

Passively transferred myasthenia gravis: protection of mouse endplates by Fab fragments from human myasthenic IgG

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SUMMARY Using the mouse passive transfer model, the effects of purified human myasthenic immunoglobulin G and of the monovalent Fab fragment on neuromuscular junctions were investigated. Treatment with IgG markedly reduced amplitudes of miniature endplate potentials. When Fab fragments were transferred alone or with subsequent addition of IgG no neuromuscular transmission block was induced. Myasthenic IgG and Fab were specifically demonstrated at the neuromuscular junctions by immunohistochemistry. On electronmicroscopy endplate structure was normal in transfer experiments using IgG for up to 30 days. It is suggested that Fab fragments bind to acetylcholine receptors without affecting transmission and protect them from the attack of complete IgG antibodies.

Circulating antibodies (AB) to the nicotinic acetylcholine receptor (AChR) at the neuromuscular junction are probably the main cause of the transmission defect in myasthenia gravis (MG) (for review see Lennon,¹ Heilbronn and Stalberg,² Drachman.³ It has been shown that on passive transfer of human myasthenic immunoglobulins or IgG to recipient mice typical features of MG can be reproduced: (1) reduced amplitudes of miniature endplate potentials (MEPP) and endplate potentials (EPP) (2) a decrease in the number of AChR measured by α -bungarotoxin (α -Butx) binding and (3) a decremental response and clinical weakness in some animals.⁴⁻⁶ Accelerated degradation of membrane bound AChR has been observed in mice treated identically.⁷

There is evidence from studies using muscle cell cultures that native IgG antibodies or bivalent (F(ab')₂) fragments are needed to induce accelerated degradation of AChR but Fab fragments are inactive.⁸ In this study we investigated

the effects of Fab fragments and those of IgG from myasthenic sera on neuromuscular junctions in vivo using the mouse transfer model. It will be demonstrated that Fab fragments (1) bind to AChR but do not reproduce the myasthenic transmission defect, and (2) completely inhibit the effect of IgG on MEPP amplitudes when transferred simultaneously.

Material and methods

Patients Serum was obtained from three patients with myasthenia gravis (MG) as established by clinical signs, abnormal EMG, positive Tensilon test and antibodies to AChR (immunoprecipitation assay.^{9,10} Serum from a non-myasthenic patient and normal IgG (AB Kabi, Sweden) served as controls. **Preparation of serum fractions** (a) Purified IgG was isolated from the MG serum by separation on exchange chromatography using a column packed with DEAE-Sephacel (Pharmacia) (0.05 M phosphate buffer, pH 8.0).¹¹ The eluates were concentrated and checked for purity on standard agar gel immunoelectrophoresis using goat anti-human whole serum (Behringwerke). The quantitative IgG concentration was measured by radial immunodiffusion (Behringwerke).

(b) In the individual control serum a crude immuno-

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globulin fraction was obtained by sterile ammonium sulphate precipitation at 33% saturation as described previously.⁵

(c) The preparation of Fab fragments was carried out according to a procedure described by Porter¹² with some modifications: IgG (0.6g) was dissolved in 40 ml buffer (0.1 M sodium phosphate, pH 7.0, 0.01 M cysteine, 0.002 M sodium-EDTA) and incubated with 6 mg mercuripapain (Serva) at 37°C for 16 hours. The solution was then dialysed against distilled water to inactivate the enzyme. For chromatography on DEAE-Sephadex A 50 (Pharmacia) the digest was dialysed with starting buffer (0.05 M TRIS-HCl, pH 8). Column dimensions were 5×35 cm, the flow rate was 30 ml/hour. A linear gradient was applied with a LKB Gradient Mixer ranging from 0.05 M to 0.5 M TRIS-HCl, pH 8, starting at 200 ml elution volume. The IgG digest was fractionated into four peaks which were found to be pure Fab (I), Fab plus Fc (II and III), and pure Fc (IV) by standard immunoelectrophoresis. After pooling, the Fab fraction was concentrated and again assessed for purity. No papain was present in the final Fab solution as demonstrated by an optical test originally described for chymotrypsin.¹³ Antiacetylcholine receptor antibody activity of the Fab fragments was measured by an immunoprecipitation assay¹⁰ with the modification that the receptor-Fab-complexes were precipitated with anti-human Fab serum (Behringwerke).

