

A Potential Role for B-Cell Activating Factor in the Pathogenesis of Autoimmune Myasthenia Gravis

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Objective: To compare serum B-cell activating factor (BAFF) levels in patients with myasthenia gravis (MG) with those in control subjects without MG.

Design: Case-control study.

Subjects: Forty-three patients with MG were compared with control subjects without MG. These included 48 healthy subjects, 25 patients with multiple sclerosis, and 3 patients with amyotrophic lateral sclerosis.

Results: In all subjects studied, there was no correlation between the serum BAFF level and the concentration of total IgG, IgA, or IgM. The BAFF levels in patients with multiple sclerosis or amyotrophic lateral sclerosis were not

significantly different from those in healthy subjects. However, BAFF levels in patients with MG were significantly higher than those of all the control subjects. There was no correlation or dependence between the serum BAFF level and the extent or severity of disease. There was a trend for BAFF levels to be higher in patients who were seropositive for acetylcholine receptor–specific antibodies.

Conclusions: We report that BAFF levels are increased in patients with autoimmune MG. Our data suggest that BAFF is likely to play a role in the pathogenesis of MG by promoting the survival and maturation of autoreactive B cells.

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AUTOIMMUNE MYASTHENIA gravis (MG) is a B cell–mediated disease in which the target autoantigen is the acetylcholine receptor (AChR) at the neuromuscular junction.¹ Most patients with generalized symptoms have circulating anti-AChR antibodies. Some patients who are seronegative for anti-AChR antibodies have circulating antibodies to muscle-specific kinase (MuSK).^{2,3} The AChR-directed antibodies can bind to the various subunits of the AChR; however, most are specific for the α subunit.⁴ There is no correlation between the serum antibody titer and disease severity in MG.⁵ The inductive signals that lead to the breakdown of immune tolerance to the AChR remain unknown.

Although the percentage of B cells in the blood of patients with MG is the same as that of healthy subjects, the frequency of B cells that express CD71 is significantly higher in patients with MG,⁶ particularly in seropositive patients. Because CD71, a transferrin receptor, is essential for the transport of iron into proliferating cells, the increased expression of

CD71 suggests that the percentage of proliferating B cells is higher in patients with MG compared with healthy controls.

In some patients, the myasthenic thymus is implicated in initiating, or contributing to, the disease process.^{7,8} The presence of germinal centers in the thymic perivascular space indicates that B-cell activation and proliferation are occurring within the thymus. Patients with MG with thymic follicular hyperplasia tend to have higher serum titers of AChR-specific antibodies.⁵ The germinal center environment also provides the necessary signals for AChR-specific B-cell survival.⁹ Germinal centers within the thymus have strong overexpression of CD23,¹⁰ a multifunctional molecule. One of its roles is to promote the survival and differentiation of germinal center B cells through a mechanism that involves upregulation of Bcl-2.¹¹ Thymic germinal center B cells do overexpress Bcl-2.^{12,13} In the MG thymus with follicular hyperplasia, the overexpression of CD23 and Bcl-2 provides strong evidence that the germinal center environment is promoting the survival and differentiation of AChR-specific B cells.

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Within germinal centers, B cells are in close proximity to and are influenced by soluble signals from dendritic cells. Dendritic cells and other myeloid cells (monocytes/macrophages) produce and secrete B-cell activating factor (BAFF).¹⁴⁻¹⁶ B-cell activating factor-transgenic animals exhibit hypergammaglobulinemia, lymphoproliferation, and B-cell hyperplasia, and they develop autoimmune disease. Conversely, in BAFF-deficient animals, there are defects in peripheral B-cell maturation and decreased levels of circulating immunoglobulins.¹⁷ Therefore, BAFF is a potent survival factor for B cells and is necessary for peripheral B-cell differentiation. B-cell activating factor regulates Bcl-2 family members in a manner consistent with pro survival.^{18,19} B-cell activating factor is an important molecule within the germinal center. Its role in promoting the survival and maturation of AChR-specific B cells has not been studied. In this study, we measured BAFF levels in the serum of patients with autoimmune MG. The BAFF levels were compared with those in control subjects without MG. These included healthy subjects, patients with multiple sclerosis (MS), and patients with amyotrophic lateral sclerosis (ALS). We report that BAFF levels were increased in patients with MG.

