Immunoenzymometric Assay and Radioimmunoassay Measure Different Populations of Antibody against Human Acetylcholine Receptor

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We used a "sandwich"-type immunoenzymometric assay (IEMA) and a radioimmunoassay (RIA) to measure antibody against the human nicotinic acetylcholine receptor in serum from individuals with myasthenia gravis, with markedly different results for certain specimens, as measured by the two techniques. In some cases, antibody concentrations were high by RIA but low by IEMA; in others, the reverse was found. Such differences persisted through 30 months after thymectomy. An investigation of potential causes of this disparity suggests that high IEMA measurements reflect specific anti-receptor antibody and are not artifactual. The IEMA is recommended as an adjunct to the RIA because some patients with myasthenia gravis who have low concentrations of anti-receptor antibodies as measured by RIA have significantly above-normal concentrations of anti-receptor antibodies as measured by IEMA.

Additional Keyphrases: myasthenia gravis · nicotinic acetylcholine receptor · autoimmune disorders

In myasthenia gravis (MG), the patient produces antibodies against the nicotinic acetylcholine receptor (AcChR), which results in loss of this receptor from the neuromuscular junction, as reflected by impaired muscular function characterized by weakness and fatigability. Typically, the concentration of such antibodies [Ab(AcChR)] in the serum, measured by RIA (1), is significantly above-normal in approximately 85% of MG patients (2, 3). For individual patients, fluctuations in Ab(AcChR) concentrations loosely parallel the patient's clinical state (4-6), but information as to the antibody titer alone is not useful in predicting the severity or progress of the disease in a given patient (2, 7, 8).

With regard to experimental autoimmune myasthenia gravis, we recently reported the development of an immunoenzymometric assay (IEMA) for the measurement of Ab(AcChR) from immunized rabbits (9). Results of such measurements paralleled those obtained by RIA, suggesting that the IEMA might provide a more rapid and sensitive means for testing MG patients' sera. We have now examined serum samples from 33 MG patients, by both assays. In some cases, antibody concentrations were high by RIA but low by IEMA; in others, the reverse was true. We report here the results of these studies and a preliminary exploration of reasons for RIA/IEMA differences we have observed.

Materials and Methods

Preparation of Receptor

Human skeletal muscle, obtained from amputations from diabetic patients, was trimmed of connective tissue and excess fat, diced, and either processed immediately or stored at -70 °C (storage at -70 °C did not change the amount of receptor that could be extracted). All extractions were done at 4 °C, as follows. The tissue (50 g wet weight) was homogenised in 200 mL of cold receptor buffer containing, per liter, 10 mmol of sodium phosphate (pH 7.4), 2 mmol of EDTA, 0.1 mmol of phenylmethylsulfonyl fluoride, and 100 mmol of sodium chloride. After centrifugation (20 000 × g, 30 min), the pellet was resuspended in an identical volume of receptor buffer, and the centrifugation was repeated. This second pellet was then resuspended in 100 mL of receptor buffer containing 20 mL of Triton X-100 surfactant per liter, stirred for 90 min, and subsequently centrifuged at 100 000 × g for 60 min. The milky top layer was manually aspirated and discarded. The remaining clear supernatant fluid was filtered through sterile glass wool. Aliquots were sealed in ampules and stored at -70 °C until assayed and used.

AcChR Assay

We measured AcChR by a method similar to that of Claudio and Raftery (10). Briefly, different dilutions of alpha-bungarotoxin (Btx), radiolabeled with 125I (New England Nuclear), in 50-μL volumes were incubated with 300 μL of each AcChR preparation for 1 h at 22 °C. Nonspecific binding of 125I-labeled Btx by AcChR was simultaneously measured in parallel at each dilution by preincubating receptor with a 60-fold excess of unlabeled Btx before reacting with 125I-labeled Btx. We then applied 80-μL aliquots of these mixtures to DEAE filter discs (Whatman, DE-81, 2.4 cm in diameter), washed the discs twice for 30 min in receptor buffer, and then measured the radioactivity in a gamma counter. Each analysis was performed in triplicate. Specific binding was saturable. AcChR concentrations ranged from 0.2 to 1.4 pmol of Btx sites per milliliter of extract.

