The Severity of Myasthenia Gravis Correlates With the Serum Concentration of Titin and Ryanodine Receptor Antibodies

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Background: Myasthenia gravis (MG) is caused by autoantibodies to the acetylcholine receptor (AChR). Non-AChR muscle autoantibodies are present in many MG serum samples, mainly from patients with thymoma or late-onset MG. The exact relationship between MG severity and several non-AChR muscle antibodies is unknown.

Objective: To study the correlation between the severity of MG and the concentration of antibodies against striated muscle tissue sections, titin, citric acid antigen, ryanodine receptor, and AChR.

Setting: The severity of MG was graded in 146 consecutive patients with MG, and their serum samples were tested for the presence of autoantibodies. Ten patients who were titin antibody positive were observed in longitudinal follow-up.

Results: No significant difference was found in MG severity between late-onset and thymoma MG. Titin, citric acid antigen, and ryanodine receptor antibodies occurred significantly more often among patients with severe MG than among patients with less severe disease. Changes in MG severity correlated with changes in titin antibody titer in the individual patient. Titin antibodies showed a better longitudinal correlation with disease severity than the AChR antibodies.

Conclusions: Non-AChR muscle autoantibodies occurred more frequently in severe MG regardless of MG subgroup. Thymoma per se does not generate a more severe MG. It may well be the presence of a humoral immune response to non-AChR muscle antigens such as titin, citric acid antigen, and ryanodine receptor that leads to a severe disease, not the presence of thymoma or a late age of onset. These antibodies can serve as important prognostic markers in MG regardless of the presence of thymoma.

Arch Neurol. 2000;57:1596-1600

ACETYLCHOLINE receptor (AChR) antibodies are present in the serum of 85% to 90% of patients with generalized myasthenia gravis (MG) and occur very infrequently in the serum of healthy individuals.1-4 Non-AChR muscle autoantibodies are present in many MG serum samples, mainly from patients with thymoma or late-onset MG.5 Assays for non-AChR antibodies such as antibodies against cross-striational components of striated skeletal or heart muscle (SH),5,7 antibodies against a citric acid extract of striated muscle (CA),8 and antibodies against the well-defined muscle proteins titin and ryanodine receptor (RyR)9,10 are widely used in MG diagnostic work because of their relationship to a thymoma. Testing for a combination of muscle autoantibodies, especially against both titin and RyR, gives the highest sensitivity and specificity for the detection of a thymoma in MG.11,12 The pathogenetic relevance of autoantibodies against antigens such as SH, titin, CA, and RyR is still unclear. However, in rather small patient materials the presence of titin antibodies seems to be a marker for more severe disease in late-onset MG,13 whereas RyR antibodies seem to be a marker for a more severe disease in thymoma MG.14,15 The relationship between MG severity and a combination of AChR and several non-AChR muscle antibodies has not been examined previously. In this study we correlate the severity of MG with the presence of SH, titin, CA, RyR, and AChR antibodies in unselected and well-defined MG patient material.

RESULTS

DISEASE SEVERITY AND MUSCLE ANTIBODIES

Mean SH antibody titers did not differ significantly among 4 MG severity groups (groups 4 and 5 combined), and neither did the proportion of patients positive for SH antibody (Table 3). Severity groups 4 and 5 combined had a significantly higher proportion of patients positive for titin anti-
PATIENTS AND METHODS

PATIENTS

Serum from 146 consecutive patients with MG (52 men, 94 women) was tested (Table 1). Mean age of MG onset was 42 years, and mean age at admission to this study with a blood sample drawn was 51 years. None of the patients received immunosuppressive drugs at that time. The diagnosis of MG was based on a typical clinical pattern, a positive edrophonium test result, and neurophysiological investigations with decrement at repetitive stimulation and increased jitter at single-fiber electromyography. The diagnosis of thymoma was based on histopathologic findings in all patients.

