Acetylcholine receptor turnover in mice with passively transferred myasthenia gravis

I. Receptor degradation

STEPHEN WILSON, ANGELA VINCENT, JOHN NEWSOM-DAVIS

From the Department of Neurological Science, Royal Free Hospital School of Medicine, London, UK

SUMMARY The *in vivo* degradation of endplate acetylcholine receptors was investigated in mice treated daily with IgG from 10 myasthenia gravis patients. Four preparations increased the rate of degradation. The number of endplate acetylcholine receptors after 7 days of IgG treatment was greater than that predicted on the basis of the increased degradation rate, suggesting a compensatory increase in receptor synthesis.

Myasthenia gravis (MG) is a disease of the neuromuscular junction characterised by weakness and fatigability of skeletal muscle. The basic defect is a reduction in the number of functional acetylcholine receptors (AChRs)^{1,2} resulting in reduced amplitude of the miniature end-plate potentials.³ This is due to the presence of anti-AChR antibodies which are detectable in 85-90% of MG patients,⁴ as determined by immunoprecipitation of solubilised human AChR labelled with radioactive alpha-bungarotoxin (a-butx), a snake toxin which binds specifically and irreversibly to muscle AChR (see ref 5).

The pathogenic role of anti-AChR was demonstrated by the passive transfer of MG to mice using IgG purified from MG patients^{6,7} and is indicated by the correlation between anti-AChR levels and clinical state during treatment of the disease in an individual.⁸ Anti-AChR is thought to cause a loss of postsynaptic AChR by several mechanisms which include complement-induced postsynaptic lysis⁹ and an increased rate of AChR degradation: MG sera or IgG were shown to accelerate degradation of ¹²⁵I-a-butx-labelled AChR in mammalian skeletal muscle cultures^{10,11} and to increase the rate of degradation of AChRs at the endplates of mice injected with MG IgG.¹² In this paper we have confirmed that some IgG preparations increase the degradation of mouse endplate AChR and we relate our results to anti-AChR levels against both human and mouse

AChR and show that some MG sera can bind mouse AChR *in vivo* without affecting the AChR degradation rate.

Methods

Patients IgG preparations were obtained from 10 different MG patients undergoing plasma exchange all of whom had elevated serum anti-AChR antibody (see table 1). All had had a thymectomy; the thymus showed hyperplasia in seven cases and thymoma in three. All were receiving alternate day steroid therapy at the time of study. Control serum was obtained from pooled human plasma and one male with Lambert-Eaton myasthenic syndrome.

Mice Two strains of mice were used. Initial experiments were done on AKR mice (Olac), and for subsequent experiments B6D2F₁ hybrids, as used by Toyka *et al.*,^{6,7} were specially bred.

Preparation of IgG

1 litre bags of plasma, removed during plasmapheresis, were stored at -20°C until required. They were thawed at room temperature and the fibrin removed by allowing the plasma to clot. The IgG was prepared and purified by the Rivanol ammonium sulphate technique.¹³ The final IgG solution was dialysed for 72 hours at 4°C against sterile Ringer lactate solution and filtered through a millipore filter system using a 0.2 µm pore size. The concentration of IgG, as determined by standard radial immune diffusion, in the final product varied between 23 and 36 mg/ml. It was diluted to 20 mg/ml in Ringer lactate before use.

Preparation of muscle extracts and assay of anti-AChR antibody

Human AChR was extracted from homogenised human muscle membranes by 1% triton X 100 in PTX buffer (0.1 M PO₄, pH 7.4, 0.1 mM phenylmethylsulphonylfluoride

Address for reprint requests: Dr A Vincent, Royal Free Hospital School of Medicine, Pond Street, London NW3 2QG, UK.

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0.02% sodium azide⁸). Mouse AChR was prepared similarly from the carcasses of skinned and eviscerated mice homogenised in PTX/1% triton X 100. The concentration of AChR in the extracts was determined by a filtration disk assay (DE 81, Whatman) using ¹²⁵I-a-buTx (20–50 × 10⁶ cpm/μg) at a concentration of 5 nM.⁸ The extracts contained between 0.1 and 2 nM human AChR and 1 to 1.5 nM mouse AChR respectively.

