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Phenotypic and Functional Analysis of Lymphocytes in Myasthenia Gravis

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Introduction

Myasthenia gravis (MG) is a disease of neuromuscular transmission which is manifested clinically by muscle weakness, frequently of a profound degree. It is one of a new class of autoimmune diseases whose pathogenesis is mediated by autoantibodies directed against tissue receptors. In myasthenia, the responsible antibodies are directed against the nicotinic acetylcholine receptors (AChR) located at postsynaptic myoneural junctions. The interaction of these antibodies with AChR disrupts neuromuscular transmission.

Although the pathogenesis of MG has been elucidated only within the past decade, immunologists had long been interested in this disease. MG occurs in association with other putative autoimmune disorders including rheumatoid arthritis and systemic lupus erythematosus [reviewed in 61] and is associated with an increased incidence of an array of autoantibodies including antinuclear factor, anti-thyroglobulin and rheumatoid factor [reviewed in 62]. Other clues pointing to the immune system were provided by several lines of evidence implicating the thymus in the pathogenesis of MG. Patients frequently showed dramatic clinical improvement after thymectomy. Thymic pathologic findings were commonly observed with germinal center hyperplasia in as many as 75% of patients and lymphoepithelial thymomas in an additional 10% [16, 55]. Cells within the thymus, including myoid and epithelial cells [25, 43, 128] and perhaps even thymocytes [29] were shown to express AChR. These observations, taken together, suggested that the thymus represented a potentially important focus for initiating or perhaps perpetuating the autoimmune response in MG.

Despite significant advances in the characterization of antibodies to AChR (hereafter called anti-AChR) and elucidation of their immunopathogenic role in MG, a wide gap remains in the understanding of those mechanisms which are responsible for the emergence of these antibodies. In an effort to delineate such

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mechanisms, several laboratories have investigated the properties of cellular components and soluble factors that are intimately involved with regulating normal immune responses, the rationale being that defects in such an immunoregulatory network might account for the emergence of autoimmunity in MG. As is the case in many clinical disorders, investigators have focused on studies of blood lymphocytes. However, since the thymus is likely involved in the pathogenesis of MG and since this organ appears to be the pivotal site for establishment of the T cell repertoire [136], considerable attention has also been directed at thymic cell populations. In this presentation, we will review the body of information that deals with lymphocyte abnormalities in human MG with particular emphasis on aberrant immunoregulation. The reader is referred elsewhere in this series for comprehensive discussions of anti-AChR antibodies and experimental MG.

Aberrant B Cell Responses

Serum Immunoglobulins

Using a sensitive radioimmunoassay which employs mammalian AChR as substrate, anti-AChR are found in the serum of 80–90% of patients with MG [53, 60]. The majority of antibodies in most patients with MG are generally not directed against the ACh binding portion of the receptor but rather to part of the externally exposed alpha subunit [123]. Of interest, the α subunit has recently been reported to share determinants with membrane constituents of ubiquitous gram negative organisms [114]. Anti-AChR are responsible for impaired neuromuscular transmission and account for the reduced numbers of AChR at neuromuscular junctions [111, 120]. Their mechanism of action is likely several fold and includes accelerated receptor degradation [6, 38, 44] interference with ACh binding [21] and complement-mediated destruction of post-synaptic folds [21, 24]. Accentuated B cell responses are not confined solely to anti-AChR, as other autoantibodies including anti-thyroid, anti-nuclear, and anti-striated muscle antibodies to name a few (Table 1) are frequently found in patients with this disorder [reviewed in 62]. Quantitation of serum immunoglobulin levels indicates that many patients have elevated serum IgG levels [64], an indication of in vivo polyclonal B cell activation.

In Vitro Analysis of B Cell Function: Peripheral Blood Cells

Further evidence for augmented in vivo polyclonal B cell activation was provided by our finding [56] of increased frequency of blood lymphocytes which spontaneously secrete immunoglobulin (Fig. 1). The frequency of such spontaneously immunoglobulin secreting cells (IgSC) is considered an index of the level of in vivo B cell activation. The finding of increased circulating IgSC levels in patients with MG is consistent with increased antecedent in vivo B cell activation. Increased numbers of IgSC were observed in one third of the patients and tended to be associated with increased clinical activity.

The percentages of B cells expressing surface immunoglobulin is normal in the peripheral blood mononuclear cells (PBM) of MG patients [58, 70]. Also, stimulation of patient PBM with pokeweed mitogen (PWM), a polyclonal B cell

Table 1. Serum autoantibodies in myasthenia gravis

Acetylcholine receptor	70–90%
Striated muscle	20–50%
Nuclear[a]	20–40%
Mitochondrial	4–6%
Smooth muscle	5–10%
Thyroid	15–40%
Gastric parietal cell	10–20%
Rheumatoid factor	10–40%
Coombs[a] test	10%
Heterophile antibodies	10%
False-positive serology	0.5–1%
Antiplatelet	5–50%[b]
Lupus erythematosus preparation	1–2%
Antilymphocyte	40–90%
Specific neuronal antinuclear	[c]
Squamous epithelium	8%

[a] Antinuclear antibodies
[b] Different assays in same study
[c] It is not certain that such antibodies exist
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activator whose action depends on the interaction between T cells, B cells, and monocytes, results in a normal pattern of polyclonal IgSC responses [58].

Studies of anti-AChR production by such PBM cultures have proven to be particularly illuminating. We have found that even unstimulated cultures of PBM from MG patients frequently elaborate anti-AChR [69] whereas anti-AChR production by unstimulated normal PBM has never been observed (Table 2). Furthermore, addition of PWM to the culture system augments the secretion of anti-AChR antibody in the majority of patients (Table 2). By contrast, PWM-induced anti-AChR responses are observed in only 5–8% of normal cultures. We found that this secretion of anti-AChR by the unstimulated or PWM-stimulated PBM of MG patients was not simply a reflection of a nonspecific in vitro B cell hyperreactivity. The levels of secreted anti-AChR did not correlate with levels of secreted Ig but did correlate significantly with the serum anti-AChR levels in individual MG patients.