Injection of experimental animals BDF₁-mice. Ten female B6D2F₁/J mice (Jackson Laboratories, Maine, USA) were injected daily with Ig fractions for 7 to 10 days and immunosuppressed with cyclophosphamide (300 mg/kg). To ensure sterility the purified IgG fraction was passed through a filter assembly (0.45 μm pore size, Sartorius) before injection.

Two groups of C57 mice (four animals in each group) were given daily ip injections of human myasthenic IgG and human control IgG respectively. Each dose contained about 10–12 mg IgG. The resulting mouse-serum concentration of human IgG as measured by Tripartigen^(R) (Behringwerke) ranged from 500 to 1000 mg/100 ml. Mice receiving treatment for more than three days were immunosuppressed by a single injection of cyclophosphamide (100 mg/kg). Two mice from each group were killed and studied after three and 30 days of treatment.

Testing of neuromuscular transmission At the end of the experimental period eight mice were anaesthetised and the left hemidiaphragm was removed, transferred to a bath containing modified Liley's solution gassed with 95% O₂ and 5% CO₂ and kept at 31°C. Intracellular recordings of MEPP were carried out by standard microelectrode techniques as described previously.⁶ In up to 24 muscle fibres 15–20 MEPP were recorded per fibre, the mean amplitudes per fibre calculated, and these values used to calculate the grand mean for all fibres in each diaphragm.

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Preparation of protein A conjugated horseradish peroxidase (prot A-HRPO)

Protein A (Pharmacia, Sweden) was conjugated to horseradish peroxidase (type IV Sigma Chemical Co. USA) according to the method of Nakane and Kawoi.¹⁴ The conjugate was separated from unreacted components of gel filtration on AcA44 ultrogel (LKB), as described by Engel *et al.*¹⁵ and concentrated to 1.0 mg/ml by ultrafiltration. The conjugate solution was divided into small aliquots containing 10 mg BSA/ml and stored at -20°C until used. In order to determine the concentration of the conjugate which gave the best specific staining with the lowest background, the conjugate was diluted stepwise with 0.15 M NaCl in 0.1 M phosphate buffer pH 7.2 (PBS) to five different concentrations (200, 100, 50, 25 and 12.5 μg/ml). These dilutions were separately incubated with 10 μm thick skeletal muscle cryosections from a rabbit with experimental autoimmune myasthenia gravis. The rabbit was earlier shown to have acetylcholine receptor antibodies bound to its motor endplates. After staining for peroxidase activity with Karnovsky's diaminobenzidine medium¹⁶ the sections were studied under light microscopy.

A conjugate concentration of 25 μg/ml was chosen for the further experiments since this gave low background stain with a still substantial endplate stain.

Anti-human Fab antibody Rabbit anti-human Fab serum was purchased from Behringwerke. The IgG fraction was separated from serum by ammonium sulphate precipitation¹⁷ and kept at +4°C after lyophilisation. Before use the lyophilised powder was dissolved in PBS to a protein concentration of 1 mg/ml. Three different dilutions (200, 100 and 50 μg/ml) were used in the experiments.

Light microscopic localisation of IgG and Fab at motor endplates Mice were killed by decapitation. The left and right tibialis anterior were excised and frozen by immersion in freon chilled in liquid nitrogen. 10 μm thick longitudinal cryosections of the muscles were cut on a microtome. After fixation for 15 min in acetone the sections were rinsed for 30 min at room temperature in two changes of PBS. For the localisation of IgG, sections were incubated with Prot A-HRPO (25 μg/ml) for 30 min at room temperature, followed by washing 2×15 min in PBS. Staining for peroxidase activity was performed with H₂O₂ and diaminobenzidine. The sections were finally rinsed 2×15 min in 50 mM Tris-HCl, dehydrated in graded series of alcohol, cleared in xylol and mounted under cover glass. In the case of Fab localisation sections were first incubated with anti-human Fab (200–50 μg/ml) followed by a 30 min rinse in PBS, and then processed as above.

Electron microscopic localisation of IgG at motor endplates Thin strips of mouse diaphragm muscle were processed for electron microscopic localisation of IgG by the method of Engel *et al.*¹⁵ After post-fixation in OsO₄ and dehydration in alcohol the

muscle strips were embedded in Epon 812 with orientation for longitudinal sectioning. 1.5 μ m semi-thin sections were cut on an LKB ultratome and studied under light microscope for peroxidase stain. When endplates were found in a section consecutive ultra-thin sections were cut for electron microscopy. A Philips 201EM was used.

Specificity controls In order to establish specificity of the system, control sections were either pre-incubated with unlabelled protein A or H₂O₂ was omitted from the staining medium.

