A bioassay for neuromuscular junction-restricted complement activation by myasthenia gravis acetylcholine receptor antibodies

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ARTICLE INFO

Keywords:
Acetylcholine receptor autoantibody
Complement
Muscle contraction
Myasthenia gravis
Neuromuscular junction

ABSTRACT

Background: Myasthenia gravis (MG) is an autoimmune neuromuscular disorder hallmarked by fluctuating fatigable muscle weakness. Most patients have autoantibodies against acetylcholine receptors (AChRs) at the neuromuscular junction (NMJ). These are thought to have three possible pathogenic mode-of-actions: 1) cross-linking and endocytosis of AChRs, 2) direct block of AChRs and 3) complement activation. The relative contributions of these mechanisms to synaptic block and muscle weakness of individual patients cannot be determined. It likely varies between patients and perhaps also with disease course, depending on the nature of the circulating AChR antibodies.

New method: We developed a new bioassay which specifically enables functional characterization and quantification of complement-mediated synaptic damage at NMJs, without interference of the other pathogenic mechanisms. To this end, we pre-incubated mouse hemi-diaphragm muscle-nerve preparations with mAb35-hG1, a humanized rat AChR monoclonal and subsequently exposed the preparation to normal human serum as a complement source. NMJ-restricted effects were studied.

Results: Clearly NMJ-restricted damage occurred. With immunohistology we showed complement deposition at NMJs, and synaptic electrophysiological measurements demonstrated transmission block. In whole-muscle contraction experiments we quantified the effect and characterized its onset and progression during the incubation with normal human serum.

Comparison with existing methods: With this new assay the complement-mediated component of myasthenic NMJ pathology can be studied separately.

Conclusions: Our assay will be of importance in detailed mechanistic studies of local complement activation at NMJs, investigations of new complement inhibitors, and laboratory pre-screening of therapeutic efficacy for individual MG patients to optimize care with clinically approved complement inhibitors.

1. Introduction

Myasthenia gravis (MG) is an autoimmune neuromuscular disorder hallmarkmarked by fatigable muscle weakness (Gilhus et al., 2019). Most patients (~80–85%) have IgG1 and IgG3 autoantibodies against acetylcholine receptors (AChRs) at the neuromuscular junction (NMJ) (Lazaridis and Tzartos, 2020). Their pathogenic actions are thought to be threefold: 1) direct block of AChR ion channel function, 2) AChR degradation through cross-linking and endocytosis (i.e. antigenic modulation), and 3) AChR density reduction due to complement-mediated focal postsynaptic membrane damage (Ruff and Lisak, 2018). The nature of the AChR antibodies, and possibly also patient predispositions, are likely determinants. Using AChR-expressing cell lines, some studies have quantified antigenic modulation activities or direct AChR ion channel-blocking properties of MG patient sera (Keefe et al., 2009; Lozier et al., 2015; Lyons et al., 1998). However, as yet it is not possible to determine the relative contributions of the pathological sub-processes at NMJs in individual MG patients in vivo.

Complement activation at the NMJ is a predominant factor in the pathomechanism of MG (Dalakas et al., 2020; Howard, 2018; Ruff and...
Lisak, 2018). Early studies showed complement depositions at NMJs of MG patients and experimental MG rats (Engel et al., 1977; Sahashi et al., 1978). Furthermore, complement-depleted or -deficient animals are less susceptible to experimental MG (Kusner et al., 2018). Membrane attack complex (MAC) locally damages the postsynaptic membrane, causing AChR density reduction which results in diminished endplate potentials (EPPs). In parallel, the density of synaptic voltage-gated Na channels becomes reduced, elevating the muscle fibre’s firing threshold. Together, these two effects lower the safety factor of neuromuscular transmission and this underlies the fatigable muscle weakness in MG (Plomp et al., 2015; Ruff and Lennon, 1998). Muscle differences in resistance to complement activation may play a role in regional weakness distribution in MG (Kusner et al., 2008).

