Differential Estrogen Receptor Expression in Autoimmune Myasthenia Gravis

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Myasthenia gravis (MG) is an autoimmune disease associated with thymic hyperplasia and is much more prevalent in women than men. In this study we investigated potential changes in estrogen receptor (ER) expression in thymic hyperplasia. We first quantified by real-time PCR the relative expression of ERα and ERβ in normal thymus and found that the ERβ to ERα ratio was inverted in thymocytes (8.6 ± 1.2), compared with thymic epithelial cells (0.18 ± 0.05). The ER transcript number gradually decreased in thymic epithelial cells during culture, indicating that the thymic environment influences ER expression. CD4+ helper T cells expressed higher level of ERs, compared with CD8+ cells, as assessed by flow cytometry in thymocytes and peripheral blood mononuclear cells. In MG patients, we found an increased expression of ERs on thymocytes and both ERs on T cells from peripheral blood mononuclear cells, indicating that the signals provided by thymic and peripheral microenvironments are distinct. Finally, activation of normal thymocytes by proinflammatory cytokines induced increased expression of ERs especially in the CD4+ subset, suggesting that an excess of proinflammatory cytokines could explain the increase of ERs expression on MG lymphocytes. The dysregulation of ER expression in MG lymphocytes could affect the maintenance of the homeostatic conditions and might influence the progression of the autoimmune response. (Endocrinology 146: 2345–2353, 2005)

The biological action of estrogens is primarily mediated by binding to one of two specific estrogen receptors (ERs), ERα or ERβ, which belong to the nuclear receptor superfamily, a family of ligand-regulated transcription factors. ERα and ERβ contain the conserved structural and functional domains typical of nuclear receptor family members, including domains involved in DNA-binding, dimerization, ligand-binding, and transcriptional activation (1). Although, ERα and ERβ share similar mechanisms of action, several differences in the transcriptional abilities of each receptor have been identified, suggesting that these receptors may regulate distinct cellular pathways (2). When ERs are coexpressed, ERβ exhibits an inhibitory action on ERα mediated gene expression (3, 4). Furthermore, these two receptors exhibit distinctive response to synthetic antiestrogen molecules (5). The transcription activation function (AF) of ERα and ERβ are mediated by an N-terminal ligand, independent AF (AF-1) and a C-terminal ligand-depend AF (AF-2). A comparison of the AF-1 domains of the two ERs has revealed that this domain is very active in ERα, but not in ERβ, under identical conditions (5), giving a possible explanation for their diversity of responsiveness to several ligands.

ERs have been shown to be involved in thymic development because ERα knockout mice have smaller thymuses than their wild-type littermates (6). In the mouse thymus, both stromal and thymocytes express ERα at the mRNA and protein levels (7). In rat, ERα and ERβ are expressed on thymocytes and stromal cells, and estrogen decreases thymus size (8). In humans, only a few studies investigated the expression of estrogen binding sites on normal and pathological thymic cells (9–11). The type of receptors and the nature of the cells expressing them are not yet clearly identified.

Autoimmune diseases are more prevalent in women than men (12, 13). The increased incidence of autoimmunity in women raises the question of the potential role of sex hormones (estrogen, progesterone, and testosterone) as mediators of these differences in autoimmunity (14). In both multiple sclerosis and rheumatoid arthritis, disease activity decreases throughout pregnancy but most profoundly during the third trimester when estrogens and progesterone levels are the highest. Conversely, a flare-up of disease activity often occurs during the postpartum period when estrogens and progesterone concentrations fall. This fluctuation of disease activity has also been explained by the hormonal environment during pregnancy, which favors a polarization of the immune response toward a Th2 response (13). Interestingly, Th1-dependent autoimmune diseases such as rheumatoid arthritis were found to improve after β-estradiol treatment (15, 16), whereas Th2-dependent diseases such as lupus erythematosus tend to exacerbate after β-estradiol treatment (17, 18). These observations highlight the functional link between sexual hormones and the immune system. ERs were reported to be expressed by macrophages (19) and T and B cells (20). Moreover, it was shown that estrogens act directly on immune cells (macrophages and T cells) by reducing the synthesis and secretion of TNFα, IL-6, and IL-1 cytokines (21–23).

