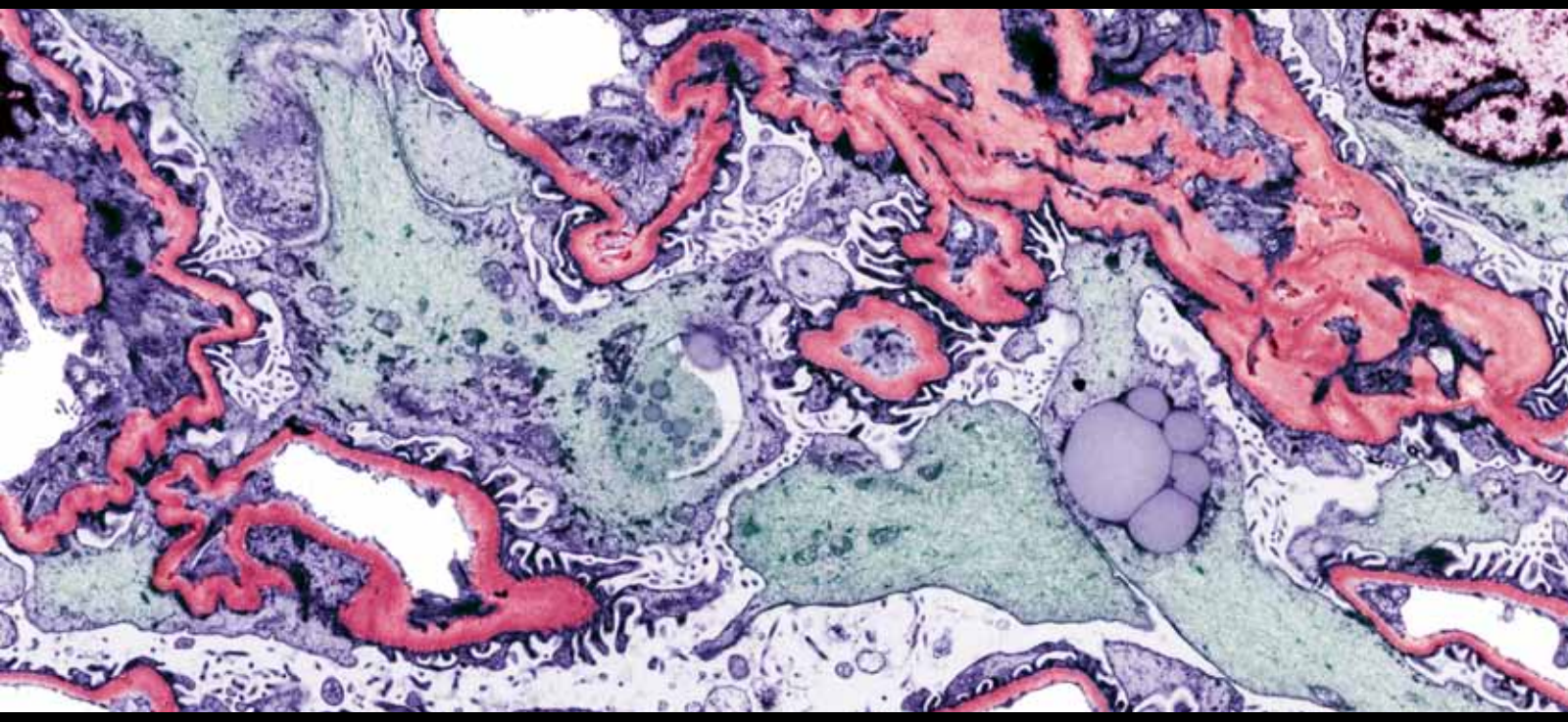
Myasthenia Gravis

Guest Editors: Johan A. Aarli, Nils Erik Gilhus, Robert P. Lisak, Renato Montegazza, and Shigeaki Suzuki



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Editorial Board

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SAGE-Hindawi Access to Research
Autoimmune Diseases
Volume 2011, Article ID 697575, 3 pages
doi:10.4061/2011/697575

Editorial
Myasthenia Gravis

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Received 5 December 2011; Accepted 5 December 2011

Myasthenia gravis (MG) is the best defined autoimmune disturbance with the production of antibodies to the *n*-acetylcholine receptor (AChR) as dominating feature. Some MG patients, who do not have AChR antibodies, have antibodies to muscle-specific kinase (MUSK). In addition, some MG patients, especially those with a tumour of the thymus gland, have antibodies to other muscle proteins such as titin, ryanodine receptor (RyR), and voltage-gated potassium receptor. The role of virus infection of thymic cells and of cytokines and CD4+ cells in the pathogenesis of MG is a potential clue to the disease mechanisms in MG.

The main focus of this special issue is the characterization of the autoimmune responses in MG, the relationship between the immunological disturbances and the clinical picture, the role of the thymus, and the specific problems related to paediatrics and anaestiology. The special issue will summarize the most recent developments in the area.

Considerable data support the involvement of thymus in the aetiology of MG. P. Cavalcante et al. address the question of whether inflammation and Epstein-Barr virus (EBV) infection are frequent pathogenic features of MG thymus. By using Low-Density Array and real-time PCR, the authors show that the MG thymic transcriptome is characterized by upregulation of genes implicated in inflammation and immune response, delineating a peculiar inflammatory and antiviral signature. By using more sensitive molecular and immohistochemical techniques, the authors have implemented and improved their previous finding of an active EBV infection in MG thymus. Hence, a new pathogenic model of virus-mediated autoimmunity in MG is proposed. EBV infection may contribute to MG-specific autoimmune responses occurring within a chronically inflamed MG thymus, through its ability to promote activation, survival, and expansion of autoreactive B cells. The overall results of the study strongly suggest that inflammation and EBV infection are key events in the intrathymic pathogenesis of MG.

In the subsequent paper, S. Ragheb and R. P. Lisak discuss the role of the B-cell activating factor (BAFF) in the pathogenesis of MG. Three independent studies have shown higher BAFF levels in the circulation of MG patients. BAFF is a potent B-cell survival factor, and it plays an essential role in B-cell homeostasis and B-cell function in the periphery.

Both normal and autoreactive B cells are BAFF dependent; however, excess BAFF promotes the survival, growth, and maturation of autoreactive B cells. When over-expressed, BAFF protects B cells from apoptosis, thereby contributing to autoimmunity. BAFF may provide new treatment options for MG patients, particularly those patients with thymic lymphoid follicular hyperplasia.

One-half of patients with cortical thymoma develop MG, while 15% of MG patients have thymomas. F. Romi has evaluated the diagnosis and treatment of MG in patients with thymoma. Titin and RyR antibodies are found in 95% of thymoma MG, and 50% of late-onset MG (MG-onset > 50 years) are associated with severe disease and may predict thymoma MG outcome. Nonlimb symptom profile at MG onset with bulbar-, ocular-, neck-, and respiratory symptoms should raise the suspicion about the presence of thymoma in MG. The presence of titin and RyR antibodies in an MG patient younger than 60 years strongly suggests

a thymoma, while their absence at any age strongly excludes thymoma. Thymoma should be removed surgically. Prethymectomy plasmapheresis/iv-IgG should be considered before thymectomy. The pharmacological treatment does not differ from nonthymoma MG, except for tacrolimus which is an option in difficult thymoma and nonthymoma MG cases with RyR antibodies.

Patients with autoimmune MG should be further classified before initiating therapy, as treatment response varies for ocular versus generalised, early onset versus late onset, and acetylcholine receptor antibody positive versus MuSK antibody positive disease. G. O. Skeie and coworkers discuss available treatment approaches in MG. Most patients need immunosuppression in addition to symptomatic therapy. Prednisolone and azathioprine represent first-choice drugs, whereas several second-choice options are recommended and should be considered. Thymectomy should be undertaken in MG with thymoma and in generalised, early-onset MG. For MG crises and other acute exacerbations, intravenous immunoglobulin (IvIg) and plasma exchange are equally effective and safe treatments. Children and females in childbearing age need special attention regarding potential side effects of immunosuppressive therapy. MG pathogenesis is known in detail, but the immune therapy is still surprisingly unspecific, without a pinpointed attack on the defined disease-inducing antigen-antibody reaction being available.

Treatment of juvenile MG raises special problems which are discussed by J. Jayawant and N. Finnis. Juvenile MG has many clinical features that are distinct from adult MG. Prepubertal children in particular have a higher prevalence of isolated ocular symptoms, lower frequency of acetylcholine receptor antibodies, and a higher probability of achieving remission. Diagnosis in young children can be complicated by the need to differentiate from congenital myasthenic syndromes, which do not have an autoimmune basis. Treatment commonly includes anticholinesterases, corticosteroids with or without steroid-sparing agents, and newer immune modulating agents. Plasma exchange and intravenous immunoglobulin (IVIG) are effective in preparation for surgery and in treatment of myasthenic crisis. Thymectomy increases remission rates. Diagnosis and management of children with juvenile MG should take account of their developmental needs, natural history of the condition, and side-effect profiles of treatment options.

The autoimmune response in MG is heterogeous, but may provide more specific and useful diagnostic information, as discussed by S. Suzuki et al. MG is caused by antibodies that react mainly with the acetylcholine receptor on the postsynaptic site of the neuromuscular junction. A wide range of clinical presentations and associated features allow MG to be classified into subtypes based on autoantibody status. Striational antibodies, which react with epitopes on the muscle proteins titin, ryanodine receptor (RyR), and Kv1.4, are frequently found in MG patients with late onset and thymoma. Anti-titin and anti-RyR antibodies are determined by enzyme-linked immunosorbent assay or immunoblot. More recently, a method for the detection of anti-Kv1.4 autoantibodies has become available, involving 12–15% of all MG patients. The presence of striational antibodies is associated with more severe disease in all MG subgroups. Anti-Kv1.4 antibody is a useful marker for the potential development of lethal autoimmune myocarditis and response to calcineurin inhibitors.

Some disease mechanisms which may occur in MG, such as the presence of matrix metalloproteinase-3 (MMP-3), may also have a specific pathogenetic effect. High MMP-3 levels in a proportion of MG patients have been reported. This is discussed by N. E. Gilhus and coworkers. MMP-3 is capable of degrading a variety of proteins, including agrin, which plays a critical role in neuromuscular signaling by controlling acetylcholine receptor clustering. A pathogenic role of MMP-3 in other neurological disorders has been suggested but not proven. We have examined the levels of MMP-3 in 124 MG patients and compared them to 59 multiple sclerosis (MS) patients, 74 epilepsy patients, 33 acute stroke patients, and 90 healthy controls. 15.3% of the patients in the MG group were MMP-3-positive (defined as higher than cut-off value 48 ng/mL) with very high mean MMP-3 concentration (79.9 ng/mL), whereas the proportion of MMP-3 positive patients in the MS (3.4%), epilepsy (6.7%), stroke (0%), and the control group (4.4%) was significantly lower. Mean MMP-3 concentration in the total MG group (25.5 ng/mL) was significantly higher than in the MS (16.6 ng/mL) and stroke group (11.7 ng/mL), but did not differ significantly from the epilepsy (19.4 ng/mL) and the control group (23.4 ng/mL).

The Lambert-Eaton Myasthenic Syndrome (LEMS) is a rare disease with a well-characterized pathogenesis. It has many clinical and immunological similarities with MG, which are discussed by N. E. Gilhus. In 50% of the patients, LEMS is a paraneoplastic manifestation and is caused by a small cell lung carcinoma (SCLC). Both LEMS patients with SCLC and those without this tumour have in 85% of cases pathogenetic antibodies of very high LEMS specificity against voltage-gated calcium channels (VGCCs) in the cell membrane of the presynaptic motor nerve terminal. Better understanding of LEMS pathogenesis has lead to targeted symptomatic therapy aimed at the neuromuscular junction and to semispecific immunosuppression. For SCLC LEMS, tumour therapy is essential.

MG patients visiting outpatient clinics frequently complain of headache. There have been few reports on the relation between chronic headache and MG. N. Suzuki and co-workers have investigated whether MG symptoms affect the development or worsening of chronic headache. Among the 184 MG patients, tension-type headache was observed in 71 (38.6%) patients and 9 (4.9%) complained of migraine. Twenty-five (13.6%) complained that headache appeared or was exacerbated after the MG onset. The investigation into differences in the clinical characteristics of the MG patients showed that women tended to suffer from MG-associated headache more often than men. Logistic regression analyses revealed that female gender and mild ocular symptoms were independently predictive of headache associated with MG.

MG is characterized by reduced muscle endurance and is often accompanied by respiratory complications. Improvement of respiratory function is therefore an important

objective in MG therapy. S. Hallebach et al. have examined long-term respiratory muscle endurance training in ten patients with mild-to-moderate MG. During the first month, they performed five training sessions per week. For the following 3 months, training frequency was reduced to five sessions per two weeks. Myasthenia score, lung function, and respiratory endurance were determined prior to training, after the first month and after 4 months. Myasthenia score improved from 0.71 ± 0.1 to 0.56 ± 0.1 ($P = 0.007$). Respiratory endurance time increased from 6.1 ± 0.8 to 20.3 ± 3.0 min ($P < 0.001$). The authors conclude that this maintenance program is feasible and is significantly beneficial for MG patients.

MG is of particular interest to anaesthetiologists because of the muscle groups affected, the pharmacology of the neuromuscular junction and interaction of both the disease and treatment with many anaesthetic drugs. Anaesthetists may encounter children with MG to facilitate treatment options or to institute mechanical ventilation in the face of a crisis. To complete the review on long-term treatment in MG, N. Bagshaw and W. Masters have reviewed the documentation pertaining to the pathophysiology and applied pharmacology of the disease and explore the relationship between these and the anaesthetic management.

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SAGE-Hindawi Access to Research
Autoimmune Diseases
Volume 2011, Article ID 939520, 10 pages
doi:10.4061/2011/939520

Review Article

B-Cell-Activating Factor and Autoimmune Myasthenia Gravis

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Received 26 July 2011; Accepted 8 October 2011

Academic Editor: Johan A. Aarli

BAFF is a potent B-cell survival factor, and it plays an essential role in B-cell homeostasis and B-cell function in the periphery. Both normal and autoreactive B cells are BAFF dependent; however, excess BAFF promotes the survival, growth, and maturation of autoreactive B cells. When overexpressed, BAFF protects B cells from apoptosis, thereby contributing to autoimmunity. Three independent studies have shown higher BAFF levels in the circulation of MG patients. BAFF may play an important role in the pathogenesis of MG. BAFF antagonists may well provide new treatment options for MG patients, particularly those patients with thymic lymphoid follicular hyperplasia.

1. Autoimmune Myasthenia Gravis

Myasthenia gravis (MG) is a relatively uncommon disease, with an estimated incidence of 100–200 per million in the United States. It is a B-cell-mediated disease in which the target autoantigen is the acetylcholine receptor (AChR) at the postsynaptic membrane of the neuromuscular junction [1–3]. Approximately 85% of patients with generalized MG have circulating anti-AChR antibodies [4–6]. These antibodies are responsible for the pathology of MG, leading to impaired neuromuscular transmission and subsequent muscle weakness that are due to fewer functional AChRs. Some MG patients who are seronegative for anti-AChR have circulating antibodies to muscle-specific kinase (MuSK) [7, 8]. Although these antibodies do not appear to fix complement, MuSK-specific antibodies are pathogenic nevertheless [9–12]. AChR-specific antibodies are heterogeneous in their specificities and can bind to the various subunits of the AChR [13]; however, most are specific for the α-subunit [14, 15]. Interestingly, the loss of functional AChRs leads to increased expression of the α-subunit. It has been suggested that this enhanced expression helps to drive the autoimmune response [16–18].

Thymic abnormalities are found in patients with autoimmune MG. Approximately 70% of MG patients have thymic follicular hyperplasia, 15% have thymomas, and the remainder have a histologically normal thymus for their age. The myasthenic thymus is implicated in initiating or perpetuating the disease process [19–23]. Hyperplasia is associated with early onset of disease. Lymphoid follicular hyperplasia primarily affects the thymic medulla. Germinal centers in the thymic perivascular space are similar to those found in lymph nodes. The presence of these germinal centers indicates that B-cell activation and proliferation are occurring within the myasthenic thymus. The fine specificities of anti-AChR antibodies produced by thymic B cells are similar to those found in patient sera, demonstrating that the thymic B-cell repertoire is the same as that in the periphery [24, 25]. It is likely that peripheral blood B cells recirculate through the thymic germinal centers, become activated or reactivated, and their immunoglobulin genes undergo somatic hypermutation and affinity maturation. Indeed, patients with thymic follicular hyperplasia tend to have higher serum titers of anti-AChR antibodies [26] and show evidence of enhanced B-cell activation [27–29]. It is thought that the thymic germinal center environment is providing signals that promote autoreactive B-cell survival, activation, and maturation. Yet, these signals are not entirely known. In human MG, the germinal center environment is providing the necessary signals for AChR-specific B-cell survival [29]. Germinal centers within the thymus have strong overexpression of CD23 [30]. CD23 is 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 [31–33]. In the MG thymus with follicular hyperplasia, germinal center B cells do overexpress Bcl-2 [34, 35], an indicator of enhanced survival. The overexpression of CD23 and Bcl-2 provides strong evidence that the thymic germinal center environment is promoting the survival and differentiation of AChR-specific B cells. Clinical improvement following thymectomy may be partially due to the removal of thymic germinal centers [36, 37].

Cell cultures from peripheral blood, lymph node, and thymus of MG patients produce AChR-specific antibodies *in vitro* [38–44]. The frequency of immunoglobulin-secreting cells in the MG thymus is higher than that in blood [45]. Thymic cell cultures also produce antibodies to tetanus toxoid (TT) [44]. Since TT is not normally expressed in the thymus, this is indirect evidence that peripheral blood-derived TT-specific B cells circulate through the thymus. MG patients clearly have AChR-specific B cells in the circulation. AChR-specific B-cells are either absent (clonally deleted) or anergic (nonresponsive) in healthy nonmyasthenics. There is some evidence that AChR-specific B cells are present in nonmyasthenic healthy subjects at a low frequency [46, 47]; yet, they are not pathogenic. Given appropriate signals, these cells might become activated, leading to the production of autoantibodies. The cellular and molecular signals that are necessary for the induction of human MG are not known. We do not fully understand the molecular signals that allow autoreactive B cells to mature and persist. One such signal is B-cell-activating factor (BAFF); its role in promoting the survival and maturation of AChR-specific B cells has not been studied.

2. BAFF and B Cells

B-cell-activating factor (BAFF), also known as B-lymphocyte stimulator (BLyS), is a member of the tumor necrosis factor (TNF) superfamily: TNFSF 13b [48, 49]. Myeloid cells (neutrophils, monocytes, macrophages, and myeloid-derived dendritic cells) are the primary producers of soluble BAFF [50–53]. A membrane-bound form of BAFF is also expressed on the surface of myeloid cells. Full-length BAFF is a 285 aa type II transmembrane protein. Within the extracellular domain, BAFF contains a furin consensus cleavage site. A furin family protease cleaves the membrane form of BAFF to generate soluble BAFF (sBAFF), which contains the extracellular 152 amino acids (aa 134–285). sBAFF is a homotrimer, and it interacts with its receptors in its trimeric form [54–56].

BAFF transgenic animals exhibit hypergammaglobulinemia, lymphoproliferation, B-cell hyperplasia, splenomegaly, and develop autoimmune disease with manifestations that are similar to those in systemic lupus erythematosus [57, 58]. As they age, BAFF transgenic mice also have a propensity to develop B-cell lymphomas [57]. In BAFF-deficient animals, there is a marked reduction in the B-cell compartment with depletion of marginal zone and follicular B cells. Defects in peripheral B-cell maturation are accompanied by hypogammaglobulinemia [59, 60]. Therefore, BAFF plays an essential role in B-cell homeostasis. It is a potent survival factor for B cells, and it plays an essential role in the maintenance and maturation of peripheral B cells [61–65]. BAFF regulates follicular B-cell numbers. Long-lived plasma cells are also dependent on BAFF for their survival [66, 67]. BAFF differentially regulates Bcl-2 family members in a manner consistent with prosurvival and attenuation of apoptosis. These antiapoptotic effects are mediated by upregulation of Bcl-2 and inhibition of Bim [68–71]. When overexpressed, BAFF protects B cells from apoptosis, thereby contributing to autoimmunity and malignancy.

Because BAFF is a crucial and potent factor for the survival and growth of B cells, both normal and autoreactive B cells compete for available BAFF. BAFF levels appear to regulate the survival threshold for B cells. Autoreactive B cells are poorly competitive for survival and they appear to be more dependent on BAFF for their survival [72–75]. An environment of excess BAFF promotes the survival and maturation of autoreactive B cells, thereby breaking immune self-tolerance. Therefore, BAFF levels can alter the selection of autoreactive B cells [76].

BAFF costimulates B-cell activation/proliferation via the B-cell receptor (BCR) or via CD40, and it mediates the survival of these activated B cells [48, 49]. Furthermore, coupling of BCR signaling and BAFF-R expression has been demonstrated [77, 78]. This leads to the intriguing concept that follicular B-cell selection, activation, and survival are linked. Therefore, the type and strength of signals that are received via the BCR, CD40, and receptors for BAFF affect and control the fate of B cells, whether they are normal or autoreactive [79–81]. Interestingly, a recent study demonstrates that interleukin-17 (IL-17) may also synergize with BAFF to enhance the survival and maturation of human B cells [82]. The important role of BAFF in the homeostasis and function of peripheral B cells is predominantly dependent on sBAFF. The role of membrane-bound BAFF is not clear. It may serve an accessory function, or it may be involved in bidirectional communication through reverse signaling mechanisms, as has been shown for other members of the TNF superfamily [83, 84].

Three independent studies have shown that serum BAFF levels in patients with MG are significantly higher than those in nonmyasthenic control subjects [85–87]. However, there is no association between the serum BAFF level and the extent or severity of disease. This is not surprising as previous studies have shown that there is no correlation between the serum titer of anti-AChR antibodies and disease severity [26, 88]. There is a trend for BAFF levels to be higher in patients who are seropositive for AChR-specific antibodies [85, 86]. In the myasthenic thymus with lymphoid follicular hyperplasia, macrophages express BAFF [89].

3. CXCL13, BAFF, and Notch

The chemokine CXCL13, also known as B-lymphocyte chemoattractant (BLC), guides B cells to follicles in secondary lymphoid organs [90, 91]. It has an important role in the formation and maintenance of B-cell follicles. Both CXCL13 and BAFF are found in inflammatory sites where there is lymphoid neogenesis [92]. A recent study demonstrates a synergy between BAFF and CXCL13 [93]. This has profound

implications for the formation of ectopic follicles and for B-cell homeostasis. Ectopic B-cell follicles are found in the MG thymus with lymphoid follicular hyperplasia. Both BAFF and CXCL13 are expressed in the MG thymus, and CXCL13 overexpression is found in the thymus with follicular hyperplasia [89, 94, 95]. Thymic epithelial cells have also been shown to produce CXCL13 *in vitro* [94]. This suggests that molecules that are essential for B-cell recruitment, survival, and maturation may be working in concert to drive the B-cell response in the MG thymus with hyperplasia.

The Notch signaling pathway regulates cell fate during lymphocyte development and differentiation. Notch signaling affects the activation and maturation of B cells into antibody-secreting plasma cells. Recent studies show that the Notch signaling pathway may cooperate with the BAFF pathway to protect B cells from apoptosis as they mature in the germinal center [96–98].

4. BAFF Production

Within the immune system, the primary source of sBAFF is the myeloid lineage. The signals that modulate BAFF expression are not fully understood. Resting monocytes constitutively express a low level of membrane-bound BAFF; this expression is upregulated by interferon-γ (IFN-γ), IFN-α, and interleukin-10 (IL-10). These cytokines augment BAFF expression in monocytes, macrophages, and dendritic cells. Bacterial components such as lipopolysaccharide (LPS) also upregulate BAFF expression [50, 51, 99]. Therefore, signals from both the innate and adaptive immune response can modulate BAFF production by myeloid cells. *In vivo* therapy in human patients has shown that IFN-α and IFN-β upregulate BAFF expression in patients with melanoma and multiple sclerosis, respectively [100, 101]. Interestingly, IFN-γ and the type I interferons (IFN-α and IFN-β), which are known to have opposite effects on myeloid cell function, have similar effects on BAFF expression.

The role that cytokines may play in regulating the myeloid/B-cell interaction in MG has been largely ignored. Myeloid cells play an important role in the development and regulation of the T-cell-dependent anti-AChR antibody response [102–104]. Furthermore, in one study that utilized AChR-pulsed dendritic cells to tolerize B cells, tolerance was associated with reduced BAFF expression [102]. Data from experimental autoimmune myasthenia gravis (EAMG), the animal model for human MG, show that IFN-γ and IL-12 are necessary for disease induction [105–107]. These results highlight the importance of T_H1-type cytokines in EAMG. However, cytokines made by T_H2 and T_{FH} cells are also important for B-cell growth and differentiation [108–110]. There are no studies that elucidate the influence of these various cytokines on BAFF expression in MG, or their influence on the survival and maturation of AChR-specific B cells in the germinal center where B cells are in close contact with BAFF-expressing dendritic cells.

Suppressor of cytokine signaling-1 (SOCS-1) plays a critical role in the negative regulation of IFN-γ signaling. In SOCS-1-deficient mice, IFN-γ-stimulated dendritic cells are hyperresponsive. SOCS-1 deficiency results in higher BAFF production by dendritic cells and leads to systemic autoimmune-like disease in mice [111].

The autoimmune regulator (AIRE) gene is primarily expressed in the thymus in medullary cells and in the periphery on antigen-presenting cells [112, 113]. AIRE plays a role in both the central and peripheral immune self-tolerance mechanisms for T cells. AIRE deficiency leads to higher numbers of antigen-presenting cells [114]. AIRE-deficient mice also have higher serum levels of BAFF than wild-type mice, and this is associated with increased expression of membrane-bound BAFF on the surface of dendritic cells. Aging $AIRE^{-/-}$ mice have a similar phenotype to BAFF transgenic mice [115, 116]. As shown recently, $AIRE^{-/-}$ mice are also susceptible to the induction of EAMG [117], and this appears to be age related. Susceptibility is associated with lower expression of AChR in the thymus and, presumably, a failure to eliminate AChR-reactive T cells; that is, a failure of central tolerance.

5. Functional BAFF Receptors

Three functional receptors for BAFF have been identified. They are BCMA (B-cell maturation antigen, TNFRSF 17, CD269), TACI (transmembrane activator and cyclophilin ligand interactor, TNFRSF 13b, CD267), and BAFF-R (BAFF receptor, BR3, TNFRSF 13c, CD268). Both BCMA and TACI can also bind to the BAFF-related molecule APRIL (a proliferation-inducing ligand). The BAFF-R binds BAFF exclusively. Cell-surface expression of the receptors is primarily restricted to B cells [118], although activated and memory T cells are reported to express TACI and BAFF-R [119, 120].

BAFF-R-deficient mice have a marked reduction in the B-cell compartment and lack both marginal zone and follicular B cells [121, 122]. B-lymphopenic A/WySnJ mice have a mutant signaling-deficient form of the BAFF-R. They have a similar phenotype to that of BAFF-deficient mice. They exhibit a loss of peripheral B cells and decreased levels of circulating immunoglobulins [123–126]. Data on receptor expression in humans and mice show that the BAFF-R is the predominant receptor on circulating B cells [120]. In B cells, the prosurvival signals of BAFF are mediated by the BAFF-R.

TACI-deficient mice have a higher number of hyperresponsive B cells in the periphery, they develop autoimmune disease, they exhibit lymphoproliferation, and they develop lymphoma [127–129]. The interaction of BAFF with TACI appears to deliver inhibitory signals such that signaling through TACI decreases the size of the B-cell pool. For humans, the role of TACI is more ambiguous. On the one hand, TACI expression is upregulated after B-cell stimulation, and TACI is found primarily on marginal zone B cells and on $CD27^+$ memory B cells [130]. TACI appears to be a negative regulator/terminator of the B-cell response. On the other hand, in humans, TACI mutations are associated with immunoglobulin deficiency [131–133]; TACI mutations are associated with familial combined variable immunodeficiency (CVID) and with selective IgA deficiency. This would appear to suggest that TACI plays a positive role in terminal B-cell differentiation.

BCMA-deficient mice lack an obvious phenotype [60, 134]. BCMA expression is restricted to the end stages of B-cell differentiation. BCMA expression is upregulated in germinal center cells and in plasmablasts, and it serves an essential survival and maturation function as B cells differentiate into plasma cells [66, 67, 135].

The signals that regulate the cell-surface expression of BAFF-R, TACI, and BCMA are not known. Mature human B cells, at all stages of differentiation, express one (or more) of the BAFF-binding receptors and are BAFF dependent [136, 137]. The BAFF-R is the main receptor that mediates BAFF signals in naïve B-cells. Following activation, and during differentiation, BAFF-R expression is down-modulated while TACI expression is upregulated. BCMA expression is upregulated at the terminal stages of B-cell differentiation and appears to be restricted to antibody-producing cells. A recent study demonstrates that IL-17 may synergize with BAFF, to enhance the survival and maturation of human B cells [82]. This study demonstrates the potential involvement of IL-17 in B-cell biology, and highlights the potential for other cytokine signals to enhance or antagonize BAFF-mediated signaling. BAFF levels, and the interaction of BAFF with its three receptors, regulate peripheral B-cell homeostasis and function and regulate the immune self-tolerance of B cells [118, 138–140]. Dysregulation of this signaling alters peripheral immune self-tolerance and leads to the development of autoimmune disease.

In autoimmune MG, in the myasthenic thymus with lymphoid follicular hyperplasia, germinal center B cells express the BAFF-R in close proximity to BAFF-expressing macrophages [89]. In the circulation, one study shows that the frequency of B cells that express the BAFF-R is higher in patients with MG [141]. However, in another study, there is no difference between MG patients and healthy controls in the percentage of B cells that express BAFF-R, TACI, or BCMA [142].

6. Signaling via BAFF-R

The BAFF-R is expressed on all peripheral B cells, and it binds BAFF exclusively. Signaling downstream of the BAFF-R leads to B-cell survival through activation of NF-κB [138]. Activation of the NF-κB transcription factor normally proceeds either through the canonical pathway which is dependent on NEMO (NF-κB essential modulator), or through the alternate pathway which is NEMO independent [143]. Both pathways have been shown to be utilized in BAFF-R signaling [144–147]. However, engagement of BAFF-R leads to weak activation of the classical NF-κB1 pathway and potent activation of the alternate NF-κB2 pathway. Recent studies show that the BAFF-R has a single TNF receptor-associated factor- (TRAF-) binding site that is specific for TRAF3. In the absence of BAFF ligand, TRAF3 binds to the NF-κB-inducing kinase (NIK) and targets NIK for proteolysis, thereby inhibiting the alternative NF-κB2 pathway. In the presence of BAFF, engagement of the BAFF-R leads to recruitment and binding of TRAF3, thereby terminating TRAF3-mediated degradation of NIK, subsequently increasing NIK levels and activating the alternative NF-κB2 pathway [148–153]. NF-κB2 is known to upregulate various prosurvival molecules, including Bcl-2.

7. BAFF and T Cells

In vitro, BAFF costimulates human T-cell activation, which has been shown to be mediated by the BAFF-R [120, 154]. *In vivo*, BAFF transgenic animals exhibit enhanced cutaneous delayed-type hypersensitivity (DTH) responses, which are considered to be classical T_H-1-mediated immune responses [155]. BAFF may also play a role in T_H-17-mediated immune responses. In mouse models of collagen-induced arthritis, both T and B cells are necessary for disease induction and progression. When Lam et al. use shRNA to silence the BAFF gene, intra-articular injection of shRNA suppresses the development of disease by inhibiting the generation of plasma cells and T_H-17 cells [156]. Furthermore, in a comparison of wild-type and IL-$17^{-/-}$ mice, recombinant BAFF exacerbates disease in the wild-type animals, but not in the IL-$17^{-/-}$ animals. These studies highlight the previously unrecognized role of BAFF in T-cell-mediated immune responses.

8. BAFF Pathway-Targeted Therapy

BAFF levels, and the extent of signaling through BAFF-R, TACI, or BCMA, regulate B-cell function and B-cell tolerance. BAFF plays a role in a diverse array of human B-cell diseases that include autoimmunity, malignancy, and immunodeficiency [157]. Four different antagonists of the BAFF pathway have been developed for clinical use thus far. The first is an anti-BAFF neutralizing antibody (LymphoStat-B, Belimumab) [158]. The second is anti-BAFF-R [159], which blocks the interaction of BAFF with the BAFF-R and also kills BAFF-R expressing cells. The third is the decoy receptor BR3-Fc, which is a humanized fusion protein of the extracellular domain of human BAFF-R with the Fc portion of human IgG1 [160]. Because BAFF, but not APRIL, binds to the BAFF-R, these three antagonists offer a method of selective BAFF blockade. The fourth antagonist is TACI-Ig (Atacicept), a fusion protein of the extracellular domain of human TACI with the Fc portion of human IgG1 [161]. TACI-Ig offers a nonselective method of BAFF blockade, because it would interfere with both BAFF and APRIL signaling.

The efficacy of Belimumab has been examined in systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) patients [162, 163]. Belimumab is now FDA approved for SLE. Two phase III trials have met their primary endpoints. They show that Belimumab is clinically effective by reducing flare rates and reducing disease activity in patients with SLE. A phase II trial in RA has shown that, although Belimumab decreases the levels of rheumatoid factor, its clinical efficacy is mild compared to the TNF antagonist drugs that are currently available. Thus, Belimumab is no longer tested in RA. Clinical trials of Atacicept are ongoing in patients with SLE and RA [164, 165]. Because BAFF blockade deprives B cells from an obligate survival factor, the effect of BAFF blockade appears to be mediated mainly via B-cell depletion. Mature B cells, at all stages of differentiation

(from naïve to plasmablast), are dependent on BAFF and are potentially susceptible to BAFF blockade. BAFF itself may be therapeutic in primary immunodeficiencies that affect the B-cell compartment [166], and BAFF may be used to enhance the efficacy of vaccines aimed at boosting the humoral immune response [167, 168].

Some MG therapies may also affect BAFF levels. Glucocorticoid effects on B cells may involve pathways that decrease BAFF levels [169], and intravenous immunoglobulin preparations contain some antibodies with both BAFF and APRIL specificities [170]. BAFF may play an important role in the pathogenesis of MG. Because BAFF levels regulate B-cell tolerance, BAFF antagonists may benefit patients with MG by increasing the apoptosis of autoreactive B cells. BAFF antagonists may provide new treatment options for MG patients, particularly for early-onset patients with thymic hyperplasia.

