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Original Article

An Increase in Circulating B Cell–Activating Factor in Childhood-Onset Ocular Myasthenia Gravis

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ABSTRACT

BACKGROUND: Myasthenia gravis is a B cell–mediated autoimmune disorder. The pathophysiology of childhood-onset ocular myasthenia gravis remains unclear. We investigated serum B cell–activating factor levels and other immunological parameters in child patients with ocular myasthenia gravis. **METHODS:** Blood samples were obtained from 9 children with ocular myasthenia gravis and 20 age-matched controls. We assayed serum concentrations of B cell–activating factor, anti-acetylcholine receptor antibody titers, 7 types of cytokines (interleukins-2, -4, -6, -10, and -17A; interferon-γ; tumor necrosis factor-α) as well as the percentages of peripheral blood CD4+, CD8+, and CD19+ cells. **RESULTS:** Serum B cell–activating factor levels were significantly higher before immunosuppressive therapy in patients with childhood-onset ocular myasthenia gravis than in controls and decreased after immunosuppressive therapy. A significant positive correlation was observed between serum B cell–activating factor levels and anti-acetylcholine receptor antibody titers in patients with myasthenia gravis. Serum B cell–activating factor concentrations did not correlate with the percentages of CD4+, CD8+, and CD19+ cells or the CD4+/CD8+ ratio. No significant differences were observed in the levels of the 7 different types of cytokines examined, including interleukin-17A, between preimmunosuppressive therapy myasthenia gravis patients and controls. **CONCLUSIONS:** Circulating B cell–activating factor may play a key role in the pathophysiology of childhood-onset ocular myasthenia gravis.

Keywords: childhood-onset ocular myasthenia gravis, B-cell activating factor, IL-17, Th17

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Introduction

B-cell activating factor (BAFF), a member of the tumor necrosis factor (TNF) superfamily, is secreted by various myeloid cells such as macrophages, dendritic cells, and neutrophils.[1] BAFF is a potent survival factor for B cells and plays an essential role in peripheral B-cell homeostasis.[2] The overexpression of BAFF protects B cells from apoptosis, thereby contributing to autoimmunity and malignancy.[3-5] Accordingly, the BAFF pathway may be a novel target for the treatment of autoimmune diseases. The clinical application of an anti-BAFF neutralizing antibody (LymphoStat-B, Belimumab) to treat systemic lupus erythematosus has already been reported.[6,7]

Myasthenia gravis (MG) is a B cell–mediated autoimmune disorder in which the target autoantigen is the acetylcholine receptor (AChR) at the neuromuscular junction. Previous studies demonstrated that serum BAFF levels were significantly higher in adult patients with MG than in controls.[8-10] Childhood-onset MG is a relatively uncommon disease, with an estimated incidence of 3-9.1 per million per year,[11] and the relationship between BAFF and childhood-onset MG remains unclear.

We examined serum BAFF levels, the frequencies of peripheral blood T-cell subsets and CD19+ cells, and levels of seven types of circulating cytokines at different clinical stages in patients with childhood-onset ocular MG.

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Materials and Methods

Subjects and sample collection

Nine children with ocular MG (three boys and six girls) and 20 age-matched control subjects (seven boys and 13 girls) with epilepsy and/or developmental retardation without any inflammatory complications were enrolled in this study. Patients were diagnosed with ocular MG by pediatric neurologists on the basis of: (1) the presence of fluctuating ocular muscle weakness with fatigability; (2) normal strength in all other muscles; (3) positive response to an anti-cholinesterase injection; and (4) absence of abnormal electrophysiological signs such as a decrement of more than 10% in compound muscle action potential on repetitive nerve stimulationof the trapezius muscle (to exclude generalized MG).[12,13] The mean age ± standard deviation (SD) of MG patients was 5.9 ± 4.4 years (range, 1.8-13.0 years) and 7.8 ± 4.5 years (range, 1.1-12.8 years) in the controls. Samples were obtained from nine patients with ocular MG who received no immunosuppressive therapy (IST; the pre-IST MG group). We immunologically tested 4 patients before and after IST (the post-IST MG group; one boy and three girls; 5.1 ± 3.7 years; range, 1.8-10.3 years). All nine patients in the pre-IST MG group achieved complete remission after IST. Written informed consent was obtained from the parents of all patients and control subjects. This study was approved by the Ethics Committee of Shinshu University School of Medicine and was performed in accordance with the 1964 Declaration of Helsinki and its later amendments.