Myasthenia gravis (MG) is a neurological autoimmune disease caused by antibodies to the acetylcholine receptor (AChR), found in the serum of 85% of patients (24). Moreover, it is unknown whether estrogens are involved in MG pathogenesis. The aim of this study was to examine estrogen receptor (ER) expression in thymus and peripheral blood mononuclear cells (PBMCs) in MG patients, and to evaluate the effects of estrogen on ER expression in thymic and peripheral microenvironments.
associated with thymic abnormalities including hyperplasia, found in 50% of patients, and thymoma (thymic tumor), evidenced in about 20% of MG patients (25–27). Thymectomy is an effective therapy for many patients (28). There is a clear relationship between thymic pathology and gender in MG. Indeed, thymic hyperplasia, characterized by the presence of lymphoid follicles, essentially affects female patients (ratio 9:1) during the fecond period of their life (29). Most patients in this subgroup present the human leukocyte antigen-B8 and -DR3 phenotype (30, 31). Modulation of the clinical symptoms of MG during pregnancy or menstruation has been reported, and this phenomenon disappears after thymectomy (32).

Because most MG patients with thymic hyperplasia are females and estrogen appears to play a role in the thymic development, we analyzed the expression of ERα and ERβ in the different thymic cell subpopulations and compared the expression of the two types of ERs in myasthenic patients and healthy controls. We found that all thymic cell subsets express ERs, but thymocytes and thymic epithelial cells (TECs) have a clear different pattern of expression. In MG patients’ lymphocytes, the expression of ERs was found to be increased in both the thymus and peripheral blood mononuclear cells (PBMCs). We further demonstrate that ER expression is increased on proinflammatory cytokine treatment. Altogether these data demonstrate a dysregulation of ERs in MG disease and suggest that these changes could be mediated by factors produced by the microenvironment.

**Patients and Methods**

**Patients**

The study included 37 MG patients with anti-AChR antibodies, 28 for the thymus study and nine for the PBMC analysis. Two patients were older than 40 yr. Thymuses were obtained from 21 females and seven males (ages 14–44 yr, median age value 25 yr) undergoing thymectomy at Marie Lannelongue Hospital. Among them, 24 were used in the real-time PCR experiments and four were studied by flow cytometry. The blood from nine MG patients (15–39 yr) was taken anyway for other routine medical tests. All patients received anticholinesterase drugs except three patients who have additionally iv immunoglobulins treatment. MG severity was graded according to the Myasthenia Gravis Foundation of America’s classification (33): grade I (ocular), one patient; grade IIa (mild generalized weakness), 10 patients; grade IIb (mild generalized weakness with bulbar muscle involvement), 21 patients; grade IIIB (moderate generalized muscle weakness, with bulbar muscle involvement), four patients; and grade IVb (severe generalized weakness with bulbar muscle involvement), one patient. Thymic hyperplasia was defined according to classification of Levine and Rosai (34) as thymuses containing germinal centers. The degree of hyperplasia was determined by the pathologists of our center by analyzing two distinct thymic sections: moderate hyperplasia for a low number of germinal centers and high hyperplasia for a high number of germinal centers (from three on one section).

The cellularity of the thymus was also determined by the pathologists using a lymphocytic to adipose tissue ratio, defined by the surface occupied by the lymphoepithelial area among the total tissue (100%: only lymphoepithelial tissue, 0%: only adipose and connective tissue). These thymic histological analyses were totally independent of the other results. Anti-AChR antibody titers were determined in serum as previously described (35). Patients without anti-AChR antibody were excluded. The level of antibody was between 0.8 and 772 nm/liter with a median value of 11 nm/liter. Patients on corticosteroid treatment and those with thymomas were excluded from the study. Normal thymuses, which would otherwise be discarded, were obtained from eight newborn infants (aged 3 d to 9 months) and 10 young adults (aged 14–46 yr) undergoing cardiac surgery in our hospital. Ten were used in real-time PCR analysis and eight were used in flow cytometry experiments.

