Antibodies to skeletal muscle in myasthenia gravis

Part 1. Diagnostic value for the detection of thymoma

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Summary

The role of anti-muscle antibodies (AMA) in the diagnosis of thymoma in patients with myasthenia gravis (MG) is evaluated. We compared ELISA and Western blot assay for antibodies to citric acid muscle extract (a-CAE) with an immunofluorescence assay (IF). Sera from 234 selected MG patients and 123 controls were tested. There was no essential difference between ELISA and IF. Western blot was superior in young onset patients but less useful in patients with an onset beyond the age of 40 years. Unusually high post-test probabilities were found by our patient selection criteria which seem most realistic for clinical practice. Western blot revealed no differences in specificity of AMA in thymoma and non-thymoma patients irrespective of age at onset of disease.

Introduction

In 1960, some 15 years before the detection of anti-acetylcholine receptor antibodies (a-AChR), antibodies to other parts of skeletal muscle ("anti-muscle antibodies", AMA) were identified in patients with myasthenia gravis (MG) (Strauss et al. 1960).

In large unselected groups of MG patients, AMA frequencies of 30–60% are reported; in groups of MG patients with thymoma percentages of 80–100% are found versus 20–30% in non-thymoma patients with MG (reviewed by Lanska (1991)). In the latter group, AMA are mainly found in patients with onset of MG in middle age or thereafter (Limburg et al. 1983; Lennon and Howard 1985; Komiyama et al. 1988). AMA in non-MG patients with thymoma have also been found (Strauss et al. 1966; Oosterhuis et al. 1976; Carrano et al. 1983). Determination of AMA is thus a test for the presence of thymoma with a great sensitivity although not very specific in MG. However, a-Str were also detected in patients with other immunological and neuromuscular diseases without thymoma (Weiner and Osserman 1966; Stern et al. 1967; McDonald et al. 1979; Carrano et al. 1983; Hauser et al. 1986) with frequencies even up to 30%.

AMA form a heterogeneous group; the most well-known are "anti-striated antibodies" (a-Str) because of the striated pattern on skeletal muscle in an IF assay. A subgroup of anti-muscle antibodies as identified by reaction with citric acid muscle extracts (Aarli 1972) binds to the sarcolemmal membrane and does not form a striated pattern. The nature and significance of these antibodies (a-CAE) has been extensively evaluated by the group of Aarli and Gilhus (Aarli et al. 1987, 1988). The specificity of a-CAE for the presence of thymoma was claimed to exceed that of a-Str (Aarli et al. 1981; Gilhus et al. 1984).

To evaluate the sensitivity and specificity of a-Str and a-CAE for the presence of thymoma in patients with MG, we examined several groups of MG patients, diseased controls and healthy persons by immunofluorescence, ELISA and Western blotting. In contrast with most other studies we used stringent inclusion criteria to preclude factors other than thymic histology from influencing test characteristics.

Patients and methods

Sera from 234 MG patients and 123 controls were used in this study. Inclusion criteria for MG patients...
TABLE 1  
DEMOGRAPHIC DATA

<table>
<thead>
<tr>
<th></th>
<th>No. of patients</th>
<th>Gender M/F</th>
<th>Median age at onset*</th>
<th>Median age at study*</th>
<th>Median duration of MG*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Thymoma</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Early onset MG</td>
<td>28</td>
<td>11/17</td>
<td>32</td>
<td>35</td>
<td>4</td>
</tr>
<tr>
<td>Medium onset MG</td>
<td>25</td>
<td>10/15</td>
<td>49</td>
<td>52</td>
<td>2</td>
</tr>
<tr>
<td>Late onset MG</td>
<td>15</td>
<td>6/9</td>
<td>67</td>
<td>68</td>
<td>1</td>
</tr>
<tr>
<td><strong>No thymoma</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Early onset MG</td>
<td>100</td>
<td>20/80</td>
<td>25</td>
<td>26</td>
<td>2</td>
</tr>
<tr>
<td>Medium onset MG</td>
<td>36</td>
<td>12/24</td>
<td>49</td>
<td>53</td>
<td>2</td>
</tr>
<tr>
<td>Late onset MG</td>
<td>30</td>
<td>17/13</td>
<td>68</td>
<td>72</td>
<td>3</td>
</tr>
</tbody>
</table>

* Years.

were seropositivity for a-AChR, no use of immunosuppressive drugs, and generalized MG for less than 10 years. Furthermore, all sera were obtained before thymectomy.

