Acetylcholine receptor turnover in mice with passively transferred myasthenia gravis

II. Receptor synthesis

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SUMMARY The *in vivo* synthesis of diaphragm endplate acetylcholine receptors was estimated in mice treated daily with IgG from eight myasthenia gravis patients. Myasthenia gravis IgG preparations which had previously been shown to increase the rate of receptor degradation also increased the rate of receptor synthesis, suggesting the existence of a compensatory mechanism serving to stabilise the number of acetylcholine receptors.

An increased rate of acetylcholine receptor (AChR) degradation is thought to play an important role in the loss of end-plate AChRs which occurs in myasthenia gravis (MG). The rate of loss of ^{125}I -a-bungarotoxin (a-buTx)-labelled AChRs is increased both in myoblast cultures^{1,2} and in mice injected daily with IgG prepared from MG patients.³ We have confirmed these results but find that the increased rate of degradation is not an invariable result of human antibodies binding to mouse AChR at the end-plate.⁴ Moreover, even in those animals in which an increased rate of AChR degradation could be demonstrated the total number of acetylcholine receptors present at the end-plate after seven daily IgG injections was reduced less than predicted from the increased degradation rate.

These observations suggested that an increased rate of AChR synthesis might compensate for the increased rate of degradation that occurred with some IgG preparations⁴ and in this paper we describe the results of experiments designed to test this possibility. End-plate AChRs were first blocked by intrathoracic injection of non-radioactive a-buTx. The animals then received daily injections of IgG. The rate of synthesis of new AChRs, free from a-buTx, was measured at various time intervals by removing the diaphragms and incubating in radioactive a-buTx *in vitro*. AChR synthesis was increased

in animals injected with IgG preparations which increased AChR degradation.

Methods

Patients The IgG preparations were obtained from the ten patients described in the preceding paper.⁴ Mice of both the AKR strain and the B6D2F1 hybrids were again used. The IgG was purified by the Rivanol ammonium sulphate method from plasma obtained during plasma exchange therapy.

Synthesis of junctional AChRs of mouse diaphragm *in vivo* Groups of animals ($n = 10$) were anaesthetised with halothane as previously described⁴ and a-buTx ($0.1 \mu\text{g/g}$ body weight) was injected into the left hemi-thorax. The amount of a-buTx was critical as high doses led to an unacceptable mortality rate, and too low a dose caused insufficient blocking of the end-plate AChR. The exact amount injected was determined from preliminary experiments in which the animals were killed four hours later and their diaphragms incubated *in vitro* in ^{125}I -a-buTx as described in the preceding paper.⁴ The amount of a-buTx injected was designed to block more than 90% of the mouse end-plate AChRs without killing the animals. The value for unblocked AChRs at 4 hours was used as R_0 (see below).

The injected animals were killed at intervals over 1-9 days and the appearance of AChRs free of a-buTx was measured by incubation of the diaphragm *in vitro* in ^{125}I -a-buTx as described previously.⁴ The number of observed AChRs (R_{obs}) was expressed as a percentage of the initial total number present in diaphragms from untreated mice. The rate of synthesis of AChR was determined using the degradation constants (K) derived from the preceding paper: if R_s is the actual number of AChRs synthesised by any particular time t , then $R_s = T \cdot r$ where r is the frac-

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tional synthesis rate per day. R_{Obs} , the observed number of AChRs, is made up of $r/K(1-\exp(-Kt))$, the number of newly synthesised AChRs which remain, and $R_0 \exp(-Kt)$ the number of residual unblocked AChRs (R_0) which are still present (see ref 5).

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

$$\text{therefore } r = \frac{(R_{Obs} - R_0 \exp(-Kt))K}{(1 - \exp(-Kt))}$$

$$\text{and } R_s = \frac{(R_{Obs} - R_0 \exp(-Kt)) K t}{1 - \exp(-Kt)}$$

The values for R_s were calculated and plotted against time and a line drawn through them by the method of linear regression.