For RNA analysis, fragments of thymic tissue were placed in RNA stabilizing and protecting solution RNA Later (QIAGEN, Courtaboeuf, France) for a week and then flash frozen in liquid nitrogen and stored at −80°C until use. Blood from nine age- and sex-matched controls were obtained from the Centre de Transfusion Sanguine. These investigations were approved by the local Ethics Committee, Comité Consultatif de Protection des Personnes dans la Recherche Biomédicale (Kremlin Bicêtre, France).

**TEC culture**

Primary TEC cultures were established as previously described (36). Briefly, small fragments of human thymic tissue were washed in RPMI 1640 medium without phenol red (Life Technologies, Inc. Invitrogen Corp., Cergy-Pontoise, France) and transferred to 75-cm² culture dishes. The culture medium was supplemented with 20% horse serum (Life Technologies, Invitrogen), 0.2% Ultraser G (Life Technologies), 2 mmol/liter t-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin and replaced twice a week. After 10 d of primary culture, the confluent monolayers were washed with PBS and then treated with 0.075% trypsin (Life Technologies) and 0.16% EDTA for 10 min at 37°C. In some experiments, TECs were collected after 3, 6, 8, or 10 d of primary culture. The epithelial nature of the cells was checked by immunocytochemical analysis of cytocentrifuged cells using an antikeratin polyclonal antibody (Clone A5575; DAKOCytomation, Trappes, France). An anticlellin III monoclonal antibody (IgG1 clone III-53; ICN, Orsay, France) was used to determine the percentage of contaminating fibroblast cells. After 10 d of culture, the proportion of keratin-positive cells was consistently greater than 70%. The cells were directly harvested in Trizol (Life Technologies) for RNA extraction and frozen at −80°C.

**Thymocyte cultures**

To study the effect of proinflammatory cytokines on ER expression, thymocytes were subcultured in 75-cm² culture dishes. The culture medium was Xvivo-15 medium without phenol red or gentamicin (Bio-whittaker, Emeraude, France), and supplemented with 2 mmol/liter t-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin. Thymocytes (4 × 10⁶/per ml) were cultured in 15 ml of medium in the presence of 1 ng/ml recombinant human IL-1β (Sigma, Saint Quentin Fallavier, France), 10 ng/ml recombinant TNFα, and 500 U/ml recombinant human interferon (IFN)-γ (Genzyme, Cergy Saint Christophe, France). After 24 h, thymocytes were harvested and centrifuged at 1200 rpm at 4°C for 10 min, washed twice, and used for flow cytometry analysis.

**Separation of lymphocytes and PMBCs**

To purify PMBCs, we used 15 ml Leucoatpe tubes (Eurobio, Les Ulis, France) according to the manufacturer’s instructions.

**Antibodies**

Antihuman-ERα (HC-20: sc-543) and anti-ERβ (H-150: sc-8974) antibodies produced in rabbits were purchased from Santa Cruz Biotechnology (Tebu, Le Perray en Yvelines, France). We assessed the specificity of these antibodies by Western blot experiments. The presence of immunoreactive protein bands was detected in thymocytes with an apparent molecular mass of approximately 67 and 53 kDa for ERα and ERβ, respectively (data not shown). A goat anti rabbit secondary antibody labeled with fluorescein isothiocyanate was purchased from Beckman Coulter (Villepinte, France). Anti-CD4 labeled with PerCP or anti-CD8 labeled with PE monoclonal antibodies (CD4 PerCP, CD8 PE, and CD3

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bior) for 45 min at 4°C. After two washings with PBS, the biotin-labeled cells were incubated with streptavidin-coupled to Quantum red for 30 min at 4°C, washed twice with PBS, and analyzed on FACSCalibur using the CELLQuest software (Becton Dickinson). For analysis of ERs that are expressed intracellularly, cells were first stained for CD4 and CD8 or CD3 surface expression, fixed, and then permeabilized using IntraPrep permeabilization reagents (Immunotech). The permeabilized cells were then labeled with anti-ER antibodies (1:10) and staining detected by polyclonal goat antirabbit immunoglobulins coupled to fluorescein isothiocyanate used at a 1:50 dilution (Beckman Coulter). For the analysis of PBMCs, PE-coupled anti-CD14 and anti-CD19 antibodies were also used (DAKOCytomation). In all the flow cytometry analyses, the results are shown as the specific mean fluorescence intensity (MFI) obtained after subtracting the background.

