Modified and improved anti-acetylcholine receptor (AChR) antibody assay: comparison of analytical and clinical performance with conventional anti-AChR antibody assay

Bruno Ferrero,1* Giuseppe Aimo,2 Roberto Pagni,2 Bruno Bergamasco,1 Maria R. Bongioanni,1 Lodovico Bergamini,1 and Luca Durelli1

We developed a modified anti-acetylcholine receptor (AChR) antibody (Ab) assay based on a radioreceptor assay and a calibration curve. We compared the analytical and clinical performances of this modified assay with those of the conventional anti-AChR Ab radioreceptor assay. Serum specimens were from patients with myasthenia gravis (MG) (n = 156) and from control subjects (n = 106). The modified assay demonstrated lower within-assay (4.0–6.6%) and between-assay (5.3–7.8%) CVs, greater linearity, lower cost, and shorter assay time than the conventional method. ROC curve analysis indicated almost identical specificity and sensitivity (>0.92) for these two anti-AChR Ab assays. The modified and conventional assays were also equivalent for blocking anti-AChR Ab assay. Moreover, the modified anti-AChR Ab assay, differently from the conventional assay, allowed us to reveal anti-AChR Ab concentration differences among different clinical grades of MG.

INDEXING TERMS: myasthenia gravis • radioreceptor assay • intermethod comparison

Myasthenia gravis (MG) is an autoimmune disease characterized by fluctuating muscular weakness that usually becomes more evident upon repetitive motor activity [1].3

Anti-acetylcholine receptor (AChR) antibody (Ab) is involved in the pathogenesis of the disease. It causes the loss of functional AChRs at the postsynaptic membrane that underlies the defect of neuromuscular transmission [2–4]. Circulating anti-AChR Ab can be detected in 75–90% of myasthenic patients [3, 5, 6], and detection of these Abs is useful for diagnosis and follow-up [7, 8]. The exact relation between antibody concentration and clinical state is not clearly established as yet, although concentrations are higher in severe generalized MG [5, 9], and clinical improvement is usually associated with a decrease of Ab concentration [7].

Conventional radioreceptor assay methods for determining anti-AChR Ab concentration are lengthy and expensive [8]. For each assay, at least four different radiolabeled human (or fetal calf) AChR amounts and four different rabbit anti-human IgG amounts, in duplicate, are usually required to immunoprecipitate the anti-AChR Ab and radiolabeled AChR complexes. For this reason, this method can be rarely used for frequently repeated determination of longitudinal Ab concentration variation. The great between-assay variation further reduces its use for serial Ab studies. We developed a faster, easier, and more reproducible technique for anti-AChR Ab detection, by interpolating unknown anti-AChR Ab values from a radioreceptor assay calibration curve [10–13]. Here we describe this technique, its analytical performance, and the results, obtained over 5 years from 156 myasthenic patients and 106 nonmyasthenic subjects. Comparison of results obtained with our technique and with the conventional method demonstrates that our technique is a reliable and cheap alternative to the conventional anti-AChR Ab assay, and suggests its use for serial anti-AChR Ab determination.

Department of 1Neurosciences, and 2 Laboratory “Baldi e Riberi”, University Hospital “Molinette”, Via Cherasco 15, I-10126, Turin, Italy.

*Author for correspondence. Fax Int +31 11 6963487; e-mail chinigo@golgi.molinette.unito.it.

3 Nonstandard abbreviations: MG, myasthenia gravis; AChR, acetylcholine receptor; Ab, antibody; BGT, α-bungarotoxin; AUC, area under the curve; and Staph A, Staphylococcus aureus Protein A.

