGTGTT (sense) and GGT CACTGTCCCAGCATCTT (antisense); IL-1β, TGAAATGCCACCTTT TGACA (sense) and GTAGCTGCCACAGCTTCTCC (antisense); IL-6, TTCCATCCAGTTGCCTT–CTT (sense) and CAGAATTGCCATTGC ACAAC (antisense); TGF-β, TGCGCTTGCAGA–GATTAAAA (sense) and GCTGAATCGAAAGCCCTGTA (antisense); and HPRT, GTTGGA–TACAGGCCAGACTTTGTTG (sense) and GAGGGTAGGCTGGCCT ATAGGCT (antisense). Values are presented as the relative amount of transcript of each sample normalized to the housekeeping gene hypoxanthine phosphoribosyltransferase (HPRT).

In vitro effect of ghrelin on RAW 264.7 monocytes treated with LPS

To examine the effect of ghrelin on monocytes, RAW 264.7 monocytes (American Type Culture Collection) were suspended in the standard culture medium supplemented with 10% FCS and cultured in 96-well flat bottom plates at 1 × 10^5/well overnight. Various concentrations of ghrelin (10^{-6} M, 10^{-8} M, 10^{-10} M) were added to the culture and 1 h later the cells were stimulated with LPS (Sigma-Aldrich) at various doses (0.1, 1, 10 μg/ml). After 2 h of incubation at 37°C, supernatants were collected and the levels of TNF-α and IL-6 were detected by using a sandwich ELISA.

Isolation of microglial cells from the CNS

The spinal cords were incubated with 35 mg/ml Liberase Blendzyme 3 (Roche Molecular Biochemicals) and 0.1 mg/ml DNaseI (Roche Molecular Biochemicals) in RPMI 1640 medium at 37°C for 30 min. Mononuclear cells were isolated on 30%–80% discontinuous Percoll gradients and were stained with FITC-CD11b mAb, PE-CD45 mAb, and allophycocyanin-CD3 mAb (BD Pharmingen). $CD11b^{high}CD45^{high}$ macrophage cells, $CD11b^{int}CD45^{int}$ microglial cells, and $CD3^+$ T cells were isolated using

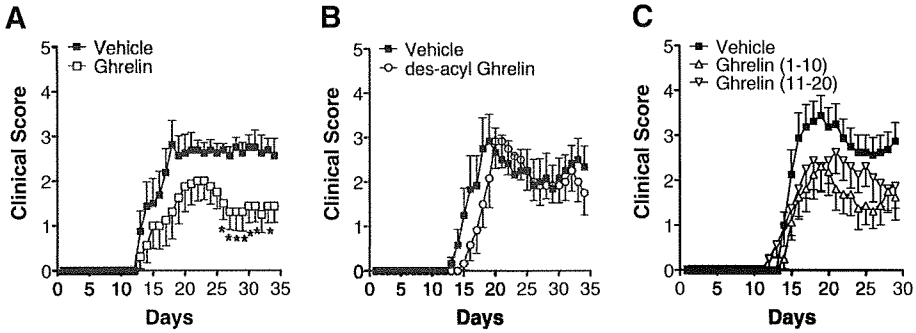

FIGURE 1. Effect of ghrelin on actively induced EAE. EAE was induced in female B6 mice (n = 8 in each group of the three experiments) by immunization with MOG_{35-55}. *A*, The mice were treated every other day starting at the day of immunization with 5 μg/kg ghrelin, while controls were administrated with the vehicle, 0.9% saline, alone. *B*, The mice were injected from day 1 every other day with 5 μg/kg des-acyl ghrelin, whereas controls were subjected to 0.9% saline injections. *C*, Following an alternative protocol, mice were treated from days 1–10 (induction phase treatment) or from days 11–20 (effector phase treatment) with 5 μg/kg ghrelin and in-between with 0.9% saline, while controls were treated every day with 0.9% saline injections. Data represent mean ± SEM. *, Significant differences between the groups ($p < 0.05$; Mann-Whitney U test).

FACSAria (BD Biosciences). The total RNA was extracted from the isolated cells and was subjected to reverse transcription and real-time PCR.

In vitro effect of ghrelin on microglia cells treated with LPS

Mononuclear cells were prepared from brains of untreated non-EAE mice incubated with Liberase Blendzyme 3 and DNase I as described above and were isolated on 40%–80% discontinuous Percoll gradients. Isolated cells were suspended in DMEM supplemented with 10% FCS and cultured in 96-well flat bottom plates at 2×10^5/well in the presence of ghrelin (10^{-6} M) overnight and later stimulated with LPS at different doses (0.01, 0.1 μg/ml). After 5 h of incubation at 37°C, supernatants were collected and the levels of TNF-α were detected by using a sandwich ELISA.

