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H. 知的財産権の出願・登録状況

１．特許取得：なし

２．実用新案登録：なし

研究成果の刊行に関する一覧表

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Selective phosphodiesterase-3 inhibitor cilostazol ameliorates experimental autoimmune encephalomyelitis

Juri Kureshiro, Katsuichi Miyamoto, Noriko Tanaka and Susumu Kusunoki

We investigated the possible therapeutic effect of cilostazol, a specific inhibitor of phosphodiesterase-3, for experimental autoimmune encephalomyelitis (EAE). Mice affected with EAE induced by inoculation with MOG_{35-55} were fed with cilostazol or vehicle control. The clinical EAE scores of the cilostazol-fed mice were lower than those of the controls. Serum level of soluble intercellular adhesion molecule-1 was significantly lower in the cilostazol-fed mice than in the controls. In the recall responses with MOG_{35-55}, proliferation and IFN-γ production by lymphocytes from cilostazol-fed mice were significantly reduced. Cilostazol may exhibit repressive effects on EAE by reducing the antigen-specific T-cell response and decreasing the expression of the adhesion molecules. Cilostazol is a hopeful choice for the treatment of multiple sclerosis. *NeuroReport* **20:718–722 © 2009 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

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Keywords: adhesion molecules, cilostazol, experimental autoimmune encephalomyelitis, intercellular adhesion molecule-1, multiple sclerosis, P-selectin, phosphodiesterases inhibitor

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Introduction

Phosphodiesterase (PDE) is an enzyme that degrades cyclic nucleotides. Eleven PDE families are generated from multiple genes and most families have multiple isoforms, thus there are over 50 PDE isoforms. These enzymes vary in their substrate specificity and affinity, tissue distribution and subcellular localization [1].

Cilostazol is an antiplatelet drug that is a specific inhibitor of PDE-3. It is commonly used for the treatment of ischemic brain disease. The inhibition of PDE-3 activity in cardiovascular tissues results in increased levels of cAMP with a consequent reduction in platelet aggregation and smooth muscle cell proliferation. Recently, it has been shown that cilostazol represses vascular cell adhesion molecule-1 gene transcription, through inhibition of NF-κB binding to its recognition sequence [2], inhibits lipopolysaccharide-induced apoptosis [3] and attenuates the production of monocyte chemoattractant protein-1 in response to tumor necrosis factor-α in vascular endothelial cells [4].

Multiple sclerosis is a chronic inflammatory demyelinating disease of the central nervous system (CNS). In multiple sclerosis, the migration of the activated lymphocytes and monocytes/macrophages into the CNS is considered to be an important step for inducing inflammation and demyelination within CNS. Adhesion molecules, such as vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), and P-selectin, play crucial roles in the migration of the immune cells into CNS.

Experimental autoimmune encephalomyelitis (EAE) is regarded as an animal model of multiple sclerosis. Some PDE inhibitors, including amrinone [5], ibudilast [6], mesopram [7], and rolipram [8,9], have been reported to inhibit EAE development by reducing antigen-mediated T-cell proliferation and to skew the T-cell cytokine profile toward a Th2 phenotype. In addition, we recently demonstrated that the selective cyclooxygenase-2 inhibitor, celecoxib, prevents EAE by inhibiting the infiltration of inflammatory cells into the CNS and reducing the expression of adhesion molecules, P-selectin, and ICAM-1 [10].

Thus, we considered that cilostazol could possibly suppress EAE by modifying the immune balance and inhibiting the expression of adhesion molecules. Therefore, we analyzed the therapeutic effect of cilostazol in EAE.

Materials and methods

Mouse

Wild-type C57BL/6 mice were purchased from Clea Japan (Tokyo, Japan). The experiments involving animals were approved by the local ethics committee in Kinki University.

Induction of experimental autoimmune encephalomyelitis

Mice were immunized subcutaneously in the flanks with 100 μg of MOG_{35-55} peptide (MEVGWYRSPFSRVV HLYRNGK) in 0.1 ml phosphate-buffered saline and 0.1 ml complete Freund's adjuvant containing 1 mg

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Mycobacterium tuberculosis H37Ra (Difco Laboratories, Detroit, Michigan, USA) and were injected intraperitoneally with 300 ng of pertussis toxin (List Biological Laboratories, Campbell, California, USA) on the day of immunization and 2 days later.

Clinical assessment of experimental autoimmune encephalomyelitis

EAE was scored on the following scale: 0 = no clinical signs; 1 = partial loss of tail tonicity; 2 = completely limp tail and abnormal gait; 3 = partial hind limb paralysis; 4 = complete hind limb paralysis; and 5 = fore-limb and hind-limb paralysis or moribund state.

Treatment with cilostazol

Mice were fed a blended solid diet with 0.1% cilostazol (Ohtsuka, Tokushima, Japan). Control mice were fed with pure solid diet.

MOG_{35-55}-specific T-cell proliferation assay

On day 12 after immunization with MOG_{35-55}, draining lymph nodes were harvested and single-cell suspensions were prepared. Cells were cultured with MOG_{35-55} for 72 h onto 96-well flat-bottom plates (1×10^6 cells/well) as described earlier [10]. To measure cellular proliferation, (^{3}H)-thymidine was added (1 μCi/well) and uptake of the radioisotope during the final 18 h of culture was counted with a β-1205 counter (Pharmacia, Uppsala, Sweden). To evaluate proliferative responses of lymph node cells to peptide, we determined the sensitive index.

Detection of cytokines

Lymph node cells from the MOG_{35-55}-immunized mice were cultured for 48 h in the presence of the different concentrations of MOG_{35-55}. The concentrations of IFN-γ, TNF-α, IL-10, and IL-17 in the supernatants were measured by using a sandwich enzyme-linked immunosorbent assay (BD Biosciences, San Jose, California, USA). All reagents, including recombinant mouse cytokines and antibodies, were purchased from BD Biosciences.