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Materials and Methods

Subjects
We studied 106 nonmyasthenic subjects and 156 myasthenic patients. Nonmyasthenic subjects (n = 106, 49 females, 57 males, median age 45 years, range 15–73) included healthy controls (n = 19), patients with nonneurologic autoimmune diseases (n = 11), and patients with nonmyasthenic neurologic diseases (n = 76). In myasthenic patients (n = 156, 87 females, 69 males, median age 38 years, range 9–74), diagnosis was established according to appropriate clinical history and positive response to anticholinesterase drugs (edrophonium in 148 patients or piridostigmine in 8). In 89 myasthenic patients, diagnosis was electromyographically confirmed by the decremental response to repetitive nerve stimulation at 3 Hz [1]. Patients were graded according to Osserman’s classification [14]: remission (n = 21 patients), grade I (pure ocular disease, n = 31), grade II-A (mild generalized disease, n = 32), grade II-B (moderate severe generalized disease, with bulbar symptoms, n = 45), grade III (acute disease, with severe bulbar symptoms, and often rapid progression to respiratory insufficiency, n = 16), and grade IV (chronic severe disease, n = 11). Among the 156 myasthenic patients, 73 had been thymectomized 1–8 years before the study (11 had a thymoma: four women, seven men, median age 57 years, range 49–74), and 85 were treated with immunosuppressive drugs. Serum aliquots obtained from myasthenic patients and controls were stored at −70 °C until used. The procedures followed were in accordance with the Helsinki Declaration of 1975, as revised in 1983.

ACHR Preparation
Fetal calf muscle was immediately dissected after sacrifice and frozen at −70 °C until used. The muscle was homogenized in a blender apparatus for 1 min in four volumes of a cold buffer containing 50 mmol/L NaCl, 2 mmol/L sodium EDTA, 1 mmol/L sodium ethylene glycol-bis (β-aminoethoxy)ethylenediaminetetraacetic acid (EGTA), 50 mmol/L Tris-HCl, pH 7.4 (buffer A), and 2 mmol/L phenylmethylsulfonyl fluoride (Sigma Chemical Co., St. Louis, MO). Homogenate was centrifuged at 40 000g for 30 min at 4 °C (J2–21 M/E Centrifuge; Beckman Instruments, Fullerton, CA). Pellets were recovered, diluted 1:2 with buffer A, rehomogenized, and then Triton X-100 was added to a final concentration of 20 mL/L. The mixture was gently stirred for 3 h at 4 °C. After the extraction, the material was centrifuged at 40 000g for 1 h at 4 °C. The supernatant was recovered and filtered through a glass wool column to remove lipids. Final ACHR content from crude extract was determined by the diethylaminoethyl cellulose (DE-81; Whatman, Maidstone, UK) disc assay and expressed as nanomoles of ACHR per liter of extract [8, 15]. We used fetal calf ACHR as antigen for the anti-ACHR Ab assay because (a) it is easier to obtain than human ACHR; (b) antigen–Ab reactivity shows little variability among different ACHR preparations lower than that of human ACHR [9]; and (c) fetal calf ACHR has been demonstrated to react with human anti-ACHR Abs to the same extent or better than the human antigen [8].

Anti-ACHR Ab assay
Anti-ACHR Abs detected in most myasthenic patients are directed against epitopes located outside the ACh binding site (binding anti-ACHR Ab) [3]. Abs directed against the ACh binding site can be detected in ~40% of myasthenic patients: These Abs block iodine α-bungarotoxin ([125I]BGT) binding to ACHR (blocking anti-ACHR Ab) [2]. Therefore, a complete serologic test for MG requires determination of both blocking and binding anti-ACHR Ab.

Conventional radioreceptor assay for binding anti-ACHR Ab. All sample sera were diluted 1:10 in PBS + 1 mL/L Triton X-100. Fetal calf ACHR, diluted at 1 nmol/L in buffer A, was radiolabeled with a 2 molar excess of [125I]BGT (specific activity 555.0 kBq/μg; DuPont, Wilmington, DE) for 4 h at room temperature and dispersed in 1-mL aliquots. Each aliquot was incubated in duplicate with increasing amounts (5, 10, 25, 50 μL) of sample serum diluted with normal serum (respectively 45, 40, 25, 0 μL) to a final volume of 50 μL to reach the same immunoglobulin concentration in all samples and to reduce the analytical imprecision due to different biological matrices [16]. Incubation was performed overnight at 4 °C. Therefore, 100 μL of a 0.75% rabbit anti-human IgG solution (Dako, Glostrup, Denmark) was added to each sample to give a complete Ab precipitation [3, 8]. After 2 h of incubation at room temperature, samples were centrifuged at 4000g for 30 min at 4 °C and then washed twice in PBS + 5 mL/L Triton X-100 to remove unbound materials. After each wash, centrifugation was repeated at 4000g for 20 min at 4 °C. Final pellet radioactivity, proportional to precipitated receptor–toxin immune complexes, was revealed by a gamma counter (LKB Multi gamma 1261; LKB, Uppsala, Sweden) and expressed as counts per minute (cpm). The slope of the straight line, obtained by plotting pellet radioactivity against the respective sample serum amount, was calculated by linear regression analysis. When the slope showed a positive value but r was <0.9, anti-ACHR Ab was always retested. Each slope, expressed as cpm/μL of serum, was multiplied by the sample serum dilution used and then divided by the cpm of 1 pmol of [125I]BGT. The result, expressed as pmol of anti-ACHR Ab/μL of sample serum, was multiplied by 106, obtaining the concentration of anti-ACHR Ab, usually expressed as nmol/L. To validate the analytical procedure, in each group of determinations four different nonmyasthenic and two myasthenic sera of well-known concentrations were tested as negative and positive controls, respectively.

