Myasthenia gravis (MG) is a human autoimmune disease mediated by anti-acetylcholine receptor (AChR) antibodies. The thymus is probably the site where the autoimmune response is triggered and maintained. Recent reports have linked various autoimmune disease with defective Fas expression. We thus analyzed Fas expression in thymocytes and peripheral blood lymphocytes (PBL) from MG patients. The proportion of a thymocyte subpopulation with strong Fas expression (Fas hi) was markedly enhanced in MG patients with anti-AChR antibodies. In peripheral blood, Fas hi lymphocytes proportion was not significantly modified in patients whatever their anti-AChR antibody titer, compared with controls. Altogether, these results indicate that Fas hi thymocytes, which accumulate in MG patients with anti-AChR antibodies, could be involved in the autoimmune response that targets the AChR.

Thymocyte Fas Expression Is Dysregulated in Myasthenia Gravis Patients With Anti-Acetylcholine Receptor Antibody

By Nathalie Moulian, Jocelyne Bidault, Frédérique Truffault, Ana Maria Yamamoto, Philippe Levasseur, and Sonia Berrih-Aknin

Myasthenia gravis (MG) is an autoimmune disease characterized by antibodies that target the acetylcholine receptor (AChR) at the neuromuscular junction and that are present in more than 85% of patients. These autoantibodies are produced under the control of major histocompatibility class (MHC) II-restricted CD4+ helper T lymphocytes. Several arguments suggest a relationship between MG and the thymus: (1) MG is often associated with morphologic thymus abnormalities. 10 to 15% of patients have thymomas and 50% to 60% have hyperplasia; these are characterized by the presence of lymphoid follicles with germinal centers and are found in young patients with high titers of anti-AChR antibodies; (2) thymectomy has a beneficial effect; 3) after thymectomy of MG patients, both anti-AChR antibody titers 4 and in vitro production of anti-AChR antibodies from stimulated peripheral blood lymphocytes (PBL) decrease; (4) isolated thymocytes from MG patients can produce anti-AChR antibodies spontaneously; (5) AChR-reactive T cells are present in the thymus of MG patients; and (6) T lymphocytes and B lymphocytes from MG thymuses are activated. Altogether these arguments indicate that the thymus is probably the site where the autoimmune response is triggered and maintained.

During intrathymic development, programmed cell death appears to be an important option at several stages. A large number of immature thymocytes die because they fail to express a T-cell receptor (TCR) with sufficient affinity for thymic MHC molecules. Cells that are not positively selected may die from “neglect” and may constitute the bulk of cells that die of apoptosis in the thymus. The same mechanism of cell death underlies the deletion of autoreactive immature thymocytes. Thymocytes bearing autoreactive TCR with a high affinity for MHC molecules and specific peptide presented by stromal cells in the thymus undergo negative selection by apoptosis, whereas cells with lower affinity can further differentiate into mature CD4+ or CD8+ cells strongly expressing TCR. In addition, some autoreactive single-positive CD4+ or CD8+ cells are deleted in the periphery.

It has been suggested that autoimmune disease is caused by a failure to eliminate self-reactive lymphocytes, essentially in the periphery, and also by defective apoptosis. In three different strains of mice that develop a disease analogous to human lupus, abnormalities have been detected in the Fas and Fas ligand genes, whose protein products interact to mediate apoptosis. Elevated serum concentrations of a soluble form of the Fas receptor that is able to inhibit Fas-mediated apoptosis have been found in human systemic lupus erythematosus and Fas antigen expression is increased in peripheral lymphocytes. Recent studies have linked defective Fas-mediated T-lymphocyte apoptosis to various Fas gene mutations in a human autoimmune lymphoproliferative syndrome.