Results

Passive transfer of IgG

The MEPP-amplitude in the control diaphragm was 0.70 \pm 0.3 mV (mean \pm standard error) which is in the normal range for mice treated with non-myasthenic Ig, or for untreated control mice.^{5,6} The mean MEPP-amplitude of the mouse treated with the myasthenic IgG fraction was markedly reduced (0.37 \pm 0.1 mV and 0.32 \pm 0.13 mV respectively). Thus it was demonstrated that the myasthenic IgG fractions were impairing neuromuscular transmission on passive transfer (table).

Localisation of IgG

All mice receiving treatment with myasthenic IgG displayed peroxidase stain at their motor endplates, indicating the presence of IgG (figs 1, 2). In contrast no stained endplates were found in muscle from control animals. Motor endplate stain was also absent in the specificity control sections. Electron microscope studies (fig 3) revealed the stain predominantly at the tips of the postsynaptic folds, in good agreement with the distribution of the acetylcholine receptors.¹⁸

Some stain was also seen at the presynaptic membrane.

Passive transfer of Fab and Fab + IgG

The mean MEPP-amplitude in the diaphragms from two mice injected with the purified Fab fragments from two individual myasthenic patients were within the normal range (0.51–1.25 mV for control mice (Toyka *et al.*,⁵ 1977, and unpublished observations) (table). At the end of the experimental period the Fab titre was measured in one of the mice and was found to be 0.64 pmol α -Butx binding sites per ml as compared to 60 pmols injected per day. Correspondingly only a weak precipitation line was seen when the mouse plasma was tested against anti-Fab serum on an Ouchterlony test. This indicates that the low AB titre was not merely due to inactivation of the receptor binding sites but rather to decreased circulating Fab concentration.

When BDF₁ mice were treated with Fab fragments first and subsequently with Fab + IgG mean MEPP amplitudes were again not reduced (table). In these experiments the Fab concentration could not be measured because the anti-Fab serum cross-reacted with the IgG present in the mouse plasma. The determination of IgG levels revealed values that were closely comparable to the plasma concentrations in mice treated with IgG alone.

Localisation of Fab at motor endplates

Fab fragments were demonstrated at motor endplates in muscle cryosections of both mice treated with human myasthenic Fab (fig 4) by incubation with rabbit anti-Fab antibody followed by

Table Amplitudes of miniature endplate potentials (MEPP) and immunohistochemical localisation of IgG and Fab fragments at motor endplates in mice treated with human immunoglobulin (Ig) fractions

Animal/strain	No of animals	Treatment	Duration of treatment	Amount of IgG or Fab + IgG (mg/day)	IgG/Fab ^u § present at motor endplate	Intensity of endplate stain for IgG/Fab §	MEPP amplitude (mV)
Mouse/C57	2	Myasthenic IgG	3 d	11–12	pos/n t	+ + +/—	n t
„	2	Control IgG	3 d	11–12	neg/n t	—/—	n t
„	2	Myasthenic IgG	30 d	11–12	pos/n t	+ + +/—	n t
„	2	Control IgG	30 d	11–12	neg/n t	—/—	n t
Mouse/B6D2F ₁ /J	3†	Myasthenic IgG	10 d	12	pos/n t	+ + +/—	0.37/0.32
„	2†	Control Ig	10 d	12	neg/n t	—/—	0.70*
„	2	Myasthenic Fab§	9 d	8	neg/pos	—/+ +	0.80/0.95
„	1	Control Fab	7 d	8	neg/neg	—/—	(0.69)‡
„	2	Myasthenic Fab + IgG§	9 d	8 + 12/8 + 16	pos/pos	+ + +/+ + +	0.80/1.14

n t = not tested.

* = mean MEPP amplitude for mice injected with control Ig 0.71 mV (n = 10) and for myasthenic Ig 0.38 mV (n = 13)⁵.

† = 3 of these 5 mice were studied with MEPP measurements and 2 with immunohistochemistry on the following day.

‡ = only 10 fibres were analysed.

§ = Ig from 2 individual myasthenic IgG fractions.