Real-time RT-PCR

Total RNA was extracted from thymus, TECs, or thymocytes in 1 ml of Trizol solution, after addition of 200 μl chloroform. Total RNA was purified using an RNeasy Kit (Qiagen Inc., Hilden, Germany), dissolved in 50 μl of isopropanol. The RNA pellet was washed in 75% ethanol, dried, dissolved in 50 μl deionized water, and stored at −80°C. Total RNA concentration was determined spectrophotometrically (Gene Quant II; Pharmacia Biotech, Cambridge, UK). RNA purity was determined by the ratio of absorbance at 260 nm to absorbance at 280 nm, which should be in the range of 1.7–2.2.

Total RNA (1 μg) was reverse transcribed by incubation for 1 h at 42°C in a total volume of 50 μl in the presence of 5 μl of 10× reverse transcription buffer [500 mM Tris-HCl (pH 8.3), 60 mM MgCl2, 400 mM KCl, 40 mM dithiothreitol], 1.5 mM deoxynucleotide triphosphates, 40 U of ribonuclease inhibitor (RNasin, Promega, Charbonnieres, France), 50 pmol of specific primers, and 2.5 U of avian myeloblastosis virus reverse transcriptase (Eurobio).

PCRs were performed on the LightCycler apparatus (Roche Diagnostics, Mannheim, Germany), using primers designed with the Oligo software (Med Probe, Oslo, Norway) and purchased from Eurobio. The sense and antisense primers were: 5′-AGA CTC CCT ACT GTG C-3′ and 5′-CCC TAT GCT TTT CTG GCT-3′ for ERα; 5′-TAG TGG TCC ATC GCC AGT TAT CAC-3′ and 5′-ACA CGC CTC TGT CTC TTC AGC-3′ for ERβ, respectively. The ERα and ERβ PCR products were 236 and 439 bp, respectively. PCRs were performed using the Faststart DNA Master SYBR Green I kit (Roche Diagnostics) using 1.5 μl cDNA and 13.5 μl Mastermix. The following LightCycler experimental run protocol was used: initial denaturation at 95°C for 10 min; amplification for 40 cycles (10 sec at 95°C and then 5 sec at the specific primer temperature (62°C for ERα) and 66°C for ERβ); final extension for 14 sec for ERα and 25 sec for ERβ; and final melting curve program for melting curve analysis. Optimal experimental parameters (hybridization temperature, elongation time, and MgCl2 concentrations) were determined for each primer pair. For each gene, the specificity of the PCR product was assessed by verifying a single peak in melting curve analysis. For complementary length verification, LightCycler PCR products were separated by electrophoresis onto a 1.5% agarose gel. For each sample, RNA concentration was also controlled by real-time RT-PCR of the 28S RNA using the following sense and antisense primers: 5′-CCG GTA AAC GGC GGG AGT AA-3′ and 5′-GGT AGG GAC AGT GGG AAT CT-3′, respectively. By comparing the 28S rRNA concentration with the concentration of the ERα or ERβ target transcript, the PCR was optimized to amplify the same number of RNA copies of ERα or ERβ as the internal reference. The difference between the cycles at half maximal amplification for the target and reference genes was determined with the LightCyclerob software.

Quantification of ER transcripts in MG thymus

To determine whether thymuses derived from MG patients exhibit dysregulation of ER expression, we analyzed ER expression in the whole thymic extracts from 24 MG patients (age median 25 yr, 17 females and seven males) and eight age-matched healthy controls (age median 20.5 yr, four females and four males) by real-time PCR. The expression level of ERα was similar in normal and MG thymuses (Fig. 2A), but a difference was observed between patients and controls in ERβ expression (Fig. 2B). Myasthenic patients with moderate hyperplasia demonstrated lower ERβ levels of expression, compared with controls and patients with high degrees of hyperplasia. Consequently, the ERβ to ERα ratio shown in Fig. 2C was significantly lower in patients with moderate hyperplasia, compared with controls and patients with high hyperplasia. Because ERβ is highly expressed in thymocytes, we wondered whether the decrease observed in ERβ level in the moderately hyperplastic samples was due to a low lymphocytic to adipose tissue ratio evaluated as described in Patients and Methods. Indeed, we found a correlation between ERβ expression and the lymphocytic to adipose

**Statistical analysis**

Differences in the ER expression in the four different thymic subsets were evaluated by a one-way variance Friedman test (for nonparametric paired data). Differences between two different groups were compared by paired (Wilcoxon test) or unpaired (Mann-Whitney U test) nonparametric tests for paired and unpaired data, respectively. All analyses were performed using GraphPad software (San Diego, CA).