In view of the crucial role of complement in MG and other autoimmune diseases, the complement cascade has increasingly become a target for new therapeutics (Garred et al., 2021; Zelek et al., 2019). For MG, multiple blocking compounds have been studied in pre-clinical models and clinical trials (Albazi et al., 2020; Dalakas et al., 2020; Zelek and Morgan, 2020). Eculizumab, an antibody targeting the C5 complement factor, thereby preventing the formation of MAC, has recently been approved for clinical use in MG (Muppidi et al., 2019). Other C5 targeting investigational agents have shown efficacy in clinical trials (Howard et al., 2021; Mantegazza et al., 2020).

Not all MG patients may be equally responsive to these new complement inhibitors (Mantegazza et al., 2020; Nishimura et al., 2014). Therefore, more knowledge on the local mechanisms of complement activation at the MG NMJ and tests to predict the efficacy of specific complement inhibitors in individual MG patients are highly needed (Mantegazza et al., 2020). We here developed a functional bioassay in mouse muscle/nerve tissue ex vivo to induce and analyze local complement activation and damage at the myasthenic NMJ. In future application, our assay might be used to characterize the effects of inhibitors of complement activity on sera from MG patients to identify individuals with the highest chance of therapeutic success.

2. Materials and methods

2.1. Mice

We used nerve-muscle tissue from 2 to 8 months-old male and female NOD.CB17-Prkdcscid/J (Nod/scid) mice, which produce no IgG and lack a functional complement system due to C5 deficiency (Shultz et al., 1995). Original breeders were from Jackson Laboratory (Bar Harbor, ME, USA) (Stock #001303). Mice were bred and housed in sterile individually ventilated cages. Sterile food and water were provided ad libitum. For one imaging experiment we used a mouse from a B6. Cg-Tg(Ty1-YFP)16Jrs/Jrm mouse (Jackson stock #003709). Experiments were performed according to Dutch law and Leiden University guidelines, including approval by National and Local Animal Experiments Committees.

2.2. Anti-AChR monoclonal mAb35-hG1

mAb35 is a rat monoclonal raised against AChR from electric eel Electrophorus electricus (Conti-Tronconi et al., 1981). It binds to the AChR of several species (including humans), at the main immunogenic region, i.e. it competes with the majority of MG patient AChR IgGs (Tzartos et al., 1982). Its Fab sequence has been identified (Noridomi et al., 2017), which we used to design a recombinant IgG with a human IgG1 Fc region. This recombinant IgG was produced and purified by LakePharma (Belmont, CA, USA). The stock solution, at 10 mg/ml, containing 100 mM HEPES, 100 mM NaCl, 50 mM NaOAc (pH 6.0), was kept at 4 °C.

2.3. Normal human serum, C5-depleted human serum and human C5

Normal human serum (NHS), C5-depleted human serum (#A320) and purified human C5 (#A120) were purchased from Complement Technology (Tyler, TX, USA). Materials were shipped on dry ice and immediately stored at – 80 °C. Shortly before use, a stock vial was thawed at room temperature. NHS and C5-depleted serum were diluted in Ringer’s medium at 33% (0.5 ml serum plus 1 ml Ringer’s), directly before application. C5 stock solution was added to the 33% C5-depleted serum to give the desired concentrations.

2.4. Myasthenia gravis patient IgG

IgG was purified from plasmapheresis waste material from an AChR antibody positive MG patient, obtained after informed consent at the Leiden University Medical Centre. The female patient, 21-year-old, had been diagnosed with AChR MG two years earlier, and presented with severe generalized MG, with an MGFA score of 4 and MG-ADL score of 14. She was treated with plasmapheresis, prednisolone and azathioprine. AChR antibody titre in the patient serum shortly before the therapeutic plasmapheresis was > 5.5 nM. Total IgG was purified using an IgGial affinity resin on a Akta Pure protein purification system (GE Healthcare), as described previously (Klooster et al., 2012). The IgG was dialyzed to phosphate-buffered saline (PBS), concentrated, filter-sterilized and stored at – 80 °C until use.

