Thymus in Myasthenia Gravis

Isolation of T-Lymphocyte Lines Specific for the Nicotinic Acetylcholine Receptor from Thymuses of Myasthenic Patients

A. Melms,* B. C. G. Schalke,† Th. Kirchner,‡ H. K. Müller-Hermelink,¶ E. Albert,§ and H. Wekerle*

*Max-Planck-Society, Clinical Research Unit for Multiple Sclerosis, Würzburg, D-8700 Würzburg, Federal Republic of Germany; †Departments of Neurology and ‡Pathology University of Würzburg, D-8700 Würzburg, Federal Republic of Germany; ¶Department of Pediatrics, University of Munich, München, Federal Republic of Germany

Abstract

The thymus is believed to play a central role in the pathogenesis of Myasthenia gravis (MG). According to various hypotheses, MG is initiated within the thymus by an immunogenic presentation of locally produced nicotinic acetylcholine receptor (AChR) T cells to potentially autoimmune T cells. Data on 10 consecutive MG patients demonstrated two critical features of MG thymuses that support the concept of intrathyhmic activation of autoreactive, AChR-specific lymphocytes. Morphologically, the thymuses showed lympho-follicular hyperplasia in nine cases and benign thymoma in one case. The paramount feature revealed by immunohistological double marker analyses was the intimate association of myoid cells (antigen producing) with interdigitating reticulum cells (potentially antigen presenting cells), both of which were surrounded by T3+ lymphocytes in thymus medulla. All 10 thymuses contained T lymphocytes reactive with AChR. This was to contrast to the peripheral immune compartment (blood) where only 3 of 10 patients, significant T cell responses to AChR were observed. AChR-specific T cell lines could be established from 8 of 10 thymuses, all members of the helper/inducer subset as indicated by the expression of markers T3 and T4.

Introduction

The thymus is profoundly involved in the pathogenesis of myasthenia gravis (MG). It is generally known that most, if not all myasthenic patients have pathological changes of their thymic tissue. Specifically, > 70% of all myasthenic thymuses show lympho-follicular hyperplasia, and at least 10% of myasthenic patients have thymic epithelial neoplasia (1).

A causal connection between thymic changes and the development of clinical MG has been suggested by the beneficial therapeutic effects of thymus extirpation. In fact, at least in MG with short duration of symptoms, associated with thymus hyperplasia, thymectomy appears to be one of the most rewarding therapeutic measures (2-5). Finally, at least an indirect role of the thymus was indicated by the demonstration of putative thymus-dependent cellular responses against the nicotinic acetylcholine receptor (AChR) in MG patients (6-8).

This clinical experience together with the observation of myogenic inducibility of thymic stem cells in culture (9) led us to postulate that in MG the thymus is the primary site of a pathogenetic autoimmune response against the postsynaptic AChR (10, 11). We proposed a four-step sequence of intrathyhmic pathogenesis: (a) myogenic induction of primitive thymic stem cells leading to synthesis and expression of AChR on thymic myoid cells; (b) release of AChR from myoid cells, perhaps due to cell death, and uptake of myoid AChR by thymic antigen presenting cells (e.g., interdigitating cells, IDC) and immunogenic presentation; (c) recognition of immunogenically presented AChR by specific autoreactive T lymphocytes, differentiating within the thymus; (d) emigration of the activated AChR specific T lymphocytes to the peripheral immune system, interaction with AChR specific B cells resulting in the production of pathogenic anti-AChR autoantibodies.

This report supports a central element of the concept of intrathyhmic generation of MG. We demonstrate that thymus tissues from myasthenic patients indeed regularly contain T lymphocytes which can recognize and react against AChR. There is evidence that AChR specific T cells are more frequent within the thymus than in peripheral compartments of the immune system, e.g., peripheral blood. We furthermore show that these T cells can be isolated and propagated as AChR specific T cell lines and that all the T cell lines recovered express membrane markers of the CD4 T cell subset.

Methods

Patients. All patients were seen in the Department of Neurology (Director, Prof. Dr. H. G. Mertens) of the University of Würzburg, and are participants in a double-blind controlled trial comparing the benefits of long term immunosuppression with cyclosporin A vs. azathioprine (12).