Serum samples were separated immediately and kept at −30 °C until later analyses.

BAFF assay

Serum BAFF levels were measured by a quantitative sandwich enzyme immunoassay using a commercial Quantikine kit (R&D Systems, USA) according to the manufacturer's instructions. Fifty microliters of a BAFF standard or sample was added to a microplate precoated with a mouse monoclonal antibody specific for BAFF and incubated for 3 hours at room temperature on a horizontal orbital shaker set at 500 ± 50 rpm. After washing, 200 μL of an enzyme-linked polyclonal antibody that was specific for BAFF was added to each well, and the plate was incubated on the shaker for an additional 1 hour. After washing, a substrate solution was added for 30 minutes, and absorbance was measured at 450 nm. The lower detection limit was 2.68 pg/mL. Samples were assayed in duplicate.

Cytokine assay

Interleukin (IL)-2, IL-4, IL-6, IL-10, IL-17A, interferon-γ, and TNF-α levels were determined using a cytometric bead array kit (BD Pharmingen, San Diego, CA, USA) according to the manufacturer's instructions. Each series of beads was coated with a specific antibody against a single cytokine. The mixture of beads detected seven types of cytokines in one sample from discrete fluorescence intensity at 670 nm. A secondary phycoerythrin-conjugated antibody stained the beads depending on the amount of bound cytokine. A standard contained a mixture of predetermined amounts of all seven cytokines. Ten serial dilutions were prepared, providing a range of concentrations from 20 to 5000 pg/mL. After fluorescence intensity calibration and electronic color compensation procedures, standard and test samples were analyzed using a FACSCalibur (BD, San Jose, USA) equipped with CellQuest (BD Pharmingen). The lower detection limits for IL-2, IL-4, IL-6, IL-10, IL-17A, interferon-γ, and TNF-α were 2.6, 4.9, 2.4, 4.5, 18.9, 3.7, and 3.8 pg/mL, respectively. Samples were assayed in duplicate.

Statistical analysis

Values are presented as the mean ± SD. To determine the significance of differences between two independent groups, we used an unpaired *t* test or Mann-Whitney's *U* test when the data were not normally distributed. Serum BAFF levels in the pre- and post-IST MG groups were compared using a paired *t* test. The relationship between two parameters was analyzed by Spearman's correlation coefficient using the rank test. A cytokine concentration below the detectable limit was defined as 1.0 pg/mL for statistical analyses. An anti-AChR antibody titer below the

TABLE 1.
Clinical and Laboratory Findings of Children with Ocular MG and Age-matched Control Subjects

	Patients With Ocular MG		Controls
	Pre-IST	Post-IST	
N	9	4	20
Age (years)	5.9 ± 4.4	5.1 ± 3.7	7.8 ± 4.5
Male/female	3/6	1/3	7/13
Oral anti-cholinesterase agent	3	2	0
Immunosuppressive treatment			
PSL	0	3	0
PSL + IAPP	0	1	0
None	9	0	0
Thymoma/thymectomy	0	0	0
Laboratory data (mean ± SD)			
White blood cells (/μL)	6901.1 ± 3013.6	9295.0 ± 2785.2	6800.5 ± 1849.5
Lymphocytes (%)	40.9 ± 16.4	52.8 ± 10.1	45.0 ± 13.0
CD4+ cells (%)	44.1 ± 9.3	40.3 ± 8.1	NA
CD8+ cells (%)	26.9 ± 3.9	30.8 ± 9.0	NA
CD19+ cells (%)	12.7 ± 4.9	7.7 ± 3.2	NA
IgG (mg/dL)	905.0 ± 145.5	670.8 ± 160.3*	NA
Anti-AChR antibody (nmol/L)	2.2 ± 2.0	2.7	NA