METHODS

SUBJECTS

Patients with MG included 29 women and 14 men with an age range of 20 to 72 years. Clinical diagnosis of MG was confirmed by electrophysiology, pharmacologic testing with edrophonium chloride, and/or serum anti-AChR and anti-MuSK antibody titers. The extent of disease and severity of symptoms were graded according to the Myasthenia Gravis Foundation of America clinical classification scale.²⁰ Patients with MG included those who were receiving no therapy or receiving pyridostigmine bromide only. Patients who were receiving any immunomodulatory therapy or had undergone thymectomy were excluded. Informed consent was obtained from all subjects. Patients with MG were compared with race-, sex-, and age-matched control subjects without MG. These included 48 healthy subjects, 3 patients with ALS, and 25 patients with MS. Patients with MS included 23 patients with relapsing-remitting disease, 1 patient with primary progressive disease, and 1 patient with secondary progressive disease. Patients with MS were untreated at the time of study. Serum samples from all subjects were stored at -70°C until the time of study.

BAFF ENZYME-LINKED IMMUNOSORBENT ASSAY

Serum BAFF levels were measured by an enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, Minnesota), which was calibrated using soluble human recombinant BAFF as a standard. Briefly, a monoclonal antibody specific for BAFF was precoated onto a microplate. The BAFF standards and serum samples were then added in duplicate and incubated for 2 hours. After washing, an enzyme-linked polyclonal antibody that was specific for BAFF was added, and the plate was incubated for an additional 2 hours. After washing, a substrate solution was added for 30 minutes. Color developed in proportion to the amount of bound BAFF. Absorbance was measured at 450 nm. The standard curve included BAFF concen-

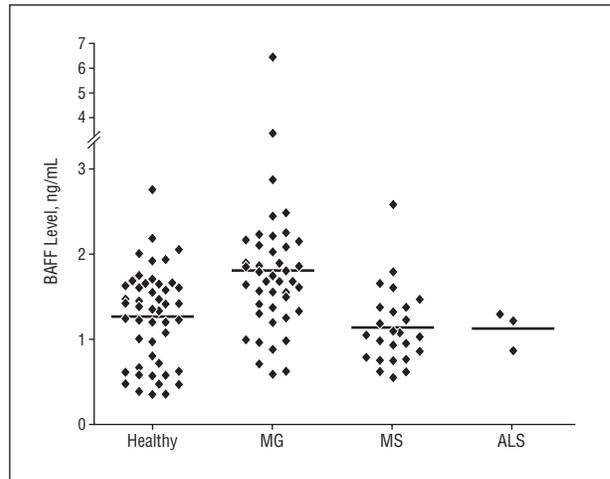


Figure 1. Serum B-cell activating factor (BAFF) levels. The BAFF levels were measured by enzyme-linked immunosorbent assay. Data are shown for 48 healthy subjects, 43 patients with myasthenia gravis (MG), 25 patients with multiple sclerosis (MS), and 3 patients with amyotrophic lateral sclerosis (ALS). Each point represents an individual. The line denotes the mean.

trations in the range of 62.5 pg/mL to 4000 pg/mL. The minimal detectable dose of BAFF (ie, sensitivity) was at 3.4 pg/mL. The goodness of fit for a representative standard curve was $r^2=0.9955$. The intraassay coefficient of variation was 4.9%; the interassay coefficient of variation was 8.0%. Using this assay, BAFF levels in healthy human serum are reported to be between 671 and 2447 pg/mL, with a mean (SD) of 1169 (283) pg/mL.

SERUM IMMUNOGLOBULIN MEASUREMENTS

Serum IgG, IgA, and IgM were measured by a radial immunodiffusion assay (The Binding Site, Birmingham, England). Briefly, serum was added to a well cut into an agarose gel containing monoclonal antibodies to IgG, IgA, or IgM. The IgG, IgA, or IgM in the serum diffused radially and a precipitin ring formed. The diameter of the ring was proportional to the concentration of IgG, IgA, or IgM in the serum sample. The assay was calibrated using IgG, IgA, and IgM standards of known concentration. The concentrations of IgG standards were 2250, 13 500, and 22 500 mg/L. The concentrations of IgA standards were 545, 3270, and 5450 mg/L. The concentrations of IgM standards were 265, 1590, and 2650 mg/L.

ANTI-AChR AND ANTI-MuSK

Titers of anti-AChR antibodies were determined by commercial laboratories at different times. Titers of anti-MuSK antibodies were determined by Angela Vincent, MD, at Oxford University.

STATISTICAL ANALYSIS

Linear regression analysis, the 2-tailed nonparametric Mann-Whitney test, and the nonparametric 1-way analysis of variance (Kruskal-Wallis test) were used. $P < .05$ was considered significant.

