Background: Acetylcholine receptor (AChR) from human muscles is the antigen used currently in radioimmunoprecipitation assays (RIPAs) for the determination of anti-AChR antibodies in the diagnosis of myasthenia gravis (MG). Our aim was to develop and validate an ELISA using TE671 cells as the source of AChR.

Methods: After TE671 cell homogenization, the crude AChR extract was used for plate coating. Anti-AChR antibodies were determined in 207 MG patients and in 77 controls.

Results: The mean intra- and interassay CVs (for two samples with different anti-AChR antibody concentrations) were 9.7% and 15.7%, respectively. Test sensitivity and specificity, for generalized MG, were 79.5% (95% confidence interval, 72.8–85.0%) and 96.1% (89.0–99.1%). The detection limit was 2 nmol/L. Anti-AChR antibody concentrations from 53 MG patients, as tested with our ELISA, showed good agreement with an RIPA with a mean difference (SD) of 1.0 (5.6) nmol/L.

Conclusion: Our ELISA is a simple screening test for the diagnosis of MG and enables rapid and inexpensive patient follow-up.

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Circulating antibodies to the acetylcholine receptor (AChR) are essentially polyclonal and heterogeneous IgG, and recognize diverse epitopes of the skeletal muscle nicotinic AChR. These antibodies are pathogenic in myasthenia gravis (MG) (1), a disease characterized by fatigability and relative weakness of voluntary muscles. Up to 90% of patients with generalized MG, and a lower percentage of those with ocular MG, have detectable serum concentrations of anti-AChR antibodies. The quantification of these antibodies is currently used as a laboratory support for diagnosis, therapy monitoring, and follow-up in MG. Antibody concentration is commonly determined by exploitation of the ability of anti-AChR antibodies to immunoprecipitate-solubilized human muscle AChR, which, in turn, is complexed with \( ^{125}\text{I}-\text{a-bungarotoxin} \) (\( ^{125}\text{I}-\alpha\text{-BuTx} \)), a potent acetylcholine antagonist. The main disadvantages of this sensitive method are the use of radioisotopes and the limited availability of human muscles. Furthermore, the presence of \( \alpha\text{-BuTx} \) deprives a part of the anti-AChR antibodies of potential binding sites.

Various authors have proposed alternative solid-phase immunoenzymatic methods to overcome some of the above-mentioned limitations. One method involves the coating of polystyrene wells directly with muscle extracts (2); another method uses muscle-derived AChR, which in turn is captured by means of precoating with \( \alpha\text{-BuTx} \) (3) or with anti-AChR monoclonal antibodies (4). The main drawbacks of these methods are the paucity of AChR in relation to other muscle proteins (2) and the inadequacy of AChR capture, whether by \( \alpha\text{-BuTx} \) or by anti-AChR monoclonal antibodies (3, 4). The effect of these drawbacks is a reduction in the diagnostic sensitivity of these methods.

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7 Nonstandard abbreviations: AChR, acetylcholine receptor; MG, myasthenia gravis; \( ^{125}\text{I}-\alpha\text{-BuTx} \), \( ^{125}\text{I}-\text{a-bungarotoxin} \); RIPA, radioimmunoprecipitation assay; SLE, systemic lupus erythematosus; and PBS, phosphate-buffered saline.
The human rhabdomyosarcoma cell line TE671, which expresses AChR on its surface (5), has been used as an alternative to human muscle as a source of AChR (6–10). Radioimmunoprecipitation assays (RIPAs) with TE671 cell-derived AChR as antigen have been developed (6–8) or reevaluated (9). Our own group previously proposed a TE671 cell-based ELISA for the detection of serum anti-AChR antibodies (10). Here we report the development and a thorough validation of this ELISA.

Materials and Methods

Patients
Serum samples were from 207 MG patients: 176 with generalized MG (120 women and 56 men; ages, 16–75 years), 31 with ocular MG (13 women and 18 men; ages, 23–72 years). The diagnosis was based on typical clinical, pharmacological, and electrophysiological features. Ocular MG patients had a disease duration of >2 years. The patients were classified and clinically monitored in accordance with the guidelines described by Osserman and Jenkins (11). Control samples consisted of sera from 30 healthy individuals (17 women and 13 men; ages, 18–55 years), 19 non-MG patients with autoimmune diseases [10 with systemic lupus erythematosus (SLE) and 9 with rheumatoid arthritis], and 28 patients with other neurological diseases (11 with multiple sclerosis, 6 with amyotrophic lateral sclerosis, 5 with polyneuropathy, 4 with dystrophinopathy, 1 with polymyositis, and 1 with pseudotumor orbitae). Serum samples were stored at −20 °C until use. Thawed samples were centrifuged to remove particulate material. The study was approved by the institution’s ethics committee.

