Anti-Titin Antibodies in Myasthenia Gravis

Tight Association With Thymoma and Heterogeneity of Nonthymoma Patients

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Background: Titin is the major autoantigen recognized by anti–striated muscle antibodies, which are characteristic of generalized myasthenia gravis (MG).

Objective: To seek a correlation between anti-titin antibodies and other features of MG patients, including histopathology, age at diagnosis, anti–acetylcholine receptor (anti-AChR) autoantibody titers, and clinical severity.

Methods: A novel, highly specific radioligand assay was performed on a large group of 398 patients with generalized MG.

Results: Among thymectomized patients, anti-titin antibodies were present in most patients with thymoma (56/70 [80%]), contrasting with only a minority of patients with thymus atrophy or hyperplasia (17/165 [10%]). They were also present in 64 (41%) of 155 nonthymectomized patients who had a radiologically normal thymus. In these patients and in those who had a histologically normal thymus, anti-titin antibodies were associated with a later age at onset of disease and with intermediate titers of anti-AChR antibodies. After controlling for these 2 variables, disease severity was not significantly influenced by anti-titin antibodies.

Conclusions: Anti-titin antibodies are a sensitive marker of thymoma associated with MG in patients 60 years and younger, justifying the insistent search for a thymoma in MG patients of this age group who have these antibodies. In nonthymoma patients, anti-titin antibodies represent an interesting marker complementary to the anti-AChR antibody titer, identifying a restricted subset of patients. These clinical correlations should prompt further studies to examine the mechanisms leading to the production of anti-titin antibodies.

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Myasthenia Gravis (MG) is an autoimmune disease of the neuromuscular junction characterized by fatigability and weakness of striated muscles. It is mediated primarily by autoantibodies directed at the muscle acetylcholine receptor (AChR) in the postsynaptic membrane of the motor end plate. These autoantibodies are present in the serum of most patients (85%) and can passively transfer the disease to rodents. Consequently, they have a major diagnostic and pathogenetic value.

In addition to anti-AChR antibodies, antibodies recognizing other components of the skeletal muscle and yielding striational immunostaining have long been detected in MG patients. The presence of these anti–striated muscle antibodies (ASMs) is important from the pathophysiological viewpoint inasmuch as it suggests that the antimuscle sensitization is not limited to AChR, posing the problem of the interrelationships between the various antimuscle immune responses. At the clinical level, ASMs have been shown to provide a sensitive marker for thymoma, a clinically severe condition that is often associated with MG. These antibodies, however, are not absolutely specific for either MG or thymoma and are heterogeneous. Taken as a whole, they bind various autoantigens, including actin, α-actinin, myosin, the ryanodin receptor, and connectin or titin. The latter protein has recently elicited particular interest in the context of MG. Titin is a giant filamentous protein with a molecular weight of 3 × 10⁶ d that extends across the length of the sarcomere and plays an essential role in muscle elasticity. Remarkably, despite the huge size and the complex structure of titin, most anti-titin antibodies recognize a restricted region with a molecular weight of 30000 d, called MGT30 (MG thymoma, 30 kd) or the main immunogenic region.
PATIENTS, MATERIALS, AND METHODS

PATIENTS

A group of 398 unrelated white patients with generalized MG was evaluated and analyzed by one of us (P.G., B.E., J.-M.W., or C.T.). Patients fulfilled the criteria for generalized MG based on clinical history and abnormal findings on electromyogram. Their main features are listed in Table 1. Criteria for disease severity were the muscle strength score and the 6-point disability score.20,21

Serum samples from a group of 237 control patients were also tested. The control group comprised 40 healthy subjects 33±14 (mean ± SD) years of age, 88 healthy patients older than 65 years of age, 80 patients with other autoimmune diseases (type 1 diabetes [n=20], systemic lupus [n=50], or Lambert-Eaton syndrome [n=10]), 29 patients with other neurological diseases (tuberculous meningitis [n=4], dementia [n=3], low-pressure hydrocephalus [n=4], Guillain-Barre syndrome [n=2], or multiple sclerosis [n=16]).

