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Effects of Cytokines on Acetylcholine Receptor Expression: Implications for Myasthenia Gravis

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Myasthenia gravis is an autoimmune disease associated with thymic pathologies, including hyperplasia. In this study, we investigated the processes that may lead to thymic overexpression of the triggering Ag, the acetylcholine receptor (AChR). Using microarray technology, we found that IFN-regulated genes are more highly expressed in these pathological thymic tissues compared with age- and sex-matched normal thymus controls. Therefore, we investigated whether proinflammatory cytokines could locally modify AChR expression in myoid and thymic epithelial cells. We found that AChR transcripts are up-regulated by IFN-γ, and even more so by IFN-γ and TNF-α, as assessed by real-time RT-PCR, with the α-AChR subunit being the most sensitive to this regulation. The expression of AChR protein was increased at the cytoplasmic level in thymic epithelial cells and at the membrane in myoid cells. To examine whether IFN-γ could influence AChR expression in vivo, we analyzed AChR transcripts in IFN-γ gene knock-out mice, and found a significant decrease in AChR transcript levels in the thymus but not in the muscle, compared with wild-type mice. However, up-regulation of AChR protein expression was found in the muscles of animals with myasthenic symptoms treated with TNF-α. Altogether, these results indicate that proinflammatory cytokines influence the expression of AChR in vitro and in vivo. Because proinflammatory cytokine activity is evidenced in the thymus of myasthenia gravis patients, it could influence AChR expression and thereby contribute to the initiation of the autoimmune anti-AChR response.


Myasthenia gravis (MG) is a human autoimmune disease characterized by muscle weakness, which is enhanced by physical effort. Autoantibodies directed against the nicotinic acetylcholine receptor (AChR) of the neuromuscular junction are found in 85% of patient sera (1, 2). The autoantigen, AChR, is a heterodimeric receptor consisting of four subunits in a molar stoichiometry α2β2γδ during the early embryonic stages or after denervation (3, 4), and α2β2δ in the adult form (5). The anti-AChR Abs are pathogenic and are able to passively transfer the disease to normal animals (6, 7) and to decrease expression of AChR in cell lines (8). In muscles of MG patients, the motor endplate is a target for anti-AChR Abs and there is a decreased number of functional nicotinic receptors, leading to an impairment of synaptic transmission and muscle weakness (9). The thymus plays a pivotal role in the pathogenesis of MG. It frequently shows abnormalities (50–60% hyperplasia, 10–15% thymoma) (10) and thymectomy is clinically beneficial (11–13). The hyperplastic thymus is one of the sites of T and B cell hyperactivation and autoreactivity to AChR (14, 15), and B cells isolated from thymic hyperplasia spontaneously produce Abs to AChR (16). Several signs of activation among T cells have been demonstrated, including an increase in Fas expression in the thymic CD4+ population showing autoreactivity (17). Nicotinic receptor protein and messengers encoding all the subunits have been observed by RT-PCR on myoid cells (18, 19) and on thymic epithelial cells (TEC) (20–22) of thymuses from normal and MG patients. Several studies have suggested the involvement of the myoid (19) and epithelial cells (23) in the primary autosensitization step in MG, even if the initial steps that lead to AChR Ab production are not known. As previously suggested (24), an attractive hypothesis is that the inflammatory environment of the hyperplastic thymus could influence the expression of AChR as well as that of genes involved in the immune response such as MHC molecules, thereby triggering the autoimmune response.

The aim of this study was to further explore the influence of proinflammatory cytokines on AChR expression. We chose to analyze the effects of TNF-α and IFN-γ, because these cytokines have been shown to be involved in the pathogenesis of MG. First, IFN-γ was shown to regulate AChR expression on human TEC (23). In addition, IFN-γ is involved in induction of experimental autoimmune MG (EAMG) (25). In IFN-γ gene receptor knockout (KO) mice, immunization with torpedot AChR does not induce MG (26). In the Lewis rat model, coinjection of IFN-γ and AChR and Freund’s adjuvant triggers an earlier onset and more severe signs.

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induces signs of myasthenia in mice (28). Because TNF-α has a synergistic action with IFN-γ and high producer alleles of TNF-α are associated with thymic hyperplasia (29), this cytokine was also tested in our study.

We first demonstrated a proinflammatory environment in MG thymic hyperplasia by using microarray technology, and showed that IFN-regulated genes and MHC class II molecules are more highly expressed in hyperplastic compared with normal thymus. We then analyzed the in vitro effects of IFN-γ and TNF-α on AChR RNA and protein levels in a myoid immortalized thymic cell line (MITC) and TEC. Our results clearly indicate that IFN-γ is a powerful regulator of AChR subunits genes in both cell types. Finally, in experimental models, we show that these cytokines influence the in vivo level of AChR expression in the thymus and the muscle.

Materials and Methods

Mouse tissues

Muscle and thymus samples were collected from nine wild-type (WT) C57BL/6 mice and nine IFN-γ gene KO mice on the C57BL/6 background (The Jackson Laboratory). All mice were 8- to 9-wk-old males. Tissue samples were immersed in liquid nitrogen and then kept at 70°C until RNA extraction.