Radioimmunoassay (RIA) for Ab(AcChR)

Following the procedure of Lindstrom (1), we incubated 50 μL of 125I-labeled Btx (0.5 pmol/L) with 100 μL of AcChR in 12 × 75 mm polystyrene conical test tubes for 1 h at 22 °C. Every batch of AcChR was pooled from four or more different preparations, each of which had been previously assayed for AcChR content and for binding by sera known to be positive for Ab(AcChR), both in the RIA and the IEMA procedures. Diluted test or control sera were added in 50-μL volumes, the contents of the tubes were vortex-mixed, and the tubes were incubated for an additional 3 h at 22 °C. To preserve uniformity, we used the same lot of commercial goat antiserum against human IgG to precipitate antigen-antibody complexes. The optimal amount of goat antihuman IgG for precipitation of human IgG was determined in separate experiments by use of a panel of MG-positive

Received October 12, 1984; accepted March 12, 1985.

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3 Nonstandard abbreviations: AcChR, acetylcholine receptor; Ab(AcChR), antibody against AcChR; Btx, alpha-bungarotoxin; IEMA, immunoenzymometric assay; MG, myasthenia gravis; SLE, systemic lupus erythematosus.

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sera. We added this volume (64 µL) to the mixture and, after vortex-mixing, covered the tubes and incubated them overnight at 4 °C. We then centrifuged the tubes for 10 min at 3000 x g. The supernatant fluid in each tube was discarded, and the pellet was washed twice with pH 7.4 phosphate-buffered saline (30 mmol of phosphate, 0.14 mol of NaCl, and 200 mg of sodium azide per liter) to which 0.5 mL of Tween 20 polyoxyethylene (20) sorbitan monolaurate detergent was added per liter. The washed precipitates were then recentrifuged, and radioactivity was measured in a gamma counter.

The same set of reference (positive and negative) serum controls were tested with each assay. We measured nonspecific trapping of label by using 50 µL of phosphate-buffered saline in place of 50 µL of diluted serum (11). Samples were assayed in triplicate; if the CV was not less than 10%, the assay was repeated. Concentrations of Ab(AcChR) in serum were calculated from the counts/min per pellet and expressed as nanomoles of precipitable Btx sites per liter of serum.

Measurement of Ab(AcChR) by IEMA

The IEMA procedure we used to measure human Ab(AcChR) parallels that described for rabbit antibody against AcChR purified from the electric organ of the Pacific ray, *Torpedo californica* (9). Flat-bottomed polystyrene microtitration plates (Immulon; Dynatech, Alexandria, VA) were coated with a 2 µg/mL solution of Btx and incubated overnight at 37 °C. The human AcChR preparation was diluted with an equal volume of receptor buffer, and 200 µL of the resulting solution was added to each of 60 central wells of the washed plate. The plates were incubated for 2 h at 22 °C, in a hummid chamber. After unbound AcChR and extraneous serum proteins were washed from the wells, serum samples diluted with receptor buffer were added, 200 µL each, to quadruplicate wells. Each plate also contained positive and negative reference sera and buffer controls. The plates were then incubated for 1.5 h at 37 °C, in a humid chamber. Goat anti-human IgG antibody conjugated to alkaline phosphatase (EC 3.1.3.1) was diluted 1000-fold in phosphate-buffered saline, and 200 µL added to each well. After overnight incubation at 4 °C, 200 µL of p-nitrophenyl phosphate (Sigma Chemical Co., St. Louis, MO), 2.7 mmol/L, were added to each well. The absorbance of the liberated p-nitrophenol anion was determined at 405 nm with a plate reader (Model EL307; Biotek, Burlington, VT). The enzymic reaction was stopped by adding 50 µL of a 3 mol/L solution of sodium hydroxide when the absorbance of the most-positive control wells reached 1.10, usually after 20 to 30 min. The reference blanks were outer wells that had received neither AcChR nor diluted human serum. Only the substrate/sodium hydroxide inhibition solution was added to these wells. We optimized the assay on the basis of results of a series of experiments in which we systematically varied the concentrations of Btx, AcChR, and alkaline phosphatase-conjugated goat anti-human IgG (9).

