

Myasthenia gravis: further electrophysiological and ultrastructural analysis of transmission failure in the mouse passive transfer model

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SUMMARY Using the mouse passive transfer model the mean amplitude of miniature endplate potentials and endplate potentials of mice treated with myasthenic immunoglobulins was markedly decreased. Miniature endplate potential frequency and quantum content of endplate potentials were normal, arguing against a major presynaptic disarrangement. Under electron-microscopy no gross structural alterations of endplates were demonstrated. It is concluded that the mouse passive transfer model closely resembles human myasthenia gravis of recent onset.

Myasthenia gravis is a neuromuscular disease characterised by weakness and abnormal fatigability of skeletal muscle based on failure of neuromuscular transmission. The cause of the transmission failure has long been thought to lie in the presynaptic nerve terminal of the neuromuscular junction (Elmqvist *et al.*, 1964). Recently, however, a large body of evidence has accumulated pointing to the postsynaptic membrane as the site of the defect. The findings indicate a reduced number of receptors available for bungarotoxin binding at the endplate region (Fambrough *et al.*, 1973; Engel *et al.*, 1977a). It has been shown that the reduced sensitivity of myasthenic muscle endplates to microiontophoretically applied acetylcholine (Albuquerque *et al.*, 1976), as well as the decreased bungarotoxin binding, can be accounted for by either loss or damage of receptors, or their occlusion by some other agent to which they will bind (Engel *et al.*, 1977a, b). Structural alterations of the postsynaptic muscle membrane (Engel and Santa, 1971) may also interfere with transmitter diffusion to the receptor or with the receptor itself.

Several lines of evidence now suggest that the reduction in receptor availability involves a

humorally mediated autoimmune reaction against the ACh-R:

1. Immunisation of animals including primates with purified ACh-R results in a myasthenia-like disease (experimental autoimmune myasthenia gravis) (Patrick and Lindstrom, 1973; Green *et al.*, 1975; Tarrab-Hazdai *et al.*, 1975; Granato *et al.*, 1976; Sanders *et al.*, 1976).
2. Antibodies against ACh-R were demonstrated in the serum of patients with myasthenia gravis in vitro (Almon *et al.*, 1974; Aharonov *et al.*, 1975; Bender *et al.*, 1975; Lindstrom *et al.*, 1976a; Mittag *et al.*, 1976) using various techniques.
3. Passive transfer of immunoglobulin G from patients with myasthenia gravis to mice reproduces typical features of the disease (mouse passive transfer model) (Toyka *et al.*, 1975, 1977).
4. Incubation of cultured fetal muscle cells with myasthenic immunoglobulins results in an accelerated degradation of ACh-R (Appel *et al.*, 1977; Kao and Drachman, 1977), and to a reduction in acetylcholine sensitivity in vitro (Anwyl *et al.*, 1977; Bevan *et al.*, 1977).
5. Immunoglobulin-like particles (Rash *et al.*, 1976), and more recently IgG and C3 (Engel *et al.*, 1977b), have been located by electron-microscopical examination at the endplate membrane in human myasthenic muscle.

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A question of interest is what specific defects of neuromuscular transmission develop in an experimental animal not producing antibodies against its own ACh-R but injected with immunoglobulin from myasthenic humans. We also wished to know whether treatment of the clinical disease would alter the response of mice injected with the patients' immunoglobulins.

The present studies confirm the electrophysiological similarities between human myasthenia gravis, experimental autoimmune myasthenia gravis, and the mouse passive transfer model. We also find that the efficacy of myasthenic immunoglobulin in producing defective neuromuscular transmission in the mouse is not predictable from the severity of the clinical disease.

Patients and methods

Thirteen patients (five female, eight male) with the diagnosis of myasthenia gravis—established by criteria used previously (Toyka *et al.*, 1975, 1977)—gave informed consent for withdrawal of up to 200 ml of blood. Ten patients with diseases other than myasthenia gravis were used as individual controls (Table 1).