Human thymic tissues

Fresh thymic fragments were obtained from immunologically normal patients undergoing corrective cardiovascular surgery (discarded tissue) or from MG patients undergoing thymectomy as a treatment of the disease at Centre Chirurgical Marie Lannelongue (Le Plessis Robinson, France).

Cultures of thymic epithelium

Primary cultures of human TEC were established as previously described (30). Briefly, small fragments of thymic tissue were washed in RPMI culture medium and transferred into 75-cm² culture dishes. The culture medium (RPMI 1640; Invitrogen Life Technologies) supplemented with 20% horse serum (Roche Diagnostics), 0.2% Ultroser (Invitrogen Life Technologies), 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin was replaced twice weekly. Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO². After 8–12 days of primary culture, the confluent monolayers were treated with 0.075% trypsin-EDTA (Invitrogen Life Technologies) for 5 min at room temperature. The epithelial nature of the cells was determined by immunofluorescence with the anti-keratin MNF116 Ab (1/50) (DakoCytomation) and FITC-conjugated sheep anti-mouse Ig (1/100) (Silenus, Eurobio). The percentage of positive cells was always higher than 80%.

Cultures of the MITC line

Establishment of the human MITC line has been previously described (19). Cells were cultured in RPMI 1640 (Invitrogen Life Technologies) containing 100 U/ml penicillin, 100 µg/ml streptomycin, and 10% of FCS (Eurobio). All cells were grown at 37°C in 5% CO².

Cytokine stimulation cultures

The cells were replated in 35-mm petri dishes at a density of 2 × 10⁶ cells/dish and cultured in RPMI medium supplemented with 20% horse serum. After 24 h, the medium was replaced by RPMI supplemented with 5% of horse serum and cytokines were added at the following concentrations: 250 U/ml for recombinant human IFN-γ (R&D Systems) or 10 ng/ml for TNF-α (R&D Systems), or both. In some experiments, IFN-γ or TNF-α alone was added overnight before addition of the second cytokone. After the appropriate incubation time (24 or 48 h), total RNA was extracted or AChR expression was determined by 125I-labeled α-bungarotoxin (125I-α-Bgt) binding or by flow cytometry.

α-Bgt binding experiments

MITCs were incubated with IFN-γ for 3–72 h at 37°C. The medium was then replaced with fresh medium containing 10 nM 125I-α-Bgt and cultures were maintained for 20 min at room temperature. Subsequently, the cells were washed four to five times with medium, and proteins were extracted in 1 ml of 0.1% Triton X-100 for 30 min. The protein extracts were counted in a gamma counter (LKB-Wallace). Background radioactivity was estimated by incubating cells with 13 µM unlabeled α-Bgt for 1 h before adding 10 nM of 125I-α-Bgt. Bound 125I-α-Bgt was considered to represent surface nicotinic AChR (nAChR).

Flow cytometry analysis

The cells were tested for their responsiveness to IFN-γ as follows. After the addition of the cytokines, the cells were incubated for 3 days, and adherent cells were washed, trypsinized, and stained with the mAb35 directed against the extracellular main immunogenic region of AChR (31) for 30 min at 4°C. This was followed by washing and labeling with FITC-conjugated anti-rat Ig (Valbiotech). For intracellular staining, the cells were permeabilized for 20 min with saponin (final concentration 0.01%) before staining with mAb35.

Expression of IFN-γ receptor was analyzed by using a mouse monoclonal anti-IFN-γ receptor Ab (R&D Systems) at a dilution 1/10 for 30 min at 4°C. After three washes in PBS, the cells were incubated with a secondary goat anti-mouse Ab coupled to FITC (DakoCytomation) for 30 min, then washed and permeabilized using IntraPrep Permeabilization reagents (Immunotech).

Flow cytometry experiments were performed on a FACSCalibur apparatus (BD Immunocytometry Systems), and CellQuest software was used for the analysis.

Sequencing of the α-AChR promoter and analysis of transcriptional elements

The hAChRα3 clone containing a 16.4-kbp genomic sequence fragment of human AChR subunit gene was digested by EcoRI and each restriction fragment was subcloned into the pBR322 plasmid (32). One 2493-bp fragment corresponding to the promoter region of the human AChR α-subunit gene was sequenced on both strands (GenBank accession number AF557345). Using Signal Scan software (33), we scanned the AChR α-subunit promoter sequence for eukaryotic cis-acting regulatory DNA elements and trans-acting factors.

Total RNA preparation

Total RNA extraction from adherent cells (TECs and MITCs line) was performed using the TRIZol kit protocol according to the manufacturer’s instructions (Invitrogen Life Technologies). Total RNA was purified by adding 0.5 vol of ammonium acetate (7.5 M) and 2.5 vol of absolute ethanol. After 1 h precipitation, total RNA was pelleted by centrifugation (15 min, 12,000 × g, 4°C), washed in 75% ethanol, dried under vacuum and stored at −80°C after dissolution in distilled water. The total RNA concentration was determined by measuring absorbance at 260 nm on a Gene Quant II spectrophotometer (Amerham Pharmacia Biotech). The purity of the RNA preparation was checked by measuring the 260/280 nm absorbance ratio. The concentration and the quality of RNA were also checked by a quantitative PCR using primers from the 28S RNA sequence. Samples exhibiting poor amplification of 28S cDNA were excluded. For microarray experiments, whole thymic samples were used. After total RNA extraction by the TRIZol extraction protocol, the RNA were purified using Qiagen columns and RNA quality was checked on the Agilent bioanalyzer.