Detection of soluble adhesion molecules

The concentrations of ICAM-1, VCAM-1, and P-selectin in the serum from mice on days 14, 21, and 30 after the induction of EAE were measured by using enzyme-linked immunosorbent assay (R&D Systems, Wiesbaden-Nordenstadt, Germany). Assays were carried out according to the manufacturer's instructions.

Pathological analysis

On days 14 and 30 after the induction of EAE, mice were perfused with formalin. The brains and spinal cords of animals were removed and fixed in 10% buffered formalin, and paraffin was embedded. Four-micrometer sections were stained with hematoxylin and eosin, Luxol fast blue and antibodies of adhesion molecule ICAM-1 (CD54), VCAM-1 (CD106), P-selectin (CD62-P) (BD Bioscience).

Statistical analysis

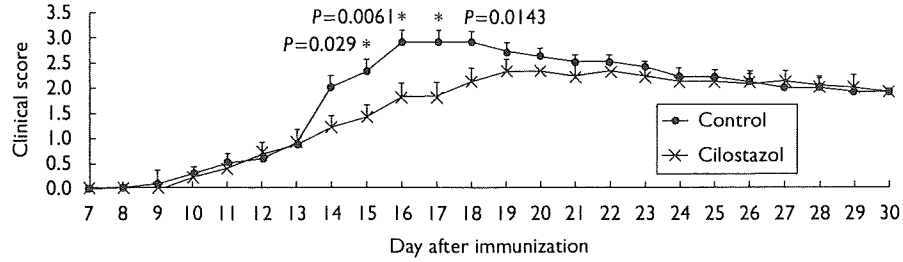
All values were expressed as means ± SEM. The day of onset, maximum clinical score, cumulative score, and incidence were evaluated by Mann–Whitney rank-sum test. Clinical EAE courses were compared by two-way analysis of variance (ANOVA). Statistically significant differences from repeated-measures ANOVA and Bonferroni's method were further analyzed by Mann–Whitney rank-sum test. T-cell proliferation and secretion of IFN-γ were compared by two-way ANOVA and Bonferroni's method and were further analyzed by Student's *t*-test. Soluble adhesion molecules were analyzed by Bonferroni's method.

Results

Effects of cilostazol on the clinical symptoms of experimental autoimmune encephalomyelitis

Control mice showed the first signs of EAE on day 13.9 ± 0.54 after immunization. The maximum clinical

Fig. 1



Effects of cilostazol on actively induced experimental autoimmune encephalomyelitis (EAE). Mice underwent oral administration of blended solid diet with 0.1% cilostazol (crosses) starting from the first day of immunization. Control mice fed with pure solid diet (closed circles). Results of the statistical analysis are shown in Table 1. The mean EAE scores in cilostazol-fed mice were significantly lower than the control mice on days 15, 16, and 17 after EAE immunization. One representative experiment of three independent experiments is presented and expressed as mean ± SEM. *$P<0.05$ vs. control.

Table 1 Clinical scores of EAE treated with cilostazol

	Max. score	Day of onset	Incidence	Cumulative score
Control	3.5 ± 0.12	13.9 ± 0.54	28/28	42.2 ± 2.70
Cilostazol	2.9 ± 0.28	17.4 ± 1.27*	23/28	36.1 ± 3.86

Each mouse was immunized with MOG_{35-55} peptide for the induction of EAE. Mice were fed with 0.1% cilostazol-blended solid diet ($n=28$) or pure solid diet as control ($n=28$). The mean ± SEM of the following parameters are shown: maximum score of EAE (Max. score), the day of EAE onset, incidence of paralyzed mice among sensitized rats (Incidence), summation of the clinical scores from day 1 to day 30 (Cumulative score).
EAE, experimental autoimmune encephalomyelitis.
*$P<0.05$ vs. control.

score was 3.5 ± 0.12 and the cumulative score on days 7–30 was 42.2 ± 2.70 (Fig. 1, Table 1). In contrast, cilostazol-treated mice showed the first signs of EAE on day 17.4 ± 1.27 after immunization. The maximum clinical score was 2.9 ± 0.28 and cumulative score on days 1–30 was 36.1 ± 3.86. On days 15, 16, and 17, the clinical scores in cilostazol-treated mice were reduced significantly compared with control mice. Brain and spinal cord sections from both groups obtained at 14 and 35 days after disease induction exhibited cellular infiltration and demyelination. There was no significant difference between the two groups (data not shown).

Proliferation of Th1 cells and cytokine secretion

To investigate the response of T-cells to MOG_{35-55} in cilostazol-treated mice, we examined the proliferative response and cytokine production of draining lymph nodes cells *in vitro*. The proliferative response of MOG-reactive T-cells in cilostazol-treated mice shows a tendency to reduce compared with control mice (Fig. 2a). The level of IFN-γ in the culture supernatants of lymph node cells obtained from mice treated with cilostazol was reduced significantly compared with that from control mice (Fig. 2b). There were no significant differences in the levels of TNF-α, IL-10, and IL-17 in the supernatants between cilostazol-treated and untreated mice (data not shown).