Statistical analysis

The differences in the clinical score between ghrelin-, des-acyl ghrelin-, and sham-treated groups were analyzed by the nonparametric Mann-Whitney U test. FACS analysis, real-time PCR, ELISA, and proliferation data were subjected to two-way ANOVA. In case of significant differences, a Fisher post hoc test was applied. Probability values of <0.05 were considered as statistically significant.

Results

Ghrelin inhibits EAE

To explore the modulatory effects of ghrelin on inflammatory demyelinating diseases, we employed a model of EAE actively induced in B6 mice with MOG_{35-55}. Although classical forms of EAE are typically characterized by acute paralysis followed by complete recovery, this EAE model shows persistent paralysis with partial recovery as a reflection of persistent inflammatory demyelination in the CNS (21, 22). In the first series of experiments, we injected 0.5, 5, or 50 μg/kg ghrelin to the mice every other day from day 1 to 35 postimmunization, while the control mice were injected with 0.9% saline. The results showed that the continuous injections of 5 μg/kg ghrelin suppressed most efficiently the clinical signs of EAE (Fig. 1*A*), whereas a lower (0.5 μg/kg) or a higher dose (50 μg/kg) showed only a marginal effect (data not shown). The treatment with 5 μg/kg ghrelin did not significantly alter either the onset or peak score of EAE. However, significant differences were noted in mean clinical score after day 25 postimmunization between the ghrelin-treated and the control mice (Fig. 1*A*).

Moreover, the effect of ghrelin on EAE was specific as des-acyl ghrelin, an acyl-modified ghrelin, which lacks the n-octanoic acid on the third serine, and consequently its binding ability to GHS-R (7) (Table I) had no modulatory effect on EAE at any concentration examined (Fig. 1*B* and Table II). Thus, the discrepant results obtained with ghrelin and des-acyl ghrelin indicate that ghrelin treatment would ameliorate the clinical course of EAE via activation of the GHS-R.

To further characterize the effects of ghrelin on EAE, we next examined if treatment lasting for a shorter duration may also be immunomodulatory in vivo. We injected 5 μg/kg ghrelin every day from day 1 to 10 postimmunization (roughly corresponding to the induction phase) or from day 11 to 20 (roughly corresponding to the effector phase). As shown in Fig. 1*C*, both protocols showed similar levels of disease suppression, although it was less notable than the continuous treatment from day 1 to 35 (Table II).

Ghrelin does not influence cellular infiltration into CNS

In the previous results on prophylactic or therapeutic treatment of EAE, clinical suppression of EAE was generally associated with a significant reduction of cellular infiltration in the CNS (23). To clarify if histological manifestation of EAE is also suppressed by ghrelin treatment, we treated MOG_{35-55}-immunized B6 mice with 5 μg/kg ghrelin or 0.9% saline every other day and prepared sections of spinal cords at the peak of disease (day 17 after immunization) (Fig. 2). Clinical signs were milder in the ghrelin-treated mice compared with saline-treated ones. However, histology of the spinal cord sections with H&E staining revealed equivalent levels of cellular infiltration in ghrelin- and saline-treated mice. To confirm this, we isolated mononuclear cells from spinal cords of the

Table II. *Clinical scores of EAE treated with ghrelin or des-acyl ghrelin following different treatment protocols*[a]

Treatment	Incidence	Mean Day of Onset ± SEM	Mean Maximal Score ± SEM	Mean Cumulative Score ± SEM
Vehicle[b]	8/8	16.38 ± 1.13	3.75 ± 0.33	55.44 ± 7.14
Ghrelin[b]	7/8	17.86 ± 1.30	3.29 ± 0.33	36.71 ± 9.99
Vehicle[b]	6/8	18.83 ± 2.55	3.67 ± 0.40	49.33 ± 12.99
Des-acyl ghrelin[b]	6/8	18.00 ± 0.71	3.80 ± 0.44	49.05 ± 8.09
Vehicle[c]	7/8	15.14 ± 0.51	4.43 ± 0.07	50.43 ± 3.10
Ghrelin (1–10)[c]	6/8	16.00 ± 0.73	3.17 ± 0.53	34.00 ± 7.25
Ghrelin (11–20)[c]	7/8	16.29 ± 1.25	3.50 ± 0.45	38.72 ± 8.79

[a] The table shows the results of three separate experiments (n = 8 mice in each group of the three experiments).

