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Acetylcholine receptor antibody as a diagnostic test for myasthenia gravis: results in 153 validated cases and 2967 diagnostic assays

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SUMMARY Anti-acetylcholine receptor (AChR) antibody was undetectable in 26/153 (17%) sera from myasthenia gravis patients assayed by standard RIA using human acetylcholine receptor. Eight of these were found to be positive with a modified protocol using a mixture of normal and denervated AChR, reducing the proportion of "negative" sera to 12%. Many of these were from patients with a short history; two such patients later developed low positive values. Anti-AChR without clinical evidence of myasthenia was found in one of three monozygotic twins of myasthenia gravis patients, and in one of thirty other first degree relatives of a further 17 patients. Anti-AChR is a valuable and highly specific diagnostic test which, with the assay used here, is positive in about 88% of patients with clinical features of myasthenia gravis

In the last eight years it has been established that anti-acetylcholine receptor (anti-AChR) antibody is implicated in the loss of functional receptors in the post-synaptic membrane that underlies the defect in neuromuscular transmission in myasthenia gravis (for reviews see refs 1, 2). This antibody is usually detected by an immunoprecipitation assay in which AChR is labelled with ^{125}I-alpha-Bungaratoxin (a-BuTx), a snake toxin that binds to AChR with high affinity. Anti-AChR antibody appears to be specific for myasthenia gravis[3]. Its clinical acceptance as a diagnostic test is suggested by the steadily increasing number of serum samples sent to us for this assay (over 4,000 since 1980). In this paper we describe our assay methods and results in 153 myasthenia gravis cases studied before treatment by thymectomy or immunosupressive drugs, and we assess the value of this assay in diagnosis.

Methods

Iodination of alpha-bungarotoxin (a-BuTx)

Alpha-bungarotoxin was obtained from the Miami Serpentarium (Florida, USA) or from Biotoxins Incorporated (California, USA), iodinated by a modification of the method of Vogel *et al*[4] using 12·5 nmoles (100μg) of a-BuTx, 5mCi Na^{125}I and 28 nmoles (55μl) iodine monochloride in 205μl 0·3M NH_4Cl/NH_4OH, pH 8·9. The reaction was terminated after two minutes at room temperature by addition of 20μl sodium thiocyanate (0·1M) followed by 20μl 0·1M potassium iodide as carrier. The reaction products were diluted into 20 ml of 3mM phosphate buffer, pH 7·4 and applied directly to 1ml of DEAE-Sephadex (Pharmacia Fine Chemicals Ltd) equilibrated in the same buffer. After loading at 5ml/h and washing briefly the iodinated toxin was eluted using a gradient of 0–0·08M NaCl/3mM phosphate. 0·7–1ml fractions were collected and stored at 4°C after addition of phenylmethyl sulphonylfluoride (PMSF, 0·1M in propan-2-ol diluted 1 in 1,000 to give to give 0·1mM final concentration) as preservative. The bimodal peak obtained consisted of varying proportions of di-iodo and mono-iodo a-BuTx. The specific activity of the di-iodo a-BuTx which was used in all the assays described was 200-500 cpm/fmole counted on a Packard Autogamma.

Preparation of muscle extracts

Human muscle from amputated limbs, used in the standard assay, was obtained as fresh as possible and stored at −70°C until required. For the modified assay, gastrocnemius/soleus muscle was obtained 3–8 hours after death from patients with no neuromuscular disease and from two patients with amyotrophic lateral sclerosis. Muscle was cleaned of fibrous connective tissue and chopped roughly before homogenisation in an MSE atomix liquidizer in two volumes of 0·1M phosphate buffer, pH 7·4 with

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0·02% sodium azide and PMSF to inhibit proteolysis. Homogenisation at top speed was performed for periods of up to half a minute until a thick slurry was obtained. A further two volumes of buffer were added and the mixture centrifuged in an MSE angle 18, 6 × 250 ml rotor for 30 minutes at 13,000 rpm. On some occasions the pellet was resuspended in buffer and recentrifuged to eliminate most of the soluble proteins. The membrane pellets were then resuspended with agitation in an equal volume of 0·02M phosphate, pH 7·4, containing 2% Triton × 100 and PMSF (0·1mM), and rotated at room temperature for two hours or overnight at 4°C. The supernatants were separated by centrifugation at 13,000 rpm and filtered through Whatman standard filter paper. Further PMSF (0·1mM) was added before storage at 4°C.