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SAGE-Hindawi Access to Research
Autoimmune Diseases
Volume 2011, Article ID 404101, 7 pages
doi:10.4061/2011/404101

Review Article

Juvenile Myasthenia Gravis: A Paediatric Perspective

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Received 1 June 2011; Accepted 10 August 2011

Academic Editor: Johan A. Aarli

Myasthenia gravis (MG) is an autoimmune disease in which antibodies are directed against the postsynaptic membrane of the neuromuscular junction, resulting in muscle weakness and fatigability. Juvenile myasthenia gravis (JMG) is a rare condition of childhood and has many clinical features that are distinct from adult MG. Prepubertal children in particular have a higher prevalence of isolated ocular symptoms, lower frequency of acetylcholine receptor antibodies, and a higher probability of achieving remission. Diagnosis in young children can be complicated by the need to differentiate from congenital myasthenic syndromes, which do not have an autoimmune basis. Treatment commonly includes anticholinesterases, corticosteroids with or without steroid-sparing agents, and newer immune modulating agents. Plasma exchange and intravenous immunoglobulin (IVIG) are effective in preparation for surgery and in treatment of myasthenic crisis. Thymectomy increases remission rates. Diagnosis and management of children with JMG should take account of their developmental needs, natural history of the condition, and side-effect profiles of treatment options.

1. Introduction

Myasthenia gravis (MG) is an autoimmune disease in which antibodies are directed at the postsynaptic membrane of the neuromuscular junction, leading to varying degrees of muscle weakness and fatigability. Where MG presents before 19 years of age, it is termed juvenile myasthenia gravis (JMG). Although JMG shares many features with the more common adult MG, there are many important differences.

In this paper we discuss the pathogenesis, epidemiology, presentation, treatment, and outcome of JMG and highlight some of the clinical features and challenges particular to paediatric patients.

2. Pathogenesis

In the majority of cases MG is caused by antibodies to the nicotinic acetylcholine receptor (AChR). Antibodies to the AChR are found in over 80% adults with generalised disease but only in 55% of adults with weakness confined to the oculomotor muscles. Patients with AChR antibodies are often referred to as seropositive. AChR antibodies are probably less frequent in prepubertal patients than in adolescent and adult patients [1, 2] (see Table 1). Antibodies to muscle-specific kinase (MuSK) and to Leucine rich protein 4 (LRP4) have been reported in some seronegative patients.

Childhood myasthenias encompass JMG, which is the subject of this paper; congenital myasthenic syndromes, a heterogeneous group of genetically inherited disorders of the neuromuscular junction [3]; transient neonatal myasthenia, which results from placental transfer of maternal AChR (or very occasionally MuSK antibodies) to infants of mothers with autoimmune MG [4].

3. Epidemiology and Clinical Features

JMG is a rare disorder of childhood, but its incidence and prevalence vary geographically. Precise data on incidence and prevalence are not known. Paediatric presentation of MG is more common in Oriental than in Caucasian populations [5]. Up to 50% of all cases of MG in Chinese populations present in childhood, mostly with ocular features, with a peak age at presentation of 5–10 years [6]. Caucasian patients, in contrast, are more likely to present in adulthood [7, 8], with prepubertal onset in less than 10% cases [2, 9].

Table 1: Comparisons of prepubertal and postpubertal features of JMG.

	Prepubertal	Pubertal	Postpubertal/ adult
Male : female ratio	M = F	F > M 4.5 : 1	F > M 4.5 : 1
Patients with AChR antibodies detected in generalised disease	50–71% [1, 2]	68–92% [1, 2]	80–90%
Ocular presentation			
Caucasian	40% [7]	9–16% [12]	28% [6]
Chinese	75% [6]		
Progression of OMG to generalised MG	8–15% [17, 18]	23–43% [16, 19]	79% [8]
Remission (spontaneous or with treatment)	42–60% [1, 2, 20]	26% [2]	38% [9]

The most frequent clinical presentation of JMG is with ptosis, which is often associated with other ocular symptoms namely unilateral or asymmetric ophthalmoplegia, strabismus, and lid twitch, which may only be elicited after sustained upgaze [10]. These symptoms cause particular problems in children as, if severe, they may cause persistent amblyopia [11]. Most children also develop generalised muscle weakness, which presents as painless fatigability of the bulbar and limb musculature, with resultant dysphonia, dysphagia, and proximal limb weakness. Weakness is often fluctuating and usually becomes more pronounced through the day and improves with rest. Children are at risk of choking or aspiration and are at increased risk of chest infection. Occasionally, impairment of the respiratory muscles necessitates ventilatory support. This is known as "myasthenic crisis".

Prepubertal children presenting with JMG have some interesting and distinct clinical features compared with those who present around or after puberty [1, 2]. Prepubertal JMG is more likely to manifest as ocular myasthenia [12]. There is an equal male: female ratio [13], in contrast to the female predominance that is seen in peri-/postpubertal children, and a better prognosis, with a higher rate of spontaneous remission in prepubertal presenters [1, 12]. Peri- or postpubertal patients presenting with JMG share more similarities with adult-onset MG (see Table 1).

Ocular myasthenia gravis (OMG) is, by definition, MG restricted to the oculomotor muscles for 2 years without becoming generalised [14]. In adult populations up to 80% patients with OMG at presentation will progress to generalised disease [8, 9, 15]. Case series in children (using a variety of treatment protocols and follow-up intervals) have reported lower rates of generalisation than adults [16]. Progression may be even less frequent in prepubertal children [17, 18].

3.1. Transient Neonatal Myasthenia. This results from transfer of maternal AChR antibodies across the placenta leading to defects of neuromuscular transmission in the neonate [4]. Not all mothers have detectable AChR antibodies and a few are asymptomatic at the time. Usually the affected baby is normal at birth, subsequently developing signs such as hypotonia, weak cry, poor suck, reduced movements, ptosis and facial weakness, and occasional respiratory insufficiency requiring mechanical ventilation. Short-term treatment with anticholinesterases is usually sufficient.

4. Diagnosis of JMG

JMG is primarily a clinical diagnosis with classical patterns of fluctuating weakness and fatigability as described above. A number of diagnostic tools are available to aid with diagnosis. In very young children it is particularly important to distinguish between autoimmune myasthenia and congenital myasthenic syndromes (CMS) as the treatment options, prognosis, and genetic implications are very different (see Table 2).

CMS usually present in the first years of childhood with variable disability. There is often a positive family history, and diagnosis is aided primarily by electrophysiology and DNA analysis and occasionally by muscle biopsy [21]. With the exception of the autosomal dominantly inherited slow-channel syndrome, the CMS are inherited by autosomal recessive mutations, which result in loss of function at the neuromuscular junction [10].

4.1. Serology. Detection of antibodies to the AChR supports the diagnosis of JMG. In young children where AChR antibodies are negative this can lead to difficulty in differentiating from CMS. Some of these children who are negative for AChR antibodies will have "low affinity" antibodies to the AChR which were not detectable using the standard assays [22]. Some children will, in fact, turn out to have CMS.

A variable percentage (0–49%) of MG patients without AChR antibodies are found to have antibodies against another neuromuscular junction protein, the muscle-specific kinase (MuSK) [23]. MuSK positive MG is rare in children, and these children represent a distinct subgroup of JMG, with a marked female predominance. MuSK antibodies appear to be associated with more severe disease with prominent facial and bulbar weakness and frequent respiratory crises [24].

Patients without antibodies to AChR or MuSK are described as having seronegative myasthenia gravis (SNMG). SNMG patients are phenotypically more similar to AChR seropositive patients than MuSK positive patients, both in clinical presentation and in response to treatment. "Low affinity" antibodies to clustered AChRs can be found in 60% of previously defined SNMG patients. These antibodies are found in all age groups [22].

Seroconversion has been described in a small number of cases of children who have developed MuSK antibodies after thymectomy for AChR seropositive MG [25]. This has not been described in adults.

Other potential antigens at the neuromuscular junction have been identified in adults with later-onset MG, but

Table 2: Differential diagnosis of JMG.

Congenital myasthenic syndromes	Usually presents in infancy but can present later
Mitochondrial cytopathies	Children frequently have additional neurological impairments or epilepsy
Myopathies	Including congenital myopathies and muscular dystrophies
Neurotoxins	For example, botulism, venoms
Guillain-Barré syndrome	
Acute disseminated encephalomyelitis	
Multiple sclerosis	
Brainstem tumour	
Hypothyroidism	

the relevance to the childhood population has not been established [26].

4.2. Pharmacological Investigation. The Tensilon test involves intravenous infusion of edrophonium, a fast-acting, short-duration cholinesterase inhibitor. This prevents the breakdown of acetylcholine, thereby increasing the concentration of the neurotransmitter at the neuromuscular junction. The patient is observed, and ideally a video recorded, looking for a transient improvement in previously documented weakness, for example, ptosis, dysphonia. This test is not without risk and should only be performed by staff experienced in paediatric resuscitation, due to the cholinergic effects of edrophonium, which can result in bradycardia, nausea, and excess salivation.

4.3. Electrophysiology. Electrophysiological testing can be invaluable in investigation of suspected JMG. Repetitive nerve stimulation in JMG will show a decrement in the compound motor action potential of >10% by the 4th or 5th stimulation.

Single fibre EMG (SFEMG) is especially useful in diagnosis of seronegative MG and congenital myasthenic syndromes. It can be technically more difficult in children due to discomfort of the procedure and the level of cooperation required. It can be done under local or even general anaesthetic. Sensitivity for a neurotransmission disorder is 97% [27]. A normal result therefore makes a diagnosis of myasthenia very unlikely [28].

4.4. Imaging. Although thymoma in children is rare, the thymus must be imaged (usually by CT) once JMG has been diagnosed. AChR seropositive MG is frequently associated with changes in the thymus, with histological changes and in vitro effects suggesting that the thymus plays a pathogenic role [47]. Thymus hyperplasia is the commonest abnormality of the thymus in JMG [20]. Thymoma is particularly rare in prepubertal children [12].

Thymus changes are not a common feature of MuSK positive disease, and thymoma has not been reported in MuSK-positive children.

Thymus abnormalities in SNMG patients have been found to be histologically very similar to the thymus hyperplasia seen in AChR seropositive MG [47].

5. Management

Management of children with JMG should be delivered by a multidisciplinary team comprising a paediatrician with support from a paediatric neurologist, physiotherapist, occupational therapist, psychologist, speech therapist and dietician. Other members of the team may also need to be involved, depending on associated comorbidities such as bulbar weakness leading to difficulty with oral feeding, or respiratory insufficiency requiring noninvasive ventilatory support, which should be managed by a respiratory paediatrician.

Treatment of JMG has largely been extrapolated from adult studies and experience with adult patients. There are few studies looking specifically at interventions in children, particularly prepubertal children (see Table 3). Some case series include paediatric patients but they are often not subdivided into prepubertal and postpubertal age groups for analysis. Given the evidence that prepubertal JMG may behave quite differently in terms of disease severity and progression, this may impact on necessity for treatment and treatment response. Side-effect profiles and considerations are not always directly comparable between adult and paediatric populations.

5.1. Acetylcholinesterase Inhibitors. Acetylcholinesterase inhibitors are first-line treatment in JMG and provide symptomatic relief. In mild cases and in some cases of ocular MG, acetylcholinesterase therapy may be sufficient. Pyridostigmine is a long-acting cholinesterase inhibitor that is commonly used. Dosing is usually 4–6 times per day and is tailored to effects. Cautious use in MuSK-positive children is advised due to risk of acetylcholine hypersensitivity [48].

5.2. Thymectomy. Because of the presumed role of the thymus in the pathogenesis of MG, thymectomy is a recognised aspect of management. Thymectomy may remove thymic germinal centres and disrupt antibody diversification [47]. A systematic review of the literature concluded that thymectomy increases the probability of remission or improvement of symptoms in AChR seropositive, nonthymomatous, autoimmune MG [49]. This paper included only one paediatric study [31]. More recent reviews of children including prepubertal patients, also suggested increased remission rates after thymectomy [30, 32]. Caution needs to be taken in early childhood due to subsequent immunosuppression and the high rates of spontaneous remission in prepubertal presenters.

Current evidence suggests that thymectomy should not be recommended in MuSK-positive disease as it is unclear whether it confers any benefit [29, 50, 51].

Table 3: Treatment options in JMG.

Treatment	Evidence of efficacy in generalised JMG	Reference
Acetylcholinesterase inhibitors	First line therapy.May be sufficient in ocular JMG or mild generalised JMG	Skeie et al., 2010 [29]
Thymectomy	Recommended to increase remission rates in postpubertal, seropositive children. Not recommended in prepubertal children	Hennessey et al., 2011 [30] Rodriguez et al., 1983 [31] Tracy et al., 2009 [32] Lindner et al., 1997 [20]
Steroids	Often used in combination with steroid sparing agents. Significant side-effect profile if used long-term at high dose	Schneider-Gold et al., 2005 Cochrane review: one JMG study, others adult or unspecified age ranges [33] Zhang et al., 1998 [6]
Steroid sparing agents		
Azathioprine	Usually used in combination with corticosteroids. Occasionally used alone.	Mertens et al., 1981 [34] includes some children but no subgroup analysis Gold et al., 2008 [35] Palace et al., 1998 [36] adult study
Cyclosporin A	As monotherapy or in conjunction with corticosteroids	Tindall et al., 1987 [37] adult study Hart et al., 2009 (Cochrane) [38] series include some children
Cyclophosphamide		De Feo et al., 2002 [39] adult population Hart et al., 2009 [38] (Cochrane) series include some children
Tacrolimus		Furukawa et al., 2008 [40] Ponseti et al., 2008 [41] both include post pubertal children Ishigaki et al., 2009 [42] prepubertal female
MMF		Hehir et al., 2010 [43] includes some peri-/postpubertal children
Rituximab		Wylam et al., 2003 [44] pediatric case
IVIG/Plasma exchange		Selcen et al., 2000 [45] Gajados et al., 2008, Cochrane database [46]

Thymectomy in pure OMG remains controversial. Whereas OMG is not life threatening, patients may be dependent on long-term immunosuppressant medications, including corticosteroids, with the resultant side effects which can be substantial in children. Persistent amblyopia can result in children as the visual system is maturing. As discussed above, we know that a proportion of children will progress to generalised disease. Thymectomy is not proven to reduce risk of progression of OMG to generalised JMG [52] and is not routinely indicated in pure OMG in children but has been performed in refractory cases.

A variety of surgical methods for thymectomy have been described: full or partial sternotomy, thorascopic, or transcervical approaches. Evidence suggests that symptom resolution is equivalent regardless of surgical approach [53, 54]. After thymectomy there is increased risk of antimuscarinic side effects of cholinesterase inhibitors, and they should therefore be used under close supervision in the postoperative period.

5.3. Immunosuppressive Therapies. Frequently some form of immunosuppression or immunomodulation is required to improve symptoms of JMG. Corticosteroids are often effective and are the mainstay of therapy but can worsen symptoms in the first few weeks of use, particularly if started at high doses [33]. Because of the numerous adverse effects associated with long-term high-dose steroids, steroids are often used in combination with a steroid-sparing immunosuppressant, for example, azathioprine. Children are at particular risk of steroid side effects, including growth failure, susceptibility to severe infection, and delay in receiving live vaccinations.

Azathioprine is a purine analogue that suppresses B and T cell proliferation. It has been found to be effective when used alone [34], but is most commonly used in combination with prednisolone as a steroid-sparing agent. Beneficial effects may take months to be seen [35] but eventually result in weaning of steroid doses [36].

Some studies have suggested that azathioprine or corticosteroids may reduce the likelihood of progression of OMG to the generalised form of disease [55, 56]. Although these studies included some children in their case series, these were not specifically paediatric studies, and given the lower rates of progression in prepubertal children anyway, these findings are of uncertain relevance in paediatric practice.

Patients unresponsive or intolerant to azathioprine should be considered for other immunosuppressive agents, which could include cyclosporin A (which has a faster

time to symptomatic benefit than azathioprine [37]) or cyclophosphamide [39]. A Cochrane review suggests that cyclosporin either as monotherapy or with corticosteroids, or cyclophosphamide in conjunction with corticosteroids, improves symptoms of MG within 1 year [38].

Mycophenolate mofetil (MMF) blocks purine synthesis by selectively inhibiting proliferation of activated T and B lymphocytes [57]. A recent retrospective study of AChR seropositive patients, which included children (age range 11–87 y), concluded some benefit of MMF when used either as monotherapy or in conjunction with prednisolone. Maximum effects may not be seen until after one year of treatment [43].

Tacrolimus inhibits interleukin 2. Efficacy studies have been carried out in adults and postpubertal children and have shown early and sustained improvement of symptoms with tacrolimus, allowing dose reduction of prednisolone and in many cases its complete withdrawal. These steroid sparing effects were seen within 6 months [40, 41]. A case has also been reported of tacrolimus being successfully used as adjunctive therapy in refractory pure ocular myasthenia in a 3-year-old girl [42].

Rituximab is a chimeric IgG monoclonal antibody that depletes B cells and has been used in refractory JMG [44].

5.4. Plasma Exchange/Intravenous Immunoglobulin (IVIG). Improvement in symptoms after plasma exchange or administration of IVIG is usually temporary, 4–10 weeks. Their use is therefore largely reserved to optimise condition for surgery before thymectomy and in management of myasthenic crisis [45, 46, 58]. A single randomised controlled trial showed no evidence for superior benefit of plasma exchange over IVIG in treatment of myasthenic crisis [59].

Efficacy studies are not available for prepubertal children.

Allogeneic hematopoietic cell transplantation has been reported as successful in treating a 17-year-old male with refractory JMG that had been diagnosed aged 11 months [60].

6. Outcome

Outcomes in JMG have improved significantly over the last decade, with better recognition, diagnosis, and more effective therapies, and long-term prognosis is good [61]. Children with JMG exhibit higher rates of remission than adults. This includes spontaneous remission and remission following a period of drug therapy. Prepubertal children have the highest rates of spontaneous remission. Remission rates also appear to be influenced by ethnic origin [1].

7. Summary

JMG is a rare, autoimmune condition of childhood that shares many characteristics of clinical presentation and management strategies with the adult form of the disease. However, as described in this paper, there are many important aspects that are specific to the paediatric population, in particular the distinct clinical features of the prepubertal presentations, differences in rates of AChR seropositivity, diagnostic challenges including differentiation from CMS, and response to therapy. Further studies looking at efficacy of therapies in pre- and postpubertal children are needed to better understand and support this distinct group of patients.

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SAGE-Hindawi Access to Research
Autoimmune Diseases
Volume 2011, Article ID 847393, 6 pages
doi:10.4061/2011/847393

Review Article

Myasthenia Gravis: A Review of Available Treatment Approaches

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Received 14 April 2011; Accepted 11 August 2011

Academic Editor: Renato Mantegazza

Patients with autoimmune myasthenia gravis (MG) should be further classified before initiating therapy, as treatment response varies for ocular versus generalised, early onset versus late onset, and acetylcholine receptor antibody positive versus MuSK antibody positive disease. Most patients need immunosuppression in addition to symptomatic therapy. Prednisolone and azathioprine represent first choice drugs, whereas several second choice options are recommended and should be considered. Thymectomy should be undertaken in MG with thymoma and in generalised, early-onset MG. For MG crises and other acute exacerbations, intravenous immunoglobulin (IvIg) and plasma exchange are equally effective and safe treatments. Children and females in child bearing age need special attention regarding potential side effects of immunosuppressive therapy. MG pathogenesis is known in detail, but the immune therapy is still surprisingly unspecific, without a pin-pointed attack on the defined disease-inducing antigen-antibody reaction being available.

1. Introduction

Myasthenia gravis (MG) has a prevalence of 150 per million, with nearly one million MG patients worldwide. The yearly incidence is 10–15 per million per year [1]. Before any treatment was available the prognosis was severe, with an expected 50% 10-years' mortality. With modern treatment facilities such as immunotherapy, thymectomy, and intensive care facilities available, population-based studies show that MG and non-MG individuals have the same life expectancy [2], but still often with reduced physical abilities, reduced quality of life, and risk of complications.

There are three key aspects of MG which define the therapeutic opportunities.

(i) MG is a well-defined autoimmune disease and thus responds to immunoactive treatment.

(ii) MG is caused by impaired acetylcholine receptor (AChR) stimulation in the postsynaptic skeletal muscle membrane and thus responds to an increase in AChR activity.

(iii) MG has muscle weakness as the only symptom, and consequently should respond to measures that increase muscle function and counteract muscle weakness.

MG treatment is firmly established as the domain of neurologists. Neurologists should be in charge even if the target organ is skeletal muscle, disease mechanisms are systemic, thymus is a target organ for diagnostic, therapeutic and scientific approach, hypoventilation is a life-threatening symptom, and diplopia often the most troublesome symptom. Ten percent of MG patients have another autoimmune disorder in addition, further supporting the need for complementary medical competence. Close cooperation with other fields of medicine provides knowledge regarding new immunoactive drugs, thus expanding the therapeutic opportunities for MG.

For complicated and rare disorders such as MG, the establishment of medical centres supervising the treatment of the majority of MG patients and of all complicated patients is important to improve treatment quality. Increased treatment experience will optimize present therapy and

facilitate the introduction of new and better treatment procedures. Centres with special competence and qualifications in MG treating the majority of patients will further enhance research, including well-controlled and prospective treatment studies.

Ideally treatment recommendations should be based on scientific evidence of high quality, preferentially more than one blinded and controlled prospective study with a sufficient number of well-defined MG patients. There are disappointingly few such studies for MG. Recommendations therefore, rely on studies of lower quality and even sometimes only on case reports, clinical experience, and knowledge from non-MG treatment. It is important for patients as well as doctors to know which treatment is supported by high-quality evidence and which is more tentative and based on clinical experience and circumstantial evidence.

2. MG Classification

The various subgroups of autoimmune MG respond differently to treatment. Thus, before deciding any treatment, all individual MG patients should be defined according to subgroups. Classification aspects reflect the investigations of each patient that are necessary to undertake [3]:

(1) early-onset MG: age at onset <50 years. Thymic hyperplasia;

(2) late-onset MG: age at onset >50 years. Thymic atrophy;

(3) thymoma-associated MG;

(4) MG with anti-MuSK antibodies;

(5) ocular MG: symptoms only from periocular muscles;

(6) MG with no detectable AChR and MuSK antibodies;

The MG group with no detectable antibodies is heterogeneous. Some of these patients have low-affinity AChR antibodies that are not detectable by the routine assays and sometimes also thymic hyperplasia [4]. Some may similarly have undetectable MuSK antibodies, and some most probably have autoantibodies against other antigen(s) in the postsynaptic membrane. There are not yet any commercial tests available for the low-affinity AChR antibodies [5]. MG patients with a thymoma have nearly always detectable AChR antibodies in serum. Necessary investigations include tests for AChR and MuSK autoantibodies and CT/MR of the anterior mediastinum. Titin and ryanodine receptor antibodies may be helpful for classification. For patients with no AChR and MuSK antibodies, it is necessary with thorough examinations to exclude other causes for their muscle weakness, including nonautoimmune myasthenic syndromes. Neurophysiological examinations with repetitive nerve stimulation and jitter measurements are important to establish the initial diagnosis, especially in patients without detectable antibodies.

MG should be classified according to severity [6]. This is important when deciding specific treatment in the individual patient. It is also important in the followup to evaluate effects of various interventions. An accurate MG severity evaluation is crucial for controlled therapeutic studies. MG represents a challenge for such evaluation due to variation among muscle groups and variation during the day.

MG in early childhood poses special treatment challenges linked to growth and development in general and of the immune system [7]. The same is true for treatment of MG women in childbearing age, mainly due to potential effects of the disease and the therapies on the developing child in utero [8]. Epidemiology differs between ethnic populations and also regarding the frequency of the various MG subgroups [9]. However, MG patients are classified in the same way universally. Nonautoimmune myasthenic syndromes (genetic, toxic) and non-MG autoimmune syndromes (LEMS, neuromyotonia) are not included in this review.

3. Treatment of Acute Exacerbations

Acute exacerbations of MG need effective and urgent life-saving treatment. Life-threatening hypoventilation is the utmost threat. Plasma exchange and intravenous immunoglobulin (IvIg) are both effective for acute MG [10]. Their beneficial and symptom-relieving effect is regarded as well proven from several studies and from widespread clinical use [11]. In contrast to most other treatment options, the clinical response is rapid, occurring already after 2-3 days and often with a dramatic effect. This treatment should be given for severe exacerbations and is mandatory for MG crisis or threatening crisis. Plasma exchange or IvIg can also be used for less severe exacerbations, before surgery or together with initiation of immunosuppressive therapy with a slower effect [12]. Severe MG exacerbations with impaired respiratory function need hospitalization and often intensive care treatment.

Plasma exchange and IvIg have a similar clinical effect, and a similar responder rate. The only controlled and randomised study did not show any difference for these two treatment options [13]. Also nonrandomised evidence favours an equal effect, although the clinical impression may be a somewhat faster and more extensive effect for plasma exchange. IvIg has less side effects and less severe side effects. Optimal technique and high experience reduce the complication rate, especially for plasma exchange. Both treatment options are expensive, but IvIg represents a simpler procedure and may be superior from a total economic perspective [11]. Patients responding to plasma exchange and IvIg are not necessarily the same. Thus, if one treatment fails, the other may well be tried. It should be more convenient to add IvIg after plasma exchange than doing the procedure the other way around, this is to avoid washing away all therapeutic immunoglobulin just given to treat the patient.

For severe MG and in an acute situation, high-dose parenteral corticosteroids can be given and also in addition to plasma exchange or IvIg [10]. An early exacerbation can be seen after initiation of corticosteroids, but with pharmacological doses, a therapeutic effect often appears very early.

4. Drug Treatment

4.1. General. Patients with the diagnosis of MG should always be considered for symptomatic as well as immunoactive drug treatment. Nearly all patients need some treatment, at least in periods where the disease shows clinical activity with permanent or intermittent muscle weakness. Symptomatic drugs have a short-lasting activity both regarding effect and side effects. Dosage can be rapidly changed and the treatment is flexible. Immunoactive drugs have an effect linked to pathogenesis, and the effect usually needs some time before it becomes manifest. Side effects are relevant and should be considered in a long-term perspective. Immunoactive drugs need special attention in children and MG women of childbearing age [6]. Thus, the considerations for patient and doctor are different for symptomatic and immunoactive drug treatment.

4.2. Symptomatic Drugs. Acetylcholine esterase inhibition at the neuromuscular junction has a symptomatic effect in myasthenia and especially in autoimmune MG [3, 10, 14–16]. Optimal dosage is adjusted according to effect and side effects. Side effects appear from the nonneuromuscular cholinergic synapses in the autonomic system, which are overstimulated. Alternative ways to increase the amount of acetylcholine at the neuromuscular end plate have been tried, but with less effect than inhibiting the degradation. Acetylcholine esterase inhibitors have a stable and predictable effect, apparently unchanged over years. No scientific comparisons have been undertaken between the various esterase inhibitors. The most commonly used is pyridostigmine and also the faster acting neostigmine. Ambenonium is used in some countries. Some MG patients with anti-MuSK antibodies are hypersensitive to an increase in acetylcholine concentration.

4.3. Immunoactive Drugs. Prednisone/prednisolone remains a first-choice drug in MG [3, 14–18]. It has a well-proven positive effect experienced through decades of clinical practice in a high number of patients. However, there are no formal trials and no scientific comparisons with other drugs. Side effects occur in most patients, and they are usually of clinical significance. Prednisone/prednisolone is regarded to be safe in pregnancy. To reduce the amount of side effects, dosing the drug every second morning is usually advocated. Most patients keep a sufficient clinical effect on the MG symptoms with this regimen and with markedly less side effects. Patients often continue to do well on a very low every second day dose, but experience an exacerbation if taking this low dose away. We recommend cautiousness regarding MG patients doing well and being stable on prednisone/prednisolone in a low dose; continued long-term treatment may be necessary.

Azathioprine is the other well-established first-choice immunoactive drug used for MG [3, 14–18]. This drug is often used in combination with prednisone/prednisolone. Formal scientific evidence for its effect in MG is lacking, but a controlled trial showing the superiority of the combination prednisolone—azathioprine over prednisolone alone is much cited [19]. Azathioprine is regarded as safe and with few side effects, also during long-term treatment. It is listed among drugs that should not be used in pregnancy, although formal evidence of teratogenic effects in MG patients is lacking. During the first few months of treatment, the numbers of leucocytes and leucocyte subgroups have to be counted weekly. The clinical effect of azathioprine is slow to appear. Improvement should not be expected to appear until after 3–6 months, and full effect of the drug first occurs after 1-2 years. This is a reason why azathioprine is usually combined with other immunoactive treatments, such as prednisone/prednisolone, and especially in the initial phase. Marked improvement on azathioprine is reported in 70–90% of MG patients in open series.

Mycophenolate mofetil is regarded as an alternative drug for mild MG [3, 14–18]. However, after promising results in open MG patient treatment, randomized and controlled trials failed to confirm a positive effect [20]. The drug has few and mild side effects and is easy to use both for patients and doctors. Despite its limitations, mycophenolate mofetil is still regarded as an alternative drug for mild MG, whereas more severe MG is usually not treated by this drug because of the negative controlled trials.

Methotrexate should be used only when first-choice immunosuppressive drugs do not have sufficient effect [3, 14–18]. Methotrexate has a good and proven effect for other autoimmune disorders, but is not formally tested for MG. Still it should be tried in selected MG patients with a marked functional deficit, partly because it is usually well tolerated.

Cyclosporine A is an inhibitor of T cells and has well-documented immunosuppressive effects after organ transplantation. A controlled prospective study with a limited number of included patients proved the effect of this drug for generalised MG [21]. Due to the danger of side effects, cyclosporine is regarded as a second-choice immunoactive drug for moderate to severe MG not responding to azathioprine and prednisolone [3, 14–18].

Rituximab is a chimaeric monoclonal antibody that targets B lymphocytes through its binding to the CD 20 molecule. MG is a prototype of an antibody-mediated autoimmune disease, and so rituximab and B-cell depletion are a very promising treatment alternative. In a recent review by Benveniste and Hilton-Jones [22], the effect of rituximab in 53 MG patients was recorded, including patients with both AChR and MuSK antibodies. The authors concluded that markedly positive effects were observed with distinct improvement of severe symptoms. Rituximab should be reserved for patients with severe MG, where treatment with prednisolone and at least two other standard immunosuppressive drugs has failed. For milder MG, the risk of progressive multifocal leucoencephalopathy and other potential long-term side effects probably outweigh its therapeutic potential. This drug seems to be particularly useful for anti-MuSK MG.

Tacrolimus (FK506) is a calcineurin inhibitor just as cyclosporine. The drug inhibits the proliferation of activated T lymphocytes, but also acts on ryanodine receptor-mediated calcium release from sarcoplasmic reticulum

in muscle cells. The drug has shown a beneficial effect in MG, and it represents an alternative second-choice drug for moderate to severe MG [3, 14–18], perhaps especially for MG patients with ryanodine receptor autoantibodies [23].

Other drugs, such as cyclophosphamide and several new and selective immunosuppressive drugs, have probably a positive effect on MG, as they have on other autoimmune disorders. However, this effect has not been documented so far or is less well documented than for the above-mentioned drugs. Potential side effects are significant.

5. Thymectomy

Thymectomy should be undertaken in all the 10–15% of MG patients with a thymoma. MG improvement sometimes occurs in such patients, but less consistently than in patients with a hyperplastic thymus. The main reason for thymectomy in thymoma patients is to remove a potentially infiltrating tumour [24]. In some patients with no or very mild MG symptoms, a severe exacerbation of MG with an increase in AChR autoantibodies has been reported after the removal of a thymoma [25].

Thymectomy should always be considered as an early therapeutic measure in early onset MG with generalised symptoms [3, 10]. Many patients benefit considerably. Thymic hyperplasia with an enlarged thymus and numerous germinal follicles is associated with improvement after thymectomy. Although no blinded and fully controlled studies have been undertaken, scientific evidence and clinical experience undoubtedly confirm thymectomy as a therapeutic option [26]. A transsternal approach and video-assisted thoracoscopy appear equally effective [27]. Postoperative improvement occurs gradually after 2–24 months. Age alone should not decide thymectomy or not. Some of the MG patients that experience their first symptoms after the age of 50 years have a hyperplastic thymus and also other features in common with the early onset MG group. Such patients are expected to respond to thymectomy.

Nonthymectomy: Late-onset MG patients should also be considered for thymectomy, but thymectomy is undertaken only in a minority of them [3, 10, 26]. Early debut age within this late-onset group (<60 years) and thymic hyperplasia on MR/CT imaging favour thymectomy. Higher age, symptoms for a longer time period, atrophic thymus, and presence of non-AChR antibodies against titin and/or ryanodine receptor all count against thymectomy.

For MG with purely ocular symptoms, thymectomy is not recommended. For MG patients with anti-MuSK antibodies, the majority of evidence points to no therapeutic effect of thymectomy. Anti-MuSK MG is probably not linked to thymic pathology.

Thymectomy: Some MG patients have no detectable muscle antibodies at repeated testing and even with generalised myasthenic symptoms. As a proportion of such seronegative MG patients have low-affinity AChR antibodies, thymectomy should be an option also for patients in this group. For patients with generalised MG, onset at relatively low age, and with a hyperplastic thymus on imaging, thymectomy should in our opinion be performed.

Thymectomy should always be undertaken in hospital units with experience in this type of surgery. Neurologists should evaluate the patients immediately before surgery and continuously in the postoperative phase. The patients should be in a stable condition at the time of surgery, and the threshold for treatment with plasma exchange or IvIg preoperatively should be low. Such treatment will secure a fast recovery and counteract postoperative complications, most importantly from the respiratory system.

6. Accessory Treatment

During acute MG exacerbations, intensive care therapy with respiratory support is life saving. Infections should be treated rigorously. The marked reduction in MG mortality is for a large part due to modern intensive care therapy [2], although also pharmacological treatment and thymectomy represent cornerstones in MG therapy.

Physical training, weight control, and sensible life style modifications should be discussed with all MG patients [10]. Seasonal flu vaccination should be recommended. Symptomatic ophthalmological treatment may be helpful for ocular MG with troublesome diplopia.

7. Treatment Principles

MG should be treated early and with vigour, after classification of subtype and MG severity. Moderate or severe myasthenic weakness represents an immediate and permanent challenge. Treatment at an early stage with thymectomy and/or immunoactive drugs improve long-term outcome. With a lack of initial response, it is not sufficient to have tried only 2-3 alternative drug options. Drugs can be combined. Longitudinal measurement of AChR antibodies can be helpful in evaluating treatment effect and also in the differentiation between MG and non-MG symptoms experienced by the patient [5]. The clinical response and evaluation is most important, but there tends to be a correlation between MG severity/activity and AChR antibody concentration in the individual patient. There are no studies systematically and prospectively examining the usefulness of repeated antibody examinations in established MG. Non-MG drugs given to patients with MG should always be checked for potential adverse neuromuscular effects.

