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Eye Muscle Antibodies in Patients with Ocular Myasthenia Gravis: Possible Mechanism for Eye Muscle Inflammation in Acetylcholine-Receptor Antibody-Negative Patients

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Myasthenia gravis is an organ-specific autoimmune disorder generally thought to be caused by an antibody-mediated attack against the skeletal muscle nicotinic acetylcholine (Ach) receptor (AchR) at the neuromuscular junction. Extraocular muscle weakness and double vision are present in about 90% of patients with myasthenia gravis and are the predominant complaints in about 20% of patients, when the condition is called ocular myasthenia gravis (OMG). While serum antibodies against the AchR are detected in most patients with generalized myasthenia gravis (GMG), they are not found in about one-third of patients with the ocular variety, and epidemiological, clinical, and serological studies suggest that OMG and GMG are two separate diseases. Both forms of myasthenia gravis are sometimes associated with thyroid autoimmunity or thyroid-associated ophthalmopathy (TAO). We have therefore tested the sera of patients with GMG and OMG by Western blotting for antibodies against porcine eye muscle membrane proteins in general, and by enzyme-linked immunosorbent assays (ELISA) specifically for reaction with two skeletal muscle antigens which are prominent marker antigens for TAO, namely, the calcium-binding protein calsequestrin and the so-called "64-kDa protein." The 64-kDa protein has recently been identified as the flavoprotein subunit of mitochondrial succinate dehydrogenase. Patients with ophthalmopathy and myasthenia were excluded. Nine of the patients had associated Graves' hyperthyroidism without evident ophthalmopathy and one had Hashimoto's thyroiditis. Antibodies against porcine eye muscle membrane antigens of M_r 15–110 kDa were detected in patients with GMG or OMG, one or more antibodies being detected in 100% of patients with GMG and in 88% of those with OMG. The most frequently found antibodies were those targeting eye muscle membrane proteins of 15, 67, and 110 kDa. Antibodies reactive with purified calsequestrin (63 kDa) were detected in 21% of patients with OMG but in no patient with GMG. Antibodies recognizing purified succinate dehydrogenase (67 kDa) were found in 42% of patients with OMG, in 100% (5 of 5) of patients with GMG, and in 48% of all patients with myasthenia gravis not associated with Graves' hyperthyroidism. There was no close correlation between any eye muscle-reactive antibody and antibodies against the AchR in either group of myasthenic patients. The findings support the notion that immunoreactivity against skeletal muscle proteins other than the AchR may play a role in the development of the muscle weakness in AchR antibody-negative patients with OMG and GMG, although it is unlikely that any of the antibodies demonstrated in this study are directly implicated. Similarly, while the demonstration of antibodies reactive with eye muscle antigens associated with TAO in patients with OMG raises the possibility that the link between the ocular lesions of myasthenia gravis and Graves' disease may be autoimmunity against a common antigen(s), it is more likely that both disorders are mediated by cytotoxic T cells recognizing another cell membrane antigen, such as the novel thyroid and eye muscle shared protein G2s, and that serum antibodies reactive with succinate dehydrogenase Fp subunit and calsequestrin are markers of an immune-mediated eye muscle reaction.

Key Words: myasthenia gravis; ocular myasthenia gravis; eye muscle; skeletal muscle: immunoblotting; thyroid-associated ophthalmopathy; 64 kDa protein; succinate dehydrogenase; calsequestrin.

INTRODUCTION

Myasthenia gravis is an organ-specific autoimmune disorder generally thought to be caused by an antibody-mediated attack against the skeletal muscle nicotinic acetylcholine (Ach) receptor (AchR) at the neuromuscu-

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lar junction (1–3). Loss of functional receptors with impairment of the neuromuscular signal transmission results in weakness and fatigability in selected skeletal muscles (2). There is also evidence of skeletal muscle inflammation (4) and production of serum antibodies reactive against a variety of muscle antigens (5, 6). Extraocular muscle weakness and double vision are present in about 90% of patients with myasthenia gravis and are the initial complaints in about 20% of cases. This latter condition is called ocular myasthenia gravis (OMG). Epidemiologic, clinical, and serologic studies have supported the notion that OMG and generalized myasthenia gravis (GMG) are different diseases (7–9). That serum antibodies against the AchR are detected in 90% of patients with GMG but in only 65% with the ocular variety (10, 11) suggests that other antibodies may play a role in the development of eye muscle weakness in OMG.