PREPARATION OF THE IMMUNOGLOBULIN (IG) FRACTION

The immunoglobulin fractions were prepared by sterile ammonium sulphate precipitation at 33% saturation as described previously (Toyka *et al.*, 1975, 1977). Dialysis was carried out three times against Ringer's solution. The IgG concentration in the dialysed crude Ig fraction was 1000 to 3000 mg per 100 ml as measured by single radial immunodiffusion (RID, Behringwerke).

INJECTION OF MICE

Thirty female B6D2F₁/J mice (Jackson Laboratories, Maine, USA) were injected daily with the Ig fraction of an individual patient for up to 12 days. The amount of IgG injected was 12–20 mg per day. The animals were immunosuppressed by a single dose of cyclophosphamide (300 mg per kg body weight) on day 2. Levels of human IgG were measured in the mouse serum at the end of the experimental period. They ranged from 800 to 1200 mg per 100 ml which is equivalent to the concentration in normal human serum.

ANALYSIS OF TRANSMISSION AT THE NEUROMUSCULAR JUNCTION

At the end of the experimental period the mice

Table 1 Clinical and electrophysiological correlations

Case number	Clinical and electrophysiological correlations						
	Sex	Age (yr)	Duration of disease (yr)	Clinical classification*	Decremental response†	Treatment‡	MEPP amplitude in mice (mV)§
Myasthenic patients							
1	F	43	2	II B	+	Mest, Pred	0.35
2	F	18	1/5	II A	+	—	0.53
3	F	31	1/2	II A	0	Mest	0.44
4	F	35	15	II B	+	Thx, Aza, Mest	0.53
5	F	22	3/2	II B	+	Thx, Mest	0.46
6	M	47	14	IV	+	Thx, Mest, Pred	0.26
7	M	48	1	III	+	Thx, Mest, Pred, Aza	0.33
8	M	76	1/5	II B	ND	Mest	0.44
9	M	26	4	II A	—	Thx, Mest	0.34
10	M	62	16	II A	0	Mest	0.28
11	M	32	5	III	—	Thx, Mest	0.31
12	M	73	1/2	II A	+	—	0.28
13	M	44	5	II A	+	—	0.26
Control subjects							
14	M	32	10	Myoblastoma	ND	—	0.80
15	M	22	1	Polymyositis	ND	Pred, Aza	0.91
16	F	19	1/5	Dermatomyositis	ND	Pred, Aza	0.66
17	F	57	5	Polymyositis	ND	Pred, Aza	0.75
18	F	37	20	Lupus erythematosus	ND	Pred, Aza	0.49
19	F	42	6	Lupus erythematosus	ND	Pred, Aza	0.68
20	F	50	1/2	Polynuropathy	ND	—	0.69
21	M	73	2	Polymyositis	ND	Pred, Aza	0.63
22	F	56	5	Amyotrophic lateral sclerosis	ND	—	0.76
23	M	51	1	Mediastinal cyst	0	—	0.73¶

*Based on Osserman's clinical classification; †indicates decrement of more than 10% at stimulation rate of 2 or 5 Hz; ND = not determined; ‡Mest = pyridostigmine (Mestinon); Thx = thymectomy; Pred = prednisone; Aza = azathioprine; §mean MEPP amplitude in diaphragms injected with the individual immunoglobulin fraction; ||mean of two experiments; ¶mean of three experiments.