For each plate we used the same positive and negative reference controls. IEMA units of Ab(AcChR) were determined relative to these standard reference serum controls by the formula:

\[
U_x = \frac{(U_p - U_o)(A_x - A_o)(A_p - A_o)}{A_x} + U_c
\]

where \( U \) is IEMA units of Ab(AcChR)

\( A \) is the absorbance at 405 nm

\( x \) identifies the assayed sample

\( c \) identifies the non-disease reference control

\( p \) identifies the positive reference control

IEMA units of Ab(AcChR) are thus relative, not absolute.

The precision of the assays was determined by calculating coefficients of variation. In both the IEMA and RIA, CVs were always less than 10%, and often less than 5%. Variations between assays performed on different days were likewise within 10% of each subject's mean value.

**Human subjects and sera.** We obtained serum samples from 33 patients at the Myasthenia Gravis Treatment Center, Beaumont Hospital, Royal Oak, MI. We also obtained serum samples from healthy controls, ages 8 to 67 years. Serum specimens were sealed in 0.5-mL aliquots and stored at −20 °C until analysis.

**Results**

Initially, we measured concentrations of Ab(AcChR) by RIA and IEMA, under assay conditions that were as nearly identical as possible: we used the same buffers for both RIA and IEMA, and kept the respective incubation times and temperatures the same. When differences between IEMA and RIA results were observed, we optimized each assay independently, on the basis of literature references and our own experience with IEMA measurement of antibody against *Torpedo* AcChR. The differences we report have persisted through the optimization procedure.

Figure 1 (left) illustrates the measurement of antibody against human nicotinic AcChR by RIA under conditions of increasing serum dilution. Antibody measurements from a control serum (subject E) established a low background at each dilution. With the same diluted serum samples and the same pooled AcChR preparation in a simultaneously performed IEMA, we found a similar relationship of Ab(AcChR) and serum dilution, as seen in Figure 1 (right). For three subjects (B, C, and E), there was agreement between the amounts of Ab(AcChR) measured by RIA and those measured by IEMA. Patient A, however, who had the highest concentration of Ab(AcChR) among those measured by RIA, appeared to have a very low concentration of Ab(AcChR) when tested by IEMA. Conversely, the RIA indicated no serum Ab(AcChR) for patient D, whereas the IEMA indicated a significant concentration of specific antibody. Then, using RIA and IEMA concomitantly, we measured concentrations of Ab(AcChR) from patients' sera collected over three years post-thymectomy. Figure 2 compares RIA and IEMA measurements of Ab(AcChR) concentration, plotted as a function of time after thymectomy. The disparity between the IEMA and RIA results for patient A persisted through nine different samples of patient A's serum, which had been obtained over the course of more than three years. This phenomenon also persisted for patient D through five samples obtained over two years.

In addition to RIA/IEMA differences in the concentration of Ab(AcChR) measured in any given serum specimen, a comparison of both parts of Figure 2 indicates widespread diversity in the patterns of Ab(AcChR) concentrations as a function of time. For example, one month after thymectomy patient B had a substantial decrease in the IEMA-measured concentration of Ab(AcChR), but a completely opposite change in the Ab(AcChR) concentration measured by RIA. Whereas the Ab(AcChR) concentration for patient F remained nearly invariant over a 20-month period according to RIA, the IEMA measurements indicated that significant changes in Ab(AcChR) concentration had occurred.

To eliminate nonspecific adherence of antibody as a cause of IEMA/RIA differences, we performed the following control experiments. Coating buffer without Btx was incubated in the wells of microtiteration plates. We then proceeded with the IEMA as usual. Absorbance readings in the range of 0.000–0.025 indicated that neither AcChR nor antibody adsorbed to the treated plastic. In other experiments, we
omitted AcChR: the resulting absorbances, below 0.025, indicated that antibody does not bind to Btx-coated wells. In the absence of serum, or with serum diluted 10 000-fold, absorbances were again within the background range, which demonstrated that enzyme-conjugated second antibody does not bind either to AcChR or to Btx.