The IgG was assayed for anti-AChR activity against human and mouse receptor by an immunoprecipitation assay similar to that described by Lindstrom *et al.*⁴ The appropriate extracts were labelled with ¹²⁵I-a-buTx (5 nM) and dilutions of serum or IgG were incubated with the extracts for 2 hours at room temperature before addition of sheep anti-human IgG (Seward Laboratories Ltd) followed by centrifugation and washing in PTX buffer.

Binding of human anti-AChR antibody to mouse AChR in vivo

Groups of mice were injected daily with 15 mg (0.75 ml) of control or MG IgG for seven days. A single dose of cyclophosphamide (300 mg/kg) was given intraperitoneally on day 1 to reduce any immunological reaction that the mice might have to the foreign protein. The animals were killed on day 7 and the AChRs extracted with 1% triton X 100 and labelled with ¹²⁵I-a-buTx as described above. The total number of AChRs present in an aliquot (25 μl) was measured by the filter disk assay. The percentage of AChRs with antibody bound in MG IgG treated animals was determined by measuring the amount of AChR which was precipitated from the same volume of extract in the presence of carrier normal human serum and excess sheep anti-human IgG. The counts precipitated in the presence of carrier human IgG, after subtraction of the values obtained from control IgG treated animals, were expressed as a percentage of the total number of counts bound to AChR measured as described above.

Measurement of the total number of junctional AChR following injection of MG IgG

Groups of ten mice were injected daily with control or MG IgG as above. At day seven the animals were killed, their diaphragms removed and incubated *in vitro* in ¹²⁵I-a-buTx (1 μg/ml).¹⁴ After 3–4 hours the diaphragms were placed in Ringer lactate solution and left overnight at 4°C. The muscles were fixed in 5% glutaraldehyde, stained for cholinesterase, and cut into 2 mm strips containing end-plates and strips containing no end-plates; the pieces were then counted in the gamma counter. The binding to the end-plate region was corrected for non-specific binding by subtracting the non end-plate binding on a weight for weight basis. The results of MG IgG treated mice were expressed as a percentage of those obtained from diaphragms treated with control IgG only.

Measurement of AChR half-life in vivo

The technique used was similar to that described by Berg and Hall,¹⁵ Chang and Huang¹⁶ and Stanley and Drachman.¹² Groups of mice were anaesthetised with halothane, and given a single intrapleural injection of ¹²⁵I-a-buTx into the left hemi-thorax (0.1 μg/g body weight in a total volume of 70 μl of Ringer lactate solution). The amount

injected was determined by preliminary experiments and was chosen to bind to approximately 90% of the total available AChR sites. Four hours later (time "0") a group of mice were killed, their diaphragms removed and washed for two hours at room temperature and then overnight at 4°C in pre-oxygenated Ringer lactate solution. The muscles were fixed in 70% Ringer and 5% glutaraldehyde, and the radioactivity associated with the end-plate region measured as above. Groups of animals were killed at intervals over the next 2–10 days, and the binding to the end-plate region expressed as a percentage of the amount present at time "0" and plotted on a semi log scale against time.

Groups of animals (10–12), injected with a-buTx as above, were given daily injections of 15 mg of IgG intraperitoneally from an individual MG or control preparation starting at time "0". Two animals were killed at each time interval over the next 2–10 days and the decline in end-plate radioactivity measured as above.

A straight line was drawn through the points on the semi-log plots using the method of linear regression, and any difference between the slope of the line in MG IgG treated and control animals was assessed by Student's *t* test. The fractional degradation rate *K* can be given by $\ln 2/t_{1/2}$ where $t_{1/2}$ was the time in days, taken from the semi-log plots, by which the radioactivity had declined to 50% of the starting value. Results are expressed as $t_{1/2}$ values.