Abbreviations:
AChR = Acetylcholine receptor
CD = Cluster of differentiation
IAPPI = Immunoadsorption plasmapheresis
IST = Immunosuppressive treatment
MG = Myasthenia gravis
NA = Not assayed
PSL = Prednisolone
SD = Standard deviation

* Significant difference between the pre- and post-IST groups ($P = 0.02$).

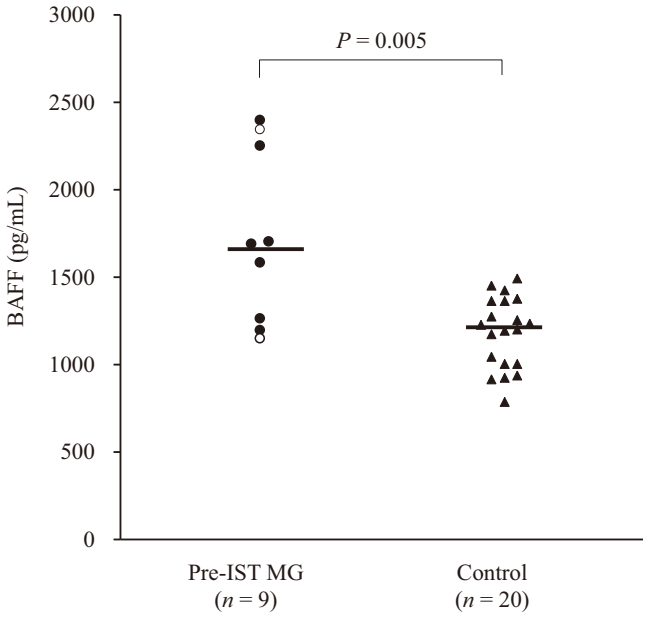

FIGURE 1.
Serum B cell–activating factor (BAFF) levels in ocular myasthenia gravis (MG) patients before immunosuppressive therapy and controls. Horizontal bars represent median values. Statistical comparisons by the Mann-Whitney *U* test. Horizontal bars represent median values. Open circles indicate negative anti-acetylcholine receptor antibody titers in pre-immunosuppressive therapy (IST) MG patients; filled circle represent positive titers.

detectable limit (0.3 nmol/L) was defined as 0.1 nmol/L. All statistical analyses were conducted using PASW statistics, version 18 (SPSS Inc., Chicago, USA). The level of significance was defined as a *P* value of less than 0.05.

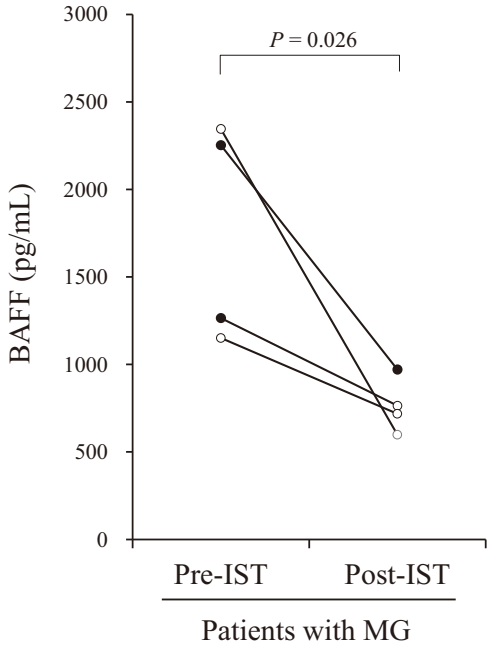

FIGURE 2.
Serum B cell–activating factor (BAFF) levels in 4 patients with ocular myasthenia gravis (MG) before and after immunosuppressive therapy. Open circles indicate negative anti-acetylcholine receptor antibody titers in pre- and post-immunosuppressive therapy (IST) MG patients; filled circles represent positive titers.