Antibody against any non-AcChR antigen remaining in IEMA wells could produce artificially high absorbance readings. To reduce nonspecific adsorption of extraneous antigen in the receptor preparations to the plate surface, we incubated bovine serum albumin at concentrations of 0.0, 0.5, and 5.0 g/L in phosphate-buffered saline (at 20 min for each of three incubations) in Btx-coated wells before adding AcChR. We then added diluted serum from 10 patients, whose Ab(AcChR) concentrations as measured by RIA differed from those measured by IEMA. The treatments made no difference at any albumin concentration, implying that extraneous antigens in the receptor preparation do not

Fig. 1. RIA (left) and IEMA (right) measurement of antibody against AcChR as a function of serum dilution. Subjects A–D have confirmed myasthenia gravis; subject E is a reference control. Bars indicate ± SD of triplicate determinations.

Fig. 2. RIA (A) and IEMA (B) measurements of antibody against AcChR as a function of time in serum specimens obtained over a period of several years. (O) patient A, (C) patient B, and (△) patient D are the same individuals as in Figure 1. Two more patients (△, □) also have confirmed myasthenia gravis. For a nondiseased reference group (n = 40), the concentration of Ab(AcChR) was 0.9 (SD 0.7) nmol/L by RIA and 2.0 (SD 0.2) antibody units by IEMA.
directly adsorb either to polystyrene or to Btx.

In the human AcChR preparation, the concentration of total protein is often 2 g/L, whereas the concentration of AcChR may be only 0.25 mg/L. This 8000-fold difference suggests that extraneous protein could be associated with the AcChR that is bound to Btx in the IEMA. To reduce such potential contamination, we adapted a procedure commonly used in the purification of Torpedo AcChR. We allow the crude receptor preparation to interact with purified toxin, which is covalently linked to an affinity resin; the resin is then washed with 1 mol/L NaCl to remove electrostatically associated proteins that are not specifically bound to the toxin. Before adding diluted patients' sera to IEMA plates that had been coated with Btx and incubated with the human AcChR, we incubated one half of each plate three times (20 min each) with 1 mol/L NaCl in receptor buffer. The other half was incubated with receptor buffer only. The plates were then washed, patients' sera were added, and the procedure was completed as usual. The differences in Ab(AcChR) concentrations measured in the high-salt vs no-salt wells were less than 10%, suggesting that extraneous protein electrostatically associated with AcChR was not responsible for IEMA/RIA differences.

The second antibody conjugated to alkaline phosphatase in the IEMA could have different binding properties than the second antibodies used for precipitation in the RIA, even though both were commercially obtained goat antibody against human IgG. To test whether differential binding of second antibody could be the source of IEMA/RIA differences, we incubated the RIA second antibody in IEMA wells with complexes of patient's antibody–AcChR–Btx. Alkaline phosphatase coupled to rabbit antibody against goat IgG then provided the enzyme marker. In a side-by-side comparison with the standard IEMA, various patients' sera gave identical results. Similarly, we performed RIAs in which we used alkaline phosphatase-conjugated goat antibody against human IgG to bind the complexes of patient's antibody–AcChR–radiolabeled Btx, then used rabbit antibody against goat IgG for precipitation. The same observed differences among patients persisted. To assure that Btx was not the source of the differences we observed, we also performed a set of IEMAs in which we first coated the plastic wells with radiolabeled Btx. Again, relative absorbances were essentially unchanged from those obtained in the conventional IEMA.

Differences between RIA and IEMA measurements of Ab(AcChR) concentration could also depend upon steric considerations. In the RIA, antibody–antigen complexes form with AcChR in a liquid phase, but in the IEMA they form with AcChR on a solid phase. Presumably, AcChR could have different conformations in the two systems. Alternatively, antibody has unrestricted access to AcChR in the RIA, but in the IEMA perhaps only a limited set of antigenic determinants on the AcChR is available for binding.

Btx is quite small compared with AcChR and may hold the extracellular face of the AcChR very near the plastic surface. We thus attempted to place AcChR–Btx complexes at a greater distance from the surface of the plate. We coated plates with diluted serum from either rabbits or mice injected with Torpedo AcChR. We applied human AcChR, and then we tested the patient's serum. We wanted either to increase the distance between AcChR and the plate or to give the adsorbed AcChR an orientation which would permit binding of antibody to its extracellular face. We obtained similar results with three different sources of antisera against Torpedo AcChR and serum samples from five individuals. None of these combinations gave results that were substantially different from those from the conventional IEMA.