The patients were divided into 5 subgroups according to their type of MG: (1) ocular MG, with purely ocular (nongeneralized) symptoms; (2) early-onset MG, with onset before age 50 years; (3) late-onset MG, with onset at age 50 years or older; (4) MG with thymoma, regardless of age at onset; and (5) AChR-antibody–negative MG (Table 2). The severity of MG was graded using a modified Oslerman scale at the time the blood sample was taken: (1) patients with purely ocular muscle weakness characterized by ptosis and/or diplopia with no signs of bulbar or generalized weakness; (2) patients with mild generalized weakness, usually with ocular muscle weakness but without bulbar involvement, and respiratory muscles are not involved; (3) patients with mild generalized weakness and a mild bulbar involvement with dysphagia and respiratory muscles are not involved; (4) patients with moderate generalized weakness, usually with moderate bulbar and respiratory muscle weakness; and (5) patients with severe generalized, bulbar, and respiratory muscle weakness.

The severity of MG was also graded using an activity of daily living scale (ADL) based on Tindall's quantitative MG scoring system scale. The ADL scale includes 8 parameters, and the severity is graded from I to III (mild to severe).

Ten patients positive for titin antibody were observed longitudinally for 1 to 6 years, with clinical follow-up and serum samples. Both AChR antibody concentration and titin antibody titer were assessed. Six of these patients were treated with prednisolone and 1 with azathioprine after the first assessment.

SKELETAL MUSCLE ANTIBODY ASSAYS

AChR Antibodies

The concentration of anti-AChR antibodies was measured by a standard radioimmunoassay method with human iodine 125–labeled AChR as the antigen and using AChR radioimmunoassay kits (IBL, Hamburg, Germany). Titin Antibodies

Titin antibodies were detected by enzyme-linked immunosorbent assay using purified titin fragment MGT-30 at a 3.5-µg/mL concentration as the antigen. Both positive and negative controls were included. The results are reported as antibodies present or not present.

CA Antibodies

Citric acid antigen was prepared from amputated human leg muscle and used in a 2-µg/mL concentration. Citric acid antibodies were detected by enzyme-linked immunosorbent assay. The OD values were obtained at 492 nm, subtracting the OD value of the control well from each serum-treated well. Only OD values above 500 were considered positive because of extensive testing of serum from healthy controls and from patients with autoimmune and muscle disorders other than MG.

RyR Antibodies

Ryanodine receptor antibodies were assayed by Western blot using crude sarcoplasmic reticulum prepared from rabbit skeletal muscle as antigen. Both positive and negative control serum samples were included. The results are reported as antibodies present or not present.

STATISTICAL ANALYSIS

The severity of MG in the different MG subgroups and at the 2 measurement times in the longitudinal study was compared using the χ² test of independence. The mean AChR antibody concentrations in the different MG severity groups and in the longitudinal study were compared using the Mann-Whitney test. The mean antibody titer in the different groups of MG severity and in the longitudinal antibody study and the mean age of onset were compared using the t test for difference between population means with unequal variances.

The proportion of patients positive for SH, titin, CA, RyR, and AChR antibodies in the different MG severity groups and in the total material was compared using the χ² test of independence with the Yates correction with 1 df. Because of the small number of patients with the most severe MG, patients in severity groups 4 and 5 and patients in ADL groups II and III were combined when comparing antibody concentrations, titers, or proportions of patients.

The serum samples were diluted beginning with 1:64 until an immunofluorescence-negative dilution or a 1:1024 dilution was reached.

Patients in severity groups 4 and 5 combined had a significantly higher mean CA antibody titer (P < .03), and these groups had a higher proportion of positive findings than patients in group 3 (P < .01) and also higher than group 2 (P < .01 for both titer and proportion) (Table 3).
Mean CA antibody titer and proportion in group 2 and 3 did not differ significantly.

Patients in severity groups 4 and 5 combined had significantly more RyR antibodies than patients in severity group 2 and in group 3 (P = 0.005 for both) (Table 3). The proportion of patients positive for RyR antibody in severity group 2 did not differ from that in group 1 or group 3. To examine whether any one of the muscle antibodies was a better marker for severe MG, the occurrence of the various muscle antibodies in MG severity groups 4 and 5 combined was compared. There was no significant difference regarding the proportion of patients positive for SH, titin, CA, and RyR antibodies; each of these antibodies occurred in 45% to 65% of the patients in severity groups 4 and 5 combined. For severity group 3, they occurred in 12% to 41% of the patients. Severity groups 4 and 5 included 8 patients with thymoma, 8 patients with late-onset MG, and only 4 other patients. Thus, non-AChR muscle autoantibodies occurred more frequently in severe disease regardless of thymoma.