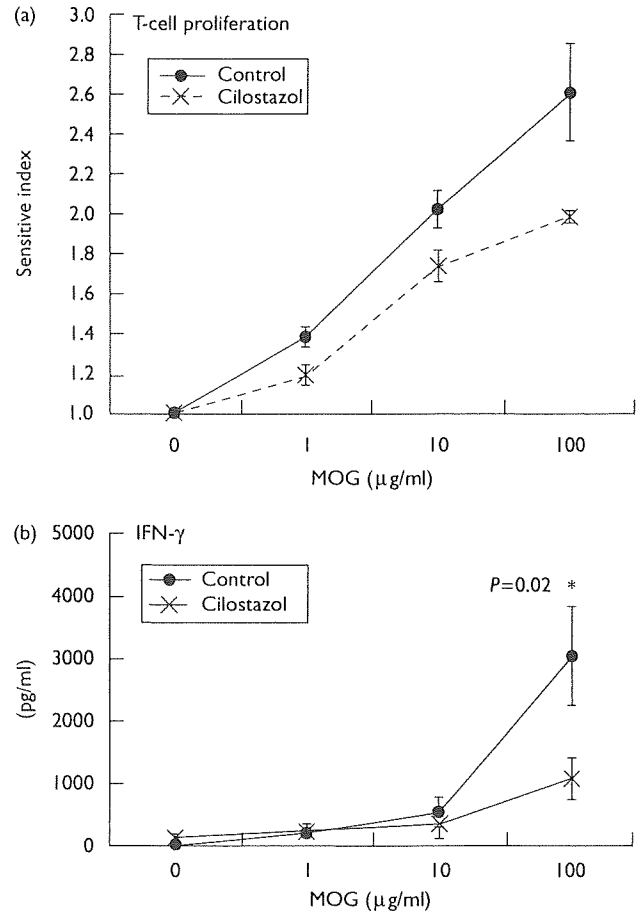
Adhesion molecules

The serum level of ICAM-1 was reduced to a significantly greater degree in cilostazol-treated mice compared with control mice (Fig. 3), although immunohistochemical investigation on the expression of those molecules showed no significant differences between them (data not shown).

Discussion

We showed that oral administration of cilostazol ameliorated the clinical signs of EAE. In the recall response, cilostazol inhibited the production of IFN-γ. This significant effect could be because of the proliferative effect of cilostazol. Some PDE inhibiters, such as mesopram, rolipram, and ibudilast, have been shown to

Fig. 2

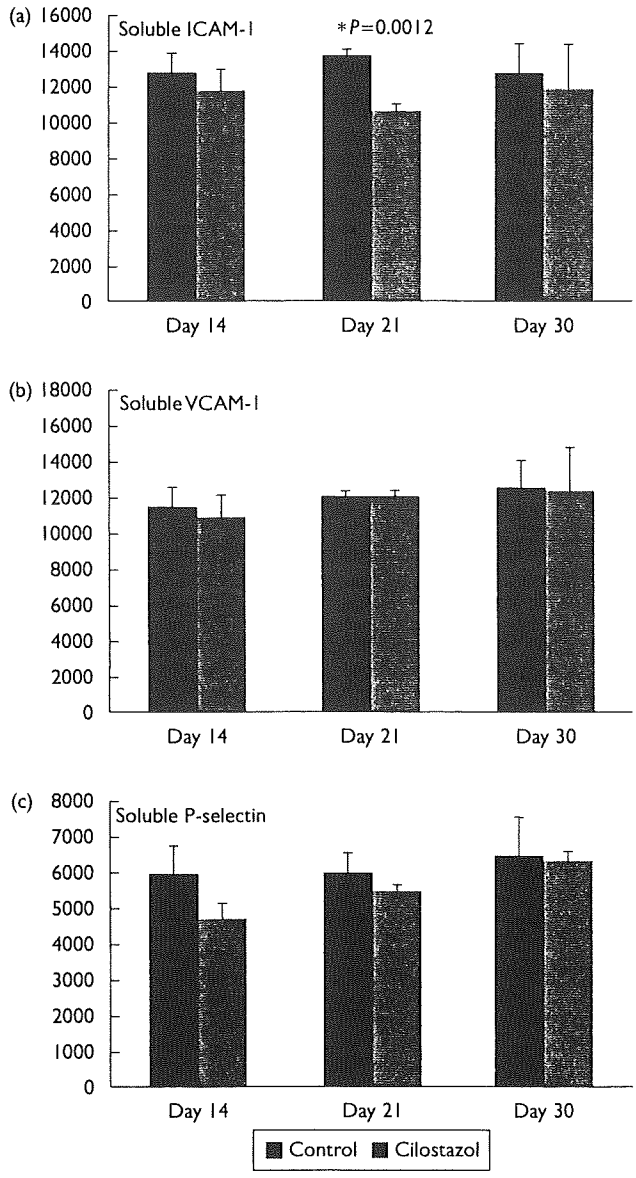


Comparison of MOG_{35-55}-specific T-cell response treatment with cilostazol. Popliteal and inguinal lymph node cells from treated and control animals were incubated in the presence of MOG_{35-55} for 48 h. The proliferative response was determined by the uptake of [^{3}H] thymidine (a), and IFN-γ was detected by enzyme-linked immunosorbent assay (b) ($n=8$ for each group). Error bars represent SEM.
*$P<0.05$ vs. control.

suppress the development of EAE [5–9] by reducing the production of Th1 cytokines, such as IFN-γ, TNF-α, and IL-2 [11–15]. Although IL-17 has recently been shown as one of the key cytokines for the development of EAE, IL-17 production was not significantly reduced in the cilostazol-treated group in our experiment. We therefore consider that cilostazol suppressed the Th1 response in the same manner as other PDE inhibitors, described above.

We recently showed that the selective cyclooxygenase-2 inhibitor, celecoxib, prevents EAE by inhibiting infiltration of inflammatory cells into the CNS, and the expression of P-selectin and ICAM-1, inhibition of cellular infiltration by celecoxib might be mediated by downregulation of the expression of adhesion molecules [10]. Thus, adhesion molecules play crucial roles in the development of EAE.