Discussion

The concentration of Ab(AcChR), as measured by RIA and IEMA, varied significantly for certain MG patients through repeated assays involving different pooled preparations of human AcChR. The disparity was not predictable a priori, in that some patients with a high concentration of Ab(AcChR) as measured by IEMA had a low Ab(AcChR) concentration detectable by RIA, whereas others with a very high antibody titer as detected by RIA were within the range of normal subjects when measured by IEMA. In contrast, Kawanami et al. (12) reported that the percentage of MG patients positive by RIA was similar to the percentage positive by IEMA, although higher IEMA titers than RIA titers for at least five of their seven patients with above-normal concentrations of Ab(AcChR) suggested to them that their IEMA system had greater sensitivity than the RIA.

Persistent differences between RIA and IEMA measurements of Ab(AcChR) concentration suggest that nonidentical but overlapping populations of antibody are measured by the two assays. If results from one assay were consistently lower than those from the other, then one assay would simply be more sensitive, as was reported by Fowler and Cheng (13), who compared a solid-phase RIA with IEMA for measuring antibodies against chromatin. IEMAs have also been compared with liquid-phase, precipitation RIA for measuring various antibodies. For example, Callahan et al. (14) used both assays to measure the concentrations of human serum antibodies against 14 distinct pneumococcal polysaccharide antigens, examining eight to 10 sera for each antigen. For two of the antigens, the correlation coefficient (r) between IEMA and RIA measurements ranged from 0.88 to 0.93. With the other 12 antigens, however, r varied between −0.23 and +0.46. For these latter 12 cases, they reported that the RIA was more sensitive than the IEMA.

With reference to autoimmune diseases, Edwins and Wilkinson (15) reported a high IEMA/RIA correlation among 283 patients with antibody against thyroglobulin. Of those patients, 16 were positive by RIA but negative by IEMA, while 32 were positive by IEMA but negative by RIA; in no instance were normal results by one assay but abnormal by the other. Nagai et al. (16) compared serum samples from 18 patients with anti-mitochondrial antibody and also found a high IEMA/RIA correlation, with only one patient with deviant results. Venables et al. (17), comparing IEMA and RIA results from sera of 124 patients with Sjögren's syndrome and systemic lupus erythematosus (SLE) and found a good IEMA/RIA correlation but suggested the IEMA was 10-fold more sensitive. RIA/IEMA measurements of antibody against double-stranded DNA have also indicated some differences. Halbert et al. (18) reported a good correlation among 64 patients, although 11 were positive on IEMA but negative by RIA, and three were positive by RIA but negative by IEMA. The extent of correlation between RIA and IEMA among 88 SLE patients examined by Miller et al. (19) depended upon disease activity and renal involvement: of their 25 patients with inactive SLE, 14 were positive by IEMA, but only six were positive by RIA, although in only one case was the IEMA value dramatically higher than the RIA value. Stokes et al. (20) reported that while RIA and IEMA generally agreed, the correlation was not always good: of 326 patients positive for antibody against DNA, 56 were positive only by IEMA; for those positive by both assays, they found r = 0.62.

Investigations of possible reasons for such RIA/IEMA differences in these various assays of autoantibodies have
not yet been reported. Marked bi-directional differences such as we have found between the assays requires an explanation other than mere sensitivity. We suggest that whereas one set of AcChR antigenic determinants will be available for antibody binding in both RIA and IEMA, another set of determinants may be available for antibody binding in the precipitation RIA that are unavailable in the IEMA.

Indirect support for the possibility that the antibody populations measured by RIA and IEMA are not identical comes from a study in which rabbit antiserum against Torpedo AcChR was fractionated by using different solid-liquid phase systems (21). When AcChR was directly coupled to Sepharose 4B in a presumably random orientation, the concentration of eluted Ab(AcChR) relative to the concentration of applied Ab(AcChR) was the same by both RIA and IEMA. When, however, AcChR was bound to neurotoxin that had been covalently coupled to Sepharose 4B, the concentration of affinity-purified antibody was selectively enhanced in IEMA measurements relative to RIA measurements.