Microarrays

Four pools of thymic RNA were prepared from: 1) MG patients with severe thymic hyperplasia (n = 5); 2) MG patients with mild thymic hyperplasia (n = 5); 3) adult normal controls (n = 5); and 4) 1-wk- to 1-year-old controls (n = 10). The three first groups were compared with the fourth one corresponding to our control reference. The first three groups were age matched (16–25 years old). Because MG hyperplasia is highly associated with females, all the samples were from females. Severe hyperplasia was defined as thymuses containing many germinal centers (more than three per section) while the mild hyperplasia was defined by a low number of germinal centers (less than two per section). The thymuses were provided from patients undergoing thoracic surgery with no immunological defects (groups 3 and 4), or from MG patients undergoing therapeutic thymectomy (groups 1 and 2). For microarray analysis, 20 µg of total RNA was labeled with cyanine 5 or cyanine 3 using the direct labeling protocol of Agilent optimized for their cDNA chips. For each array, the RNA control pool (group 3 and 4) was crossed with RNA from the other pools (group 1, 2, or 3) and these comparisons were repeated five times. Labeled cDNA were finally hybridized overnight onto the human 1 cDNA arrays from Agilent (G4100A; 12,814 unique clones) and scanned using the 428 Affimetrix scanner (MWG). The images were analyzed with GenePix pro V4.0 (Axon Instruments). Raw data were then corrected by a nonlinear transformation.
(Lowess algorithm) using the TIGR Microarray Data Analysis System (www.tigr.org).

**RT-PCR**

A reverse transcription reaction mixture (total volume, 50 µl) containing 1 or 2 µg of total RNA from TECs and MITCs, 10 µl of 10× reverse transcriptase buffer (Eurobio), 1.5 mM dNTPs, 10 U of RNase OUT (Invitrogen Life Technologies), 50 pmol of 3′ primer, and 4 U of avian myeloblastosis virus reverse transcriptase (Eurobio) was incubated at 42°C for 60 min and then quickly chilled on ice. Standard PCR was conducted in a total volume of 100 µl containing 1 µl of reverse transcriptase reaction mixture, 10 µl of PCR buffer (670 mM Tris-HCl pH 8.8; 160 mM (NH₄)₂SO₄; 0.1% Tween 20), 0.5 µM each primer, 200 µM each dNTP and 2.5 U of EUROBIOTAQ II DNA polymerase (Eurobio). The sequences of the primers used for IFN-γ receptor were 5′-ATTGTGCTG TATGCCGAGATG and 5′-CAGATGATACACGCTAAG for the forward and reverse primers, respectively.

The mixture was overlaid with mineral oil and then amplified using a PHC3 thermal cycler (Techne) for 30 cycles as follows: denaturing step, 94°C for 1 min; annealing step at the primer hybridization temperature for 1 min; extension step, 72°C for 2 min. The final elongation step lasted 10 min at 72°C. PCR products were analyzed on 1.5% agarose gel containing ethidium bromide.

Quantitative RT-PCR were performed on a LightCycler apparatus (Roche Diagnostics), using primers designed with Oligo software (Med Probe). The primers were purchased from Eurobio and the human sequences were previously described (34). For the mouse β-AChR, the sequences of the primer set used were: 5′-CAAGGCACCATGCTCAGCTC and 5′-TCAGAGCAGCTACGAGGTCAT. PCR were performed using the Faststart DNA Master SYBR Green I kit (Roche Diagnostics). The LightCycler mastermix (13.5 µl) was placed in the LightCycler glass capillaries and 1.5 µl of cDNA was added as PCR template. Capillaries were closed, centrifuged, and placed in the LightCycler rotor. The following LightCycler experimental run protocol was used: denaturation program (95°C for 10 min), amplification and quantification program and melting curve program (60–95°C with a heating rate of 0.1°C per second and 95°C for 10 min), amplification and quantification program and melting curve program and finally cooling to 40°C. 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 that there was a single peak in melting curve analysis and by checking the size of the fragments by electrophoresis in an agarose gel during the set up of the experiments. Most results are expressed as fold change.

**Effect of TNF-α on AChR expression by rat muscle**

Ten Lewis rats were injected i.p. with a solution containing the anti-AChR mAb 35 at a dose of 12.5 pmol per 100 g of body weight and PBS-BSA 0.5% added to 1 ml. Five of the treated rats received a supplementary s.c. injection of 150 µg/kg of TNF-α, solubilized in PBS to a total volume of 1 ml. An additional five control rats received an injection of 1 ml of PBS. After 24 h, animals were sacrificed and extensor digitorum longus muscles were removed and AChR was quantified by RIA as previously described (35).

**Statistical analysis**

Results are expressed as mean ± SEM. The nonparametric Mann-Whitney U test was used for unpaired data and the paired t test was used for paired data.

