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A modified assay for antibody against the nicotinic acetylcholine receptor in myasthenia gravis

D. S. DWYER, R. J. BRADLEY, S. J. OH & G. E. KEMP *Neurosciences Program and Departments of Psychiatry and Neurology, The University of Alabama in Birmingham, The Medical Center, Birmingham, Alabama* 35294, *USA*

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SUMMARY

Myasthenia gravis is an autoimmune disease which may be detected by the presence of serum antibodies against the nicotinic acetylcholine receptor at the neuromuscular junction. Immunoprecipitation assays have been developed to measure these immunoglobulins and calculate titres. These assays require the labelling of the receptor with ^{125}I-α-bungarotoxin which binds irreversibly. However, the standard immunoprecipitation assay may significantly underestimate the titres of some myasthenic patients. We have discovered patients with antibodies specific for the α-bungarotoxin binding site of purified rat muscle receptor. If labelled toxin is already present on the receptor, these antibodies are unable to bind to the protein. This phenomenon may lead to underestimates of the actual antibody titre. To circumvent this problem, we have designed a modified immunoprecipitation assay to evaluate titres.

INTRODUCTION

The autoimmune origin of the neuromuscular disease myasthenia garvis (MG) is now widely accepted (Drachman, 1978a,b). Pathogenesis appears to involve an antibody-mediated response (IgG) against the nicotinic acetylcholine receptor (AChR) of the muscle endplate, with impairment of neuromuscular transmission (Heinemann *et al.*, 1977; Kao & Drachman, 1977; Anwyl, Appel & Narahashi, 1977). Various groups (Almon, Andrews & Appel, 1974; Aharonov *et al.*, 1975; Lindstrom *et al.*, 1976) have detected antibodies against AChR in the serum of patients with MG. We have found measurable antibodies against purified rat muscle AChR in 88% of the myasthenic patients tested (Bradley *et al.*, 1978).

Anti-AChR antibodies have been assayed by two procedures: immunoprecipitation, a form of radioimmunoassay (Monnier & Fulpius, 1977; Lindstrom *et al.*, 1976) and inhibition of ligand (^{125}I-α-bungarotoxin, α-BGT) binding (Bender *et al.*, 1975; Almon & Appel, 1975). The immunoprecipitation assay involves labelling the receptor protein with ^{125}I-α-BGT which binds irreversibly. Antibodies from MG patients may combine with this toxin–receptor complex and can then be precipitated by adding the appropriate anti-human IgG. Titres are derived from immunoprecipitation values and are usually expressed as the number of toxin binding sites precipitated per litre of serum.

This method, however, may significantly underestimate the titre of antibodies in some patients. We have discovered a sizeable population of myasthenics whose IgG-AChR binding is inhibited by the presence of ^{125}I-α-BGT. Previously, we have reported one patient whose antibody is totally excluded from the receptor if toxin is already bound (Bradley *et al.*, 1979), Thus, bound toxin may alter or sterically occlude the antigenic determinants recognized by a population of myasthenic IgG. We now report this finding in a series of patients and a modified immunoprecipitation assay for determining titres.

Correspondence: D.S.Dwyer, Neurosciences Program, The University of Alabama in Birmingham, The Medical Center, Birmingham, Alabama 35294, USA.

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MATERIAL AND METHODS

Patients. The patients with MG reported here are part of a larger population of more than fifty patients who are under study in our laboratory. Four patients were selected for this study based on the fact that their specific IgG titre obtained by the conventional immunoprecipitation assay was significantly lower than the α-BGT inhibition titre.

Acetylcholine receptor. Purified AChR from denervated rat hind limb muscle was prepared according to the method of Kemp *et al.*, (1979). This procedure involves a combination of cobra toxin affinity chromatography, ion exchange chromatography and gel filtration. The specific activities of these receptor preparations were between 5 and 7 pmol of toxin bound per μg of protein.

Assay procedures. For the toxin inhibition assay varying amounts of serum were incubated with 20 fmol AChR for 16 hr at 4°C. Then, 0·2 pmol ^{125}I-α-BGT (New England Nuclear, sp. act. 135,000 Ci/mol) were added and incubation was continued for 1 hr at 25°C. The total volume of each sample was 200 μl. The mixture was passed over a CM-Sephadex C-50 column (Pharmacia) to separate the receptor–toxin complex from unbound toxin. The AChR-^{125}I-α-BGT was then counted in a gamma counter (Scientific Products). Values were expressed as the precentage inhibition of toxin binding as compared to control serum.

The immunoprecipitation assay was conducted by first labelling the receptor with ^{125}I-α-BGT and then removing excess toxin on a C-50 column as above. Labelled AChR was then incubated with serum for 16 hr at 4°C. Sera from non-myasthenic patients were used as controls. An optimal amount of goat anti-human IgG (ICN Pharmaceuticals) was then added for 6 hr at 4°C and the complex was precipitated by centrifugation at 100 *g*. Radioactivity in the pellet was counted and titres were calculated. The data may be presented as the maximum percentage of AChR precipitated per assay or alternatively as moles of ^{125}I-α-BGT precipitated per litre of serum. The latter value is taken from the linear portion of the precipitation curve.

In the modified precipitation assay, unlabelled AChR (20 fmol) was first exposed to serum (16 hr at 4°C) and precipitated as described. Receptor remaining in the supernatant was drawn off and incubated with 0·2 pmol of ^{125}I-α-BGT for 1 hr at room temperature. The mixture was passed through a C-50 column and counted. The amount precipitated by the serum was derived by subtracting the supernatant AChR from the total AChR used in the assay. In control experiments where serum from non-myasthenic patients was substituted, all toxin binding activity was recovered in the supernatant.