Myasthenia gravis is linked with autoimmune thyroid disease in that approximately 5% of the patients also have Graves' hyperthyroidism or Hashimoto's thyroiditis (12, 13). Thyroid-associated ophthalmopathy (TAO) is an autoimmune disorder of the extraocular (eye) muscle and orbital connective tissue closely associated with Graves' hyperthyroidism (reviewed in 14). OMG and TAO share some clinical features and may occur in the same patient. In a recent study, Marino *et al.* (15) showed that when myasthenia gravis was associated with thyroid autoimmunity, it tended to have a milder clinical expression with preferential ocular involvement and lower frequencies of thymic disease and AchR antibodies. This association was particularly strong in patients with ophthalmopathy and thyroid autoimmunity (15). We have thus studied patients with GMG and OMG for serum autoantibodies reactive with eye muscle antigens, including two recently identified as being associated with TAO, namely, the Fp subunit of mitochondrial succinate dehydrogenase (the "64 kDa protein") (16) and calsequestrin, a 63-kDa calcium-binding protein localized in the sarcoplasmic reticulum of the skeletal muscle fiber (17). We demonstrated antibodies against one or more eye muscle proteins of M_r 15–110 kDa in 100% of patients with OMG and 88% of patients with GMG, and against succinate dehydrogenase Fp in 42% of patients with OMG and 100% with GMG.

CLINICAL SUBJECTS AND METHODS

Clinical Subjects

The studies concerned patients with the following:

(i) Generalized myasthenia gravis (GMG). Five males and two females, of whom two had associated Graves' hyperthyroidism, were studied. The diagnosis was made from the typical history and clinical examination, including ophthalmologic assessment, and confirmed by the tensilon test. Only one patient had detectable serum antibodies against the AchR.

(ii) Ocular myasthenia gravis (OMG). Sixteen males and 17 females, of whom 7 had associated Graves' hyperthyroidism and 1 had Hashimoto's thyroiditis, were also studied. The diagnosis was made from the typical history and clinical examination, including ophthalmologic assessment and confirmed by the tensilon test. Eighteen of the patients had detectable serum antibodies against the AchR.

Patients with ophthalmopathy and myasthenia were excluded from the study.

(iii) Normal subjects. Twenty-one males and 33 females, aged-matched with myasthenic patients, with no personal or family history of myasthenia gravis, thyroid disease, ophthalmopathy, or other autoimmune disease, were recruited from ancillary hospital and laboratory staff.

The study was IRB approved and informed written consent was obtained from all patients and normal subjects studied.

SDS–PAGE and Western Blotting

Antibodies reactive with porcine eye muscle membrane proteins and purified beef heart succinate dehydrogenase Fp subunit were detected following standard Laemmli SDS–PAGE (18) using an 8.5% separating gel and a 4% stacking gel in a minigel apparatus, as reported previously (19, 20). Primary antibodies were patients' sera diluted 1/0 and a rabbit anti-flavoprotein subunit antiserum diluted 1/2000, and secondary antibody was an alkaline phosphatase-conjugated anti-human IgG (γ chain specific) antiserum diluted 1/2000, for patients' sera, or anti-rabbit IgG (whole molecule) antiserum diluted 1/2000, for the anti-Fp antiserum. Tests were read by two observers and results were expressed as band density. A band density of + or greater was taken as a positive test.

Isolation of Purified Beef Heart Muscle Succinate Dehydrogenase

Succinate dehydrogenase containing the Fp and Ip (iron–sulfur protein) subunits was solubilized by perchlorate treatment (21) of succinate:coenzyme Q oxidoreductase (complex II of the respiratory chain), which had been isolated from beef heart mitochondria by the method of Baginsky and Hatefi (22). The enzyme preparation was >90% pure based on gel analysis and content of covalently bound flavin adenine dinucleotide. Pure enzyme was excised from SDS–polyacrylamide gels according to the method of Merli *et al.* (23) and used as antigen in Western blotting and ELISA.