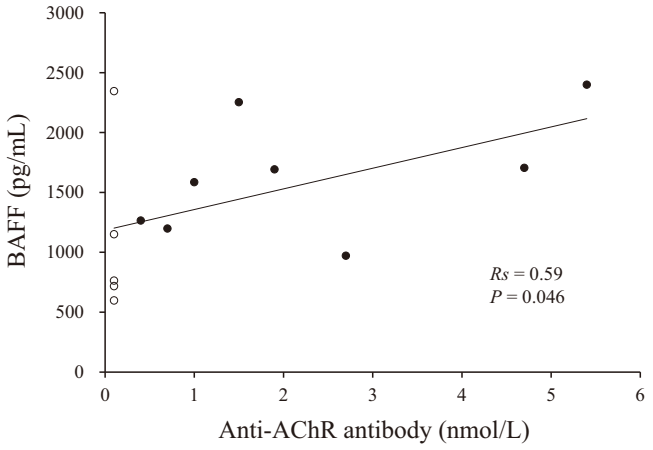

FIGURE 3.
Positive correlation between B cell–activating factor (BAFF) levels and anti-acetylcholine receptor (AChR) antibodies in pediatric patients with ocular myasthenia gravis (MG). Open circles indicate negative anti-AChR antibody titers in pre- and post-immunosuppressive therapy MG patients; filled circles represent positive titers.

Results

Clinical characteristics

The clinical data of children with ocular MG are presented in Table 1. No significant differences were observed in age, gender, or white blood cell/lymphocyte counts between the pre-IST MG group and controls. No patients had thymoma. Of the nine patients in the pre-IST MG group, three were treated with anti-cholinesterase drugs at the time of analysis. In the post-IST MG group, three patients received oral prednisolone with or without an anti-cholinesterase agent. The remaining patient underwent two courses of immunoadsorption plasmapheresis followed by the administration of oral prednisolone. The mean duration from the start of prednisolone therapy to immunological analysis was 36.5 ± 12.4 (28-55) days. Two of nine patients in pre-IST group tested negative for the anti-AChR antibody throughout their clinical course. Another one of nine patients tested positive in the pre-IST period and turned negative in the post-IST period. In total, 7 patients in the pre-IST MG group and one post-IST MG patient were positive for the anti-AChR antibody; their titers were 2.2 ± 2.0 nmol/L and 2.7 nmol/L, respectively. Serum immunoglobulin G levels were significantly lower in the post-IST MG group than in the pre-IST MG group ($P = 0.02$). Additionally, the percentages of CD4+, CD8+, and CD19+ cells in the pre-IST MG group were not significantly different from those in the post-IST MG group.

Serum BAFF levels in children with ocular MG before and after IST

As shown in Figure 1, serum BAFF levels were significantly higher in the pre-IST MG group (1732.7 ± 494.7 pg/mL) than in the control group (1182.8 ± 203.5 pg/mL; $P = 0.005$). Serum BAFF levels could be compared before and after IST in 4 patients and were found to be markedly decreased by IST ($P = 0.026$), as shown in Figure 2. On the other hand, about serum anti-AChR antibody, two of four

