TNFA and TNFB Polymorphisms in Myasthenia Gravis

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Background: Tumor necrosis factor (TNF) α and TNF-β are proinflammatory cytokines thought to be involved in the pathogenesis of myasthenia gravis (MG).

Objective: To examine whether TNF polymorphisms are associated with MG, MG subgroups, and the presence of titin and ryanodine-receptor antibodies.

Patients and Methods: We did genotyping on 30 patients with MG and 92 healthy blood donors for 2 biallelic TNFA polymorphisms (G to A at positions −238 and −308) and 1 TNFB polymorphism (Nco1 digestive site) using methods based on the polymerase chain reaction.

Results: Patients with thymoma were typically homozygous for both the TNFA*T1 and TNFB*2 alleles, but patients having an early onset of MG without thymoma were carriers of the TNFA*T2 and TNFB*1 alleles. Patients without thymoma who had the titin antibody had the same high frequency of TNFA*T1 and TNFB*2 as patients with thymoma, whereas patients without the titin antibody carried the same allele, TNFA*T2 and TNFB*1, regardless of age and thymic disease. No association was found with acetylcholine-receptor levels or disease severity for any of the TNFA or TNFB polymorphisms.

Conclusion: Patients having MG, including those with thymoma, who have the titin antibody are most often homozygous for the TNFA*T1 and TNFB*2 alleles, whereas the presence of the TNFA*T2 and TNFB*1 alleles correlates with early-onset MG and the absence of titin antibodies.

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PATIENTS AND METHODS

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The study included 30 patients with MG and 92 healthy blood donors, all white Norwegians. The diagnosis of MG was based on typical clinical features, the presence of AChR antibodies in all patients, a positive result on the edrophonium chloride test, and the findings of neurophysiological examinations (decrement >10% at 3 Hz after repetitive motor nerve stimulation and increased jitter on single-fiber electromyography). Seven patients had a thymoma (lymphoepitheloma). 13 had late-onset nonthymoma MG (onset of MG symptoms at 40 years or older), and 10 had early-onset MG (onset at younger than 40 years). All patients with MG who had a thymoma had had a thymectomy. The 5 patients with early-onset MG who had had a thymectomy had thymic hyperplasia, and the 7 patients with late-onset MG who had had a thymectomy had thymic atrophy. A thymoma was excluded by computed tomographic scan of the mediastinum in the 11 patients who had not had a thymectomy. Seventeen patients used immunosuppressive treatment—prednisone in 10 patients and prednisone plus azathioprine in 7 patients. Four patients had an additional autoimmune disease: Sjogren disease (n = 1), diabetes mellitus (n = 1), rheumatoid arthritis (n = 1), and systemic lupus erythematosus (n = 1).

The patients were classified according to the severity of MG at the peak of illness (worst symptom excluding death). They were grouped into the following 6 categories modified by Oosterhuis: 0 indicates complete remission without medication; 1, minor symptoms (purely ocular or minor generalized symptoms); 2, mild generalized symptoms (mildly disabled with obvious weakness at appropriate testing but without substantial bulbar symptoms); 3, moderate generalized symptoms (restricted in daily activities and with substantial bulbar dysfunction); 4, severe generalized symptoms (requiring hospital admission because of ventilatory failure or dysphagia); and 5, MG death (death occurred after the deterioration of MG, with respiratory distress and final respiratory failure or cardiac arrest).

ACHR, TITIN, AND RYANODINE-RECEPTOR ANTIBODY DETERMINATIONS

Antibodies to AChR were determined in all patient serum samples by immunoprecipitation of human AChR labeled with iodine 125-ß-bungarotoxin. Serum samples were tested for the presence of titin antibodies by enzyme-linked immunosorbent assay using the epitope myasthenia gravis titin 30-kd (MGT-30) antigen, as previously described, and for ryanodine-receptor (RyR) antibodies in Western blot using crude sarcoplasmic reticulum as antigen. This preparation has the same sensitivity and specificity for detecting RyR antibodies in Western blot as fully purified RyR.

DNA PREPARATION

Genomic DNA from each person tested was extracted from whole blood with a blood kit (QIAamp; Qiagen GmbH, Hilden, Germany) as described by the manufacturer.

TNFA GENOTYPING

Two polymorphic loci in the promoter region of the TNFA gene were studied. Both polymorphisms involve a G- to-A transition, one at position −238 and the other at position −308. The TNFA region incorporating these 2 sites was amplified by polymerase chain reaction (PCR) using a PCR kit (GeneAmp; Perkin Elmer, Norwalk, Conn) and the following primers: 5′-AGGCAATAGGT'TTGAGGCCCAT-3′ and 5′-ACACTCCCCATCCTCCCCGGCT-3′. The PCR was performed using a gene amplifier system (model 9600, Perkin Elmer), programmed for 35 cycles of incubation at 95°C for 15 seconds and at 60°C for 30 seconds.