Cell Culture

TE671 cell line (American Type Culture Collection) was cultured at 37 °C, 5% CO₂ in RPMI-1640 supplemented with 2 mmol/L glutamine, 100 kU/L penicillin, 10 mg/L streptomycin, 25 mmol/L HEPES (all products from Sigma Chemical Co.), and 100 mL/L fetal calf serum. Confluent cells were harvested every 5–7 days.

Preparation of Crude AChR Extract
Cells were washed in homogenization buffer containing 5 mmol/L Tris (pH 7.8), 10 mmol/L EDTA, 10 mmol/L ethyleneglycol-bis-(β-aminoethylether)-N,N,N′,N′-tetraacetic acid, 0.1 g/L phenylmethysulfonyl fluoride, and 0.2 g/L sodium azide, and then removed with a cell scraper. Cells were centrifuged at 500g for 15 min and then resuspended in homogenization buffer at 4 °C. After incubation for 30 min, cells were homogenized with a Polytron for 30 s on ice. Homogenized cells were centrifuged at 500g for 15 min at 4 °C. Supernatant containing membrane debris was centrifuged at 40 000g for 45 min at 4 °C. The pellet was resuspended in homogenization buffer at 4 °C, sonicated for 3 min on ice, and frozen in aliquots at −80 °C.

TE671 Cell-Based ELISA Optimization
We optimized the coating concentration of the AChR preparation by testing different dilutions of the preparation (total protein concentration, 5 mg/L to 5 g/L) against a reference serum (anti-AChR antibody concentration of 120 nmol/L, positive control) and against a pool of sera from 10 blood donors (negative control). The total protein concentration of the AChR preparation was taken as an index of its AChR content.

We evaluated the ability of the TE671 preparation to bind specifically to 125I-α-BuTx by testing with moniodinated 125I-α-BuTx (0.5–550 fmol/well; Amersham) in microwells coated with 100 μL of the AChR preparation, which contained a total protein concentration of either 0.50 or 0.75 g/L, or with 50 fmol 125I-α-BuTx/well in microwells coated with 100 μL of serially diluted AChR preparation (total protein concentration, 5 mg/L to 5 g/L). The specific activity of 125I-α-BuTx was ~2000 Ci/mmol. The AChR preparation was diluted with phosphate-buffered saline (PBS). Single microwells were coated with 100 μL AChR preparation/well and incubated overnight at 4 °C. After wells were washed with PBS, 200 μL of 50 g/L nonfat dry milk in PBS was added to each well to block nonspecific binding sites. After a 1-h incubation at 4 °C, wells were washed three times. We used 5 g/L nonfat dry milk in PBS to make the appropriate dilutions of 125I-α-BuTx. Each dilution was tested twice. Microwells were incubated for 2 h at room temperature. After a triple washing cycle, each microwell was inserted into appropriate tubes, and the radioactivity was measured.

We also evaluated the effect on assay specificity of antibodies directed against other non-AChR autoantigens that the AChR preparation could contain. We performed a study of preadsorption on four serum samples: two samples, from MG patients, had anti-AChR antibody concentrations of 40 and 12 nmol/L; the other two samples, from SLE patients, were positive for anti-AChR antibodies at a low concentration (see Results). Briefly, PC12 cells (clone 16A, courtesy of Dr. E. Clementi, Department of Pharmacology, University of Milan, Italy) were cultured at 37 °C, 5% CO₂ in RPMI-1640 supplemented exactly as for TE671 cell culture, with the addition of 50 mL/L horse serum. Cells were harvested at confluence every 3–4 days, and crude extraction of the PC12 cells was carried out as for TE671 cells. Each serum sample (50 μL) was diluted twofold in 20 g/L nonfat dry milk in PBS and incubated overnight at 4 °C with 50 μL of the PC12 cell preparation. This preparation had a total protein content of 5 g/L. Membranes were spun down (10 000g for 15 min), and the supernatant was re-incubated for 1 h at 4 °C with 50 μL of the PC12 cell preparation. After centrifugation, the supernatant was tested for the presence of anti-AChR antibodies with the TE671 ELISA. In a similar study, the same four serum samples were also tested for anti-AChR antibodies after preadsorption with a TE671 cell preparation (total protein
content of 5 g/L), using the same procedure as for PC12 cells.

