Enzyme Immunoassay for Measuring Antibodies against Skeletal Muscle in Patients with Myasthenia Gravis

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We developed a highly sensitive enzyme immunoassay (EIA) for measuring IgG and IgM antibodies against human skeletal muscle (SM) component and tested sera from 100 patients with myasthenia gravis (MG), 59 with thymoma and 41 without thymoma. We found that the frequency of anti-SM IgG antibodies was significantly higher in MG patients with (81%) than without (37%) thymoma. The titers of the anti-SM IgG antibodies measured by EIA correlated well with those measured by RIA (r = 0.81, P < 0.01). We also found that 12% of the myasthenic patients with thymoma and 15% without it had anti-SM IgM antibodies. There was no correlation between the titers of the IgG and IgM antibodies. Our EIA provides a measure of anti-SM antibodies that is of comparable sensitivity to that of RIA.

Additional Keyphrases: anti-acetylcholine receptor antibodies • thymoma • radioimmunoassay compared

Autoantibodies against the nicotinic acetylcholine receptor (AChR) have been detected specifically in sera from patients with myasthenia gravis (MG), and obviously are involved in the pathogenesis of MG. Therefore, the determination of the presence of anti-AChR antibodies is necessary for a definitive diagnosis of MG. MG patients have both anti-AChR and anti-skeletal muscle (SM) antibodies. Anti-SM antibodies usually have been measured by the indirect immunofluorescence technique (1–4). We recently examined with an improved radioimmunoassay (RIA) for measuring anti-SM antibodies a large number of serum samples from myasthenic patients and showed that patients with MG and thymoma have both anti-SM and anti-AChR antibodies (5). We here report the development of a conventional enzyme immunoassay (EIA) and its evaluation for use in routine diagnostic procedures. We also measured the concentrations of anti-SM IgM antibodies in sera from MG patients.

Materials and Methods

Subjects: Serum samples were taken from 100 MG patients (69 with and 41 without thymoma), 58 patients with other neurological disorders (12 with muscular dystrophy, 14 with amyotrophic lateral sclerosis, 12 with Parkinson’s disease, five with polyneuropathy, 11 with multiple sclerosis, and four with neuro-Bechet’s disease), and from 100 healthy controls. The diagnosis of MG was confirmed from the patient’s typical history, clinical signs, and electrophysiological findings, and was classified clinically as ocular or generalized and whether in the mild, moderate, or severe stage, according to Pattee’s classification. Serum samples were kept at −80°C until used.

Preparation of antigens: Human gastrocnemius muscle was obtained surgically from amputated limbs and stored at −70°C. The preparation of antigen from skeletal muscle was previously described (5). Briefly, minced muscle was homogenized in 3 mL of cold phosphate-buffered saline (PBS; 25 mmol/L phosphate buffer, pH 7.7, containing 0.2 g of Na2HPO4 and 1 mmol of phenylmethylsulfonyl fluoride per liter) per gram of tissue. After centrifugation at 10 000 × g for 60 min to precipitate the membrane containing AChR, the supernate was passed through a Protein G-Sepharose resin (Pharmacia, Uppsala, Sweden) to eliminate endogenous Protein G-binding materials (mainly IgG in tissue and blood). The eluate was used as the antigen source for the anti-SM antibody assay.

Enzyme immunoassay: Anti-SM antibodies were measured by solid-phase EIA with a Vectastain ABC kit (Vector Laboratories Inc., Burlingame, CA). The PBS extracts were diluted with 25 mmol/L phosphate buffer, pH 7.7, to a concentration of 50 mg/mL. Round-bottom polyvinyl chloride microtiter plates (Dynatech Laboratories Inc., Alexandria, VA) were coated with 100 μL of diluted antigen for 1 h at room temperature. After the plates had been washed three times with washing buffer (10 mmol/L phosphate buffer, pH 7.7, containing 5 g of bovine serum albumin and 1 mL of Tween 20 per liter), each well was blocked for 2 h at room temperature with washing buffer that contained 100 mL of Blockace (Dainippon-siyaku Co., Ltd., Japan) per liter.

The tested serum samples were diluted with dilution buffer (50 mmol/L Tris · HCl buffer, pH 7.4, containing 0.1 mol of NaCl and 2 mL of Triton X-100 per liter), 4000-fold for the measurement of IgG and 1000-fold for IgM antibodies, then incubated on the antigen-coated plates overnight at 4°C. After five washes, 100 μL of a 1000-fold-diluted biotinylated anti-human IgG or IgM antibody was added to the wells, after which the plates were incubated for 30 min at room temperature. After washing the plates as before, we added 100 μL of a 1000-fold-diluted avidin–peroxidase complex (Vector Laboratories), then incubated the samples for 30 min at room temperature. After three washes, a substrate mixture of 50 μL each of ABTS and H2O2 solution (peroxidase substrate kit, cat. no. 172-1064; Bio-Rad Laboratories, Richmond, CA) was added. The reaction was stopped with 50 μL of 2.5 mol/L H2SO4 reagent, after which the absorbance at 415 nm was recorded on an EIA reader (Biomek 1000; Beckman Instruments, Berkeley, CA).

In preliminary experiments, we had determined the optimal conditions for the detection of anti-SM antibodies. In addition, we confirmed the specificity of this EIA by

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5 Nonstandard abbreviations: EIA, enzyme immunoassay; SM, skeletal muscle; MG, myasthenia gravis; AChR, acetylcholine receptor; ABTS, 2,2'-azinodi(3-ethylbenzthiazoline sulfonate) and PBS, phosphate-buffered saline.