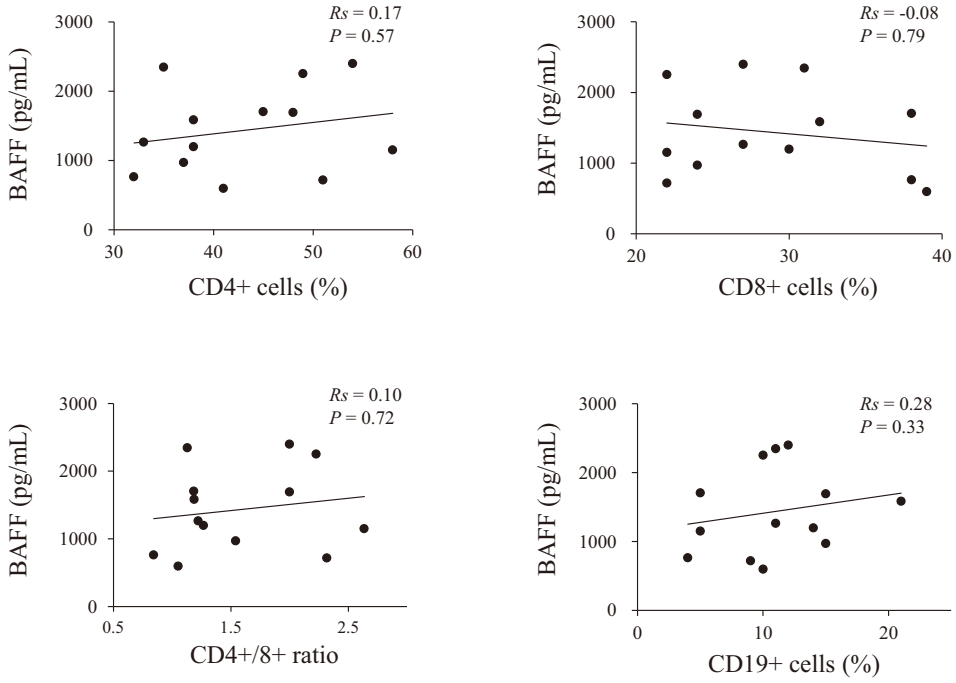

FIGURE 4.
B cell–activating factor (BAFF) levels did not correlate with the percentages of CD4+, CD8+, and CD19+ cells or the CD4+/CD8+ ratio in pediatric patients with ocular myasthenia gravis.

patients in post-IST group were seronegative, one of the seropositive patients turned seronegative after IST, and the other patient remained seropositive whose titer got higher after IST (1.5-2.7 nmol/L).

Relationship between serum BAFF levels and the humoral/cellular immune status

To determine the relationship between serum BAFF levels and the humoral/cellular immunity status, we simultaneously assessed serum anti-AChR antibody titers; the percentages of peripheral blood CD19+ , CD4+, and CD8+ cells; and CD4+/CD8+ ratios in the pre- and post-IST MG periods. As shown in Figure 3, a significant positive correlation was observed between serum BAFF levels and anti-AChR antibody titers in patients with MG (R values = 0.59, $P = 0.046$). On the other hand, serum BAFF concentrations did not correlate with the values of the four other parameters (the percentages of CD4+, CD8+, and CD19+ cells; and CD4+/CD8+ ratio), as shown in Figure 4.

Serum cytokine profiles before and after IST

Serum cytokine profiles before and after IST are shown in Table 2. No significant differences were observed in the serum concentrations of IL-6, IL-17A, and interferon-γ between the pre-IST MG and control groups. The levels of these cytokines became undetectable after IST. On the other hand, IL-2, IL-4, IL-10, and TNF-α were below detectable levels in both the pre- and post-IST periods. Serum cytokine levels did not correlate with BAFF levels (data not shown).