Measurement of dissociation of ¹²⁵I-a-buTx from mouse end-plates

The contribution of dissociation of a-buTx from the labelled diaphragms to the total loss of ¹²⁵I-a-buTx *in vivo* was assessed by *in vitro* culture of ¹²⁵I-a-buTx labelled diaphragms in the presence of 10% control IgG or MG IgG (MG7 and MG10) for 72 hours at 37°C. 0.2 nM dinitrophenol was added to prevent active degradation.

Calculation of the predicted number of AChRs after IgG treatment

The number of AChRs present at the endplates of MG IgG treated mice at *t* days (*R_t*) was predicted for *t* = 7 days using the equation given by Linden and Fambrough¹⁷

$$R_t = \frac{r}{K}(1 - \exp(-Kt)) + R_0 \exp(-Kt)$$

where *r* is the fractional synthesis rate per day in the absence of increased degradation (taken as equal to the control degradation rate 0.095/day, see *Results* and ref 18), *K* is the measured degradation rate per day and *R₀* is the number of AChRs present at time 0. Results were expressed as a percentage of *R₀*.

Results

Serum anti-AChR titres in the ten MG patients measured against human receptor gave little indication of the titre measured against mouse AChR (tables 1 and 2) which varied considerably for both species (*r* = 0.57, ns) although it was very similar between the two mouse strains. There was, however, some correlation between the titre measured against

Table 1

Case	Age/sex		Grading	Thymoma (T) Hyperplasia (H)	Anti-human AChR antibody titre $\times 10^{-9}$ M
MG/1	49	M	IV	T	116.0
MG/2	20	M	IIb	H	29.4
MG/3	32	F	IV	H	48.0
MG/4	22	F	IIb	H	22.8
MG/5	50	M	IV	T	25.0
MG/6	50	F	IV	H	79.0
MG/7	55	F	IV	T	78.0
MG/8	21	M	III	H	13.0
MG/9	31	F	III	H	2.2
MG/10	40	F	IV	H	16.0

Table 2 The effects of IgG preparations on the percentage of AChR with antibody bound and the degradation rate related to anti-mouse AChR titres

	Anti-mouse AChR $\times 10^{-9}$ M	Percentage of extractable AChRs with bound antibody on day 7 (n = 2)	AChR degradation $t_{1/2}$ in days [†]
No IgG	—	0	7.0 $\pm$ 0.5, n = 3
Control 1	—	0	7.2 $\pm$ 0.3, n = 4
		0	7.0 $\pm$ 0.4, n = 4
		0	7.1 $\pm$ 0.2, n = 3
MG/1	5.8	0 $\pm$ 0.24	7.7 $\pm$ 0.44
	5.2	26 $\pm$ 0.35	6.5 $\pm$ 0.24
MG/2	0.4	4 $\pm$ 1.1	6.6 $\pm$ 0.38
	0.2	4 $\pm$ 1.3	5.3 $\pm$ 0.18*
MG/3	10.6	68 $\pm$ 8.1	6.7 $\pm$ 0.38
	10.1	56 $\pm$ 0.2	8.7 $\pm$ 0.76
MG/4	4.1	67 $\pm$ 8.5	5.5 $\pm$ 0.22‡
	4.3	63 $\pm$ 1.9	4.8 $\pm$ 0.16‡
MG/5	2.3	31 $\pm$ 2.8	7.25 $\pm$ 0.17
	2.5	15 $\pm$ 0.8	7.1 $\pm$ 0.38
MG/6	4.7	76 $\pm$ 0.4	4.25 $\pm$ 0.11‡
	5.5	70 $\pm$ 0.1	7.1 $\pm$ 0.32
MG/7	14.8	82 $\pm$ 0.5	4.1 $\pm$ 0.29‡
	13.3	3.2 $\pm$ 1.9	3.25 $\pm$ 0.1‡
MG/8	0.4	0	7.0 $\pm$ 0.97
	0.3	2.8 $\pm$ 1.6	6.7 $\pm$ 0.28
MG/9	0.0	36 $\pm$ 0.9	6.4 $\pm$ 0.37
	0.0	0 $\pm$ 3	6.4 $\pm$ 0.27
MG/10	4.6	84 $\pm$ 0.7	5.3 $\pm$ 0.19‡
	4.6	84 $\pm$ 2.5	6.0 $\pm$ 0.19*