IMMUNOLOGICAL ASSAYS

Anti-AChR antibody titers were determined with a radioimmunoprecipitation assay as described by Lindstrom,22 with modifications,23 using an antigen extract prepared from the AChR-expressing rhabdomyosarcoma human cell line TE671.24 Briefly, TE671 extracts were complexed to iodinated α-bungarotoxin (α-BGT), and α-BGT–AChR, 100 fmol/L, was incubated with 1 µL of serum. The antibody titer was calculated from the amount of α-BGT–AChR precipitated and expressed as nanomoles per liter of serum. All assays were performed in duplicate, in parallel with serial dilutions of positive and negative control serum samples.

Anti-titin antibodies were detected using human MGT30 antigen and methionine labeled with sulfur 35. Briefly, the complementary DNA encoding this epitope (pET8C-MGT30) was transcribed and translated in vitro (37 MBq/mL) using TE671.24 Briefly, TE671 extracts were complexed to iodinated α-bungarotoxin (α-BGT) and α-BGT–AChR. 100 fmol/L, was incubated with 1 µL of serum. The antibody titer was calculated from the amount of α-BGT–AChR precipitated and expressed as nanomoles per liter of serum. All assays were performed in duplicate, in parallel with serial dilutions of positive and negative control serum samples.

Anti-titin antibodies were detected using human MGT30 antigen and methionine labeled with sulfur 35. Briefly, the complementary DNA encoding this epitope (pET8C-MGT30) was transcribed and translated in vitro in the presence of [35S]methionine (37 MBq/mL) using the TNT protocol (Promega Corp, Madison, Wis).

Unincorporated [35S]methionine was removed by chromatography through a NAP-5 column (Pharmacia Biotech Inc, Piscataway, NJ). This step decreased the background substantially. Aliquots (30000 cpm) of labeled MGT30 antigen were incubated overnight at 4°C with 2 µL of serum. The amount of labeled MGT30 protein bound by serum immunoglobulins was quantitated in a Microbeta Trilux counter (EGC Instruments, Richardson, Tex) after IgG isolation with 1 mg of Protein A–Sepharose (Pharmacia Biotech Inc). All samples were tested blindly in duplicate. The intra-assay and interassay coefficients of variation were 12% and 15%, respectively. In pilot experiments, the MGT30 antigen and immune complexes were migrated using denaturing 10% polyacrylamide gel electrophoresis (not shown). The radiolabeled material appeared as a single species with a molecular weight of 47 kd, slightly larger than the predicted molecular weight, as previously described.13 The specificity of anti-titin antibodies was also verified in an inhibition experiment by preincubating the serum with cold recombinant MGT30 antigen produced in bacteria.

Anti–striated muscle antibodies were assayed by indirect immunofluorescence on a rat diaphragm. Antibody titers were determined after serial dilution of serum. Titers above 1:20 were considered positive.

DATA ANALYSIS

Values for age at diagnosis and normal strength scores were normally distributed. Titers of anti-AChR autoantibodies were normalized by a logarithmic transformation after an arbitrary small value of 0.01 nmol/L was assigned to null results. Means were compared with the Student t test or, in case of variance inequality detected with the Levene test, with the nonparametric Mann-Whitney test. Comparison of means after controlling for confounding factors was carried out with an analysis of variance/multivariate analysis of variance module (Statistica for Windows; Statsoft Inc, Tulsa, Okla).

The positive predictive value was the proportion of subjects with thymoma among those with positive assay results. The negative predictive value was the proportion of subjects without thymoma among those with negative assay results.

RESULTS

ASSOCIATION OF ANTI-TITIN ANTIBODIES WITH THYMOMA IN THYMECTOMIZED MG PATIENTS

The presence of anti-titin antibodies was assessed in 243 patients with MG who had undergone thymectomy and in 149 control subjects, including 40 healthy persons, 80 patients with autoimmune diseases other than MG, and 29 patients with other neurological diseases (Figure 1). Antibody assay results were negative in all control subjects; the amount of radioactivity retained by control serum samples was, without exception, at the background level (mean±SD, 66±22 cpm). A positivity threshold of 132 cpm/µL of serum, corresponding to the
mean ±3 SDs, was adopted. Of the 235 samples of serum from subjects with MG with known histopathologic conditions, 73 (31%) tested positive. Most blood samples had titers at least 10 times above the positivity threshold. In various neurological and neurological disease controls, anti-titin antibodies were not found. Together with their absence in healthy subjects, this absence of anti-titin antibodies demonstrated their high specificity for MG, comparable to that of anti-AChR autoantibodies.