[b] After induction of EAE with MOG_{35-55}, mice were treated in two different experiments following the standard protocol of every other day s.c. treatment with 5 μg/kg ghrelin or 5 μg/kg des-acyl ghrelin. The controls were injected with 0.9% saline (vehicle).

[c] Following an alternative protocol, we treated the mice from days 1–10 (induction phase treatment) or from days 11–20 (effector phase treatment) with 5 μg/kg ghrelin and in-between with 0.9 % saline, while controls were injected every day with 0.9% saline only. Data represent mean ± SEM.

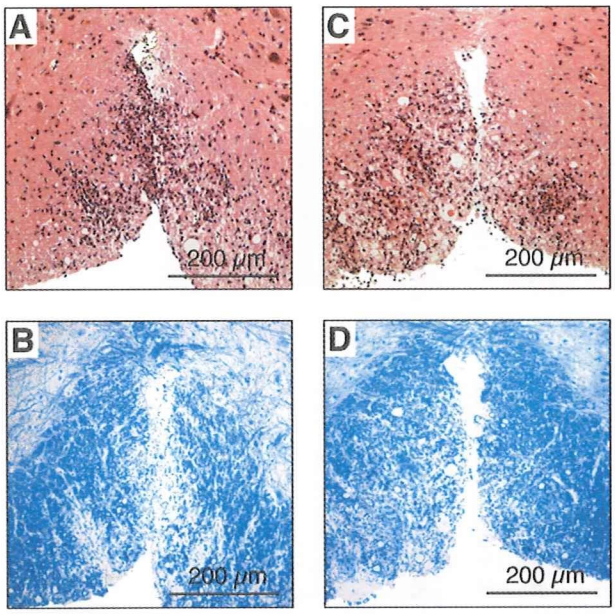

FIGURE 2. Histopathological assessment of the spinal cord of EAE mice. Spinal cords from EAE mice (n = 5/group) were removed on day 17 postimmunization as described in *Material and Methods*. The spinal cord sections from sham- (*A* and *B*) and ghrelin-treated (*C* and *D*) mice were stained in with H&E in the upper panels or Luxol fast blue in the lower ones. Representative sections are shown.

mice at the peak of disease and enumerated the number of the lymphoid cells. Notably, the total cell number was slightly elevated in the ghrelin-treated mice (1.40×10^6/mouse) compared with the saline-treated mice (1.05×10^6/mouse). To further analyze the effects of ghrelin on the formation of CNS inflammation, we evaluated the cellular composition of the CNS-derived lymphocytes by using FACS. Although there was a trend that $CD4^+$ and $CD8^+$ T cell numbers are increased in the lesions of ghrelin-treated mice as compared with saline-treated mice (Fig. 3*A*), it did not reach the level of statistic significance. It was also noted that ghrelin treatment did not alter the number of NK cells

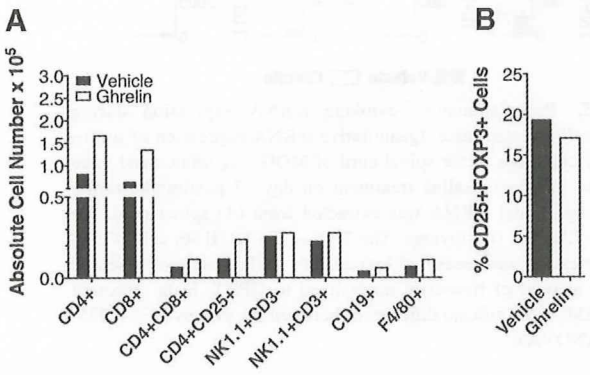

FIGURE 3. Quantification of spinal cord cellular infiltrates by flow cytometry. *A*, The cells were isolated from spinal cords of ghrelin- and sham-treated mice on day 17 postimmunization and subjected to flow cytometer analysis as described in *Materials and Methods*. Data are representative of two independent experiments and presented as absolute cell number (n = 8 mice/group in each experiment). *B*, The proportion of $CD25^+FOXP3^+$ cells in the $CD4^+$ T cell population isolated from spinal cord mononuclear cells was analyzed by flow cytometry 20 days after immunization. Data represent two independent experiments (n = 5).