Production of anti-AChR by PWM-stimulated PBM of MG patients has been reported by others [75]. However, Newsome-Davis and colleagues [83, 84] have found little or no in vitro synthesis of anti-AChR by PBM of MG patients with or without stimulation with PWM. In their study, anti-AChR production by patient PBM was markedly augmented when they were co-cultured with autologous thymus cells. In fact, the observed response was greater than that seen when the PBM were incubated with PWM. This phenomenon was observed only when the cell populations were obtained from patients whose thymus showed changes of germinal center hyperplasia on pathologic analysis. Furthermore, the enhancing effect of thymic cells appeared relatively specific for anti AChR-production by PBM since neither production of total IgG nor anti-influenza antibody were significantly augmented. It is not known whether this enhancement by the addition of

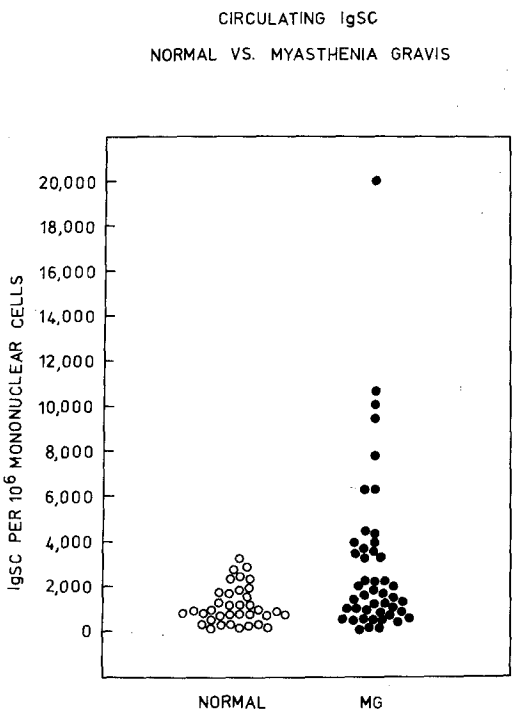


Fig. 1. Circulating IgSC in blood of normal and myasthenic subjects. Reproduced by kind permission of the Editor of Neurology

thymocytes reflects presentation of an AChR-like material present in the thymus of the action of particularly effective helper T cells in the thymocyte population. Also, it is not yet known if thymus cells from non-MG subjects induce or augment anti-AChR production by PBM from patients with MG. In addition, it is not known if thymus cells from patients with MG induce anti-AChR production by normal PBM. More recently, these investigators have found that enzymatically dispersed lymph node cells from patients with MG synthesize anti-AChR [131].

Table 2. In vitro synthesis of anti-AChR antibodies and IgG

	Anti-AChR$_c$ [a]	IgG$_c$ [b]	Anti-AChR$_{PWM}$ [a]	IgG$_{PWM}$ [b]	Serum Anti-AChR [d]
Myasthenia	3.9 ± 1.1 [e]	448 ± 68	28.1 ± 6.7 [e]	$2{,}197 \pm 235$	21.3 ± 3.1 [f]
Controls	0.4 ± 0.2	442 ± 100	0.87 ± 0.5	$2{,}440 \pm 249$	5.8 ± 0.3

[a] fmol ^{125}I-alpha-bungarotoxin bound/10^7 cultured cells (mean $\pm$ SEM); c: nonstimulated cultures; PWM: pokeweed mitogen
[b] ng/ml secreted (mean $\pm$ SEM)
[d] pmol ^{125}I-alpha-bungarotoxin bound/ml serum (mean $\pm$ SEM)
[e] $p < 0.001$ versus normal
[f] $p < 0.01$ versus normal

Intrathymic B Cells

Frequency and Localization

In light of the striking presence of germinal centers in MG thymus, considerable attention has been focused on the antibody secreting potential of the cells from this organ. The thymus is not generally considered an organ of B cell activity. Indeed, prominent intrathymic germinal center formation has been described in only two other clinical settings, e.g., SLE [15, 73] and thyrotoxicosis [107]. Smaller and less numerous follicles may be present in thymus glands of healthy individuals [77, 126]. Germinal center formation in peripheral lymph nodes occurs in response to the local trapping of antigen. It is possible that germinal center formation in the MG thymus occurs on an analogous basis and represents a response to locally trapped antigen, e.g., intrathymic AChR. Antigen trapping is facilitated when the antigen is complexed with antibody [47, 74]. Since the thymus is a source of anti AChR- (see below) both antigen and antibody are theoretically available in this organ, perhaps insuring the maintenance of germinal center formation. In turn, such germinal centers may provide a rich source of memory B cells [47, 116].

A number of approaches have been used to explore the phenotypic properties and the possible biologic significance of these germinal centers. In an early histopathologic study, they were shown to contain EAC rosetting cells, consistent with B cell composition [110]. Recent immunohistologic studies indicated that there were few if any surface Ig positive B cells in the thymic cortex of MG patients or control subjects [17, 89, 115]. The cellular constituents of the germinal center were similar to that seen in peripheral lymph nodes [51]. That is, IgM + IgD + cells were observed in the mantle zone whereas IgM + IgD − cells comprised the follicular center (Fig. 2). Occasional IgM + and IgD + cells were found in the medulla outside the germinal follicles in MG and non-MG patients.

B cells have also been enumerated in cell suspensions of thymus tissue obtained from patients with MG. Earlier studies, relying on EAC rosetting techniques and detection of surface Ig positive cells with reagents available at that time, suggested greater frequency of B cells in the thymus of MG patients than in normals [1, 2, 19, 65, 86, 88, 103]. More recently, using improved anti-Ig reagents, we [58, 70] reported a considerably lower frequency of surface Ig positive B cells in MG thymus than we and others had previously identified. Indeed, we observed no significant difference between percentages of B cells in suspensions of MG and control thymus cells. This observation was all the more surprising since several of the suspensions were prepared from tissue which demonstrated follicular hyperplasia on histologic analysis. We believe that the discrepancy between our new findings and the earlier reports largely reflects current improvements in the immunofluorescence technique employed for identification of B lymphocytes; these have led to lower percentages of cells now counted as bonafide surface Ig-positive. Furthermore, there was no correlation between the degree of germinal center hyperplasia in individual MG thymus specimens and the percentage of B cells in the cell suspension from that tissue. This apparent paradox did not reflect error introduced by tissue sampling, since cell suspensions were prepared from representative tissue fragments and comparable results were obtained when suspensions were prepared from minced