Increased absorbance in the IEMA is most immediately caused by an increase in the amount of bound second-antibody–enzyme conjugate. Binding of conjugate to the plate, to Btx, or to AcChR, however, was negligible, yielding absorbance readings well within background values. Increases in bound second antibody thus appear to be related to increases in bound primary serum antibody. No basis for this binding of primary antibody has yet been discovered. We showed that primary serum antibody does not bind to the plate or to Btx. Nonpecific binding, if any, of serum antibody would have to be to non-AcChR antigens in the system. We applied albumin in an extra incubation step before adding AcChR, to reduce or prevent extraneous proteins in the receptor preparation from binding nonspecifically to any available sites on Btx-coated plates. The absence of any change in the results in the presence of albumin suggests that irrelevant antigens bound to the plates are not causing an artificial increase in IEMA results.

A second possible explanation of high IEMA/low RIA measurement of antibody concentration in an individual serum is that membranous components associated with AcChR could act as nonspecific antigens in the IEMA system. Such antigenic components, which might remain in the wells by electrostatic association with the AcChR bound to Btx, could react with primary antibody in the patient’s diluted serum that was not Ab(AcChR). This possibility is unlikely, inasmuch as several high molarity NaCl incubations did not remove or reduce IEMA/RIA differences (in purification of AcChR, such a procedure removes membrane protein contaminants loosely associated with AcChR). Moreover, such AcChR-bound antigens, should they exist, should also lead to falsely high values by RIA.

Another possible reason for this type of disparity—high RIA value but low IEMA for a given subject—is the location of the Btx binding site on the extracellular surface of AcChR. When Btx (8000 Da) is bound to a solid surface, the AcChR will bind face-down. Binding AcChR in this inverted posture may bring portions of its surface close to the plate that certain antigenic determinants are sterically prevented from binding serum antibody. Alternatively, restriction of antibody binding to AcChR could result from conformational changes imposed when AcChR binds to Btx on a solid-phase support. Differences in confirmation between solid- and liquid-phase AcChR could account for IEMA/RIA disparities in either direction. Dwyer et al. (22) used a monoclonal antibody against AcChR to hold the AcChR at some distance from the solid support. Nine of their 10 MG patients had similar results for Ab(AcChR) by both IEMA and RIA. This issue could be resolved by using a panel of monoclonal antibodies that bind determinants either on the cytoplasmic or on the extracellular surfaces of AcChR. Using such a procedure, Tzartos et al. (23) showed that the pattern of Ab(AcChR) specificity defined with monoclonal antibodies remained unique but constant for eight individuals over several years. This finding may also reflect the RIA/IEMA differences seen in our Figure 2.

We have shown that the high IEMA concentrations of Ab(AcChR) we found, with concomitant low or negative RIA values, are not artificial. The enzyme-conjugated second antibody does not bind to the plastic plate, to Btx, or to AcChR. Antibody in MG serum does not bind to the plastic or to Btx; it is unlikely to bind to extraneous protein, as discussed above. It is also unlikely that antibody from MG patients but not from healthy controls would bind nonspecifically to human AcChR only in an IEMA procedure. Our previous findings (9) with rabbit Ab(AcChR) contradict any explanation of RIA/IEMA differences based on the possibility that the IEMA is more sensitive than the RIA. We therefore suggest that concentrations of Ab(AcChR) measured preferentially by IEMA are probably related to the selectively enhanced availability of certain AcChR determinants in the IEMA system relative to the RIA.

Using the IEMA to measure Ab(AcChR) as an adjunct to the RIA is advisable because potentially between 10% and 15% of MG patients give negative results in the RIA (2, 9). We have found several patients who are negative for Ab(AcChR) by RIA but who have above-normal Ab(AcChR) concentrations when measured by IEMA. We have also found that some patients who have had symptoms of MG for less than two years have above-normal Ab(AcChR) concentrations only by IEMA (unpublished observations). These findings clearly warrant both the application of the IEMA method and further study of IEMA/RIA differences.

This work was supported by NIH grant NS-14491 and by grants from the Muscular Dystrophy Association of America and by the Myasthenia Gravis Association of Detroit. C.M.K. was the recipient of a Vieta Fellowship from the National Myasthenia Gravis Foundation.

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