**Results**

**Evidence of proinflammatory environment in MG thymic hyperplasia**

To further our understanding on the role of the thymus in the etiology of MG, we first used a gene array to analyze the pattern of gene expression associated with inflammation in hyperplastic thymuses of patients diagnosed with MG. We analyzed separately the expression of IFN-induced genes (excluding MHC genes), MHC class II genes, and MHC class I genes. A significant increase of the level of expression of IFN-induced genes was associated with severe MG thymic hyperplasia (p < 0.007 and p < 0.0001 for IFN-α and IFN-γ, respectively) (Fig. 1A), while expression of these genes was not modified in patients exhibiting mild MG thymic hyperplasia (data not shown). The data also show a significant increase of the expression of MHC class II genes (p < 0.0001, paired t test). Indeed all the genes of this category spotted in the array were increased in MG severe thymic hyperplasia (11 of 11) while four of the five MHC class I genes were found increased, but the difference with controls was not significant probably because of the low number of genes (Fig. 1B). An intermediate increase in class II gene expression was also observed in thymuses from patients with a mild hyperplasia (data not shown). These observations support the inflammatory state of MG thymic hyperplasia.

We then asked whether this inflammatory environment could influence AChR expression on thymic cells. To this end, we analyzed the effects of proinflammatory cytokines such as IFN-γ and TNF-α on AChR expression. To confirm such a possible role of IFN-γ, we first determined whether myoid and epithelial cells express the IFN-γ receptor, and searched for IFN and TNF responsive elements on the α-AChR promoter.
Evidence of IFN-γ receptor transcripts on MITC and TEC cells and identification of IFN responsive elements on the human α-promoter

We analyzed the expression of IFN-γ receptor on MITCs and TECs by RT-PCR and we observed a band corresponding to the expected size of cDNA. Messengers encoding IFN-γ receptor were clearly observed in RNA extracted from MITCs and TECs (Fig. 2A). Protein expression analysis by flow cytometry evidenced IFN-γ receptor expression at the cell surface on both MITCs and TECs (Fig. 2B).

To analyze whether IFN-γ may be able to directly modulate AChR gene transcription, we sequenced a fragment of the human AChR-α sequence upstream from the initiation site of transcription of the clone AChRα31 to identify potential IFN-responsive elements. One 2493-bp fragment corresponding to promoter region of human AChR-α gene was sequenced on both strands, and the sequence is now available on GenBank (accession number AY557345).

Using Signal Scan software (33), we scanned promoter AChR-α sequence for eukaryotic cis-acting regulatory DNA elements and trans-acting factors. We identified 14 putative IFN-γ-responsive element motifs (CWKKANNY) known to bind IFN-γ-induced transcription factors and to trigger transactivation of several genes, including class II MHC genes (36). We also identified six putative “W-box” elements (WGNAMCYK). One of them is separated by three nucleotides from the γ-interferon response element (γ-IRE) element at position 1397–1415 whereas a second W-box overlaps the γ-IRE element by three nucleotides at position 1467–1479. These two motif arrangements have been previously reported in the promoter region of several class II MHC genes, including the α chain of HLA-DP gene (36). Thus, the AChR-α promoter could be activated by IFN-γ (Fig. 3).

Binding of TNF-α to its receptor triggers activation of several trans-acting factors such as AP1 and NF-κB via the Ras/ERK pathway as shown for the metalloproteinase-9 gene (37) or both NF-κB and NF-IL6 (C/EBP/β) as demonstrated for the cyclooxygenase gene (38). Study of the AChR-α promoter region allowed us to identify four putative AP1 motifs, known to bind the Fos/Jun complex, at positions 183, 258, 1368, and 1405; two NF-κB binding motifs located at position 951 and 1897; and nine NF-IL6 motifs at positions 26, 41, 157, 192, 354, 696, 1431, 1851, and 1935, suggesting that TNF-α may control AChR-α subunit transactivation (Fig. 3).

Because the required regulatory elements are present in the upstream region of the AChR-α gene, IFN-γ and TNF-α could potentially mediate the direct regulation of AChR expression in thymic epithelial and myoid cells.