The product of 117 base pairs (bp) was digested with NcoI and fragments resolved by electrophoresis on a 10% polyacrylamide gel. In the presence of the A (T2) allele, the 117-bp fragment is digested to give 2 fragments, 70 bp and 47 bp. The restriction enzyme Ncol does not cleave this region in the presence of the G (T1) allele. Heterozygous persons (T1 and T2) were detected by the presence of all 3 fragments. The biallelic polymorphism at position −238 was detected by NlaIV restriction enzyme digestion of the amplified 117-bp DNA. The allele TNF* (A-238) yields 2 fragments of 70 bp and 47 bp, whereas the allele TNF*G (G-238) shows 3 fragments of 50 bp, 47 bp, and 20 bp. Heterozygous persons showed all 4 fragments.

TNFB GENOTYPING

The TNFB genotyping was done using the PCR-restriction fragment length polymorphism technique. The 368-bp sequence in the first intron was amplified by PCR using the following primer pairs: TNF 502 and TNF 302, 5′-CTCCCTGCACTGCGCTGGACATC-3′ and 5′-GAAGAGACGTTCAGGTGGTGTCAT-3′, respectively. Amplification was undertaken by 35 cycles of incubation at 95°C for 15 seconds and at 65°C for 30 seconds. Fragments obtained after the digestion of PCR products with Ncol were detected by electrophoresis in 6% polyacrylamide gels. The TNFB*1 yields 2 fragments of 235 bp and 133 bp, whereas the TNFB*2 is uncleaved by Ncol and shows a single 368-bp fragment.

STATISTICAL ANALYSIS

The χ² test with the Yates correction, the Fisher exact test, and the Student t test were applied to compare groups statistically. Differences were considered significant at P<.05.

RESULTS

TNFA POLYMORPHISMS

The distribution of the TNFA A/G polymorphism at position −308 (T1 and T2) is shown in Table 1. Although the gene frequencies are similar when comparing all patients with MG with controls, analysis of the MG subgroups shows that the TNFA* T2 allele is found more often in patients who have early-onset MG (7 of 10) compared with all other patients with MG (11 of 30) (χ²=5.2, P=.03; Fisher exact test, P=.01) and, in par-
than controls (32 of 90) (\(x^2 = 5.8, P = .02\); Fisher exact test, \(P = .01\)). All patients with MG who have a thymoma are suboptimal.

No significant difference was found between patients (including subgroups) and controls for the TNFA A/G polymorphisms at position −238 (A/G) (Table 1).

**TNFB POLYMORPHISMS**

The TNFB alleles show a similar distribution in the total group having MG and controls (Table 2). Patients having early-onset MG, however, were more often (4 of 10) homozygous for the TNFB*1 allele than other patients with MG (0 of 20) (\(x^2 = 6.1, P = .02\); Fisher exact test, \(P = .01\)), whereas patients with MG who do not have the titin antibody were more often (9 of 15 vs 2 of 15, respectively) carriers of the TNFA*T2 allele (\(x^2 = 5.2, P = .03\); Fisher exact test, \(P = .01\)) (Table 3). Titin antibodies were found in serum samples from 13 of 19 patients homozygous for the TNFA*T1 allele compared with only 2 of 10 heterozygous patients (\(x^2 = 4.2, P = .05\); Fisher exact test, \(P = .10\)). The 1 patient homozygous for TNFA*T2 had no detectable titin antibodies. The 3 patients who had RyR antibodies were homozygous for TNFA*T1.

No obvious associations were found between the level of AChR antibodies or the presence of RyR and titin antibodies for the TNFA A/G polymorphisms at position −238 (A/G) (Table 3 and Table 4), but group sizes were suboptimal.

**TNFB Polymorphisms**

Patients having titin antibodies were more often (11 of 15) homozygous for the TNFB*2 allele than those (4 of 15) who did not have such antibodies (\(x^2 = 4.8, P = .04\); Fisher exact test, \(P = .01\)) and than controls (32 of 90) (\(x^2 = 7.1, P = .02\); Fisher exact test, \(P = .005\)), but patients who did not have the titin antibody were more often (11 of 15) carriers of the TNFB*1 allele (\(x^2 = 4.8, P = .04\); Fisher exact test, \(P = .04\)) (Table 3). Titin antibodies occurred in 11 of 15 patients with MG who were homozygous for the TNFB*2 allele compared with 4 of 11 heterozygous patients with MG and 0 of 4 patients who were homozygous for the TNFB*1 allele.