2.5. Ex vivo muscle/nerve preparations and mAb35-hG1/human serum incubation

Mice were euthanized by CO2 inhalation. The diaphragm muscle with phrenic nerves was quickly dissected and placed in Ringer’s medium containing (in mM): NaCl 116, KCl 4.5, CaCl2 2, MgCl2 1, NaH2PO4 1, NaHCO3 23, glucose 11 (pH 7.4) at room temperature (20–22 °C), bubbled with 95% O2/5%CO2. The diaphragm was separated in left and right hemi-diaphragm. The left hemi-diaphragm was further cleaned and trimmed for use in the contraction experiment (see below). The preparation was stretched out with small pins in a 35 mm diameter plastic petri-dish with a silicone rubber-lined bottom, and the phrenic nerve was placed over a bipolar platinum stimulation electrode. Stereomicroscopical pictures were taken and the muscle was inspected for contraction upon electrical nerve stimulation. Then, incubation was started in mAb35-hG1, diluted in 2.5 ml pre-bubbled Ringer’s medium to the desired concentration. The petri-dish was covered and placed in a water bath at 32 °C for 3 h. Thereafter, mAb35-hG1 was washed away with several volumes of Ringer’s medium and used in electrophysiological or muscle contraction experiments (see below), where they were exposed to NHS or C5-depleted human serum, as described. From some mice, levator auris longus (LAL) muscle strips and epitrochleocoeus (ETA) muscles were also dissected, and pinned out in Ringer’s medium, and then pre-incubated with mAb35-hG1 and used for immunostainings or microscopical time-lapse video recording (see below).

2.6. NMJ micro-electrode electrophysiology

Intracellular recordings of miniature endplate potentials (MEPPs) at the NMJ of hemi-diaphragm-phrenic nerve preparations were made in Ringer’s solution at 26–28 °C in phrenic nerve-hemi-diaphragms. For details, see (Klooster et al., 2012).

2.7. Muscle contraction experiments

Nerve stimulation-evoked tetanic contraction force of the mAb35-hG1 pre-incubated left phrenic nerve-hemi-diaphragm was measured in 1.5 ml Ringer’s medium at room temperature. A force transducer (type K30, Harvard Apparatus, Hugo Sachs Elektronik GmbH, March-Hugstetten, Germany), connected to an amplifier TAM-A 705/1 (Hugo
Sachs Elektronik), was used. The output signal was digitized with a Digidata 1440 A digitizer (Axon Instruments/Molecular Devices, Union City, CA), connected to a PC running Axoscope 10 (Axon Instruments). With a Vernier control, the optimal basic stretch tension was determined and maintained, i.e. the tension that gave maximal contraction upon 40 Hz supramaximal phrenic nerve stimulation during 1 s. Then, the phrenic nerve was stimulated supramaximally (usually 10 V) once every 5 min with 280 stimuli of 100 µs duration at 40 Hz, i.e. for 7 s. The stimulator used was a Master8-cp (AMPI, Jerusalem, Israel). After equilibrating in Ringer’s medium, with several washes, the medium was replaced by 1.5 ml 33% NHS or C5-depleted human serum (with a concentration range of added C5 in a series of experiments), and contractions were evoked every 5 min for at least 2 h. The medium was bubbled very gently with 95% O₂/5%CO₂, paying particular attention to limit the frothing when serum was present in the 1.5 ml incubation bath. The area under each contraction curve was determined in later off-line analyses, using Clampfit 11 (Axon Instruments).

2.8. Immunostaining and fluorescence microscopy

Binding of mAb35-hG1 and deposition of human C6 and MAC at NMJs was assessed with immunofluorescence microscopy. mAb35-hG1/ NHS incubated muscles were fixated with 1% paraformaldehyde in PBS for 15–30 min at room temperature and, if necessary, stored at 4 °C in the fixative. Next, the specimen was washed extensively with PBS for 30 min. The specimens were incubated with either 4 µg/ml AlexaFluor594-conjugated goat anti-human IgG (H+L) (Invitrogen A11014), 40 µg/ml goat anti-human C6 (Quiidel, A307) or 0.14 µg/ml mouse anti-human MAC (Dako, M0777). For C6 and MAC staining, a secondary incubation (3 h) was done with, respectively, AlexaFluor594-conjugated donkey anti-goat IgG (Invitrogen A11058) or 4 µg/ml AlexaFluor488-conjugated goat anti-mouse IgG (H+L) (Invitrogen A11001). To stain AChRs, 1 µg/ml α-bungarotoxin (BTx), conjugated with either AlexaFluor-488 or – 594, where appropriate, was included in the final antibody incubation. Then, the specimens were washed extensively with PBS for 1 h and mounted in ProlongGold (Invitrogen) anti-fading medium on a microscopic slide. NMJs were visualized under a Zeiss Axioskop epifluorescence microscope using an oil-immersion 63x objective. Pictures were taken using Axiovision software (Zeiss).