As shown in Figure 1 (inset) and in Table 2, the great majority of positive serum samples (56/73) were from MG patients with thymoma. Conversely, only 12 (16%) of 74 patients with MG with thymus atrophy and 5 (5%) of 91 patients with MG with thymus hyperplasia had anti-titin antibodies, confirming the preferential presence of anti-titin antibodies in thymoma patients. No difference in serum levels of anti-titin antibodies, however, was noted according to thymus histopathologic findings.

**PREVALENCE OF ANTI-TITIN ANTIBODIES IN NONTHYMECTOMIZED MG PATIENTS WITH LATE ONSET OF THE DISEASE**

A series of 155 blood samples from patients with MG who had not been thymectomized were then tested (Table 2). Anti-titin antibodies were found in a group of 64 patients (41%), among whom men outnumbered women (40 men, 24 women). In light of the tight association of anti-titin antibodies with thymoma in thymectomized patients mentioned above, the possibility was raised of an undetected thymoma in some of these patients. In fact, none of these patients showed radiological (computed tomography) evidence of thymoma.

When these patients were subgrouped by their age at the time of diagnosis, most of the serum samples that tested positive (n = 53) appeared to be from patients with a late age of onset, older than 60 years (Table 2). To rule out the possibility that anti-titin antibodies are autoantibodies that spontaneously arise with aging, blood samples from 88 healthy subjects older than 65 years were assayed (Figure 1). None of the samples tested positive for anti-titin antibodies.

**DIFFERENT FEATURES OF MG PATIENTS WITH ANTI-TITIN ANTIBODIES**

On the basis of the close association of anti-titin antibodies with thymoma, the above data raise the question of the significance of anti-titin antibodies in nonthymomomatous patients and more precisely in nonthymectomized patients. As shown in Table 3, such patients with anti-titin antibodies were significantly older than those without anti-titin antibodies. Their titers of anti-AChR autoantibodies were also 10 times higher on average. This difference was essentially due to the absence of anti-titin antibodies in patients with low anti-AChR antibody titers (Figure 2). Among patients lacking anti-AChR antibodies, all but one tested negative for anti-titin antibodies. Remarkably, the distribution of titers of anti-AChR antibodies was significantly more homogeneous in anti-titin antibody–positive patients, as shown by the comparison of the variance, which was significantly smaller in these patients compared with those lacking anti-titin antibodies (Table 3).

Similar differences were observed among thymectomized patients with a normal or atrophic thymus; anti-titin antibodies were associated with an older age at diagnosis and with higher and more homogeneously distributed titers of anti-AChR autoantibodies (Table 3). When titers of anti-AChR autoantibodies were plotted against the age at diagnosis, nonthymectomized patients and those with a normal thymus who tested positive for anti-titin antibodies formed a homogeneous group of patients with late age of onset and intermediate titers of autoantibodies. In contrast, the age at onset of disease and the titers of anti-AChR autoantibodies of pa-

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Table 1. Features of 398 Myasthenia Gravis Patients

<table>
<thead>
<tr>
<th>Category</th>
<th>No. (Male/Female)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male/female</td>
<td>146/252</td>
</tr>
<tr>
<td>Age at diagnosis, y</td>
<td></td>
</tr>
<tr>
<td>0-20</td>
<td>37 (4/33)</td>
</tr>
<tr>
<td>21-40</td>
<td>150 (31/119)</td>
</tr>
<tr>
<td>41-60</td>
<td>113 (46/67)</td>
</tr>
<tr>
<td>&gt;60</td>
<td>98 (85/13)</td>
</tr>
<tr>
<td>Anti-AChR titer, nmol/L</td>
<td></td>
</tr>
<tr>
<td>High (&gt;100)</td>
<td>31 (4/27)</td>
</tr>
<tr>
<td>Intermediate (10-100)</td>
<td>162 (62/100)</td>
</tr>
<tr>
<td>Low (1-10)</td>
<td>128 (57/71)</td>
</tr>
<tr>
<td>Negative or uncertain (&lt;1)</td>
<td>77 (23/54)</td>
</tr>
<tr>
<td>Associated autoimmune diseases</td>
<td>55 (15/40)</td>
</tr>
<tr>
<td>Thymectomized</td>
<td>243 (73/170)</td>
</tr>
<tr>
<td>Thymus histopathologic findings</td>
<td></td>
</tr>
<tr>
<td>Hyperplasia</td>
<td>91 (15/76)</td>
</tr>
<tr>
<td>Normal or atrophy</td>
<td>74 (21/53)</td>
</tr>
<tr>
<td>Thymoma</td>
<td>70 (36/34)</td>
</tr>
<tr>
<td>Unknown</td>
<td>8 (1/7)</td>
</tr>
</tbody>
</table>

*AChR indicates acetylcholine receptor.