Regulation of AChR expression by cytokines in MITC

To determine the ability of proinflammatory cytokines to regulate AChR expression, we analyzed AChR expression at the cell membrane of the myoid cell line (MITC) using mAb35, directed against the α-AChR subunit. After a 3-day incubation of MITC with 1 TCACTTCGCA ATTTGAGAAG GGGGCGGGA GAGAGGGCC CTGCTGAGA 5' 51 CTGCGCAGTA GATGCGGCA CTGGCTCCAG GTTGCTGGC TGGAGAAG 101 AGAACGACCA AGAGCGTCA TCTTGTGATA CAATGGAGA TATAAGGAG 151 GACAGAGCT CGAGCTGAA CTGGATTGCA TTCTCTGTGAA TTTGTTACA 201 ATTGCTTCC CACCTTCGGA ATGCTGCTA GACACATGC CTGCCATT 251 TTTGTGTAAG TATGGCCTTA GGGCTATGAA CAGCTATGAA CACCTATGAA 301 ACATGCTAGA CGAGCTGAA CAGTTACGTA CAGGCTGAA AAGATGAGA 351 CTGGAGATGA AAGAGAGAGT TTGACTGCT TGTCTGCTAG AAGAGATGA 401 CTGGTTTTCG AAGAGGCGA CAGGGCGTGG AGTGGCTGGA CGTGGTCTAG 451 CAATCGCTAG TGTGAATAC AAGGAGGTGA CAGAGTGAGT CACGCTGAA 501 GAGGATGAGA TTGAGGACTG AAGAGGAGG TTTGTTAGG TCACTGAGA 551 GCTTGAGACG GAGGCTTCGT TGAGCTGCTA GACAGATGGA ACAAGGGAGA 601 AGGGCACTG CAGCAATGGA AGAGGTTGGA CAGGGCGTGG AGTGGCTGGA 651 ACATGCCAGA TTGAGGACTG AAGAGGAGG TTTGTTAGG TCACTGAGA 701 TACTCCACGA CAGTCTGAGA AGAGGTTGGA CAGGGCGTGG AGTGGCTGGA 751 ACATGCCAGA TTGAGGACTG AAGAGGAGG TTTGTTAGG TCACTGAGA 801 AACCACTGCC TTAGTTCAAA TTAAGGGG GAAGAGGAGG TTTGTTAGG TCACTGAGA 851 TACTCCACGA CAGTCTGAGA AGAGGTTGGA CAGGGCGTGG AGTGGCTGGA 901 ACATGCCAGA TTGAGGACTG AAGAGGAGG TTTGTTAGG TCACTGAGA 951 TGAGTACAGA CGAGCTGCTA GACAGATGGA ACAAGGGAGA 1001 AGGGCACTG CAGCAATGGA AGAGGTTGGA CAGGGCGTGG AGTGGCTGGA 1051 ACATGCCAGA TTGAGGACTG AAGAGGAGG TTTGTTAGG TCACTGAGA 1101 TACTCCACGA CAGTCTGAGA AGAGGTTGGA CAGGGCGTGG AGTGGCTGGA 1151 ACATGCCAGA TTGAGGACTG AAGAGGAGG TTTGTTAGG TCACTGAGA 1201 TACTCCACGA CAGTCTGAGA AGAGGTTGGA CAGGGCGTGG AGTGGCTGGA 1251 AACCACTGCC TTAGTTCAAA TTAAGGGG GAAGAGGAGG TTTGTTAGG TCACTGAGA 1301 GAGGATGAGA TTGAGGACTG AAGAGGAGG TTTGTTAGG TCACTGAGA 1351 TTTTACCTCA CTGAGTCTGA AGAGGAGG TTTGTTAGG TCACTGAGA 1401 AGGGCACTG CAGCAATGGA AGAGGTTGGA CAGGGCGTGG AGTGGCTGGA 1451 GAGGATGAGA TTGAGGACTG AAGAGGAGG TTTGTTAGG TCACTGAGA 1501 TTTTACCTCA CTGAGTCTGA AGAGGAGG TTTGTTAGG TCACTGAGA 1551 AACCACTGCC TTAGTTCAAA TTAAGGGG GAAGAGGAGG TTTGTTAGG TCACTGAGA 1601 AGGGCACTG CAGCAATGGA AGAGGTTGGA CAGGGCGTGG AGTGGCTGGA 1651 ACATGCCAGA TTGAGGACTG AAGAGGAGG TTTGTTAGG TCACTGAGA 1701 TACTCCACGA CAGTCTGAGA AGAGGTTGGA CAGGGCGTGG AGTGGCTGGA 1751 ACATGCCAGA TTGAGGACTG AAGAGGAGG TTTGTTAGG TCACTGAGA 1801 TACTCCACGA CAGTCTGAGA AGAGGTTGGA CAGGGCGTGG AGTGGCTGGA 1851 GACTCTCCGA TGTGAATAC AAGGAGGTGA CAGAGTGAGT CACGCTGAA 1901 GCACTGTGCA CGGCGAGCTA CTGGCTGCTA TTTGTTAGG TCACTGAGA 1951 CAGGCTGCA CGGCGAGCTA CTGGCTGCTA TTTGTTAGG TCACTGAGA 2001 AGGGCACTG CAGCAATGGA AGAGGTTGGA CAGGGCGTGG AGTGGCTGGA 2051 ACATGCCAGA TTGAGGACTG AAGAGGAGG TTTGTTAGG TCACTGAGA 2101 TCTGGTTCGC AGAGGTTGGA CAGGGCGTGG AGTGGCTGGA 2151 ACAATCTTGTC AATGGTTTCA AAGGAGGTTGGA CAGGGCGTGG AGTGGCTGGA 2201 TCTGGTTCGC AGAGGTTGGA CAGGGCGTGG AGTGGCTGGA 2251 CATTGTGTGA AGGGGTTGGA CAGGGCGTGG AGTGGCTGGA 2301 CTGGCTGCA CGGCGAGCTA CTGGCTGCTA TTTGTTAGG TCACTGAGA 2351 GCCCTACGCA AGAGGTTGGA CAGGGCGTGG AGTGGCTGGA 2401 TCCTGGACCG AGAGGTTGGA CAGGGCGTGG AGTGGCTGGA 2451 TCTGGTTCGC AGAGGTTGGA CAGGGCGTGG AGTGGCTGGA

FIGURE 2. Expression of IFN-γ receptor in MITC and TEC cultures. A, mRNA expression was assessed by RT-PCR. In both types of cells, one unique band was observed at the expected size. B, IFN-γ receptor was analyzed by flow cytometry in MITCs and TECs. The left histogram represents cells incubated with the secondary Ab.