Fig. 3



Soluble adhesion molecules in experimental autoimmune encephalomyelitis (EAE)-induced mice. Serum from EAE mice ($n=10$ for each group) was collected on day 14, 21, and 30 after immunization EAE with MOG_{35-55}. Soluble adhesion molecules, such as intercellular adhesion molecule-1 (ICAM-1) (a), vascular cell adhesion molecule-1 (VCAM-1) (b), and P-selectin (c), were measured by enzyme-linked immunosorbent assay as described in Materials and methods. In cilostazol-fed mice, the soluble level of ICAM-1 on day 21 after immunization EAE was significantly reduced compared with the control. The results of one experiment that is representative of two independent experiments are expressed as mean ± SEM. $*P<0.017$ vs. control.

The early infiltration of autoreactive T-cells requires complex molecular interactions between immune cells and cerebral endothelial cells that constitute the blood–brain barrier. Bullard *et al.* [16] reported that ICAM-1 null mutant mice, which express no isoforms of ICAM-1, exhibited significantly attenuated EAE characterized by markedly reduced spinal cord T-cell infiltration and IFN-γ production by these cells. They suggested that ICAM-1 expression by T-cells might be critical for modulating the effecter T-cell response. In addition, the expressions of ICAM-1 and VCAM-1 were elevated in endothelial cells [17], and the levels of sICAM-1 and sVCAM-1 in serum in multiple sclerosis patients were also increased [18,19].

There have been many reports describing the effects of cilostazol on the expression of adhesion molecules. In patients with noninsulin-dependent diabetes mellitus, cilostazol decreased the plasma levels of sP-selectin, sICAM-1, and sVCAM-1 [20]. Cilostazol also inhibited high glucose-mediated endothelial–neutrophil adhesion by decreasing ICAM-1 and P-selectin expression [21]. Cilostazol suppresses VCAM-1 gene transcription by inhibiting NF-κB binding to its recognition sequence [2]. In our study, the serum level of ICAM-1 was decreased in cilostazol-treated mice. This result is compatible with previous reports. These results suggest that cilostazol can ameliorate EAE not only by suppression of the Th1 response, but also by prevention of T-cell migration into the CNS, because of the effects on the adhesion molecules.

Cilostazol is a drug that can be administered orally. Our present investigation indicates that cilostazol is potentially a new therapeutic drug for preventing the relapse of multiple sclerosis.

Conclusion

We observed that PDE-3 inhibitor cilostazol might exhibit repressive effects on EAE by reducing the antigen-specific T-cell response and decreasing the expression of the adhesion molecule, ICAM-1. Although further investigation is needed to clarify the detailed mechanisms of therapeutic effects in EAE, cilostazol is a hopeful choice for the treatment of multiple sclerosis.

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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).

Asialo-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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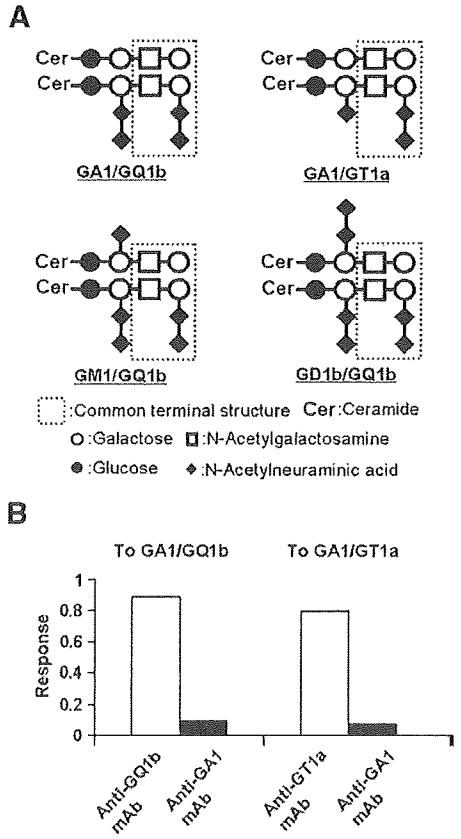

Fig. 1. (A) Pattern diagrams of four glycolipid complexes containing GQ1b and GT1a. GA1/GQ1b, GM1/GQ1b, GD1b/GQ1b and GA1/GT1a have a common terminal structure, which is shown by a dotted box. (B) In the left graph, the white bar shows the response (0.88) of monoclonal anti-GQ1b antibody to GA1/GQ1b, 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 (Joukahainsenkatu, 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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Upregulation of water channel aquaporin-4 in experimental autoimmune encephalomyeritis

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ABSTRACT

Aquaporin-4 (AQP4) is a water channel protein that plays an important role in water movement in the central nervous system (CNS). Recently, presence of anti-AQP4 antibody has been reported in the sera from patients with neuromyelitis optica. AQP4 is therefore a possible target for inflammatory mechanisms in CNS. In the present investigation, we performed semi-quantitative analysis of AQP4-mRNA in brain and spinal cord from mice affected with experimental autoimmune encephalomyelitis (EAE) using real-time PCR. AQP4-mRNA expression was increased in EAE; reaching a peak in the spinal cord at 14 days, and in the brain at 21 days after first inoculation. Immunohistochemical analysis showed that AQP4 is expressed on astrocytes, indicating that the increase in AQP4 expression may correlate with astrocytic activation. This is the first study to demonstrate upregulation of AQP4 in EAE. The upregulation of AQP4 could be involved in the development of inflammation in the acute phase of EAE.