Table III. *Cytokine production and proliferation of MOG_{35-55}-specific T cells after ghrelin treatment*[a]

Treatment	CPM ± SEM	Cytokine Production (pg/ml) INF-γ ± SEM	IL-17 ± SEM	IL-4 ± SEM
Vehicle	47,590 ± 10,988	2,087 ± 487	820 ± 211	ND
Ghrelin	36,663 ± 9,058	2,883 ± 615	674 ± 148	ND

[a] Mice were immunized with MOG_{35-55} and treated with 5 μg/kg ghrelin or 0.9% saline everyday from day 1 to 10 (n = 3/group). Popliteal and inguinal LN cells were harvested on day 11 after immunization and stimulated with 10 μg/ml MOG_{35-55}. CPM marks the proliferative response to MOG_{35-55}. The cytokines were measured in the supernatant by sandwich ELISA after 72 h of stimulation. Data represent mean ± SEM of duplicate samples from one out of three independent experiments. ND, Not detectable.

($NK1.1^+CD3^-$), NKT cells ($NK1.1^+CD3^+$), B cells ($CD19^+$), or macrophages ($F4/80^+$) in the spinal cord lesions. The proportions of $CD25^+FOXP3^+$ cells in the $CD4^+$ T cell population isolated from spinal cords were not altered in ghrelin-treated mice (Fig. 3*B*). In parallel, we also examined the composition of lymphoid cells obtained from spleen, LN, and thymus. Again, we could not reveal any significant change in the subsets of lymphocytes in ghrelin-treated mice (data not shown). Concordant with the histological findings, these data imply that ghrelin did not ameliorate clinical EAE by reducing the numbers of inflammatory cells in the CNS, but rather by regulating the inflammatory potential of the CNS infiltrates.

Ghrelin does not inhibit the induction of MOG_{35-55}-reactive T cells

To elucidate the immunomodulatory mechanism of ghrelin, we examined the cytokine production and proliferative response of draining LN cells to MOG_{35-55} that were obtained from MOG_{35-55}-sensitized mice treated for 10 days every day with ghrelin or saline. The LN cells were collected on day 11 after immunization and stimulated with MOG_{35-55} in vitro. Accordingly, we harvested the supernatant and measured the levels of IFN-γ, IL-17, and IL-4 by using ELISA. Although the IL-4 concentration was under the detection level, IFN-γ and IL-17 could be detected in the MOG_{35-55}-stimulated culture supernatant (Table III). There was no significant difference in the level of IFN-γ and IL-17 when we compared ghrelin-treated and saline-treated groups. Furthermore, ghrelin-treated mice did not differ from saline-treated mice in the proliferative response of the draining LN cells to MOG_{35-55}. We also examined the frequency of $CD4^+CD25^+$ $FOXP3^+$ regulatory T cells in the lymph nodes and spleens using flow cytometry and did not find significant differences between ghrelin-treated and saline-treated mice (data not shown). These results indicate that in vivo ghrelin treatment did not inhibit the induction of MOG_{35-55}-reactive T cells.

Ghrelin does not affect induction of pathogenic autoimmune T cells

To further confirm that MOG_{35-55}-reactive T cells are normally induced in ghrelin-treated mice, we evaluated if the ability of the MOG_{35-55}-sensitized lymphoid cells, obtained from MOG_{35-55}-immunized mice, to transfer EAE into naive mice could be affected by in vivo ghrelin treatment. To this aim, we immunized donor mice with MOG_{35-55} and treated them every day with ghrelin or saline from immunization up to day 10. Next day, we pooled lymphocytes from spleen and LN and cultured them in the presence of MOG_{35-55}. Three days later, $CD4^+$ T cells were purified and injected into recipient mice as described in *Materials and Methods*. It was theoretically possible that in vivo ghrelin treatment does not

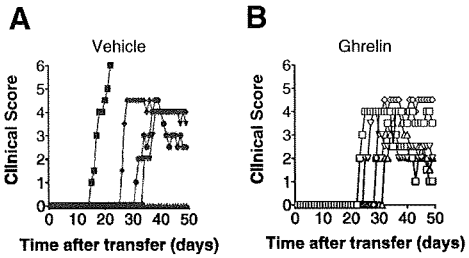

FIGURE 4. Effects of ghrelin treatment on the induction of encephalitogenic T cells. MOG_{35-55}-sensitized lymphoid cells were derived from MOG_{35-55}-immunized and (*A*) saline- or (*B*) ghrelin-treated mice (n = 15/group). The cells were stimulated with MOG_{35-55} and $CD4^+$ T cells were separated 3 days later for passive transfer of EAE into naive mice (n = 5/group). Data represent individual EAE score for each mouse.

inhibit induction of MOG_{35-55}-reactive T cells, but would prohibit the ability to cause EAE in vivo. In postulating that this could happen, $CD4^+$ T cells from ghrelin-treated donors should be less encephalitogenic than those from saline-treated mice. The results showed that transfer of activated $CD4^+$ T cells either derived from saline- or ghrelin-treated donors induced passive EAE in the recipients, showing approximately the same clinical course and severity (Fig. 4). Thus, it can be concluded that ghrelin treatment does not affect the induction of encephalitogenic MOG_{35-55}-reactive $CD4^+$ T cells.