Most MG patients are in the need of long-term therapy. For patients in a stable remission when on immunoactive drugs, a conservative policy regarding full drug withdrawal is recommended. A low-dose prednisolone, azathioprine, or other immunoactive drugs can in such patients be sufficient to maintain the stable condition, but also necessary to avoid new exacerbations. Younger patients in particular, not least after thymectomy, can obtain a full clinical remission without any need for continued drug treatment. Late-onset MG patients and thymoma MG patients usually need life-long treatment.

In 10% of MG patients, the onset is before age 18 years [6]. The disease is very rare in infancy. In Asian populations, up to 50% of cases present in adolescence [9]. Most children

with MG have AChR antibodies. Those without antibodies should be thoroughly checked for non-MG myasthenic syndromes. The response to thymectomy is usually very favourable, and thymectomy should be done early. Immunosuppressive drugs are used for moderate and severe cases in the same way as for adults, but chronic administration of such drugs in children usually leads to significant side effects. Children more often obtain full remission after thymectomy, so that withdrawal of immunosuppressive drugs should be tried after a couple of years, especially if a marked reduction of AChR antibody titre has occurred.

Pregnancy and giving birth for MG women is usually uncomplicated, although operative intervention during birth (Caesarean section, forceps use) occurs more frequently than in controls, related to prolonged labor [28]. Anticholinesterase drugs and prednisolone are considered to be safe during pregnancy. Azathioprine and other immunosuppressive drugs should be withdrawn before a planned pregnancy and should be avoided in the fetal organ-developing period. Arthrogryposis occurs with increased frequency in children of MG mothers, caused by movement inhibition in utero due to transplacental transfer of mother's AChR antibodies and thereby reduced function of the fetal AChR [28, 29]. 10–15% of the children of MG mothers experience a transient neonatal MG, usually mild and lasting only a few days. This risk is influenced by AChR antibody subclass and antigen specificity [7]. Lactation is recommended by most neurologists irrespective of mother's immunosuppressive MG drug treatment. Previous thymectomy does not influence pregnancy and giving birth negatively and could have a protective effect on neonatal MG [8]. Father's MG is not known to have any influence on the child, apart from the increased risk for MG and other autoimmune disease due to genetic factors.

MuSK-antibody associated MG has a phenotype that differs from non-MuSK MG [14]. The patients tend to have more severe symptoms, and the therapeutic response is more variable. Cholinesterase inhibitors should be tried, but considered less beneficial for this subtype. Most patient series report no confirmed effect of thymectomy. Immunosuppressive drugs should be tried for the same indications as in non-MuSK MG. Prednisolone/prednisone and azathioprine have lower success rate in patients with MuSK antibody MG. As patients with this MG subtype often have severe and progressive symptoms and from bulbar and respiratory muscles, effective and intense therapy is necessary. Most patients use corticosteroids. From many case reports, rituximab and plasma exchange seem to be important alternatives often used, and the same is probably true for IvIg [10, 11, 14, 22].

8. Future Treatment

It is a paradox that MG treatment is still so unspecific. MG is the best characterised autoimmune disease with well-defined pathogenetic antibodies that impair function through the destruction and inhibition of muscle cell AChR. Still our therapeutic immunosuppression is aimed at general mechanisms of the immune system. The external causes for MG are totally unknown, apart from those 10–15% of patients with a paraneoplastic condition linked to thymic neoplasia.

The ultimate aim of eradicating MG by removing the cause of the disease seems still far away.

Antigen-specific treatment of MG should be promising, and such strategies work in animal models. Induction of specific immunological tolerance to AChR or MuSK, shifting the immune response from harmful to nonharmful is theoretically possible [3, 30, 31]. Strategies involving antigen-presenting cells are considered for treatment of autoimmune disorders, and manipulation of this process could be antigen-specific. T-cell receptor vaccination may be less promising, since T-cell receptor usage is not very restricted in human MG [32]. A sensible approach would be to remove the pathogenic autoantibodies specifically, or remove the plasma cells and/or B lymphocytes producing these antibodies [3]. So far such treatment has not been cost-effective. The effect has not been superior to today's standard treatment, costs have been higher, and procedures more complicated. Administering nonpathogenic AChR (or MuSK) antibodies to MG patients and thereby blocking the action of the patient's own pathogenic antibodies would be an alternative experimental approach.

MG is improved by the inhibition of acetylcholine esterase. Other non-AChR molecules could theoretically be influenced therapeutically to improve the neuromuscular function. So far no such additional treatment has been established as effective in the clinical situation.

New and more selective immunoactive drugs are marketed worldwide. These drugs are established as first- or second-line treatment for an increasing number of autoimmune and inflammatory disorders, due to their proven superior clinical effect. For MG, these drugs have not been evaluated in prospective and controlled trials. The present treatment has reasonably good effect in the large majority of MG patients, so that most patients have a high level of daily function and with few and modest side effects of the treatment. However, there is a need for better and more focused treatment. Such treatment should be established through formal multicenter trials in MG networks, not by random and individual off-label use of the drugs. As pathogenesis differs in MG subgroups, the immunoactive treatment needs to be individualised. Subgroups of MG will respond differently to various treatment alternatives. In the future, the detailed evaluation of each MG patient will hopefully have distinct therapeutic consequences, so that the treatment regime is tailored according to the specific autoimmune dysfunction.

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SAGE-Hindawi Access to Research
Autoimmune Diseases
Volume 2011, Article ID 973808, 5 pages
doi:10.4061/2011/973808

Review Article

Lambert-Eaton Myasthenic Syndrome; Pathogenesis, Diagnosis, and Therapy

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Received 26 May 2011; Accepted 4 August 2011

Academic Editor: Johan A. Aarli

Lambert-Eaton Myasthenic Syndrome (LEMS) is a rare disease with a well-characterized pathogenesis. In 50% of the patients, LEMS is a paraneoplastic manifestation and caused by a small cell lung carcinoma (SCLC). Both LEMS patients with SCLC and those without this tumour have in 85% of cases pathogenetic antibodies of very high LEMS specificity against voltage-gated calcium channels (VGCCs) in the cell membrane of the presynaptic motor nerve terminal. Better understanding of LEMS pathogenesis has lead to targeted symptomatic therapy aimed at the neuromuscular junction and to semispecific immunosuppression. For SCLC LEMS, tumour therapy is essential.

1. Introduction

The neuromuscular synapse represents a predilection site for disease. Autoimmune, genetic, and toxic disorders are linked to the neuromuscular junction. The dominating symptom of all such disorders is muscular weakness. The disorders interfere with the acetylcholine-mediated transmission of the signal from the presynaptic nerve to skeletal muscles, impairing muscle contraction. Both the autoimmune, genetic and toxic conditions can effect either pre- or postsynaptically. Mutated genes leading to a change in protein function result in myasthenic syndromes of various types, the postsynaptic acetylcholine receptor most often the target, or also proteins in the postsynaptic membrane functionally linked to this receptor. Toxins exert their function pre- or postsynaptically and will paralyze either attacker or prey in nature's fight for survival. Such toxins are widely used in medicine, both therapeutically and for diagnostic and research purposes.

Lambert-Eaton Myasthenic Syndrome (LEMS) represents one of the distinct autoimmune disorders at the neuromuscular junction. In 1956, Lambert and coworkers reported 6 patients with atypical myasthenia, lung carcinoma, and a specific response to repeated nerve stimulation differing from myasthenia gravis [1]. During recent years, disease mechanisms have been thoroughly elucidated for LEMS, so that this disorder can now be characterized as a model disease for other autoimmune and paraneoplastic disorders. LEMS is caused by pathogenic autoantibodies to presynaptic voltage-gated calcium channels (VGCCs) in the membrane of the motor nerve terminal, impairing acetylcholine release, and thereby causing distinct weakness of striated skeletal muscles. The challenge now is to transfer this detailed pathogenetic knowledge into even more effective therapy.

2. Epidemiology

LEMS fulfils the criteria for a rare disease. In a study from South Holland, Wirtz et al. [2] found a LEMS prevalence of 2.3 per million and an annual incidence rate of 0.5 per million. This incidence was 1.4 times lower than what they found for myasthenia gravis. A low prevalence relative to incidence reflects the poor survival of LEMS patients with the paraneoplastic type of disease. 60% of the LEMS patients were males. Mean age of debut was 58 years. There seems to be two peaks for age of onset, one around 40 years and one at a higher age, similar to what is seen for myasthenia gravis [3].

LEMS is subclassified into two main subgroups; LEMS combined with small cell lung carcinoma (SCLC), and LEMS

with no SCLC. The no-SCLC LEMS group is dominating regarding prevalence as this group has a near normal survival rate. No-SCLC LEMS patients have a lower age of debut than SCLC LEMS [3, 4]. LEMS with SCLC shows a male preponderance, reflecting smoking habits. The frequency of LEMS among the total SCLC patient population is reported between 0.5 and 3% [2, 5]. LEMS-related autoantibodies occur in a higher proportion of SCLC patients, but without leading to manifest neuromuscular disease. SCLC patients with LEMS tend to be younger than those without LEMS [6].

3. Clinical Picture

Muscle weakness represents the hallmark of LEMS. This weakness starts nearly always in proximal muscle groups, especially in the legs. 80% of LEMS patients experience proximal weakness in both arms and legs [4, 7, 8]. Also facial weakness, eye muscle complaints, bulbar muscular weakness, and distal pareses are relatively common. LEMS with SCLC tends to have more severe muscle weakness and with a distinct progression. Areflexia is a common finding.

Autonomic dysfunction is the second typical symptom of LEMS. Such symptoms are milder and have less functional significance than muscular weakness. However, it affects a large majority of LEMS patients. Dry mouth, dry eyes, erectile dysfunction, constipation and reduced sweating are frequently confirmed when examining LEMS patients, and to the same degree for patients with and without SCLC.

4. Pathogenesis

LEMS is caused by autoantibodies to VGCC in the presynaptic neuronal cell membrane. Such antibodies show a high sensitivity, as they can be detected in 85% of all LEMS patients. The LEMS specificity in patients with distinct muscle weakness is nearly 100%. Among SCLC patients without any symptoms of muscle weakness or autonomic dysfunction, 3–5% have VGCC antibodies. VCCC antibodies are hardly ever found in other control groups, but have been described in patients with clinically pure cerebellar ataxia.

The presynaptic release of acetylcholine is a complex process. The VGCC antibodies in LEMS lead to a reduction in the quantal release of acetylcholine [9]. A direct pathogenetic effect of the autoantibodies has been shown by injection in experimental animals and supported by the patients' clinical and electrophysiological response to plasma exchange with removal of the autoantibodies. The number of VGCC is reduced in LEMS patients, caused by antibody-mediated cross-linking of the ionic channels. Research groups in Oxford and at the Mayo Clinic have been very active in elucidating these disease mechanisms.

The autonomic dysfunction in LEMS is probably caused by the same VGCC antibodies that cause the muscle weakness. The antibodies impair transmitter release from parasympathetic and sympathetic neurons through down-regulation of the receptors [10, 11].

VGCC mediates calcium influx into the nerve terminal. This influx activates presynaptic signalling pathways. Synaptotagmin, synaptobrevin, synthaxin, and SNAP-25 are molecules taking part in the interaction between the increased intracellular calcium concentration and the release of acetylcholine from preformed synaptic vesicles. With calcium influx being hampered by the VGCC antibodies, presynaptic compensatory mechanisms influence acetylcholine release. This complex interaction has recently been reviewed by Takamori [11]. Non-VGCC molecules influencing presynaptic acetylcholine release have been examined as potential targets for autoantibodies in LEMS patients without VGCC antibodies. By a similar approach, MuSK was identified as an alternative antigen target in myasthenia gravis. For LEMS, alternative antigens have been suggested but not finally proven

VGCC represents multisubunit ionic channels, and comprising 4 or 5 subunits. Membrane depolarisation opens the central pore for calcium influx. Electrical signals are thereby coupled to neurotransmission, secretion, and other events in various cell types. Nonvoltage-gated calcium channels respond to other types of stimuli, for example mechanical stretch. The role of VGCC for neuromuscular synaptic transmission and disease was recently nicely reviewed by Urbano et al. [12], that review concentrating on the molecular processes related to VGCC subtypes. VGCC were initially grouped according to tissue where they were detected, and/or their pharmacological properties; L, P/Q, N, K, T. The LEMS autoantibodies are directed selectively against the P/Q subtype of VGCC. More recently VGCC has been grouped in an alternative way according to the gene name of their alpha 1 subunit, which also reflects their protein structure. VGCC properties have now been examined in detail and linked to molecular sequence. The VGCC recognized by the autoantibodies in LEMS are of the Ca_v 2.1 subtype, both at the motor and autonomic axon terminals. The antibodies may to some degree bind also to other VGCC subtypes, especially to Ca_v 2.2 [13]. However, the primary target and the cause of the down-regulation is binding to Ca_v 2.1 [12, 14]. Although VGCC downregulation represents the main mechanism for dysfunction, also a direct antibody-mediated channel block has been reported. Most VGCC antibodies in LEMS are directed against the alpha-1 subunit. The exact pattern of epitope reactivity seems to differ between LEMS patients with and without SCLC [15].

The role of T lymphocytes has not been established in LEMS. T cells do not aggregate around the presynaptic terminal. In contrast to myasthenia gravis, no morphological or functional disturbances have been reported in thymus or other lymphoid organs. However, the expression of T cell markers in LEMS patients suggested a down-regulation of immunosuppression in SCLC patients with LEMS, compared to such patients without LEMS [16]. T cell immunoregulation may therefore facilitate or counteract the development of LEMS. T cell activity in the SCLC tissue may be relevant for the induction of LEMS.

A genetic susceptibility has been established for nearly all autoimmune disorders, both from family history and from susceptibility genes, especially HLA-antigens. LEMS without SCLC is significantly associated with HLA-B8 (HLA- class I),

and HLA -DR3 and -DQ2 (HLA-class II) [3, 17]. About two-thirds of nontumour LEMS patients compared to one-third of controls have this HLA-pattern. This is not surprising, as the same HLA genotypes are found with increased frequency in most autoimmune disorders, including myasthenia gravis. The clinically well-known autoimmune overlap manifests through this joint genotype. A unique observation is the report of monozygous twins, one with LEMS and VGCC antibodies, the other with myasthenia gravis and acetylcholine receptor antibodies [18].

In contrast, no relation has been found for SCLC LEMS and HLA [3]. This indicates a pathogenetic difference between the two LEMS subtypes. The same observation is true for myasthenia gravis, where there is no HLA-association for the paraneoplastic, thymoma-associated subtype. Nor do paraneoplastic disorders in general show a consistent HLA-pattern. Patients with paraneoplastic disorders do not have an increased frequency of the HLA-genotypes that are associated with nonparaneoplastic autoimmunity. Tumour tissue from SCLC LEMS patients expresses a reduced amount of HLA class I antigens compared to tissue from SCLC patients without LEMS [19].

For the 50% of LEMS patients with a SCLC, the tumour represents the initiating LEMS event. VGCC are expressed on the surface of the SCLC cells [20]. This expression of cancer-related neoantigens induces the autoantibody production, and the autoantibodies cross-react with presynaptic VGCC antigens. This induction of autoimmunity usually takes place early in tumour development, in most patients before a SCLC diagnosis has been established, and before even a malignant or lung disease has been suspected.

In the remaining LEMS patients, that is, those with no SCLC, no initiating event can be identified. Such patients do not develop a SCLC or any another malignant or lung disorder later, linked to their LEMS. This is again similar to other autoimmune disorders. Cross-reactivity of antibodies occurring as a response to a clinical or subclinical infection would have been a plausible explanation, but has been impossible to confirm.

Antibodies against SOX proteins (sry-like high-mobility group box) represent a specific serological marker for SCLC [6]. No pathogenetic role has been established for the SOX antibodies. However, they occur more frequently in SCLC LEMS (67%) than in SCLC no-LEMS (36%).

5. Diagnosis

The LEMS diagnosis is suspected from typical clinical symptoms; the triad of muscle weakness with a typical distribution, areflexia, and autonomic dysfunction. Presence of VGCC autoantibodies confirms the LEMS diagnosis, due to the very high antibody specificity. Absence of detectable VGCC antibodies does not rule out LEMS. Neurophysiological tests with adequate repetitive stimulation undertaken in relevant muscles strongly support a diagnosis of LEMS. Therapeutic response to drugs increasing acetylcholine availability at the postsynaptic receptor is expected, but has no strong diagnostic value, less than for myasthenia gravis. The most frequent misdiagnosis is probably seronegative and atypical myasthenia gravis or unspecified myasthenic syndromes.

Once a diagnosis of LEMS has been confirmed, or even suspected, starts the search for a SCLC. Smoking markedly increases this risk, but nonsmokers should undergo the same diagnostic program. Extensive imaging is necessary, and if necessary including PET. If the initial search is negative, the screening should be repeated after 3 months, and then every 6 months up till 2 years after LEMS debut, this according to recent EFNS guidelines [21]. SOX antibodies represent an additional marker of diagnostic value in LEMS patients, as they have high specificity for SCLC, although lower sensitivity [6]. Two national cohorts (Dutch and English) with a total of 219 patients were recently used to develop a clinical score for predicting SCLC in LEMS [22]. Age at onset, smoking, weight loss, general well-being, bulbar involvement, male sexual impotence, and SOX antibodies were all independent predictors for SCLC in LEMS.

6. Associated Disease

The most important disease association is the one between LEMS and SCLC. One half of LEMS patients have a paraneoplastic disorder. Other tumours do probably not occur with any increased frequency in LEMS. Whereas SCLC can be linked to various paraneoplastic disorders and various autoantibodies, LEMS is linked to a SCLC only.

LEMS patients with no SCLC have an increased occurrence of other autoimmune disorders, at least in part due to a genetic predisposition for autoimmune reactivity.

An increasing number of rare disorders of the central nervous system have been found to be associated with serum autoantibodies to ion channels, receptors, or associated proteins in the cell membrane. Some of these autoantibodies are also pathogenic. Encephalitis can be caused by antibodies to voltage-gated potassium channels (VGKC) and to NMDA-receptors, and antibodies to GAD and aquaporin 4 are associated with distinct neurological syndromes. VGCC antibodies and LEMS can also coexist with central nervous system disease. Among SCLC patients diagnosed with paraneoplastic subacute cerebellar degeneration, 16% were found with concomitant LEMS, and 24% with increased VGCC antibody levels [23, 24]. Cerebellar ataxia has been reported also in a few nonparaneoplastic LEMS patients with VGCC antibodies [25].

7. Therapeutic Principles

LEMS pathogenesis points directly to potential treatment principles. The reduced quantal content release of acetylcholine can be counteracted by symptomatic therapy. Acetylcholine esterase inhibition will increase the amount of acetylcholine in the synaptic cleft. Therapy with pyridostigmine and similar inhibitors has usually a positive effect, but less so and less predictably than in myasthenia gravis. Acetylcholine esterase inhibition should be tried, but is perhaps not first-line therapy [26, 27]. 3,4 diaminopyridine

is an aminopyridine that blocks presynaptic voltage-gated potassium channels and thereby prolongs the duration of the presynaptic action potential. The amount of acetylcholine released increases. The positive clinical effect of 3,4 diaminopyridine is well documented [26, 28]. The 3,4 diaminopyridine phosphate salt has recently been marketed [9].

If symptomatic LEMS treatment is insufficient, immunosuppressive drug therapy should be initiated. A combination of prednisone/prednisolone and azathioprine is best documented [26, 29, 30]. For other immunosuppressive drugs, there are mostly limited series and case reports published. Mycophenolate and cyclosporine have been recommended, probably also because they are used for myasthenia gravis. Rituximab is a monoclonal antibody that specifically targets B lymphocytes. This drug should therefore be promising for all autoantibody-mediated disorders, including LEMS. A positive treatment result in a few patients has recently been reported [28, 31]. Intravenous immunoglobulin is used for several paraneoplastic disorders, and it has a well-proven effect for acute exacerbations of myasthenia gravis as well. A beneficial short-term effect has been reported for LEMS, probably to the same degree for paraneoplastic and no-tumour LEMS. An EFNS guideline concludes that intravenous immunoglobulin may be tried in LEMS [32]. Plasma exchange has probably a similar effect, but is less useful as a long-term therapy [30].

Physical training can be carried out safely in mild and moderate LEMS [26]. Overweight should be avoided. All complicating disorders, such as respiratory infections, should be vigorously treated. Drugs with a potential negative impact on neuromuscular transmission should be avoided. Standard vaccination programmes are recommended also for LEMS patients. Treatment with intravenous immunoglobulin during pregnancy should be considered due to the risk of fetal arthrogryposis, similar to what is seen in myasthenia gravis. Transient neonatal LEMS due to transplacental transfer of IgG antibodies has been described [33].

Effective treatment for the SCLC can improve the paraneoplastic LEMS as well. For LEMS patients with SCLC, the anticancer treatment is crucial. Survival for patients with SCLC and LEMS is slightly better than for SCLC patients without LEMS [3]. However, presence of VGCC antibodies without manifest LEMS does not seem to increase survival. Nor is presence of SOX antibodies in paraneoplastic LEMS linked to any increase in survival [6].

8. Future Perspectives

For one group of LEMS patients, the cause of the disease is known to be a SCLC. But even if LEMS represents an early symptom of a causative tumour, the prognosis for survival is not good. More effective cancer treatment is the main challenge for this patient group.

For the other half of LEMS patients, the cause of the disease is unknown. However, the pathogenesis is very well understood, and the pathogenic antibodies have been characterized in detail. Still the therapy is immunologically unspecific or semispecific and combined with symptomatic treatment. Antigen-specific treatment should be an aim, suppressing or modulating the immune response against VGCC specifically. The rarity of LEMS hampers research. The new and more selective immunoactive drugs already on the market and with a proven effect for less well characterized disorders, have not been tried in controlled studies for LEMS. Even uncontrolled observations are few. Multicentre evaluation for rare disorders such as LEMS is very welcomed.

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SAGE-Hindawi Access to Research
Autoimmune Diseases
Volume 2011, Article ID 213092, 17 pages
doi:10.4061/2011/213092

Research Article

Inflammation and Epstein-Barr Virus Infection Are Common Features of Myasthenia Gravis Thymus: Possible Roles in Pathogenesis

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Received 30 May 2011; Accepted 23 June 2011

Academic Editor: Nils Erik Gilhus

The thymus plays a major role in myasthenia gravis (MG). Our recent finding of a persistent Epstein-Barr (EBV) virus infection in some MG thymuses, combined with data showing that the thymus is in a proinflammatory state in most patients, supports a viral contribution to the pathogenesis of MG. Aim of this study was to gain further evidence for intrathymic chronic inflammation and EBV infection in MG patients. Transcriptional profiling by low density array and real-time PCR showed overexpression of genes involved in inflammatory and immune response in MG thymuses. Real-time PCR for EBV genome, latent (EBER1, EBNA1, LMP1) and lytic (BZLF1) transcripts, and immunohistochemistry for LMP1 and BZLF1 proteins confirmed an active intrathymic EBV infection, further supporting the hypothesis that EBV might contribute to onset or perpetuation of the autoimmune response in MG. Altogether, our results support a role of inflammation and EBV infection as pathogenic features of MG thymus.

1. Introduction

Myasthenia gravis (MG) is a well-characterized autoimmune disorder of the neuromuscular junction. In most cases (>80%), the disease is associated with the production of autoantibodies against the acetylcholine receptor (AChR), which impair neuromuscular transmission resulting in muscle weakness and disabling fatigability. Less frequently, MG is associated with the presence of antibodies against the muscle specific kinase (MuSK) receptor [1]. The remaining MG patients—referred as seronegative—are negative for anti-AChR and anti-MuSK antibodies, although a proportion of them (66%) has recently been found to have low-affinity anti-AChR antibodies [2].

A wealth of data supports the involvement of thymus in the pathogenesis of MG with AChR autoantibodies. Marked pathological alterations of thymus occur in over 80% of AChR-positive patients [1], comprising thymic hyperplasia observed in 50–60% of AChR-positive cases and variable proportion of seronegative cases [3–5], and thymoma present in 10–15% of cases. Thymus with hyperplasia contains B-cell infiltrates that can organize into ectopic germinal centers (GCs) forming B-cell follicles (follicular hyperplasia) or be distributed throughout thymic medulla (diffuse hyperplasia, also called thymitis) [3]. Ten to 20% of AChR-positive cases have an atrophic thymus very similar to that of age-matched controls with regard to the amount of adipose tissue and epithelial space and characterized by the

presence of infiltrating B cells, in some cases forming GCs in the residual islands of medullary parenchyma [3, 4, 6], indicative of thymic hyperplasia and immune activation.

The thymus of AChR-positive MG patients contains all the components required to initiate and sustain the autoimmune response: the autoantigen, expressed on muscle-like myoid cells [7] and thymic epithelial cells (TECs) [8], professional antigen-presenting cells [9], AChR-specific T cells [10], and plasma cells producing anti-AChR antibodies [11]. As sign of thymic involvement in MG pathogenesis, thymectomy results in stable remission in a high proportion of AChR-positive patients (see [12] and references included).

Both genetic and environmental factors are involved in the etiology of MG. Viral infections are the prime environmental factors suspected to play a role in the development of autoimmunity through mechanisms which include general activation of the host immune system and molecular mimicry [13]. In the former process, pathogens act as promoters of autosensitization mainly by initiating an innate immune response that in turn stimulates inflammation and activates the host immune system [13]. Striking evidence of chronic inflammation of thymus in most MG patients [14, 15] makes plausible the hypothesis that persistent viruses or other microbial agents may contribute to intrathymic etiologic mechanisms of the disease. Our recent findings provided indication of a viral contribution to onset or maintenance of the intrathymic autoimmune response in MG patients [6, 16]. In a study, we found evidence of a chronic poliovirus infection in the thymus of some (14.7%) MG patients, suggesting that persisting viruses, which stimulate innate immune responses and chronic inflammation, might be responsible for immunological alterations and autosensitization in the thymus [16]. In another study, we identified an abnormal accumulation of Epstein-Barr virus- (EBV-) infected B cells and plasma cells in MG thymuses but not in normal control thymuses [6]. We found viral DNA and both viral latency and lytic gene mRNAs and proteins in most of the examined MG thymuses, indicating EBV persistence and reactivation [6]. Since EBV has the unique ability to disrupt B-cell regulatory checkpoints and to interfere with the B-cell differentiation program [17, 18], our finding suggested that EBV infection may contribute to chronic B-cell activation and persistent autoimmune response in this organ in MG patients [6].

Herein, we searched for new evidence of inflammation and EBV infection in MG thymus. Our objectives were (a) to characterize MG thymus for the expression of genes involved in biological processes related to immune response, including genes encoding for proinflammatory molecules, regulators of immune response, and antiviral agents; (b) to gain further evidence of EBV infection in MG thymus by extending the search for EBV presence from the 17 MG thymuses examined in our previous study [6] to an additional 19 MG thymuses.

2. Material and Methods

2.1. MG Patients, Thymic Tissues, and Control Cell Lines. The study included pathological thymuses from MG patients who underwent thymectomy as therapeutic treatment and nonpathological thymuses obtained during heart surgery in babies and adult cardiopathic subjects. Written informed consent was obtained from all patients for thymectomy and use of thymus for research purposes. The study was approved by the Ethics Committee of the Carlo Besta Neurological Institute.

Thymic tissues from 10 AChR-positive MG patients (Patient Group 1), including 3 with follicular hyperplasia, 3 with thymitis, and 4 with thymic involution (see Table 1 for clinical features), were individually used for TaqMan Low-Density Array (LDA) Immune Panel analysis. Two nonpathological thymuses from babies aged 0.5 and 10 months were used as reference tissues in the LDA analysis. Real-time RT-PCR, performed to confirm LDA data, was carried out on MG thymuses from the 10 patients included in the LDA and from additional 17 patients (Patient Group 2, Table 1), including 6 with follicular hyperplasia, 6 with thymitis, and 5 with thymic involution, previously resulted positive for intrathymic EBV infection [6]; as control, the 2 nonpathological thymuses examined by LDAs and additional 5 EBV-negative nonpathological thymuses (mean age: 31.4 ± 17.3) [6] were analyzed.

Real-time PCR for EBV DNA and RNA detection was carried out on thymuses from 19 MG patients (Patient Group 3, Table 1), including 15 AChR-positive MG patients (6 with follicular hyperplasia, 4 with thymitis, and 5 with thymic involution) and 4 seronegative MG patients (3 with follicular hyperplasia and 1 with thymitis). Thymuses from 2 adult healthy subjects, previously resulted EBV-negative [6], were analyzed to test the specificity of the PCR procedures described in what follows.

For each thymus, some fragments were fixed in 10% formalin for histopathological classification; other fragments were snap-frozen and stored at -80°C. EBV-positive lymphoblastoid JY and EBV-negative human Jurkat T-cell lines were cultured at 37°C in 5% CO_2 in RPMI 1640 (Euroclone, Pero, Italy) with 10% foetal bovine serum (Invitrogen, Carlsbad, CA), 2 mM sodium pyruvate (Invitrogen), 2 mM L-glutamine, and 100 U penicillin/streptomycin (all from Euroclone) and used as controls in molecular analyses and immunohistochemistry.

2.2. Transcriptional Profiling

2.2.1. RNA Isolation and cDNA Synthesis. Total RNA was extracted by 20–50 mg of frozen thymic fragments using the TRIzol method (Invitrogen) and treated with DNase I (Ambion Applied Biosystems, Foster City, CA). Random-primed cDNA was prepared using Superscript II reverse transcriptase (Invitrogen) following the manufacturer's instructions. As a control of retrotranscription efficiency, β-actin gene was amplified in the same samples.

2.2.2. TaqMan Low-Density Array (LDA). cDNAs prepared from 10 MG thymuses (Patient Group 1) and 2 control thymuses were analysed by TaqMan Low-Density Immune Profiling Array, product number 4342510 (Applied Biosystems), a microfluidic card containing predesigned primer

Table 1: Clinical features of MG patients included in the study.

Patient Group 1	Hyperplasia ($n = 3$)	Thymitis ($n = 3$)	Involuted ($n = 4$)
Sex (F/M)	3/0	2/1	2/2
Age at disease onset (years) mean ± SD	23.3 ± 9.3	30.9 ± 3.7	29.1 ± 16.3
Age at surgery (years) mean ± SD	26.3 ± 11.0	32.0 ± 3.6	31.5 ± 15.2
Ab AChR-positive	2	3	4
Seronegative	1	0	0
Immunosuppressive therapy	2	2	4
Patient Group 2	Hyperplasia ($n = 6$)	Thymitis ($n = 6$)	Involuted ($n = 5$)
Sex (F/M)	5/1	5/1	4/1
Age at disease onset (years)[a] mean ± SD	23.6 ± 7.2	23.4 ± 12.6	30.0 ± 10.2
Age at surgery (years) mean ± SD	25.5 ± 6.0	25.2 ± 11.9	33.0 ± 11.2
Ab AChR-positive	6	4[b]	4
Seronegative	0	1	1
Immunosuppressive therapy	5[c]	4	5
Patient Group 3	Hyperplasia ($n = 9$)	Thymitis ($n = 5$)	Involuted ($n = 5$)
Sex (F/M)	8/1	4/1	3/2
Age at disease onset (years)[d] mean ± SD	26.4 ± 9.2	31.0 ± 3.8	30.7 ± 14.4
Age at surgery (years) mean ± SD	30.0 ± 10.9	33.4 ± 4.0	32.20 ± 11.8
Ab AChR-positive	6	4	5
Seronegative	3	1	0
Immunosuppressive therapy	3	4	5

[a]Age at disease onset was not available for two patients, one with hyperplasia and one with thymitis. [b]Information on autoantibody presence in serum was not available in one patient. [c]Data on the therapy before thymectomy were missing in one patient. [d]Age at disease onset was not available for two patients, one with hyperplasia and one with involuted thymus.

probe sets specific for 90 genes, implicated in the immune response (e.g., cytokines/chemokines and their receptors, transcription factors, stress response, cell surface receptors, and signal transduction), and for 6 housekeeping genes (e.g., β-actin and glyceraldehyde 3-phosphate dehydrogenase, GAPDH). To run the array, the cDNA was added to the PCR master mix (Applied Biosystems) and loaded into the eight sample-loading channels of LDA. Each channel with 48 wells contains primer probe sets for 12 different genes tested in quadruplicate. After a brief centrifugation, the arrays were run on an upgraded Applied Biosystems 7900HT Real-Time PCR System (performed at Cogentech, Consortium for Genomic Technologies c/o IFOM-IEO Campus, Milan, Italy). GAPDH was used to normalize the results. For each target gene, relative expression was calculated from the formula $2^{-\Delta\Delta Ct}$ using as calibrators normalized values obtained from control thymuses.

2.2.3. Real-Time RT-PCR. cDNA samples prepared from thymic fragments of Patient Group 1 and 2, and 7 control thymuses, were subjected to real-time PCR for IL-6, IL-10, IFN-1β, IFN-γ, MxA, and HLA-DRα genes. Predesigned functionally tested TaqMan gene expression assays (Applied Biosystems) were used: assay ID Hs00174131_m1 for IL-6; assay ID Hs00174086_m1 for IL-10; assay ID Hs01077958_s1 for IFN-β; assay ID Hs00174143_m1 for IFN-γ; assay ID Hs00182073_m1 for MxA; assay ID Hs00740413_g1 for HLA-DRα. Each cDNA was amplified in triplicate using 7500 Fast Real-time PCR system (Applied Biosystems) in a PCR volume of 20 μL containing 10 μL of TaqMan Fast Universal PCR Master Mix and 1 μL of TaqMan gene expression assays (all from Applied Biosystems). GAPDH mRNA was analyzed as endogenous control by using TaqMan Predeveloped Assay Reagents Human GAPDH (Applied Biosystems). The omission of cDNA was taken as no template control. Data analysis followed the same method ($2^{-\Delta\Delta Ct}$ method) as that in LDA.

2.2.4. Statistical Analysis. One-way ANOVA with Bonferroni multiple comparison post hoc test was performed to assess the significance of differences in transcriptional profiling LDA data. In real-time RT-PCR analysis, Mann-Whitney U test was used to compare IL-6, IL-10, IFN-β, IFN-γ, MxA, and HLA-DRα transcript levels in control and MG thymus. P values < 0.05 were considered significant. GraphPad PRISM version 4.0 (GraphPad Software, San Diego, CA) was used for data elaboration and statistical analysis.

2.3. Tissue Processing for EBV Detection

2.3.1. DNA and RNA Isolation. For DNA and RNA isolation aimed to detect EBV genome and transcripts, snap-frozen thymic specimens from the donors belonging to the Patient Group 3 and from 2 control thymuses were used. For each OCT-included snap-frozen thymus, a total of 18 serial sections were obtained using a cryostat (Leica Microsystems, Nußloch, Germany) for alternate DNA, RNA isolation, or immunohistochemistry. 30-μm sections 1, 4, 7, 10, 13, and

16 were collected for DNA extraction, 30-μm sections 3, 6, 9, 12, 15, and 18 for RNA extraction, and 6-μm sections 2, 5, 8, 11, 14, and 17 for immunohistochemistry.