In the immune system, the Fas/Fas ligand system is involved in two mechanisms: T-cell cytotoxicity and activation-induced cell death. The involvement of Fas in thymic apoptosis (and particularly in negative selection) is controversial. In lpr mice, in which systemic autoimmune disease is related to a Fas gene defect, and in Fas-null mice, negative selection is normal in the thymus while activation-induced cell death of activated peripheral lymphocytes is impaired. However, an accumulation of immature cells (CD4+CD8+TCR low) resistant to apoptosis has been de...
Table 1. Characteristics of the Patients Studied

<table>
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<tr>
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<th>No. of Patients</th>
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<tr>
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<td></td>
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<tr>
<td>≤35 yr</td>
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<tr>
<td>&gt;1 yr</td>
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<td>36</td>
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<tr>
<td>&gt;10</td>
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Thymus. All the patients were on anticholinesterase treatment and 4 were on steroids and immunosuppressive drugs. Three groups of patients were distinguished according to the anti-AChR antibody titer: high titer > 10 nmol/L (10 patients); intermediate titer, 1 to 10 nmol/L (11 patients), and negative or borderline titer, < 1 nmol/L (10 patients). Anti-AChR antibody titers were determined in serum as previously described.37,38

Blood from the same patients (except for 8 patients) was collected just before thymectomy, and PBL were isolated over Ficoll gradients.

Normal thymuses were obtained from young adults (age range, from 10 to 25 years) undergoing heart surgery. Blood from healthy adult volunteers was also used in control experiments.

Thymocyte isolation and selection. Thymocytes were mechanically isolated by gently scraping fresh thymic tissue, filtering the cells through sterile gauze, and washing them once in Hanks’ balanced salt solution (HBSS). When the quantity of cells (counted by using the Trypan blue dye exclusion method) was sufficient, aliquots of 20 million cells were stored in liquid nitrogen.

In some experiments, cells were thawed and CD8-depleted thymocytes were isolated. After washing, total thymocytes were mixed with anti-CD8 magnetic beads (Immunotech, Marseille, France) in phosphate-buffered saline (PBS)-30% human AB serum to separate CD8+ cells. Anti-Fas antibody (UB2) was fixed on goat-antimouse–coated magnetic beads (Immunotech) following the manufacturer’s instructions. Using these beads CD8-depleted thymocytes were depleted in Fas+ cells. CD8-depleted cells and CD8- and Fas-depleted cells were cultured (0.2 × 10^6 cells/200 μL/well) in RPMI 1640 medium supplemented with 2 mmol/L L-glutamine, 25 mmol/L HEPES, 100 IU/mL penicillin, 100 μg/mL streptomycin, and 2% human AB serum in the absence or in the presence of peptides (5 μg/mL) from the AChR α-subunit, p168-181 (1H) and p351-368 (3H). After a 6-day culture period, 1 μCi of [3H]-thymidine (NEN, Les Ulis, France; 6.7 Cie/mmol) was added to each well. After 20 hours, cells were obtained and [3H]-thymidine incorporation was determined by counting the radioactivity on filters. Cultures were performed in 4 to 8 samples.

Immunofluorescence studies. Thymocytes and PBL were labeled with the following monoclonal fluorochrome-coupled antibodies (Immunotech): anti-CD4, anti-CD8, anti-CD3, anti-CD25, and anti–HLA-DR. FITC-anti Vβ5.1 (LC4 clone) and FITC-anti Vβ6.7 (OT145 clone) were obtained from T Cell Sciences Inc (Cambridge, MA). Three-color flow cytometry was used to examine the relationship between Fas and other markers, PBL and thymocytes (10^6) were first incubated with anti-Fas (anti-CD95) monoclonal antibody (clone UB2; Immunotech) for 30 minutes at 4°C, then washed twice in HBSS supplemented with 5% fetal calf serum (FCS), stained with goat-antimouse IgG antibody, washed twice, and incubated with Cy-chrome-labeled streptavidin (Pharmingen, San Diego, CA) and membrane fluorescein (FITC)– or phycoerythin (PE)-coupled antibodies.