Ghrelin decreases mRNA levels of proinflammatory cytokines in the CNS

After demonstrating that ghrelin does not suppress the infiltration of inflammatory cells in the spinal cord, we wondered whether the cytokine milieu in the ghrelin-treated mice could be significantly altered. To answer the question, we analyzed the mRNA levels of pro- and antiinflammatory cytokines (IFN-γ, TNF-α, IL-1β, IL-6, IL-4, IL-10, and TGF-β) in the spinal cord, spleen, LN, and thymus of ghrelin- and saline-treated mice at the peak of disease (day 17) by using quantitative PCR. Although ghrelin treatment had no effect on the mRNA levels of IL-4, IL-10, and IFN-γ in the spinal cord, spleen, LN, and thymus (data not shown), we found significantly reduced levels of TNF-α ($p < 0.0015$), IL-1β ($p < 0.025$), and IL-6 ($p < 0.025$) in the spinal cord of ghrelin-treated mice, compared with saline-treated ones (Fig. 5*A*). In contrast, the level of TGF-β showed a trend for slight elevation in the spinal cord. We also found a diminished level of TNF-α mRNA ($p < 0.0001$) in the spleen of ghrelin-treated mice (Fig. 5*B*), whereas we saw no significant change in any of the cytokines that we measured in LN or thymus of ghrelin-treated mice (Fig. 5, *C* and *D*). Because TNF-α, IL-1β, and IL-6 mRNAs were selectively down-regulated in the spinal cord, we suspected that monocytes could be potential target cells in the ghrelin-mediated EAE suppression. This idea was consistent with the fact that ghrelin treatment did not inhibit the induction of MOG_{35-55}-reactive T cells.

Ghrelin suppresses the proinflammatory cytokine production of LPS-stimulated monocytes

To verify the postulate that in vivo treatment with ghrelin may ameliorate EAE by targeting monocytes, we examined in vitro effects of ghrelin on the monocytic cell line RAW 264.7 that robustly produce proinflammatory cytokines when stimulated with LPS. The RAW 264.7 line cells were first exposed to various doses of ghrelin for 1 h and then stimulated with LPS. We harvested the supernatant 2 h later and measured the levels of TNF-α and IL-6 by ELISA. The results revealed that prior exposure to ghrelin