2.9. Time-lapse video

A LAL muscle from a Thy1-YFP transgenic mouse was dissected and pre-incubated ex vivo for 2.5 h in 25 µg/ml mAb35-hG1 in Ringer’s medium. Then, it was washed in Ringer’s and placed in the epifluorescence microscope and viewed with a 40x water immersion objective. Guided by the YFP expressing motor axons and nerve terminals, an area with a few clearly visible NMJs and only a few muscle fibres thickness was chosen. Using Axiovision software (Zeiss), time-lapse brightfield images were taken every 30 s during a 3 h incubation period with 33% NHS, and were converted to a 36 s duration time-lapse mp4 video.

3. Results

3.1. mAb35-hG1 and normal human serum induce severe, complement-mediated damage at the ex vivo mouse NMJ

Based on earlier used methods (studying complement-dependent pathogenic effects at the ex vivo mouse NMJ of neuropathy-related anti-ganglioside antibodies (Plomp et al., 1999)), we pre-incubated a Nod/Scid mouse diaphragm with a concentration of 50 µg/ml mAb35-hG1 for 3 h at 32 °C, to allow for diffusion into the muscle and synaptic clefts of the NMJs, aiming for saturating AChR binding. Before and after the incubation, MEPPs at NMJs were measured. No statistically significant changes occurred (from 1.00 ± 0.10 before to 0.86 ± 0.12 mV after the incubation, n = 15 NMJs sampled per condition, p = 0.38, t-test). Nerve stimulation-evoked contraction was clearly visible before as well as after the mAb35-hG1 incubation. Thus, mAb35-hG1 binding apparently did not directly inhibit AChR function in this acute setting. Then, 33% NHS was added during 1 h at room temperature, with the aim to induce complement activation by the pre-formed antigenic complexes at the NMJ. Next, NHS was replaced by Ringer’s medium. During the NHS incubation, myofiber damage appeared within the whole muscle, however clearly restricted at the NMJ-rich midline zone (Fig. 1A-B). Stimulation of the phrenic nerve no longer induced a visible contraction of the muscle. Recording of MEPPs was attempted but revealed greatly depolarized muscle fibres and absence of detectable MEPPs at NMJs. Immunostaining showed that, as expected, mAb35-hG1 had bound to the NMJ (Fig. 1C). Binding was particularly intense at the outer edges of the AChR area (stained for with AF488-BTx) (Fig. 1 C). Localized myofiber damage (hypercontractions) were clearly visible in the brightfield image (Fig. 1C). In contrast, the AChR area seemed intact, at least no clear fragmentation was observed (Fig. 1 C). In a separate staining experiment using fixed ETA muscle, mAb35-hG1 binding at the NMJ was confirmed (Suppl Fig. S1). Immunostaining of human C6 and MAC clearly showed AChR co-localized depositions of these complement factors at NMJs of damaged diaphragm muscle fibres (Fig. 2). MAC deposition was further confirmed and visualized in damaged NMJs of ETA and LAL muscles (Suppl Fig. S2). We also imaged a mAb35-hG1 pre-incubated ex vivo LAL muscle from a Thy1-YFP transgenic mouse, to facilitate NMJ identification (Fig. 3). In the presence of 33% NHS, a time-lapse microscopic video recording clearly showed the development of severe local damage of the muscle fibres at the position of the NMJ (Suppl Video 1). After an incubation period of ~75 min, local fibre contraction/movement appeared within the area and an apparent complete digestion of intracellular content of the muscle fibre underneath the NMJ occurred within a period of 10–20 min. Presynaptic nerve terminal structures seemed to remain relatively intact (Fig. 3).