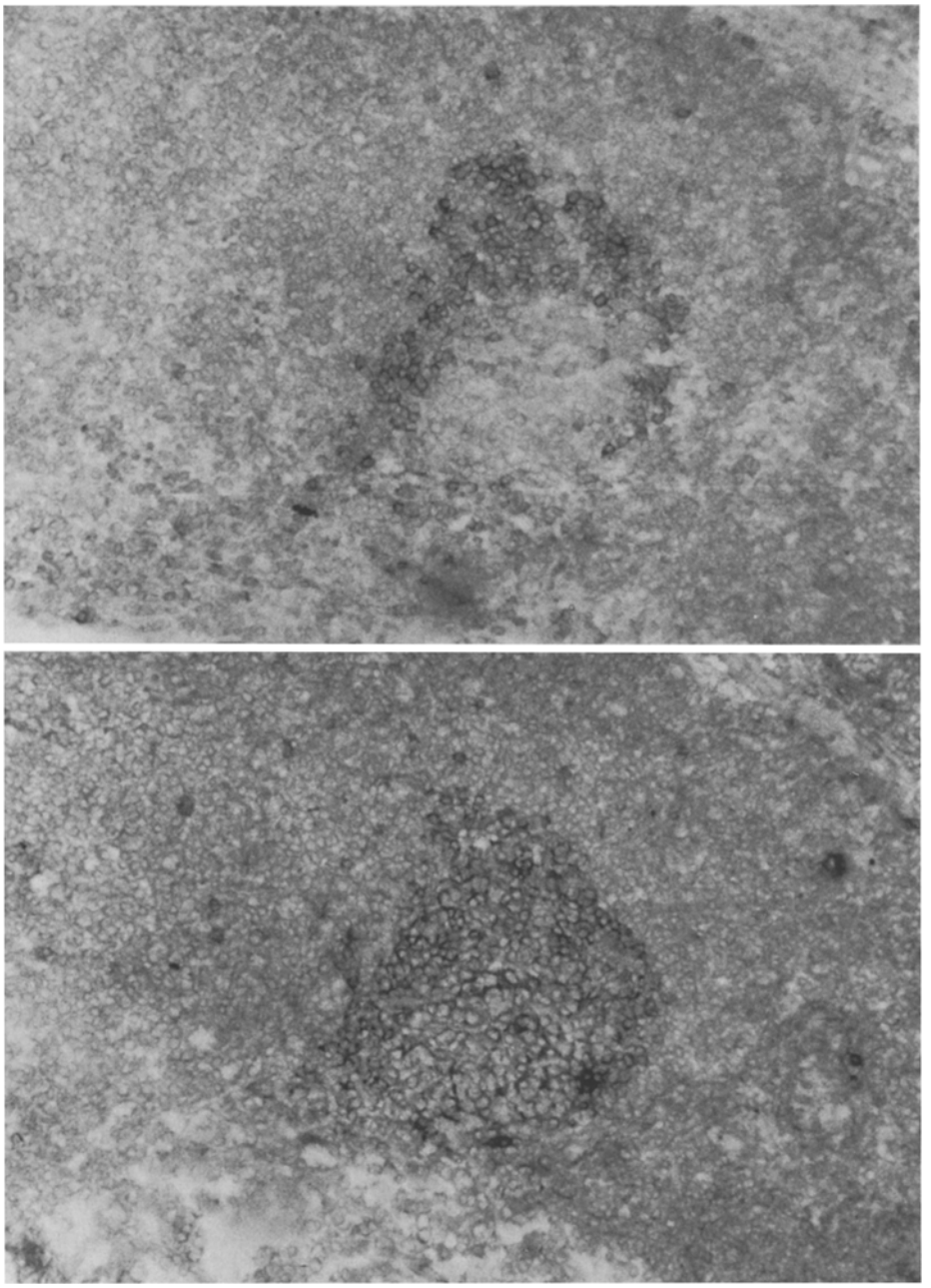


Fig. 2. Frozen section of MG thymus with germinal center. (*Top*) Immunoperoxidase staining demonstrates IgD positive cells in the mantle zone. (*Bottom*) This section demonstrates IgM positive cells in the mantle zone and follicular center. Scattered IgD and IgM positive cells are present in the medulla outside of follicles (hematoxylin counterstain, x400). Reproduced with kind permission of the Editor of the American Journal of Pathology

thymic fragments incubated with collagenase prior to cell dispersion. Rather, we suggested that the B cells present in such germinal centers were diluted by the tremendous excess of thymic non-B cells in the cell suspensions.

Antibody Activity

Studies have been undertaken to assess the possible functional significance of thymic B cells. Smiley et al. reported that thymic tissue fragments from MG patients secreted Ig when cultured in vitro [108]. We [58] found an increased frequency of spontaneous IgSC in myasthenic thymus cell suspensions compared to control thymus cell suspensions (obtained from subjects undergoing cardiac surgery), a finding indicative of enhanced in vivo B cell activation in myasthenic thymus. In a sizeable group of MG patients the frequency of IgSC was higher in thymus cell suspensions then in suspensions of PBM, a pattern not observed in the control group. There was no correlation between the frequency of spontaneous IgSC and the histologic appearance of the thymus. This suggested that accentuated B cell activation may occur in vivo in MG thymus in the absence of formation of germinal centers. We speculated that this apparent compartmentalized B cell response might reflect (1) the action of a local B cell activator not found in normal thymus, perhaps altered AChR, and/or (2) a local immunoregulatory milieu that favors activation of B cells once they are triggered.

Some light was shed on the latter point by studies carried out in PWM-stimulated cultures [58]. Thymocytes from both MG and control subjects differentiated prominently into cells secreting Ig following culture with this polyclonal activator. Surprisingly, these thymocyte responses frequently exceeded those in the PWM-cultured autologous PBM, even though the B cell percentages were uniformly much less in the thymocyte suspensions than in the PBM at the initiation of culture. Thus, it is possible that the thymus of most adult humans has the capacity for prominent humoral immune responses despite a relative paucity of B cells. The thymus, therefore, may represent an extremely favorable milieu (relative to blood) for B cell differentiation. It is also possible that the B cells present in thymus constitute a particularly reactive B cell subset (relative to peripheral blood B cells). We reasoned that this property of thymic B cells would not be manifested in vivo unless they were provided with a stimulus, as might be provided by AChR in the myasthenic thymus.

Studies initially described by Newsome Davis et al. address the specificity of thymic cell antibody secretion [83, 84, 102, 127, 131]. They reported in vitro synthesis of anti-AChR by thymic lymphocytes of patients with MG, but only when the thymus showed the histologic appearance of germinal center hyperplasia [83, 102]. They found that: (a) peak production of anti AChR occurred in the first four days in unstimulated cultures; (b) addition of PWM uniformly inhibited anti-AChR synthesis in cultures extended for eight days; (c) thymic lymphocytes produced more anti-AChR than did PBM of the same subject; (d) thymic lymphocytes produced more anti-AChR in vitro when previously exposed to collagenase and protease during preparation of the cell suspensions [131]. The mechanism of PWM-induced suppression of anti-AChR antibody production was not investigated. In particular, it is not known if PWM had a similar effect on total IgG synthesis.

This phenomenon, i.e., inhibition of Ig production by a polyclonal B cell activator, is seen in other clinical disorders where B cells have presumably undergone prior in vivo activation. Examples include: (1) inhibition of Ig production by PWM-stimulated PBM of SLE patients [57]; (2) inhibition of IgE synthesis by PWM-stimulated PBM of patients with hyper-IgE syndromes [99], and (3) inhibition of rheumatoid factor production by PWM [121] or peptidoglycan [59] stimulated PBM of patients with rheumatoid arthritis.

We have studied the in vitro synthesis of anti-AChR by thymic lymphocytes of 11 patients with MG who did not have thymoma [68]. We found that (a) cells cultured for 8–9 days secreted more anti-AChR than those cultured for only 4–5 days, similar to our earlier findings with cultured PBM; (b) cycloheximide inhibited anti-AChR secretion indicating that the secreted anti-AChR was actively synthesized in vitro; (c) in some patients, peak anti-AChR synthesis by thymic lymphocytes was greater than that by PBM, although PBM always synthesized more IgG; and (d) addition of PWM had a variable effect on anti-AChR production by thymocytes, – inhibition, enhancement, or no effect. By contrast, addition of PWM to cultures of PBM, as mentioned earlier, always increased anti-AChR secretion. In general, investigators have observed a strong correlation between thymocyte in vitro anti-AChR secretion and serum anti-AChR in individual MG patients, highlighting the possible biologic relevance of this in vitro activity. Calculations made in one study suggest that the thymus anti-AChR secretion represents a small component of the total in vivo anti-AChR response [102].