Diagnosis of MG was based on clinical examination, amplitude decrement during repetitive EMG stimulation, positive edrophonium and/or curare test. Clinical grading was done according to Osserman (13). In 9 of 10 patients anti-AChR antibodies were determined (Dr. I. Kalies, Erlangen). Transthoracic thymectomy was performed at the Department of Thoracic Surgery of the University of Würzburg. All patients gave written consent for their specimens to be used for research.

Antigens. A preservative free preparation of tetanus toxoid (TT) was used (Behring-Werke AG, Marburg, FRG; lot no. 831832, 2860 IU/ml) at a final concentration 0.1-1.0 IU/ml. AChR of Torpedo californica electric organ (Pacific Biomarine Lab., Venice, CA) was prepared as described (14). Some preparations were done according to Rüchel et al. (15). Electroplax tissue was

Address reprint requests to Dr. Wekerle, Max Planck Society, Clinical Research Unit for Multiple Sclerosis, P.O. Box 61 20, D-8700 Würzburg, FRG.

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1. Abbreviations used in this paper: AChR, acetylcholine receptor; MG, myasthenia gravis.

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minced in the presence of enzyme inhibitors PMSF (Sigma Chemical Co., St. Louis, MO, 10–4 M) Iodoacetamide (Sigma Chemical Co., St. Louis, MO, 5 × 10–4 M), EDTA (10–3 M; Sigma Chemical Co.) in cold 50 mM phosphate buffer pH 7.4. After centrifugation (30,000 g, 30 min) the pellet was resuspended in 5 mM phosphate buffer pH 7.4 containing 2% Triton X-100 (Merck AG, Darmstadt, FRG) and extracted on a stirrer for 2 h. After a second centrifugation the supernatant that contained solubilized AChR was stored at −80°C or applied to a α-cobratoxin linked CNBr Sepharose column. After washing with buffer, AChR was competitively eluted with 0.2 M carboxymethylcholine (Sigma Chemical Co.) or 0.1 M hexamethoniumbromide (Fluka, Buchs, Switzerland). AChR was concentrated on a DEAE-A 25 column (Pharmacia Fine Chemicals, Freiburg, FRG) and eluted in a small volume by buffer containing 0.5 M NaCl. Before use in tissue culture, the detergent Triton X-100 had to be reduced by dialysis for 7 d in 5 mM phosphate buffer, pH 7.4. AChR had a specific activity of 4.8–8.0 nM α-bungarotoxin binding sites/mg protein as determined by a 125I-α-bungarotoxin binding assay (16). Concentration in tissue culture was 1 μg/ml (∼ 4 × 10–7 μM AChR).

HLA-typing. Typing of HLA-A, -B, -C as well as HLA-DR, -DQ antigen was done by the National Reference Laboratory (Prof. Dr. E. Albert, Munich, FRG) using standard typing material as defined by the 6th Workshop on HLA Typing 1984 (17).

Isolation of PBMC. PBMC from EDTA blood were isolated by density centrifugation on Lymphoprep gradients (Nygaard, Oslo, Norway) for 30 min at 800 g. After being washed in Ca2+-Mg2+-free PBS, 2 × 10⁷ cells/well were cultured in 96 well microtiter plates (Nunc, Roskilde, Denmark) in RPMI 1640 medium (Gibco Laboratories, Grand Island, NY) plus 2% pooled human serum, 2 mM glutamine (Gibco), 100 U/ml penicillin and 100 µg/ml streptomycin (Biochrom, Berlin, FRG) i.e. complete medium (CM) in the presence or absence of antigen or mitogen (TT 1.0 IU/ml, AChR 1 µg/ml, PHA-P 5 µg/ml). Cultures were incubated 95% air. 5% CO2 for 5 d. We pulsed the cultures with 0.2 µCi ([3H]thymidine) [3H]Tdr, Amer- sham-Buchler, Braunschweig; specific activity 5 Ci/mM) for 16 h. before harvesting onto glass fiber filters by a Skatron Cell Harvester. Incorporated [3H]thymidine was measured by liquid scintillation counting in a beta-counter (Kontron, Basel, Switzerland).