were anaesthetised with pentobarbitone (60–70 mg per kg body weight). From each animal the left hemidiaphragm was removed, preserving a 1 cm stump of the left phrenic nerve. It was then transferred rapidly to a modified Liley's solution containing 10 mmol magnesium per litre and gassed with 95% O₂/5% CO₂. The diaphragm was pinned at resting length in a flow-chamber on a piece of silicone rubber and left to equilibrate for at least 30 min. Temperature was maintained at 31°C. Stimulation of the phrenic nerve was carried out through a suction electrode by a Grass S 88 stimulator at 3 Hz (0.2 ms, 5–7 V). Intracellular recordings were made with conventional microelectrodes filled with 3 M KCL (4–12 MΩ). The electrical signals were amplified by a WPI M4A preamplifier and displayed on a Tektronix storage oscilloscope. Recordings were made on a Siemens Mingograph (frequency range 0–1000 Hz). The membrane resting potentials (MRP) of all cells were measured and only cells with MRPs over –60 mV at the end of the recording period were used. Fifteen to 20 miniature endplate potentials (MEPP) with rise times less than 1 ms were measured per muscle fibre, and the amplitude was then corrected to a MRP of –75 mV. Miniature endplate potential frequencies were also recorded in each muscle fibre. Electrical stimulation of the phrenic nerve was performed in 23 of the 30 diaphragms. Thirty or more endplate potentials (EPP) were recorded and the last 20 were measured. The mean amplitudes of MEPP and EPP and the mean MEPP frequency were calculated for each fibre, and these mean values were used to calculate the grand mean for all fibres in each diaphragm (usually 12 to 20 per diaphragm). The average quantum content of EPP was estimated according to the formula (Boyd and Martin, 1956)

$$\text{quantum content (m)} = \frac{\text{mean EPP amplitude}}{\text{mean MEPP amplitude}}$$

No correction was made for nonlinearity as EPP amplitudes were usually small.

Statistical analysis was carried out using the nonparametric Wilcoxon test (Diem and Lentner, 1968). P values smaller than 0.05 were considered significant.

PREPARATION OF MUSCLE SPECIMEN FOR ELECTRONMICROSCOPIC EXAMINATION

Strips of diaphragms were fixed with fresh cold 2.5% glutaraldehyde in 0.1 M cacodylate, pH 7.2. The regions known to be rich in motor endplates were trimmed down to blocks 1 mm of edge. The blocks were washed overnight in buffer, postfixed with OsO₄, and embedded in epoxy resin. Ultra-thin sections were cut with an LKB microtome,

double stained with uranyl acetate and lead citrate, viewed with a Zeiss 9A electron microscope, and photographed at primary magnifications ranging between ×1900 and ×41 000. About 250 serial and random sections from 20 to 30 endplates were examined.

Results

The amplitudes of miniature endplate potentials in control animals ranged from 0.50 mV to 0.91 mV with a mean of 0.71 mV. The range of EPP amplitudes was 0.50 mV to 0.85 mV with a mean of 0.74 mV.

The main abnormalities in mice injected with myasthenic immunoglobulins were a 48% reduction of MEPP amplitudes ($P < 0.005$) and a 33% reduction of EPP amplitudes ($P < 0.01$), as shown in Figs. 1 and 2. Mean quantum content of EPPs and the membrane resting potentials (MRP) were not significantly different in the two groups (Table 2). The mean MEPP frequency appeared to be decreased in the myasthenic diaphragms by 25%, but in fibres with small MEPPs a greater proportion of these potentials was obscured by the background noise (usually 100–150 μV). When electrodes with a particularly favourable signal-to-

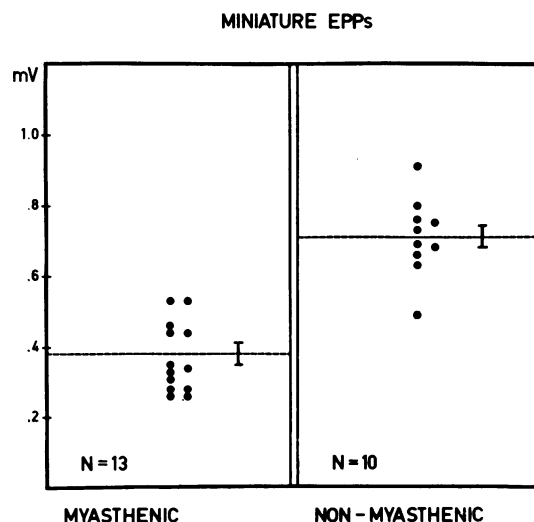


Fig. 1 Amplitudes of miniature endplate potentials (mV) in mice treated with immunoglobulin fractions from patients. Each data point represents the mean amplitude of a diaphragm. Five of the data points are means of duplicate or triplicate experiments. Horizontal bar indicates the arithmetic mean, vertical bar the standard error of the mean. N=number of patients.