Mean AChR antibody concentration and the proportion of patients with AChR antibodies did not differ significantly between severity groups 2 and 3 (Table 3). Patients in severity groups 2 and 3 combined had significantly higher AChR antibody concentrations than those in severity groups 4 and 5 combined (P = 0.05), but the proportion of patients positive for AChR did not differ significantly. No significant difference was observed in AChR antibody concentration or proportion of affected patients between severity groups 1 and 2.

The longitudinal study of 10 patients (Figure) showed a statistically significant overall reduction in disease severity scores at the second assessment (either as a result of immunosuppressive therapy or spontaneously) (P = 0.01). At the first assessment, there were no patients in severity groups 1 or 5; 2 in group 2; 7 in group 3; and 1 in group 4. The mean ± SD AChR concentration was 24.1 ± 21.5 nmol/L; and the titin titer, 3360 ± 4053. At the second assessment, there were no patients in severity groups 3, 4, or 5; 6 in group 1; and 4 in group 2. The AChR concentration was 13.8 ± 11.2 nmol/L; and the titin titer, 720 ± 976. This clinical improvement was statistically significant for the decrease in mean titin antibody titer (P = 0.05); however, the decrease in AChR antibody concentration did not reach statistical significance (P = 0.10).

**DISEASE SEVERITY ASSESSED BY THE ADL SCALE AND MUSCLE AUTOANTIBODIES**

Patients in ADL severity group I had significantly higher AChR antibody concentrations than those in ADL severity groups II and III combined (P = 0.05), but the propor-

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**Table 1. Characteristics of Patients With Myasthenia Gravis* Included in the Study in Each of the 5 Severity Groups of the Modified Osserman Scale**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>18 (12)</td>
<td>42 (29)</td>
<td>66 (45)</td>
<td>16 (11)</td>
<td>4 (3)</td>
<td>146 (100)</td>
</tr>
<tr>
<td>Male</td>
<td>9 (50)</td>
<td>12 (29)</td>
<td>23 (35)</td>
<td>6 (38)</td>
<td>2 (50)</td>
<td>52 (36)</td>
</tr>
<tr>
<td>Female</td>
<td>9 (50)</td>
<td>30 (71)</td>
<td>43 (65)</td>
<td>10 (62)</td>
<td>2 (50)</td>
<td>94 (64)</td>
</tr>
<tr>
<td>Age at onset, y, mean ± SD</td>
<td>37 ± 14</td>
<td>39 ± 20</td>
<td>41 ± 20</td>
<td>52 ± 19</td>
<td>66 ± 9</td>
<td>42</td>
</tr>
<tr>
<td>No. of patients undergoing thymectomy</td>
<td>1</td>
<td>14</td>
<td>52</td>
<td>15</td>
<td>2</td>
<td>84</td>
</tr>
</tbody>
</table>

*Unless otherwise indicated, data are number (percentage) of patients.

**Table 2. The Number of Patients With Myasthenia Gravis in the 5 Severity Groups of the Modified Osserman Scale for Each of the 5 Myasthenia Gravis Subgroups**

<table>
<thead>
<tr>
<th>Myasthenia Gravis Subgroup</th>
<th>Ocular (n = 12)</th>
<th>Early Onset (n = 52)</th>
<th>Late Onset (n = 40)</th>
<th>Thymoma (n = 20)</th>
<th>AChR* Negative (n = 22)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>12</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>18</td>
<td>11</td>
<td>4</td>
<td>9</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>32</td>
<td>21</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>2</td>
<td>5</td>
<td>7</td>
<td>2</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

*AChR indicates acetylcholine receptor.