Isolation of Purified Porcine Eye Muscle Calsequestrin

Pig eye muscle was homogenized in a prechilled blender and centrifuged at 13,000*g* for 30 min. Ammonium sulfate was added to the supernatant to a final concentration of 92% and the pH was adjusted to 4.7 with phosphoric acid. The precipitate from this step was collected by centrifugation at 13,000*g* for 30 min, dissolved in Tris–phosphate buffer, and dialyzed against the same buffer. The sample was then applied to a DEAE–Sephacel column and preequilibrated with buffer A (0.1 M potassium phosphate, pH 7.1, 1 mM EGTA, 50 mM NaCl). Buffer B (0.1 M potassium phosphate, pH 7.1, 1 mM EGTA, 700 mM NaCl) was then applied and 3-ml fractions were collected, monitoring absorbance at 280 nM. Two-hundred-microliter aliquots were taken from every third fraction and subjected to Western blot analysis to check reactivity with an anti-calsequestrin monoclonal antibody in order to identify those tubes containing calsequestrin. Purified calsequestrin was used as antigen in ELISA.

Enzyme-Linked Immunosorbent Assay

The method has been described in previous publications from this laboratory (24, 25). The optimal antigen concentration was found to be 1 μg/ml for both calsequestrin and succinate dehydrogenase and the optimal serum dilution was 1/25. The second antibody is an alkaline phosphatase-labeled goat anti-human IgG (1/1500 dilution). Results are expressed as optical density (OD) at 410 nm and positive reactivity against test antigen is taken as mean + 2 SD for normal subjects tested concurrently.

Statistical Analysis

Differences in mean (±SE) values between patient groups and normals in ELISA was assessed statistically using the Student *t* test. Differences in prevalences of serum autoantibodies reactive with eye muscle membrane antigens in immunoblotting, and with Fp and calsequestrin in ELISA, between patient and control groups, were assessed using χ^2 tests.

RESULTS

We tested sera from 7 patients with GMG, 33 patients with OMG, and 54 normal subjects, as controls, for antibodies against porcine eye muscle membrane antigens utilizing SDS–PAGE and Western blotting, and against succinate dehydrogenase Fp and calsequestrin in ELISA. Patients with OMG were further classified as anti-AchR antibody positive (18 patients; group I) or anti-AchR antibody negative (15 patients; group II). Seven patients with OMG had associated Graves' hyperthyroidism but no ophthalmopathy. Patients with GMG, of whom only one was anti-AchR antibody positive and two had associated Graves' hyperthyroidism, were studied as a single group (7 patients; group III). The results are summarized in Fig. 1 and Table 1. One or more serum antibodies against porcine eye muscle membrane proteins of M_r 15–110 kDa were detected in 100% of patients with GMG and in 88% with OMG (Table 1). Next, we tested for serum antibodies against purified Fp and calsequestrin in ELISAs. Anti-Fp antibody levels in the three groups of patients and the normals are depicted in Fig. 1. Only 29 of the sera were available for Fp antibody testing. Results are expressed as OD at 410 nm. Mean (±SE) values were 0.327 (±0.068) for patients of group I, which was significantly greater than that for normals (0.146 ± 0.016, *t* test, $P < 0.05$), 0.250 (±0.062) for group II (P = NS) and 0.584 (±0.129 for patients of group III ($P < 0.001$). Taking an OD of 0.285 (mean + 2 SD for normals) as the upper limit of normal, tests were positive in 6 of 12 (50%) patients of group I tested, 4 of 12 (33%) of group II, and 5 of 5 (100%) patients of group III, but in none of 18 normal subjects. Taking an OD of 0.49 (mean + 2 SD for normals) as the upper limit of normal, antibodies against calsequestrin were detected in 6 of 18 patients of group I (33%), 1 of 15 patients of group II (7%), in no patient of group III (0%), and in 2 of 54 (4%) normal subjects (results not shown). Mean (±SE) values for patient groups were significantly different compared to normals only for patients of group I ($P < 0.05$). Overall, antibodies against calsequestrin were demonstrated in 21%, and against succinate dehydrogenase Fp in 42%, of all patients with OMG, and antibodies against succinate dehydrogenase Fp subunit were demonstrated in 45% of all patients with OMG or GMG not associated with Graves' hyperthyroidism. There was no significant correlation between any eye muscle antibody and (a) past or present Graves' hyperthyroidism or (b) antibodies against the AchR, in either group of myasthenic patients.