The evidence accumulated to date underscores the considerable heterogeneity among patients with respect to in vitro assessment of B cell function. Further work is clearly needed to delineate the responsible factors and to correlate findings with clinical features of disease, including response to thymectomy. Nevertheless, these findings have provided new insights into the thymus as a potential source of antibody production.

Cell-Mediated Immunity (CMI)

Several investigators have addressed the integrity of the T cell limb of the immune response with often conflicting results. This is particularly true of studies assessing delayed hypersensitivity skin test responses in MG patients. Some investigators [5, 97] have found decreased capacity to become sensitized to dinitrochlorobenzene, a primary antigen, and decreased cutaneous responsiveness to recall antigens, whereas others have found normal or supernormal responses [35, 50, 109]. In our experience, MG patients not treated with corticosteroids or immunosuppressive agents, demonstrated a pattern of delayed hypersensitivity comparable to that observed in normals.

In vitro responses that are thought to be correlates of CMI have also been examined in patients with MG, again with variable results. Some investigators have found depressed responses to phytomitogens in non-thymectomized MG patients [41, 45, 105] whereas others [30, 40, 97] including ourselves [63] did not confirm this abnormality. The response to standard mitogens has been tested using indirect

macrophage migration [9] and indirect leukocyte migration inhibition [35]. No difference was found between the degree of sensitivity of patients with MG and controls to streptokinase-streptodornase or PPD.

In earlier studies, blood T cells were enumerated by their capacity to rosette with sheep erythrocytes. On the basis of this criterion, most reports indicated normal relative and absolute numbers of peripheral blood T cells in MG (see Analysis of Immunoregulatory Mechanisms for further discussion).

Studies have also been carried out to search for evidence of CMI to muscle and AChR antigen in patients with MG. When blood lymphocytes were stimulated with muscle extracts, variable results were obtained. Thymic lymphocytes from patient specimens in general did not show enhanced proliferative responses in the presence of muscle extract [63]. However, thymic cells were found to enhance PHA-induced cytotoxicity to muscle cells in culture [7], an effect not seen with thymic cells from non-myasthenic controls. The exact target specificity of these muscle-directed responses is unknown. In vitro cytoxicity against muscle cells may represent the effect of local antibody synthesis and accompanying antibody-dependent cellular cytotoxicity.

Attempts to determine if AChR preparations elicit in vitro proliferative responses have been confounded somewhat by the nature of the preparation used. Detergent-soluble preparations in general have displayed toxic effects on lymphocytes [93]. On the other hand, the magnitude of proliferative responses to water soluble preparations has been modest at best [4, 18]. Richman et al. observed greater PBM responses to AChR in more severely affected patients with thymoma [93, 94]. This response did not appear to be directed at the portion of the ACh-binding site. Others have observed prominent in vitro responses of cells of myasthenic patients to AChR in the presence of autologous serum [18]. This observation suggests that the lymphocytes may be responding to AChR-containing immune complexes that are formed in vitro. In a recent study, McQuillen et al. reported that human AChR stimulated higher in vitro proliferative responses of MG PBM than normal PBM [76].

In a limited study, Richman et al. observed in vitro proliferative responses of thymic cells cultured with AChR [93]. The significance of this finding is unclear since concomitant studies of blood lymphocytes were not reported; nor was the response of thymic cells to conventional antigens, e.g., candida and tuberculin, examined.

PBM from some MG patients proliferate when co-cultured with autologous thymus cells [2, 86, 93]. This phenomenon is predominantly observed when the cells are obtained from patients with thymic hyperplasia [2], and is not seen when thymus cells and autologous PBM from controls are co-cultured. The stimulus for this "autologous mixed lymphocyte reaction" is not known, although expression of a neoantigen, elaboration of a soluble activator, or perhaps presentation of altered AChR by the thymus cells have been entertained as possibilities.

In summary, a broadbased defect in cellular immunity has not been demonstrated in patients with MG. The variable results studied likely reflect heterogeneity of patient populations as well as differences in in vitro techniques. There does appear to be in vitro evidence of peripheral and perhaps thymic T cell cell-mediated immunity towards AChR. However, the bulk of the evidence suggests

that such autoaggressive T cells play little if any direct pathogenic roles. They may represent markers for T cells which help specific anti-AChR responses.

Analysis of Immunoregulatory Mechanisms

A large body of evidence has emerged in the past 10 years indicating that immune responses are regulated by cellular components and soluble macromolecules. It has therefore been reasoned that autoimmune responses may be caused by aberrant immunoregulation. The components of this immunoregulatory network are too numerous and complex to be detailed here. Therefore, we will focus on those aspects of immunoregulation that have been examined in MG. The bulk of the work has been focused on T cells since they subserve a central role in the immunoregulatory system. Investigators have examined phenotypic properties of T cells in the blood and thymus in an effort to identify if the appropriate mix of cellular components is present. More importantly, studies have been undertaken to examine the function of these subsets of immunoregulatory cells. Recent attempts have also been made to search for idiotype/anti-idiotype interactions in MG since the idiotype/anti-idiotype network hypothesis proposed several years ago by Jerne [42] now looms as an important mechanism for controlling immune responses.

Phenotypic Characterization of Cells in MG: Blood

Early studies enumerated subsets of cells expressing receptors for the Fc fragment of IgG (T γ cells) and cells expressing receptors for the Fc fragment of IgM (T μ cells). These cell populations, upon activation, were shown to mediate suppression (T γ) and help (T μ), respectively. We [67] found increased numbers of T γ cells and normal numbers of T μ cells. This experience has largely been confirmed by others [87, 118] although Harfast et al. [36] found, that as a group, their MG patients had normal levels. The significance of the elevated number of T γ cells was confounded by the controversy engendered by the report that the T γ cell subset may contain cells with monocyte characteristics in addition to T cells [92].

Several investigators have enumerated circulating T cells with monoclonal antibodies although a clear picture has not emerged. We [106] found a modest decrease in OKT8+ (putative suppressor/cytotoxic) cells in MG patients as a group with the most impressive changes observed in early-onset patients (less than 35 years old) with no apparent influence of prior thymectomy. Berrih et al. [10] originally reported depressed percentages of T8 cells but this was not confirmed in an expanded study subsequently reported [11]. In the latter study, they found moderately increased percentages of T4 cells, normal percentages of T8 cells, and increased T4/T8 ratios. Of interest, some patients demonstrated significant numbers of cells that expressed both OKT4 and OKT8 determinants, confirming an observation they had made in their earlier report [10]. The significance of these double-labeled cells was unclear. In a limited study, Haynes et al. [37] reported that MG patients with atrophic thymus had a decrease in absolute levels of blood OKT4+ cells with normal numbers of OKT8+ cells. Miller et al. [78] reported normal percentages of OKT4+ cells, increased percentages of OKT8+ cells, and

depressed OKT4/T8 ratios. The latter was particularly apparent in patients whose disease was poorly controlled.