FIGURE 3. Nucleotide sequence of AChR-α subunit 5′ flanking region. The putative sites for transcription factors are underlined: γ-IRE (bold line), W box (single line), AP1 (double line), NF-κB (small dotted line), and NF-IL6 (large dotted line).
IFN-γ, we observed a 70% increase in AChR expression at the cell surface (Fig. 4A). IFN-γ did not induce death of myoid cells (data not shown), suggesting that the increase in AChR expression after IFN-γ treatment was due to an up-regulation of AChR expression at the cell level and not to a selection of a minor subset of cells expressing high level of AChR. AChR expression was also analyzed using 125I-α-Bgt that binds mainly to the complete AChR. The analysis was performed after treatment with IFN-γ during 3–72 h. Similar to the flow cytometry results, we observed a significant increase of ~70% in AChR expression at the cell surface after a 48- or 72-h incubation (Fig. 4B). Thus, IFN-γ is a powerful regulator of AChR expression on thymic myoid cells.

**Up-regulation of AChR expression triggered by cytokines in TECs**

AChR transcripts are detectable in TECs, although at a much lower level than in MITCs (34). We thus tested the effects of cytokines on the transcript levels of AChR subunits in these cells. TECs were incubated for 24 or 48 h with IFN-γ and/or TNF-α. Ratios were then calculated by comparison with the transcript level observed in untreated cells. After 24 h of incubation, AChR-α transcript level was strikingly increased by IFN-γ (ratio 13.7 ± 3.7) and even more by the mixture of IFN-γ and TNF-α (20.1 ± 5.6), while TNF-α alone had no effect (Fig. 5A). It should be underlined that in all the experiments (five of five), AChR-α transcript level was higher when cultured with the IFN-γ/TNF mixture than with IFN-γ alone. When IFN-γ was added before TNF-α, the increased ratio was the same (15.3 ± 4.2), whereas when TNF-α was added first, the up-regulation of AChR-α subunit was less marked (8.8 ± 3.9). These results suggest that TNF-α needs a prior activation by IFN-γ. One can hypothesize that, as was previously demonstrated (39), IFN-γ induces the increased expression of the TNF-α receptor at the cell surface leading to an enhanced response to TNF-α and an additional effect of this cytokine compared with IFN-γ alone. After 48 h of incubation, the level of up-regulation of AChR was the same whether IFN-γ was added before, at the same time, or after TNF-α (Fig. 5B). These results show that the regulation by IFN-γ and TNF-α mixture is sustained.

We also investigated the effect of these cytokines on the mRNA expression of the other AChR subunits because TECs express mRNA for all AChR subunits (34). As IFN-γ and TNF-α mixture was the most efficient for the up-regulation of AChR transcripts, we analyzed all the subunits using these conditions. We observed that the cytokine mixture induced a significant increase of all the AChR subunits (Fig. 6), although the effect was much less marked than for the α subunit (Fig. 5).

We then investigated whether this up-regulation of AChR subunit mRNA was followed by an increase in the AChR protein expression. AChR was undetectable at the cell surface of TECs, either by toxin binding or by Ab binding, even after treatment with the cytokine mixture (data not shown). We then analyzed the intracytoplasmic AChR expression in fixed cells by flow cytometry. We observed a moderate but significant increase in cytoplasmic AChR subunit expression of ~1.7 ± 0.2-fold by IFN-γ/TNF-α mixture after 24 h of incubation (p < 0.04) (Fig. 7). Thus, the mixture of IFN-γ and TNF-α increases significantly the expression of AChR transcripts in TECs, while the protein was hardly increased in the cell cytoplasm.

**FIGURE 4.** IFN-γ induces increased AChR expression in MITCs. A, MITC cultures were untreated or incubated with IFN-γ for 3 days. AChR surface expression was analyzed by flow cytometry using mAb directed against α-AChR subunit. The left histogram represents cells incubated with the secondary Ab. B, AChR expression was analyzed using 125I-α-Bgt binding in MITC cultures incubated with IFN-γ. Results are presented as percentage of toxin binding compared with untreated cells.