Modified radioreceptor assay for binding anti-ACHR Ab. A sample of pooled sera from 15 healthy subjects was
reassayed >20 times by the conventional anti-AChR Ab method. Being always negative for anti-AChR Ab, it was chosen as the zero calibrator and used for diluting patient sera. A sample of pooled sera from six myasthenics with high-concentration anti-AChR Ab (median value 71.7 nmol/L, range 52.2–112.1) was serially diluted ninefold from 1:10 to 1:2560 with the zero calibrator. Each dilution was stored in 50-μL aliquots at −70 °C, and anti-AChR Ab concentration (ranging from 76.8 nmol/L for the 1:10 dilution to 0.3 nmol/L for the 1:2560 dilution) was calculated by taking the mean of 10 conventional Ab determinations. Mean and SD of anti-AChR Ab concentrations (nmol/L) of these nine calibration dilutions were, respectively, 76.8 ± 6.51, 38.4 ± 2.95, 19.2 ± 1.33, 9.6 ± 0.89, 4.8 ± 0.41, 2.4 ± 0.19, 1.2 ± 0.11, 0.6 ± 0.05, and 0.3 ± 0.03. These nine myasthenic serum dilutions, in duplicate, were used to obtain a calibration curve, by plotting, for each dilution, the mean of the duplicate values of revealed antibody concentrations: If duplicate antibody concentrations differed from their own mean by >10% (mean ± 3 SD of the within-assay precision of the modified anti-AChR Ab assay; this value was chosen to exclude incorrect Ab values with the probability of 99%), the sample was always retested. The anti-AChR Ab assay was repeated if only one of the three positive control serum Ab concentrations, measured by the modified assay, differed by more than the mean ± SD of Ab concentrations determined by 10 conventional assays (i.e., if the Ab concentration of the 3.5 ± 0.31 nmol/L control serum is >3.29 nmol/L or <3.81 nmol/L, the modified assay must be repeated). Patient anti-AChR Ab concentrations were determined by using Wiacalc (Pharmacia-LKB, Uppsala, Sweden) software, by comparing the unknown sample radioactivity (average of duplicate values) with the same radioactivity on the calibration curve and interpolating the corresponding Ab value (expressed in nmol/L). This modified anti-AChR Ab assay method includes a four-parameter polynomial logistic regression statistical model [17] to construct the calibration curve. The equation utilized by this fitting algorithm has the general form: response = D + (D − A)/(1 + (concentration/C)^B), where A, B, C, D are, respectively, the reference estimate (cpm), the slope factor, the turning point (nmol/L), and the estimated blank (cpm); the response is expressed as measured cpm and the concentration as nmol/L. This curve is fitted to the means of the measured calibration points by means of a weighted least-squares method; such an analysis procedure minimizes errors related to an excessively theoretic or experimental calibration curve [11, 13]. Fig. 1 shows an example of the calibration curve obtained by a zero calibrator and nine calibration points (from 0.3 to 76.8 nmol/L).