**Table 3. Patients With Myasthenia Gravis With Antibodies Against AChR, SH, Titin, CA, and RyR in the Various Severity Groups of the Modified Osserman Scale (Groups 4 and 5 Combined) and in the Total Material**

<table>
<thead>
<tr>
<th>Substance</th>
<th>1 (n = 18)</th>
<th>2 (n = 42)</th>
<th>3 (n = 66)</th>
<th>4 and 5 Combined (n = 20)</th>
<th>Total (N = 146)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AChR</td>
<td>13 (72)</td>
<td>33 (79)</td>
<td>60 (91)</td>
<td>18 (90)</td>
<td>124 (85)</td>
</tr>
<tr>
<td>Concentration, nmol/L</td>
<td>45 ± 201</td>
<td>73 ± 142</td>
<td>64 ± 150</td>
<td>21 ± 36</td>
<td>58 ± 131</td>
</tr>
<tr>
<td>SH</td>
<td>1 (6)</td>
<td>10 (24)</td>
<td>27 (41)</td>
<td>11 (55)</td>
<td>49 (34)</td>
</tr>
<tr>
<td>Titer</td>
<td>1024 ± 0</td>
<td>435 ± 430</td>
<td>220 ± 222</td>
<td>273 ± 302</td>
<td>292 ± 313</td>
</tr>
<tr>
<td>Titin</td>
<td>3 (17)</td>
<td>9 (21)</td>
<td>24 (36)</td>
<td>13 (65)</td>
<td>49 (34)</td>
</tr>
<tr>
<td>CA</td>
<td>1 (6)</td>
<td>8 (19)</td>
<td>16 (24)</td>
<td>12 (60)</td>
<td>37 (25)</td>
</tr>
<tr>
<td>Titer</td>
<td>600 ± 0</td>
<td>731 ± 750</td>
<td>1155 ± 1206</td>
<td>2925 ± 2501</td>
<td>1622 ± 1853</td>
</tr>
<tr>
<td>RyR</td>
<td>1 (6)</td>
<td>2 (5)</td>
<td>8 (12)</td>
<td>9 (45)</td>
<td>20 (14)</td>
</tr>
</tbody>
</table>

*AChR indicates acetylcholine receptor; SH, striated skeletal or heart muscle; CA, citric acid extract of striated muscle; and RyR, ryanodine receptor. Unless indicated otherwise, data are number (percentage) of patients or mean ± SD.
tion of patients positive for AChR did not differ significantly (Table 4). The proportion of patients positive for titin, CA, and RyR antibodies in ADL severity groups II and III combined was higher than that in ADL severity group I (P = .01 for each antibody). Patients in ADL severity groups II and III combined had significantly higher CA antibody titer than patients in ADL severity group I (P = .01). No significant difference was found between patients in ADL severity group I and in groups II and III combined regarding titer of SH and titin antibodies and proportion of patients positive for SH antibody.

DISEASE SEVERITY IN MG SUBGROUPS

No significant difference in the severity of MG was found between late-onset MG and thymoma MG, or between late-onset MG and early-onset MG (Table 2). Patients with thymoma MG had a more severe MG than patients in the early-onset MG subgroup (P = .005). Myasthenia gravis was more severe in its early-onset type than in the AChR-negative type (P = .005). Patients negative for AChR MG had a more severe MG than patients in the ocular MG subgroup (P = .005).

Patients in severity group 4 had significantly higher age of MG onset than patients in severity groups 2 and 3 (P = .03 for both). Also patients in severity group 5 had significantly higher age of MG onset than patients in severity groups 2 and 3 (P = .005 for both). Accordingly, later onset of MG correlated with more severe disease and early onset with less severe disease.

**COMMENT**

The occurrence of titin, CA, and RyR autoantibodies is highest among patients with thymoma and late-onset MG.6,11,25 We have shown that MG is more severe in patients with thymoma and in those with late-onset MG, and that titin, CA, and RyR antibodies occur significantly more often among patients with severe MG than among patients with less severe disease. Eighty-five percent of patients with thymoma MG have autoantibodies with specificity against at least 4 different muscle antigens.11 This study clearly shows that the presence of muscle autoantibodies is associated with more severe disease in all MG subgroups, although the frequency of these antibodies is highest in thymoma and late-onset MG.