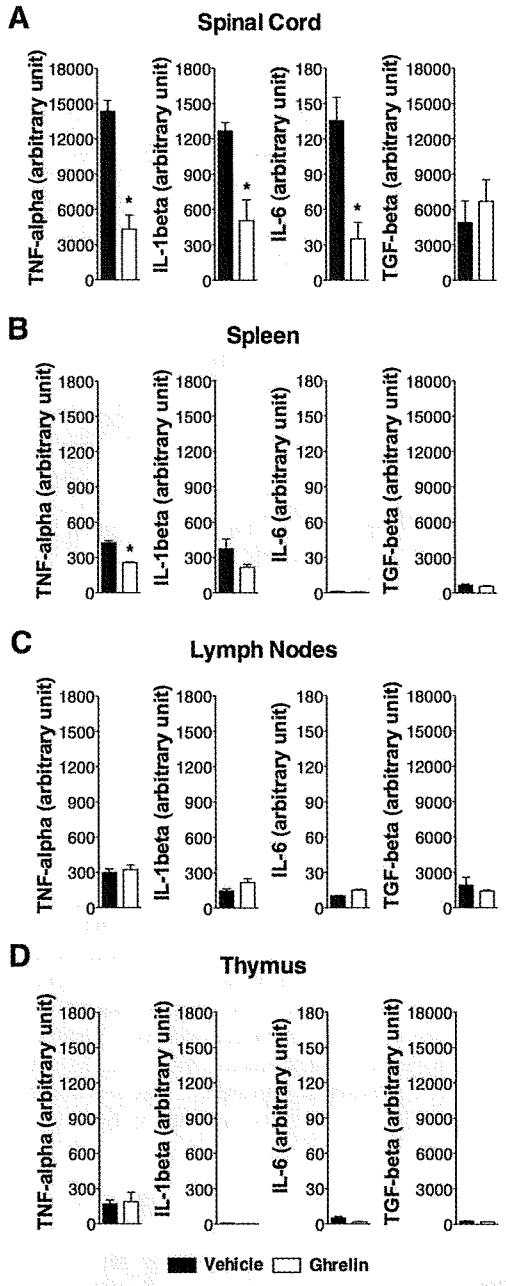

FIGURE 5. Proinflammatory cytokine mRNA expression during EAE in ghrelin-treated mice. Quantitative mRNA expression of proinflammatory cytokines in the spinal cord of MOG_{35-55}-immunized mice subjected to ghrelin or saline treatment on day 17 postimmunization (n = 5/group). Total mRNA was extracted from (*A*) spinal cord, (*B*) spleen, (*C*) LN, and (*D*) thymus. The TNF-α, IL-1β, IL-6, and TGF-β mRNA expression was measured by real-time PCR. Data are presented as relative amount of transcript normalized to HPRT. Data represent mean ± SEM. *, Significant differences between the groups ($p < 0.025$; two-way ANOVA).

would significantly suppress the production of TNF-α ($p < 0.02$) and IL-6 ($p < 0.05$) by LPS-stimulated RAW 264.7 cells in a dose-dependent manner (Fig. 6). The inhibitory effect of ghrelin was very potent, as in addition to the effects on LPS-stimulated monocytes, even the basal production of TNF-α ($p < 0.008$) and IL-6 ($p < 0.03$) was significantly reduced by in vitro ghrelin treatment. Given that in vivo treatment with ghrelin could suppress the

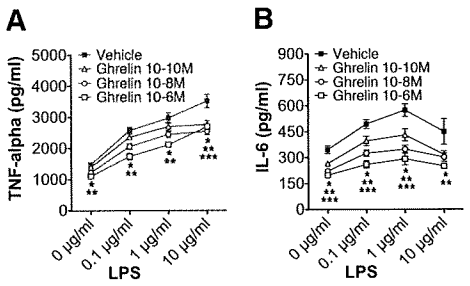

FIGURE 6. Effect of ghrelin on the proinflammatory cytokine production of LPS-stimulated monocytes. The monocytes were treated with various concentrations of ghrelin (10^{-6} M, 10^{-8} M, 10^{-10} M) 1 h before stimulation with 0.1, 1.0, and 10 μg/ml LPS. The (*A*) TNF-α and (*B*) IL-6 production was measured 2 h after LPS stimulation by sandwich ELISA. Data represent mean ± SEM of duplicate samples from one out of three independent experiments. Significant differences at 10^{-6}, 10^{-8}, and 10^{-10} M ($p < 0.05$; two-way ANOVA) are depicted as *, **, and ***, respectively.

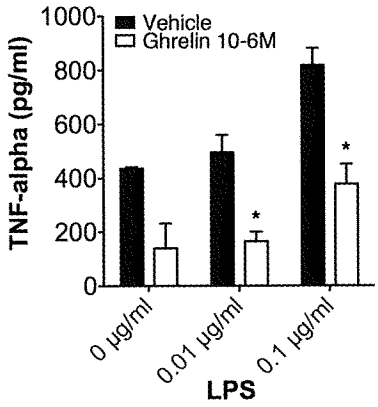

FIGURE 8. Effect of ghrelin on the proinflammatory cytokine production of LPS-stimulated microglia. The microglia cells were treated with ghrelin (10^{-6} M) overnight and later stimulated with 0.01 and 0.1 μg/ml LPS. Five hours after stimulation, the TNF-α production was measured using ELISA. Data represent mean ± SEM of duplicate samples from one out of two independent experiments. *, Significant differences between the groups ($p < 0.05$; two-way ANOVA).

development of EAE without altering histological EAE or T cell-derived cytokine balance, the ghrelin-mediated suppression of monocyte-produced TNF-α and IL-6 would strongly support the postulate that monocytes are the main target cells in ghrelin-mediated suppression of EAE.

Ghrelin inhibits the expression of proinflammatory cytokines in microglia

The proinflammatory cytokines are known to be produced not only by CNS-infiltrating macrophages but also by T cells and microglia in the course of EAE. To investigate which cells are important in the ghrelin-mediated suppression of EAE, we first examined the expression of proinflammatory cytokines in macrophages. Unexpectedly, the mRNA of IL-1β, IL-6, and TNF-α did not alter in CNS-infiltrating macrophages of ghrelin-treated mice compared with the control mice (Fig. 7*A*). We next examined the expression of these cytokines in other cell types also known as a source of inflammatory cytokines and found reduced expression of these cytokines in microglia (Fig. 7*B*). Additionally, the expression of inflammatory cytokines was decreased in CNS-infiltrating T cells (Fig. 7*C*). Hence, these results suggest that microglia might play a crucial role in ghrelin-mediated inhibition of EAE.