Preparation of thymocytes cell suspension. Thymus specimens were obtained at surgery and kept in cold Hepes buffered DME (Dulbecco’s modified Eagle’s medium; Gibco). Connective tissue of the fibrous capsule, septa and blood vessels were carefully removed. The trimmed thymus lobules were rinsed with DME to further minimize red blood cell contamination. Coarse mincing by scissors was followed by homogenization in a loosely fitting glass tissue homogenizer (Belco, Vineland, NJ). The cell suspension was filtered through nylon wool to remove large particles. Aliquots of this single cell suspension were used for culture experiments, immunostaining, or were stored in liquid nitrogen. For tissue culture and staining experiments thymocytes could be fractionated on a discontinuous BSA gradient (Sigma Chemical Co.; density 1.081 g/ml) into low density (LD) and high density (HD) thymocytes. Most culture experiments were done with the LD-fraction that appeared to be enriched for DR* and surface Ig+ cells as compared to the original cell suspension (Table II).

Thymocyte cultures. 3–4 × 10⁷ LD-thymocytes were cultured in triplicates in CM (conditions see culture of PBMC) for 7–10 d without adding exogenous IL-2. Activated cells were separated by density gradient centrifugation and expanded in IL-2 containing growth medium (5–10 U/ml lymphocult HP; Biotest Diagnostics, Frankfurt, FRG). At 10–14-d intervals cells were restimulated with antigen presented by thawed autologous LD-thymocytes as described below. Microproliferation assay. 2 × 10⁶ antigen activated T lymphoblasts (from PBMC or thymus culture) were cultured in doublecultures or triplicates with or without 2 × 10⁶ irradiated (40 Gy) autologous PBMC or in the case of thymus cultures 3–4 × 10⁷ irradiated (40 Gy) autologous LD-thymocytes in 0.2 ml CM in the presence or absence of antigen or mitogen (see PBMC cultures). After 72 h cells were pulsed with [3H]-Tdr and harvested 16 h later.

Analysis of lymphocyte populations with a FACS. Cytological analysis of cell populations was performed by means of indirect immunofluorescence with fluoresceine conjugated Fab(ab)2 fragment of goat anti-mouse IgG (Medac, Hamburg, FRG; dilution 1:100) on an Ortho 30/50 system (Ortho, Neckargemünd, FRG) using a linear scale.

Reagents Leu 3a, Leu 2a, anti-HLA-DR, anti-IL-2-receptor were purchased from Becton-Dickinson (Heidelberg, FRG), OKT 3, OKT 4, OKT 6, and OKT 8 were from Ortho, anti-human Ig-FITC was from Miles (Münich). All reagents were diluted 1:200. Background fluorescence activity (usually <2%) was determined by incubation with the second antibody alone. This value was subtracted from the individual results.

Immunohistochemistry. For a double marker analysis, combined staining with immunoperoxidase and alkaline phosphatase was applied to 5-µm thick cryostat sections of fresh thymus tissue. The sections were fixed with acetone for 10 min at room temperature and air dried. The monoclonal antibodies used were: (a) OKT 3, diluted 1:100; (b) KiM 1 (18), diluted 1:4000; (c) KiM 4 (19), diluted 1:5000; (d) anti-desmin (Laboserv, Giessen, FRG), diluted 1:5. All antibodies were diluted with 0.1 M PBS, pH 7.4.

Demonstration of the first antigen was performed by the indirect immunoperoxidase technique using a three stage procedure, as described by Stein et al. (20). After this reaction the immune staining of

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<th>Table 1. Clinical Data</th>
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<td><strong>Patient</strong></td>
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* Less than 0.4 nM/1-α-bungarotoxin binding sites.
the second antigen was achieved by the alkaline phosphatase method following the description of Feller et al. (21).