^u = staining for IgG with prot A-HRPO and for Fab with anti-Fab-IgG and then prot A-HRPO.⁷

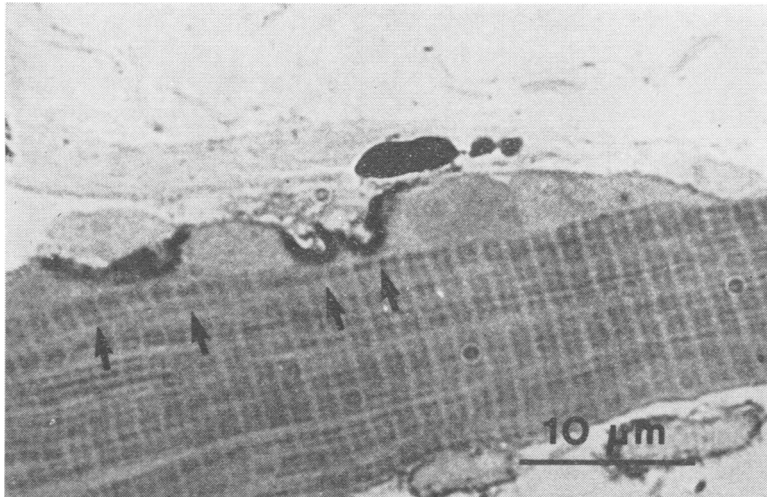


Fig 1 1.5 μm thick Epon-section of a muscle fibre from a mouse treated with myasthenic IgG for 3 days. Nerve terminals stained heavily for peroxidase activity (arrows). A stained erythrocyte can be seen above the endplate ($\times 2500$).



Fig 2 Photograph of cryosection demonstrating the localisation of IgG at motor endplates of a mouse treated with myasthenic IgG for 10 days ($\times 710$).

prot A-HRPO. Sections from the same animals incubated with only prot A-HRPO were negative. Of the three different dilutions of anti-Fab antibody tested, 50 $\mu\text{g}/\text{ml}$ was used since the higher concentrations produced excessive background stain. The stain produced at endplates of Fab-treated mice was less intense than the stain at endplates of mice treated with IgG. Fab could not be demonstrated at motor endplates of the one mouse receiving control Fab.

Discussion

The results obtained in this study suggest that treatment with monovalent Fab fragments obtained from myasthenic IgG protect the neuro-

muscular junction from the effect of the native IgG antibody on MEPP amplitudes. Passive transfer of Fab fragments alone was ineffective in bringing about the neuromuscular transmission defect that was found in mice after transfer of IgG from the same patients. In previous investigations it was established that reduced MEPP amplitudes are a sensitive and valid parameter for assessment of the transmission defect in MG and its animal models.^{19-21 4-5}

The presence of Fab fragments and IgG was clearly demonstrated by specific immunohistochemical staining at the neuromuscular junction of animals injected with myasthenic immunoglobulin fractions, but not in control animals.

No major ultrastructural changes were observed

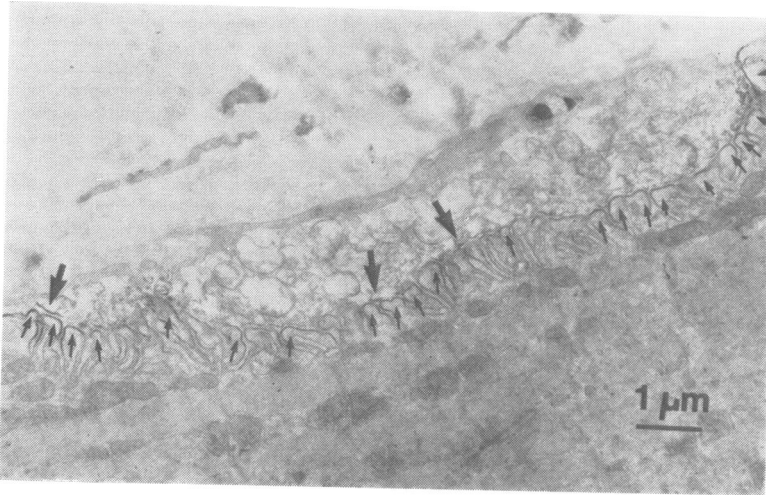


Fig 3 *Electron micrograph of motor endplate from a mouse treated with myasthenic IgG for 30 days. Stain predominantly at the tips of the postsynaptic folds (thin arrows) which appear structurally normal. Some stain also visible at the presynaptic membrane. Regular counterstaining has been omitted to avoid obscuring peroxidase stain.*