Ghrelin inhibits the proinflammatory cytokine production of LPS-stimulated microglia

We next examined the effect of ghrelin on microglia. To test whether ghrelin directly affects microglia, we isolated mononuclear cells from the brains of untreated mice. In untreated non-EAE mice, most (~77%) of the brain mononuclear cells were $CD11b^+$ cells, and the majority of $CD11b^+$ cells (~95%) were considered as $CD45^{low}$ microglia cells. Among these mononuclear cells, $CD19^+$ B cells were <0.1% and $CD3^+CD45^+$ T cells were 1–1.5%. We cultured the isolated mononuclear cells in the presence of ghrelin overnight and stimulated them with LPS in different doses for 5 h. The TNF-α levels in the culture supernatant were measured by using ELISA. In the presence of ghrelin, the TNF-α levels were significantly reduced (Fig. 8). These results suggest that ghrelin directly affects microglia by reducing the production of inflammatory cytokines.

Discussion

Starvation is known to have immunosuppressive effects (24–26). Although little was known about the mechanistic link between starvation and immunity, recent studies have shed light on the immunomodulatory potency of a range of feeding regulatory hormones such as leptin and NPY. For example, serum leptin is decreased after acute starvation in parallel with immunosuppression or Th2 bias, whereas exogenous leptin would correct the altered Th1/Th2 balance toward Th1 (27, 28). In contrast, NPY is increased after starvation. Exogenous NPY would shift the Th1/Th2 balance toward Th2 and can ameliorate the severity of EAE (29). Interestingly, both peptide hormones are linked to ghrelin in an endocrine feedback system (30). Ghrelin itself is increased after

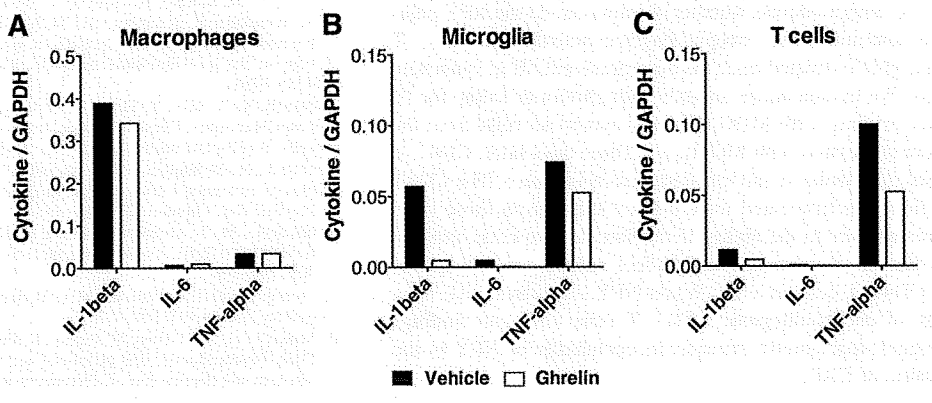

FIGURE 7. Effect of ghrelin on proinflammatory cytokine mRNA expression in infiltrating cells and microglia. Total mRNA was extracted from (*A*) macrophages, (*B*) microglia, and (*C*) T cells obtained on day 20 postimmunization from the spinal cords of MOG_{35-55}-immunized mice treated with ghrelin or saline. The IL-1β, IL-6, and TNF-α mRNA expression levels were measured by real-time PCR. Data are presented as relative amount of transcript normalized to the housekeeping gene GAPDH.

starvation, and it can potently stimulate the release of NPY in the CNS (12). Moreover, ghrelin shows antagonistic effects against leptin (31). Although the available data on the action of ghrelin on leptin or NPY may not be extrapolated to speculate about its role in the immune system, we decided to explore whether ghrelin may exhibit beneficial effects in the modulation of EAE. Furthermore, ghrelin was reported to have protective effects on endotoxic shock in rats (32). Additionally, the wide range of GHS-R expression within the immune cells strongly suggested the immunomodulatory potential of ghrelin (6). Considering its endocrine interactions, ghrelin becomes an interesting candidate for the in vivo modulation of EAE.

To evaluate the effects of ghrelin on the immune system in vivo, we used the representative EAE model induced with MOG_{35-55} in B6 mice. Subcutaneous injections of ghrelin significantly suppressed EAE severity, especially after the peak of disease, while the EAE onset occurred almost similarly in both ghrelin- and sham-treated mice. Priming phase treatment (days 1–10) as well as effector phase treatment (days 11–20) also showed disease-suppressing effects, suggesting a modulatory role of ghrelin during all phases of disease. The unacylated ghrelin form, des-acyl ghrelin, failed to suppress EAE, demonstrating that the disease suppression was mediated by the GHS-R.