Results

Myasthenia patients. We analyzed peripheral blood lymphocytes and thymus cells from 10 MG patients who consecutively underwent transsternal thymectomy for therapeutic reasons (Table I). All were female. With one exception, their symptoms were present for < 2 yr with clinical severity corres-

Figure 1. Double immunostaining of MG thymus. (a) Localization of desmin-positive myoid cells (blue) in the medullary area of OKT 3-positive T cells (brown) around a germinal center (G). The medulla contains a Hassall’s corpuscle (H) (× 190). (b) Demonstrating desmin-positive myoid cells (blue) near a Hassall’ corpuscle (H) and outside the network of KiM four-positive dendritic reticulum cells (brown) of germinal centers (× 150). (c) Close contact of a desmin-positive myoid cell (blue) and KiM 1-positive interdigitating cells (brown) (× 600).
thymic lymphofollicular hyperplasia is thought to be linked to a mildly enhanced disease susceptibility (22). None of our patients was demonstrated to be homozygous in the DR region.

Association of myoid cells, dendritic cells and T3+ lymphocytes in thymus medulla. All thymuses with lymphofollicular hyperplasia showed marked expansion of the perivascular space, and marked “peripheralization” of the medullary areas. Germinal centers were frequent and surrounded by T lymphocyte areas of peripheral phenotype. A hallmark of all MG thymuses was the intimate association of myoid cells with IDCs, and their localization in areas occupied by “mature” T3+, thymic lymphocytes. In contrast, myoid cells were never seen in germinal centers. (Fig. 1). Thymic tissue from non-MG patients obtained by heart surgery (seven cases) or autopsy (three cases) also contained myoid cells, but they were only very rarely associated with IDCs (23).

Primary antigen reactivity of peripheral blood and thymic lymphocytes. Lymphocyte suspensions (PBL) were prepared from peripheral blood samples and, in parallel, from specimens of freshly excised thymus tissue (Fig. 2). Specific reactivity against AChR and against TT was assessed in vitro in primary microcultures. With the exception of three patients (J.S., J.B., D.E.) all peripheral blood lymphocytes cultures responded by proliferation to TT. In contrast, reactivity of the same PBL cultures to AChR was much lower in each case.

Significant reactions were seen in three of nine patient cultures only.

The pattern of antigen reactivity in primary thymus cell cultures did not principally differ from the one in PBL cultures. Two features appear, however, worth mentioning. First of all, like PBL cultures, most primary thymus cultures showed reactivity against TT (7/8). There was, however, a relative increase of the primary AChR reactivity as compared to the PBL cultures. 9 of 10 thymuses contained cells that reacted primarily against AChR, and, remarkably, the amplitude of AChR reactions in some cases approached or even surpassed the one against TT, which was in striking contrast to the very low AChR reactivity in PBL cultures. Second, in thymus cultures more often than in PBL cultures, T cell proliferation was observed in the absence of any added exogenous (auto-)antigen.

T lymphocyte lines specific for AChR or TT from myasthenic thymus and peripheral blood. After confrontation with either AChR or TT in primary cultures activated T lymphoblasts were isolated by density gradient centrifugation, cultured in the presence of IL-2 containing media, and were then again restimulated with antigen presented by autologous antigen-presenting cells. Cyclic alternation of antigen stimulation and IL-2–dependent T cell propagation is the principle for establishing antigen monospecific T lymphocyte lines. We selected AChR and TT specific T cell lines both from thymic and from PBL primary cultures. In contrast to the low success rate in obtaining AChR specific T cell lines from myasthenic peripheral blood (3/10 attempts), it was remarkable that T lines were selected from 8 of 10 myasthenic thymuses (Fig. 3). Furthermore, AChR specific T cell lines could be derived not only from freshly processed thymus samples but also from frozen samples as well. All these cell populations were strongly reactive against the AChR, but had lost their reactivity to TT. Reactivity against accessory cells alone, independent of the presentation of AChR (i.e., self-mixed lymphocyte reaction) varied between individual T lines. TT specific T cell lines were successfully selected from 7 of 8 thymus specimens (data not shown). They were highly reactive against the selecting antigen, TT, but ignored AChR. SMLC activity was seen in three of these cultures. Completely similar T lines were derived from peripheral blood at high success rate (4/6 attempts). All T lines investigated, irrespective of their antigen specificity or organ origin, expressed the mature CD 4 membrane phenotype. Staining with a standard set of monoclonal antibodies and appropriate fluorescent reagents established expression of T 3 and T 4 determinants on all the lines but not T 8, T 6 nor membrane immunoglobulins (Table II).