They are therefore important prognostic factors in patients with MG. Whether the presence of non-AChR antibodies is the result of severe disease, late age of onset, the presence of thymoma, or all these factors together is still unclear. Thymoma has traditionally been regarded as an unfavorable prognostic marker in MG.26 However, we have demonstrated that thymoma per se is not associated with a more severe MG; mean disease severity in thymoma MG and late-onset nonthymoma MG did not differ significantly, in line with another recent study.27

Our study indicates that there is a correlation between the severity of MG and titin antibody titer in the individual patient. Kuks et al25 showed that non-AChR muscle antibody concentrations tend to fluctuate together with the AChR antibody concentration, clinical course, and immunosuppressive therapy in patients with MG. The correlation of titin antibodies with more severe disease in late-onset MG,13 and RyR antibodies with more severe disease in thymoma MG14,15 has been demonstrated in studies based on selected MG patient materials. In this longitudinal titin and AChR antibody study, we have shown that titin antibodies correlate better with disease severity than do AChR antibodies. In interindividual comparisons, a high AChR antibody concentration correlates with less severe MG and earlier age of onset. Titin antibodies, combined with RyR antibodies, are also an important marker for the diagnosis of thymoma in MG.11 Titin and RyR antibodies should therefore both be tested for initially and at follow-up in patients with MG.

Although age 40 years has traditionally been the cutoff between early-onset and late-onset MG, recent epidemiological data support using age 50 years.28,29 Using age 40 years would have moved 4 of our patients from the early-onset to the late-onset MG subgroup, but without changing any conclusions.

In thymoma-associated MG, muscle autoantibodies are most probably directly induced by the thymoma. In such patients MG is a paraneoplastic disease with true paraneoplastic antibodies.7,30 Two properties of thymomas are probably fundamental for the induction of antibodies: (1) the

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Table 4. Patients With Myasthenia Gravis With Antibodies Against AChR, SH, Titin, CA, and RyR in the 3 ADL Severity Groups (Groups II and III Combined) and in the Total Material

<table>
<thead>
<tr>
<th>ADL I (n = 126)</th>
<th>ADL II and III (n = 28)</th>
<th>Total (N = 146)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AChR Concentration, nmol/L</td>
<td>64 ± 140</td>
<td>21 ± 36</td>
</tr>
<tr>
<td>SH Titer</td>
<td>38 (30)</td>
<td>11 (55)</td>
</tr>
<tr>
<td>Titin Titer</td>
<td>298 ± 322</td>
<td>273 ± 302</td>
</tr>
<tr>
<td>CA Titer</td>
<td>36 (29)</td>
<td>13 (65)</td>
</tr>
<tr>
<td>RyR Titer</td>
<td>5255 ± 7126</td>
<td>5200 ± 4716</td>
</tr>
</tbody>
</table>

*ADL indicates activities of daily living; AChR, acetylcholine receptor; SH, striated skeletal or heart muscle; CA, citric acid extract of striated muscle; and RyR, ryanodine receptor. Unless indicated otherwise, data are number (percentage) of patients or mean ± SD.
aberrant expression of a nonreceptor cytoplasmic protein with an AChR-like epitope, and similarly titin- and RyR-like epitopes and (2) thymuslike morphologic characteristics associated with the maintained function of the tumors to provide homing for immature thymocytes. Abnormal selection inside the thymoma and somatic mutations of the lymphocytes may initiate autoimmune due to the aberrantly expressed muscle antigen-like self-peptides in the neoplastic epithelium, perhaps combined with altered immunoregulatory mechanisms. Some patients with MG, especially those with thymoma and late-onset MG, may develop a myasthenic myopathy. A recent study showed that the presence of titin antibodies correlated with electromyographic evidence of myopathy. The frequent occurrence of non-AChR muscle autoantibodies in thymoma and late-onset MG, and in MG with myopathy indicates that these antibodies are correlated to the pathogenesis of MG both at thymic and muscular levels, in addition to being markers for disease severity and prognosis.

A number of grading systems have been developed to quantify MG severity. Similar results were obtained in this study using a modified Osserman scale and an ADL scale. The ADL scale has well-defined descriptive terminology and parameters but a limited number of grades compared with the modified Osserman scale. The disadvantage of a limited number of grades is more prominent when assessing patients with less severe MG. An ADL-based scale with a higher number of grades would therefore be optimal.

In conclusion, we have demonstrated that the presence of antibodies against titin, CA, and RyR in the serum of patients with MG indicates a more severe disease. These antibodies should be used as prognostic markers in MG, regardless of the presence of a thymoma.

Accepted for publication May 22, 2000.

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