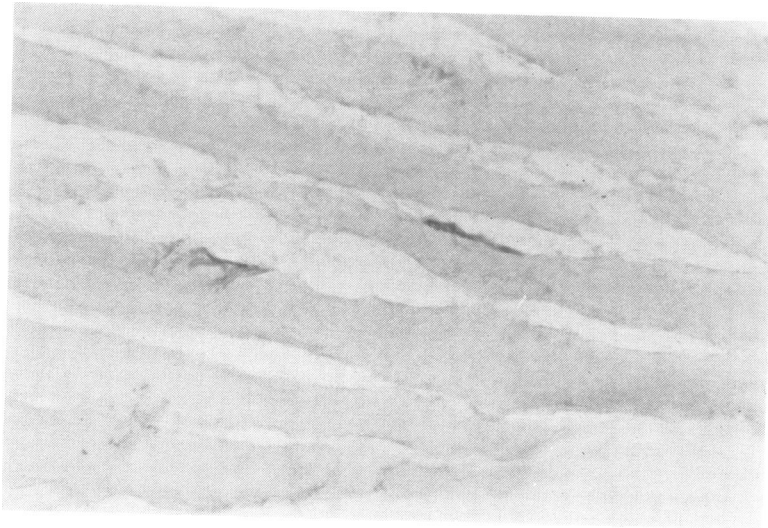


Fig 4 *Photograph of cryo-section demonstrating the localisation of Fab at motor endplates of a mouse treated with myasthenic Fab ($\times 710$).*

at motor endplates of mice exposed to MG-IgG for 30 days. The experiment was, however, designed for ultrastructural localisation of IgG, therefore the tissue-preservation was probably not good enough to allow minor alterations of motor endplate structure to be discerned. Similar findings of unchanged morphology were reported in a preliminary study in 8 to 10 day experiments.⁶ This is at variance with observations in human MG where widening of synaptic folds and a reduction of the postsynaptic surface area were reported.²² A complement mediated lesion of the postsynaptic membranes is likely to be the basic mechanism for these morphological alterations, since IgG and complement components (C3 and C9) were identified in the synaptic cleft.²³ The role of complement has

further been documented by passive transfer of chronic EAMG to syngeneic recipients. After deprivation of the third component of complement no clinical weakness ensued in these animals.²⁴ In the mouse transfer model the lack of endplate damage even after 30 days of transfer may be due to incomplete complement activation across the species barrier as has been suggested by findings in earlier experiments.⁵

There are two possible explanations why Fab fragments did not impair neuromuscular transmission. The monovalent Fab fragment is unable to activate complement²⁵ and to cross-link receptor binding sites.⁸ Using muscle cell cultures it has been demonstrated that intact IgG antibodies from myasthenic serum accelerate the process of AChR degradation²⁶⁻²⁷ while Fab

fragments were inactive.⁸ In the latter study, after cross-linking Fab by a second antibody (anti-Fab) an increased rate of degradation was again observed. These experiments were performed in the absence of complement.

In a recent biochemical study Fab fragments from rabbits with experimental autoimmune MG were found to bind to AChR from *Torpedo californica* and to inhibit further binding of the IgG fraction of identical sera.²⁸

The staining of endplates from mice after passive transfer of Fab fragments was less intense than that observed after IgG from myasthenic sera. Furthermore, the concentration of circulating Fab was found to be less than one twentieth of the corresponding levels in IgG-treated animals, although equivalent amounts of antigen-binding sites were injected per day. This is in agreement with the observation that heterogeneous Fab fragments are rapidly distributed and eliminated by the kidney.²⁹ This in turn leads to a shorter plasma half-life of Fab than is seen after passive transfer of IgG in rabbits and baboons.³⁰ Therefore the possibility cannot be excluded that the available number of antigen-binding sites carried by Fab fragments in our experiments was too low to reduce MEPP amplitudes.

If the amount of Fab fragments combining with receptor sites was indeed much lower than corresponding IgG concentrations, then it is surprising that pre-treatment with Fab protected the neuromuscular junction against IgG. We suggest that Fab fragments acted by interfering with the binding of IgG and by effectively blocking the cross-linking of IgG with AChR binding sites. If cross-linking were the crucial first step in antibody-receptor interaction only a portion of the receptor binding sites has to be blocked by a ligand in order to inhibit the antibody effect. The hypothesis has been proposed that a network of antibody molecules connects adjacent AChR binding sites ultimately allowing the activation of complement.³¹ Blocking of only a small portion of binding sites by Fab might then prevent the myasthenic transmission defect. We conclude that Fab fragments may protect the receptor from antibody attack at low concentrations. It is not known whether Fab fragments could also show this effect after binding of antibody to AChR has occurred. In this case treatment with homologous Fab fragments might provide a mode of specific experimental immunotherapy.

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