Discussion

This report demonstrates AChR reactive T lymphocytes in thymic tissues obtained from a series of 10 thymectomies of MG patients presenting successively at our clinic. Primary T cell stimulation was corroborated by the establishment of AChR specific T cell lines from 8/10 cases. We thus show that myasthenic thymuses regularly harbor significant numbers of autoreactive T lymphocytes specific for AChR.

Which stimulus could activate and expand the intrathymic AChR specific T cell clones? The most plausible possibility is AChR produced locally and presented in an immunogenic way. AChR is synthesized in rich amounts by intrathymic

Figure 2. Primary antigen induced proliferation of 2 x 10^6 PBMC (a) and 3-4 x 10^6 LD-thymocytes (b); [3H]thymidine incorporation, mean of triplicates±SD. DE, NC; not tested.
myoid cells, which can also be induced in vitro to develop from primitive precursor cells (24, 25). As any antigen to be recognized by T cells, AChR has to be taken up, processed and reexpressed in the context of MHC antigens on the membranes by "professional" antigen presenting cells (26). The thymic medulla contains high numbers of such presenting cells, e.g., IDCs (27, 28). Three observations are in support of such a presentation mechanism. (a) Non-T, non-B thymic cells enhance anti-AChR Ig production by autologous PBL (29); (b) in some cases thymic cells could enhance proliferation of autologous PBL in myasthenic patients (30, 31); (c) as emphasized in our present studies, myasthenic thymus medulla contains ample amounts of AChR expressing, desmin-positive myoid cells (23), which are often in intimate contact with IDCs, and the latter are surrounded by mature T3+ T lymphocytes.

These findings provide support for an active role of the thymus in the immuno-pathogenesis of MG. In addition, they are compatible with, but do not prove the concept of an intrathymic first phase of myasthenic pathogenesis. Still, our present data do not definitely establish the origin of AChR specific thymic T cells. It should be noted that, in contrast to former views, the normal thymic medulla is by no means completely secluded from the peripheral immune cell traffic. This is certainly also the case for the thymus in MG. A hallmark of MG thymus tissue is the "peripheralization" of its medulla. Most MG thymuses contain significant numbers of germinal centers with B lymphocytes and enlarged regions of immunocompetent mature T lymphocytes (32-36). Some of the B cells seem to be in an activated state (37) and can be shown to synthesize autoantibodies against AChR (38, 39), and permanent AChR specific B cell lines have been derived from MG thymuses (40). One may assume quite confidently that most, if not all B cells specific for AChR or other antigens in myasthenic thymuses are the progeny of B lymphocytes immigrated from the peripheral immune system. Moreover, recent experimental evidence suggests, that even in normal rodent thymuses activated T lymphocytes will immigrate via the blood circulation. This has been shown by tracing immune T cells to the thymus either after active immunization (41, 42) or after injection of activated T line cells (43). The immune status of the thymus is further complicated by the observation that soluble antigen can enter the medulla and be immunogenically presented by local presenter cells (44). Indeed, in our series of myasthenic thymuses, besides AChR reactive T cells we also demonstrated T lymphocytes reacting against TT as a control (foreign) antigen. It is thus open, whether TT specific T cells count among those T lymphocytes that have immigrated to the thymus after encountering their antigen in the periphery. In principle, the AChR specific T cells demonstrated in our study may well be secondary immigrants as well rather than the direct progeny of intrathymically differentiating precursor

Figure 3. Antigen-induced T cell proliferation of \(2 \times 10^5\) T line cells from peripheral blood (a) and from thymus (b) in the presence of irradiated, autologous antigen-presenting cells; \([\text{H}]\)thymidine incorporation, mean of triplicates±SD. NC(4), ER, DE, NC(B); not tested; KP, HR, JS, JB, DE, PC(4); 0, attempted but failed.