1. Introduction

Aquaporin (AQP) is a family of water channel proteins, which are present on the plasma membrane at the boundary of various tissues, and provide a major pathway for osmotically driven water transport through cell membranes. Some members of the aquaporin family have been identified in the central nervous system (CNS) [1]. AQPs play important roles in the dynamic regulation of brain water homeostasis and in the regulation of cerebrospinal fluid production. AQP4 and AQP1 are the major species that are expressed abundantly in the brain [2,3]. AQP1 is restricted to the apical domain of choroid plexus epithelial cells [4–6]. AQP4 is abundantly present in astrocyte foot processes and cells lining the subarachnoid space and ventricles, whereas AQP9 is localized in astrocyte processes [7–9]. Astrocytes are the major components of the blood–brain barrier (BBB) and have roles as essential effectors in inflammatory reactions in the brain. The distribution of AQP4 suggests the involvement of AQP4 in the movement of water between blood and the brain and between the brain and cerebrospinal fluid (CSF).

Upregulation of AQP4 has been observed in the post-ischemic state [10], brain tumors [11], bacterial meningitis and brains with contusion [12,13]. Furthermore, AQP4 has been shown to be upregulated in experimentally-induced ischemia [14], injury or hyponatremia in rodent brain [15–17], suggesting that the regulation of AQP4 level is associated with the development of brain edema regardless of its cause.

In mice lacking AQP4, brain swelling is reduced following water intoxication and focal cerebral ischemia [18]. AQP4 deletion likely protects against cytotoxic edema in this model by slowing the entry of water into the brain parenchyma. On the other hand, remarkably increased elevations in intracranial pressure in AQP4-deficient mice have been reported in vasogenic edema models, intraparenchymal fluid infusion [19], focal cortical freeze injury [20,21], and tumor cell implantation [22,23]. Thus, in those cases, AQP4 expression may reduce vasogenic brain edema [24].

Recently, the presence of antibodies against AQP4 has been reported in patients affected with Caucasian neuromyelitis optica (NMO) and some Japanese optico-spinal type multiple sclerosis (OS-MS) patients [25–28]. NMO is an idiopathic inflammatory, necrotizing disease characterized by selective involvement of the optic nerves and spinal cord [29]. Misu et al. reported that the loss of AQP4 immunostaining was observed in dymyelinating lesions of NMO patients [30]. Therefore, AQP4 may be involved in the pathophysiological mechanism of inflammatory demyelinating disorder.

Herein, we analyzed AQP4 expression in inflammatory disease of the CNS using experimental autoimmune encephalomyelitis (EAE), an animal model of MS. We performed semi-quantitative analysis of AQP4-mRNA in brains and spinal cord from EAE-induced mice using real-time PCR. We also performed immunohistochemical analysis.

2. Materials and methods

2.1. Mouse

Wild type C57BL/6 mice were purchased from Clea Japan (Tokyo, Japan). The mice were maintained under specific pathogen-free conditions. All mice for experiments were aged 8–12 weeks. The

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experiments involving animals were approved by the local ethics committee in Kinki University.

2.2. Peptides

MOG_{35-55} (single letter amino acid code: MEVGWYRSPFSRVVH-LYRNGK) was synthesized by Tore Research Institute (Tokyo, Japan). The peptides were >90% pure, as determined by HPLC.

2.3. Induction and assessment of EAE

Mice were injected subcutaneously in the tail base bilaterally with 200 μl of inoculum containing 100 μg of MOG_{35-55} and 0.5 mg of *Mycobacterium tuberculosis* H37Ra (Difco Laboratories, Detroit, MI) in incomplete Freund's adjuvant. Pertussis toxin (List Biological Laboratories Inc., Campbell, CA, 200 ng) was injected intravenously on post-inoculation days 0 and 2 [31]. Immunized mice were examined daily and scored as follows: 0, no clinical signs; 1, limp tail; 2, partial hind leg paralysis; 3, total hind leg or partial hind and front leg paralysis; 4, total hind leg and partial front leg paralysis; 5, moribund or dead. Mice were examined daily for signs of EAE in a blinded fashion.

2.4. Pathological analysis

On days 0, 7, 14, 21, 28, and 35 after immunization for EAE, five mice per day were sacrificed under anesthesia with diethyl ether, and the brains and spinal cords were removed after perfusion with PBS. The whole brain was cut into three pieces by coronal sections, and the thoracic cord also was cut into three pieces by axial sections. The middle part of each was used for PCR analyses, and the other parts were analyzed histopathologically. For pathological analysis, thinly sliced (10 μm) frozen sections were fixed with acetone and stained with hematoxylin and eosin, and Luxol fast blue to assess inflammation and demyelination. Immunostaining with anti-AQP4 (Santa Cruz biotechnology, Santa Cruz, CA) or anti-glial fibrillary acidic protein (GFAP, BD Biosciences, San Jose, CA) antibodies was also performed.

2.5. Reverse transcriptase-PCR for identification of AQP4

For RT-PCR analysis, brain and spinal cords from each mouse were homogenized in RNAzol™ B (Biotecx laboratories, Friendswood, TX) for total RNA extraction. After isolation of total RNA, oligo (dT)-primed cDNA was prepared with a First-Strand cDNA Synthesis Kit (Amersham Pharmacia Biotech, Buckinghamshire, England). We performed quantitative PCR with a commercial kit (Light Cycler-DNA Master SYBR Green I, Roche Molecular Biochemicals, Mannheim, Germany) using the Light-Cycler™ quantitative PCR system [32]. The PCR amplification was repeated 45 times (at 95 °C for 15 s, at 56 °C for 7 s, and at 72 °C for 15 s). The PCR primers used were as follows: AQP4-sense, TGGTGTTCAC-TATTTTTGCC and antisense, GATCAAGTCTTCCGTCTCCA.

2.6. Statistical analysis

The expression level of AQP4 was analyzed by Mann–Whitney-*U* test.