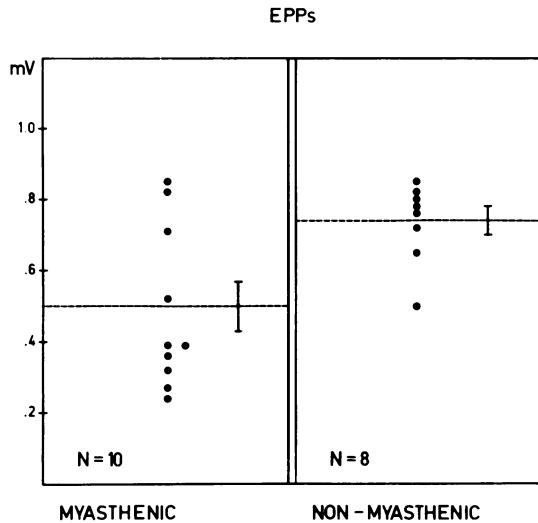


Fig. 2 Amplitudes of endplate potentials (mV) in mice treated with immunoglobulin fractions. Each data point represents the mean of one diaphragm. Horizontal bar indicates the arithmetic mean, vertical bar the standard error of the mean. N=number of patients.

Table 2 Mean values \pm standard error of the mean of membrane resting potentials (MRP, -mV), MEPP frequency (sec^{-1}), and quantum content of EPPs in 10 mM Mg solution. Diaphragms of mice treated with immunoglobulin fractions from patients with myasthenia gravis or non-myasthenic diseases

Treatment	MRP	MEPP frequency	Quantum content of EPPs (at 3 Hz stimulation)
Myasthenic immunoglobulins (N=15)	74.7 \pm 0.7	1.5 \pm 0.25	1.2 \pm 0.05 (N=12)
Control immunoglobulins (N=13)	75.6 \pm 0.6	1.9 \pm 0.50	1.1 \pm 0.10 (N=12)
P value	NS	< 0.05	NS

N=number of diaphragms tested. P values calculated by Wilcoxon test, NS=not significant.

noise ratio were used MEPP frequency was in the same order of magnitude as in controls. A similar phenomenon has been observed in human myasthenia gravis (Rash *et al.*, 1976).

In 11 of 13 tested patients transfer of the immunoglobulin fraction caused a lowering of the average miniature endplate potential below the lowest control value (0.50 mV) (Table 1). In two experiments we found an average MEPP amplitude of 0.53 mV, which was in the lower range of the control values. In the present series all of eight sera from male patients and three of five females gave a positive result. Two mice injected

with the serum of one patient (number 6) showed mild clinical signs of muscle weakness with dragging gait and reduced strength of the forelimbs when holding on to the grid of the cage. Neither the remaining myasthenic sera nor any of the control sera caused similar signs in any animal.

We were unable to demonstrate a clear-cut correlation in our material between MEPP amplitudes in the test preparations and the clinical conditions of the patients from whom the serum was obtained. For example patient 10 was clinically in almost complete remission, but his serum produced a definite lowering of MEPP amplitude, while serum from clinically more affected patients often produced only a moderate reduction (patient 8) or no decrease at all (patient 4).

Electronmicroscopical examination of serial and random sections of the endplates of two markedly affected diaphragms after treatment with myasthenic immunoglobulins revealed essentially normal nerve and muscle junctional components (Fig. 3). In a number of endplates some regions of the postsynaptic membrane showed electron-dense material the density of which approximates that of the inner part of the basal membrane. It appeared as a series of five to 10 variably shaped spikelets located at the tips of the synaptic folds measuring 50–150 Å in length and up to 50 Å in width and abutting on the outer surface of the postsynaptic membrane (Fig. 3, inset). In a few control endplates similar material was also observed.

Discussion

In confirmation of previous reports it was shown in the present series of experiments that transfer of the immunoglobulin fraction of sera from myasthenic patients to mice produces electrophysiological and, in some animals, clinical signs similar to those found in the human disorder.