Next, a range of lower mAb35-hG1 pre-incubation concentrations was tested to roughly determine a concentration-effect relationship. A semi-diaphragm was cut into five longitudinal strips, which were pre-incubated with either 0, 6.25, 12.5, 25 or 50 µg/ml mAb35-hG1. Thereafter, they were incubated with 33% NHS for 2 h and 20 min. Stereomicroscopical inspection revealed that 6.25 µg/ml Mab35-hG1 already induced localized myofiber damage (Suppl Fig. S3). The visually inspected damage in the strips pre-incubated with the higher concentrations seemed more intense, an appeared sooner (i.e. started to be observable after 45 min 33% NHS incubation).

In a further attempt to assess the effect electrophysiologically, pre-incubation was repeated with 6.25 µg/ml mAb35-hG1. However, the NMJ zone myofiber damage was still of such magnitude that no MEPPs could be detected in most NMJs, with severe depolarization of the myofiber. In the few small areas that appeared not damaged, normal membrane potentials (about ~75 mV) and MEPPs (about 1 mV) could be measured. In a further attempt to dampen the damaging effect, pre-incubation was done with only 1 µg/ml mAb35-hG1, and the 33% NHS incubation thereafter was limited to 1 h. This resulted in only very limited spots of myofiber damage, dispersed within the preparation, and only partial loss of nerve stimulation-evoked contraction. In the damaged areas, again greatly depolarized fibres were encountered and no MEPPs could be detected. In intact adjacent areas, however, MEPPs of normal amplitude and frequency could be readily measured. Thus, it appeared not feasible to observe a partial effect at individual NMJ level with electrophysiological analysis. Apparently, once the complement-mediated damage sets in, it acutely leads to major myofiber depolarization and thereby the inability to detect MEPPs.

3.2. mAb35-hG1/normal human serum-induced NMJ damage can be quantified in muscle contraction experiments

These effects were subsequently studied in muscle contraction experiments, characterizing the NMJ damage at a higher functional
integration level. After pre-incubation with 10 µg/ml mAb35-hG1, the
diaphragm was mounted in Ringer’s medium in the contraction
measurement set-up and the phrenic nerve was supramaximally stimu-
lated at 40 Hz for 7 s every 5 min, until stable. No tetanic fade was
observed, again indicating that mAb35-hG1 most likely does not inhibit
AChR function directly in this acute setting (Fig. 4A). Then, the Ringer
medium was replaced by 33% NHS and the contraction was further
monitored. After 1 h, the contraction had been reduced by ~50% and
after 2 h, at the end of the measuring period, the reduction was >95%
(N = 4, Fig. 4A-B). Apparently, NMJs became blocked irreversibly,
because subsequent replacement of the 33% NHS with Ringer’s medium
did not restore contraction (data not shown). Clear damage of muscle
fibres at the NMJ zone was observed upon stereomicroscopical in-
spections of these preparations at the end of the experiment. In a
negative control experiment (in which mAb35-hG1 was omitted during
the pre-incubation), subsequent 33% NHS incubation did not induce any
loss of contraction force (Fig. 4A), and no NMJ zone damage became
visible.

We next assessed whether this ex vivo MG model might be suitable to
quantify the contribution of individual complement factors to the NMJ
damage. Using C5-deficient human serum and purified human C5 we
determined the concentration-effect relationship for complement factor
C5. In a first experiment, incubation of a mAb35-hG1 pre-incubated
mouse diaphragm with 33% C5-deficient serum during 90 min remained
without any effect. When purified C5 (at 67 µg/ml) was then added to
the C5-deficient serum, contraction became almost completely blocked
within 75 min (Suppl Fig. S4). Medium replacement with Ringer’s did
not cause recovery of contraction. In a further series of experiments the
concentration-effect relationship for complement factor C5 was estab-
lished (Fig. 4C). The concentration of C5 that inhibited the contraction by 50% after 2 h was ~5 µg/ml (Fig. 4D).

3.3. Application of myasthenia gravis patient IgG in the bioassay

We also tested whether purified IgG from a MG patient was capable
of triggering complement-mediated and NMJ-restricted damage in the
developed bioassay. A mouse diaphragm was pre-incubated with
23.4 mg/ml purified MG patient IgG, dialyzed to Ringer’s medium.
During the subsequent incubation with 33% NHS for 2 h, the contraction
gradually diminished by ~70%, and myofibre damage in the NMJ zone
was clearly visible (Fig. 5).