Figure 1. Anti-titin antibodies in myasthenia gravis (MG) patients and control subjects. Anti-titin antibodies were assayed with a radioligand assay and are expressed in amounts of radioactively labeled MGT30 protein bound per microliter of serum. MG-TX indicates thymectomized MG patients; MG-NTX, nonthymectomized MG patients; AID, autoimmune diseases other than MG (see the “Patients, Materials, and Methods” section); ND, neurological diseases other than MG; NS, normal serum samples; and AS, aged healthy subjects (≥65 years). Inset, Breakdown of anti-titin antibodies in thymectomized MG patients by thymus histopathologic findings. H indicates hyperplasia; A, atrophy; and T, thymoma.
tients who tested negative for anti-titin antibodies varied broadly (Figure 2).

The very few patients with thymus hyperplasia who tested positive for anti-titin antibodies were also older at the time of diagnosis than the patients who tested negative (Table 3). Titers of AChR antibodies, however, were not significantly different on average, although their distribution was, again, more homogeneous in patients with anti-titin antibodies. In contrast, in patients with thymoma, the various features were hardly influenced by the presence of anti-titin antibodies. The age at diagnosis was even slightly younger in thymoma patients with anti-titin antibodies.

It has been argued that anti-titin antibodies are associated with more severe clinical symptoms in nonthymoma patients. Univariate analysis of our data set also detected a significant decrease of the muscle strength score at peak of illness (Table 3), as well as an increase of the disability score (not shown) in nonthymectomized patients and in patients with normal thymus histologic findings. However, after adjustment for the age at diagnosis, these effects were no longer seen.

### COMPARISON OF ANTI-TITIN ANTIBODIES AND ASMAs

Anti-titin antibodies are among the cluster of autoantibodies detected by indirect immunofluorescence on skeletal muscle sections. Their presence showed a high correlation with the results of ASMA immunofluorescence tests (Table 4). Twenty-six patients, however, were discordant for anti-titin antibodies and ASMAs. Interestingly, their clinical features were markedly different (detailed data not shown): the age of onset of disease was significantly increased in patients with anti-titin antibodies alone compared with those with ASMAs alone ($P<.01$, Mann Whitney test). Likewise, anti-AChR antibody titers were increased in these patients ($P=.03$).
Three patients with anti-titin antibodies alone but none of those with ASMAs alone had a thymoma; conversely, only 1 (6%) of 16 patients with anti-titin antibodies but 4 (40%) of 10 with ASMAs had thymus hyperplasia.

Altogether, both anti-titin antibodies and ASMAs had a good predictive value for the presence of thymoma, particularly in subjects 60 years of age or younger (Table 5). Cases of thymoma without anti-titin antibodies or ASMAs were uncommon.

The identification and use of molecularly defined antigens are essential to a better understanding of the complexity of autoimmune responses. Although the presence of ASMAs in the serum of MG patients has long been known,20 relatively few studies have investigated the molecular components recognized by these antibodies. The identification of titin as the major target of ASMAs and of a 30-kd region of this giant protein as its major epitope constituted considerable progress in this context.13 For the detection of these antibodies, we set up a novel radioligand assay characterized by a low background noise. It uses a totally defined and pure radiolabeled substrate obtained by in vitro transcription and translation of the complementary DNA encoding the antigen of interest. This assay has recently been successfully used for a number of autoantibodies, notably in insulin-dependent diabetes mellitus, systemic lupus erythematosus, celiac disease, and autoimmune hepatitis.15-18 In all these cases, the combination of the remarkable assay specificity for the autoantigen (linked to the use of a recombinant antigen) with high sensitivity and a precise quantitation of antibody concentration explains the superiority of this technique to conventional assays, even those using recombinant antigens. The recombinant antigen is used in an unlabeled form in enzyme-linked immunosorbent assay, which is much less specific and quantitative than radioligand assay (data not shown).