Cell labeling was analyzed on a FACScan flow cytometer (Becton Dickinson, Grenoble, France) using Lysis II software that allows the analysis of data obtained with a Becton Dickinson flow cytometer. A gate was set on intact cells using forward- and side-scatter analysis; 10^4 cells were analyzed in this gate.

Thymocyte culture and anti-Fas antibody assay. After the isolation of thymic cells, 0.5 × 10^6 were cultured in the presence of 5 μg/mL anti-Fas antibody (clone CH-11; Immunotech) immobilized on 96-well plates, in RPMI 1640 medium supplemented with 10% FCS, 2 mmol/L L-glutamine, 25 mmol/L HEPES, 100 IU/mL penicillin, and 100 μg/mL streptomycin. After 18 hours the cells were harvested and labeled; Fas expression was analyzed as previously described.
Fig 1. Comparison of Fas expression in thymocytes from controls and MG patients. Freshly isolated thymocytes were stained with anti-Fas antibody, then with goat-antimouse IgG antibody, and finally with Cy-chrome-labeled streptavidin. Using the Lysis II program, a marker was set to define the proportion of Fas hi thymocytes. (A) Representative analysis of one control and three MG thymuses. The percentage of Fas hi thymocytes is indicated, as well as age and the anti-AChR antibody titer (nmol/L). Fas expression is clearly increased in patients with positive anti-AChR antibody titers (MG2 and MG3) but not in the patient with a negative titer (MG1). (B) The proportion of Fas hi thymocytes was determined in 26 MG patients and 7 control subjects. Two groups of patients (anti-AChR antibody titer ≥ 1 nmol/L and < 1 nmol/L) are distinguished and compared with controls by using the Mann-Whitney test. The bar represents the mean value. Only the group of patients with positive anti-AChR antibody titers differed from the controls.

Activating state of Fas hi thymocytes. We first compared the activation state of Fas hi and Fas lo cells by means of two-color immunofluorescence with anti-Fas and anti-interleukin-2 (IL-2) receptor α (anti-CD25) antibodies or anti-Fas and anti-HLA-DR antibodies on freshly isolated thymocytes from 6 MG patients and 6 control subjects. A representative analysis of 1 MG patient is presented in Fig 2A. In all the thymuses (Fig 1A) about 90% of thymocytes displayed low Fas expression (Fas lo). In age-matched control thymuses a small proportion of thymocytes (0.4% to 2.8%) showed strong Fas expression (Fas hi). The major peak of fluorescence was composed of Fas lo cells. A second peak, at the end of the major peak, was composed of Fas hi cells; its fluorescence level was above 102. In thymuses from patients with negative or borderline anti-AChR antibody titers (<1 nmol/L), the proportion of Fas hi thymocytes was not significantly different from control values (1.3% ± 0.2% vs 1.5% ± 0.3% in controls). By contrast, MG patients with positive titers (≥1 nmol/L) had far higher proportions of Fas hi thymocytes (0.7% to 14.2% of total thymocytes; mean 5.6% ± 0.7%; P < .0003 compared to controls and P < .0001 compared to patients with negative or borderline titers (Fig 1B). Furthermore, this increase in the proportion of Fas hi cells correlated with the autoantibody titer (4.1% ± 0.7% in thymuses from MG patients with an intermediate titer, P < .006 compared to controls; and 7.0% ± 1.1% in thymuses from MG patients with a high titer, P < .0004 compared to controls).