**FIGURE 5.** Effect of cytokines on α-AChR mRNA level in TEC cultures. α-AChR mRNA levels were analyzed in epithelial cell RNA extracts by real-time RT-PCR. TEC cultures were untreated or incubated with IFN-γ, TNF-α, or with the two cytokines consecutively or simultaneously for 24 (A) and 48 h (B). Results are presented as the ratio of α-AChR mRNA level compared with untreated cells. The results are expressed as the mean ± SEM of five different experiments.
In vivo regulation of nicotinic receptor AChR expression by IFN-γ

Because IFN-γ was able to regulate the AChR expression in thymic cells, we wondered whether AChR expression would be modified in IFN-γ KO mice. We expected to observe a reduction of AChR expression in the KO mice; therefore, we decided to analyze first the expression of the β-AChR which is much more expressed in the thymus than the other subunits (34). We investigated its expression by real-time RT-PCR in thymus and hind limb muscles of nine controls WT and nine IFN-γ KO mice (25). We observed a significant decrease of the AChR-β level in the thymus of KO mice relative to the WT animals (33.3 ± 2.7 arbitrary units in control mice vs 17.1 ± 1.7 in KO mice, p < 0.0003) (Fig. 8A). However, there was no significant modification of the AChR-β subunit transcripts level in the hind muscle of these KO mice (mean 20.5 ± 2.8 arbitrary units) compared with the control (17.4 ± 3.3) (Fig. 8B). A similar analysis of the α-subunit showed no modification in the muscle, while in the thymus the results were not interpretable, probably because of the very low expression of this subunit in the thymus (data not shown). Thus, IFN-γ appears to influence the expression of AChR in the mouse thymus, but not in muscle.

In vivo modulation of Ab-induced AChR loss by TNF-α

To examine whether TNF-α could influence AChR expression in the muscle, we used the EAMG model induced by monoclonal anti-AChR Ab (mAb 35). Injection of this mAb induces clinical symptoms of MG and a significant loss of AChR expression in the muscles. The simultaneous injection of TNF-α and mAb35 led to a less severe AChR loss in the extensor digitorus longus rat muscles compared with rats injected with mAb 35 alone. As shown in Fig. 9, AChR expression in mice injected with mAb35 alone was 45% of controls, while in mice coinjected with mAb35 and TNF-α, it reached 70% (p < 0.0002), indicating that TNF-α influences the expression of muscle AChR at least in animals presenting myasthenic symptoms.

Discussion

The aim of this study was to examine the expression of proinflammatory cytokines in the thymus of MG patients and to analyze their influence on AChR expression. The main results of this study are as follows: 1) a large number of IFN-α- and IFN-γ-regulated genes are highly expressed in the thymus of MG patients; 2) the α-AChR promoter contains several IFN and TNF responsive elements; 3) IFN-γ triggers a large increase in AChR membrane expression in thymic myoid cells; 4) in TEC, IFN-γ and TNF-α strikingly increase the expression of AChR α-subunit mRNA, and moderately increase expression of the other subunits; these agents also induce significant up-regulation of the cytoplasmic AChR protein expression; 5) KO mice lacking IFN-γ gene expression exhibit a 2-fold decrease of AChR-β transcript expression in the thymus; and 6) TNF-α protects muscle from AChR loss induced by anti-AChR mAb.

Significance of IFN-inducible genes in thymic hyperplasia

In this study we have shown that a large number of non-MHC and MHC genes up-regulated by IFN-γ are highly expressed in hyperplastic MG thymuses. Although these results suggest the increased expression of IFN-γ in the thymus of these patients, such an increase has not been reported. Analysis of expression of IFN did not show any significant increase in peripheral mononuclear cells (40, 41) or in the thymus (42) from MG patients. These data are similar to that observed in peripheral blood cells of patients with severe lupus, where patients showed deregulated expression of genes in the IFN pathway (43, 44) while IFN protein expression was not increased in the sera, nor was IFN mRNA elevated in mononuclear cells. To explain the apparent contradiction between the absence of increased expression of these cytokines in the patients and signs of IFN activity, it is possible that this IFN signature reflects another cytokine or stimulus that engages IFN-signaling pathways. For example, IL-18 has been identified as an IFN-γ inducing factor and IL-18 levels were significantly higher in the sera of MG patients, in correlation to the disease severity (45). Another hypothesis is that the overproduction of these cytokines could be transient. They may only increase at the onset of the disease. Indeed it is known that natural IFNs have a half-life of only few hours (46). In our study, the thymus is collected at least several months after MG initiation and then the levels of these cytokines may be decreased to normal values. A possible increase in IFN levels at the onset of the disease may be sufficient to induce activation of many other genes whose expression remains elevated over a longer time frame.

It should be noted that in our microarray analyses, we observed a significant increase of MHC class II Ags, but not of class I Ags. This increase could be due to a higher number of cells expressing HLA class II Ags in the pathological tissues. Indeed, the microarray analysis was performed on whole thymic extracts, and it is clear that thymuses from MG patients contain more B cells and follicular dendritic cells than normal thymuses (10), and because B and follicular dendritic cells express high levels of MHC class II, they may count in the increased expression of the thymic class II
Ags in the whole thymic extracts. However, we cannot exclude that the significant increase of MHC class II Ags in the MG thymuses could also be due to the overexpression of the transcription factor CIITA that plays a central role in the control of MHC class II transcription (47). Indeed, CIITA was found to be slightly increased in MG thymic hyperplasia in the microarray experiments (data not shown). Therefore, its overexpression could influence the expression of class II Ags in all cells expressing them, including TECs and activated thymocytes.