3.4. Protocol optimization

During the course of these experiments we noted that the diaphragm
preparations, including the negative controls without mAb35-hG1, often
deteriorated to some extent from the pre-incubation procedure itself
(3 h at 32 °C, without being oxygenized). Some muscles showed diffuse
mild muscle fibre damage and produced rather low initial 40 Hz tetanic
contraction forces of sometimes less than 10 g (instead of the ~15 g
usually pulled by untreated hemi-diaphragms). Apparently, these incu-
bation conditions were not optimal for the ex vivo preparation. There-
fore, we tested an alternative protocol, i.e. the mAb35-hG1 pre-
incubation was performed for 3 h at room temperature instead of 32 °C,
while the incubation medium was being continuously bubbled with 95%
O₂/5% CO₂, albeit at a very low intensity to minimize evaporation from
the (small volume) medium. This resulted in clearly better-preserved
specimens, with initial tetanic contraction forces often being >15 g.
Subsequent incubation with 33% NHS induced the usual severe re-
ductions in contraction force (Suppl Fig. S5) and the clearly visible
myofibre damage in the NMJ zone. Therefore, this optimized protocol
is recommended for future studies.

4. Discussion

In this bioassay, the sequential incubation of ex vivo mouse muscle/nerve preparations with AChR monoclonal mAb35-hG1 and 33% NHS
induced severe damage at myofibres at the position of the NMJ. The
resulting block in neuromuscular transmission was quantified in hemi-
diaphragm contraction experiments. We provide evidence that this
was a complement-mediated effect by showing its absence when using C5-depleted NHS, and an immunohistological demonstration of C6 and MAC at the damaged NMJs which was co-localized with AChRs and the bound mAb35-hG1. Since mAb35-hG1 binding was required for this complement-mediated effect and MAC deposition was shown, the classical complement pathway is most likely fully involved. MAC is a complex of C5b, C6, C7, C8 and multiple C9 molecules that form a membrane pore. One main effect of sub-lytical MAC formation in cellular membranes is uncontrolled influx of Ca\(^{2+}\) ions (Morgan et al., 2017). At the NMJ this may lead to amplification of Ca\(^{2+}\) release from intracellular stores through activation of inositol 1,4,5-triphosphate receptor 1, such as also shown with excessive postsynaptic Ca\(^{2+}\) influx due slow-channel AChR mutations (Zhu et al., 2011). The resulting overall Ca\(^{2+}\) overload activates Ca\(^{2+}\)-dependent apoptotic proteases such as calpains and caspases (Groshong et al., 2007; Zhu et al., 2014), which then digest subsynaptic intracellular proteins. This is compatible with our real-time observations in the time-lapse video recordings and stereomicroscopical inspections of the damaging effect at myofibres in the sub- and peri-synaptic region.

The strict spatial restriction of the complement activation at the native NMJ makes our new model highly relevant for MG. One other study used a (non-muscular) cellular system that expressed densely clustered AChRs to investigate the complement activating properties of (clustered) AChR specific IgGs from MG patients that were AChR antibody seronegative in conventional tests (Jacob et al., 2012). A modest correlation was found between (histologically quantified) complement activation in the cellular assay and the jitter level in the clinical single-fibre electromyography of the patients. A complicating factor was

![Deposition of complement factors and associated myofibre damage at mouse diaphragm NMJs after ex vivo mAb35-hG1/33% NHS incubation.](https://example.com/fig2.png)

**Fig. 2.** Deposition of complement factors and associated myofibre damage at mouse diaphragm NMJs after ex vivo mAb35-hG1/33% NHS incubation. (A) Epi-fluorescence microscopy picture of C6 deposition (red), co-localized with AChRs (green) and (B) MAC (green) deposition, co-localized with AChRs (red). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
the requirement to block complement regulators CD55 and CD59 with antibodies to enable high enough complement activation to be detected. A serological study has indicated an inverse relationship between AChR antibody titre and the serum levels of complement factors such as C3 and C4, suggesting increased complement consumption in high-titred MG individuals (Romi et al., 2005). However, this was not confirmed by others, who instead found a positive correlation of C5a and MG severity (Aguirre et al., 2020). It would be interesting to test a series of sera of a well-defined MG cohort in our new assay (using the patient serum instead of the NHS during the contraction measurements) to investigate the correlations between the specific NMJ damage in the test and the levels of several complement factors, AChR antibody titre and the clinical severity. Obviously, great care should be taken to obtain sera in a standardized and careful way to preserve the vulnerable complement (Lachmann, 2010).