RESULTS

Thymocytes with strong Fas expression (Fas hi) accumulate in MG patients with positive anti-AChR antibody titers. Fas expression was compared between thymuses from control subjects and MG patients by using immunofluorescence on freshly isolated thymocytes (Fig 1). In all the thymuses (Fig 1A) about 90% of thymocytes displayed low Fas expression (Fas lo). In age-matched control thymuses a small proportion of thymocytes (0.4% to 2.8%) showed strong Fas expression (Fas hi). The major peak of fluorescence was composed of Fas lo cells. A second peak, at the end of the major peak, was composed of Fas hi cells; its fluorescence level was above 102. In thymuses from patients with negative or borderline anti-AChR antibody titers (<1 nmol/L), the proportion of Fas hi thymocytes was not significantly different from control values (1.3% ± 0.2% vs 1.5% ± 0.3% in controls). By contrast, MG patients with positive titers (≥1 nmol/L) had far higher proportions of Fas hi thymocytes (0.7% to 14.2% of total thymocytes; mean 5.6% ± 0.7%; P < .0003 compared to controls and P < .0001 compared to patients with negative or borderline titers (Fig 1B). Furthermore, this increase in the proportion of Fas hi cells correlated with the autoantibody titer (4.1% ± 0.7% in thymuses from MG patients with an intermediate titer, P < .006 compared to controls; and 7.0% ± 1.1% in thymuses from MG patients with a high titer, P < .0004 compared to controls).

Activation state of Fas hi thymocytes. We first compared the activation state of Fas hi and Fas lo cells by means of two-color immunofluorescence with anti-Fas and anti-interleukin-2 (IL-2) receptor α (anti-CD25) antibodies or anti-Fas and anti-HLA-DR antibodies on freshly isolated thymocytes from 6 MG patients and 6 control subjects. A representative analysis of 1 MG patient is presented in Fig 2A. In MG patients (Fig 2B), a higher proportion of Fas hi thymocytes expressed the IL-2 receptor α and HLA-DR, relative to Fas lo thymocytes. Similar results were obtained in controls (Fig 2B).
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Thymocytes were also simultaneously labeled with anti-Fas and anti-CD3 antibodies. Although Fasl0 thymocytes showed a wide range of CD3 expression, Fashi thymocytes uniformly showed intermediate CD3 (CD3int) expression (ie, between low and high). The same characteristics were found in control thymuses. Three representative analyses from three MG patients with no anti-AChR antibody, with an intermediate anti-AChR antibody titer are shown. In these representative analyses, the percentage of HLA-DR+ or CD25-expressing cells is strikingly higher in Fashi thymocytes than in Fasl0 thymocytes. Such analyses were performed on 6 controls and 6 MG patients. Data presented are mean ± SEM. The increase in HLA-DR or CD25+ cells in Fashi thymocytes compared with Fasl0 thymocytes were similar in controls and in MG patients.

In the same labeling experiments we examined CD4 and CD8 markers in Fashi thymocytes from MG patients and controls to characterize their maturation state. As previously described by Debatin et al., Fashi cells had a CD4/CD8 profile similar to that of the total thymocyte population. By contrast, Fasl0 cells were mainly in the CD4 lineage, ie, CD4+ and CD4+CD8+ cells in MG patients and in control subjects (data not shown). Thymocytes were also simultaneously labeled with anti-Fas and anti-CD3 antibodies. Although Fasl0 thymocytes showed a wide range of CD3 expression, Fashi thymocytes uniformly showed intermediate CD3 (CD3int) expression (ie, between low and high). The same characteristics were found in control thymuses. Three representative analyses from three MG patients with no anti-AChR antibody, with an intermediate anti-AChR antibody titer are shown. In these representative analyses, the percentage of HLA-DR+ or CD25-expressing cells is strikingly higher in Fashi thymocytes than in Fasl0 thymocytes. Such analyses were performed on 6 controls and 6 MG patients. Data presented are mean ± SEM. The increase in HLA-DR or CD25+ cells in Fashi thymocytes compared with Fasl0 thymocytes were similar in controls and in MG patients.

Patients with positive anti-AChR antibody titer, but not in patients with negative or borderline titer, the proportion of CD4+Fashi and CD4+CD8+ Fashi thymocytes were significantly increased, relative to the control subjects (P < .002 for both subsets). No significant increase was observed in CD8+Faslo cells, whatever the anti-AChR antibody titer. In patients with positive anti-AChR antibody titers, the significant increase in double-negative Fashi thymocytes was related to the presence of B cells in this population; indeed, the proportion of Fashi cells was about 30% of total thymic B cells (data not shown).