RESULTS

The BAFF levels in patients with MG were compared with those in patients with MS, a disease with an autoimmune pathogenesis that is considered to be T cell initiated.²¹ The BAFF levels in patients with MG were also

Table. Mean BAFF Levels^a

Subjects	ng/mL	
	BAFF Level, Mean (SD) (SEM)	95% Confidence Interval
Healthy subjects	1.264 (0.56) (0.08)	1.102-1.426
Patients with MG	1.810 (0.93) (0.14)	1.525-2.095
Patients with MS	1.141 (0.45) (0.09)	0.955-1.328
Patients with ALS	1.130 (0.23) (0.13)	0.562-1.698
All control subjects ^b	1.201 (0.51) (0.06)	1.091-1.312

Abbreviations: ALS, amyotrophic lateral sclerosis; BAFF, B-cell activating factor; MG, myasthenia gravis; MS, multiple sclerosis.

^aPatients with MG vs healthy subjects, $P < .001$; patients with MG vs patients with MS, $P < .001$; patients with MG vs patients with ALS, $P = .050$; patients with MG vs all control subjects, $P < .001$.

^bAll control subjects includes healthy subjects, patients with MS, and patients with ALS.

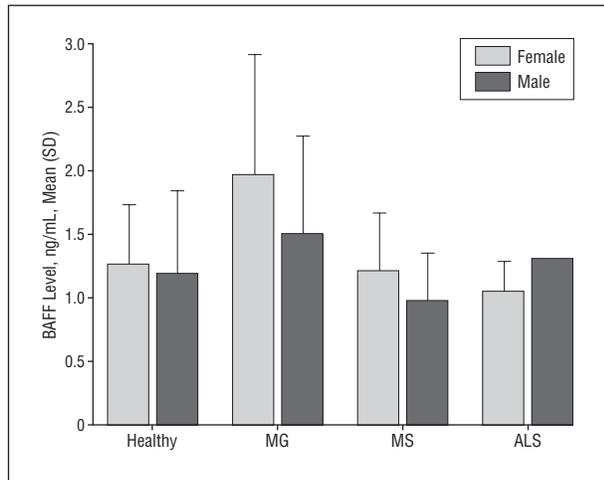


Figure 2. Effect of sex on serum B-cell activating factor (BAFF) levels. MG indicates myasthenia gravis; MS, multiple sclerosis; and ALS, amyotrophic lateral sclerosis.

compared with those in patients with ALS, a neurodegenerative disorder whose pathogenesis is unknown but is not considered to be immune mediated.²² **Figure 1** shows the serum BAFF levels in patients with MG, MS, and ALS in comparison with those in healthy subjects.

As the **Table** shows, BAFF levels in patients with MS or patients with ALS were not significantly different from those in healthy subjects. However, BAFF levels in patients with autoimmune MG were significantly higher than those in healthy subjects ($P < .001$) and higher than those in patients with MS ($P < .001$) and ALS ($P = .050$). When patients with MG were compared with all the control subjects (healthy subjects, patients with MS, and patients with ALS together), BAFF levels in the serum of patients with MG were significantly higher ($P < .001$). The mean (SD) (SEM) for patients with MG was 1.810 (0.93) (0.14) ng/mL with a 95% confidence interval of 1.525 to 2.095 ng/mL. The mean (SD) (SEM) for all control subjects was 1.201 (0.51) (0.06) ng/mL with a 95% confidence interval of 1.091 to 1.312 ng/mL. As **Figure 2** shows, for patients with MG, BAFF levels were slightly higher in female patients compared with their male counterparts; however, the difference was not statistically significant