The use of PMSF (0·1mM) as a protease inhibitor during preparation and storage of the extracts was crucial to prevent proteolysis and loss of AChR activity.

Acetycholine receptor assay

5–100 μl aliquots of muscle extracts were incubated at room temperature for at least two hours with dilutions of ^{125}I-a-BuTx (di-iodo) ranging from 0·5 to 5nM, and binding of toxin assessed by one of two methods: (a) binding of AChR-^{125}I-a-BuTx to DE 81 filter discs (Whatman Ltd) as described by Schmidt and Raftery[5]; (b) precipitation with anti-AChR. In (a), 25μl of the labelled extract was diluted into 250μl PTX buffer (20mM phosphate pH 7·4, 0·1% triton X 100), applied to presoaked double filter discs and washed with 10ml of PTX buffer. The discs were counted on a Packard Autogamma. In (b) the labelled extract was incubated with excess of a positive myasthenia gravis serum for two hours followed by incubation with excess antihuman IgG overnight (see below). The precipitate was pelleted, washed and counted as described below. As controls for both methods, a duplicate aliquot of extract was preincubated in cold a-BuTx, before addition of ^{125}I-a-BuTx, and the counts subtracted. Results were expressed as pmoles of toxin binding sites/ml of extract.

Standard anti-acetycholine receptor antibody assay

One hundred and fifty-three patients (103 F, 50 M) were examined by one of us (JND) and diagnosed as clinically definite myasthenia gravis on the basis of typical clinical features and responses to anti-choline esterase medication. These patients included 26 who clinically had only ocular muscle involvement, and 15 who had a thymoma. None of these patients had been treated by thymectomy, immunosuppressive drugs or plasma exchange before the serum sample was obtained. Sera were stored at −20° until required.

Myasthenia gravis sera were incubated at 1, 2·5 and 10 μl with 10–20 fmoles of ^{125}I-a-BuTx binding sites (labelled with ^{125}I-a-BuTx to about 80% saturation) in a volume of 75 μl. For the two smaller volumes, sera were diluted 1 in 20 in PTX buffer and 20 and 50 μl added to the 25 μl labelled muscle extract. The 10 μl assay was set up by adding 10 μl directly to the labelled extract. The volumes were made up with PTX buffer. After two to four hours at room temperature anti-human IgG (Seward Laboratories Ltd; 15–30 μl diluted 1:3 in PTX) was added to the 1 and 2·5 μl assays and the tubes left overnight at 4°C. The precipitates were pelleted, washed and counted as above. To the 10 μl asay an equal volume of 16% polyethylene glycol (PEG) was added and after overnight incubation and centrifugation the pellets were washed twice very briefly with 1 ml of PTX buffer (see ref 6) and counted.

Control incubations were performed with sera from normal healthy persons or neurological controls. The mean results from three control incubations were subtracted from each of the test assays. One high titre serum and one low titre (0·5–1·0 nmoles/l) serum were included as positive controls. Results were expressed as nmoles of ^{125}I-a-BuTx, binding sites precipitated/litre of serum.

The results were given as positive only if all three tests (1, 2·5 and 10 μl serum) were positive and consistent with each other. If there were inconsistencies, or the values obtained were less than 1·0 nmoles/l, the serum was retested. Titres greater than 0·5 nmoles/l were given as positive and based on the value obtained with 1 μl of serum, or with 2·5 μl in the case of relatively low titres (for example <2·0 nmoles/l). 1 μl of serum often precipitated most or all of the available AChR in which case the titre was given as a minimum value.

The mean cpm of three control sera was subtracted from these values, which in some test sera gave negative results. Sera from other normal controls or non-myasthenic neurological patients gave values between −0·3 and 0·3 nmoles/l (based on 2·5 μl serum). However, on repeat testing no control serum consistently gave values over 0·2 nmoles/l (see also ref 7). Thus sera whose values repeatedly fell in the range 0·2 to 0·5 nmoles/l were designated as equivocal.