In the same labeling experiments we examined CD4 and CD8 markers in Fashi thymocytes from MG patients and controls to characterize their maturation state. As previously described by Debatin et al., Fashi cells had a CD4/CD8 profile similar to that of the total thymocyte population. By contrast, Fasl0 cells were mainly in the CD4 lineage, ie, CD4+ and CD4+CD8+ cells in MG patients and in control subjects (data not shown). Thymocytes were also simultaneously labeled with anti-Fas and anti-CD3 antibodies. Although Fasl0 thymocytes showed a wide range of CD3 expression, Fashi thymocytes uniformly showed intermediate CD3 (CD3int) expression (ie, between low and high). The same characteristics were found in control thymuses. Three representative analyses from three MG patients with no anti-AChR antibody, with an intermediate anti-AChR antibody titer are shown. In these representative analyses, the percentage of HLA-DR+ or CD25-expressing cells is strikingly higher in Fashi thymocytes than in Fasl0 thymocytes. Such analyses were performed on 6 controls and 6 MG patients. Data presented are mean ± SEM. The increase in HLA-DR or CD25+ cells in Fashi thymocytes compared with Fasl0 thymocytes were similar in controls and in MG patients.
titer, and with a high titer are shown (Fig 4). CD3<sup>int</sup>Fas<sup>hi</sup> thymocyte proportion was all the more increased as the patients’ antibody titer was high.

**T-cell repertoire in Fas<sup>hi</sup> thymocytes.** In previous analyses of TCR V<beta> gene segments in thymic cells, we found that the percentage of V<beta>5.1-expressing cells, but not that of cells expressing V<beta>6.7, was increased in MG patients<sup>39,41</sup>; this increase involved both mature single-positive thymocytes and their CD3<sup>+</sup>CD4<sup>-</sup>CD8<sup>-</sup> late precursors. This study suggested a bias in intrathymic selection. In addition, expansion of V<beta>5.1-expressing cells was observed in the presence of selected peptides from the AChR α subunit in CD4<sup>+</sup> mature thymocytes from MG patients; V<beta>5.1-expressing cells were thus enriched in potentially autoreactive cells (Cohen-Kaminsky et al, manuscript submitted, January 1997). We examined the expression of V<beta>5.1 and V<beta>6.7 in Fas<sup>hi</sup> thymocytes in the present study (Fig 5) in isolated CD4<sup>+</sup> thymocytes. This analysis was done on thawed thymocytes from six MG patients, after checking that the Fas expression level was not modified in CD4<sup>+</sup> cells by cryopreservation. CD8-depleted thymocytes were selected after separation with magnetic CD8-coated beads. Three-color immunofluorescence analysis showed that the proportion of cells expressing V<beta>5.1 was significantly enhanced among CD4<sup>+</sup>Fas<sup>hi</sup> thymocytes relative to CD4<sup>+</sup>Fas<sup>lo</sup> cells (P < .04). By contrast, V<beta>6.7 expression was not significantly increased among CD4<sup>+</sup>Fas<sup>hi</sup> thymocytes from MG patients.

![Figure 5](https://example.com/figure5.png)

**Fig 5.** Comparison of V<beta>5.1 and V<beta>6.7 expression in CD4<sup>+</sup>Fas<sup>lo</sup> and CD4<sup>+</sup>Fas<sup>hi</sup> thymocytes from six MG patients. CD8-depleted thymocytes were labeled with anti-Fas, PE-coupled anti-CD4, FITC-coupled anti-V<beta>5.1 or anti-V<beta>6.7. After setting gates to define Fas<sup>+</sup> and Fas<sup>lo</sup> cells, V<beta>5.1 and V<beta>6.7 expression was analyzed in these gates and compared by using the Wilcoxon test. V<beta>5.1-expressing cells were enriched in Fas<sup>hi</sup> cells compared with Fas<sup>lo</sup> cells, whereas V<beta>6.7-expressing cells were equally represented in both populations.