The effect of MG and control IgG upon the rate of synthesis of junctional AChRs in vivo was assessed by injecting 15 mg/day of IgG intraperitoneally into each

animal starting after the intrathoracic injection of a-buTx. Cyclophosphamide (300 mg/kg) was given as a single dose on day 1 to suppress the immunological response to the foreign protein and the preparations of IgG were the same as those used in the previous study.⁴

Results

In this study we have taken advantage of the essentially irreversible nature of a-buTx binding to block existing AChRs at the mouse diaphragm end-plates by intrathoracic injection, and have measured the appearance of AChRs free from bound a-buTx over a period of days by incubation in ¹²⁵I-a-buTx in vitro. The non-endplate bound ¹²⁵I-a-buTx, which was subtracted, was always less than 10% of the control treated-endplate binding and did not differ between control (uninjected), control IgG and MG IgG treated animals.

An example of results from an experiment performed with control IgG is shown in fig 1A, and from three using MG IgG preparations in fig 1B,C,D. The rate of synthesis in control IgG treated animals was similar to that derived from experiments in non-IgG treated animals and with the measured rate of degradation in untreated animals⁴ (see table 1). The rate of appearance of newly synthesised AChRs in end-plates of MG IgG treated animals was variable and in some cases did not differ from control values (table 1). In contrast, some preparations, for example, MG 4, 6, 7 and 10 (see fig 1) caused a significant increase in the rate of appearance of unlabelled AChRs.

For each experiment the synthesis rate constant was derived using the equation given in the methods. The effect of MG IgG on the calculated rate of synthesis of AChR was not apparently related to the clinical state of the patient, to the anti-AChR titre against human or mouse AChR, or to the binding of human antibodies to the mouse AChR in vivo. However, those IgG preparations which had no effect upon the rate of AChR degradation in the previous experiments⁴ also had no effect on the rate of synthesis determined here, and the IgG preparations which had caused an acceleration of degradation also brought about an increased synthesis of new AChRs. This increased synthesis rate was clearly not due simply to the use of an increased degradation rate in the calculation since the actual observed number of newly synthesised AChRs was also increased (see fig 1). When the results of experiments in both AKR and B6D2F1 mice were combined there was a significant positive correlation between the rates of degradation and synthesis ($p < 0.01$; fig 2).

Fig 1 Experiments designed to show AChR synthesis in mice injected with control or MG IgG preparations. In each case the closed symbols represent the experimental observations (R_{Obs}) and the open symbols the calculated number of AChRs synthesised (R_s) as a percentage of the total number present initially.

A AKR mice injected with control IgG.

B B6D2F1 injected with MG 10 IgG.

C AKR (Δ) and B6D2F1 ($\circ$) mice injected with IgG 7.

D AKR mice injected with IgG 4. Note that the observed appearance (R_{Obs}) of new binding sites as well as the calculated synthesis (R_s) is increased in these MG IgG treated animals.

Table 1 The effect of IgG upon the rate of degradation and synthesis of AChRs

Patient	AChR degradation %/day†	AChR synthesis %/day
Control 1	9.5 ± 0.4 (± SE n = 4)	9.4 ± 0.4 (SE n = 4)
Control 2	—	9.7 (n = 2)
MG 1	8.5	—
MG 2	10.0	—
MG 3	9.8	7.8
MG 4	11.9*	18.2‡
MG 5	9.1	9.7
MG 6	15.0‡	12.3*
MG 7	15.7*‡	13.4/14.8*
MG 8	9.4	—
MG 9	10.2	—
MG 10	12.2*	15.5‡
Control 1	9.4 ± 0.4 (± SE n = 4)	9.2 ± 0.2 (± SE n = 3)
MG 1	10.2	—
MG 2	12.2*	—
MG 3	7.6	7.8
MG 4	13.5‡	14.6‡
MG 5	9.4	9.6
MG 6	9.4	10.7
MG 7	19.3‡	15.9‡
MG 8	9.8	8.4
MG 9	10.1	8.5
MG 10	10.8*	16.7‡

*p < 0.01, ‡p < 0.001, (Student's t test)

†Results derived from ref 4.

Fig 2 Correlation between AChR synthesis and AChR degradation rates for AKR (circles) and B6D2F1 (triangles) mice.

Open circles are from control IgG treated animals.