Conventional radioreceptor assay for blocking anti-AChR Ab. The procedure to assay serum Ab directed against the ACh binding site (able to block BGT binding to AChR) [3, 5] was very similar to the binding anti-AChR Ab assay described above. The only difference was that the blocking anti-AChR Ab concentration was determined by incubating AChR overnight at 4 °C with increasing amounts (5, 10, 25, 50 μL) of myasthenic serum, and then with a 2-molar excess of [125I]BGT for 4 h at room temperature (in opposite order to the binding anti-AChR Ab assay). Toxin–receptor–Ab complexes were then precipitated by rabbit anti-human IgG and counted as described above. Ab concentration (nmol/L) was calculated by linear regression analysis by using the four different dilutions in duplicate. This value is the total anti-AChR Ab concentration diminished by the amount of blocking anti-AChR Ab; therefore, the blocking anti-AChR Ab concentration can be calculated as the difference between the binding anti-AChR Ab concentration and this value.

Modified radioreceptor assay for blocking anti-AChR Ab. AChR was incubated overnight at 4 °C with 50 μL of myasthenic serum, and then with a 2-molar excess of...
values were compared by Student’s t-test. If data distribution was gaussian and variances equal, statistical analyses were done with the Astute statistics computer program (DDU software; The University of Leeds, Leeds, UK). The calibration curve was obtained with the weighted least-squares method by using the Wiaalc computer program.

**Results**

**Muscle AchR content**

AchR content in the crude Triton X-100 extract varied, with different muscle samples, between 2.2 and 8.5 nmol/L. To determine the optimal AchR concentration to be used as antigen for the anti-AchR Ab assay, three different myasthenic sera (whose anti-AchR Ab concentrations were around the mean of the majority of myasthenic sera concentrations) were tested in quadruplicate with different dilutions (from 0.25 to 4 nmol/L) of three different fetal calf AchR samples. The best AchR concentration needed to precipitate all the anti-AchR Ab in myasthenic serum was between 0.75 and 1.5 nmol/L, in keeping with other reports [5, 8]. For this reason we always used the AchR concentration of 1 nmol/L.

**Analytical evaluation of the two different binding anti-AchR Ab assay methods**

**Precision.** Anti-AchR Ab concentrations of three Ab-positive myasthenic sera (at low, middle, and high Ab concentration) were assayed by both the conventional and modified methods in 10 replicates to assess within-assay precision. In addition, the same serum samples were run in 15 consecutive assays over >120 days to assess between-assay precision. These variabilities are evaluated as mean CVs (Table 1). Within-assay CVs ranged from 8.4% to 11.6% (median 9.9%) with the conventional method, and from 4.0% to 6.6% (median 5.5%) with the modified method. Between-assay CVs ranged from 12.2% to 14.7% (median 12.3%) with the conventional method, and from 5.3% to 7.2% (median 6.6%) with the modified method. Fig. 2 shows the precision profiles of the two assay methods. The three highest ranges of anti-AchR Ab concentrations (2.2 nmol/L (0–0.4, 0.4–0.9, and 0.9–2.2 nmol/L) showed CVs that decreased from 19.7% to 9.8% with the conventional method and from 14.2% to 6.1% with the modified method. The reproducibility of the modified procedure for the remaining part of the range of the test (2.2–245.6 nmol/L) was always much lower (range 3.8–5.9%) than that obtained with the conventional procedure (range 8.5–10.5%).

**Reproducibility of the modified assay.** With the modified method, a high calibration curve reproducibility was shown in >98% of assay runs. In fact, only 2 of 148 calibration curves, over 5 years of assays, were rejected (and therefore repeated) because only one of the positive control Ab values differed by more than ± SD from the average value of 10 conventional assays on the same sample.