For DNA isolation, thymic sections were resuspended in 300 μg/mL proteinase K in digestion buffer, homogenized with TissueLyser LT (Qiagen, Valencia, CA), and incubated at 50°C overnight; DNA was extracted following the standard phenol-chloroform protocol. RNA was isolated from thymic sections by using the TRIzol method (Invitrogen), after homogenization with TissueLyser LT (Qiagen). RNA integrity was checked on ethidium bromide containing 1% agarose gel in Tris-borate/EDTA buffer. All RNA samples were treated with DNase I (Ambion Applied Biosystems). Concentration of DNA and RNA was estimated by Nanodrop 2000 c Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE).

2.3.2. Real-Time PCR for EBV DNA. Real-time PCR specific for the *Bam*HI-W repeated multiple splices [19] was performed on each DNA sample. Genomic DNA (0.8 μg) was amplified in a final volume of 25 μL containing 12.5 μL of TaqMan Universal PCR Master Mix (Applied Biosystems), 900 nM each primer, and 175 nM probe. Primers and probe used were as in [19]. Following two steps at 50°C for 2 min and 95°C for 10 min, 50 cycles of 1 sec at 95°C and 1 min at 60°C were carried out by a 7500 Fast Real-time PCR System (Applied Biosystems). Real-time PCR reactions were performed in duplicate, including a no template control consisting in the omission of DNA. A threshold cycle (Ct) value was calculated by determining the point at which the fluorescence exceeded a threshold limit (10 times the standard deviation of the baseline). Samples were defined positive for Ct values lower than 38 cycles. DNA integrity and amplification efficiency was checked by amplifying a fragment of the β-globin gene from each DNA preparation.

To test sensitivity and efficiency of the real-time PCR assay, dilution series (0.5 to 5×10^3 copies of EBV genome) of the DNA isolated from the EBV-positive JY cells [20] were analyzed in triplicate. The standard curve was obtained automatically by using the 7500 Fast System software. As negative control, DNA derived from Jurkat cells was amplified.

2.3.3. Real-Time RT-PCR for EBV Latency Transcripts. Real-time RT-PCR for the detection of EBV-encoded small RNA (EBER) 1, EBV nuclear antigen (EBNA) 1, and latent membrane protein (LMP) 1 transcripts was performed on DNase-treated RNA (0.5 μg) from thymic sections. TaqMan PCR primers and probes for EBER1, EBNA1, and LMP1 were as in [19, 21]. EBNA1 and LMP1 primers and probes were incorporated into TaqMan Gene Expression Assays by Applied Biosystems. RNA was amplified in a final volume of 20 μL containing 5 μL of 4x TaqMan Fast Virus 1-Step Master Mix and 1 μL of TaqMan Gene Expression Assay for EBNA1, LMP1, and GAPDH or 1.25 μM each primer and 0.18 μM probe for EBER1 (all from Applied Biosystems). TaqMan Fast Virus 1-Step Master Mix (Applied Biosystems) is designed for high-sensitivity virus detection and performs reverse transcription and PCR all in one reaction.

Real-time RT-PCR reactions were incubated on 7500 Fast Real-Time PCR System (Applied Biosystems) at 50°C for 5 min and 95°C for 15, followed by 50 cycles at 95°C for 15 sec and 60°C for 1 min. Real-time RT-PCR reactions were performed in duplicate, including a no template control consisting in the omission of RNA. Detection of GAPDH transcript (Applied Biosystems) served as control for the presence of template RNA and efficiency of real-time RT-PCR. A threshold cycle (Ct) value was calculated as described above for EBV DNA detection. Samples were defined positive for Ct value lower than 38 cycles.

To test sensitivity and efficiency of the real-time RT-PCR assays, dilution series (from 0.1 to 10^5 cells per reaction) of the RNA obtained from the EBV-positive cell line JY were amplified in presence and absence of 1 μg of RNA from the EBV-negative Jurkat T-cell line. The standard curve was obtained automatically by using the 7500 Fast System software. As negative control, RNA derived from Jurkat T cells was amplified. Each point of standard curve was run in triplicate. PCR product identity was checked by sequencing on an ABI 3100 Genetic Analyzer (Applied Biosystems).

2.3.4. Real-Time RT-PCR for BZLF1 Lytic EBV Transcript. For the detection of EBV lytic transcript BZLF1, DNase-treated RNA obtained from thymic sections was retrotranscribed into random-primed cDNA by using SuperScript Vilo cDNA Synthesis kit (Invitrogen). cDNA corresponding to 500 ng of RNA was amplified with the BZLF1-out forward and reverse primers previously reported [22]. Amplification was performed in a final volume of 50 μL consisting of 1x PCR buffer (Finnzyme, Espoo, Finland), 0.2 mM dNTPs (Applied Biosystems), 0.4 μM of each primer, and 1 U of DNAZyme (Finnzyme). After a predenaturation step at 95°C for 5 min, 40 cycles were repeated at 95°C for 1 min and 59°C for 1 min followed by an extension step of 7 min at 72°C. For each sample, 5 μL of PCR product were subjected to real-time PCR in a volume of 20 μL containing 10 μL of *Power* SYBR Green PCR Master Mix (Applied Biosystems) and 0.8 μM each of BZLF1-inn primers [22] and incubated on 7500 Real-time PCR System (Applied Biosystems). As a control of retrotranscription efficiency, β-actin gene was amplified in the same samples. cDNA from JY and Jurkat cell lines was amplified as positive and negative control, respectively, and PCR product identity was checked by sequencing on an ABI 3100 Genetic Analyzer (Applied Biosystems).

2.3.5. Immunohistochemistry. Immunohistochemistry was performed on 6-μm sections from snap frozen thymic tissues belonging to Patient Group 3 ($n = 19$). All the 19 thymuses were immunostained with antibodies specific for human CD20 (1 : 300; clone L26, Dako, Glostrup, Denmark) and CD138 (1 : 50; clone MI15, Dako). Sections from 8 MG thymuses (4 follicular hyperplasia, 2 thymitis, and 2 involuted thymuses) were immunostained with antibodies for latent EBV protein LMP-1 (ready-to-use, clone CS 1-4, isotype IgG1, Dako) and lytic EBV protein BZLF1 (1 : 10; isotype IgG2, Lifespan Biosciences Inc., Seattle, WA). Sections were fixed with 4% paraformaldehyde and incubated for 10 min

in 1.5% hydrogen peroxide in methanol, to eliminate endogenous peroxidase activity. For BZLF1 immunostaining, sections were treated with 0.1%Triton X 100 for 10 min. To block nonspecific binding, sections were incubated for 1 hour in 5% BSA. Incubations with primary antibodies were performed overnight at 4°C. Sections were then incubated with DakoCytomation EnVision + System Labelled Polymer-HRP Anti-Mouse (Dako) for 1 hour. Peroxidase reaction was visualized with 3,3′ diaminobenzidine (DAB) plus substrate buffer (Dako). All sections were counterstained with hematoxylin, visualized by optical microscopy (Nikon, Germany), and examined using Image Proplus (Media Cybernetics, Silver Spring, MD). Immunohistochemistry specificity was controlled by omitting the primary antibodies or replacing them with isotype-specific nonimmune IgG (Dako).

3. Results

3.1. Transcriptional Profiling of MG Thymus. To characterize the thymic transcriptome of MG patients for the expression of genes implicated in inflammation and immune response, we preliminarily used LDA approach on a small series of MG patients and found that gene expression profile of MG thymuses were distinct from that of control thymuses, delineating a thymic condition characterized by chronic inflammation and active immune response, as previously reported [14, 15]. Starting from our LDA results and from the published data [14, 15], we selected six genes (IL-6, IFN-γ, HLA-DRα, IL-10, IFN-β, and MxA), expected to be dysregulated during inflammation and immune response, and analysed their expression by real-time RT-PCR on a higher number of MG and control thymuses, including EBV-positive MG thymuses and EBV-negative nonpathological thymuses [6]. The results confirmed inflammatory state and immune response activation in MG thymus. LDA and real-time RT-PCR results are described in detail in what follows.

3.1.1. LDA Data Reflect Inflammatory State and Immune Activation in MG Thymus. The ninety genes included in the LDA assays were successfully amplified in MG and control thymuses. The analysis of variance identified 21 genes whose expression was significantly different among the MG and control sample groups ($P < 0.05$). The other genes were expressed at similar level in all groups except some of them (e.g., Bcl-2-like protein 1, a potent inhibitor of cell death; angiotensin II type-2 receptor, a protein belonging to the G-protein coupled receptor 1 family involved in programmed cell death; CD38, a transmembrane glycoprotein involved in cell adhesion, signal transduction, and calcium signalling) whose expression levels were lower in MG thymuses compared to controls, although the differences were not statistically significant. In Table 2, we reported LDA data for genes that were significantly upregulated in at least one of the three MG thymus subgroups versus normal thymuses. MG thymuses with thymitis showed the highest number of upregulated genes, prevalently cytokines and chemokines (Table 2). Among cytokines, IL-6—a well-known highly inflammatory cytokine implicated in chronic inflammatory and autoimmune diseases [23]—was the most overexpressed gene in each MG thymus subgroup compared to normal thymus, followed by IL-1β, a proinflammatory cytokine mainly produced by myeloid cells and also involved in various inflammatory and autoimmune diseases [24] (Table 2). Transcriptional levels of other molecules able to modulate inflammatory process, including colony-stimulating factor (CSF) 1, IL-7, IL-10, IL-12p35, TNF-α, and IFN-γ, were also higher in MG thymic conditions with respect to control thymuses (Table 2). A significant overexpression of IL-10—a Th2-produced cytokine having inhibitory properties on Th1 function and promoting humoral immune response [25, 26]—was identified whatever MG thymic subgroup was considered (Table 2).

Among chemokines, the chemokine receptor CXCR3, involved in recruitment and maintenance of activated T cells in the inflammatory site [27], was significantly upregulated in follicular hyperplasia and thymitis (Table 2), according to previous data [28]. Transcriptional levels of RANTES (CCL5), IL-8, monocyte chemoattractant protein- (MCP-) 1, macrophages inflammatory protein- (MIP-) 1α—monocyte chemotactic factors that are highly produced during microbial infection [29]—were increased in each thymic MG subgroup (Table 2).

We found that mRNA level of vascular endothelial growth factor A (VEGF-A)—a growth factor mediating vascular permeability and vasculogenesis [30]—was significantly upregulated in thymitis compared with normal thymuses (Table 2).

Moreover, LDA identified upregulation in MG thymus of some genes involved in immune response and antigen presentation. As expected, we observed increased expression of CD19—marker of B cells—in each MG thymus subgroup (Table 2), reflecting B cell infiltration characteristic of MG thymus [3, 6]. Transcriptional level of CD152 (CTLA-4) was higher in MG than normal thymuses, especially in thymitis. CD152 is a surface molecule mostly considered as a negative regulator of T-cell activation [31]; recently, it has been demonstrated that CD152 signalling may play a role in antimicrobial infection by endowing effector T cells with the capacity to migrate to sites of inflammation and lymph nodes [32].

In line with previous data [15], we found that MG thymuses had high expression levels of HLA-DRα (Table 2) and complement component C3 mRNA levels.

Among the 21 upregulated genes in MG thymus, there were also two genes, the SMAD family member 7 (SMAD7)—a nuclear regulator of transforming growth factor-β whose expression is altered in inflammatory diseases [33]—and the endothelial converting enzyme 1 (ECE)—a metalloprotease involved in proteolytic processing of endothelial precursors [34].

Differences between hyperplasia, thymitis, and involuted thymus were significant for CSF1, RANTES, VEGF-A, CXCR3, CD19, CD86, HLA-DRα, and MADH7 mRNAs, which were significantly higher in thymitis than involuted thymus; IL-1β and MCP-1 mRNAs, which were significantly higher in thymitis than hyperplasia; IL-7, IL-12p35, TNF-α, IFN-γ, IL-8, and CD34 mRNAs, which were

Table 2: Upregulated genes in follicular hyperplasia, thymitis, and involuted thymus versus normal thymus identified by TaqMan low-density arrays (LDAs).

Gene symbol	Biological function	Fold changes[a]		
		Hyperplasia	Thymitis	Involuted
CSF1	Cytokine	3.61 (1.23)	7.16 (2.78)*	2.22 (1.15)
IL-1β	Cytokine	11.29 (13.44)	51.72 (30.57)*	10.92 (10.92)
IL-6	Cytokine	29.56 (14.44)	1621.00 (513.70)**	72.60 (20.25)**
IL-10	Cytokine	5.93 (0.41)*	7.64 (1.12)*	7.14 (0.61)**
IL-12p35	Cytokine	4.09 (3.47)	15.42 (6.55)*	3.42 (2.11)
TNF-α	Cytokine	2.97 (1.61)	8.92 (2.95)*	3.27 (1.10)
IFN-γ	Cytokine	2.21 (0.78)	15.24 (4.89)*	1.78 (2.30)
RANTES	Cytokine	4.90 (1.07)*	6.79 (1.26)*	3.31 (0.95)
IL-7	Growth factor	5.25 (2.27)	21.18 (8.36)*	2.78 (0.92)
VEGF-A	Growth factor	5.01 (2.57)	20.07 (9.74)*	4.62 (3.02)
IL-8	Chemokine	3.90 (3.25)	16.22 (5.61)*	3.57 (2.00)
CXCR3	Chemokine receptor	4.56 (0.50)*	6.15 (1.19)*	2.57 (0.70)
MCP-1	Chemokine	4.41 (1.36)	31.08 (5.68)*	20.53 (1.60)**
MIP-1α	Chemokine	17.93 (11.38)	35.01 (11.34)*	16.11 (7.89)
CD19	CD antigen	4.37 (0.17)*	6.91 (1.41)*	3.51 (1.25)
CD86	CD antigen	1.82 (0.09)	3.82 (1.52)*	1.05 (0.44)
CD152	CD antigen	3.38 (0.49)	7.87 (3.37)*	2.97 (1.77)
HLA-DRα	MHC Class II	2.12 (0.39)	4.15 (1.05)*	1.58 (0.78)
C3	Complement component	10.66 (4.75)	29.12 (6.87)*	12.83 (10.88)
SMAD7	Cell signaling	2.53 (0.87)	4.78 (0.62)**	1.61 (1.06)
ECE1	Metalloprotease	2.57 (0.97)	6.34 (2.51)*	2.42 (1.51)

[a]For each gene, mean fold change (±SD) for the different MG thymus subgroups compared to normal thymuses is given. Fold change was calculated from the formula $2^{-\Delta\Delta Ct}$; $*P < 0.05$, $**P < 0.01$ (Bonferroni test). The genes in bold were further analysed by real-time RT-PCR.

significantly higher in thymitis than hyperplasia and involuted thymus.

3.1.2. Real-Time RT-PCR Shows Upregulation of Genes Involved in Inflammation and Antiviral Response in MG Thymus. Data derived from LDA were validated on a higher number of patients (Patient Group 2) and controls by conventional real-time PCR. We selected IL-6, IFN-γ, and HLA-DRα genes, known to be upregulated during inflammatory response against microbial infection and resulted upregulated in MG thymus by LDA (Patient Group 1, erefTabletab1). We also investigated the expression of IL-10, for its role in modulating B cell function and humoral responses [26], type I IFN-β, which plays a pivotal role in the host immune response against viral infections [25], and MxA, an important mediator of type I IFNs [35].

We found a significant upregulation of IL-6 in each MG thymus subgroup compared with normal thymuses (Figure 1(a)), confirming LDA data. An increased expression of IL-10 was also observed in all MG thymuses; in particular, IL-10 expression was significantly increased in hyperplasia and thymitis cases (Figure 1 (b)). Transcriptional level of IFN-β was significantly higher in thymitis and involuted thymuses (Figure 1(c)), whereas IFN-γ transcript was significantly up-regulated in hyperplasia and thymitis cases (Figure 1(d)). A significant increase in MxA expression was detected in each MG thymic subgroup, supporting the hypothesis of an ongoing antiviral response in MG thymus (Figure 1(e)). HLA-DRα also showed a significant upregulation in each MG thymic pathology, again supporting the inflammatory state of MG thymus (Figure 1(f)).

3.2. Characterization of MG Thymus for the Presence of EBV DNA, RNA, and Proteins. To confirm previous evidence of EBV infection in MG thymus [6], we investigated thymic tissues from 19 MG patients (Patient Group 3, Table 1) and two adult healthy donors for the presence of EBV DNA (*Bam*HI-W repeat region), RNA (EBER1, EBNA1, LMP1, and BZLF1), and proteins (LMP1 and BZLF1).

3.2.1. Presence of Infiltrating B Cells and Plasma Cells in MG and Control Thymuses. MG thymuses were initially examined for the presence of lymphoid B cell infiltrates and plasma cells, in order to verify that the thymic fragments under investigation contained B cells/plasma cells potentially positive to EBV. Presence of lymphoid B cell infiltrates (diffuse or organized in GCs) and plasma cells was found in all MG thymus specimens examined (Figures 2(a) to 2(f)); thus, alternate sections collected from these specimens were used for further analysis of EBV DNA and RNA presence.

3.2.2. Detection of EBV DNA. We used real-time PCR to detect EBV genome (*Bam*HI-W repeat region) in MG thymuses. We first validated our real-time PCR assay by

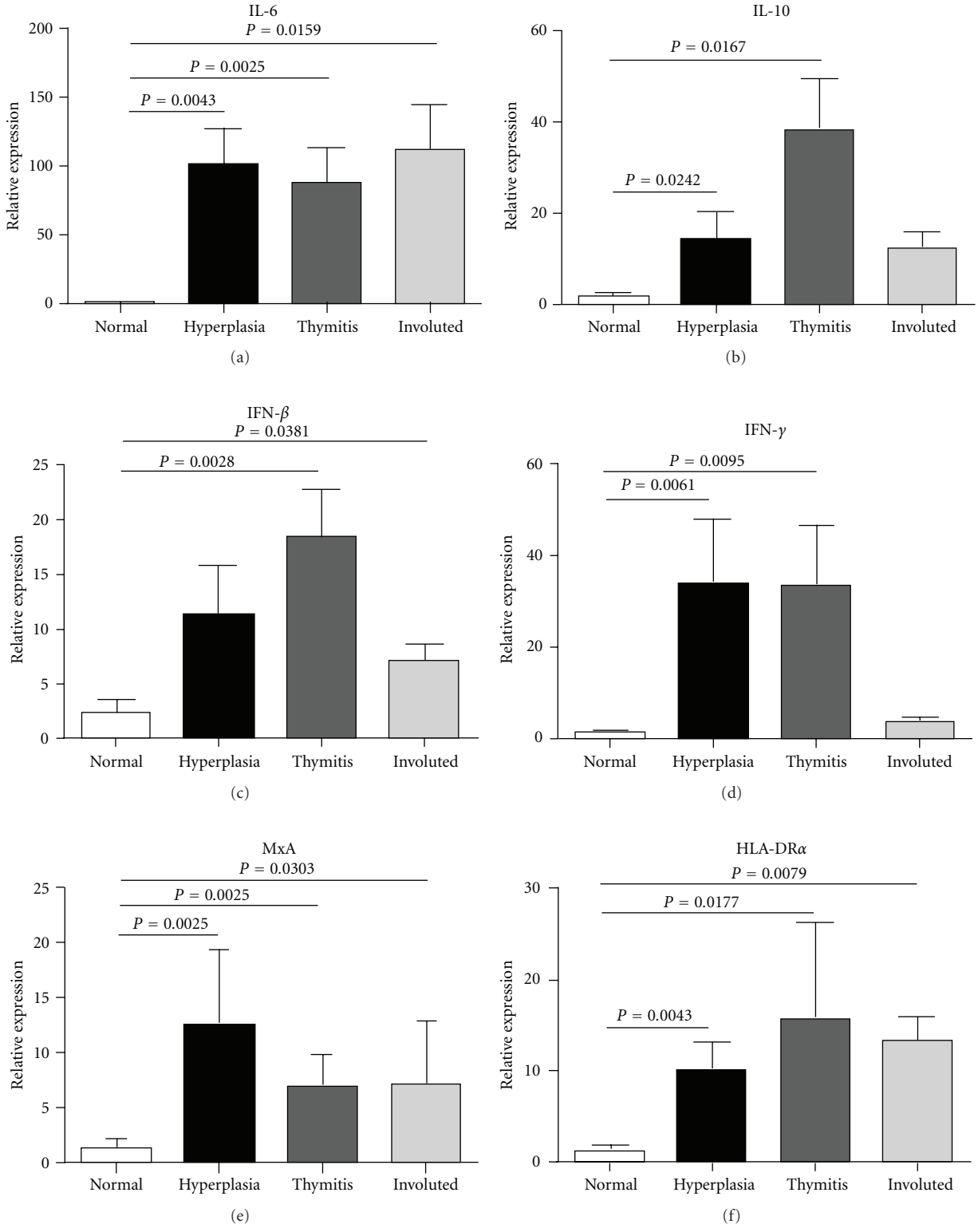

Figure 1: Relative expression of IL-6 (a), IL-10 (b), IFN-β (c), IFN-γ (d), MxA (e), and HLA-DRα (f) in the thymus of MG patients. The transcripts were analysed by real-time PCR analysis starting from total RNA extracted from the thymus of MG patients with hyperplasia ($n = 9$), thymitis ($n = 9$), and thymic involution ($n = 9$), and 7 healthy subjects. Relative expression of the 6 genes was normalized to GAPDH and calculated as $2^{-\Delta\Delta Ct}$; normalized values for nonpathological thymuses were used as calibrator. Values shown are means ± SEM of duplicate determinations. P values were obtained by the Mann-Whitney U test.

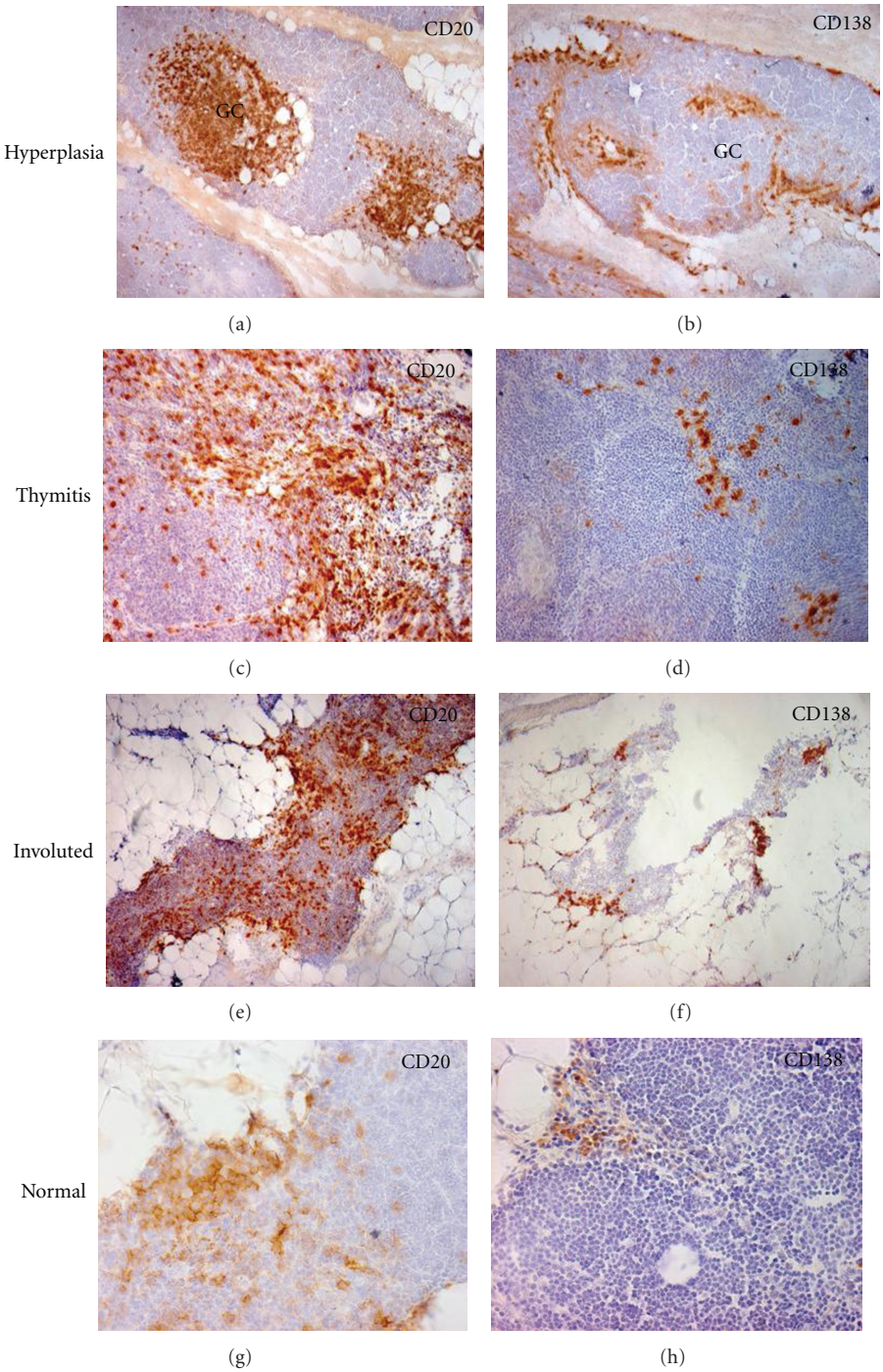

Figure 2: Presence of B cell lymphoid infiltrates and plasma cells in MG and control thymuses analysed for EBV detection. (a, b) Thymus with follicular hyperplasia (MG1). CD20+ B cells aggregate to form a germinal center (GC) in the thymic medulla (a). Many CD138+ plasma cells are present at the periphery of a GC (b). (c, d) Thymus with thymitis (MG11). Many CD20+ B cells (c) and CD138+ plasma cells (d) are sparse throughout the medullary infiltrates. (e, f) Involuted thymus (MG17). The residual thymic parenchyma contains lymphoid infiltrates with numerous CD20+ B cells (e) and CD138+ plasma cells (f). (g, h) Normal thymus from an adult healthy subject. Some CD20+ cells (g) and rare CD138+ plasma cells (h) are present in the thymic parenchyma. Magnifications: ×10 (a, b, e, f); ×20 (c, d); ×40 (g, h).

demonstrating that it was able to detect EBV DNA with high sensitivity and specificity. By amplifying dilution series of DNA from the EBV-positive JY cells [20], we obtained a standard curve showing linearity in the range from 0.5 to 5×10^3 copies per reaction, with 0.99 regression coefficient (R^2) and −3.56 slope, corresponding to 90.98% efficiency. We argued that the molecular system resulted in high and constant amplification efficiency and that >0.5 copy of

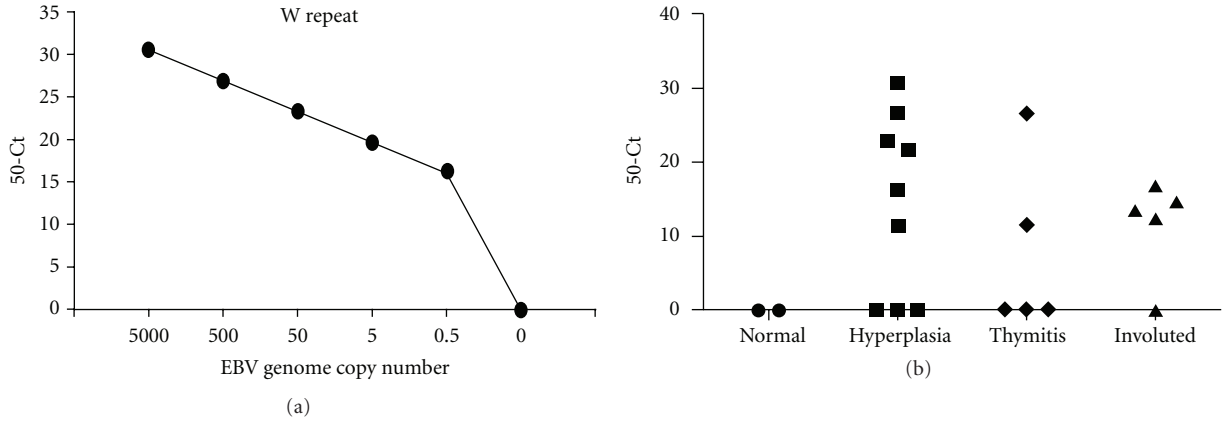

Figure 3: Real-time PCR for EBV genome detection (*Bam*HI-W repeats). (a) Dilution series of DNA (ranging from 0.5 to 10^5 copies of EBV genome per reaction), extracted from the JY lymphoblastoid cell lines, were analysed as described in Section 2. Real-time PCR resulted in high and constant amplification efficiency for >0.5 copies of EBV genome per reaction. (b) Genomic EBV W repeats were detectable in 6/9 hyperplastic thymuses, 2/5 thymitis, and 4/5 involuted thymuses, but not in two nonpathological control thymuses. Real-time PCR was performed for 50 cycles, and results are expressed as 50-Ct.

Table 3: Detection of EBV DNA and RNA transcripts in MG thymus by real-time PCR.

Patient	Thymic pathology	Anti-AChR antibodies[a]	EBV DNA[b]	Latent markers[b]			Lytic marker[b]
				EBER1	EBNA1	LMP1	BZLF1
MG1	Hyperplasia	Positive	+	+	+	−	+
MG2	Hyperplasia	Positive	+	+	+	+	+
MG3	Hyperplasia	Positive	−	+	+	−	−
MG4	Hyperplasia	Positive	−	+	+	−	+
MG5	Hyperplasia	Positive	+	+	+	+	+
MG6	Hyperplasia	Positive	+	+	+	+	+
MG7	Hyperplasia	SN	+	−	−	+	+
MG8	Hyperplasia	SN	−	−	+	−	+
MG9	Hyperplasia	SN	+	+	+	−	+
MG10	Thymitis	Positive	−	−	−	+	+
MG11	Thymitis	Positive	−	+	+	−	+
MG12	Thymitis	Positive	+	−	−	−	−
MG13	Thymitis	Positive	+	+	+	−	−
MG14	Thymitis	SN	−	−	+	+	+
MG15	Involuted	Positive	+	+	−	−	+
MG16	Involuted	Positive	+	+	+	+	+
MG17	Involuted	Positive	+	+	+	+	+
MG18	Involuted	Positive	+	+	+	+	+
MG19	Involuted	Positive	−	+	+	−	+
Ctr1[c]	Normal	SN	−	−	−	−	−
Ctr2[c]	Normal	SN	−	−	−	−	−

[a] SN: seronegative (AChR- and MuSK-negative) patients. [b] EBV genome and transcripts were analysed by real-time PCR techniques as described in Section 2. Results are expressed as follows: + detected (Ct value <38); − not detected (Ct value >38). [c] Ctr: nonpathological control thymus.

genome for reaction could be quantified with an acceptable level of accuracy (Figure 3(a)). β-globin control gene was detected at similar level in all MG and control samples (data not shown). EBV genome was detected in 12/19 (63.2%) MG thymuses (6/9 hyperplasia, 2/5 thymitis, and 4/5 involuted thymuses) but not in control thymuses (Figure 3(b) and Table 3) and EBV-negative Jurkat T-cell line.

3.2.3. Detection of Latent EBV Transcripts. We used real-time RT-PCR to analyse latent EBER1, EBNA1, and LMP1 transcripts in MG and control thymuses. We first validated our real-time RT-PCR assays by performing a number of control experiments. By using RNA from the EBV-positive JY cells, we demonstrated that our assays were able to detect the target transcripts in RNA from one EBV-positive JY cell for

reaction (Figures 4(a) to 4(c)) but not in the EBV-negative Jurkat T-cell line. Standard curves for the detection of the three targets showed R^2 always higher than 0.99 and slope ranged from −3.22 and −3.47, corresponding to efficiencies higher than 94%. Real-time RT-PCR were also able to detect the target RNA from one EBV-positive JY cell in the presence of 1 μg of RNA from the EBV-negative Jurkat T-cell line (~100,000 cells), indicating that our molecular systems could detect 1 positive cell/~100,000 negative cells.

EBER1 (Figure 4(d)), EBNA1 (Figure 4(e)), and LMP1 (Figure 4(f)) transcripts were detected in 14/19, 15/19, and 9/19 MG thymuses, respectively, but not in control thymuses (Figures 4(d) to 4(f) and Table 3). Housekeeping gene GAPDH was detected in all the specimens analysed (Figure 4(g)).

3.2.4. Detection of Lytic BZLF1 EBV Transcript. BZLF1 transcript was detected in 16/19 MG thymuses but not in normal control thymuses (Figure 5(a)). In all MG and control cDNA specimens, the housekeeping gene β-actin was efficiently amplified (Figure 5(b)).

3.2.5. Detection of Latent LMP1 and Lytic BZLF1 EBV Proteins. To confirm results of molecular analysis at protein level, we performed immunohistochemistry assays to detect LMP1 and BZLF1 proteins, markers of EBV latency and reactivation, respectively, on 8 MG and 2 control thymuses. Cells expressing LMP1 were detected in 7/8 MG thymuses analysed but not in normal thymuses (Figure 6 and Table 4). Immunoreactivity for LMP1 was mainly detected in GC and perifollicular areas in hyperplasia (Figures 6(e) and 6(f)) and within medullary infiltrates in thymitis and involuted thymuses (Figures 6(g) and 6(h)). We found cells expressing the early lytic phase EBV protein BZLF1 in thymic medulla of most MG thymuses examined (7/8) but not in normal thymuses (Figures 6(l) to 6(p) and Table 4), thus suggesting productive, not only latent, EBV infection in the thymus of MG patients.

Table 4: Detection of EBV latent LMP1 and lytic BZLF1 proteins in MG thymus by immunohistochemistry.

Patient	Thymic pathology	Anti-AChR antibodies[a]	EBV proteins[b]	
			LMP1	BZLF1
MG1	Hyperplasia	Positive	+	+
MG5	Hyperplasia	Positive	+	+
MG6	Hyperplasia	Positive	+	+
MG9	Hyperplasia	SN	+	+
MG11	Thymitis	Positive	−	+
MG13	Thymitis	Positive	+	−
MG16	Involuted	Positive	+	+
MG17	Involuted	Positive	+	+
Ctr1[c]	Normal	SN	−	−
Ctr2[c]	Normal	SN	−	−

[a] SN: seronegative (AChR- and MuSK-negative) patients. [b] Results of immunostaining for LMP1 and BZLF1 are expressed as follows: + presence of positive cells; − absence of positive cells. [c] Ctr: nonpathological control thymus.

4. Discussion

This study confirms and extends previous evidence of inflammation and viral infection in the thymus of MG patients.