* $p < 0.01$

[†]Only one experiment was performed for each MG preparation and the SE is the standard error of the regression line derived from results using 8–10 mice. For controls the results are given as mean $\pm$ SD of n experiments. Results from MG IgG treated animals are given for both AKR (above) and B6D2F₁ (below) strains. Student's *t* test comparing the slope of the semi-log plots with that of the control experiment.

‡ $p < 0.001$.

AKR mouse AChR and the percentage of extractable AKR AChRs which had human anti-AChR attached (table 2; $r = 0.6$, $p = 0.05$) although none in the case of B6D2F₁ mice ($r = 0.26$, ns).

The $t_{1/2}$ for labelled AChRs in animals not receiving injected IgG did not differ from that in animals injected with control IgG in either strain of mice (see table 2). On the other hand, the effects of MG IgG varied with the individual donor. Four IgG preparations caused a significant decrease in the $t_{1/2}$ of AChRs in AKR mice, and four had a significant effect upon B6D2F₁ mice (table 2). An example of an experiment in which there was no increased degradation is shown in fig 1a and one in which an

MG IgG caused accelerated degradation is shown in fig 1b. The decrease in $t_{1/2}$ for ¹²⁵I-a-buTx labelled AChRs found in animals treated with different IgG preparations correlated inversely with the percentage of AChRs which had human antibody bound for AKR ($r = -0.81$, $p < 0.01$) but not for B6D2F₁ mice ($r = -0.26$, ns).

It was possible that during the long time scale of these experiments ¹²⁵I-a-buTx was displaced from the AChR by the injected MG IgG antibodies. The reversibility of ¹²⁵I-a-buTx from labelled mouse diaphragms was investigated in cultured diaphragms kept for 72 hours at 37°C in the presence of 10% control IgG and 2,4-dinitrophenol to prevent

Fig Loss of a-butx from the endplates of mice injected with control (open symbols) or MG (closed symbols) IgG. The half life for control IgG injected animals is 7.1 days. (A) Acetylcholine receptors in animals injected with MG 5 IgG preparation (triangles) had a normal degradation rate. (B) AChR degradation in animals injected with MG 6 IgG preparation (squares) was increased with a half life of about 4.25 days. Each point represents one hemidiaphragm.

Table 3 The observed total number of AChRs after 7 daily injections of IgG compared with the number predicted from the accelerated AChR degradation rate in AKR mice

Source of IgG	Observed AChRs at day 7 as a percentage of control IgG injected animals	Predicted AChRs at day 7 as a percentage of initial number*
MG/4	95.5	89.3
MG/6	88.4	75.8
MG/7	77.5	74.1
MG/10	90.1	86.8

*These values are derived by calculation (see methods).

degradation. Reversibility of a-butx was only 5% of the total bound during this period and was not greater in the presence of MG IgG preparations 7 and 10 (results not shown). Both these preparations increased the loss of radioactivity from diaphragms *in vivo*.

There was no apparent correlation between increased degradation of AChRs and either thymic pathology, age at onset, clinical severity or anti-AChR titre against human receptor.

The number of junctional AChRs present in animals which had been injected with IgG over a period of 7 days was measured after incubation *in vitro*. The total number of endplate AChRs present was always higher than that which would be predicted

taking into account the increased rate of degradation (see table 3). In mice injected with IgG preparations which did not increase degradation the total number of AChRs at day 7 was between 97 and 101% of that present in control treated animals. There was no increase in extrajunctional binding in any IgG-treated animals compared with non-treated controls.