The variability in the above results defy cogent explanation. It is likely that levels of T cell subsets are influenced by a number of factors which are not uniform throughout these studies. Factors likely to be important include age of the patient, activity of disease, treatment regimens, and history of thymectomy, to name a few. Furthermore, it is important to realize that information gleaned from studies which analyze only phenotypic properties of T cells will likely not uncover the immunoregulatory defect in MG. What is needed are approaches which explore functional properties of these immunoregulatory subsets of cells.

Phenotypic Characterization of Cells in MG: Thymus Cell Suspensions

Abnormalities in distributions of thymic lymphocytes have also been reported. In an early report, we found increased percentages of T γ and T μ cells in MG thymus cell suspensions compared to thymus cell suspensions from control (cardiac) surgery subjects [67]. With regard to expression of markers detected by monoclonal antibodies, it is known that thymic cells normally undergo an orderly process of differentiation progressing from cortex to medulla [91]. The fact that thymic cells may normally display more than one differentiation marker (depending on the stage of differentiation) complicates attempts to enumerate subsets of cells in MG thymus when single-labeling approaches are undertaken. Notwithstanding this caveat, we [70] have found similar percentages of cells reacting with OKT4 and OKT8 in myasthenic and age-matched cardiac surgery control thymus cell suspensions. This experience is similar to that of Tindall [118] and Haynes et al. [37] but different from that of Newsome-Davis et al. [83] whose data suggested an imbalance in the overall OKT4/OKT8 ratios in MG thymocyte suspensions.

Although we [70] observed no significant differences between MG and control subjects in the percentages of either T cell subsets or of B cells, we have detected a significant increase in the percentage of Ia+ cells in thymic cell suspensions of patients with MG. Ia+ cells were not seen in suspensions of normal thymus by others [12]. The identity of these Ia+ cells is not clear. Although a sizeable percentage of monocyte/macrophages is Ia+, we found a low percentage of monocyte/macrophages in MG thymocyte suspensions as assessed by the presence of cytoplasmic acid naphthyl esterase of phagocytic capacity. Most B cells are Ia+. Our double-labeling studies with anti-Ia and anti-IgM indicated that B cells accounted for some of the Ia positivity. However, in our comparative studies the percentage of Ia+ cells was almost always considerably higher than the percentage of surface IgM+ cells in individual MG thymocyte suspensions. Moreover, in the double-labeling studies, a sizeable number of cells were surface Ia+ but negative for surface IgM. It is possible that some of these Ia+ cells are B lymphocytes which do not bear detectable surface IgM. Therefore, we are currently also analyzing such thymocyte suspensions using recently developed monoclonal antibodies thought to detect B lymphocyte-specific surface determinants other than Ig (e.g., B1).

Human T cells express Ia upon activation [90, 132, 134] and such cells might be expected to be increased in frequency in MG thymus. However, in cell separation

studies, the Ia + cells do not segregate in the T cell (SRBC rosetting) fraction but are enriched in the non-T cell fraction.

Other possibilities to account for the increased Ia + cells are dendritic cells and epithelial cells. Dendritic cells are Ia +, surface IgM −, esterase negative, and do not ingest latex. These cells perform important roles in antigen presentation and are potent stimulators of allogenic and autologous mixed leukocyte reactions [112, 113, 125, 133]. We [51] and others [89, 115] have evidence from studies of tissue sections that such cells might contribute to Ia reactivity in thymus cell suspensions (see below).

Phenotypic Characterization of Cells in MG: Tissue Sections

We have also examined the distribution of mononuclear cells in sections of thymus from MG subjects and age-matched cardiac controls [51] (Fig. 3). Cortical thymocytes expressed OKT11 + (sheep red blood cell receptor), OKT4 +, OKT8 +, and OKT6 + in both MG and control specimens. In the medulla of MG patients and controls, 50% of the cells were OKT11 + and OKT4 + with a third being OKT8 + and about 10% being OKT6 +. Cell suspensions likely represent a mixture of cortical and medullary cells. The OKT4 + cell predominance observed in the medulla is likely diluted out in cell suspensions of thymus of MG patients and controls. The important point, however, is that in both suspensions and sections we found no difference in the percentage or distribution of T cell subsets in MG versus controls.

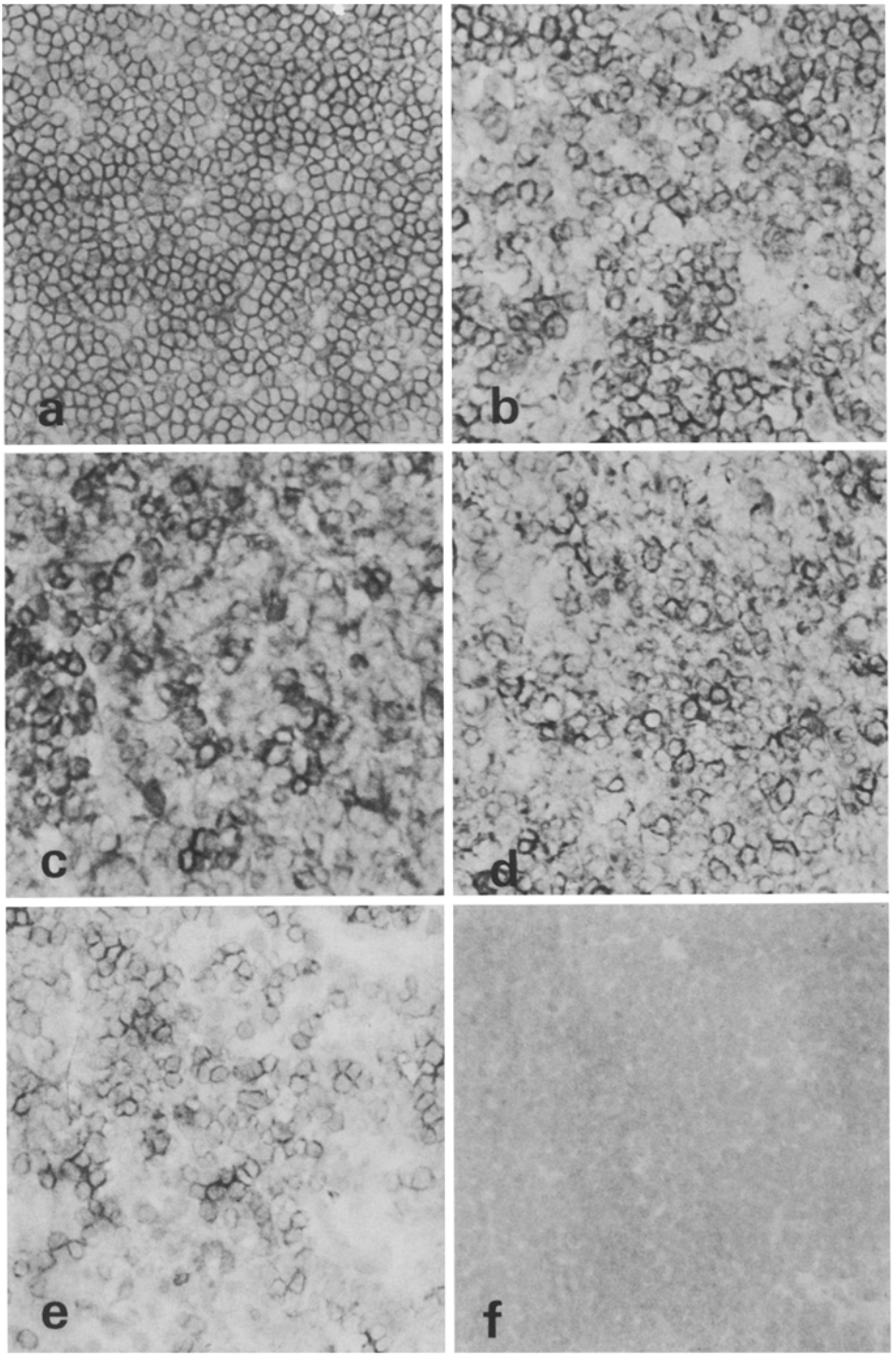
On light microscopy, Ia was seen predominantly on cortical cells with long interdigitating processes and in a confluent pattern in the medulla. This pattern was observed in both MG and control thymus. Others, using indirect immunofluorescence, have described an increased frequency of Ia + interdigitating dendritic cells in sections of MG thymus sections [115].