Modified Assay

Some sera, particularly those with low anti-AChR titres, have recently been shown to react preferentially with AChR extracted from normal leg muscle or extraocular muscle[8,9] rather than with denervated leg AChR. In order to improve detection of such antibodies in those sera which were negative with the standard assay as described above, we used 50 μl of normal postmortem AChR and 5–10 μl of highly denervated AChR for each assay. In addition both membrane preparations were given an extra wash in

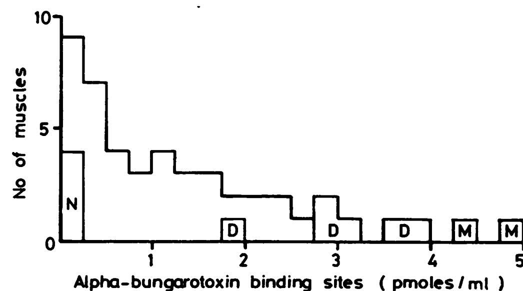

Fig 1 *Distribution of AChR concentrations in extracts from amputated muscles, including those with clinical diabetic neuropathy (D), compared with that for normal postmortem muscle (N) and postmortem muscle from patients with motor neurone disease (M).*

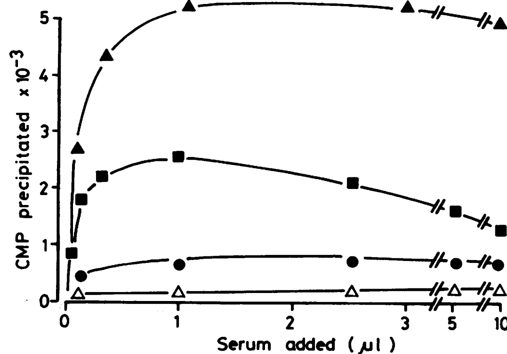

Fig 2 *Examples of titrations of three myasthenia gravis sera (filled symbols) and one control serum (open symbols) against a constant amount of ^{125}I-a-BuTx-AChR (5400 cpm). Some sera (for example ■ and to a lesser extent ▲) do not appear to precipitate all the available AChR and at antibody excess displace ^{125}I-a-BuTx from the AChR so that the cpm precipitated decrease. Another (●) appears to react with a subpopulation of the AChR preparation, in this case the normal AChR which represented less than 20% of the total in this partially denervated muscle extract.*

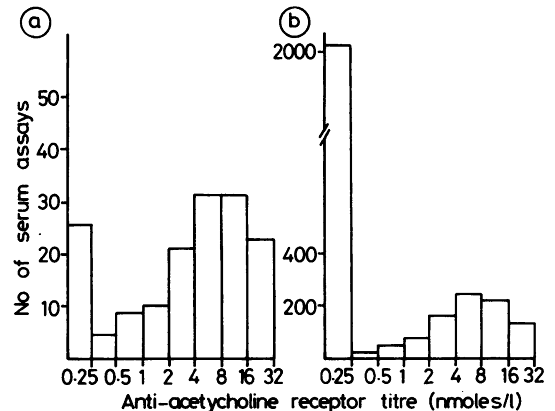

Fig 3 *Distribution of results of assays from 153 validated myasthenia gravis cases (3a) and of 2967 diagnostic assays (3b). Note the log_2 scale of the abscissa.*

buffer before extraction to reduce the protein and IgG content; a longer incubation (2 hours at room temperature and overnight at 4°C) was used; and after addition of anti-IgG and formation of a visible immune precipitate 200 μl—1 ml of PTX buffer was added to each tube to reduce non-specific precipitation.

Results

The anti-AChR assay

The majority of muscles used in the assays (n = 47) were from ischaemic limbs and the range of acetycholine receptor concentrations was wide (fig 1). The denervation present in muscle from patients with diabetic neuropathy and/or with ischaemia increased the yield of AChR, but amputated muscles from patients without clinical evidence of peripheral neuropathy also frequently contained a higher concentration of AChR than was found in the normal post-mortem muscles. Typically, the atrophied, discoloured muscles produced the highest concentration of AChR, whereas healthy looking, bulky muscles gave poor yields. The time elapsed (up to 8 hours) between amputation or death and removal of the muscle did not appear to influence the yield of AChR.

In the standard assay, three different serum concentrations were used. Anti-IgG was used to precipitate antibody AChR complexes because it gave lower non-specific precipitation than anti-Ig. We found no evidence of non-IgG antibodies which would have been detected by the 10 μl, 8% PEG precipitation.

The precipitation of CPM at 10 and 2·5 μl was in some sera substantially less than that at lower serum concentrations (fig 2) suggesting the presence of "toxin displacing antibodies".[10] These would have led to a false negative result if only high serum/AChR ratios had been used. Figure 2 also shows an example of a serum which reacted only with the normal AChR in the partially denervated muscle extract.