**Involvement of Fas<sup>hi</sup> thymocytes in the proliferative response to peptides from the AChR.** CD8-depleted and CD8<sup>-</sup>- and Fas<sup>hi</sup>-depleted cells were obtained as previously described. The depletion of Fas<sup>hi</sup> cells targeted around 90% of total CD4<sup>+</sup>Fas<sup>hi</sup> cells (Fig 6A). CD8-depleted and CD8<sup>-</sup> and Fas<sup>hi</sup>-depleted cells were cultured in the absence or in the presence of peptides from the AChR. CD4<sup>+</sup>CD8<sup>-</sup> cells constitute a source of antigen-presenting cells. 1H (169-181) and 3H (351-368) peptides were previously shown to stimulate the proliferative response in MG patient but not in control subject lymphocytes<sup>42</sup>. Firstly, thymocytes depleted in Fas<sup>hi</sup> cells spontaneously proliferate less efficiently than CD8-depleted cells in three MG patients (Fig 6B). Secondly, in these patients 1H and/or 3H peptides induce a significant proliferation response in CD8-depleted cells. This response was abrogated when Fas<sup>hi</sup> cells were depleted (Fig 6B). These experiments indicate that cells involved in the proliferative response to peptides from the AChR are Fas<sup>hi</sup> cells.

**Effect of an agonistic anti-Fas antibody on Fas<sup>hi</sup> thymocytes.** We have previously found that Fas<sup>hi</sup> thymocytes accumulate in the thymus of MG patients with positive anti-AChR antibody titers. Here we investigated whether the Fas receptor of Fas<sup>hi</sup> cells was functional and whether Fas<sup>hi</sup> cells were eliminated by an agonistic anti-Fas antibody. Immobilized CH-11 anti-Fas (5 μg/mL) was used on freshly isolated thymocytes from three MG patients. Fas and CD3 expression were analyzed after 20 hours of culture. We first checked that Fas staining with UB2 anti-Fas antibody was not blocked or dysregulated in the presence of CH-11 anti-Fas antibody on PBL which were previously shown to be resistant to an agonist antibody during a 1-day culture.<sup>31</sup> Human PBL were cultured during 20 hours in the presence of immobilized CH-11 anti-Fas antibody and Fas expression was not significantly modified (data not shown). In MG patients, the proportion of Fas<sup>hi</sup> cells was similar on freshly isolated and cultured thymocytes in control conditions. In the presence of CH-11 anti-Fas antibody the proportion of Fas<sup>hi</sup> thymocytes was reduced by about 80% (Fig 7A). We compared living cell numbers (measured by Trypan blue assay) obtained with and without CH-11 anti-Fas antibody. The total cell number obtained in the presence of CH-11 anti-Fas antibody was not significantly modified compared with control conditions. Cell numbers in CD3<sup>+</sup>Fas<sup>hi</sup> and CD3<sup>+</sup>Fas<sup>lo</sup> thymocyte populations were calculated from percentages obtained by immunofluorescence analysis. Although CD3<sup>+</sup>Fas<sup>hi</sup> were not sensitive to an agonist anti-Fas antibody, most CD3<sup>+</sup>Fas<sup>lo</sup> were eliminated in these conditions (Fig 7B).

**Expression of Fas in PBL.** Fas expression was analyzed, as described above for thymocytes, on PBL isolated from...
the same MG patients at the time of thymectomy and from healthy volunteers (Fig 8). We examined the proportion of CD4⁺ and CD8⁺ peripheral cells and Fas expression in these subsets, then we calculated the proportion of CD4⁺ Fas⁺ and CD8⁺ Fas⁺ cells among total lymphocytes. CD4⁺ and CD8⁺ peripheral cells from controls contained 37.4% ± 0.9% and 32.1% ± 4.8% of Fas⁺ lymphocytes, respectively, in keeping with previous results. Whatever the anti-AChR antibody titer of MG patients, the proportion of CD4⁺ Fas⁺ or CD8⁺ Fas⁺ lymphocytes was not significantly modified compared with control subjects. In both groups of MG patients, some individuals (25%) had significant increases (higher than the mean + 2 SD of control values) in the proportion of CD4⁺ Fas⁺ peripheral lymphocytes. A similar result has been obtained with lymphocytes from patients with systemic lupus erythematosis. Therefore, there was no major modification in the Fas⁺ cell proportion among peripheral lymphocytes, contrary to thymocytes.