Discussion

The effect of MG IgG on AChR degradation was assessed in mice with passively transferred MG by labelling the diaphragm end-plate AChRs with ^{125}I -a-butx and determining the loss of radioactivity

during daily injections of IgG. Loss of radioactivity with time is accepted as an approximation of the rate of junctional AChR degradation^{12 15-17} in normal animals although it includes simple dissociation of a-buTx from the receptor *in vivo* which results in an over-estimation of degradation rates. In MG IgG treated animals loss of radioactivity could also be due to complement-dependent lysis of AChR-containing membranes resulting from the interaction of human antibodies with mouse complement, and displacement of a-buTx from the AChRs by anti-AChR antibodies. Complement does not appear to be involved since Stanley and Drachman¹² found an increased loss of a-buTx from the endplates of MG IgG treated mice when cultured *in vitro* (in the absence of complement), and no ultrastructural signs of complement-induced damage were found in mice treated with MG IgG for up to 30 days.⁷ Moreover, we exposed a-buTx-labelled mouse diaphragms to control and MG IgG for 72 hours at 37°C *in vitro* in the presence of 2-4-dinitrophenol to prevent degradation and found that the loss of a-buTx was similar, and less than 5%, in each case. It is therefore reasonable to suppose that an increased rate of loss of bound a-buTx *in vivo* in MG IgG treated animals is neither due to displacement by antibody nor to complement-induced lysis of post-synaptic membrane.

The $t_{1/2}$ s which we obtained for ¹²⁵I-a-buTx labelled endplate AChRs in the two strains of mice treated with control IgG were similar and did not differ significantly from those found in untreated animals, or previously determined by other workers.^{12 15-17} The effect of MG IgG on the $t_{1/2}$ varied with the individual donor; some preparations caused a significant decrease but others did not. This effect was not related to the clinical state of the patient nor to the serum level of anti-(human) AChR antibody. These results contrast with those reported by both Conti-Tronconi *et al*¹⁹ and Drachman *et al*²⁰ who found a correlation between the clinical state and the effect upon AChR degradation of cultured rat myoblasts. The lack of correlation between increased degradation and anti-AChR antibody that we found is, however, not surprising since neither the titre⁴ nor the characteristics²¹ of anti-AChR correlate well with severity of disease between different individuals.

Some of the lack of effect of individual preparations could be related to the limited cross reactivity of anti-AChR with mouse receptor. This had a mean value of 11.1% and varied between IgG preparations as has been shown previously.^{21 22} There was, however, a just significant correlation ($p = 0.05$) between the anti-AChR titre measured against AKR mouse AChR *in vitro* and the degree of bind-

ing of human antibodies to AKR AChR *in vivo* at seven days. Nevertheless, even when anti-AChR reactivity with mouse AChR is taken into account the increase in AChR degradation is limited to only some of the MG IgG preparations. It seems that the binding of antibody to the AChR is not necessarily sufficient to bring about an increased rate of AChR degradation. In order to increase degradation anti-AChR antibodies have to cross-link acetylcholine receptors.²³ It may be that some anti-AChR antibodies bind to mouse AChR *in vivo* without cross linking them, as has been shown with passively transferred monoclonal antibodies.²⁴ It is, therefore, probable that not all human anti-AChR antibodies induce accelerated AChR degradation and in these cases complement-induced lysis⁹ of the post synaptic membrane may play an important role in causing loss of AChRs in MG.

It was interesting that the total number of junctional AChRs after seven days in mice injected with IgG preparations that increased the rate of degradation was greater than that predicted from the degradation rate. Both Toyka *et al*^{6 7} and Oda *et al*²⁵ found reduced miniature endplate potentials in mice treated between one and three days with MG IgG. Our results at seven days suggest a delayed, compensatory increase in AChR synthesis operating in those animals in which degradation was increased. The accompanying paper¹⁸ describes experiments designed to measure the rate of synthesis of acetylcholine receptors during passive transfer of MG.

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