TE671 CELL-BASED ELISA
We determined the total protein concentration of the AChR preparation from TE671 cells (BCA Protein Assay; Pierce). The preparation was diluted with PBS at the optimal coating concentration (0.5 g/L). Microwell plates (Polysorp; Nunc) were coated with 100 μL diluted AChR preparation/well and incubated overnight at 4 °C. After the wells were washed with 100 μL PBS/well (a volume that was maintained for subsequent washing cycles), 200 μL of 50 g/L nonfat dry milk in PBS was added to each well to block nonspecific binding sites. After that 4 °C, the wells were washed with 100 μL nonfat dry milk in PBS, we prepared 1:100 and 1:1000 dilutions for each sample and control. Each dilution (100 g/L nonfat dry milk in PBS, we prepared 1:100 and 1:1000 dilutions for wells were washed three times. Using 20 g/L nonfat dry milk/PBS to minimize nonspecific binding of the antibody to protein-free sites of the AChR preparation (e.g., nonfat dry milk/PBS to minimize nonspecific binding of the antibody to protein-free sites of the AChR preparation (e.g., through their Fc portions). After an incubation of 1 h at room temperature, wells were washed five times. p-Nitrophe- nylphosphate (Sigma) at the concentration of 1 g/L in diethanolamine buffer (pH 9.6) was added (100 μL/well). After 1 h of incubation, or when the calibrator diluted 1:10 reached the absorbance value of 1.4–1.5, the absorbances of the reaction products were read at 405 nm.

CALIBRATION
The assays for the detection of antibodies to AChR are based on the immunoprecipitation of the 125I-labeled α-BuTx/anti-AChR antibody complex. Anti-AChR antibody concentrations were expressed as picomoles of 125I-labeled α-BuTx precipitated by 1 mL of serum. The calibrator for the present ELISA was a serum sample with high anti-AChR antibody concentration (120 nmol/L) from an MG patient. The patient had no serum paraproteins and had concentrations of IgG, IgA, and IgM within the health-related reference intervals. Previously, this sample had been tested repeatedly with a reference RIPA, with human muscle as the source of antigens, and then aliquoted and stored at −80 °C. The serum was diluted serially (1:10 to 1:100 000) in 20 g/L nonfat dry milk in PBS. Anti-AChR antibody concentrations were calculated by comparison between the mean absorbance of each sample and that generated by the serially diluted calibrator serum (calibration curve). Another serum sample with known anti-AChR antibody concentration (10 nmol/L) from another MG patient was used as positive control. A pool of sera from 10 blood donors was the negative control.

STATISTICAL ANALYSIS
The following methods were used for the statistical analysis: log regression analysis for the evaluation of calibration curves; the calculation of coefficients of variation (CVs) for within- and between-run imprecision; a plot for the assessment of the agreement between two methods in accordance with Bland and Altman (12); and an ROC curve (13) for the evaluation of the diagnostic accuracy. Ninety-five percent confidence intervals are provided in parentheses for the indices of diagnostic accuracy and for data on imprecision.

Results
TE671 CELL-BASED ELISA OPTIMIZATION
The optimal coating concentration of the AChR preparation was 0.50 g/L. At this concentration, the calibration serum, but not the pool of negative sera from blood donors, yielded a linear response at dilutions in the range 1:100 to 1:10000. The statistical evaluation of this response is discussed later in this section.

When tested at the total protein concentrations of 0.50 g/L (Fig. 1A) and 0.75 g/L (Fig. 1B), the AChR preparation showed specific binding with 125I-α-BuTx. This specific binding was also confirmed by tests on the AChR preparation at serial dilutions (Fig. 1C). All correlations were significant (P <0.05). The concentration of 125I-α-BuTx-binding sites can be estimated as ~35 fmol/well at the total protein concentration of the AChR preparation used in the ELISA.