**DISCUSSION**

**Dysregulation of Fas expression in MG patients with anti-AChR antibody.** The main finding in this study is that Fas antigen expression is dysregulated in thymocytes from patients with myasthenia gravis, a human autoimmune disease, who had positive anti-AChR antibody titer. We showed that thymocytes strongly expressing Fas were present in the thymus of control subjects but at a lower proportion than in MG patients. This is in agreement with reports from Debatin et al and Yonehara et al, who described, in the normal thymus, a thymocyte subpopulation strongly expressing Fas and representing less than 4% of total thymocytes. This population had the same characteristics in control subjects and MG patients: Fas⁺ thymocytes are enriched in activated cells and are mainly in the CD4 lineage (CD4⁺ and CD4⁺CD8⁺), and express intermediate levels of CD3. This phenotype characterizes cells in a transitional state during intrathymic maturation. No Fas expression modification was observed in the thymocytes from MG patients with a negative or borderline anti-AChR antibody titer. MG disease in which anti-AChR antibody is absent is unlikely to be related to thymus function. This is compatible with the absence of thymic hyperplasia and thymoma in seronegative forms of the disease, in which thymus is very often normal or involuted. Possible involvement of Fas⁺ thymocytes in the anti-AChR autoimmune response. Previous work has suggested that the autoimmune response against AChR could take place in the thymus of MG patients, given that autoreactive T cells are present; in addition, thymic B lymphocytes isolated from hyperplastic thymuses can produce anti-AChR antibodies. Interestingly, we found that the increase in the proportion of Fas⁺ thymocytes correlated strongly with serum anti-AChR antibody titers in MG patients. In addition, among CD4⁺CD8⁻ cells, Fas⁺ thymocytes preferentially expressed the Vβ5.1 TCR segment but not the Vβ6.7 segment. Previous studies of MG thymocytes have indicated that Vβ5.1-expressing cells are potentially autoreactive (Cohen-Kaminsky et al, manuscript submitted, January 1997) and that their proportion is enhanced among mature single-positive thymocytes and their CD3⁺CD4⁺CD8⁻ late precursors. Previous work on Fas⁺ thymocytes in humans has suggested that this thymocyte subpopulation could be autoreactive. In addition, we observed that (1) Fas⁺ cells from MG patients were responsible for the spontaneous proliferation in autologous conditions, and (2) the proliferative response of MG patient thymocytes to two different AChR peptides was abolished when Fas⁺ cells were depleted. Altogether our findings suggest, therefore, that Fas⁺ thymocytes comprise autoreactive T cells that induce the autoimmune response against the AChR in the MG thymus.
verse transcription-polymerase chain reaction on total thymic mRNA rather than isolated thymocytes. Fas is also expressed on human thymic epithelial cells (unpublished results, January 1996), and this could explain the apparent discrepancy with our results. Further experiments comparing Fas protein and mRNA expression should involve isolated thymocytes and thymic epithelial cells.