The average endplate potential amplitude in animals treated with Ig was reduced by 33%, the average miniature endplate potential amplitude by 48%. The quantum content of each EPP was, therefore, not significantly changed compared to healthy animals (Table 2). Miniature endplate potential frequency—another indicator of pre-synaptic function (Hubbard *et al.*, 1969)—was not grossly changed in myasthenic animals.

Similar results have been obtained in human myasthenia gravis (Elmqvist *et al.*, 1964; Albuquerque *et al.*, 1976; Rash *et al.*, 1976) and in the chronic phase of experimental autoimmune myasthenia gravis in rats (Lambert *et al.*, 1976). In human myasthenic muscle a decreased junctional sensitivity to ACh has recently been demonstrated

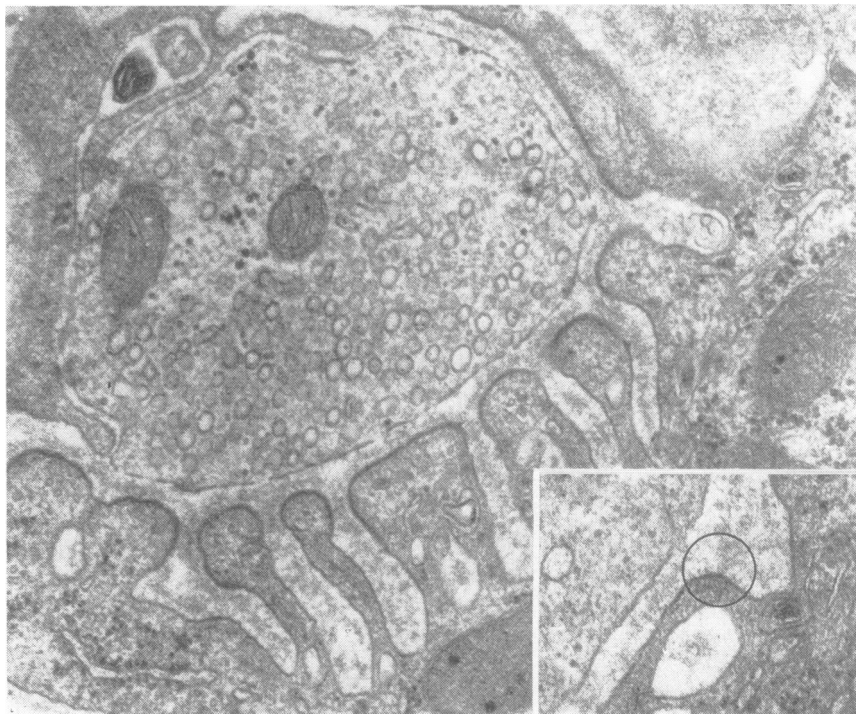


Fig. 3 Endplate of a mouse diaphragm after passive transfer of human myasthenic immunoglobulin. This essentially normal profile is representative of many neuromuscular junctions: the material in the junctional cleft is of medium to low density, being generally densest equidistant between the opposing cell surfaces. $\times 54\,000$. Inset: detail of another normal looking endplate: an orderly series of spikelets (circle) project from the outer surface of the postsynaptic membrane into the primary cleft. $\times 72\,000$.

adding support to the concept of a postsynaptic defect in myasthenia gravis, which had earlier been proposed on the basis of a decreased number of bungarotoxin binding sites (Fambrough *et al.*, 1973). Except for ACh sensitivity which was not measured by us in these experiments, all parameters of the MPTM are in good agreement with those reported for human myasthenia gravis and chronic experimental autoimmune myasthenia gravis. Unless heterogeneous and homogeneous immunoglobulins have different actions at the neuromuscular junction we think it likely that a postsynaptic effect explains our findings.

By contrast, in the acute phase of experimental autoimmune myasthenia gravis (Lambert *et al.*, 1976), or after passive transfer of serum from rats with the chronic form of this disease to normal recipient animals (Lindstrom *et al.*, 1976a), the quantum content of EPPs and MEPP frequency are greatly reduced thus adding a presynaptic component to the transmission failure in these animals.