Table II. Surface Membrane Phenotype of Fresh Separated Thymocytes and of a Thymus-derived AChR-specific T cell Line (FACS = Analysis)

<table>
<thead>
<tr>
<th>Cells</th>
<th>Control</th>
<th>T 3</th>
<th>T 4</th>
<th>T 6</th>
<th>T 8</th>
<th>HLA-DR</th>
<th>IL 2R</th>
<th>RAHIG</th>
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<tr>
<td>Thymocytes</td>
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<td></td>
<td></td>
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<td></td>
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<tr>
<td>Nonseparated</td>
<td>2</td>
<td>ND</td>
<td>55</td>
<td>78</td>
<td>47</td>
<td>2</td>
<td>1.2</td>
<td>0.2</td>
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<tr>
<td>High density</td>
<td>2</td>
<td>36</td>
<td>51</td>
<td>78</td>
<td>60</td>
<td>0.2</td>
<td>0.8</td>
<td>0.2</td>
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<tr>
<td>Low density</td>
<td>2</td>
<td>32</td>
<td>49</td>
<td>79</td>
<td>21</td>
<td>6.6</td>
<td>1.6</td>
<td>1.7</td>
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<tr>
<td>AChR specific</td>
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<tr>
<td>T cell line</td>
<td>2.8</td>
<td>95</td>
<td>95</td>
<td>3</td>
<td>3</td>
<td>95</td>
<td>51</td>
<td>ND</td>
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Control: FITC conjugated F(ab)2 fragment of goat anti-mouse IgG, without primary typing mABs. RAHIG, rabbit anti-human immunoglobulin—FITC conjugated.
cells. It should, however, be noted that the tissue distribution of AChR and TT specific T cells differed quite markedly. TT specific T cells could be isolated from peripheral blood of immune donors almost unfailingly. Within MG thymuses they were also demonstrable, but seemed to be somewhat less frequent than among peripheral blood lymphocytes. In contrast, the establishment of AChR specific T lines from peripheral blood lymphocytes, in agreement with other workers (45), was difficult. AChR specific T lines were, however, regularly isolated from the thymus tissues from the same myasthenic patients of our series.

Taken together, these data provide circumstantial evidence for a relative enrichment of AChR specific T cells within the myasthenic thymus, which could reflect either local expansion of peripheral, immigrant AChR specific T cells, or primary reactions of AChR specific T cells newly formed within the thymus.

Most of the thymuses in our series showed lympho-follicular hyperplasia and were derived from patients within the first two years of symptoms. An interesting exception was patient DE, who suffered from myasthenia for 20 years, was intermittently treated with azathioprine, and was found to have a benign thymoma. As reported in other studies (2, 46), the neo-plastic tissue portion was joined by residual thymus tissue with lympho-follicular hyperplasia and marked germinal center formation and preponderant mature T3+ lymphocytes. Almost all of the thymomatous thymocytes had the immature, “cortical” T6+ phenotype, as described in other thymomas (47). Judging from their mature T3+, T6+ phenotype, the AChR specific T cells should have been derived from the perithymomatous thymus residuum rather than from the tumor tissue. It will be of interest to determine, whether this is true for other cases of thymic neoplasia and myasthenia.

What is the functional role of thymic AChR specific T cells? First, no doubt, the pathogenesis of MG is based on anti-AChR autoantibodies which interfere with functional postsynaptic AChR (48). Direct T cellular immune effector mechanisms have not been proven so far. Most, if not all pathogenic autoantibodies have γ1-isotypes (49), thus their synthesis depends on interactions of T-helper with B cells. All our thymus-derived T lines expressed the CD 4, “T-helper” membrane phenotype. They thus were analogous to T lines isolated from MG peripheral blood by ourselves and others (45). Indeed, Hohlfeld et al. showed that antigen recognition by blood-derived AChR specific T lines was restricted by HLA class II determinants (50) and that these cells were able to enhance in vitro production of anti AChR immunoglobulins by autologous blood B lymphocytes (51). Although these observations do not prove that all AChR specific T cells are indeed involved in the production of pathogenic autoantibodies, it seems reasonable to assume that the AChR specific T lines contain at least some T helper cells indirectly involved in the pathogenesis of MG. Our finding that the thymus apparently contains impressive numbers of such cells is compatible with an active role of the thymus in MG pathogenesis. It remains to be established whether the thymus indeed is the primary site of the myasthenogenic anti-AChR T cell reaction, as postulated by us before, or whether it acts as an amplifier or a depot of pathogenic T helper cells. In either case, however, thymectomy at an early stage of disease would be justified as a radical and rational therapy.

Acknowledgments

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