A potential deficiency of the assay is that antibodies directed against the a-BuTx binding site on the acetylcholine receptor are not detected because their binding site is occupied by the toxin. However, as we have shown,[11 12] if the acetylcholine receptor is only partially saturated (ideally 75–80%) with a-BuTx, the presence of a-BuTx site antibodies can be detected, and the lower concentration of ^{125}I-a-BuTx required reduced non-specific precipitation of radioactivity.

Anti-AChR in validated myasthenia gravis cases.

Figure 3a shows the distribution of serum titres using the standard anti-AChR assay in the 153 validated cases of myasthenia gravis. The results were bimodal with 26 sera (17%) clearly within the control range (see *Methods*). The highest values obtained (32 nmoles/l) were limited by the amount of serum used (1 μl) and the amount of AChR (20–50 fmoles); titres in these cases are expressed as a minimum value (for example >32 nmoles/l; much higher dilutions are required to achieve an accurate value in sera with high titres). Titres greater than

Table 1 *Distribution of results in 153 validated myasthenia gravis cases and 2967 diagnostic assays*

	Total	*Negative (< 0·2 nmoles/l)*	*Equivocal (0·2–0·5 nmoles/l)*	*Positive (> 0·5 nmoles/l)*
Myasthenia gravis cases (%)	153	26 (17%)	4 (3%)	123 (80%)
Diagnostic	2967	2037	22	872
Predicted*	1084	184	28	872

*Calculated by multiplying the observed number of validated myasthenia gravis cases in each group by the ratio of the number of diagnostic assays (872) to the number of +ve assays in the validated myasthenia gravis cases (123) (= 7·09).

0·5 nmoles/l were given as positive. The 4 (3%) sera whose titre lay between 0·20 nmoles/l and 0·5 nmoles/l on repeated testing were given as equivocal (see *Methods*), and those <0·20 nmoles/l as "negative".

Anti-AChR as a diagnostic test

During the years 1980–1983, 2967 sera were sent to us for anti-AChR assay. The results are presented in fig 3b. These include the 153 sera from validated myasthenia gravis cases described above. Eight hundred and seventy-two sera were positive, 22 were given as equivocal and 2073 were negative. Using the distribution of anti-AChR values in the validated cases, we calculated the expected incidence of myasthenia gravis in the sera sent for diagnostic assays (see table 1). This indicated that about 1084 samples (36%) came from "true" myasthenia gravis cases. The number of equivocal titres (22) accords well with the calculated value of 28 and suggests that most sera with equivocal titres came from patients with myasthenia gravis. In addition, about 184 of the sera which were negative would also have come from myasthenia gravis patients.

Further study of negative sera

The sera from 26 validated cases that had been negative using the standard assay were subsequently reassayed using a modified protocol (see methods) designed to detect anti-AChR of restricted specificity (that is reacting only with normal AChR) and of low affinity. Low positive titres (0·26–0·67 nmoles/l) were found in eight of the 26 MG sera which were previously negative, reducing the overall proportion of sera with undetectable anti-AChR to 12%.

Two further cases with ocular myasthenia gravis, in whom the first serum sample was negative on standard testing, developed low positive anti-AChR values by the modified assay when followed serially over a period of months (fig 4a). These cases were interesting because both had had an episode of ocular symptoms 11–15 years previously which had remitted spontaneously.

Five other anti-AChR negative cases were followed serially for 2–11 months. Anti-AChR was detected in only one who transiently showed an equivocal serum titre.

In general, patients whose sera were negative even with the modified protocol (n = 18) tended to have disease of short duration or symptoms restricted to ocular muscles. Only four anti-AChR negative patients had generalised disease of duration greater than one year.

Three patients with generalised myasthenia gravis (not included in the 153 validated cases), whose sera had been sent for diagnostic assay, were found to have negative titres on initial testing but moderately high levels subsequently. Their results are plotted in fig 4b. One of them, who had a steep rise in anti-AChR antibody over a period of one month, showed a similarly steep fall following thymectomy.