In humans with myasthenia gravis no equivalent phenomenon has yet been observed.

We find that serum from male and female patients was equally active on mouse preparations, and we, therefore, do not favour the hypothesis that the pathogenetic mechanism is basically different in male and female myasthenic patients (Feltkamp *et al.*, 1974; Pirskanen, 1976). This does not rule out the possibility that genetic factors may play an important role at an earlier stage of the autoimmune process (Wekerle and Ketelsen, 1977).

The data indicate that the presence or absence of the "myasthenogenic" immunoglobulin is not clearly related to the mode and duration of treatment. The percentage of a positive mouse passive transfer assay was not different in thymectomised and nonthymectomised patients, nor did medication with immunosuppressive agents in the patients influence the ratio of positive results. It appears that the mouse transfer assay is correlated only

with the presence of signs of generalised myasthenia gravis. Further studies dealing with myasthenia gravis patients in complete remission with or without medication are needed to evaluate this correlation in greater detail.

The bioassay appears to be specific for myasthenia gravis as judged by the individual disease controls in this study.

The *in vitro* assay of binding antibodies against ACh-R extracted with detergent from human or mammalian muscle has been reported to be slightly more sensitive than the *in vivo* mouse response and highly specific for myasthenia gravis (Lindstrom *et al.*, 1976b). However, tests measuring antibodies which block bungarotoxin binding to ACh-R are much less sensitive (Almon *et al.*, 1974; Lindstrom *et al.*, 1976b; Mittag *et al.*, 1976). The variation in the sensitivity may in part be due to the difference in the physicochemical properties of the ACh-R after being disrupted from the junctional membrane with detergent (Changeux *et al.*, 1976; Lennon, 1976).

Other differences in the model system response may indicate variations in the cross-reactivity between myasthenic Ig and receptors from different mammalian species (mouse versus rat versus human muscle). The fact that human "myasthenogenic" immunoglobulins act across a species barrier demonstrates that both human and mouse ACh-R share a sufficient number of antigen-binding sites at or close to the ACh-binding site to allow for a decrease in the response to the transmitter.

Preliminary evidence from a limited number of endplates from clearly affected diaphragms after passive transfer of myasthenic immunoglobulins indicates that no gross abnormality can be demonstrated. The endplates in these muscles do not look like those from patients with chronic myasthenia gravis (Engel and Santa, 1971) or from rats with chronic experimental autoimmune myasthenia gravis (Engel *et al.*, 1976) under the electron-microscope. They resemble rather the ultrastructural findings described in patients with myasthenia gravis of recent onset (Rash *et al.*, 1976; Engel *et al.*, 1977a) where the authors found virtually normal junctional components despite markedly decreased MEPP amplitudes. The linear densities we have observed in some endplates of the affected diaphragms but, on occasion, also in control endplates, seem to be nonspecific. Similarly shaped material has been demonstrated in rabbits with experimental autoimmune myasthenia gravis and in control animals (Thornell *et al.*, 1976).

At present our findings indicate that the alterations in the mouse passive transfer model may

closely resemble human myasthenia gravis of recent onset at a stage before irreversible structural damage has occurred. This hypothesis gains indirect support from earlier experimental observations in the mouse passive transfer model (Toyka and Pestronk, unpublished) that both reduced MEPP amplitudes and ACh-R binding to bungarotoxin may return towards normal values three to four weeks after treatment with immunoglobulins has been stopped, indicating the reversibility of the transmission defect. The exact nature of the mechanism of the interactions between myasthenic antibodies and ACh-receptors still remains to be determined. Recent evidence from *in vitro* studies using cultured rat muscle cells supports the view that antibodies block transmission by increasing the rate of degradation of receptor molecules (Anwyl *et al.*, 1977; Appel *et al.*, 1977; Bevan *et al.*, 1977; Kao and Drachman, 1977). This does not necessarily imply a similar mechanism in junctional ACh-R of intact endplates.

The fact that the mouse passive transfer model combines a marked neurophysiological defect with lack of gross structural alterations makes it a suitable tool for studies on the myasthenic transmission failure.

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