4.1. Inflammation and Active Immune Response Characterize the Thymus of MG Patients. Previous analyses of the genes characterizing the hyperplastic thymus using microarray and real-time PCR approaches showed that transcripts of a large number of genes associated with inflammation and immune response were significantly upregulated in hyperplastic MG thymuses compared to controls [14, 15]. The upregulated genes included IFN-regulated genes, MHC Class II molecules, Ig family, and B cell-related genes, whose increase reflected an inflammatory state and a generalized B cell infiltration in hyperplastic MG thymus [14, 15].

In the present study, we used LDA approach to characterize the thymic transcriptome in 10 MG patients (Patient Group 1) whose thymuses had histopathological features of hyperplasia, thymitis, and thymic involution, and in non-MG subjects having normal thymuses.

LDA, a TaqMan quantitative PCR based on microfluidic systems, represents a valuable approach for sensitive and quantitative gene expression profiling that enables high throughput screening in functional genomics by simultaneously analysing mRNA expression of multiple genes in human tissues [36]. LDA technology allowed us to analyse mRNA of 90 genes belonging to different biological categories, including genes involved in inflammatory and immune responses. All the target transcripts were detected in MG and control thymuses. However, 21 genes were upregulated in MG thymuses compared to controls (Table 2): (a) proinflammatory cytokines, able to activate immune cells and having antiviral properties (i.e., IL-6, IL-1β, CSF, IL-7, IL12p35, TNF-α, and IFN-γ); (b) cytokines, chemokines, and molecules involved in migration, homing, and survival of lymphocytes in biological site of inflammation or infection (i.e., IL-6, RANTES, IL-8, MCP-1, MIP-1α, CXCR3, and CD152); (c) B-cell-related genes and genes related or potentially related to antigen presentation and humoral response (i.e., IL-10, HLA-DRα, CD19, and complement component C3).

The expression of proinflammatory cytokines reached the highest values in the thymitis cases (Table 2). Most of these cytokines are known to work in synergy and to promote inflammation and immune response during host defence, especially against viral infections [25]. Some of them are potent inflammatory molecules mainly involved in acute inflammation (i.e., IL-6, IL-1β, TNF-α, and CSF); others are mainly involved in establishing chronic inflammation and promoting humoral and cellular immune response (i.e., IL-7, IL-10, IL-12, and IFN-γ) [25]. Upregulation of IL-6 and the chemokine RANTES in MG compared to normal thymus was in line with previous studies showing that these genes were abnormally overexpressed in MG TECs either at basal condition [37] or (IL-6) when stimulated

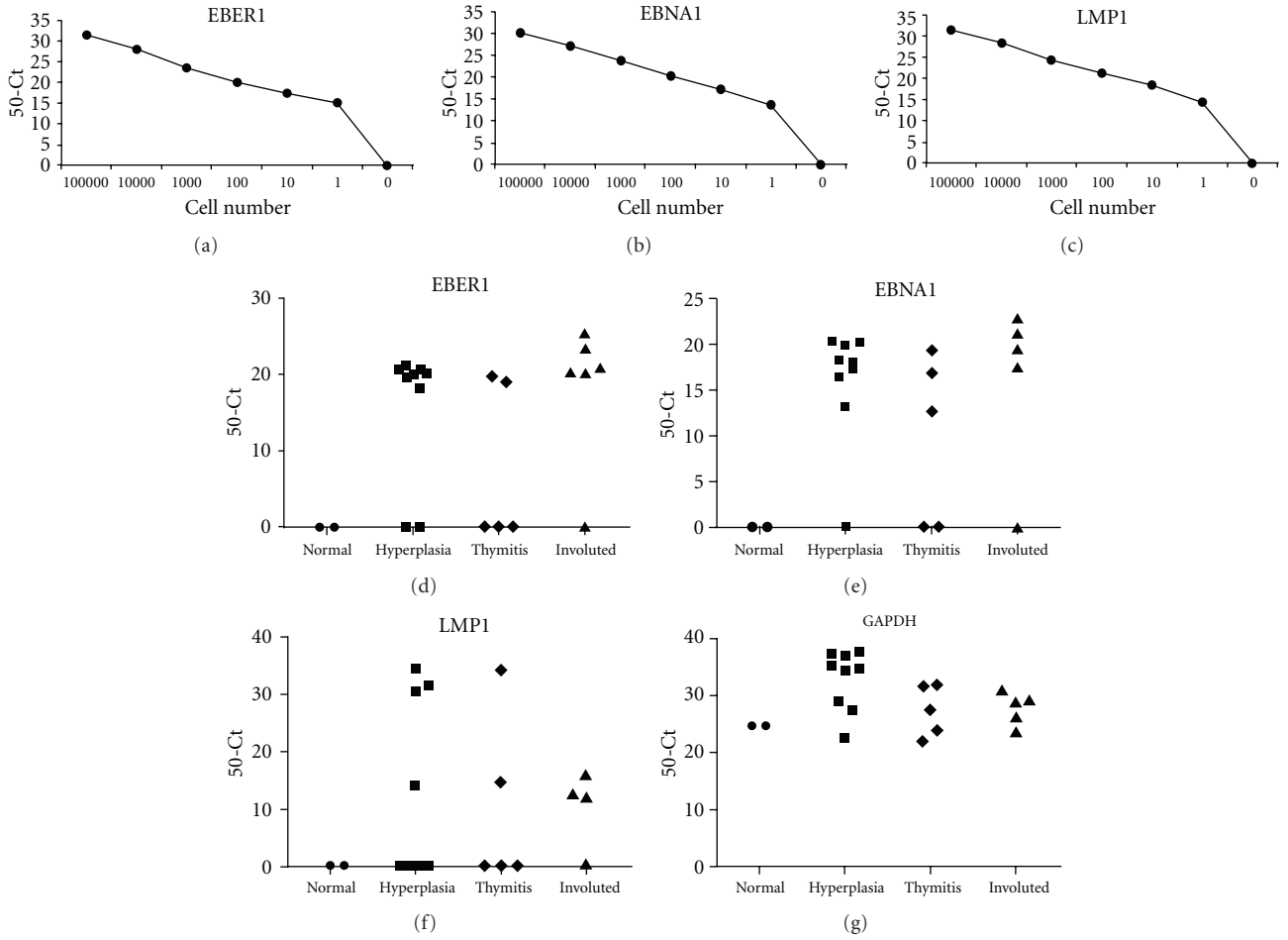

Figure 4: Real-time RT-PCR for the detection of latent EBER1, EBNA1, and LMP1 transcripts. (a, b, c) Analysis of sensitivity of the assay for EBER1 (a), EBNA1 (b), and LMP1 (c) showed that the three EBV latent transcripts could be detected in RNA extracted from a single JY EBV-infected cell. (d, e, f, g) EBER1 (d), EBNA1 (e), and LMP1 (f) were detected in most of the examined MG thymuses but not in normal control thymuses. All MG and control thymuses analysed showed high signals for endogenous control GAPDH amplification (g). Real-time RT-PCR was performed for 50 cycles, and results are expressed as 50-Ct.

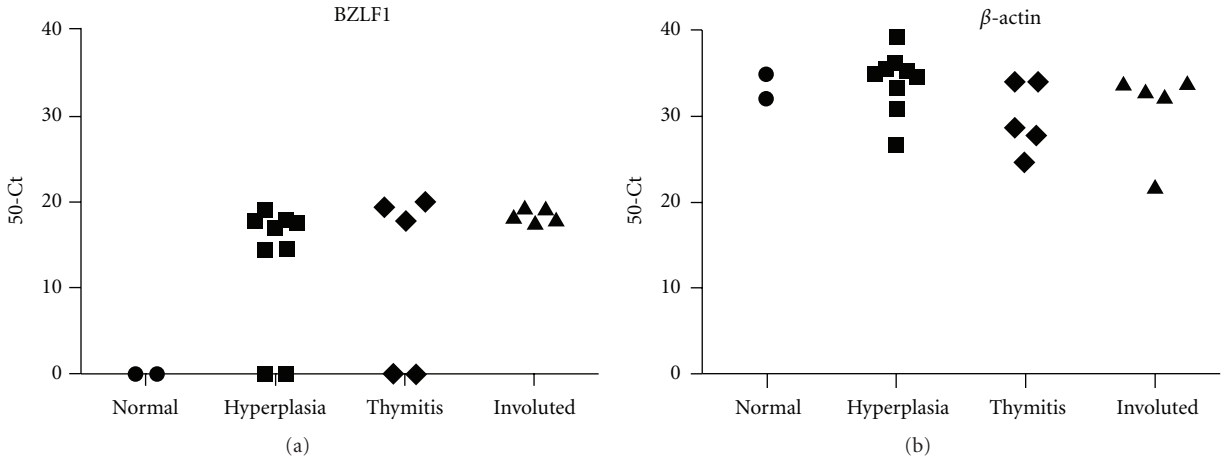

Figure 5: Real-time RT-PCR for the detection of lytic BZLF1 transcript. (a) Real-time RT-PCR for BZLF1 could detect the lytic transcript in 7/9 hyperplastic thymuses, 3/5 thymitis, and 5/5 involuted thymuses, but not in normal control thymuses. (b) All MG and control thymuses analysed showed high signals for endogenous control β-actin amplification.

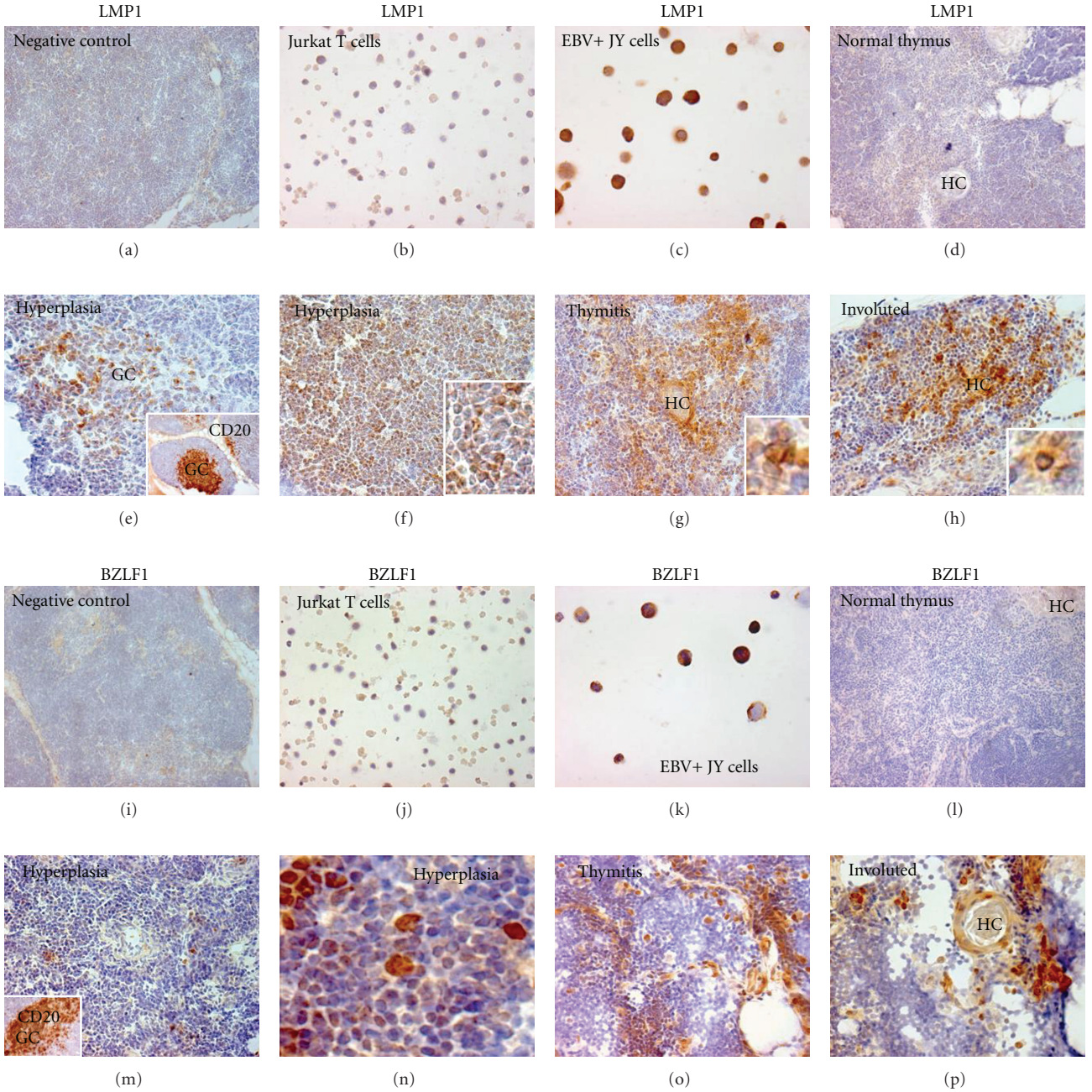

Figure 6: Immunohistochemistry for latent LMP1 (a)–(h) and lytic BZLF1 (i)–(p) EBV proteins. (a) to (h) LMP1 immunostaining. No signal of immunoreactivity was observed in negative control performed by incubating sections with isotype-specific nonimmune IgG (Dako) (a). LMP1 was not detected in EBV-negative Jurkat T-cell line (b) but was readily detectable in EBV-positive JY cells (c). Normal thymuses showed no immunoreactivity for LMP1 antibody (d). In hyperplastic thymuses (MG1 is shown), LMP1+ cells were detected in areas containing CD20+ B cells organized in germinal centers (GCs) ((e) and inset in (e)) or were diffused throughout the highly infiltrated medullary region (f). Numerous LMP1+ cells were identified in thymitis cases (MG13 is shown), that were diffused in thymic medulla and frequently located around Hassall's corpuscles (HCs), where often concentrate B cells (g). In involuted thymuses (MG16 is shown), numerous LMP1+ cells were scattered in the residual thymic parenchyma and frequently located in thymic infiltrated areas around HCs (h). Inset in (e) shows CD20 immunostaining of the same area of the main panel in a serial section. Insets in (f), (g), and (h) show areas of the main panels at higher power of magnification to reveal membrane localization of LMP1. (i) to (p) BZLF1 immunostaining. No signal of immunoreactivity was observed in negative control performed by incubating sections with isotype-specific nonimmune IgG (Dako) (i). BZLF1 was not detected in EBV-negative Jurkat T-cell line (j) but was readily detectable in EBV-positive JY cells (k). Normal thymuses showed no immunoreactivity for BZLF1 antibody (l). In hyperplasia (MG1 is shown), BZLF1+ cells were often detected at the edge of GCs ((m) and inset in (m)) or were scattered in thymic medulla (n). Inset in (m) shows CD20 immunostaining of the same area of the main panel in a serial section. In thymitis (MG11 is shown) (o) and involuted thymuses (MG17 is shown) (p), BZLF1+ cells were present in thymic medulla, in some cases located within medullary infiltrates in proximity to HCs. Magnifications: ×20 (a, d, i, l); ×40 (b, c, e, f, g, h, j, k, m, o, p); ×80 (n).

by lipopolysaccharide (LPS) [38], a major activator of Toll-like receptor (TLR) 4 known to be upregulated in MG thymus [39]. IL-6 is a well-known proinflammatory agent with pathological regulatory function on growth and differentiation of T- and B-cells [40]; RANTES has been observed to regulate the transepithelial migration of T cells [41]. Thus, overexpression of IL-6 and RANTES could support the migration of peripheral lymphocytes to thymus and their survival there, contributing to the pathological remodeling of the gland typical of MG [37]. Overexpression of IL-10 (Table 2) was also of pathogenic relevance, as IL-10 is a B-cell-related cytokine which modulates inflammatory processes and determines the antibody response by influencing B cell activation, proliferation, and differentiation [26]. IL-10 was found to be upregulated in serum of MG patients after immunoadsorption, indicating that this cytokine might be linked with the IgG synthesis or resynthesis process in MG [42]. Moreover, it has been shown that EBV infection through EBER signalling is able to induce the expression of IL-10 in B lymphocytes, where this cytokine could act as autocrine growth factor [43]. Thus, the observed IL-10 upregulation may be also explained by the previously observed intrathymic EBV infection in MG thymus [6] and implicated in B cell abnormalities which characterize this organ in MG patients.

IFN-γ is a type II interferon which exhibits strong antiviral properties and ability to enhance MHC Class I and II expression on nucleated cells [25]. In our LDA data, both IFN-γ and HLA-DRα were upregulated in MG thymuses (Table 2), supporting an antiviral reaction and a local proinflammatory environment. Increased expression of MHC Class II molecules was previously observed by Le Panse and colleagues [15], whose results indicated that overexpression of these molecules in MG hyperplastic thymus was not directly related to the increased B cell number but could be due to the proinflammatory state of MG thymus.

Our transcriptome analysis showed also overexpression of IL-8, MCP-1, and MIP-1α (Table 2); these are inflammatory chemokines linked to innate immune responses and acting as leukocyte chemotactic factors [29]; their expression was higher in MG thymuses than in controls (Table 2), suggesting that they could be implicated in abnormal recruitment of T and B cells. Chemokine receptor CXCR3, known to drive migration and homing of activated T cells in inflammatory site [27], was particularly upregulated in hyperplasia and thymitis (Table 2) confirming previous observations of increased expression of CXCR3 and its ligand IFN-γ-inducible protein 10 in the thymus of MG patients [28].

The idea that thymic microenvironment in MG patients is favourable to abnormal migration of T cells is further supported by the observation that the expression of CD152 (CTLA-4) and its CD86 ligand was increased in MG thymuses compared to controls (Table 2). The CD152 molecule is generally considered as a negative regulator of T-cell activation [31]; however, a recent study showed that CD152 signalling does not simply silence T cells but endows their capacity to migrate to sites of infection and secondary lymphoid organs [32], by upregulating the expression of CCR7 on T cells [32]. Therefore, the high expression level of CD152 in MG thymus might be due to the enrichment of CCR7 + CD152 + T cells within the inflamed MG thymus as a consequence of chemokine expressed on specialized lymphatic vessels, for example, the CCR7 ligand CCL21 [44]. The increase of VEGF-A, a growth factor mediating vascular permeability and vasculogenesis [30], and ECE, a metalloprotease implicated in proteolytic processing of endothelial precursors [34], might be associated with the abnormal lymphocyte recruitment and the angiogenic processes occurring in MG thymus [44].

Our LDA data showed increased expression of CD19, marker of B cells, in each MG thymus subgroup, thus reflecting the presence of significant B cell infiltration in MG thymus; these results are in line with previous microarray data obtained from Le Panse and colleagues [15].

We also found increased levels of complement component C3 mRNA in MG compared to normal thymuses (Table 2), consistent with previous observation of persistent complement attack on AChR-expressing thymic epithelial and myoid cells in MG hyperplastic thymus; this attack might be responsible for an increased level of autoantigen presentation to dendritic cells sustaining autoimmune reaction [45].

To confirm the MG thymic inflammatory state suggested by LDA data, we performed real-time PCR analysis of six genes, selected for playing key roles in inflammation and host defence responses against infections, in a total of 27 MG (Patient Groups 1 and 2) and 7 control thymuses. These genes were IL-6, IL-10, IFN-γ, and HLA-DRα, previously analysed by LDA, and IFN-β and MxA. IFN-β was chosen to search for evidence of action of type I IFNs in MG thymus, as this type of IFNs plays key roles in host immune response against viral infections and has been widely implicated in autoimmune conditions [46]; MxA was investigated as it is an important mediator of type I IFNs in the innate antiviral response [35].

Real-time PCR analysis confirmed upregulation of IL-6, IL-10, IFN-γ, and HLA-DRα in MG thymuses compared to controls (Figure 1). Interestingly, IFN-β and MxA genes were also overexpressed in MG thymuses, supporting the hypothesis of an ongoing antiviral and inflammatory response in MG pathological tissues. Overexpression of IFN-γ and IFN-β was in agreement with previous data [14] showing that large number of type I and type II IFN-induced genes were significantly upregulated in hyperplastic MG thymuses compared to controls. Previous transcriptional profile analysis of thymus from untreated and steroid-treated MG patients showed that the inflammatory state was reduced upon treatment [47]; in particular, the expression of type I IFN-induced genes, but not of type II IFN-induced genes, was normalized, suggesting that inflammation downmodulation by steroids occurs through type I IFN-pathways [47]. Our thymic transcriptome analysis by LDA and real-time PCR underlines a generalized thymic inflammatory state in MG patients, with increased expression of inflammatory genes being observed even for patients treated with corticosteroid before thymectomy (Table 2, Patient Group 1 and 2). This suggests that inflammatory condition does not completely disappear or is maintained after immunosuppressive treatment. However, the number of steroid-untreated patients

we analysed were low (2/10 patients in LDA and 5/27 in real-time PCR analysis); thus, further studies are needed to understand whether immunosuppressive treatment is able to reduce the MG intrathymic proinflammatory condition and establish whether other genes, besides type I IFN-induced genes [47], undergo normalization.

The overall results of our transcriptional profiling confirm that MG thymus is characterized by a chronic inflammatory state. Whether this state is the consequence of viral infection events remains to be clarified. Our previous study showing increased expression of TLR 4—key member of innate immunity—in MG thymuses with thymitis and thymus involution [39], together with the finding of a persistent poliovirus infection in the thymus of some MG patients [16], strongly supports a role of viral infections and innate immune system activation as trigger events for inflammation and intrathymic autosensitization in MG.

4.2. EBV Infection Is Commonly Found in MG Thymus. In our previous study, we demonstrated active EBV infection in 17/17 nonneoplastic MG thymuses investigated, irrespective of thymic pathology, whereas no evidence of EBV infection was found in 6 control thymuses from adult healthy subjects [6]. Specifically, in the MG thymuses analyzed, we found (a) a high frequency of EBV-infected B cells by in situ hybridization for EBERs and immunohistochemistry for latent (EBNA2, LMP1, LMP2A) and lytic (BFRF1, BMRF1, gp350/220, p160) EBV proteins; (b) expression of latent (EBNA1, LMP2A) and lytic (BZLF1) genes by nested PCR reactions on cDNA; (c) presence of EBV DNA by real-time PCR specific for LMP1 gene [6].

In the present study, we addressed whether EBV infection is a characteristic feature of MG thymus by extending our search for EBV-associated nucleic acids and proteins in additional 19 MG thymuses (Patient Group 3). We decided to apply different molecular approaches from those previously used [6], in order to verify whether we were equally able to detect EBV DNA and RNA in MG thymus (see Section 2).

Consistent with our previous findings [6], all 19 MG thymuses investigated showed signs of EBV infection (Table 3). EBV DNA was detected in 12/19 MG thymuses (Figure 3 and Table 3); EBV latent or lytic transcripts (often both) were present in all, except one (MG12), MG thymuses, whereas no sign of infection was found in two nonpathological controls (Figure 4 and Table 3). The one MG sample (MG12) negative for EBV transcripts, but harbouring infiltrating B cells by immunohistochemistry, had detectable EBV DNA genome, suggesting that the degree of EBV infection in this thymic specimen could be low (or confined to few cells).

EBV-encoded RNA called EBER1 is expressed at high levels in EBV-infected cells during latency [17, 18]; most MG patients were positive to EBER1 (Figure 4), consistent with results of our previous study in which the use of in situ hybridization for EBERs allowed us to identify a high proportion of EBERs-positive cells in most of the examined MG thymuses irrespective of the thymic pathology [6]. Here, the application of real-time RT-PCR to detect EBER1, as well as the use of independent real-time PCR assays to detect EBV DNA, LMP1, and EBNA1, strongly confirm evidence for EBV latency in MG thymus.

To establish latent infection, EBV uses four different latency gene programs (latency III, II, I, and 0), each characterized by expression of a set of viral genes that provide activation, growth, and survival signals to infected B cells [17, 18]. In this study, we detected in MG thymuses EBNA1, which is expressed in all EBV latency programs, and LMP1, which is expressed in latency III (or growth program) and latency II (or default program). In our previous study [6], we searched also for LMP2A (both transcript and protein), which is expressed in latency III and II, and EBNA2 (protein), the first latency protein to be synthesized after infection of naïve B cells and expressed only in latency III (or growth program) [17, 18]. We detected LMP2A, whereas EBNA2 was rarely detected, being identified only in rare cells in few MG thymuses, likely newly infected cells [6]. These previous results, together with those presented here, seem to suggest that EBV mainly uses the latency II to establish a latent infection in MG thymus.

Of the EBV lytic genes, we analyzed the immediate early lytic gene BZLF1, which encodes a transactivator protein regulating expression of early lytic genes [17, 48]. Consistent with our previous results [6], BZLF1 transcript was detected in most (16/19) of the examined pathological thymuses but in none of controls (Table 3 and Figure 4), indicating productive viral infection in MG thymus that may result in new infection events and propagation of EBV infection within MG thymus.

We demonstrated that our real-time PCR analysis could detect EBV transcripts in RNA extracted from a single JY EBV-infected cells (Figures 3 and 4). However, we were unable to detect all the viral transcripts analysed in all the MG thymuses investigated, although all patients were positive for at least one transcript. As suggested by Aloisi and colleagues [49], this may be due to the fact that the quality of RNA extracted from fragments of bioptic tissue sample cannot be exactly compared with that of viable, highly replicating lymphoblastoid cells that contain multiple copies of EBV genome and display high transcriptional activity. Moreover, successful detection of EBV nucleic acids within a highly heterogeneous cell population, which is a feature of a human tissue, may be difficult to achieve [49].

To confirm at the protein level the results of molecular analysis, we performed immunostaining for LMP1 and BZLF1, a latent and a lytic marker (Table 4). As in our previous study [6], we found numerous LMP1-expressing cells in the thymic medulla of MG thymuses with hyperplasia, thymitis, and thymic involution, in areas corresponding to lymphoid infiltrates and (in hyperplasia) to GCs (Figure 6). LMP1 was not detected in the normal thymuses analysed (Figure 6). In the same tissue, immunohistochemistry also revealed the presence of cells positive to BZLF1 in each MG thymic subgroup but in none of the control thymuses (Figure 6). LMP1 and BZLF1 proteins were not detected in 2 of the 8 MG thymuses analysed (LMP1 in MG11 and BZLF1 in MG13), in which the corresponding transcript was also not detected.

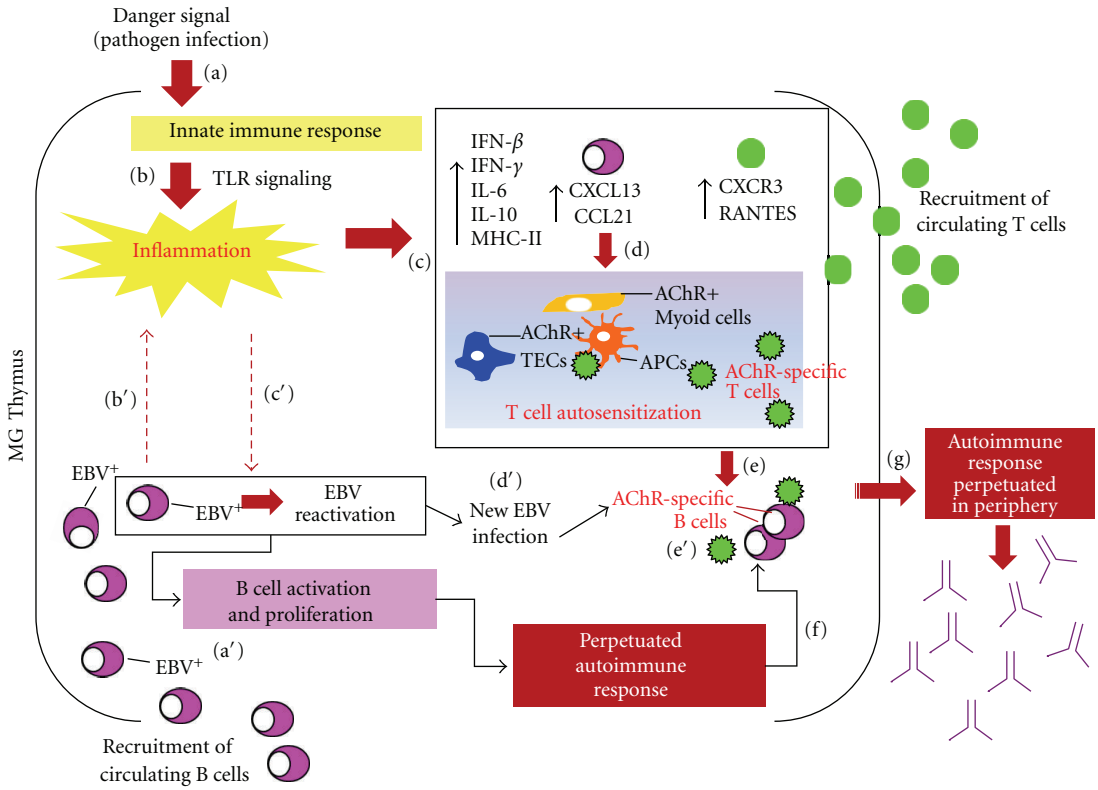

Figure 7: Proposed model of virus-induced autoimmunity in MG. A "danger signal" (e.g., pathogen infection) stimulates Toll-like receptor- (TLR-) mediated innate immune responses (a), whose dysregulated or persistent activation leads to the chronic inflammation characteristic of MG thymus (b). The chronically established thymic inflammatory state (c), characterized by overexpression of proinflammatory cytokines (e.g., IL-6, IL-10), type I and II IFNs, and T- and B-lymphocyte-attracting chemokines (e.g., CXCR3, RANTES, CXCL13, CCL21), is essential, in the context of a genetically predisposing background, for the establishment of mechanisms (d) contributing to T-cell autosensitization, including presentation or cross-presentation of "self-epitopes" by TECs or myoid cells expressing the autoantigen; upregulation of MHC genes; activation of antigen-presenting cells (APCs); as well as the constant priming of autoreactive T cells, which in turn promote autoimmune response by autoreactive B cells (e). B cell attractants CXCL13 and CCL21 recruit circulating B cells to thymus, including those harboring EBV (a′). EBV infection itself contributes to thymic inflammation (b′). EBV reactivation, influenced by the inflammatory state (c′), results in EBV propagation to uninfected B cells (d′) including AChR-specific B cells (e′). The chronically established inflammation and EBV infection promote the maintenance within the thymus of the autoimmune response (f), which may be thus perpetuated in periphery (g).

Most of the examined patients (12/19) underwent immunosuppressive therapy before thymectomy (Table 1). Of the remaining 7 patients, 6 (MG1, MG3, MG5, MG8, MG9, and MG13) were only treated with acetylcholinesterase inhibitors and one (MG4) was untreated. We found evidence of EBV infection also in the thymus of these 7 patients, thus suggesting that intrathymic EBV dysregulation is not the consequence of immunosuppressive therapy. However, we cannot exclude that immunosuppressive drugs could amplify an established intrathymic EBV infection.

In conclusion, the findings here presented strengthen the idea that EBV is implicated in the intrathymic pathogenesis of MG. EBV infection might result in the maintenance of the autoimmune response in MG thymus by contributing to chronic B cell activation and promoting survival and expansion of autoreactive B cell clones. Whether active intrathymic EBV infection is a primary event in MG or the consequence of an underlying intrathymic process that results in both attraction of circulating EBV-infected cells and EBV reactivation in MG thymus needs to be clarified. The high prevalence of EBV infection in the population and low incidence of MG suggests that other factors (genetic or environmental, or both) must intervene together with EBV to cause MG. It is possible that a preexisting inflammatory state might be necessary for colonization of thymus by EBV-infected B cells and subsequent reactivation of these cells, and in turn a chronic EBV infection itself might play a role in sustaining chronic intrathymic inflammation in MG creating a vicious circle.

5. Conclusions

Inflammation is an important contributor factor in the development and progression of autoimmune diseases. The results of transcriptional profiling here presented, by confirming previous data showing inflammation and active immune response in MG thymus, strongly support the

idea that the creation of a local proinflammatory state is a pathogenic feature of thymus in MG patients.

We postulate that a chronically established thymic inflammation may be essential, in the context of a genetically predisposing background, for the establishment of mechanisms contributing to MG autoimmunity including presentation of "self-epitopes"; upregulation of MHC genes, of type I IFNs, of proinflammatory cytokines, of adhesion, and of costimulatory molecules on antigen-presenting cells; as well as the constant priming of autoreactive T cells [13] (Figure 7). Evidence of persistent viral presence in MG thymuses, derived from our recent studies [6, 16], suggests that an initial pathogen infection might be responsible for the observed inflammatory signature and the subsequent autoantigen sensitization in MG thymus. In particular, our recent finding [6], here confirmed and reinforced, of an active EBV infection in the intrathymic B cell component in MG patients suggests that EBV infection, together with inflammation, may be a key step in the intrathymic pathogenesis of MG. Inflammation triggered by an endogenous or exogenous (e.g., microbial infection) danger signal may drive the colonization of thymus by EBV-harbouring B cells and the subsequent EBV reactivation (Figure 7). Persistent EBV infection itself may contribute to maintain a chronically inflamed thymic microenvironment. In the inflamed thymus, EBV may promote disruption of B-cell tolerance checkpoints and result in expansion of autoreactive B cell clones (Figure 7). EBV infection thus could explain how the autoimmune response can be perpetuated in MG thymus, since EBV is potentially able to immortalize B cells that are producing AChR antibodies.

By adding new evidence for inflammation and EBV infections as common feature of MG thymus, our findings may have relevant therapeutic implications: they reinforce the rationale for current therapeutic approaches, particularly anti-inflammatory drug use and thymectomy to remove the site of infection, and also suggest future rationale preventive and therapeutic measures for MG, such as EBV vaccination [50] or regulation of the existing EBV infection by the use of antiviral agents.

Conflict of Interests

None of the authors have any financial conflict of interests.

Acknowledgments

The authors thank Professor A. Lanzavecchia (Institute for Research in Biomedicine, Bellinzona, Switzerland) for the kind gift of the EBV-positive JY lymphoblastoid cell line. They thank the Italian Association against Myasthenia (AIM: Associazione Italiana Miastenia e Malattie Immunmodegenerative) for financial support. This work was also supported by 7th Framework Programme of the European Union FIGHT-MG (Grant no. 242210) and by the Italian Ministry of Health Annual Research Funding (RC2010/LR5 and RC2011/LR5).

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SAGE-Hindawi Access to Research
Autoimmune Diseases
Volume 2011, Article ID 250561, 6 pages
doi:10.4061/2011/250561

Review Article

Anaesthetic Considerations in Paediatric Myasthenia Gravis

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Received 31 May 2011; Accepted 2 August 2011

Academic Editor: Shigeaki Suzuki

Myasthenia gravis is of particular interest to anaesthetists because of the muscle groups affected, the pharmacology of the neuromuscular junction, and interaction of both the disease and treatment with many anaesthetic drugs. Anaesthetists may encounter children with myasthenia either to facilitate treatment options or to institute mechanical ventilation in the face of a crisis. This paper reviews the literature pertaining to the pathophysiology and applied pharmacology of the disease and explores the relationship between these and the anaesthetic management. In addition to illustrating the tried-and-tested techniques, some newer management options are explored.