Discussion

Although pediatric MG shares many features with adult MG, many differences exist between the two groups. Childhood-onset MG is more likely to manifest as the ocular type with a lower rate of generalization and higher percentage of patients who are seronegative for the anti-AChR antibody. There are two reports regarding the positivity for anti-AChR antibodies in childhood-onset ocular MG: one reported 43% (3/7)[14] and the other documented 80% (4/5).[15] The ratio of positivity of the antibody in this study was 78% (7/9). Furthermore, no significant differences have been reported in the ratio of males to females in childhood, which is in contrast to the female predominance observed in adult MG. The presence of thymoma is exceedingly rare in pediatric populations. Pediatric MG also spontaneously resolves at a higher rate than

TABLE 2.
Serum Cytokine Profiles of Patients with Ocular MG

Cytokines (pg/mL)	Patients With Ocular MG		Controls (n = 20)
	Pre-IST (n = 9)	Post-IST (n = 4)	
IL-2	ND	ND	1.80 ± 1.19
IL-4	ND	ND	ND
IL-6	6.53 ± 15.05	ND	5.39 ± 14.70
IL-10	ND	ND	ND
IL-17A	5.22 ± 8.39	ND	14.91 ± 18.59
TNF-α	ND	ND	ND
IFN-γ	1.59 ± 1.77	ND	1.29 ± 1.30

Abbreviations:
IL = Interleukin
IFN = Interferon
IST = Immunosuppressive treatment
MG = Myasthenia gravis
ND = All samples were below the detectable limit
TNF = Tumor necrosis factor

adult MG.[12,15-19] Nevertheless, immunological differences between childhood-onset and adult MG remain unclear.

Recent studies reported the importance of BAFF in the homeostasis and functions of B cells. In animal models, the overexpression of BAFF led to the development of several autoimmune abnormalities such as hypergammaglobulinemia, B-cell hyperplasia, and a disease resembling certain aspects of systemic lupus erythematosus.[3,4] Additionally, increased levels of serum BAFF have been detected in patients with autoimmune disorders including systemic lupus erythematosus, rheumatoid arthritis, Sjögren syndrome, Wegener granulomatosis, and adult MG.[4,8,20-22] Thus, BAFF appears to protect autoreactive B cells from apoptosis and contribute to the development of autoimmune diseases.[9,23,24] On the other hand, circulating BAFF kinetics have not been reported in childhood-onset MG. In the present study, serum BAFF levels were significantly higher before IST in children with ocular MG than in controls; these decreased after IST. Furthermore, a positive correlation was observed between serum BAFF levels and anti-AChR antibody titers. These results are consistent with previous findings obtained from adult MG patients.[8-10] Thus, BAFF may play a key role in the pathogenesis of pediatric MG as well as adult MG. In addition, BAFF could be surrogate marker for therapeutic effect.

Previous studies demonstrated that the production of the anti-AChR antibody was T cell–dependent in MG.[25-29] Th17, a new T-helper subset, is considered to play an important role in the pathophysiology of MG. Mu et al. reported that the percentage of Th17 cells to CD4+ cells increased with disease progression and was accompanied by the up-regulation of IL-17 in animal models of autoimmune MG.[30] Zheng et al. showed that serum levels of IL-17 were significantly higher in adult patients with MG than in controls. They also demonstrated a positive correlation between circulating IL-17 values and anti-AChR antibody titers in adult MG subjects.[31] Th17 cells affect the production of autoantibodies by influencing the Th1 and Th2 cytokine balance in MG patients.[32] IL-17 has been shown to synergize with BAFF to promote the survival and maturation of human B cells.[33,34] In this study, no significant difference was observed in the serum levels of IL-17A between pediatric MG patients in the pre-IST period and control subjects, which differed from that in adult MG patients.[31,35] These results suggest an apparent difference in immunological backgrounds between pediatric and adult MG. The absence of coincident increases in serum BAFF and IL-17A levels may be related to negative or low anti-AChR antibody titers in childhood-onset ocular MG.

In conclusion, serum BAFF levels were higher before IST in patients with childhood-onset ocular MG than in the controls and decreased after IST. A positive correlation was observed between serum BAFF levels and anti-AChR antibody titers. These results suggest that BAFF may play a key role in the pathophysiology of childhood-onset ocular MG.

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