DISCUSSION

We studied patients with GMG and OMG for serum autoantibodies reactive with porcine eye muscle membrane antigens, including two antigens closely associated with TAO, namely, the succinate dehydrogenase Fp subunit, which is incorrectly referred to in the literature as the 64-kDa protein, and calsequestrin, a 63-kDa calcium-binding protein. To summarize the main results, antibodies reactive with native calsequestrin were detected in 21% of patients with OMG but in no patient with GMG, while antibodies recognizing succinate dehydrogenase Fp, the 64-kDa protein, were demonstrated in 42% of patients with OMG and 100% of patients with GMG. Because patients with thyroid au-

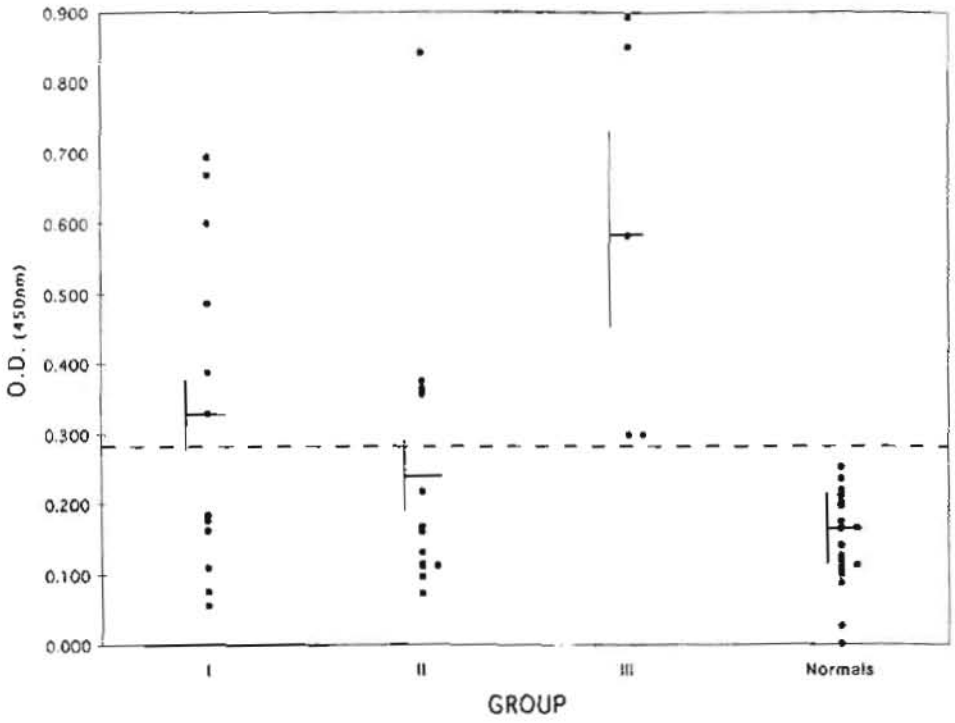

FIG. 1. Serum antibodies against purified flavoprotein subunit of succinate dehydrogenase in patients with myasthenia gravis and normal subjects determined using an enzyme-linked immunosorbent assay (ELISA). Results are expressed as optical density (OD) at 410 nm. The broken horizontal line, at 0.285 nm, is the upper limit of normal, defined as mean + 2 SD for normal subjects. Mean (±SE) values are indicated for each group. Group I, patients with ocular myasthenia gravis (OMG) and detectable serum antibodies against the acetylcholine receptor (AchR); group II, patients with OMG negative for anti-AchR antibodies; group III, patients with generalized myasthenia gravis.

toimmunity and overt ophthalmopathy were excluded from the study, it seems highly likely that the antibodies were associated with myasthenia rather than TAO.