The 3 patients who had RyR antibodies were homozygous for TNFB*2.

**CORRELATION WITH TITIN, RyR, AND AChR ANTIBODIES**

**TNFA Polymorphisms**

In addition to having a thymoma, 3 of the patients had RyR antibodies. None of the patients with early onset had titin or RyR antibodies. Patients with MG who have the titin antibody are more often (13 of 15) homozygous for the TNFA*T1 allele than patients with MG who do not have the antibody (6 of 15) (\(x^2 = 5.2, P = .03\); Fisher exact test, \(P = .01\)), whereas patients with MG who do not have the titin antibody were more often (9 of 15 vs 2 of 15, respectively) carriers of the TNFA*T2 allele (\(x^2 = 5.2, P = .03\); Fisher exact test, \(P = .01\)) (Table 3). Titin antibodies were found in serum samples from 13 of 19 patients homozygous for the TNFA*T1 allele compared with only 2 of 10 heterozygous patients (\(x^2 = 4.2, P = .05\); Fisher exact test, \(P = .13\)). The 1 patient homozygous for TNFA*T2 had no detectable titin antibodies. The 3 patients who had RyR antibodies were homozygous for TNFA*T1.

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Table 4. Acetylcholine-Receptor (AChR) Antibody Concentration and Disease Severity in Patients With Myasthenia Gravis (MG) According to TNFA and TNFB Genotypesa

<table>
<thead>
<tr>
<th>Genotype</th>
<th>AChR Antibody, nmol/L</th>
<th>MG Severity†</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNFA T1/T1 (n = 19)</td>
<td>5.8 ± 5.7</td>
<td>2.9 ± 0.9</td>
</tr>
<tr>
<td>TNFA T1/T2 (n = 10)</td>
<td>3.7 ± 3.1</td>
<td>2.7 ± 0.9</td>
</tr>
<tr>
<td>TNFA T2/T2 (n = 4)</td>
<td>4.7</td>
<td>2.0</td>
</tr>
<tr>
<td>TNFA A/G (n = 2)</td>
<td>5.1 ± 1.2</td>
<td>3.0 ± 1.4</td>
</tr>
<tr>
<td>TNFA G/G (n = 28)</td>
<td>4.1 ± 2.7</td>
<td>2.7 ± 0.9</td>
</tr>
<tr>
<td>TNFB 1/1 (n = 4)</td>
<td>3.9 ± 1.1</td>
<td>1.8 ± 1.0</td>
</tr>
<tr>
<td>TNFB 1/2 (n = 11)</td>
<td>6.2 ± 6.8</td>
<td>3.0 ± 0.7</td>
</tr>
<tr>
<td>TNFB 2/2 (n = 15)</td>
<td>3.8 ± 1.2</td>
<td>3.0 ± 0.9</td>
</tr>
</tbody>
</table>

* Data are given as mean ± SD. Since there is only 1 patient in the TNFA T2/T2 group, there is no SD.†For the classification of disease severity, see the “Patients and Controls” subsection of the “Patients and Methods” section.

The TNFA and TNFB polymorphisms show no correlation with the concentration of AChR antibodies (Table 4).

COMBINATIONS OF TNFA AND TNFB POLYMORPHISMS

The different TNFA and TNFB polymorphisms are contained in a limited number of haplotypes. We, therefore, analyzed different allele combinations. Of 10 patients having early-onset MG, 7 were carriers of both the TNFA*T2 and TNFB*1 alleles compared with 0 of 7 patients having MG who had a thymoma (χ² = 5.7, P = .02; Fisher exact test, P = .01).

Of 15 patients with MG (regardless of thymic disease) who had the titin antibody, 11 were homozygous for TNFA*T1 and TNFB*2 compared with 4 of 15 patients with MG who did not have the titin antibody (χ² = 4.8, P = .04; Fisher exact test, P = .01) and 32 of 92 controls (χ² = 7.1, P = .01; Fisher exact test, P = .005). Patients with MG who did not have the titin antibody were similar to patients with early-onset MG because 9 of 15 were carriers of both the TNFA*T2 and TNFB*1 alleles, this combination being found in only 2 of 15 patients with MG who had the titin antibody (χ² = 5.2, P = .03; Fisher exact test, P = .01).

No differences were noted when the group with MG as a whole was compared with the controls for TNFA and TNFB patterns.