The effects on the anti-AChR antibody concentrations of preadsorption with PC12 or TE671 cell preparations showed that (a) in serum samples from the two seropositive MG patients, these concentrations were slightly decreased (~10%) after preadsorption with the PC12 cell preparation and markedly decreased (~60%) after preadsorption with the TE671 cell preparation; and (b) in serum samples from the two SLE patients, the concentrations were below the detection limit after preadsorption with either PC12 or TE671 cell preparations.

CALIBRATION CURVES AND DETECTION LIMIT
The calibration curve obtained by serial dilutions of the reference serum is shown in Fig. 2. Each point corresponds to the mean absorbance, for the relative dilution, of eight different assays. A good log-linear regression (r² = 0.997) was found for the intervals corresponding to dilutions of 1:10 to 1:100 000. Measured values of up to 32-fold dilutions of two serum samples with high anti-AChR antibody concentrations ranged from 95% to 115% of the expected values. The detection limit was 2 nmol/L, which is the concentration corresponding to a signal 3 SD above the mean of four replicates of the pool of control sera. The calibration curves of the ELISA presented here are constructed with a reference serum that has been tested previously for the presence of anti-AChR antibodies with a human muscle-based immunoprecipitation RIPA. This enables a quantitative correlation between two different methods. We stored our calibrator in large quantities at −80 °C.
Within- and between-run imprecision was calculated for samples at low and high anti-AChR antibody concentrations. Each sample was tested 15 times per analytical run and in triplicate in 15 analyses performed at a monthly interval. At the low anti-AChR antibody concentration (5.1 nmol/L), the respective intra- and interassay CVs were 12.6% (7.1–21.2%) and 20.0% (12.7–29.2%). At the high anti-AChR antibody concentration (64.0 nmol/L), the respective intra- and the interassay CVs were 6.9% (2.9–13.9%) and 11.5% (6.0–19.1%). There were no significant differences in precision when the relative data of our assay were compared with those of the reference RIPA (data not shown).

ANALYTICAL ACCURACY
A plot of the respective differences of the two methods vs the means of the two methods for anti-AChR antibody determination is shown in Fig. 3. For this purpose, 53 of the 207 serum samples of MG patients were tested. When two sera with low anti-AChR antibody concentrations were each mixed with a serum with a high anti-AChR antibody concentration, the recovery was 94% and 90% of the expected calculated value. Polyclonal IgG, lipids,
hemoglobin, and bilirubin showed no interference with anti-AChR antibody determination: sera with high concentrations of these substances, mixed with a serum with anti-AChR antibody concentration of 25 nmol/L, had negligible effects on the expected values (data not shown).

**DIAGNOSTIC ACCURACY**

Anti-AChR antibody concentrations above the detection limit were found in 146 of 176 (79%) patients with generalized MG, and in 17 of 31 (55%) patients with ocular MG (Fig. 4). Patients with ocular MG had lower anti-AChR antibody concentrations than those with generalized disease ($P = 0.02$). Samples from five MG patients with anti-AChR antibody concentrations of <2 nmol/L in the reference RIPA were negative in ELISA. Anti-AChR antibody concentrations showed no statistically significant correlation either with the severity of symptoms or with the presence of thymoma in patients with generalized MG. When the control group was considered into the two SLE patients and the patient with pseudotumor orbitae showed low concentrations (<4.0 nmol/L) of serum anti-AChR antibodies.

The ROC curve for anti-AChR antibodies, which we plotted by matching the positive “diseased” group (all MG patients, $n = 207$) and the negative “control” group (all non-MG patients, $n = 77$) is shown in Fig. 5. The area under the curve was 0.868 (0.823–0.905). Correlated diagnostic accuracy indexes were as follows: sensitivity, 75.8% (69.4–81.5%); specificity, 96.1% (89.0–99.1%); and positive likelihood ratio for the point of the greatest efficiency of the test, 19.5 (8.3–45.7). If the two groups were taken separately, sensitivity for generalized and ocular MG was, respectively, 79.5% (72.8–85.2%), and 54.8% (36.0–72.4%). The specificity was 96.1% (89.0–99.1%) for both groups. The respective positive likelihood ratios were 20.4 (8.7–47.9) and 14.1 (6.0–33.0).