3. Result

3.1. Quantitative analysis of AQP4-mRNA

A significant increase of AQP4-mRNA was observed only in EAE-affected mice. In EAE mice, AQP4-mRNA expression in the spinal cord began to increase on post-inoculation day 7 and reached a peak on day 14. In contrast, the increase of AQP4-mRNA expression in the brain was delayed compared with that in the spinal cord; reaching a peak on post-inoculation day 21 (Fig. 1A). AQP4-mRNA expression at the peak

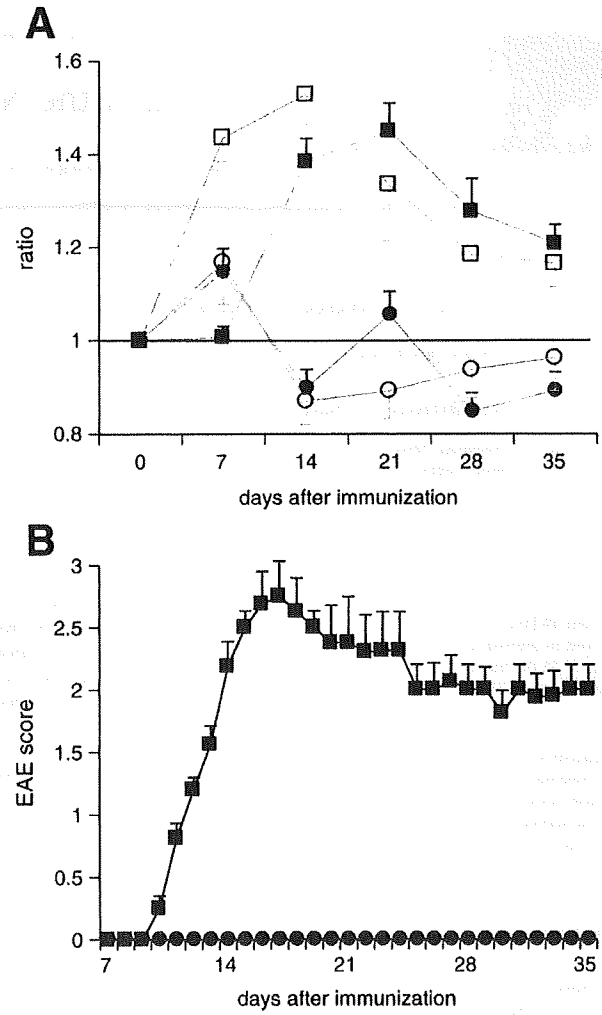

Fig. 1. Quantitative analysis of AQP4-mRNA in EAE. (A) EAE was induced in female B6 mice by immunization with MOG_{35-55} in CFA, as described in the Materials and methods. Expression of AQP4-mRNA in CNS from each mouse was analyzed by LightCycler™, as described in the Materials and methods. To compare AQP4-mRNA levels among the groups, the expression level was expressed as a relative value. We determined the expression level on day 0 in each group as 1.0. In EAE mice, AQP4-mRNA expression in the spinal cord began to increase on post-inoculation day 7 and reached the peak on day 14 (□). The increase of AQP4-mRNA expression in the brain was delayed in comparison with that in spinal cord and it reached the peak on post-inoculation day 21 (■). On the other hand, no remarkable elevation of AQP4-mRNA level was observed in brain (●) and spinal cord (○) from control mice. (B) EAE scores from three independent experiments are expressed as mean±S.E.M. (■). Control mice were immunized with CFA without MOG_{35-55} (●).

was 152% in the spinal cord and 145% in brain, compared with the pre-inoculation levels. The expression decreased gradually in both spinal cord and brain during subsequent weeks. The clinical score of EAE reached its peak on post-inoculation day 17 (Fig. 1B).

3.2. Histopathological analysis

Histopathological analysis showed that demyelination and cell infiltration were most severe on post-inoculation day 21, when the clinical manifestations were also severe. Next we performed immunohistochemical staining to analyze the protein expression of AQP4 in CNS of the animals with EAE on post-inoculation day 21. AQP4 was expressed in both spinal cord (Fig. 2A) and brain (Fig. 2B), and the staining with anti-AQP4 antibody was colocalized with anti-GFAP