Another important factor in the relative contribution of complement to disease severity in individual MG patients is the ability of the AChR-specific antibodies to activate complement at the NMJ. The first step in classical pathway complement activation, i.e. C1q binding to antigen-IgG complexes, requires formation of hexameric IgG structures through Fc-Fc interaction (Diebolder et al., 2014). IgG polymorphisms and variations in post-translation modifications may influence this ability to form hexamers (Lubbers et al., 2020). In individual MG patients these factors may relate to disease severity. In a first test, we demonstrated the ability of a purified IgG from an MG patient to induce complement-mediated NMJ-restricted damage in our assay during the subsequent 33% NHS incubation. Unfortunately, we do not know the concentration of AChR-specific IgG in this total-IgG purification material. Thus, our new assay could be used to study the inter-patient variabilities in complement activation potency by AChR-specific IgG in a large and clinically diverse cohort of MG patients. However, it should be realized that some patients might have a component of direct AChR blocking activity in their IgG, which could interfere and limit the interpretation of the results in this specific experimental setting.

For the relevance to MG, some weaknesses of our model system have to be considered. Before addition of NHS as complement source in our model, the AChR antibodies are already pre-bound at very high density in the NMJs of the mouse muscle preparation. In MG patients, the level of NMJ-bound AChR antibodies will depend on serum titre and will most likely build up gradually, especially during the early disease phase. Complement will already be activated concomitantly, even when the level of bound AChR antibody is still low. Thus, while the temporal separation of AChR antibody binding and complement activation in our bioassay forms an interpretational advantage, it is not representative for the pathophysiological sequels at the MG patient NMJ. Furthermore, due to high level pre-bound AChR antibodies and the stepwise addition of NHS, complement activation in our bioassay is probably extremely fast and high-level, as compared to the patient situation. The resulting very severe and complete sub-synaptic myofibre damage may be the reason why we could not detect NMJs with an intermediate phenotype, i.e. reduced MEPP amplitudes, such as encountered in MG patient biopsy NMJs and in NMJs of in vivo animal MG models (Plomp et al., 2015). Moreover, our bioassay makes use of mouse tissue and therefore the activation of the human complement cascade might be less dampened by mouse membrane-bound complement regulatory factors, such as e.g. CD55 or CD59 (Kusner et al., 2013; Morgan et al., 2006). Obviously, this heterology issue does not apply to the MG patient NMJ.

In conclusion, we have developed a bioassay for complement-mediated pathological damage at the myasthenic NMJ. With this ex vivo MG model, NMJ-restricted antigenic complex-triggered complement activation and damage can be studied without interference of other potential pathological effects of AChR autoantibodies, i.e. functional block and/or cross-linking of AChRs. We believe this bioassay can be of importance in 1) further mechanistic studies on the local effects of complement activation at the NMJ, 2) exploratory studies of new complement inhibitors, and 3) specialized laboratory pre-screenings of the therapeutic potency for individual MG patients of clinically approved complement inhibitors.

**CRediT authorship contribution statement**

**Jaap Plomp:** Conceptualization, Methodology, Investigation, Visualization, Writing – original draft, Funding acquisition. **Maartje Huibers:** Resources, Investigation, Writing – review & editing. **Jan...
Disclosure of Conflict of interest

None of the authors has any conflict of interest to disclose.

Acknowledgements

This study was financially supported by Alnylam Pharmaceuticals, Cambridge, MA, USA. We thank Leendert Trouw for contributing anti-human C6 and MAC antibodies, and Kasra Samareh Talebi for help with IgG purification.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jneumeth.2022.109551.

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