Raised anti-AChR without clinical evidence of myasthenia gravis

(a) Family studies Three of our female myasthenia gravis patients had a monozygotic twin; in each case,

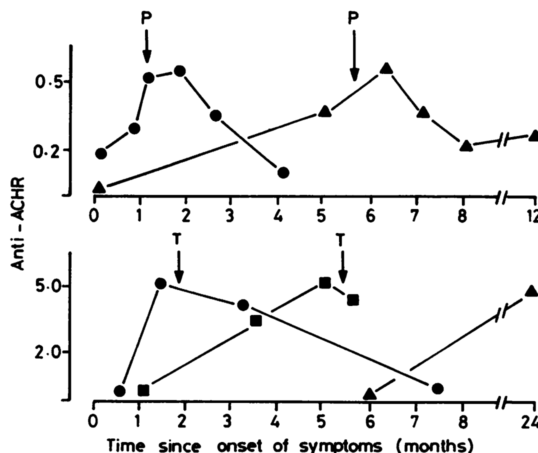

Fig 4 *Appearance of anti-AChR in sera from five patients all of which were negative on initial standard testing. (a) Two patients with previous histories of a single period of ocular weakness many years previously. Their results are plotted against the duration of the present symptoms. They were both started on prednisolone (P). (b) Three patients with generalised disease whose anti-AChR values rose substantially over a period of 1–6 months. Two patients underwent thymectomy (T); the other was a 2-year-old boy.*

Table 2 *Family studies*

	Proband		*Relatives*		
	Sex	*Anti-AChR (nmoles/l)*	*Examined*	*Clinical myasthenia gravis*	*Anti-AChR > 0·2 nmoles/l*
1	F	262	Mzt	−	24·5
2	F	470	Mzt	−	
3	F	22·8	Mzt	−	
4	F	93·7	S	+	18·0
5	F	46·9	F	+	8·3
6	M	16·0	B, M, F, S	+	3·0
7	F	1030	B	−	
8	F	229	M, F, B	−	
9	F	216	S, B	−	
10	F	143	S	−	
11	F	103	M, F	−	
12	F	93·6	M, F, B	−	
13	F	71·7	M, F	−	
14	F	62·0	S, B	−	
15	F	19·0	D	−	
16	M	19·0	F	−	
17	F	13·3	D	−	
18	F	7·4	S	−	
19	M	6·2	B, B	−	
20	F	4·4	F, M	−	0·45
21	F	1·8	M	−	
22	F	1·5	M, F	−	
23	F	< 2·0	M, F, B	−	

Mzt = Monozygotic twin; B = Brother; S = Sister; M = Mother; F = Father; D = Daughter. Underlining indicates raised anti-AChR titre. Three individuals (4, 5 and 6B) also had clinical myasthenia gravis.

the twin was unaffected, but in one of them anti-AChR was detected at a titre that was about 10% of that in the symptomatic twin (table 2). Three other myasthenia gravis patients had one other family member affected in whom serum anti-AChR was also detected; in one of these families, sera from other family members (mother, father, sister) were negative. Sera from 30 first degree relatives of a further 17 randomly selected myasthenia gravis cases were also analysed. An equivocal titre was found in one, the asymptomatic mother of a 16-year old girl who had a 10 year history of ocular myasthenia gravis. All other family sera were negative.

(b) Thymoma We have found titres of 0·4 and 0·6 nmoles/l in two patients with thymoma who had no clinical evidence of myasthenia gravis.

Discussion

The anti-AChR assay we have used is a modification of the method first reported in detail by Almon and Appel 1975,[13] Lindstrom 1976[3] and Lindstrom *et al.*[14] It differs in the relatively high concentration of serum used (up to 10 μl in 75–100 μl compared with 5 μl in 1ml), although not in the overall concentration of AChR (1–2 $\times 10^{-10}$M). Like Lindstrom[3] we use crude human AChR extract rather than partially purified AChR (see for example ref 15). We use a range of serum concentrations to avoid false negative results caused by displacement of toxin by antibodies,[10 16] and only partially label the AChR with a-BuTx in order to detect anti-a-BuTx site antibodies.[11 12] Our results in 153 validated myasthenia gravis cases (12% negative), which included 26 with purely ocular symptoms, are similar to those reported by others[3 7 14 15 17–19] using human antigen. The percentage of positive values in clinically definite cases of myasthenia gravis varies between 80 and 90% when human AChR is used as antigen but drops substantially if rat AChR is used.[20 21] On the other hand baboon AChR appears antigenically similar to human[22] and fetal calf AChR has also been used successfully.[23]

A proportion of patients (about 3%) with clinically definite myasthenia gravis have equivocal titres in the standard anti-AChR assay. The titres, although very low (0·20–0·5 nmoles/l), are not found in normal healthy controls and we conclude that an anti-AChR titre in this range indicates that the diagnosis of myasthenia gravis is likely.