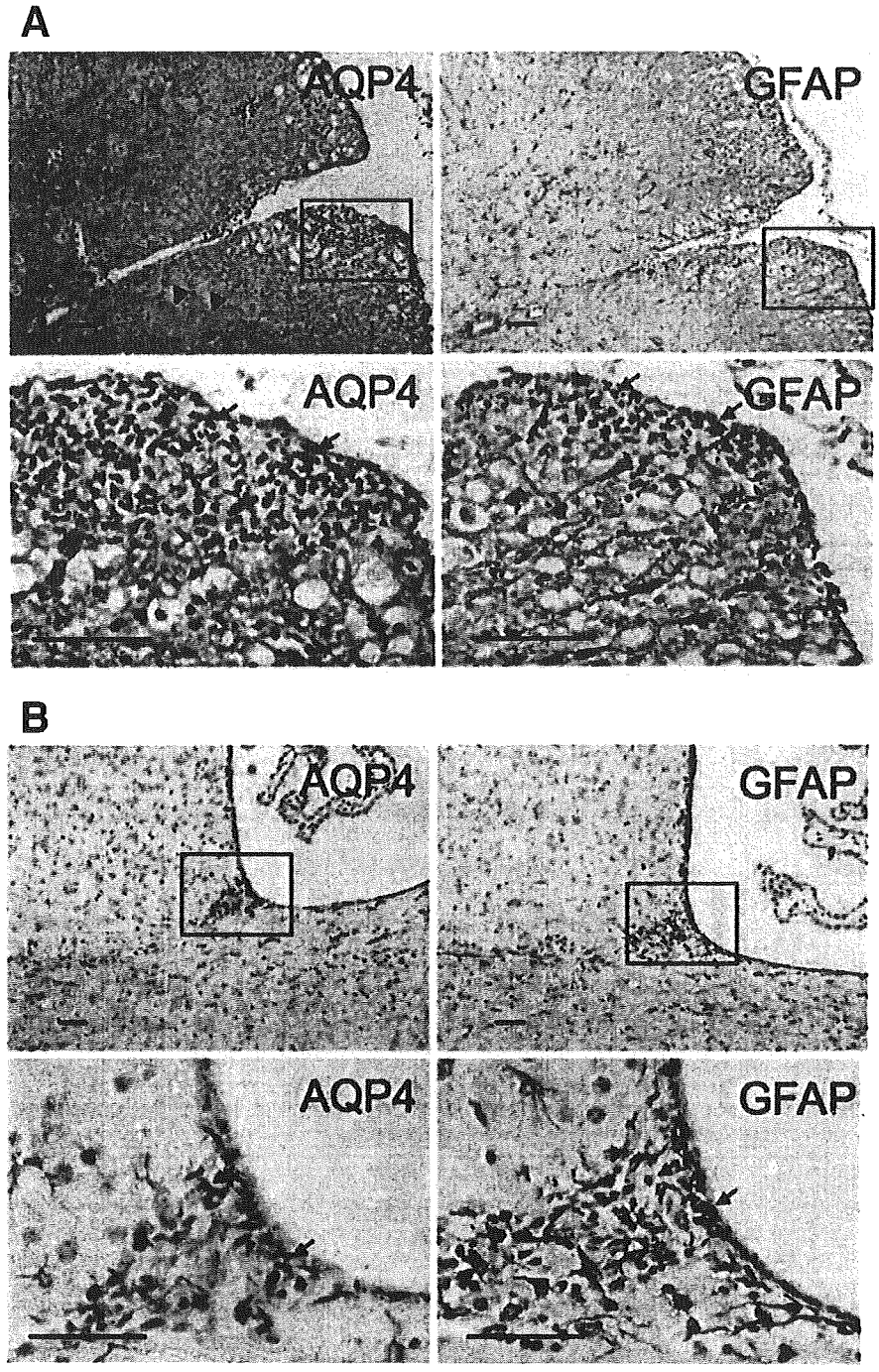

Fig. 2. Histopathological analysis. The histopathological analysis on post-inoculation day 21 is shown. Both AQP4 and GFAP were expressed (brown) in spinal cord (A) and brain (B). Each lower panel showed the enlarged square area in the upper panel. The cellular infiltration (arrows) was seen in both spinal cord (A) and brain (B). In the spinal cord, AQP4 staining in the gray matter is stronger than that in white matter (arrow head). Staining with the anti-AQP4 (green) antibody was colocalized with that of the anti-GFAP (red) antibody (C). (D) Showed the staining pattern of AQP4 and GFAP in brain from control mice immunized without MOG. Bar = 100 μm.

antibody (Fig. 2C). This suggests that AQP4 expression may correlate with astrocytic activation.

4. Discussion

We demonstrated that AQP4-mRNA expression was elevated in the CNS from mice affected with EAE in the acute phase. Immunohistochemical analysis showed that AQP4 was expressed in both spinal cord and brain in EAE mice. The distribution of the staining with anti-AQP4 antibody was similar to that of anti-GFAP antibody. AQP4 therefore may be expressed in activated astrocytes. This is the first study to investigate the regulation of AQP-4 in EAE.

The peak expression of AQP4 mRNA was detected on day 14 in the spinal cord, and on day 21 in the brain after immunization for EAE. It is unclear why the expression in the spinal cord reached its peak earlier than that in the brain. We consider that the time course of AQP4-mRNA

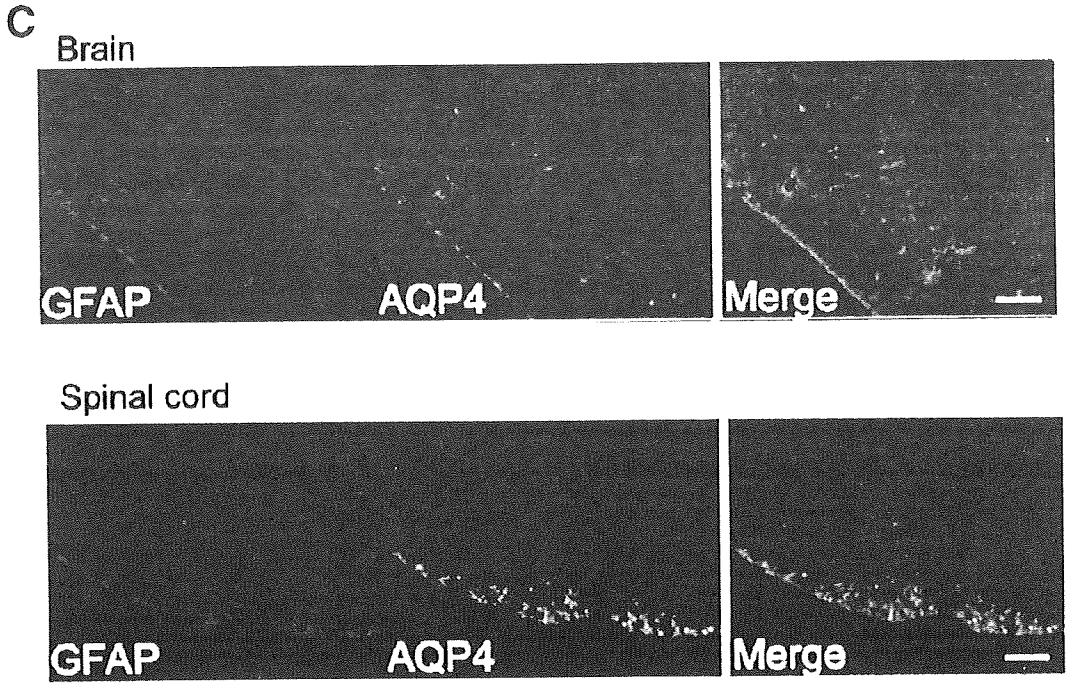

Fig. 2 (*continued*).