Patients with an onset of MG before the age of 40 are referred to as 'early onset' patients, those with onset between 40 and 60 years as 'medium onset', and the others as 'late onset'. Demographic data are given in Table 1. Disease activity was evaluated without the knowledge of antibody levels by a 6-point clinical score as described before (Oosterhuis 1984).

The diagnosis of thymoma was made by histological examination in the patients with early onset MG. Patients with medium and late onset MG are not routinely operated upon in our hospital if there is no radiological evidence of thymoma. Only 7 of these patients have been operated upon, and all had thymic atrophy. In the remaining patients CT scans or planigrams were made to rule out a thymoma.

Serum, obtained by venepuncture, was stored at -80°C without preservatives. Control sera were obtained from patients with other autoimmune diseases (polymyositis, systemic lupus erythematosus; n = 37), other non-immunological muscle diseases (myotonic dystrophy, facioscapulohumeral muscular dystrophy. n = 30), and healthy controls (n = 56). The ages of healthy controls were equally divided over the decades between 20 and 80 years.

Antibodies to acetylcholine receptor protein were measured by the radioimmunoprecipitation test with antigen from muscles of amputated human legs as described before (Limburg et al. 1983). Levels of >1 nmol/l were considered positive.

Muscle for immunofluorescence was obtained from guinea pig tongue. Sections of 4 μm were fixed with acetone for 10 min and incubated at room temperature for 45 min with test sera diluted in phosphate-buffered saline (PBS) and subsequently with fluorescein-isothiocyanate-labelled sheep anti-human γ-globulin (CLB Amsterdam). The striatal pattern was used for identification of AMA. Titres of ≤1:20 were considered negative.

Human muscle was obtained from amputated legs, and citric acid extract (CAE) was prepared according to Aarli et al. (1981). Protease inhibitors were added to prevent degradation of antigen. The final acetone precipitate was extracted twice in 0.05 M citric acid and the supernatants of both extracts were pooled and dialyzed against 0.01 M Na₂HPO₄ at 4°C. After centrifugation at 27 500 × g for 1 h the supernatant was dialyzed against distilled water for 72 h. After centrifugation at 27 500 × g for 1 h the supernatant was freeze-dried and a solution in PBS was made at a concentration of 4 mg protein per ml.

**Immunoblotting methods**

Citric acid extract (CAE) was denatured at 2.5 mg/ml protein in 0.04 M Tris-HCl, pH 6.8, 1.2% sodium dodecyl sulfate (SDS) and 3% β-mercaptoethanol (final concentrations) at 100°C for 5 min. Denatured CAE and reference proteins were separated on 10% polyacrylamide/0.1% SDS gels at 0.15 mg CAE/cm gel and subsequently blotted onto nitrocellulose membranes (Schleicher and Schuell) by means of a semi-dry electroblotter (Ancos, Denmark) according to general procedures. These Western blots were blocked in 0.01 M Tris-HCl, pH 8.0, 0.15 M NaCl, 3% BSA for 2 h and incubated with test sera 1:100 diluted in 0.01 M Tris-HCl, pH 8.0, 0.1% Triton X-100, 0.15 M NaCl, 0.3% BSA for 2 h in an incubation apparatus (Westgeest et al. 1986). Bound antibodies were detected with horseradish peroxidase-labelled goat anti-human IgG antibodies (Kallestadt, Austin, Texas) diluted 1:1000 in incubation buffer, and staining with 4-chloronaphthol.

**CAE-ELISA**

Labstar F microtitre plates were coated with CAE at 10 μg/ml in 0.1 M carbonate for 90 min at 37°C. The plates were then washed 5 times with buffer (0.05% Tween-20, 150 mM NaCl and 0.01 M Tris/HCl pH 8). Test sera were diluted 1:100 to 1:2700 in incubation buffer (0.01 M Tris/HCl, pH 8.0, 0.15 M NaCl, 0.05% Tween-20, 1% BSA) and incubated for 60 min at 37°C. After 5 washings the plates were incubated for 30 min at 37°C with 5:5000 diluted peroxidase labelled goat anti-human IgG antibodies (Kallestadt, Austin, Texas) and subsequently stained with H₂O₂/OPD for 20 min. Finally, an equal volume of 2 N H₂SO₄ was added and the OD₉₂ was measured in a multispec. Serial dilutions of a positive reference sample, arbitrary set at 100 units/ml (U/ml), were included in all assays.

**Statistics**

Prior chances of the incidence of a thymoma in MG were calculated from pooled estimates as given in a
recent study (Lanska 1991): 12.2% for the unselected group, 7.1% for early onset patients, 26% for medium onset patients and 17% for late onset patients. The positive (chance of a thymoma in case of seropositivity for AMA) and negative (chance of no thymoma in case of no AMA detectable) post-test probability can be calculated following Bayes' theorem with help of the sensitivity and specificity (Vecchio 1966; Sox 1986).