Better resolution at the ultramicroscopic level showed Ia mainly on nonlymphoid cells with no differences observed between MG and control thymus. These Ia + cells were identified as interdigitating dendritic cells and epithelial cells (Fig. 4). Lymphoid cells generally had Ia-positivity only on that portion of the plasma membrane in close opposition to Ia + nonlymphoid cells. This pattern of Ia localization is similar to that reported in murine thymus [26, 124] and normal human thymus [98].

In summary, the distribution of T cell subsets in MG thymus cell suspensions and tissue specimens appears to be normal. Cells bearing surface Ia are increased in frequency in thymus cell suspension whereas the pattern of Ia reactivity in MG tissue sections appears comparable to that seen in normal thymus tissue. In suspensions, these Ia cells are enriched in the non T-cell fraction of the thymus and

▶

Fig. 3. MG thymus frozen sections demonstrating localization of lymphocyte subsets. **A** Thymic cortex examined for T11. Virtually all cortical lymphocytes are T11 positive. T11 negative cells appear to be non-lymphoid. This same pattern is seen in the cortex T11, T4, T6, and T8. **B–E** Thymic medulla stained for T11 (**B**), T4 (**C**), T6 (**D**), and T8 (**E**). **F** Thymic cortex stained for IgM (negative control) (hematoxylin counterstain, × 400). Reproduced with kind permission of the Editor of the American Journal of Pathology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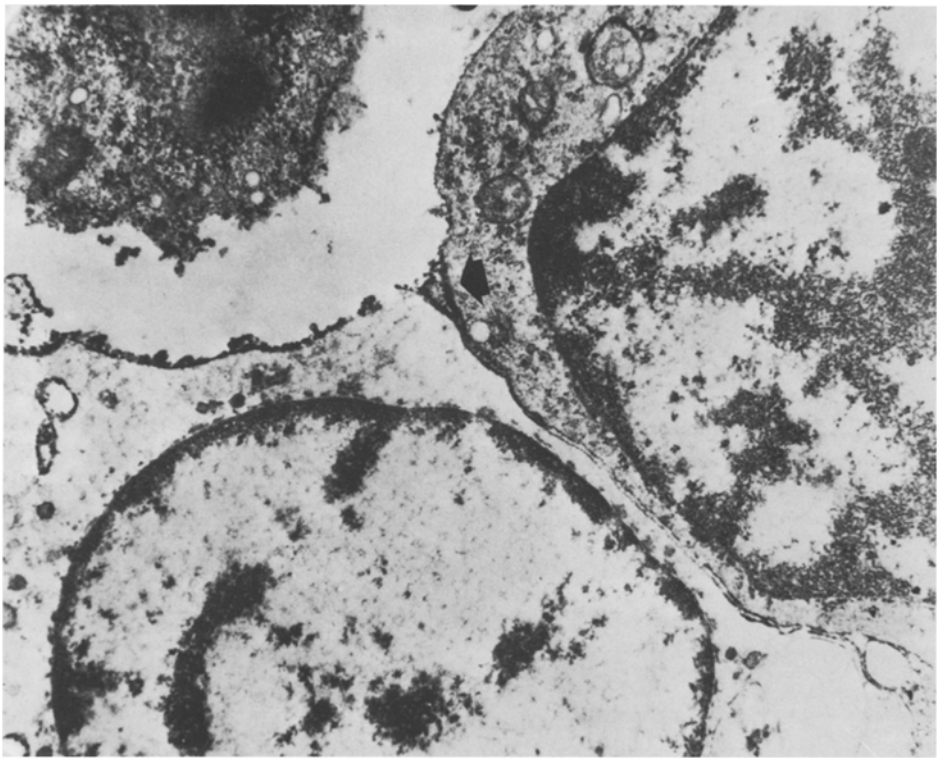

Fig. 4. Electron micrographs of MG thymus tissue sections demonstrating localization of Ia antigen. Ia positive epithelial cell (*lower left*) and adjacent thymic lymphocyte are shown. Lymphocyte has Ia positivity only on membrane adjacent to epithelial cell (*arrows*) (× 11, 140). Reproduced with kind permission of the Editor of the American Journal of Pathology

are accounted in part by surface membrane IgM positive B cells. As yet, the increased frequency of Ia-bearing cells has not been correlated with clinical parameters. Studies are currently underway to determine if they correlate with in vitro B cell responses of thymus cells.

Immunoregulation: Functional Analysis

Lacking reliable in vitro approaches to examine AChR triggered specific antibody production, most investigators have concentrated on nonspecific probes of immunoregulation. Accordingly, the bulk of data published to date cannot necessarily be extrapolated to the regulation of anti-AChR production.

There are reports consistent with impaired suppressor T cell function in MG. Taking advantage of the relative radiosensitivity of suppressor T cells in the PWM-driven Ig synthesis, Kelley et al. [46] provided evidence for diminished suppressor T cell activity in the PBM of nonthymectomized MG patients but not thymectomized patients. Mischak and Dau [81] reported impaired mitogen-induced suppressor T cell activity whereas a prominent defect (using the same in vitro approach) was not

demonstrated by Koethe et al. [48]. These investigators did, however, observe reduced suppressor T cell activity in a subset of patients in association with HLA B8. Diminished mitogen-induced suppressor activity had previously been reported in association with HLA B8/DRW3 in MG patients [135], an interesting observation in light of the increased frequency of the B8 determinant in other autoimmune diseases. Contrasted with the above observations are the findings of Birnbaum and Tsairis (using a different assay) which were interpreted as showing increased suppressor cell activity in MG patients [13].