1. Introduction

Myasthenia gravis (MG) is an autoimmune disease of the neuromuscular junction characterised by weakness and fatiguability of skeletal muscles. It can present significant challenges to the anaesthetist, not only because of the nature of the disease itself but also because of the treatment that patients may be on and the interaction of these treatments with many anaesthetic drugs.

MG affects children in three main forms—juvenile, neonatal, and congenital. Juvenile is the commonest form of the disease representing 10–15% of all cases of myasthenia in both children and younger adults [1]. It is caused by IgG autoantibodies which are directed towards the acetylcholine receptors on the postsynaptic membrane of the neuromuscular junction. This causes both a decrease in receptor numbers as well as a conformational change in the motor endplate, resulting in fewer synaptic folds and a wider synaptic cleft [2]. As a consequence, the usual physiological excess of acetyl choline is deranged and a nerve action potential may not result in muscular contraction. In juvenile MG the thymus gland is often hyperplastic, and although the exact relationship between the gland and the disease remains obscure, certain patients are known to benefit from thymectomy [3, 4].

Neonatal MG affects 10% of babies born to mothers suffering from MG and is caused by the placental transfer of maternal antiacetylcholine receptor antibodies. Recovery tends to occur spontaneously within about 6 weeks. In some cases mechanical ventilation and treatment with anticholinesterases may be required [5].

Congenital MG is a rare heterogeneous disease which can affect the whole neuromuscular junction. It is classified into presynaptic, synaptic, and postsynaptic forms, and treatment and clinical picture depend on the subtype [6]. Owing to its rarity and the fact that anaesthetic experience is extremely limited, we shall not consider it further. In this paper we shall concentrate on the juvenile form of the disease as this is the form which is most likely to be encountered by anaesthetists.

Anaesthetic management depends upon sound understanding of the pathophysiology of the disease process as well as the pharmacology of the neuromuscular junction.

2. Clinical Picture

The clinical picture is one of weakness and fatiguability of skeletal muscles. A common presentation is of ocular weakness resulting in ptosis, diplopia, or variable strabismus. Presentation is however variable and may involve ocular signs, generalised weakness, or a combination [7–10]. Bulbar weakness is often a prominent feature and may result in feeding difficulties as well as dysarthria. Respiratory muscles are affected in about 20% of myasthenic patients though rarely in isolation [5]. Myasthenic crises (see below) may

occur during times of physiological stress such as infection, surgery, or heightened emotion and result in an acute deterioration in muscle strength necessitating mechanical ventilation [11].

3. Diagnosis

Confirmation of a diagnosis involves 3 tests [11–13]. Administration of edrophonium—a short acting acetylcholinesterase inhibitor—inhibits the breakdown of acetylcholine at the neuromuscular junction (NMJ). Thus, the physiological excess of acetylcholine is restored. The test, if positive, results in a rapid improvement in muscle strength which occurs after about 30 s and lasts for about 5 minutes. This is often known as the Tensilon test. Antibodies to the acetylcholine receptor itself can be detected in the blood of certain individuals with MG. The test is highly specific and becomes increasingly sensitive with increasing age and increasing duration of disease such that about 56% of prepubertal children and up to 82% of peripubertal children with juvenile MG will have detectable antibodies against the acetylcholine receptor [14]. Single fibre EMG is a definitive way of elucidating neuromuscular transmission disorders but is not specific for MG and may well not be tolerated in children [15].

4. Treatment

There are 2 main approaches to the treatment of children with myasthenia gravis [16]. The first strategy is to pharmacologically enhance the amount of acetylcholine at the NMJ. The second strategy is to modulate the immune system. Most patients require elements of both.

Acetylcholinesterase inhibitors work by inhibiting the actions of acetylcholinesterase—an enzyme present at the NMJ which breaks down acetylcholine. During a normal muscular contraction, acetylcholine released from the presynaptic terminal diffuses across the NMJ combining with acetylcholine receptors on the motor endplate. Conformational change of the receptor causes influx of sodium ions resulting in depolarisation, release of calcium from the sarcoplasmic reticulum, and subsequent muscular contraction. Acetylcholine at the NMJ is broken down by acetylcholinesterase thus terminating its actions and allowing for closure of sodium channels and subsequent repolarisation of the motor end plate. Acetylcholinesterase inhibitors work by inhibiting the breakdown of acetylcholine meaning that more is available to facilitate muscular contraction. They can be categorised according to their duration of action. Edrophonium (see above) is a short-acting drug, useful in diagnosis but too short acting to be useful in treatment. Of the longer-acting agents, pyridostigmine is generally preferred as it has a longer duration of action and less muscarinic side effects than neostigmine [7]. Most patients will respond well to administration of acetylcholinesterase inhibitors.

Second-line treatment involves administration of corticosteroids in an attempt to modulate the immune system and consequently reduce the serum level of antibodies against the acetylcholine receptor. If used, they are generally used in relatively large doses carrying the risk of significant side effects—all of which are well documented in children [15]. Other immunomodulatory drugs such as cyclosporine, azathioprine, and cyclophosphamide are rarely used, due to their significant side-effect profile and limited experience in the paediatric setting.

In the event of an acute deterioration administration of immunoglobulins (IVIGs) or plasma exchange may help to stave off mechanical ventilation [15, 17]. Of the two, plasma exchange is the more effective but also the more laborious to perform, requiring large bore central venous access. For this reason, administration of immunoglobulins is generally the preferred first-line approach, with plasmapheresis available for the more serious cases.

The thymus gland is implicated in a high proportion of children with juvenile MG. Thymectomy has been shown to bring about improvement in symptoms in between 60–90% of patients undergoing the procedure [9, 15]. The effects of thymectomy may take a period of months or even years to bring about such improvements. The procedure is generally performed when children are at least 10 years old [18]. If performed too early the chances of remission are higher and there are theoretical concerns regarding removal of the thymus gland at a period when the immune system is still developing.

5. Crises

Myasthenic patients can suffer an acute deterioration in their neuromuscular function sometimes necessitating mechanical ventilation. These episodes are known as crises and take two main forms—myasthenic and cholinergic [11]. A myasthenic crisis is brought about by a relative decrease in the amount of acetylcholine at the NMJ resulting in muscular weakness. It may be precipitated by stress, surgery, or infection, and treatment depends on the administration of anticholinesterases or in more severe cases IVIG or plasma exchange transfusion.

Cholinergic crisis is brought about by excessive administration of acetylcholinesterase inhibitors resulting in an excess of Ach at the motor end plate. In addition to muscular weakness, the child may also demonstrate other cholinergic signs such as sweating, salivation, diarrhea and blurred vision. Treatment is based upon withholding administration of further acetylcholinesterase inhibitors and supportive measures.

The two crises are difficult to distinguish from each other clinically but can be differentiated by the administration of edrophonium. This will bring about an improvement in a myasthenic crisis but worsen a cholinergic one [7]. Evidently, full resuscitative equipment must be on hand prior to its administration.

6. Pharmacological Considerations Specific to Anaesthesia

6.1. Neuromuscular Blocking Agents. Myasthenic patients have a variable response to muscle relaxants depending

on the type of muscle relaxant used. Decreased density of Ach receptors at the motor end plate means that paediatric myasthenic patients may require up to 4 times the calculated dose of suxamethonium in order to bring about a depolarising muscle block [7]. In addition, suxamethonium is metabolised by acetylcholinesterase, and as a result, treatment with acetylcholinesterase inhibitors causes a reduction in its metabolism and a prolongation of its action [19]. For this reason suxamethonium should be avoided in myasthenic patients.

The nondepolarising muscle relaxants on the other hand have a significantly enhanced activity as well as a significantly prolonged duration of action [20]. Unfortunately, the degree to which sensitivity is increased is unpredictable and depends on interaction between disease severity and efficacy of treatment [7, 21–23]. In adults, the most extensively studied muscle relaxants are atracurium and vecuronium. In children, Brown recommended atracurium as the relaxant of choice, due to its metabolism which has been shown to be noncumulative [7]. Rocuronium has been shown to be safe in adults with myasthenia gravis [24], and there are also several reports of its actions being successfully terminated by the administration of sugammadex [25, 26]. Sugammadex negates the need to administer acetylcholinesterase inhibitors to reverse residual neuromuscular blockade and does not carry the associated risks of precipitating a cholinergic crisis. To date, the authors are unaware of any reports in the literature highlighting the use of either rocuronium or sugammadex in paediatric myasthenic patients.

6.2. Anaesthetic Agents. Volatile anaesthetic agents are already known to inhibit neuromuscular transmission, and these effects are thought to be exaggerated in myasthenic patients [27–32]. However, no clinically significant postoperative neuromuscular depression has ever been demonstrated with isoflurane, sevoflurane, or desflurane [27, 32, 33]. These neuromuscular deficits are not a feature of propofol, making total intravenous anaesthesia (TIVA); theoretically at least, the technique of choice for these patients [29].

Remifentanil is metabolised by nonspecific esterases, and as a result, concern has been expressed regarding a prolonged duration of action in patients treated with acetylcholinesterase inhibitors [34]. This however has not been shown to be the case. Similarly, plasma exchange transfusion is thought to decrease the concentration of plasma esterases. Prolongation in the clearance of remifentanil has however never been demonstrated following plasma exchange transfusion—possibly as it is metabolised to a significant extent by tissue esterases [35].

6.3. Anticholinesterases. As already mentioned these drugs bring about a significant prolongation of the duration of action of suxamethonium (and also mivacurium by the same action [36]). In addition, patients receiving preoperative treatment with anticholinesterases may show a decreased response to the administration of neostigmine intraoperatively [12]. This may make reversal of residual neuromuscular blockade even more challenging. Furthermore, intraoperative administration may precipitate cholinergic crisis, potentially confusing the issue of the patient with postoperative weakness.

7. Anaesthetic Management

Anaesthetists may become involved in the management of these children for several reasons. Patients may suffer a crisis necessitating institution of mechanical ventilation or the siting of large-bore central venous access to facilitate plasma exchange transfusion. They may require thymectomy, or they may require surgery unrelated to their myasthenia—either in an elective or an emergency situation. Below are some of the general principles that apply to the management of these children, with further detail pertaining to those requiring thymectomy at the end.

7.1. Preoperative. Severity of weakness and muscle groups affected should be carefully noted and documented with particular focus on respiratory and bulbar function. Respiratory function tests may be useful in determining the degree of respiratory involvement and the likely requirement for post-operative ventilation. In children, these are frequently not possible, irrespective of whether in the elective or emergency setting. For elective surgery, pre-perative consultation with the child's neurologist should be sought, in order to optimise treatment. Thorough preoperative assessment and pre-optimisation has been shown to improve the frequency of post-operative complications [37]. In the case of severe weakness, pre-operative administration of IVIG or even exchange transfusion may be required to improve neuromuscular function. The child should be placed first on the morning list and have their morning dose of anticholinesterases omitted [38]. The exception to this may be the child with severe weakness who may require continuation of this treatment in order to prevent a significant deterioration.

Clearly in the emergency setting, these are luxuries that one is unlikely to be able to afford. Nevertheless, due consideration should be given towards the level of pre-optimisation that may be achievable within the time constraints imposed by the urgency of surgery.

7.2. Anaesthetic Technique. Technique will be influenced significantly by the extent and nature of planned surgery, whether emergency or elective and whether there is risk of a full stomach. For all but the most minor surgery in the stable myasthenic without significant respiratory or bulbar compromise, an endotracheal tube and intermittent positive pressure ventilation are likely to be required. If possible, intubation of the trachea should be performed without the use of muscle relaxants. In the paediatric setting, this is already well described and used frequently. Tracheal intubation with volatiles alone or following an iv induction with propofol and a short-acting opioid are well described in myasthenic children [39–42]. The use of propofol and remifentanil in the form of target-controlled infusion (TCI) is well described in adult myasthenics [43, 44]. TIVA without TCI has also been described in the paediatric literature, but until more recently

pump technology has not allowed this technique to be used successfully in children under the age of 16 years [35, 45]. It will be interesting to see whether reports of its use appear in the literature over coming years.

If muscle relaxants are used, they should be used in smaller doses and should always be used in conjunction with effective neuromuscular monitoring [38]. This should be in the form of a nerve stimulator with an accurate, objective assessment of muscle response such as acceleromyography, mechanomyography, or electromyography.

Children on high-dose corticosteroids will require supplementation in the perioperative period [13].

The situation of the myasthenic patient requiring rapid sequence induction because of full stomach is a difficult one. Suxamethonium should be avoided because of the reasons already mentioned. Although rocuronium could theoretically be used, determining the dose to give is more difficult. Sugammadex has been used to reverse neuromuscular blockade following administration of $0.5\,\text{mg}\cdot\text{kg}^{-1}$, but there is no experience of its use following the higher dose required for rapid sequence induction [26]. Intravenous induction with propofol ($3\,\text{mg}\cdot\text{kg}^{-1}$), remifentanil ($1.25\,\text{mcg}\cdot\text{kg}^{-1}$) and lidocaine ($1.5\,\text{mg}\cdot\text{kg}^{-1}$) has been described in a 14-year-old girl requiring rapid sequence induction because of a full stomach [46]. It should be noted that this patient had severe preexisting neuromuscular dysfunction, though the authors noted that excellent intubating conditions were obtained after 60 seconds. There are no other reports in the literature of rapid sequence induction of either adult or paediatric patients.

7.3. Postoperative Considerations. Planning for the postoperative period requires meticulous communication between surgeon, anaesthetist, neurologist, and intensivist. Even following a minor procedure in a stable patient, a period of close observation in the recovery area is required to ensure that neuromuscular function—especially pertaining to respiratory and bulbar function—has returned to the preoperative state. Accordingly, the more extensive the surgery, the greater the duration of procedure, and the greater the exposure to drugs that interfere with neuromuscular function, the more important this period becomes. Those at risk of significant reduction in neuromuscular function, or those who have significant respiratory or bulbar impairment preoperatively, may require observation on an intensive care unit. Postoperative ventilation, assuming reasonable respiratory function preoperatively, is rarely required even following extensive surgery [13, 47].

In the event of a significant deterioration in neuromuscular function postoperatively, attention needs to be given towards ascertaining whether one is dealing with a myasthenic or cholinergic crisis. Cholinergic crisis, even if acetylcholinesterase inhibitors have been administered at the end of the procedure, is very rare. If there is any doubt as to the exact nature, edrophonium as opposed to neostigmine should be used due to the rapid onset and offset. Provision should always be made for elective reintubation if respiratory function is deemed inadequate.

7.4. Thymectomy. Thymectomy can be performed via an open sternotomy or via a thoracoscopic approach. Thoracoscopic thymectomy is associated with less chest wall trauma and may be associated with lower post-operative morbidity and shorter hospital stay [12, 48]. Surgery itself, however, may be prolonged, and because the mediastinum is accessed via the chest wall, selective one-lung ventilation required. Commonly, the right lung is isolated. A variety of methods have been used, including endobronchial intubation, bronchial blocker, double lumen endotracheal tube, and standard endotracheal tube with carbon dioxide insufflation of the left hemithorax [48]. The potential haemodynamic consequences of such techniques and the effects on gas exchange necessitate invasive arterial monitoring.

Analgesia is another important issue in these patients—especially those undergoing open sternotomy. Epidural analgesia has been described in adult myasthenics undergoing median sternotomy [49]. The requirement for high thoracic block has often deterred paediatric anaesthetists from using this technique due to the possibility of haemodynamic compromise (hypotension and bradycardia) and respiratory embarrassment. More recently, reports have appeared in the literature of using high thoracic epidural to great effect—providing post-operative analgesia without any haemodynamic or respiratory compromise [45]. In cases where epidural analgesia is not used, remifentanil infusion intraoperatively followed by administration of a longer-acting opioid towards the end of the procedure and PCA has been described [38].

Patients should be extubated as soon as possible at the end of the procedure to reduce the incidence of respiratory complications. Prolonged invasive ventilation is associated with greater respiratory morbidity [50]. If the patient is usually taking anticholinesterases but has omitted them on the morning of surgery, a dose of neostigmine is likely to be required towards the end of surgery. Anticholinesterases should be continued in the post-operative period, and dose should be titrated towards effect. Corticosteroids can be weaned down with a view towards stopping [38].

8. Conclusions

In this paper we have presented some of the main issues pertaining to the anaesthetist in the management of the child with juvenile MG. We have looked at the underlying pathophysiology of the disease process and how this relates to the applied pharmacology of drugs affecting the neuromuscular junction. We have examined the controversial issue of neuromuscular blocking drugs and explored some of the alternatives to their use. These alternatives often render neuromuscular blocking agents obsolete. In addition, the role of TIVA as the anaesthetic of choice has been explored, and we look forward to hearing about its use in conjunction with TCI more frequently in the future as experience with newer pump technology continues to grow. Finally, we have examined some of the key points in the management of patients presenting for thymectomy.

Optimal management of any child with myasthenia gravis, irrespective of surgery performed, requires a sound

understanding of disease pathophysiology and pharmacology, meticulous planning, and close consultation between surgeon, anaesthetist, and neurologist.

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SAGE-Hindawi Access to Research
Autoimmune Diseases
Volume 2011, Article ID 808607, 7 pages
doi:10.4061/2011/808607

Research Article

Long-Term Respiratory Muscle Endurance Training in Patients with Myasthenia Gravis: First Results after Four Months of Training

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Received 31 May 2011; Accepted 24 June 2011

Academic Editor: Nils Erik Gilhus

Myasthenia gravis (MG) is characterized by reduced muscle endurance and is often accompanied by respiratory complications. Improvement of respiratory function is therefore an important objective in MG therapy. A previous study demonstrated that respiratory muscle endurance training (RMET) over four weeks increased respiratory muscle endurance of MG patients to about 200% of baseline. The purpose of the present study was to establish an appropriate maintenance training and to test its effects over four months. Ten patients with mild to moderate MG participated in this study. During the first month, they performed five training sessions per week. For the following 3 months, training frequency was reduced to five sessions per two weeks. Myasthenia score, lung function, and respiratory endurance were determined prior to training, after the first month, and after 4 months. Myasthenia score improved from 0.71 ± 0.1 to 0.56 ± 0.1 ($P = 0.007$). Respiratory endurance time increased from 6.1 ± 0.8 to 20.3 ± 3.0 min ($P < 0.001$). In conclusion, this RMET maintenance program is feasible and is significantly beneficial for MG patients.

1. Introduction

Myasthenia gravis (MG) is an autoimmune disease characterized by blockade of the neuromuscular synapse. Hence, muscle strength and, particularly, endurance are reduced, ensuing in increased muscular fatigue [1, 2]. In most MG patients, the entire muscular system is concerned, and this may also involve respiratory muscles. Despite normal spirometric values, patients with generalized MG often present a characteristic "myasthenic pattern" with decreasing respiratory volumes during MVV [3] and reduced respiratory muscle endurance [2]. Respiratory muscle dysfunction can further deteriorate patients' physical fitness and evoke upper airway obstruction [4], sleep apnea [5, 6], or even respiratory failure as the characteristic feature of myasthenic crisis [7, 8]. Improvement of respiratory muscle function is therefore an important objective in MG therapy.

In addition to pharmacological or operative treatment, exercise therapy can be used as an adjuvant method in therapy of MG [9, 10]. Besides general exercise programs, specific respiratory muscle training could be beneficial especially for patients with compromised respiratory function. Positive effects of respiratory muscle training on respiratory muscle strength and endurance in patients with pulmonary disorders were demonstrated for the first time by Keens et al. [11]. Likewise, in patients with neuromuscular diseases respiratory dysfunction due to inadequate function of respiratory muscles is a strong rationale for a specific training of respiratory muscles. Numerous studies on respiratory muscle training have been performed in patients with spinal cord injury [12, 13], postpolio syndrome [14], or with neuromuscular disorders such as Duchenne's muscular dystrophy or spinal muscular atrophy [15, 16] demonstrating

improvement of lung function and of respiratory muscle strength and/or endurance.

In contrast, there is only little experience with specific respiratory training in MG patients [18–20]. These studies reported beneficial effects of respiratory training on respiratory muscle strength and/or lung function. None of these studies applied sustained hyperpnea for training. However, maintenance of an elevated level of ventilation over a longer period of time such as in situations of increased physical activity would be an appropriate training for patients with increased respiratory muscle fatigue.

Several years ago, we used a normocapnic hyperpnea training that had been previously applied in healthy untrained and trained subjects [21–23] as well as in patients with chronic obstructive pulmonary disease (COPD) [24, 25]. This type of respiratory muscle endurance training (RMET) has also been applied in MG patients in a previous study [26]. Four weeks of this normocapnic hyperpnea training in MG patients resulted in a more than twofold enhancement of respiratory muscle endurance as reflected by time to exhaustion (T_{Lim}) and total ventilated volume (V_{Lim}) in a respiratory endurance (RE) test. However, this gain in respiratory muscle endurance reduced after termination of the training period. Maintaining improved respiratory muscle endurance requires to regularly continue RMET. This might be hampered by strenuousness and expenditure of time associated with the training. Therefore, the aim of the present study was to establish a maintenance training program and to test it for feasibility and benefit with respect to respiratory muscle endurance, MG symptoms, and lung function.

2. Methods

2.1. Subjects. The patients involved in this study were regularly consulting two neurologists specialized in MG who were involved in this study (IB, PK). We chose 27 patients with mild to moderate generalized MG (degree II according to MGFA classification [17], degree 1–3 according to Oosterhuis classification [1]) as possible participants. They have been suffering from MG for 1–39 years. Patients with ocular symptoms only and hospitalized patients were excluded. Eleven of the preselected patients resigned from participation in the training study due to problems with transportation or with their time schedule (5 patients), or because they felt no need of respiratory training (6 patients). Six patients tried the use of the training device but did not manage the rebreathing technique, the use of the training device, and the training frequency. In the end, 10 patients (5 male, 5 female, average age 60 ± 4.2 y) participated in the study. Characteristics of these patients are given in Table 1. Five of them had already participated in our first RMET study several years ago [26]. All participants had experienced respiratory symptoms or problems in the past due to their myasthenia and, for this reason, were motivated to perform the respiratory endurance training. Seven patients had additional chronic diseases; five suffered from arterial hypertension, two of them additionally from diabetes mellitus. One patient had a coronary heart disease, and one other patient had a Lupus erythematodes. All patients were free from chronic respiratory diseases. None of the patients smoked at present. Two patients were ex-smokers but had ceased smoking at least fifteen years before. All participating patients gave their written informed consent. The study was approved by the local ethics committee.

2.2. Study Protocol. The study consisted of two phases. Phase 1 included a four-week training period; during phase 2, the training was continued for another three months. The protocol of phase 1 was the same as applied in the first study [26] but without a detraining period. In brief, all patients including those who had already participated in the previous study received a detailed explanation and demonstration of all testing and training details and then practiced the use of the training device at home for one week 10 min per day. The pretraining tests (baseline, B) were performed 6–8 weeks later. They contained an MG score (Besinger score [17, 27]), lung function testing, and an RE test. For lung function tests including spirometry and maximal voluntary ventilation (MVV) and for the RE test, we used a metabolic cart (MetaMax 3B, Cortex Biophysik GmbH, Leipzig, Germany). Additionally, respiratory muscle strength (maximum inspiratory pressure, $\mathrm{PI}_{\mathrm{max}}$) at residual volume (RV) was determined ($\mathrm{resPI}_{\mathrm{max}}$, Andos, Hamburg, Germany). For the RE test, patients connected their training device to the metabolic cart and breathed with a tidal volume (V_T) ranging between 50 and 75% of VC at a rate of 25–40 breaths per minute at normocapnic conditions. This set-up was intended to induce test termination after a maximum of 10–12 min. Criteria to terminate the test were patients' perception of exhaustion or reduction in ventilation ($\dot{V}_E$) by more than 10% of the target for 1 min. This test was accomplished at least two times on separate days with the best test being evaluated. We measured endurance time (T_{Lim}: time until test termination) and endurance volume (V_{Lim}: total volume breathed during the test, calculated as T_{Lim} multiplied by $\dot{V}_E$).

The normocapnic hyperpnea training started after completion of the baseline tests. During phase 1, all patients accomplished 20 training sessions in a period of 4–6 weeks with about five training days and two resting days per week. Each training session lasted 30 min. Patients achieved isocapnia by using a portable rebreathing device as described in detail by Markov et al. [23], thus performing partial rebreathing. Patients were carefully coached beforehand to be attentive to sensations of air hunger or dizziness as symptoms of hypercapnia or hypocapnia. Additionally, we repeatedly performed pCO_2 measurements in the laboratory to assure normocapnia. Target values of $\dot{V}_E$, V_T, and breathing rate (f_R) were defined in the same range as in the previous study [26], that is, $\dot{V}_E$: 50–60% of individual MVV, V_T: 50–60% of VC, f_R: 25–35 min^{-1}. Patients were instructed to perform the training at home always at the same time of a day and at a constant time interval after medication. After each training session, they had to fill in a short questionnaire regarding changes in MG symptoms (see Appendix) and to assess occurrence and degree of air hunger and respiratory effort using visual analogue scales. Moreover, after each training

Table 1: Characterization of patients participating in respiratory endurance training.

Patient	Gender	Age (y)	BMI (kg/cm^2)	MG degree		Diagn. (y)	Medication	
				MGFA	OO		ChEI (mg/d)	IT (mg/d)
1	F	43	29.3	IIa	2	2	180	
2	M	62	27.4	IIa	2	9	420	100
3	F	33	19.4	IIb	3	11	240	
4	M	66	32.4	IIa	2	9		100
5	M	75	25.9	IIa	2	4	300	
6	F	54	23.9	IIa	2	15	105	
7	F	63	30.7	IIa	2	39	240	
8	M	68	26.4	IIa	1	2		150
9	F	73	26.6	IIa	2	2	420	
10	M	67	30.5	IIa	2	2	310	

M: male; F: female; BMI: body mass index; MG degree: degree of myasthenia gravis according to classifications of MGFA (Myasthenia Gravis Foundation of America [17]) and OO (Oosterhuis [1]); Diagn.: years since MG diagnosis; ChEI: cholinesterase inhibitors; IT: immunotherapy with azathioprine.

session they documented date, time of day, duration of session, estimated breath volume, pacing frequency, and, if necessary, problems or remarks.

During training phase 1, patients came at least two times to the laboratory and performed a training session with the device connected to the metabolic cart to assure correct performance and normocapnia during training. Additionally, we contacted all patients twice a week to ask for problems with the training or with their MG symptoms.

At least 10 days after completion of the last training session, a posttraining test series (P1) was carried out in the same way as the baseline test series. All examinations were performed at the same time of day as the pretraining tests. For RE test, $\dot{V}_E$, V_T, and f_R were set to the same values as in the baseline test.

Training phase 2 started after conclusion of all P1 tests. In phase 2, training frequency was reduced to 5 training sessions per two weeks. Training settings were the same as in phase 1. One training session per month was performed in the laboratory to check correctness of training performance. Patients were phoned once per week to ask for possible problems, for MG symptoms, and for their subjective experience with training. At the end of phase 2, patients performed another posttraining test series (P4) identical to B and P1 tests. The patients were then asked to continue the training, and all participants agreed.

2.3. Data Analysis. All values are given as mean values ± SEM. We evaluated Besinger score, vital capacity (VC), forced expiratory volume in 1 s (FEV_1), peak expiratory flow (PEF), MVV, PI_{max}, T_{Lim}, and V_{Lim}. Comparisons between the three test periods (B, P1, P4) were performed using a repeated measures analysis of variance with posthoc multiple comparisons according to the Holm-Sidak method. Additionally, when a significant difference was detected, a multiple linear regression was performed to describe the relationship of this respective variable with time (days since baseline tests) and with training (cumulated volume breathed during training).

3. Results

3.1. Training Course. All patients completed at least 50 training sessions with 30 min training time per session as required. No complications were reported during the total observation time. In 8 patients, MG was stable throughout the time without change in medication or outpatient care by their neurologist. One patient (no. 7) got a respiratory infection at the beginning of phase 2 and received additional cholinesterase inhibitors for 4 weeks. In one other patient (no. 10) MG symptoms had slightly deteriorated 5 weeks prior to the training period so that he transiently needed a higher dose of cholinesterase inhibitors. During training, his symptoms gradually improved, and his medication could be adequately reduced.

3.2. Besinger Score of MG Symptoms. Besinger score ranges from 0 to 3 with 0 meaning the best value, that is, no myasthenic symptoms, and 3 meaning most severe symptoms [17, 27]. The participants of the study achieved a baseline Besinger score of 0.71 ± 0.12. Training significantly improved the score ($P = 0.007$). After phase 1 training period, the improvement was not significant (0.63 ± 0.11, $P = 0.09$), but after phase 2, we found a significant score reduction to 0.56 ± 0.10 ($P = 0.002$, Figure 1). A multiple linear regression showed no significant correlation with training ($P = 0.09$) or with time ($P = 0.19$). No deterioration in myasthenia symptoms related to respiratory training was reported in the training questionnaire.

3.3. Lung Function. Baseline lung function was normal for all patients with VC being 95.5 ± 3.7%, FEV_1 90.5 ± 3.6%, PEF 86.9 ± 4.5%, MVV 93.8 ± 6.6%, and maximal inspiratory pressure (PI_{max}) 75.1 ± 5.4% predicted. RMET induced mild but not significant increases in VC (P1: 95.9 ± 3.5%, P4: 99.2 ± 4.1%, $P = 0.39$), FEV_1 (P1: 93.1 ± 2.7%, P4: 96.3 ± 3.8%, $P = 0.28$), PEF (P1: 90.0 ± 4.8%, P4: 95.7 ± 5.4%, $P = 0.07$), MVV (P1: 95.6 ± 6.0%, P4: 101.0 ± 6.5%, $P = 0.24$), and PI_{max} (P1: 79.2 ± 5.4%, P4: 78.0 ± 6.4%, $P = 0.42$). Absolute values are given in Table 2.

Table 2: Lung function data.

Patient	VC (L)			FEV_1 (L)			PEF (L s^{-1})			MVV (L min^{-1})			PI_{max} (kPa)		
	B	P1	P4	B	P1	P4	B	P1	P4	B	P1	P4	B	P1	P4
1	3.8	4.1	4.2	3.1	3.3	3.9	7.1	6.2	7.6	142	150	133	9.1	9.4	9.5
2	3.9	3.8	4.0	3.1	3.1	3.1	8.6	8.0	8.4	138	131	188	9.5	10.9	11.4
3	3.1	3.4	3.3	2.3	2.6	2.4	4.5	5.1	4.7	74	73	84	5.6	6.9	7.0
4	3.7	3.7	3.6	2.9	3.2	2.9	7.6	9.4	9.0	130	152	144	8.9	8.9	8.3
5	3.7	3.4	3.5	2.8	2.5	2.5	7.7	7.8	8.9	124	121	114	4.3	5.3	5.3
6	3.9	3.6	4.4	2.7	2.6	3.5	4.7	5.3	7.2	73	89	105	6.6	5.8	6.3
7	2.8	2.7	2.6	2.1	2.1	2.0	4.9	5.1	4.4	91	91	97	5.6	6.6	5.1
8	4.6	4.7	4.4	3.1	2.9	2.9	7.0	7.4	7.4	162	154	155	8.1	9.0	9.3
9	2.6	2.6	2.8	2.1	2.4	2.3	4.6	4.4	5.1	76	75	81	5.9	5.1	4.6
10	2.7	2.9	3.5	1.9	2.2	2.5	7.1	7.6	7.5	94	92	90	5.9	5.8	6.1
Mean	**3.5**	**3.5**	**3.6**	**2.6**	**2.7**	**2.8**	**6.4**	**6.6**	**7.0**	**110**	**113**	**119**	**6.7**	**7.1**	**7.1**
SEM	0.20	0.20	0.20	0.15	0.13	0.18	0.49	0.52	0.53	10.3	10.3	11.1	0.57	0.67	0.75

VC; vital capacity; FEV_1; forced expiratory volume in 1 s; PEF; peak expiratory flow; MVV; maximal voluntary ventilation; PI_{max}; maximal inspiratory pressure. B; baseline values; P1; posttraining test after 4 weeks of training (phase 1); P4; posttraining test after 4 months of training (phase 2).

3.4. Respiratory Endurance Tests. In the baseline RE test, patients achieved an average time to exhaustion (T_{Lim}) of 6.1 ± 0.8 min at an average ventilation of 58.9 ± 4.7 L min^{-1} corresponding to 54.7 ± 2.5% MVV. The patients breathed during T_{Lim} a total volume (V_{Lim}) of 382 ± 78 L. RMET significantly increased T_{Lim} and V_{Lim} ($P < 0.001$). After four weeks, T_{Lim} reached 15.1 ± 2.8 min and V_{Lim} 995 ± 243 L. Prolonged training further increased T_{Lim} to 20.3 ± 3.0 min (Figure 2) and V_{Lim} to 1316 ± 275 L (Figure 3). A multiple linear regression showed significant correlation with training and with time ($P < 0.001$) for V_{Lim}. T_{Lim} was significantly correlated with training ($P = 0.005$) but not with time ($P = 0.10$).

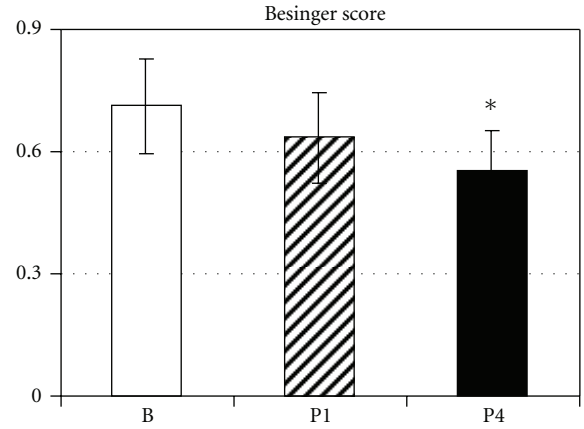

Figure 1: Besinger score of myasthenic symptoms before (B) and after 4 weeks (P1) and 4 months (P4) of respiratory endurance training. Data are presented as mean ± SEM; *significant difference versus B.

4. Discussion

In the present study, we established and evaluated a home-based RMET program appropriate for long-term application in patients with mild to moderate myasthenia gravis. A tight control consisting of a careful training documentation and self-reported questionnaires, frequent phone calls, and regular laboratory tests ensured adequate training performance. The results demonstrated that 30 min normocapnic hyperpnea training 2-3 times per week over 3 months induced further improvement of respiratory muscle endurance additionally to the gain achieved after a 4-week intensive training period (phase 1).

The results of phase 1 confirm our previous results obtained with the same training program, that is, 30 min normocapnic hyperpnea training 5 times per week over 4 weeks [26]. Most of the participants of the previous study felt the number of training sessions per week being too high to perform the RMET program regularly over a long time. The reduced training frequency applied in phase 2 was acceptable for all of the participants; therefore, all agreed to continue the RE training for at least 3 further months.