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performing the inhibition test simultaneously on serum samples that had been incubated for 1 h at room temperature with the above antigens at a final concentration of 10 g/L (data not shown).

RIA: Anti-SM antibodies were also measured by solid-phase RIA as described previously (5). Protein G (Sigma, St. Louis, MO) was labeled with Na[125I] by the Chloramine T method. Briefly, the tested serum samples were diluted 1600-fold, then incubated on the antigen-coated plates overnight at 4 °C. After four washes, 100 μL of 125I-labeled Protein G (100 000 counts/min; specific activity, 20 Ci/g) was added to the wells and the plates were incubated for 2 h at 37 °C. After being washed as before, the wells were cut out from the plates and counted for radioactivity. Anti-SM antibody titers were expressed arbitrarily as a ratio to the mean value of normal control sera. A ratio >1.8 (1 + 3SD) was considered positive.

Results

We tested the sera of patients and controls by our EIA, expressing anti-SM antibody titers arbitrarily as ratios to the mean absorbance of the normal control sera. From anti-SM IgG antibody titers of normal controls, a ratio >1.3 (1 + 2SD) is considered positive. No anti-SM IgG antibodies were detected in any of the normal controls. The assay was reproducible, with within- and between-run CVs of <8.8% and <9.0%, respectively. Anti-SM results for patients with other neurological disorders were within the normal range, except for four patients, one each with muscular dystrophy, amyotrophic lateral sclerosis, Parkinson's disease, and polyneuropathy (data not shown).

The values for anti-SM IgG antibody in the sera from MG patients are shown in Figure 1. Both frequency and mean titer of the thymoma group are significantly higher (frequency: 81%, mean titer: 2.19) than those of the nonthy- monoma group (frequency: 37%, mean titer: 1.31). The mean titers of the anti-SM antibody do not differ substantially for the different MG classifications.

A comparison of the individual anti-SM antibody titers obtained by EIA and by RIA is shown in Figure 2. There is good correlation (r = 0.81) for the titers of anti-SM IgG antibody obtained by EIA and RIA.

Anti-SM IgM antibody titers in sera from MG patients with and without thymoma are shown in Figure 3. A ratio >1.6 (1 + 2SD) is considered positive. No anti-SM IgM antibodies were detected in any of the normal controls. Anti-SM IgM antibodies were detected in 12% of the thymoma and 15% of the nontymoma groups, but there was no correlation between the titers of the anti-SM IgG and IgM antibodies (Figure 4).

Discussion

The presence of autoantibodies directed against skeletal muscle in sera from patients with MG first was reported by Strauss et al. (1). Since then, many reports have shown that MG patients with thymoma frequently have anti-SM antibodies, as detected by immunofluorescence and other immunological techniques. We recently reported (5) that RIA showed markedly high anti-SM antibody titers (mean titer, 10.1) and frequency (92%, 59 of 64) in MG patients with thymoma, but low or negative titers and lower frequency (24%) in nontymoma patients. A comparison of the frequency detected by RIA with that detected by EIA shows the latter values were slightly low for the thymoma group and slightly high for the nontymoma group. For some MG patients, anti-SM antibodies could be detected only with EIA (Figure 2). The titers obtained by EIA, on the whole,
development of MG. The findings that some patients with juvenile and ocular MG may have IgM antibodies, and that some MG patients have anti-SM antibodies that are increased only in the EIA, suggest that the EIA can be used to investigate the nature of MG. Major advantages of our EIA is that it does not use radioactive chemicals, which are hazardous to laboratory workers, and its sensitivity is the same as or better than that of RIA.

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References


Abbott TDx Monoclonal Antibody Assay Evaluated for Measuring Cyclosporine in Whole Blood
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We report here the evaluation of the Abbott TDx assay with a monoclonal antibody for selectively quantifying cyclosporine (CsA) in whole blood. Over the clinically relevant concentration ranges, results with this assay demonstrated within- and between-run CVs of <2.5% and 5%, respectively; sensitivity of 25 μg/L; good analytical recovery (100.3%); and linearity with whole-blood specimens. The percentage cross-reactivity of the major CsA metabolites varied from 15.3% for AM9 (M-1), 8.2% for AM1 (M-17), and 3.7% for AM4N (M-21), to <3% for the other metabolites tested. Results with the TDx assay (y) correlated well with those by the Sandimune selective RIA (x; Sandoz) with blood specimens from 44 renal-transplant recipients (n = 44, x = 187.3, y = 198.9, y = 5.49 + 1.03x, r = 0.967). The TDx values were on average 24% higher than those by HPLC (x') with the same patients' specimens (n = 44, x = 159.9, y = 198.9, y = 15.9 + 1.14x', r = 0.967). We conclude that the Abbott TDx monoclonal antibody assay provides a rapid, precise, and accurate means for quantifying CsA in whole blood.

Monitoring concentrations of cyclosporine (CsA) in body fluids has been used as a guide for the appropriate adjustment of dosage to reduce the frequency of side effects and yet maintain the immunosuppressive potential (1-4). Routine monitoring of the drug in whole blood has been recommended with a selective assay that specifically measures the parent CsA and not its metabolites (1-3). At present, this can be done by either HPLC or RIA (4). The former procedure is laborious and not well suited for routine use in clinical laboratories. Currently two RIAs that involve a selective monoclonal antibody, which exhibits minimal cross-reactivity with CsA metabolites, are commercially available (5-8).

As requests for CsA analysis increase, the need for commercially available, reliable automated assays for selectively measuring CsA in whole blood increases. A few