Discussion

This method for estimating the rate of synthesis of end-plate acetylcholine receptors in vivo has not been described previously and depends on certain assumptions. First, the rate of dissociation of unlabelled a-buty from the AChRs in vivo must be

relatively small and not affected by the injected IgG preparations. The rate of dissociation of toxin under culture conditions at 37°C has previously been demonstrated for mouse and rat muscle by Heinemann *et al*⁶ and Reiness *et al*⁷ who inhibited AChR degradation with dinitrophenol⁶ or sodium cyanide.⁷ They obtained rate constants for dissociation of a-buty of about 4% per day. Under similar conditions we found a value of less than 2% per day.⁴ If this value is taken into account in the loss of endplate a-buty binding, the values obtained (in percent/day) for both degradation and synthesis would be lower, but the relative increase in degradation or synthesis in MG IgG treated animals would in fact be a little higher. We found no evidence that MG IgG preparations could by a direct effect increase the rate of dissociation of a-buty.⁴

The calculation of Rs, the actual number of AChRs synthesised, depends critically on the rate of degradation. Since degradation experiments could not be performed on the same animals as the synthesis experiments, this could be a source of error. The calculation also assumes that newly synthesised AChRs are degraded at the same rate as those labelled with ¹²⁵I-a-buty in the degradation experiments. This is difficult to prove but the apparent first order kinetics for loss of ¹²⁵I-a-buty from mammalian endplates observed by ourselves and others in degradation experiments^{6,7} suggests the existence of a single turnover rate rather than a mixture of different rates.

One further factor is that the end-plate could become functionally inactive as a result of injection of the almost saturating amounts of a-buty combined with MG IgG; this might affect the rate of degradation and synthesis of the receptors which is now known to increase when end-plates are experimentally denervated by cutting the nerve.^{8,9} However there was no evidence of any denervation effect as judged by the lack of extra-junctional ¹²⁵I-a-buty binding in both these experiments and those reported in the preceding paper.⁴

The rate of synthesis of acetylcholine receptors found in uninjected control animals was very similar to that determined in mice injected with control IgG or with IgG from MG patients that did not increase degradation. However the rate of AChR synthesis was increased in those animals in which an increased rate of degradation had previously been found.⁴ The increased appearance of newly synthesised AChRs was evident even before the correction for calculation of the true rate of synthesis, which included the degradation rate constant, had been applied. This indicates that the increased rate of synthesis was not due merely to the use of a larger degradation constant in the calculations. There was a significant correlation between the rates of degradation and rates of synthesis in animals treated with the individual MG IgG preparations.

The extra-junctional AChRs which appear after denervation or during in vitro culture, show a rate of synthesis which is controlled independently of the fractional degradation rate which is constant.⁵ One previous study found no increase in the synthesis of AChRs on cultured myoblasts during the six hours immediately after MG IgG had been removed.¹⁰ On the other hand, evidence of increased AChR synthesis was found in chick myoblast cultures between 6 and 10 hours after MG IgG treatment.¹¹ As far as the intact neuromuscular junction is concerned synthesis and degradation may not be independent. The increased fractional degradation rate which has recently been described for endplate AChRs after denervation of the mouse diaphragm⁸ appears to be balanced by an increased synthesis since the total number of endplate AChR declines only slowly after denervation.

Our findings may have important implications for the mechanisms of AChR receptor loss in MG. The fact that human anti-AChR antibody can bind to mouse AChR without affecting either the rate of degradation or the rate of synthesis may be at least partly due to cross-reactivity between the species and may not indicate that these patients' antibodies fail to degrade their own receptors in vivo. However, the rate of synthesis of AChRs was consistently increased in animals treated with those MG

preparations which also increased degradation. This finding suggests the existence of a compensatory mechanism at the mouse post-synaptic membrane that serves to stabilise the number of AChRs. A similar mechanism existing in human MG muscle would tend to limit the overall loss of acetylcholine receptor.

Any effects of MG anti-AChR antibodies in humans, however, would be complicated by the existence of other mechanisms of functional AChR loss. For instance, MG patients have humoral factors that directly block pharmacological activation of the AChR in vivo.^{12,13} More importantly, there is ample evidence that complement plays a major role in loss of AChR containing post-synaptic membrane^{14,15} and it is not possible at present to determine the rates of degradation or synthesis in human endplates which have undergone morphological changes. However such experiments have been performed on rats with experimentally induced myasthenia gravis. AChR incorporation into damaged endplates appeared to be reduced, probably due to lack of available post synaptic membrane.¹⁵ Therefore if a compensatory increase in synthesis does occur in human MG endplates, its relevance may be restricted to those at which structural damage is not too severe.

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