expression depends on the clinical course of EAE. The clinical signs of EAE induced by active-immunization with MOG_{35-55} in the present study usually appear first in tail, followed subsequently by the hind limbs and ore limbs are affected. The pathological changes also appear first in the lumbar spinal cord. We generally inoculate the mice at the lower part of the body, tail base or hind limbs. Thus, the inflammation begins at the lower part of the spinal cord, and then ascends gradually up to the brain. Astrocytic activation also may occur first in the spinal cord, followed by the brain. The astrocytic activation could also be related to AQP4 expression, as described above.

Previous studies in AQP4-deficient mice have shown reduced cellular brain edema following water intoxication and ischemic stroke [18]. On the other hand, higher intracranial pressure and brain edema have been reported in AQP4-deficient mice after continuous intraparenchymal fluid infusion [24]. Thus, the effect of AQP4 deletion is difficult to predict for each situation. Future investigations should determine whether the increased expression of AQP4 in EAE aggravates or ameliorates the inflammatory edema.

In human MS, AQP4 immunoreactivity was well preserved and stained strongly in the demyelinating plaques [33]. AQP4-positive

astrocytes were more abundant at the periphery of plaques than in their center, as seen in ischemic foci. AQP4 stained from the very acute phase of necrosis to the chronic stage of astrogliosis. In contrast, the immunoreactivities of AQP4 and GFAP in NMO were consistently lost from the early stage in NMO lesions. These distinct features suggest that the different mechanisms of initiation and progression between MS and NMO. Thus, astrocytic impairment associated with the loss of AQP4 and humoral immunity may be important in the pathogenesis of NMO lesions [30]. Our results show that the regulation of AQP-4 levels in EAE is similar to that of MS but not of NMO.

Our results showed that the upregulation of AQP4 could be involved in the development of inflammation in the acute phase of EAE. Future studies using EAE to examine whether the anti-AQP-4 antibody has any effect on astrocytes with enhanced AQP-4 expression may be warranted. Such investigations may provide us with an important clue to solve the pathogenesis of NMO.

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EXPERT REVIEWS

Guillain–Barré syndrome: update on immunobiology and treatment

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Growing experimental and clinical data have shed light on the pathophysiology of Guillain–Barré syndrome (GBS) and have further promoted the development of novel therapeutic strategies for the disorder. Elevated titer of antiganglioside antibodies is a characteristic of GBS. This may determine the clinical features of each case by binding to the sites where a target ganglioside antigen is localized. In experimental models of GBS and its variants, complementary inhibitory agents may exert neuroprotective efficacy by inhibiting antiganglioside antibody-mediated activation of the classical pathway. Complement-mediated disruption of the voltage-gated sodium channel cluster has been shown to be a principal cause of conduction failure in the model of acute motor axonal variants of GBS, protected by a complement inhibitor. Anti-GQ1b antibody-mediated injury at motor nerve terminals is also protected by complement inhibitors. In the future many kinds of drug candidates that inhibit activation of the complement system at various stages will be used in models of autoimmune neuropathy, in future applied to clinical trials for GBS and its variants. Complement-independent blockade of voltage-gated calcium channels or the apoptotic mechanism of neurons should also be considered. The pathogenic effect of antiganglioside antibody depends upon the local glycolipid environment in the nerve membrane as well as the antibody specificity.

KEYWORDS: antibody • complement inhibitor • ganglioside • ganglioside complex • Guillain–Barré syndrome • voltage-gated sodium channel

Guillain–Barré syndrome & antiganglioside antibodies

In the last 10 years there has been tremendous progress on immunobiological mechanisms in the acute immune-mediated polyradiculoneuropathy, Guillain–Barré syndrome (GBS), and novel treatments are currently in the process of development. Clinical *in vitro*, *ex vivo* or *in vivo* studies on pathophysiological action of antiganglioside antibodies in GBS have greatly contributed to this progress.

Gangliosides, N-acetylneuraminic acid (sialic acid)-containing glycosphingolipids that concentrate on the surface of neurons with the oligosaccharide portion expressed on the cell surface, are considered to be involved in many nerve cell functions [1]. Gangliosides are thought to be organized in clusters, and to form membrane microdomains together with cholesterol and glycosylphosphatidylinositol (GPI)-anchored proteins [2]. These microdomains are used synonymously with lipid rafts or detergent-resistant membranes. Glycosynapses, which are enriched in tetraspanin, cholesterol-independent and involved in glycosylation-dependent cell adhesion are also included in the microdomains [3]. The microdomains cluster together to form larger platforms where protein participants in signal transduction events can assemble, and facilitate a variety of membrane-mediated functions such as cell adhesion and signal transduction [1,2,4].

Approximately 60% of patients with GBS have antibodies to various gangliosides in sera during the acute clinical phase of the disease [5]. Antiganglioside antibodies play a crucial role in the development of GBS and its variants by binding to the sites where target ganglioside antigens are specifically localized [6], although cellular immune response also impacts on the pathophysiology of GBS. In this article, we present an overview of recent progress on immunobiology and treatment in GBS and its variants, mainly focusing on antiganglioside antibody-mediated pathophysiology.

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