We had expected the training program in phase 2 to maintain respiratory endurance expressed by T_{Lim} and V_{Lim} at the enhanced level achieved after phase 1 (about 250% of baseline). However, even with the lower training frequency, T_{Lim} and V_{Lim} further increased during the following 3 months to about 340% of baseline.

Moreover, myasthenia symptoms indicated by Besinger score improved significantly compared to baseline. After four weeks of training (phase 1), we only observed a tendency to improvement, thus confirming results of our previous study [26]. During phase 2, enhancement of Besinger score progressed and reached significance after 4 months of RMET. Correspondingly, patients reported subjective improvement of their general state, reduced exhaustion in many activities of daily life, and attenuation of myasthenia symptoms. Recent reviews on exercise therapy, especially respiratory muscle training, in neuromuscular disease demonstrated limited positive effects of training therapy on pulmonary rehabilitation, exercise tolerance, and quality of life [28, 29].

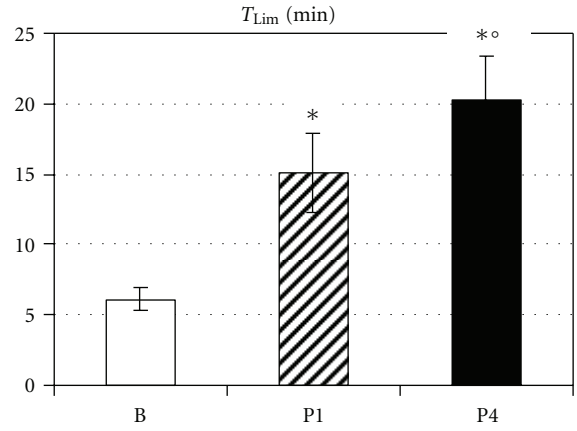

Figure 2: Respiratory endurance time (T_{Lim} (min)) before (B) and after 4 weeks (P1) and 4 months (P4) of respiratory endurance training. Data are presented as mean ± SEM; *significant difference versus B, °significant difference between P1 and P4.

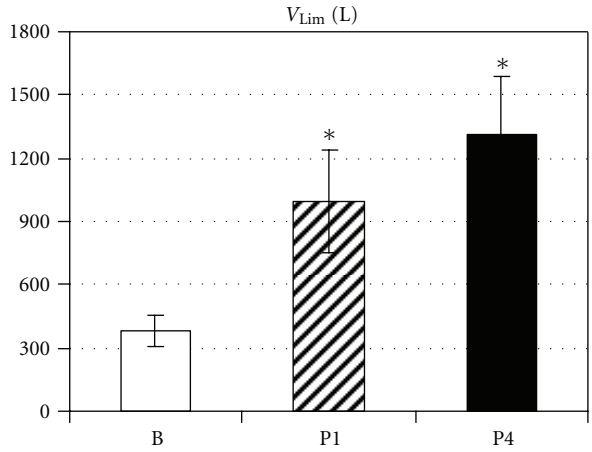

Figure 3: Total volume ventilated during respiratory endurance test (V_{Lim} (L)) before (B) and after 4 weeks (P1) and 4 months (P4) of respiratory endurance training. Data are presented as mean ± SEM; *significant difference versus B.

RMET improved lung function (VC, FEV_1, PEF, MVV, PI_{max}) of MG patients slightly but not significantly. Lung function parameters such as VC, FEV_1, PEF, and PI_{max} are based on short maneuvers requiring maximal effort. These abilities are usually not reduced in patients with mild to moderate MG. All of our patients had normal lung function at baseline reflecting a moderate degree of respiratory muscle weakness. A significant reduction of total lung capacity and hence, of VC can be expected when inspiratory muscle force is reduced by about 50% [30]. In healthy subjects, respiratory endurance training had no effect on lung function [21, 22]. Training effects also depend on duration and intensity of training. This is expressed by significant correlation of T_{Lim} and V_{Lim} with cumulated volume breathed during total training time. In MG patients, respiratory muscle training at 6 days per week over 3 months significantly improved static lung volumes such as VC and FEV_1 [18]. Accordingly, we observed further increase in these volumes during phase 2 of RMET in our patients. An 8-week inspiratory muscle training performed three times per week did not significantly change FVC and FEV_1 but significantly improved MVV and PI_{max} [20]. The increase in MVV was 8% in their study which was in a similar range as in our study.

The different effects of respiratory muscle training on lung function might also be explained by the training specificity of respiratory muscle training as already described by Leith and Bradley [31] who showed that respiratory strength training mainly improved maximal force. This is reflected in the two studies mentioned above [18, 20]. Their training programs were predominantly directed on respiratory muscle strength training, and both groups found a significant increase in PI_{max}. On the contrary, respiratory endurance training predominantly improves endurance which may be accompanied by a mild positive effect on force. Improved respiratory endurance is even more important than improvement of lung function parameters in MG patients. Weakness and fatigue of respiratory muscles is responsible for dyspnea and reduced exercise tolerance and thus, can compromise quality of life and increase the risk of respiratory failure [32]. In healthy subjects, RMET reduced respiratory muscle fatigue and increased cycling endurance in those subjects who had presented more than 10% of diaphragm or abdominal muscle fatigue in a pretraining exhaustion test [33]. As increased muscular fatigue is a characteristic feature of MG, a similar outcome had been expected for MG patients and was reflected in enhanced T_{Lim} and V_{Lim}. Moreover, all our patients perceived benefit of the training in terms of improved respiration and relief of respiratory symptoms. None of them reported any adverse effects. This is reflected best in the fact that all participants agreed to continue the training study.

Limitations of the Study. The main limitation of this study is the lack of a control group. The study program was strenuous and, particularly in phase 1, time consuming. Even the control program would have needed much time and effort as control patients also had to complete all laboratory tests. Hence, those patients who had resigned from training also refused to serve as controls. Participation in the RE training study required high motivation. Six patients who were asked for participation in this study refused as they did not see a necessity to perform this training. On the other hand, patients who had respiratory symptoms or had experienced respiratory disturbances in their past were highly motivated to perform the respiratory endurance training but were not willing to serve as nontraining controls. For these reasons, no control group could be formed.

Moreover, RMET cannot be applied to all MG patients. Some patients may not cope with the technique, and patients with severe MG are not able to perform this training at sufficient intensity. For patients with mild to moderate MG, this normocapnic hyperpnea training is appropriate if patients are motivated to learn the technique and to subject to the time need and effort of the training. These patients can considerably improve their respiratory muscle endurance.

A long-term endurance training program is expected to improve muscle endurance by inducing muscular hypertrophy. In the present study, we could not clarify whether reduced perception of respiratory effort rather than true respiratory muscle hypertrophy caused the improvements observed in the study. A recent reevaluation of 15 years of RMET experience in healthy subjects revealed that enhanced muscle endurance after RMET was unlikely due to reduced adverse respiratory sensations [34]. However, this does not preclude other factors such as improved neuromuscular coordination that may contribute to the RMET effect.

In conclusion, the study demonstrated that respiratory endurance training can be performed safely in patients with mild or moderate MG over several months. It indicates that this training program could be appropriate for long-term, ideally life-long, application resulting in improvement of respiratory muscle endurance and myasthenia symptoms.

Appendix

Self-Reported Training Questionnaire

The original questionnaire as presented to the patients was in German.

Training session no.: . . . Date: . . .

Have symptoms of your myasthenia changed after this training?

Yes
No

If yes, which symptoms/functions have deteriorated?

◯ Ptosis
◯ Double vision
◯ Swallowing problems/dysphagia
◯ Chewing problems
◯ Pursing lips/whistling
◯ Neck strength (e.g., difficulties with holding your head up?)
◯ Arm strength (e.g., problems with hair-drying?)
◯ Leg strength (e.g., difficulties with climbing a staircase?)

How long did this deterioration continue? . . .

Was additional medication (cholinesterase inhibitors) necessary?

Yes
No

Did you have to interrupt or break off this training due to deterioration of MG symptoms?

Yes
No

Further remarks concerning your current state: . . .

Acknowledgments

The authors gratefully acknowledge the participation of all our subjects. They wish to thank Mrs. Ursula Vogt and Mrs. Brigitte Mix for their technical assistance in the laboratory tests. The paper was supported by the Deutsche Myasthenie-Gesellschaft.

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SAGE-Hindawi Access to Research
Autoimmune Diseases
Volume 2011, Article ID 474512, 5 pages
doi:10.4061/2011/474512

Review Article

Thymoma in Myasthenia Gravis: From Diagnosis to Treatment

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Received 10 April 2011; Accepted 24 June 2011

Academic Editor: Robert P. Lisak

One half of cortical thymoma patients develop myasthenia gravis (MG), while 15% of MG patients have thymomas. MG is a neuromuscular junction disease caused in 85% of the cases by acetylcholine receptor (AChR) antibodies. Titin and ryanodine receptor (RyR) antibodies are found in 95% of thymoma MG and 50% of late-onset MG (MG onset ≥50 years), are associated with severe disease, and may predict thymoma MG outcome. Nonlimb symptom profile at MG onset with bulbar, ocular, neck, and respiratory symptoms should raise the suspicion about the presence of thymoma in MG. The presence of titin and RyR antibodies in an MG patient younger than 60 years strongly suggests a thymoma, while their absence at any age strongly excludes thymoma. Thymoma should be removed surgically. Prethymectomy plasmapheresis/iv-IgG should be considered before thymectomy. The pharmacological treatment does not differ from nonthymoma MG, except for tacrolimus which is an option in difficult thymoma and nonthymoma MG cases with RyR antibodies.

1. Thymoma in Myasthenia Gravis

Thymomas in myasthenia gravis (MG) are neoplasms derived from thymic epithelial cells, and are usually of the cortical subtype (WHO type B) [1]. 50% of thymoma patients develop MG (hereafter referred to as thymoma MG in this paper) [2, 3]. Cortical thymomas usually have some morphological similarities with thymic cortex; they share the capacity to propagate the maturation of immature naive CD4 T cells and export mature naive T cells into the periphery. Thymomas lacking this ability do not induce MG [4]. Thymomas with histological similarities to medullary thymic tissue or thymomas lacking developing T cells are seldom associated with MG [4]. Other thymoma characteristics that can cause reduced self-tolerance include defective epithelial expression of the autoimmune regulator (AIRE) gene and/or of major histocompatibility complex class II molecules, absence of myoid cells, failure to generate FOXP3(+) regulatory T cells, and genetic polymorphisms affecting T-cell signalling [5].

Histologically, thymomas are epithelial neoplastic cells surrounded by maturing T cells. The epithelial cells are capable of expressing epitopes cross-reactive with skeletal muscle proteins, such as acetylcholine receptor (AChR), titin, and ryanodine receptor (RyR) [6, 7]. The muscle-like epitopes are presented to T cells together with costimulatory molecules [7]. Autoreactive T cells specific for AChR and titin are found both in thymomas and in thymoma MG patients' sera [8]. Thymoma epithelial cells present AChR peptides to T-cell lines in thymoma MG patients, facilitating intrathymic immunization [9].

The patient's genetic profile and the thymic ability to export autoreactive T cells are equally important in developing MG. MG has a genetic association to HLA-DR3 or ancestral haplotype 8.1 in early-onset MG (MG onset before age 50 years) with thymic hyperplasia and several weaker associations to polymorphisms in immunoregulatory genes such as FcγR, TNF-α/β, GM-phenotypes, CTLA-4 [10], HLA, and PTPN22 $*$ R620W [11]. The chance of having a thymoma increases with the number of thymoma-associated polymorphisms in an MG patient, indicating that thymoma MG is a polygenic disease and that thymoma patients with a particular genetic profile run higher risk of developing MG [11].

2. Thymoma MG

MG is a neuromuscular junction disease characterized by muscular weakness and fatigability, caused in 85% of the

Table 1: The occurrence of the various muscle autoantibodies (ab) in the different subgroups of MG [13].

MG subgroup	AChR ab	MuSK ab	Titin ab	RyR ab
Early onset (non MuSK nonthymoma)	Positive in all patients	Negative in all patients	Positive in 10% of the patients	Negative in all patients
Late onset (non-MuSK nonthymoma)	Positive in all patients	Negative in all patients	Positive in 58% of the patients	Positive in 14% of the patients
MuSK positive (regardless onset age)	Negative in all patients	Positive in all patients	No information available	No information available
Seronegative (regardless onset age)	Negative in all patients	Negative in all patients	Negative in all patients	Negative in all patients
Thymoma (regardless of onset age)	Positive in all patients	May occur in some patients	Positive in 95% of the patients	Positive in 70% of the patients

cases by AChR antibodies [12]. When MG occurs together with a thymoma, MG is a paraneoplastic disease caused by the presence of the thymoma. Thymoma MG accounts for around 15% of all MG cases [13].

The immune response against an epitope expressed on thymoma cells spills over to neuromuscular junction components sharing the same epitope [14]. In thymoma MG, epitopes are shared between the thymoma and muscle proteins.

3. Antibodies in Thymoma MG

AChR antibodies are the main cause of muscle weakness in thymoma MG [15]. Additional non-AChR muscle autoantibodies reacting with striated muscle titin and RyR antigens are found in up to 95% of MG patients with a thymoma and in 50% of late-onset MG patients (MG onset at age of 50 years or later) [16]. These antibodies are usually associated with more severe MG [13, 17–19]. Striational antibodies demonstrated in immunofluorescence are largely made up of titin antibodies [20].

Titin is the largest known protein, with a molecular mass of 3000 kD stretching throughout the sarcomere, providing a direct link between mechanical muscle strain and muscle gene activation [21]. Myositis and myopathy with muscle atrophy are seen in some thymoma MG patients [22]. Sera from MG patients also induce degenerative changes in muscle cell cultures where both apoptosis and necrosis are implicated [23].

The RyR is the calcium channel of the sarcoplasmic reticulum (SR). Upon opening, the RyR releases Ca^{2+} into the sarcoplasm resulting in muscle contraction. In vitro, RyR antibodies can inhibit Ca^{2+} release from the SR [24]. There is also a rat model with thymoma and MG with RyR antibodies but no AChR antibodies, indicating that RyR antibodies may cause MG symptoms irrespective of AChR antibodies [25]. There are also several reports of excitation-contraction coupling defects in thymoma MG [26].

4. Recognizing the Clinical and Serological Pattern of Thymoma MG

MG patients with RyR antibodies are characterized by frequent involvement of bulbar, respiratory, and neck muscles at MG onset and a more severe disease. Neck weakness at MG onset is a distinctive feature of patients with RyR antibodies, while respiratory symptoms are also found in patients with titin antibodies with and without RyR antibodies. Limb involvement with few or no bulbar signs is typical at MG onset in RyR-antibody-negative MG [27]. Since many thymoma MG patients have RyR antibodies, neck weakness and nonlimb bulbar distribution of MG symptoms are initial characteristic features associated with thymoma MG. Such symptom distribution should always raise the suspicion about the presence of a thymoma in an MG patient.

Thymoma MG is equally frequent in males and females and occurs at any age with a peak onset around 50 years [28]. Thymoma MG and late-onset MG share similar serological profile with high prevalence of titin and RyR antibodies and lower AChR antibody concentrations compared to early-onset MG [29]. About 95% and 70% of thymoma MG patients have titin and RyR antibodies, respectively (Table 1). Around 58% and 14% of late onset MG patients have titin and RyR antibodies, respectively (Table 1) [13].

Late MG onset age, similar serological profile, favorable pharmacological treatment response, severe MG, frequent use of immunosuppressive drugs, and the occurrence of MG related mortality are common features among thymoma MG and late-onset MG patients [29]. This profile differs from early-onset MG [30], that has higher AChR antibody concentrations, almost no titin or RyR antibodies, low need for immunosuppressive drugs, less severe MG, very low MG mortality rates, and a favorable thymectomy outcome [29].

Thymoma MG tends to be more severe than early-onset nonthymoma MG [29]. In one study, MG patients with thymoma or thymic atrophy (i.e., chiefly late-onset MG) had worse prognosis than MG patients with thymic hyperplasia (i.e., early-onset MG) [31]. The presence of a thymoma per se does not give a more severe MG. Thymoma MG patients and age-matched nonthymoma MG patients share similar MG long-term prognosis [19]. The presence of titin and RyR antibodies is associated with more severe disease in thymoma MG and in late-onset MG [29]. The AChR antibody serum concentration does not correlate with MG severity, mainly because of individual variations in AChR epitope specificity [32].

5. Verifying the Diagnosis of Thymoma MG

The diagnosis of MG is based on clinical disease history and typical clinical findings. MG can be confirmed pharmacologically by edrophonium (Tensilon) test which is positive in 90% of MG patients, giving an immediate but transitory improvement of MG signs [33]. The diagnosis of MG should be confirmed by the detection of AChR antibodies, present in most MG cases. These antibodies are present in virtually all patients with a thymoma [29]. In two thirds of MG patients, failure of neuromuscular transmission in leads to decremental response to repetitive nerve stimulation by electromyographical (EMG) examination [34]. Increased jitter on single-fiber EMG is even more sensitive than repetitive nerve stimulation when performed on affected muscles [34].

In addition to MG, a thymoma should be demonstrated in order to fulfill the criteria of thymoma MG diagnosis. The diagnosis of a thymoma in MG is finally established by histopathological examination postsurgery. Titin and RyR antibodies and radiological examination of the anterior mediastinum share similar sensitivity for the presence of a thymoma in MG [29, 35, 36]. However, the presence of titin and RyR antibodies in a MG patient younger than 60 years strongly suggests a thymoma, while the absence of such antibodies at any age strongly excludes thymoma [13, 37]. Retesting for these antibodies and a new radiological examination should always be considered whenever clinical deterioration is seen over time, to minimize the risk of a previously undetected thymoma in a MG patient.

6. Surgical Treatment of Thymoma MG

When the diagnosis of a thymoma in a MG patient is established, the neoplasm should be removed surgically, and it is crucial to ensure radical excision of the neoplasm. Thymectomy can be performed transternally or through a video-assisted thoracoscopic approach, usually with similar outcome [38]. Radical excision of a thymoma does in most cases cure the thymic neoplasia, but patients will continue to suffer from MG after thymectomy, emphasizing the need of continuing followup and pharmacological treatment. When the thymoma invades the pleura or the pericardium, radical excision will not be possible and further oncological treatment is necessary. Presurgery plasmapheresis or intravenous infusion of immunoglobulin (iv-IgG) removes a great deal of circulating pathogenic antibodies [36]. In our department we give plasmapheresis or iv-IgG treatment to all patients with thymoma MG prior to thymectomy, to minimize the risk of postthymectomy MG exacerbation and myasthenic crisis. This practice varies however from department to another, and there is no consensus on this issue. Iv-IgG should be considered as first choice in patients at high risk of developing cardiopulmonary failure secondary to fluid overload caused by plasmapheresis [39]. MG outcome after thymectomy is generally less favorable in patients older than 45 years (i.e., mostly late-onset and thymoma MG patients) [40].

7. Treatment of MG Crisis in Thymoma MG

Plasmapheresis and immunoglobulin treatments are also indicated in severe cases of thymoma MG regardless of thymectomy, such as in MG crisis and in severe MG cases with poor response to standard pharmacological treatment [41]. Parallel to plasmapheresis and immunoglobulin treatment, the pharmacological treatment should be intensified in these patients as explained in the next chapter.

8. Pharmacological Treatment of Thymoma MG

The first pharmacological choice in the treatment of thymoma MG is acetylcholinesterase inhibitors. The second choice is immunosuppressive drugs whenever additional pharmacological treatment is needed before or after thymectomy. Several immunosuppressive drugs are available, such as corticosteroids, azathioprine, cyclophosphamide, cyclosporine, methotrexate, mycophenolate mofetil, rituximab, and tacrolimus. Steroids such as prednisolone are frequently given on alternate days, by gradually raising the dose to 60–80 mg initially and then with slowly tapering to 20 mg or lower. If long-term treatment with steroids is regarded necessary, nonsteroid immunosuppressants such as azathioprine should be introduced in addition (usually 100–150 mg a day). While the steroid effect appears rapidly, the clinical effect of other immunosuppressants may take a few weeks to several months to develop [29]. Overall, about 80% of MG patients and 95% of thymoma MG patients need immunosuppressive drug treatment for more than one year [42].

Tacrolimus, which is an immunosuppressant and enhancer of RyR-related sarcoplasmic calcium release, may be especially beneficial in MG patients with RyR antibodies that in theory might block the RyR interfering with its function. Since most patients with thymoma MG have RyR antibodies, tacrolimus may act specifically in these patients. It may have a purely symptomatic effect in addition to its immunosuppressive impact [40]. Tacrolimus has demonstrated favorable effects in the treatment of MG, both as monotherapy and as add-on to prednisolone [43, 44]. Patients should undergo a thorough cardiological investigation prior to commencing tacrolimus treatment.

Long-term observation of thymoma MG and age-matched nonthymoma MG patients showed no difference in MG severity over time, and both groups improved to the same degree after MG diagnosis as a result of pharmacological treatment and thymectomy. The need for immunosuppressive treatment in the two groups was similarly high. A thymoma that has been completely removed surgically does not necessarily mean worse MG prognosis in thymoma MG [19].

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SAGE-Hindawi Access to Research
Autoimmune Diseases
Volume 2011, Article ID 151258, 4 pages
doi:10.4061/2011/151258

Clinical Study

Matrix Metalloproteinase-3 in Myasthenia Gravis Compared to Other Neurological Disorders and Healthy Controls

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Received 10 April 2011; Accepted 16 June 2011

Academic Editor: Shigeaki Suzuki

MMP-3 is capable of degrading a variety of proteins, including agrin, which plays a critical role in neuromuscular signaling by controlling acetylcholine receptor clustering. High MMP-3 levels in a proportion of myasthenia gravis (MG) patients have been reported. A pathogenic role of MMP-3 in other neurological disorders has been suggested but not proven. We have therefore examined the levels of MMP-3 in 124 MG patients and compared them to 59 multiple sclerosis (MS) patients, 74 epilepsy patients, 33 acute stroke patients, and 90 healthy controls. 15.3% of the patients in the MG group were MMP-3-positive (defined as higher than cutoff value 48 ng/mL) with very high mean MMP-3 concentration (79.9 ng/mL), whereas the proportion of MMP-3 positive patients in the MS (3.4%), epilepsy (6.7%), stroke (0%), and the control group (4.4%) was significantly lower. Mean MMP-3 concentration in the total MG group (25.5 ng/mL) was significantly higher than in the MS (16.6 ng/mL) and stroke (11.7 ng/mL) groups, but did not differ significantly from the epilepsy (19.4 ng/mL) and the control group (23.4 ng/mL). MMP-3 may have a specific pathogenic effect in MG in addition to being associated with autoimmune diseases in general.

1. Introduction

Matrix metalloproteinase-3 (MMP-3) is a matrix enzyme capable of breaking down various extracellular components, including collagens (types III, IV, V, IX, and XI), matrix proteins, and proteoglycans. MMP-3 can also activate other MMPs such as MMP-9 [1, 2]. Agrin is a substrate for the endopeptidase MMP-3, stromelysin1 [3]. MMP-3 null mice have alterations to their neuromuscular junctions, including increased acetylcholine receptor (AChR) staining at the endplate and an increased number of junctional folds. An increased concentration of agrin occurs at the neuromuscular junction of such mice [4]. These observations indicate that MMP-3 is involved in controlling the structure of the neuromuscular junction via regulation of agrin levels.

Myasthenia gravis (MG) is an autoimmune disorder which causes skeletal muscle weakness. Antibodies targeting the AChR are present in 85% of patients with generalized MG (GMG) and in about 50% of patients with ocular MG (OMG) [5, 6]. MG with AChR antibodies is known as seropositive MG (SPMG). Thymoma is present in approximately 15% of MG patients [7]. Antibodies to AChR impair neuromuscular transmission by complement-mediated postsynaptic membrane damage, direct blockade of ligand-receptor interaction, and/or by an increased degradation of AChR [5, 8]. MG without detectable antibodies to the AChR is termed seronegative MG (SNMG) [9]. Antibodies to muscle-specific kinase (MuSK) are observed in a proportion of SNMG patients [10, 11]. MuSK is a key signaling protein controlling AChR clustering and the formation of the neuromuscular junction. These processes are triggered by the release of agrin from the nerve terminals. Agrin interaction with MuSK leads to the phosphorylation of AChR [12, 13].

Elevated serum MMP-3 levels have previously been reported in the autoimmune disorders systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) [14, 15]. A pathogenic role of MMP-3 in these connective tissue disorders could be related to its ability to degrade extracellular matrix components. SLE and RA have an increased incidence

in MG patients [16, 17], indicating shared pathogenic mechanisms. These disorders are associated with localized autoimmune diseases such as Hashimoto's thyroiditis and Graves' disease [18].

In MG patients, an elevated MMP-3 level, in addition to promoting autoimmune disease in general, may cause damage to the neuromuscular junction due to increased degradation of agrin, and thereby contribute to an increased muscle weakness. In a previous study [19], we demonstrated a significant elevation in MMP-3 levels in a proportion of both SPMG and SNMG, indicating a pathogenic role for MMP-3 in MG. Little is known about MMP-3 in other autoimmune and nonautoimmune neurological disorders. We have therefore examined the levels of MMP-3 in sera from MG patients and compared them to those in multiple sclerosis (MS) patients, epilepsy patients, acute stroke patients, and healthy controls.

2. Materials and Methods

2.1. Patients and Sera. Serum samples from 124 MG patients (104 SPMG and 20 SNMG), 59 MS patients, 74 epilepsy patients, 33 acute stroke patients, and 90 healthy controls available in our biobank, taken from different individuals, were included (Table 1). The MG and MS sera were collected randomly at various disease phases from in and outpatients admitted to our department. The sera from the epilepsy patients and the acute stroke patients were obtained within 24 hours after last seizure or acute stroke, respectively, from inpatients admitted to our department. The MG group included patients with early-onset MG (MG onset before age 50 years), late-onset MG (MG onset at or after age 50 years), thymoma MG and nonthymoma MG, and patients with various antibodies including antiAChR, antiryanodine receptor and antititin antibodies, but none with MuSK antibodies. None of the patients had any confounding diseases, such as SLE or RA. The control sera were collected randomly from healthy individuals. All sera were stored at −20°C.

2.2. Serum MMP-3 Levels. Total MMP-3 levels were assessed using the Quantikine MMP-3 Elisa kit as per manufacturer's instructions (R&D Systems Europe Ltd. Abingdon, UK). Patient sera were assayed at a dilution of 1 : 10 in duplicate wells. A standard curve (0.156 ng/mL–20 ng/mL MMP-3) was produced and the MMP-3 concentration (ng/mL) determined.

The cut-off level between a normal and a high MMP-3 level (hereafter referred to as MMP-3-positive) was defined as the mean MMP-3 concentration for the 90 controls + 2 SDs, which equals 48 ng/mL (Figure 1).

2.3. Statistical Analyses. The numbers of patients in the various groups were compared using the Chi-square test. The MMP-3 concentrations in the different groups were compared using the *t*-test for difference between population means.

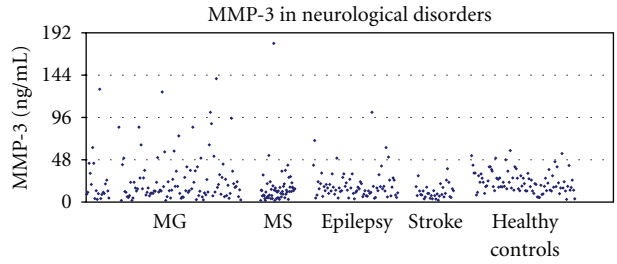

Figure 1: Scatter plot of the MMP-3 concentrations in patients with neurological disorders and healthy controls. The 2 SD cut-off at 48 ng/mL is indicated.

3. Results

Nineteen (15.3%) MG patients were MMP-3-positive, whereas the numbers of MMP-3-positive patients in the MS, epilepsy and control groups were very low (3%, 6%, and 4%, resp.). There were no MMP-3-positive patients in the acute stroke group (Table 1, Figure 1). The number of MMP-3-positive patients in the MG group was significantly higher than the number of MMP-3-positive patients in the MS group ($P = 0.034$), the acute stroke group ($P = 0.036$), and the control group ($P = 0.021$), but did not reach significance for the epilepsy group ($P = 0.118$) (Table 1).

The MG group had the highest mean MMP-3 concentration. It was significantly higher than the mean MMP-3 concentration in the MS group ($P = 0.013$), the epilepsy group ($P = 0.026$), and the acute stroke group ($P < 0.001$), but did not differ significantly from that in the control group ($P = 0.225$) (Table 1). Mean MMP-3 concentration of the 19 MMP-3-positive MG patients was significantly higher than mean MMP-3 concentration in the total MG group ($P < 0.001$) and in the total control group ($P < 0.001$). Mean MMP-3 concentration of the 19 MMP-3-positive MG patients was also significantly higher than mean MMP-3 concentration of the four MMP-3-positive controls ($P = 0.001$) but did not differ significantly from mean concentration of the five MMP-3-positive epilepsy patients ($P = 0.148$) (Table 1). Mean MMP-3 concentration was lowest in the acute stroke group. It was significantly lower than mean MMP-3 concentration in the MG group ($P < 0.001$), the epilepsy group ($P < 0.001$), and the control group ($P < 0.001$) and tended to be lower than that in the MS group ($P = 0.078$) (Table 1). The MS group had significantly higher mean MMP-3 concentration than the controls ($P = 0.024$), in contrast to the epilepsy group that had lower mean MMP-3 than the controls ($P = 0.042$) (Table 1).

4. Discussion

A proportion of MG patients (15.3%) had markedly increased MMP-3 levels. This was unique as compared to patients with other neurological disorders in this study. Similarly, the mean MMP-3 concentration in the MG group was increased. In a previous study [19], we demonstrated

Table 1: MMP-3 concentrations (ng/mL) in patients with neurological disorders and healthy controls.

	MG	MS	Epilepsy	Stroke	Healthy controls
Number of patients	124	59	74	33	90
Mean MMP-3 concentration	25.5 (±27.4)	16.6 (±23.9)	19.4 (±16.4)	11.7 (±8.1)	23.4 (±12.3)
Number of MMP-3-positives	19 (15.3%)	2 (3.4%)	5 (6.7%)	0	4 (4.4%)
Mean MMP-3 concentration of MMP-3-positives	79.9 (±27.9)	116.5 (±89.8)	67.0 (±21.2)	0	54.0 (±3.6)

that elevated MMP-3 levels are present in both SPMG and SNMG and thus is linked to MG in general.

MMP-3-positive MG patients exhibited high mean MMP-3 concentrations (79.9 ng/mL) compared to the whole MG group (25.5 ng/mL) and controls (23.4 ng/mL), indicating that these patients represent a subgroup with differences in pathophysiology and/or clinical presentation. Our cohort comprised patients at different age, gender, disease severity, and disease types (SPMG, SNMG, early-onset MG, late-onset MG, thymoma MG, and nonthymoma MG). It was not possible to correlate MMP-3-positive patients with a specific subset of MG patients, perhaps due to low number of MMP-3-positive MG patients and large variation in disease entities. A similar subgroup with high MMP-3 levels in another MG cohort was recently reported, containing GMG, OMG, SPMG, and SNMG patients [20].

None of our acute stroke patients had elevated MMP-3 levels, and mean MMP-3 concentration was lower than in any other group examined. MMP-3 may play a role in the pathophysiology of acute stroke [21]. Thromboembolic mechanisms and MMP-3 consumption may be the cause of the low MMP-3 concentrations seen in acute stroke.

In an earlier study, Kanesaka et al. found elevated MMP-3 levels during the relapsing phase of relapsing-remitting MS compared to the remission phase [22]. In our study, MS and MG patients were included regardless of disease phase or severity. This may explain the discrepancy between our results and those of Kanesaka et al. Our conclusion is that MG patients have higher MMP-3 levels than MS patients when disease phase and severity are not taken into consideration.

Very high mean MMP-3 concentrations of 258 ± 35 ng/mL and 187 ± 14 ng/mL have previously been reported in SLE and RA patients, markedly higher than in our MG patients [14]. However, mean serum MMP-3 concentration in our control group is approximately half that observed for the controls in that previous study. Methodological variation may therefore explain this difference between MG and SLE/RA. Serum levels of MMP-3 may not represent the concentration of MMP-3 at the site(s) of tissue damage, since MMP-3 can be produced locally and there have a concentration higher than observed in the general circulation. Serum MMP-3 levels may be higher in systemic diseases such as SLE and RA compared to MG with more focal damage.

Since agrin is required for the clustering of AChR in the neuromuscular junction, increased degradation of agrin and subsequent disruption of MuSK signaling, may have important consequences for the correct formation and function of the neuromuscular junction and lead to a reduction in the safety factor for successful neuromuscular transmission. Agrin is also capable of inducing the expression of the ϵ subunit of the AChR [23, 24]. MMP-3 null mice show alterations to their neuromuscular junctions [4], probably via an effect on agrin.

An increased MMP-3 level as a cause of MG is unlikely, since MMP-3 levels are increased in patients with SLE and RA who do not have MG [14, 15]. The mechanism by which MMP-3 is upregulated and the significance of an increased level of MMP-3 in MG, SLE, and RA is unknown. By expanding our patient database, we hope to identify clinical or immunological associations with the observed increase in MMP-3, further elucidating mechanisms and significance.

Some MG patients have high MMP-3 serum concentrations, and this may reflect a subgroup with different pathophysiology and/or different clinical presentation. Dysregulation of MMP-3 could be a common feature of autoimmune diseases, including MG, SLE, and RA, but not MS as examined in this study. The mechanism(s) by which altered MMP-3 levels in MG and acute stroke occurs, and its pathological significance remain to be established. MMP-3 testing might be useful as an indicator of acute cerebral ischemia in its very early phase.

Abbreviations

AChR: Acetylcholine receptor,
MG: Myasthenia gravis,
MMP-3: Matrix metalloproteinase-3,
MS: Multiple sclerosis,
MuSK: Muscle specific kinase,
RA: Rheumatoid arthritis,
SLE: Systemic lupus erythematosus,
SNMG: Seronegative myasthenia gravis,
SPMG: Seropositive myasthenia gravis.

Acknowledgments

This study was funded by a grant from the Norwegian Foundation for Muscle Disease Research. The authors declare no conflict of interests.

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SAGE-Hindawi Access to Research
Autoimmune Diseases
Volume 2011, Article ID 840364, 4 pages
doi:10.4061/2011/840364

Clinical Study

Headache Associated with Myasthenia Gravis: The Impact of Mild Ocular Symptoms

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Received 5 April 2011; Revised 6 June 2011; Accepted 6 June 2011

Academic Editor: Johan A. Aarli

Myasthenia gravis (MG) patients visiting outpatient clinics frequently complain of headache. However, there have been few reports on the relation between chronic headache and myasthenia gravis (MG). We aimed to investigate whether MG symptoms affect the development or worsening of chronic headache. Among the 184 MG patients who were followed at the MG clinics, tension-type headache was observed in 71 (38.6%) patients and 9 (4.9%) complained of migraine. Twenty-five (13.6%) complained that headache appeared or was exacerbated after the MG onset. The investigation into differences in the clinical characteristics of the MG patients showed that women tended to suffer from MG-associated headache more often than men. Logistic regression analyses revealed that female gender and mild ocular symptoms were independently predictive of headache associated with MG. Our results suggest that treatment of chronic headache should be required to improve the quality of life in MG patients.