**Test sensitivity and specificity.** Before developing the modified anti-AchR Ab method described here, we established the anti-AchR Ab cutoff value for our laboratory...
with the conventional assay and 70 control sera (19 healthy and 51 nonmyasthenic patients). The Ab values distribution was approximately normal, with a range of 0–0.54, mean 0.072, and SD 0.079 nmol/L. Therefore we chose 0.4 nmol/L (mean ± 1 SD) as the cutoff value of the assay, the Ab range below such a cutoff value including >99.9% of the healthy subjects. We recalculated the cutoff value both for the conventional and modified assays by using the data of the 106 nonmyasthenic subjects presented here: The cutoff value of 0.4 nmol/L was always effective for both methods. On the basis of this cutoff value, all sera were divided into anti-AChR Ab “positive” (>0.4 nmol/L) or “negative” (<0.4 nmol/L). Mean, SD, and range of anti-AChR Ab concentrations of 156 myasthenic patients and 106 nonmyasthenic subjects determined with the two assay methods are presented in Table 2. Test percent sensitivity, i.e., the percentage of myasthenic sera positive for anti-AChR Ab, was 125 of 156 patients (80.1%), being higher in patients with active disease (114 of 135 patients: 84.4%) than in patients in remission (11 of 21 patients: 52.4%) (P < 0.001). It was also higher in patients with generalized MG (96 of 104 patients: 92.3%) than in patients in remission (11 of 21 patients: 52.4%) (P < 0.001) or in pure ocular myasthenic patients (18 of 31 patients: 58.1%) (P < 0.005). The difference between the number of positive sera in patients with or without MG was highly significant (P < 0.001). There was no significant difference in test percent sensitivity between the conventional and modified assays. Although Ab concentration range of different MG clinical grades stretched over two to three orders of magnitude, anti-AChR Ab concentration of patients in remission or with pure ocular MG was lower (P < 0.01) than that of patients with generalized MG, with both methods. The anti-AChR Ab concentration of mild generalized myasthenic patients (grade II-A) was lower (P < 0.05) than that of moderately severe generalized myasthenic patients (grade II-B) with only the modified method. Between the other grades of generalized MG, Ab concentrations were not significantly different. Test specificity can be expressed as the ratio between the number of nonmyasthenic sera without detectable anti-AChR Ab and the total number of nonmyasthenic sera tested. Among 106 subjects without MG, a positive (“false positive”) anti-AChR Ab serum was found in one rheumatoid arthritis patient treated with penicillamine [5, 8], with both methods. As shown in Table 2, specificity of both methods was 99.1% (105 of 106 control subjects).

Linearity. The myasthenic calibration serum was serially diluted ninefold from 1:10 to 1:2560 in the zero calibrator to reach the same immunoglobulin concentration in all samples. Anti-AChR Ab assay was performed for all dilutions in triplicate with both anti-AChR Ab assay methods. The recoveries of calibrator myasthenic serum dilutions, i.e., (anti-AChR Ab measured/expected) × 100, were better for the modified method (ranging between 96% and 107%) than for the conventional method (ranging between 87% and 123%). Linear regression analysis of observed vs expected Ab concentrations revealed highly significant correlation (r > 0.99) and a slope near unity for both methods.

ROC curve analysis. Fig. 3 represents the ROC curves and the corresponding AUCs for the conventional and modi-
We determined, by both the conventional and modified methods, the blocking Ab concentration of 106 nonmyasthenic and 156 myasthenic sera (already assayed for binding anti-AChR Ab). The number of myasthenic sera positive for blocking anti-AChR Ab, with both methods, was 97 of 156 patients (62.2%), being higher in patients with active disease (90 of 135 patients: 66.7%) than in patients in remission (7 of 21 patients: 33.3%) (P <0.001), and in patients with generalized MG (77 of 104 patients: 74.0%) than in patients in remission (7 of 21 patients: 33.3%) (P <0.001) or in pure ocular myasthenic

patients (13 of 31 patients: 41.9%) (P <0.01). Among 106 nonmyasthenic subjects, no blocking Ab-positive (“false positive”) serum was found (specificity 100%). The difference between the number of positive sera in patients with or without MG was highly significant (P <0.001). The Ab concentration of patients in remission or with pure ocular MG was lower (P <0.05) than that of patients with generalized MG, regardless of the method used. As for the other grades of generalized MG, Ab concentrations were not significantly different.

**Discussion**

The conventional anti-AChR Ab assay method [3, 5, 8] is a radioreceptor assay that requires a reaction between an
excess of radiolabeled antigen (125I]BGT-AChR) and various dilutions (usually four or five in duplicate) of an antibody (anti-AChR Ab) of unknown concentration. After washing unbound radiolabeled antigen, the antibody concentration can be calculated from the slope of a regression line obtained by plotting the precipitated radioactivity vs microliters of sera used. We describe a modified radioreceptor anti-AChR Ab assay method based on a calibration curve: The concentration of an unknown radiolabeled analyte is interpolated by referring the precipitated radioactivity to a calibration curve obtained by plotting radioactivity vs concentration of samples of known concentration.