The possibility that T cells bearing nicotinic AChR are involved in immunoregulation has received particular attention. This hypothesis is based on the following evidence: (1) anti-AChR bind to thymocytes of normal mice [25], (2) labeled α-bungarotoxin which binds to AChR appears to bind to human PBM [82], and (3) functional nicotinic AChR exist on human PBM and their perturbation appears to activate suppressor cells [95].

Furthermore, Shore et al. [104] have reported that patients with childhood MG have both reduced number and functional activity of a subpopulation of theophylline-sensitive suppressor T cells. These patients also had a serum antibody which appeared to bind to nicotinic AChR on PBM of normal subjects and reduced suppressor cell activity. These data, along with those of Richman et al. [95], suggested that a subset of suppressor cells, presumably T cells, express nicotinic AChR. These observations raised the exciting possibility that anti-AChR may not only react with receptor material at the myoneural junction but also with receptor on the surface of immunoregulatory T lymphocytes. Accordingly, anti-AChR could interfere with suppressor T cell function as suggested by the data of Shore et al. [104] and contribute to the perpetuation of autoantibody production. This hypothesis, originally proposed some 5 years ago, remains to be definitively tested. A critical first step would be the documentation of nicotinic AChR on human T cells in carefully performed binding studies. There is certainly evidence other than that provided by Shore et al. for the presence of anti-lymphocyte antibodies in the serum of patients with MG [66, 135]. However, it is not known if such antibodies preferentially react with T cell subsets, cross-react with anti-AChR, and modify physiologic immunoregulatory mechanisms in MG.

In recent studies, we have begun to address the immunoregulation of anti-AChR production. We first sought to determine if the failure of PWM to stimulate cultures of PBM from healthy donors, reported by us and others, was due to potent suppressor T cell activity. Such potent suppressor T cell activity appears to account for the failure in some normal subjects of PWM to stimulate synthesis of another autoantibody, rheumatoid factor in cultures of PBM [96]. We [72] observed that PBM of normal subjects synthesized no anti-AChR even when OKT8+ cells were depleted prior to culture. Such OKT8+ cell depleted cultures did, however, show an increase in total IgG production (Table 3). These findings suggest that the failure to synthesize and secrete significant amounts of anti-AChR in vitro by PBM of normal subjects is not due to suppressor cells within the OKT8+ population. We suspect that these results most likely reflect an insufficient number of memory B cells in the peripheral blood of normal subjects.

In preliminary studies we have found that anti AChR production by PWM stimulated cells from MG patients is enhanced when such cultures are depleted of

Table 3. Effect of removal of OKT8+ cells on anti-AChR and IgG synthesis

	Unsorted PBM		OKT8 depleted PBM	
	Anti-AChR	IgG	Anti-AChR	IgG
Unstimulated	0.11 ± 0.11	265 ± 32	0.58 ± 0.39[a]	299 ± 103[a]
PWM-stimulated	1.6 ± 0.52	$1{,}732 \pm 402$	0.75 ± 0.52[a]	$2{,}058 \pm 638$[b]

[a] Not significantly different from unsorted PBM by two-tailed paired t test analysis
[b] $p < 0.05$ versus unsorted PBM by two-tailed t test analysis
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OKT8+ cells [71]. These results indicate that T cells suppressing anti-AChR are present in the blood of MG patients and that AChR-specific B cells are sensitive to their influence.

Evidence for Id/Anti-Idiotype Reactions in Human MG

As previously mentioned, a large body of data has accumulated supporting Jerne's network hypothesis of id/anti-id regulatory interactions. The reader is referred elsewhere for detailed reviews of this important area [52]. In general, the available information has been derived from experiments in which anti-idiotypic responses were generated intentionally and often in unphysiologic model systems.

In man, this area has understandably been more difficult to examine. Early reports provided evidence that heterologous anti-id antibodies could inhibit in vitro idiotype production [14, 49]. Complexes of id/anti-id were found in some patients with cryoglobulinemia [32]. Geha found evidence of an anti-id response (anti/anti-tetanus antibody) in normal subjects following a booster injection of tetanus toxoid [31]. Furthermore, his data suggested that the emergence of the anti-id response correlated with the disappearance of the idiotype in that same serum. Abdou and colleagues [3] were the first to implicate an impaired anti-id response in human autoimmune disease. They found evidence supporting the presence of anti-id antibodies (anti-anti-DNA antibody) in the serum of SLE patients in remission. Of great interest, anti-id antibodies were absent from relapse serum, results that have subsequently been confirmed by others.

To date, there have been few studies addressing the network hypothesis in MG. Dwyer and colleagues [22] have described what appears to be anti-anti-AChR Ab activity in serum of some patients with MG. It is not clear that this is true anti-idiotypic antibody and, unlike Abdou's findings in SLE, their presence did not correlate inversely with clinical severity. Others [39] found no evidence for such functionally important anti-anti-AChR Ab. It has been possible to demonstrate that idiotypic antibodies and networks to anti-AChR Ab can be produced in experimental models, although the protective capacity of such anti-anti-AChR antibody in animals with experimental autoimmune myasthenia gravis remains controversial [27, 28, 54].

Thymectomy Associated Changes in Immune Response

The beneficial effect of thymectomy is widely accepted especially in patients without thymoma, although this is not universal and has never been demonstrated in a prospective random controlled study. The reasons for this beneficial effect are not known. However, it is suspected that alteration of immune responses is responsible since the thymus is an important central organ in the immune system. In addition, thymectomy removes a source of potential autoantigen, i.e., AChR displayed on various populations of thymic cells.

In MG, thymectomy does not appear to impressively cause a state of broadbased immunosuppression. In humans, thymectomy has only been performed in patients with clinical disorders, so the effect on the immune response in normal man cannot be determined. In the mouse, adult thymectomy has little effect on cellular immunity [80]. By contrast, neonatal thymectomy causes prominent depression of cellular immunity [34, 79]. In man, there is clearly no readily demonstrable effect on Ig levels [23] or antibodies to standard antigens [101, 117]. It has been reported by one group [100, 101] that thymectomy results in a return to normal of previously depressed peripheral blood T cell cytotoxic function and K-cell reactivity, but other groups cannot confirm these findings [129]. Birnbaum and Tsairis reported changes in allogeneic MLC which they felt represented a decrease in suppressor activity [13]. Fukawa et al. [30] have reported that patients who are destined to respond to thymectomy have a pre-operative hyperimmune state (including increased delayed-type hypersensitivity skin tests) which normalizes post-thymectomy. This report is difficult to interpret in the face of the findings of Sorensen et al. [109] that thymectomy results in: (a) an increase in the size of the response to the primary skin test antigen DNCB and (b) an increase in in vitro mitogen-induced proliferative responses and a decrease (using a different assay than Scadding et al.) in T cell-mediated lympholysis. Some report a decrease in skin tests and decreased response to mitogens, whereas others found increased PWM-induced proliferation. In some, there is no consistent evidence for demonstrable thymectomy-induced changes in T cell function as measured by in vivo or in vitro testing.