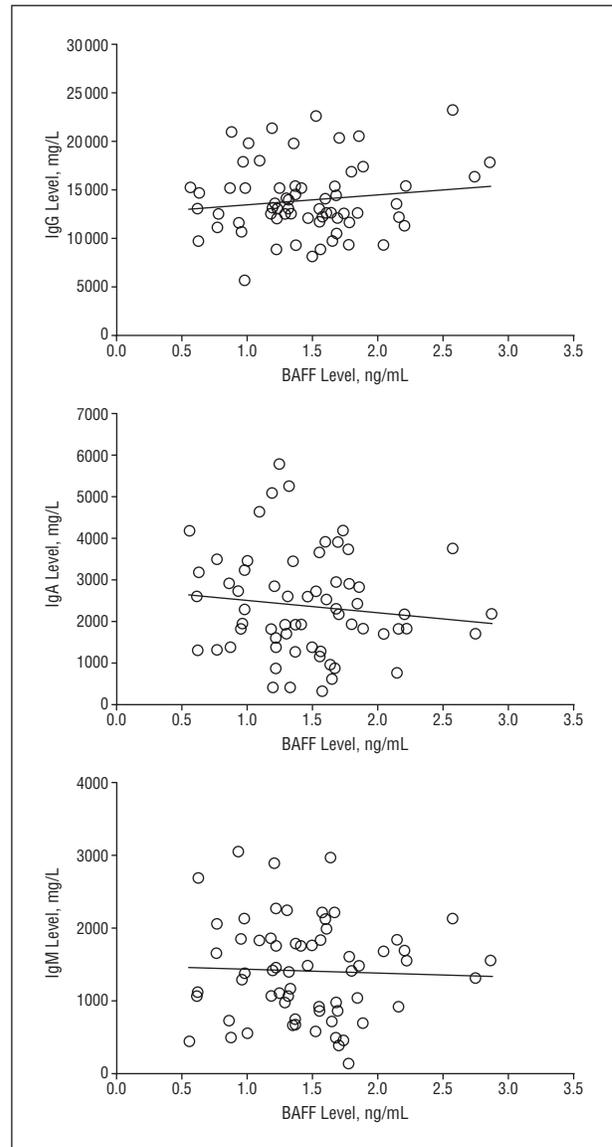


Figure 3. Correlation of B-cell activating factor (BAFF) levels with IgG, IgA, and IgM levels. The BAFF levels were measured by enzyme-linked immunosorbent assay. IgG, IgA, and IgM levels were measured by radial immunodiffusion. Each point represents an individual. The goodness of fit by linear regression analysis was IgG, $r^2 = 0.0190$; $P = .28$; IgA, $r^2 = 0.0140$; $P = .35$; and IgM, $r^2 = 0.0015$; $P = .76$

($P > .05$). For female patients with MG ($n = 29$), the mean (SD) BAFF level was 1.96 (0.96) ng/mL. For male patients with MG ($n = 14$), the mean (SD) BAFF level was 1.50 (0.78) ng/mL.

To determine whether there was a correlation between the serum BAFF level and immunoglobulin concentration, we measured IgG, IgA, and IgM levels in the serum. Of the 119 subjects included in this study, 64 sera were randomly chosen. There was no correlation between the serum BAFF levels and the serum IgG, IgA, or IgM levels in any of the subject groups. **Figure 3** shows the correlation of BAFF levels with serum immunoglobulin levels for all subject groups together. Linear regression analysis showed that the goodness of fit of BAFF levels with the serum immunoglobulin levels was IgG, $r^2 = 0.0190$; IgA, $r^2 = 0.0140$; and IgM, $r^2 = 0.0015$.

Patients with autoimmune MG were divided into groups by the extent and severity of their clinical signs and symptoms (**Figure 4**). For each class, the mean (SD) (SEM) BAFF level was class 1, 1.69 (0.47) (0.21) ng/mL; class 2, 1.49 (0.51) (0.14) ng/mL; class 3, 2.01 (0.62) (0.16) ng/mL; and class 4, 1.50 (0.41) (0.18) ng/mL. There was no correlation or dependence between the serum BAFF level and the extent or severity of disease (analysis of variance, $P=.14$). However, patients who were seropositive for anti-AChR antibodies tended to have higher serum BAFF levels than seronegative patients (**Figure 5**). This trend did not reach statistical significance ($P=.13$). For seronegative patients with MG, the mean (SD) (SEM) BAFF level was 1.59 (0.46) (0.11) ng/mL with a 95% confidence interval of 1.37 to 1.82 ng/mL. For seropositive patients with MG, the mean (SD) (SEM) BAFF level was 2.13 (1.22) (0.28) ng/mL with a 95% confidence interval of 1.54 to 2.72 ng/mL. Three of the seronegative patients were seropositive for anti-MuSK antibodies. There was no correlation between the serum BAFF level and anti-MuSK antibody titer (data not shown; $r^2=0.0920$; $P=.80$).

COMMENT

In human autoimmune disease, patients with systemic lupus erythematosus, rheumatoid arthritis, Sjögren syndrome, and celiac disease are reported to have increased serum levels of BAFF.²³⁻²⁶ In this study, we demonstrate that serum BAFF levels are increased in patients with MG. We compared patients with autoimmune MG with healthy subjects, patients with MS (an immune-mediated disease with a major role for a T cell-initiated pathogenesis), and patients with ALS (a nonimmune-mediated peripheral nervous system neurodegenerative disease). Patients, regardless of diagnosis, who were receiving immunomodulatory therapy were excluded from the study. Our data show that BAFF levels in the serum of patients with MG were significantly higher than those of all the control subject groups.