We were concerned at the proportion of negative anti-AChR titres (< 0·2 nmoles/l) in patients with clinically definite myasthenia gravis, several of whom had responded to plasma exchange (unpublished observation) suggesting the presence of a humoral immune factor, and also to immunosuppressive drug treatment. Since about half of these patients had purely ocular symptoms, and there was evidence that this subgroup have antibodies which react better with normal AChR,[9] we re-assayed the

sera against a mixture of normal and denervated receptor using conditions designed to optimise the reaction and reduce background precipitation. A further eight patients were shown to have low anti-AChR and these modifications have now been included in our standard assay. Interestingly Oda and Ito[24] also improved the sensitivity of their assay by concentrating normal muscle AChR.

Some patients with myasthenia gravis of recent onset may not show a raised serum anti-AChR titre because the available anti-AChR is bound to the endplate receptors. The high affinity of anti-AChR[8] makes this likely, and indeed in the passive transfer model, human anti-AChR antibody can be bound to mouse endplate receptors in the absence of detectable serum levels in the patient (or in the mouse) (Mossman, Vincent and Newsom-Davis, unpublished observations). Such a mechanism might account for the negative anti-AChR titres in eight of our cases, whose symptoms were of recent onset (< 1 year).

The possibilities therefore exist that in some cases either serum anti-AChR never becomes high enough to detect *in vitro*, or that the antibodies present react with determinants which are not present on detergent-solubilised AChR. A further interesting possibility is that some patients have antibodies directed against a different component of the neuromuscular junction. Preliminary results of passive transfer of Ig from anti-AChR negative myasthenia gravis patients suggest that all three mechanisms may operate in different patients (Mossman *et al*, unpublished observations).

A serum titre of anti-AChR > 0·20 nmoles/l does not necessarily imply current clinical evidence of myasthenia gravis, and indeed raised titres are commonly found in known myasthenia gravis patients in remission.[7] Low positive results have also been reported in a proportion of subjects without clinical evidence of myasthenia gravis: in relatives of myasthenia gravis patients,[25] thymoma cases,[26] tardive dyskinesia[27] or elderly and Down's syndrome Japanese individuals.[28] In addition, we found two low positive titres in 56 patients undergoing penicillamine treatment for rheumatoid arthritis,[29] and we have also detected anti-AChR in three of 40 elderly Caucasian patients selected for high anti-thyroid autoantibodies, and who were thus predisposed to autoimmune disease.[30] However, no anti-AChR was detected in 53 elderly Caucasian subjects with miscellaneous disorders or in 30 individuals with Down's syndrome.[30] Moreover, in the small family study described here, anti-AChR (at equivocal titres) was found in only one of 30 unselected first degree relatives of myasthenia gravis patients. This is far lower than the incidence reported by Pirskanen *et al* in a similar number of Scandinavian subjects[25] and we cannot account for this difference. The only high value we found was in the monozygote twin sister of a patient whose own value was ten times higher. Two other identical twins of myasthenia gravis patients were negative. Our results confirm the presence of very low titres in two cases of thymoma without evidence of myasthenia gravis, but in none of eight polymyositis cases (unpublished results).

Our experience suggests that determination of anti-AChR is now the single most useful clinical test for myasthenia gravis. Using our current modified assay it is positive in 88% of patients with validated myasthenia gravis, but only in about 60% of patients with purely ocular symptoms. It is negative in all cases of congenital myasthenia in which immunological factors are not implicated.[31] The assay should be repeated after about six months in patients with suspected myasthenia gravis in whom it is negative. Some of these cases, however, may have antibodies which cannot be detected using the assay described here.

Technical false positives in this assay appear to be very infrequent. Biological false positives (that is patients without clinical evidence of myasthenia gravis) are very rare but may occur in first degree relatives of myasthenia gravis patients, in elderly patients with a pre-disposition to autoimmunity, in non-myasthenic thymoma patients and in those undergoing pencillamine treatment. In each of these groups, there is, of course, the possibility of later development of myasthenic symptoms.

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