Using our sensitive assay, anti-titin antibodies are highly specific for MG. They were notably absent from control patients with a number of other autoimmune and neurological diseases and were not found in elderly subjects without MG. Among MG patients, anti-titin antibodies and ASMAs are both associated with the presence of thymoma. A small proportion of patients, however, was discrepant for these 2 autoantibodies. Comparison of their clinical features showed that thymoma was present among anti-titin–positive, ASMA-negative patients but not in anti-titin–negative, ASMA-positive patients, who instead shared features with anti-titin–negative patients. This finding strengthens the value of anti-titin antibody positivity for suspecting the presence of a thymoma, especially in patients 60 years of age or younger. Anti-titin antibody testing could thus provide a complementary tool for the diagnosis of thymoma, given that small tumors may be missed by chest computed tomography. In a retrospective analysis of 134 MG patients with thymoma, we found that x-ray imaging had been normal in 12 cases (our unpublished data). There is therefore a need to carefully evaluate the respective contributions of x-ray imaging and of anti-titin antibody testing to the suspicion of thymoma.

Our findings are at variance with those recently reported by Voltz et al,14 who demonstrated that anti-titin antibodies perfectly predicted the presence of a thymoma in a series of thymectomized MG patients. This discrepancy could be explained by differences (1) in the number of patients investigated, as we included 398 patients, of whom 243 had been thymectomized, vs the 44 patients included in the study of Voltz et al, and (2) in the patient characteristics, as older patients are rarely operated on, unless they show signs of a thymic tumor. Exclusive analysis of thymectomized patients, as performed in the study by Voltz et al, therefore introduces a bias in favor of patients with an earlier onset of disease, which increases the specificity of anti-titin antibodies for thymoma, since anti-titin antibody positivity in nonthymomatous patients is essentially seen in elderly subjects.
Most strikingly, anti-titin antibodies in nonthymoma patients are associated with a specific phenotype, including a late age of disease onset and intermediate titers of anti-AChR antibodies. Our findings thus extend to a large number of thymectomized patients and to nonthymectomized patients. Skeie et al.²⁵ observed a significant prevalence (9/21 [43%]) of anti-titin antibodies among patients with late-onset MG without thymoma. However, unlike Skeie et al., we found no evidence for an association of anti-titin antibodies with more severe clinical symptoms after adjustment for confounding factors, notably, the age at onset of disease. In support of the concept that nonthymoma patients with anti-titin antibodies might represent a pathogenetically distinct subset, recent work from our laboratory indicates that they display a specific association with the HLA-DR7 haplotype (M. Giraud, MSc, G. Beauvirain, PhD, A.M.Y., et al, unpublished results, 2000). Nonthymoma patients with anti-titin antibodies had features clearly distinct from those of group C described in the study by Compston et al.,²³ which included predominantly male patients with a late age of onset of disease with no thymus anomaly, a notable prevalence of ASMs, and a preferential association with the HLA-A3, -B7, and -DRw2 antigens.²⁶ In marked contrast to nonthymoma patients with anti-titin antibodies from our study, patients from group C displayed low titers and a low prevalence of anti-AChR autoantibodies and were preferentially affected with mild symptoms or occult MG. It is quite possible that autoantigens other than titin may be recognized by ASMs in these patients. More generally, antibodies against other molecular components of the striated muscle might be present in different subgroups of MG patients, which would help further clarify the heterogeneity of MG.

At the pathophysiological level, the clinical correlations discussed above elicit 2 types of questions. The first deals with the nature of events that lead to the parallel production of anti-AChR and anti-titin antibodies (as well as other ASMs) in MG patients. An attractive hypothesis is antigen spreading, according to which an initial local insult (whether or not of immunological nature) drives the local recruitment of autoreactive T cells specific for the various striated muscle constituents.²⁸ The particular association of anti-titin antibodies with aging might be related to the pattern of immune dysregulation present in this age group. The second question relates to the correlation of anti-titin antibodies with thymomas. One may wonder whether the occurrence of thymomas alters titin presentation to T cells. It is interesting that these 2 questions both draw attention to the modes of presentation to T cells of striated muscle antigens, particularly titin.

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