The histological findings at day 17 were similar in all animals regardless of the applied treatment. The inflammatory cell infiltration and demyelination occurred in both groups, suggesting a ghrelin effect independent of cell trafficking at the peak of disease. Moreover, we found by FACS analysis that the number of mononuclear cells isolated from the spinal cord and their composition did not significantly alter among ghrelin- and sham-treated mice at the same time point. Our data showed no statistically significant changes in the examined cell subsets, which supported the histological findings of unaffected immune cell traffic to the CNS. This discrepancy between analogous inflammatory status in the spinal cord on the one hand and less severe disease on the other hand in ghrelin-treated mice was remarkable, suggesting cytokine regulation as the possible mechanism of EAE suppression.

Leptin and NPY both influence the Th1/Th2 balance in opposing directions (27–29). Since ghrelin is the most potent NPY-releasing hormone and NPY suppresses EAE by a Th2 bias (29), we examined whether ghrelin affects the Th1/Th2 balance similar to NPY and if its potential mechanism of EAE suppression is primarily mediated on immune cells or secondarily through NPY release. To investigate the effect of ghrelin on the cytokine balance, we measured the cytokine responses of MOG_{35-55}-primed T cells from mice treated with ghrelin or saline. The evaluated IFN-γ, IL-17, and IL-4 levels as well as the proliferative response did not significantly alter between ghrelin- and sham-treated mice. Underlying these observations, we conclude that the suppression of EAE mediated by ghrelin does not affect the T cell-derived cytokine balance. To further address whether ghrelin acts via the NPY pathway, we determined the encephalitogenic potential of $CD4^+$ T cells from ghrelin-treated mice to cause passive EAE in syngeneic recipients. We treated donor animals with ghrelin or saline for 10 days after priming with MOG_{35-55}, and lymphoid cells from the mice were stimulated with MOG_{35-55}. Three days later, $CD4^+$ T cell blasts were isolated and transferred to naive mice. The $CD4^+$ T cells from ghrelin-treated mice did not differ from those from saline-treated mice in the ability to mediate passive EAE, indicating that ghrelin does not primarily affect induction of encephalitogenic $CD4^+$ T cells in vivo. While NPY attenuates EAE by a Th2 bias of encephalitogenic $CD4^+$ T cells (29), our findings likely suggest that ghrelin interacts independently of NPY in the amelioration of EAE.

To further clarify the mechanism of ghrelin-mediated EAE suppression, we examined the mRNA levels of several cytokines of ghrelin- and sham-treated mice at the peak of disease. Our data demonstrate significantly reduced levels of the proinflammatory cytokines TNF-α, IL-1β, and IL-6 in the spinal cord and lower levels of TNF-α in the spleen of ghrelin-treated mice. In contrast, the level of TGF-β showed a trend for slight elevation in the spinal cord. The importance of TNF-α for initiating and sustaining inflammation is well described, as well as its essential role in the development of acute EAE (33, 34). The proinflammatory role of IL-1β and IL-6 in the immunopathology of EAE is also generally accepted (35–38). Thus, the inhibition of TNF-α, IL-1β, and IL-6 must be considered as an important mechanism in the ghrelin-mediated EAE suppression.

Given the selective down-modulation of the proinflammatory cytokines, we suspected that monocytes could be potential target cells in the ghrelin-mediated EAE suppression. However, the analysis of infiltrating cells and residential microglia revealed that the suppression of proinflammatory cytokines was prominently led by microglia. A decreased expression of these cytokines was also observed in infiltrating T cells. Considering that the transfer of T cells obtained from ghrelin-treated mice induced a similar disease course compared with control mice, the reduction of proinflammatory cytokines in microglia might be important in the ghrelin-mediated suppression of EAE.

In conclusion, the present study demonstrates for the first time to our knowledge that the gastric hormone ghrelin suppresses actively induced EAE by inhibiting production of the proinflammatory cytokines TNF-α, IL-1β, and IL-6 with microglia as the main target cells. These findings support an antiinflammatory property of ghrelin, shedding light on its role in immune-endocrine interactions. Consequently, we speculate that ghrelin may serve as an antiinflammatory drug to control human CNS pathology involving the production of proinflammatory cytokines.

Disclosures

The authors have no financial conflicts of interest.

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