Results

a-CAE ELISA

Levels of antibodies to citric acid muscle extract (a-CAE) as measured in the ELISA ranged from < 1–4000 U/ml (Fig. 1). Serum levels of controls ranged from < 1–10 U/ml. Controls with other autoimmune diseases had mostly higher levels (4–10 U/ml) than healthy persons and controls with other non-immunological neuromuscular diseases. Differences between thymoma and non-thymoma patients were only striking in the early onset groups: a-CAE levels were elevated in 22 of 28 thymoma patients compared to only 2 of 100 non-thymoma patients. There was no relation (Kendall's rank correlation test, Mann-Whitney U-test) between a-CAE serum levels on the one hand, and clinical state, gender, duration of MG and thymic state (thymectomized or not) and a-AChR serum levels on the other in each of the patient subgroups.

Western blotting

An example with blots from several groups is given in Fig. 2. In both controls and patients a weak reaction was found with three proteins of 77, 97 and 100 kDa. In 5 controls with other immunological disorders and 1 control with facioscapulohumeral muscle dystrophy an additional reaction with proteins of 30, 120 and 135 kDa was found. This reaction pattern was also found in 4 non-thymoma patients with early onset MG. Blots with these patterns were considered as ‘negative’. In most thymoma patients and a large number of patients with medium and late onset MG without thymoma a diffuse staining pattern with multiple bands was found. This reaction pattern was considered ‘positive’ and was never found in non-MG controls. We found no differences in reaction patterns in thymoma and non-thymoma patients with medium or late onset MG. In 15 patients who were a-CAE-negative by ELISA, a positive Western blot was found (Table 2).
**TABLE 2**

**DIAGNOSTIC VALUE OF SEVERAL SINGLE ASSAYS AND COMBINATIONS OF TESTS FOR DETECTING THYMOMA**

<table>
<thead>
<tr>
<th>Test</th>
<th>Thymoma</th>
<th>No thymoma</th>
<th>( \pi + / \pi - )</th>
</tr>
</thead>
<tbody>
<tr>
<td>( n ) TP FP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total group</td>
<td>IF</td>
<td>68 58 166 44 30 97</td>
<td></td>
</tr>
<tr>
<td>(12.2%)</td>
<td>EL</td>
<td>68 57 166 39 33 97</td>
<td></td>
</tr>
<tr>
<td></td>
<td>WBlot</td>
<td>68 64 166 47 31 98</td>
<td></td>
</tr>
<tr>
<td></td>
<td>IF + EL</td>
<td>68 59 166 44 31 97</td>
<td></td>
</tr>
<tr>
<td>Early onset</td>
<td>IF</td>
<td>28 22 100 2 81 97</td>
<td></td>
</tr>
<tr>
<td>group (10%)</td>
<td>EL</td>
<td>28 21 100 2 80 97</td>
<td></td>
</tr>
<tr>
<td></td>
<td>WBlot</td>
<td>28 26 100 2 83 99</td>
<td></td>
</tr>
<tr>
<td></td>
<td>IF + EL</td>
<td>28 23 100 2 82 98</td>
<td></td>
</tr>
<tr>
<td>Medium onset</td>
<td>IF</td>
<td>25 23 36 15 43 95</td>
<td></td>
</tr>
<tr>
<td>group (26%)</td>
<td>EL</td>
<td>25 23 36 13 47 95</td>
<td></td>
</tr>
<tr>
<td></td>
<td>WBlot</td>
<td>25 24 36 17 41 97</td>
<td></td>
</tr>
<tr>
<td></td>
<td>IF + EL</td>
<td>25 23 36 15 43 95</td>
<td></td>
</tr>
<tr>
<td>Late onset</td>
<td>IF</td>
<td>15 13 30 27 16 78</td>
<td></td>
</tr>
<tr>
<td>group (17%)</td>
<td>EL</td>
<td>15 13 30 24 18 87</td>
<td></td>
</tr>
<tr>
<td></td>
<td>WBlot</td>
<td>15 14 30 26 17 83</td>
<td></td>
</tr>
<tr>
<td></td>
<td>IF + EL</td>
<td>15 13 30 27 16 78</td>
<td></td>
</tr>
</tbody>
</table>

* a Prior chance of a thymoma.