Previous studies have shown that the frequency of B cells in the circulation is not increased in patients with autoimmune MG.⁶ In this study, we found no difference in the serum concentrations of immunoglobulins (IgG, IgA, and IgM) between patients with MG and controls without MG (data not shown). Furthermore, there was no correlation between BAFF levels and the concentration of IgG, IgA, or IgM in the serum. Therefore, although BAFF-transgenic animals exhibit hypergammaglobulinemia, the increased BAFF levels in patients with autoimmune MG do not result in increased levels of circulating immunoglobulins.

We found no association between the serum BAFF level and the extent or severity of disease in patients with MG. This was not surprising, as previous studies have shown that there is no correlation between the serum titer of anti-AChR antibodies and disease severity.⁵ There was a trend for BAFF levels to be higher in anti-AChR-seropositive patients, although the difference in BAFF levels between seropositive and seronegative patients did not reach statistical significance. We did not attempt to cor-

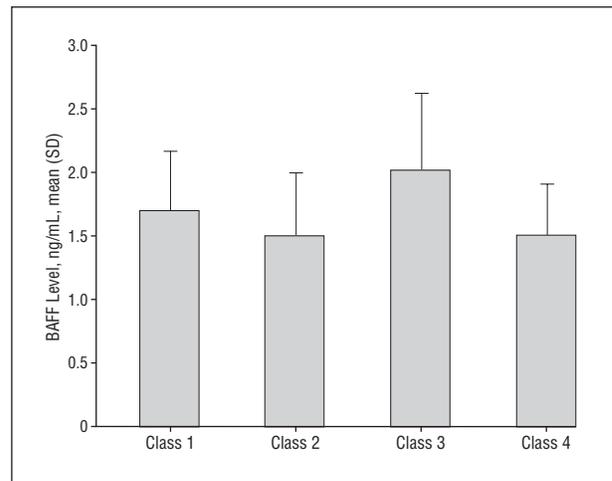


Figure 4. Effect of disease extent and severity on serum B-cell activating factor (BAFF) levels. The extent of disease and severity of symptoms were graded according to the Myasthenia Gravis Foundation of America clinical classification scale.

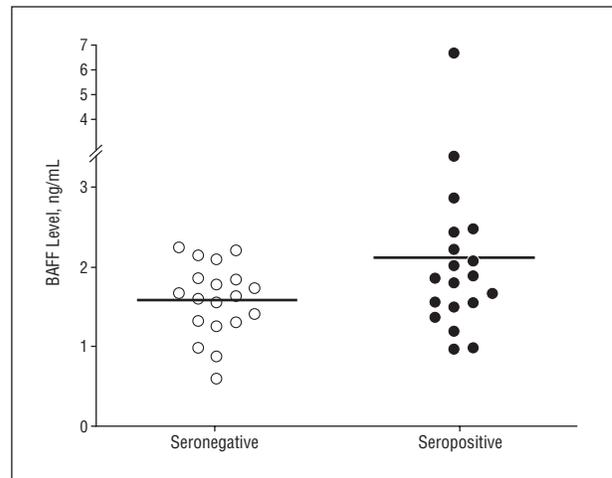


Figure 5. Effect of absence or presence of serum anti-acetylcholine receptor antibodies on serum B-cell activating factor (BAFF) levels. Each point represents an individual patient with myasthenia gravis. The line denotes the mean.

relate the serum BAFF level with the titer of anti-AChR antibodies because the titers were determined by several different commercial laboratories. Based on 3 patients who were seropositive for anti-MuSK antibodies, there was no correlation between the BAFF level and the anti-MuSK antibody titer.

In autoimmune MG, dysregulation of immune signals promotes the survival, activation, and maturation of autoreactive AChR-specific B cells. Data from several laboratories demonstrate enhanced B-cell activation in patients with MG, particularly those with thymic follicular hyperplasia.^{5,6,12,13,27,28} Follicular dendritic cells, and other myeloid cells, control B-cell growth, survival, and differentiation, but their role in the pathogenesis of autoimmune MG has not been thoroughly investigated. The mechanism(s) by which BAFF and its receptors regulate human B-cell function and tolerance is not known. Because autoreactive B cells are poorly competitive for survival, they are likely to have an increased depen-

dence on BAFF for survival.^{29,30} In patients with thymic follicular hyperplasia, it is thought that the germinal center environment is providing signals that promote AChR-specific B-cell survival and activation. Yet these signals are not known. A recent study shows that the myasthenic thymus does express BAFF.³¹ Our data on serum BAFF levels show that BAFF is likely to play a role in the pathogenesis of the disease. Furthermore, the frequency of B cells that express the BAFF receptor appears to be higher in patients with MG.³² We propose that dysregulation of the BAFF/receptor system in MG allows autoreactive B cells to survive and mature.

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