**Results**

**ER transcripts are expressed in human thymocytes and TECs**

ER expression was analyzed in whole thymic extracts as well as the two main thymic cell populations, TECs, which were enriched in culture, and thymocytes. Real-time PCR was performed for ERα and ERβ, using an external standard curve to quantify the number of transcripts. Both types of receptors were expressed in both cell types, but the ERα to ERβ ratio was inverted in thymocytes, compared with TECs (Fig. 1A). ERβ was 8.6-fold more highly expressed in thymocytes than ERα, whereas it was the opposite in TEC cultures in which ERα was 5.3-fold more strongly expressed than ERβ. Whole thymus, which included a mixture of thymocytes, TECs, and many other cells, had a pattern similar to that of thymocytes (Fig. 1A) and had a similar ER expression in males and females (Fig. 1B). Thus, TECs express predominantly ERα, and thymocytes express ERβ.

We noticed that TECs expressed a lower absolute level of ERs than thymocytes as assessed by the number of RNA molecules per microgram of total RNA (Fig. 1A). To examine whether the relatively weak expression of ER by TECs was due to the culture conditions, we analyzed ERα and ERβ expression in TECs harvested after 3, 6, 8, and 10 d of primary culture. As shown in Fig. 1C, the amount of mRNA representing both ERs was much higher at the beginning of the culture period and decreased over time in culture, although ERα was always more highly expressed than ERβ (Fig. 1C). These results suggest that the expression of ERs on TECs is not constitutive.

**Preparation of the ER standards**

To prepare the ER standards, PCR was performed using the primers for ERα and ERβ, as described above, by omitting the SYBR Green reagent. The PCR products were isolated after migration on agarose gel and then purified using the QIAQUIK kit (QIAGEN) and quantified by spectrophotometry. These reagents were then used as external standards to establish a standard curve and determine the number of molecules of mRNA in the experimental samples for ERα and ERβ.
tissue ratio, indicating that low ERβ expression in moderate hyperplasia was probably due to the low number of lymphoepithelial tissue in these thymuses (data not shown).

We did not observe any correlation between ER expression and the other parameters of the disease, such as the severity or onset of the disease or the anti-AChR antibody titer.

**ERs are expressed on normal thymocytes**

Because thymocytes expressed the highest levels of ER mRNA, we analyzed ER expression at the protein level in the different thymocyte subpopulations by flow cytometry using commercial available polyclonal antibodies. Because ERs are cytoplasmic receptors, flow cytometry experiments were performed on permeabilized cells. To analyze the possible influence of gender on ER expression on thymocytes, the results obtained by flow cytometry analysis were graphed separately for males and females (Fig. 3, A and D). The MFI of ERα and ERβ was not significantly different in males and females, although a tendency toward higher expression of ERs in females was observed. This result is not surprising because it was shown that little or no variation by gender was observed in nonreproductive tissues. One explanation is that nonreproductive tissues are responsive to either class of sex steroid irrespective of gender (39, 40).

To determine which subpopulation of thymocytes express ERα and ERβ, we performed tricolor immunofluorescence combining anti-ERs with anti-CD4 and anti-CD8 (Fig. 3, B and E). We first verified that the intracytoplasmic staining did not modify the detection of CD4 and CD8 markers. As shown in Fig. 3C, the ERα MFI was significantly different from one cell population to another (P < 0.0001, one-way variance test). It decreased from the highest levels in CD4 single positive cells to intermediate levels in CD4+CD8− and CD8 single positive populations to the lowest expression in CD4−CD8− cells (Fig. 3C).

A similar analysis with anti-ERβ indicated that the MFI was significantly different in the four thymocyte subpopulations (P < 0.0001, one-way variance test) (Fig. 3F). The mean fluorescence was similar in the CD8+ and CD4+CD8− populations but was lower in CD4−CD8− cells and higher in the single positive CD4 thymocytes (Fig. 3, E and F).