1. Introduction

Headache is one of the most common neurological symptoms. At our clinics, we have found that some myasthenia gravis (MG) patients complain of headache more than the known MG-related symptoms. In these cases, the headaches could have a more negative impact on the quality of daily life than the other MG manifestations. It seemed that certain MG symptoms induce the development or worsening of headache, but there have been only a few case reports concerning headache in MG patients [1, 2]. On the other hand, some major neurological diseases, including Parkinson's disease [3, 4] and multiple sclerosis [5, 6], have been reported to be associated with headache. In this paper, to investigate the relationship between headache and MG, we examined whether each of the MG symptoms could be a cause of headache in MG patients.

2. Methods

2.1. Patient Selection and Diagnosis of Headaches. We studied 184 patients with MG (60 men and 124 women; mean ± SD age, 55.9 ± 17.0), who were followed at the MG clinics of 2 institutions, Keio University Hospital and Hanamaki General Hospital in Japan, from November 2007 to April 2008 and had been followed up for at least 1 year. The diagnosis of MG was made based on the following criteria: typical history and signs of fluctuating weakness of voluntary muscles, presence of serum antiacetylcholine receptor antibodies (AChR Ab), definite clinical improvement on injection of the cholinesterase inhibitor, edrophonium, and decremental pattern on repetitive nerve stimulation [7]. The mean ± SD onset age and duration after onset in the 184 patients were 45.9 ± 18.0 years old and 8.4 ± 8.1 years, respectively. First, among MG patients, we diagnosed primary headaches according to the International Classification of Headache Disorders: 2nd edition (ICHD-II), based on physical and neurological examinations and head CT and/or MRI. Secondly, we examined the MG symptoms and characteristics of their headaches in detail in addition to using the questionnaire as a reference.

2.2. Division of the QMG Score. To evaluate which symptoms of MG appeared when their headache occurred, MG

symptoms at the outpatient clinic were graded according to a quantitative MG scoring system (QMG score) consisting of 13 items [8]. As Bhanushali et al. separated three of the QMG score items as a discrete ocular component (ocular-QMG score) in order to monitor the severity of ocular symptoms independently, we divided the test items of the QMG score into four subgroups according to their proximity in the body [9]. That is, we defined double vision on lateral gaze, ptosis, and facial muscle weakness as "ocular symptoms," swallowing and dysarthria after counting aloud from 1 to 50 as "bulbar symptoms," outstretching of either an arm or leg, or gripping of either hand as "limb symptoms," and vital capacity % predicted and head lifting as "trunk symptoms." All data and clinical information were obtained after the patients had given their informed consent, and the study was approved by the institutional review board of each hospital.

2.3. Statistical Analyses. Statistical assessments in this study, including Student's *t*-test, chi-square test, and logistic regression analyses, were performed using a statistical software program (StatView 5.0; SAS Institute Inc., Cary, NC). Values of $P < 0.05$ were considered to indicate statistical significance.

3. Results

3.1. Analysis of Headache in MG Patients. First, with respect to primary headache, 71 of the 184 MG patients (38.6%) were diagnosed with tension-type headache, and 4.9% (9/184) were diagnosed with migraine based on the ICHD-II [10]. Next, in our examination of the relationship between the onset of MG and headache, 13.6% (25/184) of the MG patients experienced headache associated with MG which appeared or was exacerbated after they were diagnosed with MG. Headache worsened in 15 patients, and headache initially appeared in 10 patients after MG onset.

The characteristics of MG-associated headache are shown in Figure 1. In regard to frequency, 48% (12/25) of the patients with MG-associated headache complained of headache 1–3 times a month. 20% (5/25) had headache every day. 16% had headache only irregularly over a certain period. 12% of patients had headache 1–4 times per week, with one patient complaining of severe pain more than twice a day (Figure 1(a)). The results in regard to the sites of headache associated with MG showed that deep in the orbit and back of the neck were the most favorite sites seen in 84% (21/25) and 60% (15/25) of the patients with MG-associated headache, respectively. Headache also often occurred in the temporal portion (32%), one side of the head (32%), the frontal portion (28%) or the whole head (20%). The surroundings of the orbit (12%), the face (12%), and the parietal portion (8%) were rarely documented as the sites of headache associated with MG (Figure 1(b)).

We also evaluated which symptoms of MG developed during the period of headache. Head dropping (68%) and ptosis (60%) were most frequently observed. General fatigue (56%) and double vision (52%) also often occurred, and limb weakness, chewing difficulty, shortness of breath, and talking difficulty were complications in 24%, 16%, 12%, and 8% of patients, respectively.

Then, we investigated the demographic and clinical features of MG patients who experienced appearance or exacerbation of headache after MG onset and those who did not (Table 1). MG symptoms at the outpatient clinic were evaluated according to the quantitative MG scoring system (QMG score) [8]. As Bhanushali et al. described previously [9], we divided the test items of the QMG score into 4 subgroups, where double vision on lateral gaze, ptosis, and facial muscle weakness belonged to the subgroup of "ocular symptoms." The results suggested that there were markedly significant differences in female gender ($P = 0.03$) between the two groups, whereas there were no differences in mean age, follow-up period, seropositivity of autoantibodies to the acetylcholine receptor, presence of thymoma, or history of treatments, including thymectomy and administration of prednisolone and calcineurin inhibitor (Table 1). Notably, there were also no significant differences in occurrence or aggravation of headache according to the severity of QMG score.

3.2. Clinical Factors Associated with Aggravation of Headache in MG Patients. Finally, we examined which types of clinical factors affected headache in the MG patients by univariate logistic regression analysis. To consider the possibility of an association between the severity of MG and headache, each of the four types of symptoms, that is, ocular, bulbar, limb, and trunk, was separated into mild and moderate subgroups based on the mean score. The clinical factors for which the probability values were less than 0.05 in the univariate regression analysis, which were age (1.0 and 0.04 for the odds ratio and *P* value, resp.), female gender (4.3 and 0.02), age at onset (0.98 and 0.04), mild ocular symptoms (4.8 and 0.002), mild bulbar symptoms (3.1 and 0.04), and total QMG score (1.1 and 0.04), were further entered into the multivariate logistic regression analysis for determination of the independent clinical factors affecting headache associated with MG (Table 2). Multivariate logistic regression analysis revealed that female gender (4.5 and 0.02 for the odds ratio and *P*-value, resp.) and mild ocular symptoms with QMG 1–3 in the ocular score (7.2 and 0.0005) were independent clinical factors linked with headache associated with MG.

4. Discussion

This study disclosed that 38.6% of the MG patients had tension-type headache and 4.9% had migraine according to the ICHD-II [10]. Additionally, it was notable that 13.6% of the MG patients experienced headache associated with MG. In regard to the general characteristics of MG patients, headache was affected by female gender but not by the age, the disease duration, or the severity of symptoms. Moreover, the logistic regression analysis suggested that both female gender and mild ocular symptoms might have influence on headache associated with MG.

Mild ocular symptoms indicate a slight degree of diplopia or ptosis in MG patients, which fluctuates dynamically and might lead to worsening of headache. In contrast, the MG

Table 1: Demographic and clinical features of patients with MG-associated headache.

	MG-associated headache (+) ($n = 25$)	MG-associated headache (−) ($n = 159$)	P value
Female gender	22 (88%)	102 (64%)	*0.03
Mean age, y (range)	49.4 ± 17.2	57.0 ± 16.8	n.s.
Mean disease duration, y (range)	9.2 ± 7.6	8.2 ± 8.2	n.s.
AChR-Ab positive	16 (64%)	118 (74%)	n.s.
Thymoma present	2 (8%)	37 (23%)	n.s.
Thymectomy	9 (36%)	74 (47%)	n.s.
PSL administration	15 (60%)	84 (53%)	n.s.
CNI administration	8 (32%)	43 (27%)	n.s.
QMG scores			
Total	8.4 ± 4.9	6.2 ± 4.6	n.s.
Ocular symptoms	2.2 ± 1.6	1.6 ± 1.8	n.s.
Bulbar symptoms	0.3 ± 0.5	0.2 ± 0.6	n.s.
Limb symptoms	4.9 ± 3.0	3.7 ± 2.8	n.s.
Trunk symptoms	1.1 ± 0.7	0.8 ± 0.8	n.s.

*P-value < 0.05.
MG: myasthenia gravis; QMG: quantitative myasthenia gravis scoring; n.s.: not significant; AChR-Ab, autoantibodies to the acetylcholine receptor; PSL: prednisolone; CNI: calcineurin inhibitor.

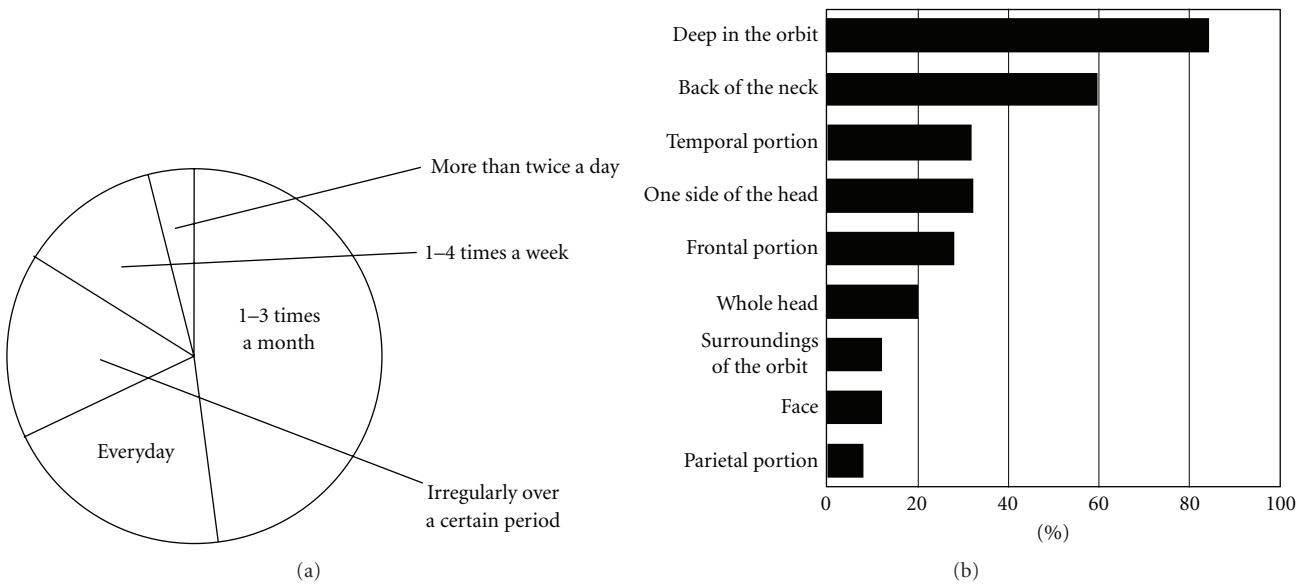

Figure 1: The characteristics of MG-associated headache. (a) The frequency of headache associated with MG ($N = 25$). (b) The rates of the patients, who complained of MG-associated headache in each site. Note that headache associated with MG occurs deeply in the orbit most frequently.

patients with severe QMG scores (scores greater than 3) would have fixed symptoms with little change, which would less frequently lead to headache associated with MG. These facts suggest that fluctuation of the MG ocular symptoms could actually underlie the headache of patients with MG. When we must classify MG-associated headache based on the ICHD-II [10], it should be most similar to secondary headache as shown in the code 11.3.3 "Headache attributed to heterophoria or heterotropia." However, MG *per se* is not included as one of the causative factors of secondary headaches in the ICHD-II.

MG is an autoimmune disease of the neuromuscular junction and does not affect the central pain pathway or other sensory tracts. We speculate that there is no direct pathologic relation between MG and headache. Indeed, since anticholinesterase agents had a partial or no effect on headache associated with MG, NSAIDs were eventually required to alleviate the pain in many cases. Based on these results, together with the results of analyses on the characteristics of MG-associated headache (Figure 1), it is difficult to distinguish MG-associated headache from tension-type headache. It was noteworthy that most of MG-associated

Table 2: Influences of clinical factors on headache associated with MG.

Variable	Odds ratio (95% CI)	*P*-value
Age (years)	1.0 (0.9–1.0)	n.s.
Female gender	4.5 (1.2–16.8)	*0.02
Age at onset (years)	1.0 (1.0–1.1)	n.s.
Mild ocular symptoms (QMG 1-3)	7.2 (2.4–21.8)	*0.0005
Mild bulbar symptoms (QMG 1-3)	2.3 (0.4–14.6)	n.s.
Total QMG score	1.1 (0.9–1.3)	n.s.

*P-value < 0.05.
MG: myasthenia gravis; n.s.: not significant; QMG: quantitative myasthenia gravis scoring; CI: confidence interval.

headache could be diagnosed with tension-type headache on the ICHD-II even when the suffered portion in 32% of the patients with MG-associated headache was restricted unilaterally (Figure 1(b)). Head dropping and fluctuation of ptosis and diplopia would accelerate visual fatigue and/or stiffness of the neck, which could be the most important precipitating factors of headache associated with MG.

It should be mentioned that this cross-sectional study has potential limitations related to recall bias and selection bias. A recall bias may have been present because patients with more severe MG symptoms may not have paid as much attention to their headache, and thus patients with mild ocular symptoms might have complained of headache more frequently. It would be another recall bias that the duration after onset of MG was as long as 8.4 years. Questionnaire asking the change of their headache characteristics between before and after the onset point had the most important role in providing the information for this study, but the duration might have been too long for completely accurate data. On the other hand, a selection bias may have played a role because the subjects of this study were limited to Japanese MG patients, who were receiving regular outpatient treatments. Due to this potential selection, neither MG patients with extremely mild symptoms nor critically severe conditions might have been included in this study. We cannot exclude these biased factors, and further investigations with a greater number of patients in all grades of MG symptoms will be needed.

In conclusion, headaches could disturb the quality of life of MG patients greatly even when their general symptoms of MG are comparatively well controlled. Careful attention to appearance or aggravation of headache in MG patients is important to improve their quality of life, especially in women with mild ocular symptoms.

Conflict of Interests

The authors declare that they have no competing interests.

Acknowledgments

The authors thank all of the patients described here. This study was supported by a Neuroimmunological Disease Research Committee Grant from the Japanese Ministry of Health, Labour, and Welfare.

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SAGE-Hindawi Access to Research
Autoimmune Diseases
Volume 2011, Article ID 740583, 7 pages
doi:10.4061/2011/740583

Review Article

Three Types of Striational Antibodies in Myasthenia Gravis

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Received 28 April 2011; Accepted 3 June 2011

Academic Editor: Johan A. Aarli

Myasthenia gravis (MG) is caused by antibodies that react mainly with the acetylcholine receptor on the postsynaptic site of the neuromuscular junction. A wide range of clinical presentations and associated features allow MG to be classified into subtypes based on *autoantibody* status. Striational antibodies, which react with epitopes on the muscle proteins titin, ryanodine receptor (RyR), and Kv1.4, are frequently found in MG patients with late-onset and thymoma. Antititin and anti-RyR antibodies are determined by enzyme-linked immunosorbent assay or immunoblot. More recently, a method for the detection of anti-Kv1.4 autoantibodies has become available, involving 12–15% of all MG patients. The presence of striational antibodies is associated with more severe disease in all MG subgroups. Anti-Kv1.4 antibody is a useful marker for the potential development of lethal autoimmune myocarditis and response to calcineurin inhibitors. Detection of striational antibodies provides more specific and useful clinical information in MG patients.

1. Introduction

Acquired myasthenia gravis (MG) in an organ-specific autoimmune disorder generally mediated by antiacetylcholine receptor (AChR) or less frequently by antimuscle-specific tyrosine antibodies at the neuromuscular junction [1]. Some MG patients have antibodies that bind in a cross-striational pattern to skeletal and heart muscle tissue sections. They were known as "striational antibodies." These autoantibodies recognize epitopes on skeletal muscle proteins including myosin, actin, actinin, and filamin [2–5]. Particularly, three types of striational antibodies including those to titin, ryanodine receptor (RyR), and Kv1.4 have been investigated by many researchers. The detection of these three striational antibodies can provide more specific clinical information and are associated with the subtypes of MG patients. In this article, we describe the characteristics of these three types of striational antibodies.

2. Molecular Structure

Titin is a giant protein (3000 kD) abundantly in the skeletal and cardiac sarcomere. Ninety percent of the titin mass is contained in a repetitive structure of 2 different 100-residue repeats [6]. Anti-titin antibody was first discovered in the serum of MG patients by Aarli et al. in 1990 [7]. Autoantibodies to titin are now determined by a commercially available enzyme-linked immunosorbent assay (ELISA). The main immunogenic region of titin is called myasthenia gravis titin-30 (MGT-30) and is situated near the A/I-band junction [8–10].

RyR is a calcium release channel located in the sarcoplasmic reticulum. There are two forms of RyR, skeletal (RyR1) and cardiac (RyR2). The RyR is a protein containing 5035 amino acids with a molecular weight of 565 kD. It is composed of 4 homologous subunits that can build a tetramer with a central channel [8]. Anti-RyR antibody was first identified by Mygland et al. in 1992 using western blot for the presence of antibodies to the protein of the sarcoplasmic reticulum from rabbit skeletal muscle [11]. Although cardiac and skeletal muscle RyRs are antigenically different, anti-RyR antibodies in MG patients cross-react with both subtypes of the receptor [12]. Several epitopes in both the N- and C-terminus of RyR1 sequence are identified and used as antigenic peptide in ELISA.

Voltage-gated K channel (VGKC) consists of four transmembrane α-subunits that combine as homo- or heterotetramers. Kv1.4 is an α-subunit with a molecular weight of

73 kD located mainly in the brain, peripheral nerves, and skeletal and heart muscles. Anti-Kv1.4 antibody was first discovered by our group in 2005 using a protein immunoprecipitation assay using ^{35}S-labeled rhabdomyosarcoma (RD) cellular extracts [13]. We cannot detect anti-Kv1.4 antibody by immunoblot or ELISA using Kv1.4 recombinant protein. This finding suggests that conformational epitopes may be necessary for the detection of anti-Kv1.4 antibody.

3. Antibodies Detection

MG can be classified into several subtypes based on the autoantibodies profile [1, 8]. Striational antibodies are principally detected only in the sera of MG patients, but not in healthy or diseased controls. Striational antibodies are rarely found in AChR antibody-negative MG. The seropositivity of striational antibodies was different in the examined populations. Generally, anti-titin antibody is detected in 20–40% of all MG patients, anti-RyR in 13–38%, and anti-Kv1.4 in 12–15% [8, 14–19]. It is well known that striational antibodies are associated with the late-onset MG subgroup. The disease onset age is eldest in MG patients with anti-titin antibodies and youngest in those with anti-Kv1.4 antibodies [8, 14–19]. It is likely that the gender ratio is almost equal in striational antibodies.

Anti-titin antibodies are closely associated with older-onset MG, and 60–80% of MG patients at disease onset older than 60 years have anti-titin antibodies [8, 14–17, 19]. Our recent study showed that 32% of late-onset MG cases without thymoma were positive for anti-titin antibodies when the cutoff age between early- and late-onset MG was defined as 50 years [20, 21]. In addition, there can be two or three of striational antibodies in a single MG patient. When we measured anti-AChR, anti-titin, and anti-Kv1.4 antibodies in 209 Japanese MG patients, we found 8 MG patients who were positive for all three autoantibodies [19].

To date, anti-titin and anti-RyR antibodies have been examined in many MG patients in the US, Europe, and Asian countries. Since common characteristics of MG patients with anti-titin and anti-RyR antibodies have been described, the clinical picture may be common across different ethnic groups and immunogenetic backgrounds. On the other hand, since anti-Kv1.4 antibodies were studied only in Japan, further examination is necessary in Caucasian MG patients.

4. Immunopathogenesis

There is no evidence that striational antibodies can really induce structural changes in skeletal muscle [8]. However, striational antibodies potentially indicate the presence of a pathological process that, in addition to the AChR antibody-mediated NMJ transmission defect, influences the muscle cell function of the patient.

Some immunological evidence including complement activation by striational antibodies and T cell proliferative response to MGT-30 have been reported [22–24]; however, these results do not prove the existence of any pathogenic role for striational antibodies in MG. The presence of titin antibodies in patients with MG correlates with the electromyographic evidence of myopathy [25]. Anti-titin antibody is associated with HLA DR7 in Caucasian MG patients [8, 15, 21]. DR3 and DR7 have opposing effects on MG phenotypes in Caucasians. DR3 has a positive association with early-onset MG and a negative association with late-onset MG, and DR7 has the opposite association [1]. DR3 and DR7 are very rare in Japanese populations. In contrast, DR9 and DR2 in Japanese MG patients have similar associations to those of DR3 and DR7, respectively, in Caucasian patients [20].

Anti-RyR antibodies cause allosteric inhibition of RyR function in vitro, inhibiting Ca^{2+} release from the sarcoplasmic reticulum [26]. Some MG patients have been shown to have impaired excitation-contraction (E-C) coupling in addition to neuromuscular transmission failure [27, 28]. The mechanisms of E-C coupling are closely related to RyR function, and anti-RyR antibody may influence muscle contraction. In this regard, a 27-year-old MG patient, who was positive for anti-RyR antibody, but not anti-AChR, was proven to be impaired E-C coupling [29]. In addition to anti-RyR antibody, autoantibodies against dihydropyridine receptor or transient receptor potential canonical type 3, which have functional interactions with RyR1 in Ca^{2+} release, were also detected in MG patients [30, 31].

On the other hand, antineuronal VGKC antibody is principally different from anti-Kv1.4 antibody (muscular VGKC). Autoantibodies to neuronal VGKC are known to be associated with acquired neuromyotonia, Morvan's syndrome, and autoimmune nonparaneoplastic limbic encephalitis. The sera of patients with these diseases mainly target the Kv1 α-subunits: Kv1.1, Kv1.2, or Kv1.6. The expression of these subunits that form Kv channels differs between the brain and muscle. Recently, the clinical spectrum of neurologic manifestations associated with neuronal VGKC autoimmunity has been expanding [32]. However, leucine-rich, glioma-inactive protein 1 was identified as a novel autoantigen in limbic encephalitis previously attributed to neuronal VGKC [33].

5. Thymoma-Associated MG

Most patients with thymoma-associated MG (T-MG) demonstrate an antibody profile with a broad striational antibody response [1, 8]. The presence of thymoma is thought to worsen the prognosis of MG, as symptoms are usually severe in these patients and are not significantly improved by thymectomy [34]. Of the 260 MG patients in our institutions, 62 (24%) had thymoma. The onset age of the thymomatous MG patient was 47 $\pm$ 12 years [19]. Bulbar involvement and myasthenic crisis were more common in patients with T-MG than in those without thymoma.

The frequencies of striational antibodies in T-MG patients are generally high. Many reports have shown that anti-titin antibodies are detected in 49–95% of T-MG, with anti-RyR found in 70–80% of cases and anti-Kv1.4 in 40–70% of cases [7, 8, 11–20]. A high frequency of striational antibodies in T-MG contributes to severe MG

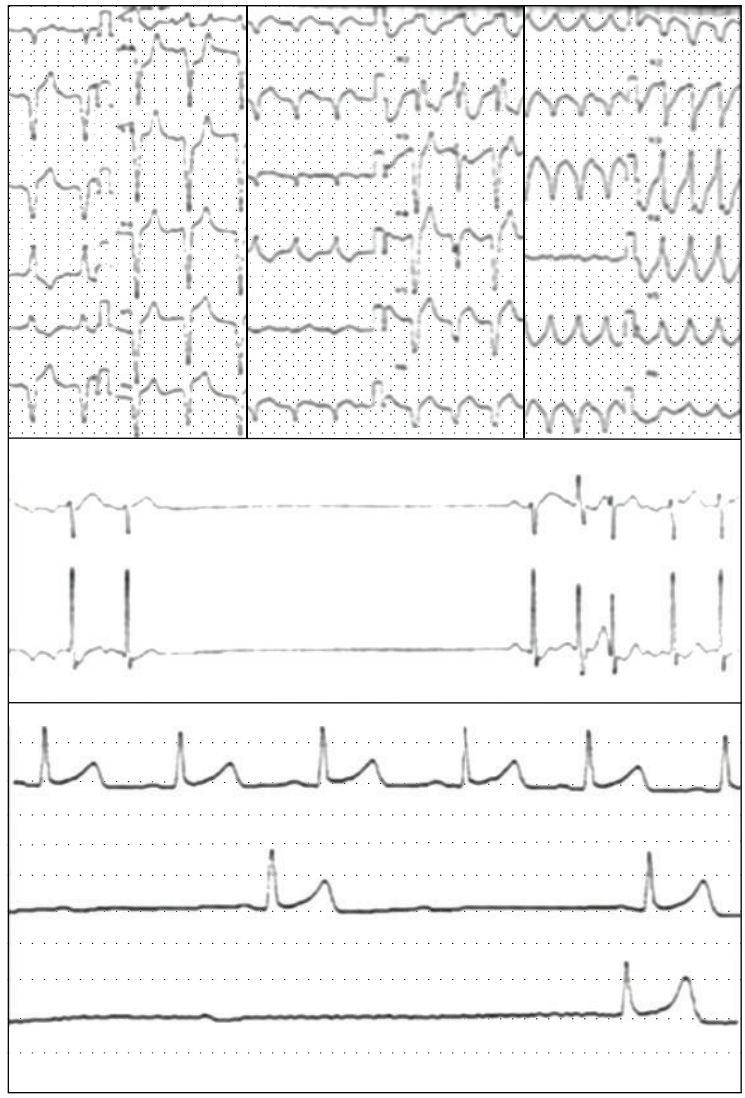

Figure 1: Electrocardiogram in myasthenia gravis patients with anti-Kv1.4 antibodies. (a) Ventricular tachycardia, (b) sick sinus syndrome, and (c) complete atrial ventricular block.

symptoms. Titin and RyR epitopes have been identified in thymoma tissue [35–38]. In addition, we also confirmed that Kv1.4 mRNA was detected in thymoma tissue [13]. It is generally believed that the aberrant immunization of T-cell against autoantigens is promoted by the pathogenic microenvironment inside the thymoma [1, 8]. IgG striational autoantibodies to titin are also produced by clonal thymic B cells established from patients with T-MG [39]. In addition, the autoantibodies for C-terminal regions in RyR1 are frequently detected in T-MG and may contribute to muscle dysfunction via the impairment of Ca^{2+} release [40]. Anti-RyR antibodies are found in spontaneous thymoma model rats [41]. Clinically, the presence of striational antibodies and computed tomographic scans of the anterior mediastinum show a similar sensitivity for thymoma in MG patients. The presence of titin and RyR antibodies in a young patient with MG strongly suggested the presence of a thymoma [8].

6. Clinical Presentation

The presence of striational antibodies is associated with more severe disease in all MG subgroups. Many studies clearly demonstrated that disease tends to be more severe in MG patients found to be positive for each striational antibody than in those found to be negative [8, 13, 17, 18, 42–44]. When the disease severity is compared between different striational antibodies, MG patients with anti-Kv1.4 antibodies show more severe symptoms than those with anti-titin antibodies [19]. Similarly, it is also reported that MG patients with anti-RyR antibodies have more severe manifestations than those with anti-titin antibodies [18]. In anti-Kv1.4-positive patients, the frequencies of bulbar involvement and myasthenic crisis were 73% and 31%, respectively [19]. In addition, patients with anti-RyR antibodies have high rates of bulbar, respiratory, and neck involvement at MG onset [18].

Table 1: Three types of striational antibodies in myasthenia gravis (MG).

Autoantigen	Titin	Ryanodine receptor (RyR)	Voltage-gated K channel (VGKC) Kv1.4
Molecular structure	Skeletal and cardiac sarcomere	RyR1: skeletal type	Brain, nerve, skeletal, and heart muscles
	Giant protein (3000 kD)	RyR2: cardiac type	Homo- or hetero-tetramers
	Repetitive structure	565 kD with 4 homologous subunits	One α-subunit (73 kD)
Original report	Aarli et al. 1990 [7]	Mygland et al. 1992 [11]	Suzuki et al. 2005 [13]
Antibodies detection	ELISA (commercially available)	ELISA, Western blot	Immunoprecipitation assay
	Myasthenia gravis titin-30 (MGT-30) near the A/I-band junction	Epitopes in both RyR1 and RyR2	35-S-labeled rabdomyosarcoma cell
		Sarcoplasmic reticulum from rabbit skeletal muscle N- and C-terminus of RyR1 sequence	Band at 70 kD
Epidemiology	20–40% in all MG patients	13–38% in all MG patients	12–15% in all MG patients
	60–80% in MG patients older than 60 years	Mean onset age: 57 years	Mean onset age: 49 years
	32% in nonthymoma MG patients older than 50 years	M : F = 1 : 1	M : F = 1 : 1
Immunopathogenesis	T cell proliferative response to MGT-30	Complement activation	Different from neuronal VGKC
	Complement activation	Inhibiting Ca^{2+} release from sarcoplasmic reticulum	QT prolongation on electrocardiogram
	Myopathy in electromyogram	Autoantibodies to dihydropyridine receptor	
	Association with DR7 in Caucasians	or transient receptor potential canonical type-3	
		Inhibiting excitation-contraction coupling	
Thymoma-associated MG (T-MG)	49–95% in T-MG	70–80% in T-MG	40–70% in T-MG
	Titin epitope expression	RyR epitope expression	Kv1.4 mRNA expression
	Production from clonal thymic B cells	Diagnosis of thymoma	
	Diagnosis of thymoma (younger than 50 years)	C-terminal regions in RyR1 as epitope	
Clinical presentation	Association with severe MG	More severe than anti-titin	More severe with anti-titin
	Concomitant with myositis	Bulbar, respiratory, and neck involvement	Bulbar involvement and myasthenic crisisMyocarditis and/or myositis
		Myocarditis and/or myositis	Lethal arrhythmias
Treatment and management	Some late-onset MG with ocular type	Early pharmacological effect of tacrolimus	Responder to calcineurin inhibitors
		poor prognosis in invasive thymoma	Sudden death

The other remarkable finding is the association between striational antibodies and myositis and/or cardiomyositis concomitant with MG [45, 46]. Autoimmune-mediated myocarditis and/or myositis developed in a few patients with MG, especially thymoma-associated MG. Inflammatory myopathies did not only lead to the deterioration of muscular weakness, but were also the most serious complications in the courses of MG patients. Since the mortality of MG itself has dramatically decreased recently, cardiac involvement in MG patients may be lethal. It is well known that myocarditis

is accompanied by thymoma-associated MG, known as "Herzmyathenie" [46, 47]. In this regard, Evoli et al. reported that 7 of 50 T-MG cases suffered sudden deaths [34]. They speculated that some of these cases were affected by myocarditis, although autopsy studies were not performed.

Our survey in 5 Japanese institutions showed that of 924 MG patients, 8 (0.9%) had inflammatory myopathies [46]. The onset age of MG was 55.3 ± 10.3 years. All patients showed severe symptoms with bulbar involvement accompanied by myasthenic crisis in 5 and invasive thymoma in 4. Myocarditis was found in 3 patients and myositis in 6. Myocarditis, developing 13–211 months after MG onset, was characterized by heart failure and arrhythmias. Histological findings of skeletal muscles showed CD8+ lymphocyte infiltration. Seven patients had at least one of three striational autoantibodies. Immunomodulatory therapy was required in all patients and was effective for both MG and inflammatory myopathies, except in one autopsy case.

We also confirmed that some MG patients with anti-Kv1.4 antibodies had a risk for lethal arrhythmias including ventricular tachycardia, sick sinus syndrome, and complete atrial ventricular block (Figure 1) [48, 49]. We emphasize that special attention should be paid to MG patients with anti-Kv1.4 antibodies. Although various Kv channels are expressed in cardiac muscles and implicated in electrophysiological function, the association between autoantibodies to Kv channels and arrhythmias is not fully elucidated [50]. We are now investigating the mechanisms of the cardiac involvement of Kv1.4 autoimmunity.

7. Treatment and Management

Since MG patients with striational antibodies have severe symptoms, they usually require strong immunosuppressive treatments. To avoid the many serious side effects of corticosteroids, we prefer to use a combination of low or medium doses of prednisone and other immunosuppressive agents. In Japan, two calcineurin inhibitors (CNIs), cyclosporine and tacrolimus, are widely used, since they are officially approved as medications for MG treatment under the national health insurance system in Japan. Since tacrolimus acts as an enhancer of RyR-related Ca^{2+} release from the sarcoplasmic reticulum, anti-RyR antibody is linked to the early pharmacological effects of tacrolimus [40]. To assess the factors associated with the response to CNIs in MG, we retrospectively analyzed the 6-month effect of CNIs in 62 MG patients [51]. Patients who achieved either a ≥3-point reduction in quantitative MG score or a ≥25% reduction in the daily dose of prednisolone were regarded as responders to CNIs. Anti-Kv1.4 antibody was proven to be associated with being a responder to CNIs.

The long-term prognosis of MG patients with striational antibodies has not been fully elucidated. Side effects of immunotherapies including infection, diabetes, stroke, ischemic heart disease, and cancers may also be more important factors in mortality associated with MG than the MG itself. The detection of striational antibodies may be potentially useful for planning therapeutic strategy. The presence of anti-RyR antibodies in T-MG and titin/RyR in nonthymoma MG indicates a less favorable prognosis [43]. However, some late-onset MG patients with anti-titin antibodies are limited to the ocular form for long periods. Further studies are necessary to prove the association between the striational antibodies and the long-term prognosis of MG.

8. Conclusion

We reviewed the characteristics of three types of striational antibodies (Table 1). Although 20 years have passed since the discovery of anti-titin antibodies in MG patient, the detection of striational antibodies is not routinely tested in the clinical management by all neurologist. Recently, several therapies for MG have emerged, including rituximab and antigen-specific apheresis whereas other treatments await clarification of efficacy and their role in MG [1]. The treatment of MG should be individualized according to clinical presentation or subtype, and requires a comprehensive assessment of the patient's functional impairment and the effect of MG on his or her daily life. The detection of striational antibodies can provide information that is useful for the classification and management of MG patients.

Acknowledgments

This paper was supported by a Grant from the Japanese Ministry of Education, Science, Sports, and Culture (no. 23591255) and a Neuroimmunological Disease Research Committee Grant from the Japanese Ministry of Health, Labour, and Welfare.

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