**CLINICAL FOLLOW-UP**

Sixteen MG patients were followed longitudinally. Their serum anti-AChR antibody concentrations varied as follows: in 7 of 16 patients, the antibody concentrations decreased in parallel with clinical improvement whether induced (5) or not (2) by immunosuppressive drugs; in 6 of 16 patients (4 clinically stationary, 2 with worsening symptoms), the antibody concentrations remained almost unchanged; in 3 of 16 patients who complained of worsening symptoms, the antibody concentrations were increased (one patient withdrew from immunosuppressive therapy).

**Discussion**

The determination of serum anti-AChR antibodies adds substantially to the other diagnostic procedures used for MG, especially when symptoms are slight and electrophysiological findings uncertain.

For the generalized form of MG, this TE671 cell-based ELISA showed a sensitivity of 79%, near the 87–88% reported in the literature (14–15). For the ocular form of MG, sensitivity decreased to 55%, which falls within the reported 45–70% range (14, 15). The method showed a detection limit of 2 nmol/L, which is higher than that of conventional RIPAs based on human muscle-derived AChR but similar to that of TE671 cell-based RIPAs (6–9). The assay precision data of our ELISA compare well with those obtained with the reference RIPA.

The TE671 ELISA correlated well with the reference human muscle-based RIPA. In contrast with previous reports on TE671 cell-based RIPAs (6–9), we found that anti-AChR antibody concentrations tested with our ELISA were not significantly lower than those tested with the reference RIPA. In those methods (6–9), which used TE671 cells as the source of antigen, AChRs were bound to $^{125}$I-α-BuTx and used for the immunoprecipitation of anti-AChR antibodies. Whereas AChR sites would be
occupied by $^{125}$I-α-BuTx in RIPAs, in our ELISA such sites are available for the binding of serum anti-AChR antibodies. However, the diagnostic sensitivity of this ELISA is lower than that of human muscle-based RIPAs, although it is comparable to that of TE671 cell-based RIPAs (6–9). The number of coated AChRs available for specific antibody binding is limited by the surface area of microwells. This limitation is probably compensated for by the availability of free α-BuTx binding sites. Such sites are important for the performance of this ELISA: we found a good dose–response relationship when we tested the specific binding between the TE671 cell-derived AChR preparation and $^{125}$I-α-BuTx. These sites, which are only a portion of the AChR sites available for anti-AChR antibody binding, probably allow our ELISA to overcome the disadvantage of microwell surface area constraints.

Regarding diagnostic specificity, two patients with SLE and one patient with pseudotumor orbitae showed low serum anti-AChR concentrations. The two SLE serum samples, after preadsorption with the PC12 or with the TE671 cell preparation, were negative for anti-AChR antibodies. Because PC12 cells are particularly rich in cytoskeletal proteins (16) and because SLE patients may present serum antibodies against these proteins (17), it is likely that the positivity of the two SLE serum samples was caused by the recognition by antibodies of non-AChR proteins, probably cytoskeletal proteins. The preadsorption of the two MG sera caused only a slight decrease in their anti-AChR antibody concentration. Sera from all healthy controls were negative for anti-AChR antibodies. Moreover, we previously had evaluated the ability of a pool of six monoclonal antibodies, each of which recognized a different region of AChR, to compete with the binding of the reference serum with the AChR preparation (10). This binding was blocked by as much as 30%. The low inhibition might depend on the heterogeneity of AChR epitopes. The pool of six monoclonal anti-AChR antibodies would only partially compete with all the naturally occurring anti-AChR polyclonal antibodies.

The advantages of TE671 cells in comparison with human muscle as a source of antigen include (a) greater homogeneity, because ischemia and other variables may influence muscle preparations; (b) easy availability; and (c) no risk of infection through the manipulation of potentially infected human tissues.

The present method for the nonradioactive detection of anti-AChR antibodies provides good overall analytical and diagnostic performance; however, it fails to detect very low anti-AChR antibody concentrations. Accordingly, it might yield more false-negative results. Data from the longitudinal follow-up of parallel antibody/clinical variations in single MG patients showed that anti-AChR antibody concentrations correlated well with clinical improvement subsequent to immunosuppressive treatment. This finding confirms the reliability of the method, which may therefore be useful, even as an addition to standard RIPAs, for easy and inexpensive monitoring of immunosuppressive therapy. We believe our ELISA can be used for specific antibody screening in MG and is particularly suitable for laboratories that are not equipped for RIPAs and/or have no access to human muscle tissue.

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