From these results, we can define a hierarchy for the expression of the ERs by the thymocytes, the single positive CD4 thymocytes being the subset with higher ER expression.

**ER expression is increased in thymocytes from MG patients**

We then analyzed the expression of ERs in freshly isolated thymocytes from four MG patients and eight healthy thymuses using flow cytometry. We observed that the MFI of ERα expression was significantly higher in thymocytes isolated from MG patients, compared with controls (Fig. 4A), whereas ERβ levels were unchanged (Fig. 4B). For ERα, the increase was statistically significant in all the thymocyte subpopulations, and the fold change was the highest in the CD4+CD8− (double negative) cells from MG patients (Fig. 4A). Because six of eight controls were prepubertal, whereas the four MG patients were postpubertal, we wondered whether the differences observed could be due to the age. The analysis of ERα in the CD4+ population indicates that ERα MFI in older controls (216 ± 94) has a tendency to be higher, compared with the prepuberty controls (161 ± 63), but it is still lower than the patients’ values (328 ± 96). Therefore, the adult controls have intermediate values between the prepuberty controls and the adult MG patients. In contrast, ERβ expression in thymocytes from MG patients was similar to controls for the different thymocyte subsets.
these differences were statistically significant in CD4+H11006182 and CD8+H925312 for ER. Expression of ERs is regulated by cytokines

Effect of cytokines on thymocyte ER expression

To determine whether the increased ER expression in thymocytes was also observed in the periphery, we performed a similar analysis in PBMCs from nine MG patients and nine sex- and age-matched controls. We observed a significantly increased ER expression in peripheral blood cells derived from MG patients vs. controls (Fig. 5, A and B). Interestingly, these differences were statistically significant in CD4+H11001 and CD8+H9252 cells but not in B cells (Fig. 5, A and B). A similar analysis in CD14+ cells (gate appropriate for monocytes) indicated no significant changes in MG patients, compared with controls (746 ± 55 vs. 754 ± 23 and 141 ± 21 vs. 182 ± 12 for ERα and ERβ, respectively) (data not shown). Thus, only T cells appear to display a higher expression of ERs in MG patients. Similar to thymocytes, CD4+ peripheral cells from controls expressed more ERs than CD8+ cells (P < 0.02 for ERα and ERβ).

Expression of ERs is regulated by cytokines

To explain the increased expression of ERs on the thymocytes of MG patients, we tested the potential influence of proinflammatory cytokines (IFNγ, IL-1, and TNFα) on ER expression. Freshly isolated normal thymocytes were incubated for 24 h with proinflammatory cytokines, and the expression of ERs was analyzed by flow cytometry in the various thymocyte subpopulations. We observed that ERα expression in the thymocyte subpopulations was increased by the cytokine mix (Fig. 6A) in both the CD4+ (P < 0.05) and CD4+CD8+ (P < 0.03) cell subsets. The up-regulation of ERβ expression on thymocytes was also observed in the CD4+ cell subset (P < 0.01) (Fig. 6B). Thus, proinflammatory cytokines up-regulate the expression of ERs in thymocytes and specifically in the CD4+ cell subpopulation.

Discussion

The aim of this study was to analyze the expression of ERα and ERβ on human thymic cell subpopulations and explore potential expression changes in the context of thymic hyperplasia exhibited by MG patients. Our major findings are as follows: (1) both ERα and ERβ transcripts are found in thymocytes and TECs, but the ERβ to ERα ratio is inverted in thymocytes, compared with TECs; (2) CD4+ thymic and peripheral lymphocytes (T-helper phenotype) express the highest levels of both types of ERs, compared with the other T-lymphocyte subsets; (3) in MG patient thymus and PBMCs, the expression of ER is up-regulated, but the increase affects only ERα in thymocytes; and (4) activation of normal thymocytes by proinflammatory cytokines increases the protein expression of ERs, especially in the CD4+ cell subset.