Several hypotheses can be raised to explain the accumulation of Fas<sup>hi</sup> thymocytes in MG patients. Firstly, it could be due to activated PBL reentering the thymus. This hypothesis is unlikely for several reasons: (1) in MG patients, the increase in Fas<sup>hi</sup> cell proportion was observed in thymocytes but not in PBL; (2) the increase of Fas<sup>hi</sup> cell proportion was observed not only in CD4<sup>+</sup>Fas<sup>hi</sup> but also in the CD4<sup>+</sup>CD8<sup>+</sup>Fas<sup>hi</sup> population that is mainly a thymic subset; and (3) Fas<sup>hi</sup> PBL, but not Fas<sup>hi</sup> thymocytes, are resistant to Fas-induced cell death in the absence of activation.43

Secondly, Fas<sup>hi</sup> thymocytes from control subjects and MG patients are enriched in activated cells, because they strongly express CD25 and HLA-DR. Activation of peripheral lymphocytes induces an increase in Fas expression.48 The sensitivity or resistance of activated cells to Fas-mediated apoptosis depends on the activation state.43 In addition, stimulation of human thymocytes through the TCR complex induces strong Fas expression51 (unpublished results, June 1996). Our team has previously found several signs of activation in the thymus of MG patients,10,13 especially increased cytokine expression.49 Taken together, these data suggest the accumulation of Fas<sup>hi</sup> thymocytes in MG patients is related to an increase in activation signals.

Thirdly, the accumulation of activated Fas<sup>hi</sup> thymocytes could be caused by a failure to eliminate these cells in the thymus. However, most Fas<sup>hi</sup> thymocytes in MG patients are sensitive to an agonistic anti-Fas antibody, showing that the

To identify any peripheral events associated with Fas<sup>hi</sup> thymocyte accumulation, we analyzed Fas expression in peripheral lymphocytes from the same MG patients collected at the time of thymectomy. We did not observe any significant modification in CD4<sup>+</sup>Fas<sup>hi</sup> or CD8<sup>+</sup>Fas<sup>hi</sup> peripheral cell proportion. Therefore, Fas expression modification in MG patients was observed in the thymic but not in the peripheral compartment. Fas ligand was previously shown to play a prominent role in the elimination of autoreactive cells in the periphery26-28; thus, the increase of Fas<sup>hi</sup> thymocytes proportion in MG patient thymuses could be corrected in the periphery.

**Why do Fas<sup>hi</sup> thymocytes accumulate in MG thymuses?** The main difference we found between control and MG thymuses was the proportion of Fas<sup>hi</sup> thymocytes, which was about four times (up to 10 times) higher in MG patients with positive AChR antibody titers than in controls. Masunaga et al<sup>46</sup> found that Fas mRNA was slightly decreased in the thymus of six MG patients, but used a nonquantitative re-
Fas receptor is functional and can transduce an apoptotic signal. We could wonder whether another Fas/Fas ligand system dysfunction could be involved in the maintenance of Fas<sup>hi</sup> thymocytes. Defective secretion of the Fas ligand or increased soluble Fas receptor levels could induce an accumulation of cells that normally die of apoptosis mediated by the Fas system. Cells able to produce Fas ligand are poorly characterized in the human thymus, although recent data suggest that thymic epithelial cell lines are good producers<sup>10</sup> (unpublished results, July 1996). Soluble Fas receptor is able to inhibit Fas-mediated apoptosis, probably in interacting with available Fas ligand; its level is increased in patients with systemic lupus erythematosus according to Cheng et al.,<sup>15</sup> although other groups disagree.<sup>51,52</sup> In addition, an increase in the level of some cytokines in MG thymuses could induce a less efficient Fas/Fas ligand-mediated cell death; indeed, it was shown that recombinant IL-12 was able to inhibit Fas-mediated apoptosis in human peripheral CD4<sup>+</sup> lymphocytes.<sup>53</sup> In conclusion, the accumulation of Fas<sup>hi</sup> cells in MG patients could be caused by an imbalance between activation and deletion of thymic cells.

Taken together, our results indicate that a subpopulation of thymocytes strongly expressing Fas antigen may comprise AChR-reactive cells. These thymocytes, enriched in activated cells, accumulate in the thymus of MG patients with positive anti-AChR antibody titers and are not totally eliminated.

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Thymocyte Fas Expression Is Dysregulated in Myasthenia Gravis Patients With Anti-Acetylcholine Receptor Antibody

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