CORRELATION WITH CLINICAL FEATURES

No correlation was found between the severity of MG and TNFA or TNFB genotypes (Table 3). No significant differences were noted in the use of immunosuppressive treatment between patients with MG with different TNFA or TNFB genotypes. Autoimmune diseases in addition to MG occurred in 3 patients heterozygous for TNFA*T1/T2 and TNFB*1/2 (diabetes mellitus [1 patient], Sjögren disease [1 patient], and systemic lupus erythematosus [1 patient]). One patient homozygous for TNFA*T1 and TNFB*2 had rheumatoid arthritis.

The main finding of this study is that patients who have MG and who have the titin antibody, including those with a thymoma, are most often homozygous for the TNFA*T1 and TNFB*2 alleles, but the presence of the TNFA*T2 and TNFB*1 alleles correlates with early-onset MG and the absence of titin antibodies. These associations seem more closely linked to the TNFB than to the TNFA locus and are stronger for the presence of titin antibodies than for the MG subgroup, the presence of a thymoma, or the early- or late-onset phenotype.

No strong HLA associations occur in patients with MG who have a thymoma, but genetic factors are known to be important. Previous studies have shown an association with Ig heavy-chain genetic markers, including GM phenotypes and allelotypes and an FcγRIIA subtype. An association with specific TNF polymorphisms in patients having a thymoma could imply that the susceptibility genes are more closely related to the major histocompatibility class III region or to the TNF genes than to the major histocompatibility class II region or genes. It has been suggested that HLA-DR3, which is found in lower frequency in patients with MG who have a thymoma, has a protective role. This is in line with our findings because HLA-DR3 is closely linked to the TNFA*T2 and TNFB*1 alleles, which did not occur in our patients with MG who had a thymoma. An increased frequency of the TNFA*T2 (A−308) and TNFB*1 alleles also has been reported in Swedish patients having early-onset MG (patients who do not have titin antibodies). Stratification analysis of the extended DR3 haplotype in those patients suggested that 2 factors conferred susceptibility to MG: the stronger located close to the TNFA*T2 locus and another in the HLA-DQ region.

The TNFA-TNFB polymorphisms are known to affect transcriptional control and could, therefore, be directly involved in pathogenesis because carriers of TNFA*T2 (A−308) and TNFB*1 alleles have an increased secretion of TNF-α. Increased levels of TNF-α and an increased number of cells expressing AChR-reactive TNFA messenger RNA are found in patients who have MG, particularly during active disease. The cytokines TNF-α and TNF-β are important B-cell growth factors, and TNF-α induces thymic epithelial cells to secrete interleukin 6. Both effects can lead to thymic hyperplasia and the increased production of autoreactive T cells that is the hallmark of early-onset MG. The immunosuppressive drug thalidomide is known to inhibit TNF-α production at the messenger RNA level and could, in theory, be of benefit in patients who have early-onset MG.

Acetylcholine-receptor antibody levels and disease severity were not associated with TNFA or TNFB polymorphisms, suggesting that the polymorphisms are more important for disease susceptibility than for disease progression and prognosis. Most of the patients who had MG in our study used immunosuppressive drugs, which may mask a potential effect of the TNF polymorphisms on the disease course. No significant differences, however, were found in the use of immunosuppressive drugs between patients with different TNFA and TNFB genotypes.

In contrast, patients with MG who had a thymoma were homozygous for TNF allelotypes (TNFA*T1 and
TNFβ *2) that are associated with low TNF-α and TNF-β secretion.8 Because TNF has antitumor activity, it is possible that the polymorphisms associated with low TNF secretion confer an increased risk for the development of thymoma. Thymoma and MG are also associated with genetic markers (both on IgG and FcγRIIIA) that correlate with high IgG2 responses and efficient immune responses to polysaccharide antigens.23-25 It is, therefore, late with high IgG2 responses and efficient immune reaction against aberrantly expressed epitopes on thymoma identical genotype, suggesting that they, too, could have body, regardless of the presence of thymoma, have an tern, suggesting a similar pathogenesis but differing in neoplastic conditions can be seen many years before a tumor is found, and sometimes a tumor is never de tected. Microscopic thymomas (0.2-0.4 mm) undetectable by macroscopic examination have been de scribed.33,34 Patients who have late-onset MG are not treated with thymectomy. Therefore, it is possible that some patients with late-onset MG who have the titin antibody have microscopic thymomas undetectable with or dinary imaging techniques. An alternative explanation could be that an immune attack against the thymoma causes tumor regression similar to what has been re ported for other tumors.35,36 Because of the close association between the presence of titin antibodies, being homozygous for TNFA*TT and TNFB*2, and having MG with thymoma, physicians should have a low threshold for thymectomy in patients with MG who have the titin antibody and who are homozygous for TNFA*TT and TNFB*2, regardless of the onset of MG and lack of abnormalities on chest computed tomography.

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REFERENCES