Significance of ER expression in the normal thymus

Our study demonstrates that human thymic cell subpopulations express ERs at both the mRNA and protein levels. In TECs we showed that ER expression decreased during culture, a finding that could reflect a gradual decline in metabolic activity. Alternatively, this result could suggest that the expression of ERs is not constitutive but depends on the thymic microenvironment, as was previously shown for expression of Fas (41) or class II human leukocyte antigens, whose decreased expression was reversible in the presence of IFNγ or thymocytes (36). Interestingly, the expression of ERα remains elevated relative to the expression of ERβ during the entire culture period. Therefore, it is likely that the thymic environment simultaneously controls the expression of both ERs in TECs.

Although several studies demonstrated a role of ERs in thymic development using mouse models lacking ER expression,
there is little information on the expression of ER on the thymic cell subpopulations. By flow cytometry analysis, we showed that the highest expression of ER\(\beta\) and ER\(\alpha\) was observed in the CD4\(^+\)/CD8\(^-\) subpopulation, compared with the other thymocyte subsets. We can thus postulate that thymocytes express the highest level of ERs at the end of their thymic maturation when they are about to leave the thymus to the periphery. Our analysis on peripheral blood lymphocytes confirms that CD4\(^+\) peripheral cells also express higher level of ERs, compared with the CD8\(^+\) cells. Together with published studies (9, 42, 43), our results substantiate the expression of ERs in the four different thymic subsets. The differences were highly significant (\(P < 0.0001\) for ER\(\alpha\) and ER\(\beta\)).

The functional role of these receptors is subject to debate. It has been shown that mice lacking ER\(\alpha\) have smaller spleens and thymuses than normal mice or mice lacking ER\(\beta\) (43). The smaller thymuses in ER\(\alpha\) knockout mice were shown to result from the lack of ER\(\alpha\) expression in radiation-resistant tissues rather than hemopoietic elements (6). Thus, the expression of the ER\(\alpha\) on thymic stromal cells is important for thymic development (6).

Dysregulation of ER expression in MG patients

Our study on total thymic extracts shows that in MG patients with moderate hyperplasia, there is shift of the ER\(\beta\)
to ERα ratio due to reduced ERβ expression. Because thymocytes express much more ERβ than ERα, this decreased ratio could reflect modification of thymic subpopulations in MG patients with mild hyperplasia. Indeed the lymphocytic to adipose tissue ratio is correlated with the ERβ to ERα ratio, thereby supporting this hypothesis.

Our analysis on thymocytes shows a significant increase of ER expression, and particularly that of ERα, in all the thymocyte populations of MG thymuses, compared with those of controls. Although the expression of the ERs was analyzed only in thymocytes, we cannot exclude changes in ER expression on the other thymic cells such as the epithelial cells. The increase of the ER expression in MG patient thymocytes is unlikely to be due to immigrant cells from the periphery because the changes observed in the thymus and PBMCs are different. In addition, the changes observed in the thymus were shown to occur in the DP population, which is thymus specific.

Other explanations for changes in ER expression in the MG thymus could be proposed. The increase of the ER expression in MG patient thymocytes could reflect a factor predisposing to the disease or could be a consequence of the disease state. A polymorphism of ERs has already been described and associated with susceptibility to lupus nephritis (48) and multiple sclerosis (49). It has also been proposed that genetic variation in hormone receptors, and particularly in the ERα gene, may play a role in migraine (50). Moreover, it was shown that certain variants of the ERβ gene are associated with an increased risk of breast cancer, particularly among women who experienced high-level and long-term estrogen exposure (51). It would therefore be interesting to determine whether specific variants of ERα and ERβ are overrepresented in MG patients and whether those variants tend to be overexpressed.

Alternatively, it is possible that the high level of ER expression in MG thymocytes could be due to components of the thymic environment able to influence ER expression. Indeed, we show in this study that activation of normal thymocytes by inflammatory cytokines increases the level of ER expression in thymocytes in vitro. Thus, the high level of ERs could be due to the proinflammatory environment. However, our data indicating that thymocytes from MG patients exhibit predominately an increase in ERα expression, whereas proinflammatory cytokines up-regulate the expression of both ERα and ERβ suggest that factors other than proinflammatory cytokines regulate ER expression as well. Other mechanisms of regulation of ER expression have been described. In several culture models, estradiol was shown to down-regulate both ERα and ERβ expression, and these effects were blocked by the partial estrogen antagonist tamoxifen (52, 53). Therefore the increase of ERs on lymphocytes from MG patients could be due to a defect in estrogen production in these patients. Although there is no direct evidence of such a defect, in vivo models of mice with