The pathogenesis of myasthenia gravis is unclear although antibodies reactive with the AchR play an important role (1). While myasthenia gravis is usually a generalized disease, the skeletal muscle autoimmune reaction and resulting weakness and fatigability may

TABLE 1

Prevalences of Antibodies against Porcine Eye Muscle Membrane Antigens in Patients with Generalized or Ocular Myasthenia Gravis by Immunoblotting

Group	Antigen				
	15 kDa[a]	30 kDa	45 kDa	67 kDa	110 kDa
AchR-Ab[b] pos. OMG[c] [group I] (n = 18)	9 (50%)[d] $P < 0.05$[e]	1 (5%) NS	8 (44%) NS	7 (39%) $P < 0.05$	5 (28%) NS
AchR-Ab neg. OMG [group II] (n = 15)	7 (47%) $P < 0.05$	9 (60%) $P < 0.05$	4 (27%) NS	7 (47%) $P < 0.05$	8 (53%) NS
GMG[f] [group III] (n = 7)	3 (43%) NS	2 (33%) NS	2 (29%) NS	6 (86%) $P < 0.001$	5 (71%) $P < 0.05$
Normals (n = 38)	4 (10%)	10 (26%)	12 (32%)	4 (10%)	11 (29%)

[a] M_r of target antigen.
[b] AchR-Ab, acetylcholine receptor antibodies.
[c] OMG, ocular myasthenia gravis.
[d] n (%) reactive with the antigen in SDS–PAGE of porcine eye muscle membranes and Western blotting with patients' serum.
[e] Statistical analyses refer to differences compared with normals determined using the χ^2 test. NS, not significant.
[f] GMG, generalized myasthenia gravis.

be limited to the extraocular muscles. Indeed, there is evidence that GMG and OMG are separate disorders with different pathogenetic mechanisms (12, 13). Several autoantibodies against non-AchR skeletal muscle components have been described in myasthenia gravis including those reactive with the ryanidine receptor [the calcium (Ca^{2+}) channel] (3) and various muscle proteins identified by immunoblotting (4), hemagglutination (5), ELISA (4), and immunofluorescence (4, 5). Since AchR antibodies are not detected in all patients with myasthenia gravis, there may be a subgroup, especially among those with the ocular variety, in whom another antibody(ies), such as those detected in the present study or, more likely, antibodies not yet identified, may be implicated in the development of the muscle weakness.

Both myasthenic syndromes occur in association with autoimmune thyroid disease and TAO. Indeed, OMG and TAO share some clinical features and may occur in the same patient. While T lymphocyte reactivity against eye muscle and orbital connective tissue antigens is likely to be the primary event in the development of ophthalmopathy, the role of circulating autoantibodies reactive with orbital antigens has been more extensively studied (reviewed in 26). Of these, a protein with a reported M_r of 64 kDa is recognized as an important autoantigen in TAO, the corresponding serum autoantibodies being good markers of the eye disorder (19, 20, 27, 28) and predictors of its development in patients with thyroid autoimmunity, especially Graves' hyperthyroidism (29, 30). The 64-kDa protein has recently been partially sequenced and identified as the Fp subunit of mitochondrial succinate dehydrogenase (16). The recalculated M_r of the 64-kDa protein is 67 kDa. In a recent study, antibodies against purified succinate dehydrogenase were detected in 67% of patients with active TAO, in 30% with stable TAO and in 30% of patients with Graves' hyperthyroidism without ophthalmopathy, but in only 7% of age- and sex-matched normal subjects, by immunoblotting (16). Another protein associated with TAO is calsequestrin, a 63-kDa calcium-binding protein which is localized in the sarcoplasmic reticulum of the muscle fiber (17). Calsequestrin is known to share an epitope with heat shock protein 60 (31). Antibodies against calsequestrin were found in 40% of patients with active TAO, but in only 4% of those with stable, "burnt out," eye disease, and in 5% of normal subjects, by immunoblotting (Gunji *et al.*, submitted). In conclusion, while antibodies against eye muscle antigens associated with TAO are found in the majority of patients with myasthenia gravis, it is likely that the muscle damage of myasthenia is mediated by $CD8^+$ (cytotoxic) T lymphocytes targeting another, cell membrane, antigen, such as the recently cloned thyroid and eye muscle shared protein G2s (32), and that the antibodies measured in this study are secondary markers of immune-mediated eye muscle damage in both disorders.

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