**Comparison of ELISA, Western blot and immunofluorescence**

Table 2 summarizes frequencies of positive tests, test characteristics and post-test probabilities for the diagnostic value of detecting a thymoma in several groups of patients. It can be seen that the power of all tests is much higher in early onset patients than in medium onset patients, while in late onset patients the diagnostic value for the detection of thymoma was very poor for any test. Western blotting appeared to be ideal in early onset patients. CAE-ELISA as a single test was optimal for the detection of a thymoma in medium onset patients followed by the Immunofluorescence test. Using Western blot introduced 4 ‘false-positive’ patients and thus reduced the diagnostic value in this category of patients. Using a combination of ELISA and Immunofluorescence yielded no additional information and the diagnostic gain of the combination was even lower in some situations by introducing ‘false-positives’. The low predictive value for the presence of thymoma of all tests beyond the age of 60 years was caused by the high rate of ‘false-positives’ in the non-thymoma group.

**Discussion**

Anti-muscle antibodies play a significant role in the exclusion of thymoma. Using several tests in our total group of 234 MG patients, we found a negative predictive value of 98%. With a 12.2% prior probability of the presence of a thymoma in an arbitrary MG patient the positive predictive value of 31% yields only a moderate diagnostic gain for the detection of thymoma. Specificities and sensitivities of AMA for the presence of thymoma vary considerably in the litera-

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**Fig. 3.** a-Str (immunofluorescence) vs a-CAE (Elisa) in 60 thymoma patients. Positive and negative results are separated by the broken lines. Although both tests are very concordant when qualitative results are considered (Table 2), no relation exists between absolute values of the tests.
ture (Lanska 1991). Thus the reported positive predictive value ranges from 21% (Komiyama et al. 1988) to 59% (Aarli et al. 1987), while the negative predictive value remains between 92% (Ashizava et al. 1987) and 100% (Oosterhuis et al. 1976). Inclusion criteria such as seropositivity for a-AChR and type of myasthenia (only generalized patients were included in our study) may account for the diversity, but we presume that these factors play only a minor role.

Our results show that the portion of early onset and late onset patients in the population under study is of substantial influence. We chose the age at onset rather than the real age at the time of study with a turning-point of 40 years because of the clinical heterogeneity between patients with an onset of MG before and after the age of 40 years (Compston et al. 1980). A second limit was set more arbitrarily (but in accordance of our previous study (Limburg et al. 1983)) at the age of 60 to divide medium onset and late onset patients because the incidence of AMA in non-thymoma patients increases with age at onset. In addition to this stratification further inclusion criteria were used. If AMA is used for the diagnosis of thymoma MG will have been diagnosed recently and the test is performed before thymectomy and the use of immunosuppressive drugs. Thus it seemed realistic to mimic these conditions by the use of certain inclusion criteria (duration less than 10 years, no thymectomy or immunosuppressive drugs).

As will be described in the following parts of our study the duration of disease, thymic state and use of immunosuppressive drugs indeed influence AMA levels so the inclusion criteria used may account for the extremely low incidence of AMA (2 of 87 patients) in our early onset non-thymoma group, and so for the high positive predictive value around 80% in early onset patients compared to the 46% reported in another study (Lanska 1991).

It is even in this population of early onset patients that AMA play a crucial role in the diagnosis of thymoma because the radiological differentiation between a thymoma and a hyperplastic thymus gland is often difficult (Keesey et al. 1980; Janssen et al. 1983; Brown et al. 1983; Williams 1989). Immunoblotting considerably improved the value of a-CAE for the diagnosis of thymoma in the early onset patients. However, addition of this qualitative test unfortunately did not improve the diagnosis in patients with onset beyond the age of 40 years.

There was no relation between titres of a-Str and serum levels of a-CAE, indicating that we are dealing with different antigens (Aarli 1972). The difference in source of antigen used (guinea pig tongue for a-Str, human skeletal muscle for a-CAE) may also be important. Nevertheless, the concordance of the two tests with respect to the diagnosis of thymoma was very high because qualitative results mostly agreed. Only in late onset patients a-Str was inferior to CAE-ELISA as has been reported by others (Gilhus et al. 1983).

Conclusion

For the clinical practice we conclude that CAE-ELISA is very specific, but not superior to the conventional immunofluorescence test, for the diagnosis of thymoma in MG. It is essential to consider early, medium and late onset patients separately in assessing the diagnostic value of AMA tests in general. CAE Western blot was the most powerful test in early onset patients but its use may be misleading for the diagnosis of thymoma in medium onset patients and here immunofluorescence or CAE ELISA as a single test are preferred. In the late onset category CAE-ELISA was superior for the exclusion of thymoma although its specificity and diagnostic gain are relatively low compared to its power in other age groups. On the whole, AMA assays are not suitable for the detection of thymoma in late onset patients.

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References