**FIG. 4.** Expression of ERs in MG thymocytes. Freshly isolated human thymocytes from MG thymuses were analyzed by three-color flow cytometry for the expression of CD4, CD8, and ERs. The results are expressed as the specific mean values ± SEM of the specific MFI of ERα (A) and ERβ (B) in the total, CD4+CD8− (double negative (DN)), CD4+CD8+ (double positive (DP)), CD4+, and CD8+ populations obtained from four MG patients (four females) and eight controls (five females and three males). Mann Whitney U test was used to compare values obtained in the patients vs. that in controls.

**FIG. 5.** Expression of ERs in PBMCs. Human PBMCs from adult controls (n = 9 including seven females and two males) and MG patients (n = 9, all females) were analyzed by three-color flow cytometry. The results are expressed as mean values ± SEM of the specific MFI of ERα (A) and ERβ (B) in the total, CD4+, CD8+, and CD19+ populations obtained from MG patients and controls. NS, Not significant.
knockouts for the gene encoding aromatase, which exhibit a defect in estrogen production, show abnormal autoimmune manifestations and elevated B lymphopoiesis (54). Together these data indicate that several physiological factors, including an excess of proinflammatory cytokines or a defect of estrogens, could modulate ER expression on the lymphocytes of MG patients.

**Relationship between germinal center formation and ER expression**

Thymic hyperplasia of MG patients is characterized by the presence of germinal centers (GCs) in the thymus (26, 55). A relationship among autoimmunity, formation of GCs, and ERs has already been reported. Indeed, ERα but not ERβ knockout mice exhibit immune complex-type glomerulonephritis, destruction of tubular cells, and severe infiltration of B lymphocytes in the kidney (56). The ERα knockout mice also show spontaneous formation of GCs in the spleen in absence of antigen challenge (56). A relationship between B cell hyperplasia and a defect in estrogen production was recently demonstrated in aromatase knockout mice that develop severe autoimmune exocrinopathy resembling Sjogren’s syndrome (54). Because a link between ERα expression and development of GCs has already been described, one can suggest a similar mechanism in MG. Combined with the increased proinflammatory activity in the thymus (57), an abnormal level of estrogens in MG patients could at the same time explain the increased expression of ER on thymic cells and the development of B cell hyperplasia, leading to the formation of GCs in the highly activated thymus of MG patients. Finally, we could speculate as to the functional consequences of the increased expression of ERα in lymphocytes of myasthenic patients, compared with healthy controls. Because estrogens were shown to influence cytokine production and the B cell response (13, 44, 45), it is possible that lymphocytes from MG patients display abnormal reactivity to estrogens and an exacerbated B cell response, thereby maintaining the autoimmune state.

In conclusion, this study shows a clear dysregulation of ER expression in thymocytes and PBMCs from MG patients, compared with healthy controls. This dysregulation could contribute to the induction, maintenance, and progression of the autoimmune response via an effect on cytokine production and the B cell response.

**Acknowledgments**

We thank Florent Crépineau, Rozen LePanse, Sandrine Lécart, and Mélénée Cuvelier for helpful discussions and critical reading of the manuscript and Shelley Schwarzbaum for editing the manuscript. We are grateful to Dr. E. Dulmet (Service d’Anatomo-Pathologie, Hôpital Marie Lannelongue, Le Plessis-Robinson) for the pathological analysis of the thymic sections and Dr. A. Serraf (Service de Chirurgie Cardiaque, Hôpital Marie Lannelongue) and Dr. Philippe Dartevelle (Service de Chirurgie Thoracique, Hôpital Marie Lannelongue) for providing the thymic samples.

Received August 2, 2004. Accepted January 14, 2005.

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This work was supported by grants from the National Institutes of Health (NS39869), the European community (QLG1-CT-2001-01918), and the Association Française Contre les Myopathies (AFM). P.N. received a fellowship from the AFM.

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Endocrinology is published monthly by The Endocrine Society (www.endo-society.org), the foremost professional society serving the endocrine community.