RESULTS

In Fig. 1 a saturation curve for all three assay systems is presented for serum from patient No. 1. It is evident that at each point the number of toxin binding sites which can be inhibited by serum is greater than the number of toxin sites which may be precipitated. However, the total amount precipitated by the modified assay is greater than the value obtained by toxin inhibition. This new estimate is in fact close to the sum of the values obtained by the other two assay systems. This would be expected if antibody binds to both toxin sites and distant sites on the AChR. For all four patients shown in Table 1 the toxin inhibition titre was greater than the precipitation titre for the same amount of serum. Titres obtained

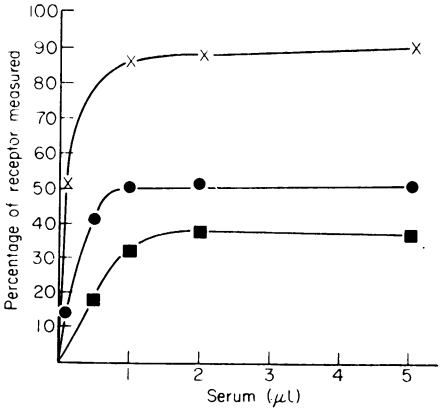

FIG. 1. Saturation profiles for three different assay systems to measure anti-AChR antibody. (●——●) ^{125}I-α-BGT binding to AChR inhibited by serum (inhibition assay). (■——■) ^{125}I-α-BGT–AChR complex precipitated after incubation with serum (conventional immunoassay). (×——×) Unlabelled AChR precipitated after incubation with serum (modified assay).

TABLE 1. Serum anti-AChR antibody titres for four patients with severe myasthenia gravis

Patient No.	Sex	Age	Titre by standard assay $\times 10^{-9}$ m/l	Titre by modified assay $\times 10^{-9}$ m/l
1	F	15	7·2	98·0
2	M	45	0	8·4
3	M	35	10·8	22·0
4	F	57	0·9	44·0

In the standard assay ^{125}I-α-BGT-AChR complex is first incubated with serum and then precipitated. In the modified assay the AChR alone is incubated with serum and precipitated. Any receptor remaining in the supernatant is assayed with ^{125}I-α-BGT.

using the modified assay were all much greater than the values derived by the standard immunoprecipitation assay. For example, patient No. 2 had no detectable titre when assayed by the standard procedure, but has a substantial value as determined by our modified method.

DISCUSSION

Patrick & Lindstrom (1973) demonstrated a relationship between anti-AChR antibodies and myasthenia-like symptoms in rabbits injected with *E. electrophorus* (electric eel) receptor. Subsequently, there have been considerable efforts to develop a reliable radioimmunoassay for the detection of antibodies in myasthenic sera. Many groups have reported the results of these assays (Lindstrom *et al.*, 1976; Mittag *et al.*, 1976; Monnier & Fulpius, 1977), but as we describe here, there may be an inherent underestimation of the actual titre due to the assay procedure. The conventional reaction is carried out by first labelling the receptor with ^{125}I-α-BGT, prior to incubation with serum and the subsequent preparation with anti-human IgG. However, when the ^{125}I-α-BGT is bound, the appearance of the AChR is changed, thus in some cases precluding the possible binding of antibody. Therefore, as we have reported, the estimated antibody titre may be dramatically altered. In the modified procedure as described in this paper, the unlabelled receptor is incubated with serum and then precipitated. The remaining AChR which does not have antibody bound is then assayed using ^{125}I-α-BGT. This method gives a more accurate estimation of the specific serum antibody activity against the AChR in MG patients.

Antibodies against the AChR may therefore be subdivided into several classes: (1) antibodies which block the binding of BGT to the AChR; (2) antibodies which will not bind to the AChR if BGT is already bound; and (3) antibodies against AChR sites not overlapping the BGT sites. It is probable that classes (1) and (2) are not mutually exclusive. Class (1) antibodies are determined by the inhibition assay, as they block the binding of α-BGT or acetylcholine but have not been shown to have any direct pharmacological effect on the receptor. This is in contrast to antibodies in Graves' disease which mimic the effects of thyroid stimulating hormone (Smith, 1976), or antibodies in insulin-resistant diabetes that bind to insulin receptors and stimulate insulin-like activity (Kahn *et al.*, 1978). The presence of class (2) antibodies is responsible for the inherent error of the conventional immunoprecipitation assay. The toxin molecule (mol. wt 8000) should be large enough to sterically hinder binding to sites on the receptor, especially if there are several toxin sites per AChR (Briley & Changeux, 1977). Patient No. 2 in Table 1 appears to have only class (2) antibodies for he shows a zero titre in the conventional precipitation assay, suggesting that no antibody can bind to the receptor if α-BGT is already bound. Class (3) antibodies are measured by the standard immunoprecipitation method, but as we have demonstrated, this may not give an accurate estimate of the anti-AChR antibody.

This communication reports clear errors in the conventional measurements of anti-AChR titres in the four patients examined. However, it must be remembered that these patients were selected for further

study because, as shown in Fig. 1 it was apparent that for a given amount of serum, the percentage of α-BGT binding which could be inhibited was greater than the percentage of α-BGT-AChR which could be precipitated. This unexpected experimental result was most easily explained by assuming that pre-bound α-BGT inhibited the binding of IgG, an hypothesis which the results from our modified technique now confirm. We are currently comparing anti-AChR titres obtained by the two precipitation assays in a larger number of patients in order to establish better the degree and frequency of antibody underestimation. Preliminary evidence suggests that the conventional precipitation assay underestimates the actual anti-AChR titres to some degree in 30–50% of all MG patients.

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