We have demonstrated that this modified method possesses higher reproducibility and lower within-assay and between-assay variabilities than the conventional method (Table 1). In addition, this modified method displayed an outstanding precision profile across the entire dynamic range, particularly in the very low concentration range (Fig. 2), the range where a low precision increases the probability of a wrong diagnosis of MG. Comparison of the two procedures revealed good correlation and agreement: The Ab concentration measured with the modified method may differ from the Ab concentration measured with the conventional method by 19.5% below to 18.2% above in 95% of cases. Other important advantages of the modified method compared with the conventional method are better linearity, lower cost, and greater rapidity (about one-fourth of reagents and assay time for a single myasthenic serum assay), with very similar sensitivity (80.1%) and specificity (99.1%) (Table 2), as confirmed by ROC curve analysis (Fig. 3).

The anti-AChR Ab modified assay method was, in addition, more useful than the conventional method in the study of the correlation of clinical state and anti-AChR Ab concentration. The relation between antibody concentration and disease severity in myasthenic patients is controversial [3, 20–23]. Although anti-AChR Ab concentrations are usually higher in severe generalized MG [9] and clinical improvement is usually associated with decreased Ab concentration [7], it is not possible to predict disease severity on the basis of Ab concentration. In individual patients, serial anti-AChR Ab determinations appear to be correlated with changes of the clinical state induced by treatments, such as thymectomy, plasma exchange, corticosteroids, cytostatic drugs, or total body irradiation [7, 24], but changes of MG severity may sometimes occur without detectable change of anti-AChR Ab concentration and vice versa [25]. The only repeatedly confirmed finding was that patients with ocular MG or patients in remission show either lower anti-AChR Ab concentration or rarer Ab positivity than patients with active generalized disease [6, 26]. Using either the conventional or modified anti-AChR Ab assay method, we confirmed a lower percentage of Ab-positive myasthenic patients and a lower anti-AChR Ab concentration in patients in remission or with ocular MG than in patients with active generalized disease. However, with the modified methodur, but not with the conventional method, we found a significant difference of anti-AChR Ab concentration among myasthenic patients of different clinical severity (namely, grade II-A and grade II-B of Osserman’s classification). Many other studies involving the conventional anti-AChR Ab method were unable to detect any anti-AChR Ab concentration difference among different clinical grades of generalized MG [3, 8, 20]. The lower within-assay and between-assay variabilities of the modified vs conventional method, reducing an important source of assay imprecision, make the modified method more useful to describe the correlation between clinical state and anti-AChR Ab concentration in several studies of individual myasthenic patients.

In addition, the same analytical and clinical performances of the modified anti-AChR Ab assay described here were maintained by using, as immunoprecipitating agent, a 100 g/L solution of Staphylococcus aureus Protein A (Sigma Chemical Co.) (Staph A) instead of rabbit anti-human IgG (data not shown) [6, 27]. Advantages of using Staph A include low cost and a short incubation time (15 min). Only such a short incubation time allows the kinetic studies needed to calculate binding avidity of anti-AChR Ab to AChR to be performed [27].
In the blocking Ab assay, we found that some myasthenic patients positive for binding Ab were also positive for blocking Ab. Vincent and Newsom-Davis [5] suggested that blocking Abs could displace BGT binding to AChR, reducing the precipitated radioactivity at the anti-AChR binding Ab assay and increasing the number of false “seronegative” myasthenic patients. Differently from Vincent and Newsom-Davis and other authors [28], we found no patient positive for blocking and negative for binding Ab. In our experience, therefore, the use of both binding and blocking Ab assays did not result in a reduced number of anti-AChR Ab “seronegative” myasthenic patients. The modified method must be, however, preferred to the conventional one also for blocking Ab assay because of its lower cost and greater rapidity, without difference in specificity and sensitivity.

In summary, the modified anti-AChR Ab assay proposed in this study displays higher precision and linearity than the conventional method, maintaining the same sensitivity and specificity. In addition, it has a lower cost, shorter assay time, and greater clinical usefulness than the conventional method. Therefore we suggest that this method be used when numerous repeated determinations are needed. For example, this method can be used for evaluating serial changes of anti-AChR Ab concentrations in the individual myasthenic patient and the possible correlation between anti-AChR Ab concentration, clinical state, and treatments.

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