**Dual effects of cytokines in the thymus and the muscle**

Our in vivo results indicate that the regulation of AChR is not the same in the thymus as in the muscle. Indeed, the expression of AChR-β was decreased in the thymus but not in the muscle of IFN-γ KO mice, indicating that the absence of IFN-γ influences the expression of AChR in the thymus but not in muscle tissue. However, we showed that TNF could directly influence AChR expression in an EAMG model induced by mAb to AChR while this cytokine alone has no effect on AChR expression on TECs or myoid cells. This apparent contradiction could be explained by the role of tissue-specific factors contributing to the AChR transcription. Indeed a combination of myogenic and non myogenic factors have been shown to be involved in AChR expression in the muscle (48) and it is possible that these factors are not expressed at the same extent in the thymus. It has been shown that herergulins regulate AChR expression in muscle cells (49) by the Ras/MAP kinase/Erk2 pathway, which is also involved in the activation of metalloproteinase-9 via TNF, indicating that TNF and herergulins could activate similar pathways. It was shown that TNF-α receptors are weakly expressed in normal muscles, while they are increased in pathological situations, such as polymyositis (50) and this increase was associated with muscle regeneration. Therefore, it is possible that the regulatory effect of TNF on AChR is associated with an increase of TNF-α receptors on muscle fibers, because of the pathological effects of the Abs to AChR. Finally, it is important to underline that the consequences of the inflammatory activity are different in the thymus and the muscle. Although in both organs, they lead to up-regulation of AChR expression, this increase is detrimental in the thymus because it could contribute to the autoimmune response while the increase of AChR in the muscle could represent a beneficial compensatory mechanism as shown in the MG patients (51).

**Intrathymic pathogenesis mechanisms in MG**

The primary events leading to the autoimmune AChR attack are not known. However, several signs of activation observed in the MG thymus have been reported. Thymic B cells from MG thymic hyperplasia are activated as demonstrated by expression of activation markers and by the ability of B cells to spontaneously produce Ab to AChR (16). Thymic T cells proliferate spontaneously to rIL-2 (14) and express high levels of Fas (17). In the present study, we also showed that the thymic environment of MG hyperplasia is activated as evidenced by increased expression of IFN-regulated genes, including both MHC and non-MHC genes. In parallel, we showed that two proinflammatory cytokines (IFN-γ and TNF-α) up-regulate AChR expression at the cell surface of myoid cells and at the cytoplasmic level in epithelial cells. Therefore the proinflammatory environment could simultaneously promote increased expression of AChR and of molecules required for antigenic presentation, leading to the enhanced presentation of AChR, and in turn to pathogenic Abs inducing myasthenic symptoms. But the mechanism by which this inflammatory environment is induced in the thymus remains enigmatic. The inflammatory response could be induced by a bacterial or a viral triggering agent. It has been shown that an inflammatory reaction to an unrelated Ag within the medulla of the thymus facilitates entry of peripheral AChR-reactive CD4+ T cells (24). Indeed, intrathymic injection of a thymotrophic leukemia virus in animals previously immunized to a protein transcribed by the viral vector showed modification of the cortical/medullary architecture and augmented entry of peripheral T cells into the thymus.

However, the consequences of an inflammatory event depends upon genetic and environmental factors. For example, the immunization of rats with AChR in combination with IFN-γ induces a mild and transient disease in WF rats, and a severe form of the disease in Lewis rats (27). The susceptibility depends upon several genes, including genes encoding several proinflammatory cytokines that are known to be polymorphic. The polymorphism of the TNF and IFN genes has already been documented (52, 53). In the case of TNF-α, the high producer allele has been associated with
MG thymic hyperplasia (29). Therefore a triggering event could probably induce higher levels of proinflammatory cytokines in susceptible patients. In support of this hypothesis, studies have shown the development of MG during treatment of chronic hepatitis C with IFN-α (54–56). Then if the concentration of proinflammatory cytokines is above a certain threshold they would induce AChR expression and presentation. Indeed, several studies have shown increased expression of AChR in MG patients. In one study, Zheng et al. (23) observed increased expression (of ~3-fold) of the two AChR subunit isoforms in MG patient thymus compared with controls, and in another study, Navaneetham et al. (22) observed increased mRNA expression of the ε-subunit. The increased presentation of AChR could occur at least by two possible mechanisms: 1) cell death of myoid cells, leading to the accessibility of AChR peptides and to their presentation by professional APCs such as the dendritic cells located in the same thymic compartment; 2) increased intracytoplasmic expression of AChR in TECs together with increased expression of MHC class II molecules, making these cells efficient APCs. Although this pathway is not classical, several reports indicate the ability of MHC class II molecules to present peptides derived from cytosolic or endogenously made Ags (57, 58).

In conclusion, this study extends previous work showing that IFN-γ up-regulates α-AChR expression in TECs (23) and demonstrates that proinflammatory cytokines influence the expression of AChR in vitro and in vivo. Because signs of proinflammation are evidenced in the thyymus of MG patients, our results support the previously reported hypothesis (24) of a pivotal role of the proinflammatory cytokines in the induction of the human MG inducing an increased thymic expression of the main autoantigen, AChR, and its presentation to restating autoreactive T cells.

Disclosures

The authors have no financial conflict of interest.

References