There have been many studies of the effect of thymectomy on the numbers of lymphocytes, T lymphocytes, and T lymphocyte subsets in PBM of patients with MG. Numbers of total lymphocytes and T cells have been reported to be decreased by some but not all groups. More recently, T cell subsets have also been studied in the blood of patients with MG who have undergone thymectomy. We found [67], in some patients, a decrease to or toward normal of a pre-thymectomy elevation of activated T cells bearing receptors for the Fc portion of IgG (T γ).

Three studies of the effect of thymectomy on T cell subsets identified with monoclonal antibodies have been reported. Berrih et al. [11] reported an increase in the OKT4/OKT8 ratio shortly after surgery. In addition, the increase in double-labeled (OKT4+ OKT8+) PBM seen prior to surgery decreased. By 6–12 months, both the total PBM T cells (OKT3+) and the OKT4/OKT8 ratio were reduced; the latter finding was observed, especially in patients who improved and in patients with thymoma. Patients studied more than two years after thymectomy frequently had very low OKT4/OKT8 ratios. The results of the study are somewhat

confounded by the lack of serial data in the patients in whom thymectomy was performed more than 2 years before and by the inclusion of patients treated with corticosteroids as well as thymectomy.

We have previously mentioned the study by Haynes et al. [37]. Twelve patients with MG including six younger patients (mean age 26.8 years) with thymic hyperplasia and six older patients (mean age 48.8 years) with involuted thymuses were studied. Of the latter group, one was receiving prednisone and one had received prednisone up to two months before thymectomy. The thymic hyperplasia group who had MG for a mean duration of 2.8 years prior to study had normal preoperative levels of total lymphocytes, T cells (E-rosette and anti-3A1), helper/inducer T cells (OKT4), and suppressor/cytotoxic cells (OKT8+) and had no significant postoperative changes in these parameters or in plasma cortisol levels despite clinical improvement in four of six patients. The atrophic thymus group, who had MG for a mean duration of 0.7 years prior to study, had a depression of lymphocyte count, T lymphocytes (by E-rosetting and monoclonal anti-3A1), and OKT4+ cells pre-thymectomy, all of which normalized after thymectomy. This was accompanied by a decrease in the pre-thymectomy levels of plasma cortisol. The significance of these findings is not clear due to: (a) the relatively small number of patients in each category studied with the monoclonal antibodies; (b) serial data are shown only for total lymphocytes in two subjects; (c) one presumes but does not know that the data presented for lymphocytes and subsets were determined at the time of last follow-up post-thymectomy.

We [106] studied seven patients (mean age 42.9 years) who had thymectomy at least three months earlier (mean, 3 years) and had not received corticosteroids. We found normal levels of OKT3+, OKT4+, OKT8+, and surface Ig+ cells with a trend ($p < 0.1$) for decreased OKT8+ cells. While older patients (onset after 35 years of age) may have a higher incidence of atrophic thymus than younger patients, we have observed notable exceptions to this pattern in both directions. It is reasonable to state that we do not know as yet whether thymectomy results in any quantitative changes in peripheral blood T cells subsets in all or any subpopulation of patients with MG. Even if changes in levels of T cells do result from thymectomy, it is not clear how this relates to levels of anti-AChR.

A second possible mechanism for thymectomy-induced improvement in patients with MG is a reduction in circulating anti-AChR. As already mentioned, thymic lymphocytes of patients with MG are capable of in vitro synthesis of anti-AChR Ab. However, a significant decrease in serum anti-AChR Ab titer concomitant with improvement has been difficult to demonstrate. It is possible that there is a change in an important subpopulation of anti-AChR.

A third possible mechanism is the removal of an important source of an immunologic stimulus. The previously mentioned immunologic abnormalities of the thymus cells and the presence of cells with AChR in the thymus have led to the suggestion of an intrathymic pathogenesis of MG. Removal of the thymus in patients in whom the thymus is serving as an important stimulus could result in improvement, but the mechanism or pathway is not clear.

An additional postulated mechanism is that thymectomy results in the elimination of the source of a material which has neuromuscular blocking activity. It has been reported that the thymus contains material, possibly quaternary

ammonium salts, which could directly affect neuromuscular transmission [85]. We know of no confirmation of these early data and this theory would run counter to the clear cut importance of anti-AChR at the endplate.

Thymectomy has been reported to result in temporary changes in circulating thymic hormone [8, 122, 130] and there is some evidence that there is an increase in the amount of thymosin α1 in the myasthenic thymus [20]. It is not clear that serum levels or levels of circulating lymphocytes binding thymosin α1 correlate chronologically with time of improvement. Moreover, changes in levels of thymic hormones should be reflected in qualitative and/or quantitative changes in T cells or T cell subsets and as reviewed above, such evidence is not clear cut.

Based on a controversial model of experimental autoimmune thymitis, Goldstein and co-workers [33] believe that "thymitis" results in the release of a thymic hormone, thymopoetin, which has both immunoregulatory function and the capacity to cause neuromuscular block. There is no good evidence for such a factor being present in the serum of patients with MG.

Conclusion

We have reviewed a large body of information that deals with the integrity of the immune system in patients with myasthenia gravis. In particular, we have addressed recently accumulated data dealing with immunoregulatory mechanisms. It is clear that the deficit in neuromuscular transmission, the clinical hallmark of this disease, is caused by antibodies with specificity for AChR. Although, AChR-reactive T cells can also be found in patients, there is no evidence that they play a primary role in muscle dysfunction. The bulk of the evidence does not indicate a broadbased deficit in T and B cell function. A clear picture has not emerged from studies of phenotypic markers on putative immunoregulatory populations of cells likely due to the heterogeneity of patient populations studied. Analysis of these cells in some functional assays has provided evidence for aberrant immunoregulation. However, the relevance of such findings to anti-AChR production in MG is unclear. Real progress in this area will likely be made persuant to the development of in vitro assays which enable delineation of mechanisms regulating AChR triggered anti-AChR responses.

Considerable progress has been made in elucidating possible roles for the thymus in the pathogenesis of MG. The possibility that sensitization to AChR occurs within the thymus remains a favored hypothesis. The existence of AChR bearing, presenting, and reactive cells within MG thymus provides support for this hypothesis. Phenotypic characterization of thymic cells has already provided some interesing information, e.g., increased Ia-bearing cells in suspensions of some MG patients. Application of newly described monoclonal antibodies (directed against the various cellular constituents of the normal thymus) to the study of MG thymus will likely yield further valuable information.

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