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3-2-2015

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Experimental Neurology 270 (2015) 3–10

Contents lists available at ScienceDirect

Experimental Neurology

journal homepage: www.elsevier.com/locate/yexnr

Guidelines for pre-clinical assessment of the acetylcholine receptor-specific passive transfer myasthenia gravis model—Recommendations for methods and experimental designs

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ARTICLE INFO

Article history:
Received 27 November 2014
Revised 13 February 2015
Accepted 17 February 2015
Available online 3 March 2015

Keywords:
Myasthenia gravis
Passive transfer model
Acetylcholine receptor antibody
Autoimmune disease
Neuromuscular junction

ABSTRACT

Antibodies against the muscle acetylcholine receptor (AChR) are the most common cause of myasthenia gravis (MG). Passive transfer of AChR antibodies from MG patients into animals reproduces key features of human disease, including antigenic modulation of the AChR, complement-mediated damage of the neuromuscular junction, and muscle weakness. Similarly, AChR antibodies generated by active immunization in experimental autoimmune MG models can subsequently be passively transferred to other animals and induce weakness. The passive transfer model is useful to test therapeutic strategies aimed at the effector mechanism of the autoantibodies. Here we summarize published and unpublished experience using the AChR passive transfer MG model in mice, rats and rhesus monkeys, and give recommendations for the design of preclinical studies in order to facilitate translation of positive and negative results to improve MG therapies.

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Introduction

The postulates of Witebsky–Rose–Koch require an antibody-mediated autoimmune response be recognized by specific characteristics; presence of autoantibody, the identification of the corresponding antigen, the ability to induce the production of the antibody in an experimental animal and demonstrate disease manifestations similar to the human disease (Witebsky et al., 1957).

These criteria still form a solid basis for defining an antibody-mediated autoimmune disease and provide for two experimental models, i) the injection of antigen to elicit an 'active' immune response and ii) the injection of antibodies as a 'passive' transfer of autoimmunity. Experimental autoimmune myasthenia gravis (EAMG) produces autoantibodies by the injection of AChR usually with an immunostimulator. Active immunization against other proteins found at the neuromuscular junction (NMJ) can also cause weakness. The passive transfer myasthenia gravis (PTMG) model is the injection of those autoantibodies into another animal, which will also demonstrate weakness. MG was one of the first diseases that fulfilled the Witebsky–Rose–Koch criteria for autoimmunity (Toyka et al., 1975, 1977). Subsequently, transfer of monoclonal AChR antibodies produced by hybridomas cloned from EAMG model induced similar disease characteristics (Lindstrom et al., 1976; Engel et al., 1979; Lennon and Lambert, 1980; Richman et al., 1980). The robustness and clear-cut phenotype of PTMG have made it a useful model for characterizing the immunopathogenesis of AChR-MG (~80% of the MG cases) and for testing medication that reduces the pathogenic effect of autoantibodies. Although PTMG with antibodies to muscle specific kinase and low-density lipoprotein receptor-related protein 4 have also been performed, the majority of PTMG studies have involved antibodies to the AChR. Over the years, the purpose of the model has shifted from the investigation of the pathology induced by AChR antibodies towards preclinical studies aimed at testing therapeutic interventions. Here, we provide recommendations for the design of preclinical studies using AChR-PTMG model (referred to as PTMG in the text below) in order to facilitate translation of positive and negative results in order to improve MG therapies in clinical practice.

Purpose of the passive transfer model of myasthenia gravis

MG is a T cell dependent-B cell mediated disease (Conti-Fine et al., 2006). Activation of $CD4^+$T cells is required for the autoimmune process

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http://dx.doi.org/10.1016/j.expneurol.2015.02.025
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by impairment of T regulatory cells, cytokine secretion and B cell activation. B cells, and in particular plasma cells, on the other hand are the source of the autoantibodies. The EAMG model utilizes the autoimmune cellular processes, the afferent arm of the immune response, to produce autoantibodies directed at the AChR, the efferent arm of the response. The PTMG model removes the highly variable response of the afferent arm thereby allowing the efferent effects of the antibodies to be studied in a reproducible way. The use of PTMG model for pre-clinical evaluation of a therapeutic is justified when the effect is limited to inhibiting the autoantibody binding or preserving the function and structure of the neuromuscular junction (NMJ) during antibody attack.

Pathophysiology of AChR antibodies

By the transfer of purified immunoglobulins from MG patients to mice and the subsequent muscle weakness developed in the mouse, Toyka and colleagues demonstrated that MG is an antibody mediated autoimmune disease (Toyka et al., 1975). Complement-activating antibodies against the extracellular domain of the AChR induced rapid, dose dependent myasthenia as early as 8 h and death by 48 h. The source of antibodies transferred to animals can be serum IgG of MG patients, polyclonal IgG from chronic EAMG animals, or monoclonal antibodies produced by B cell hybridomas or by heterologous expression (Lennon and Lambert, 1980; Richman et al.; van der Neut Kolfschoten et al., 2007). The main immunogenic region (MIR) on the alpha subunit of the AChR binds a high proportion of antibodies from MG patients (Tzartos and Lindstrom, 1980; Whiting et al., 1986), and it is the target recognized by monoclonal antibodies that produce PTMG. Furthermore, the α subunit antibodies are more pathogenic than the antibodies against the β subunit (Kordas et al., 2014) probably because the alpha subunit is represented twice among the five AChR subunits.

The antibody effector mechanisms are antigenic modulation and complement-mediated focal lysis of the postsynaptic membrane (Lennon et al., 1978; Tzartos et al., 1985; Loutrari et al., 1992). Transfer of IgG from MG patients to mice reduced the number of functional AChR, although AChR synthesis rose to compensate (Wilson et al., 1983a,b; Sterz et al., 1986). Monovalent AChR antibodies without complement binding capacity are not pathogenic unless they interfere with ion channel function (Lagoumintzis et al., 2010). The PTMG model revealed that the influx of mononuclear cells into the NMJ was antibody and complement-dependent, an event also seen during the acute phase (occurring one week after AChR induction in rats) of EAMG. The deposition of IgG and complement components was associated with a large influx of macrophages and a loss of both AChR and postsynaptic folds (Lindstrom et al., 1976; Engel et al., 1979).

PTMG has been used to determine the effects of sex, strain and age on susceptibility to antibody-mediated AChR loss (Hoedemaekers et al., 1998), the importance of the expression levels of AChR-associated proteins like rapsyn in the susceptibility of the AChR to antigen modulation (Losen et al., 2005), and the beneficial effects of complement inhibitors (Morgan et al., 2006; Kusner et al., 2013).

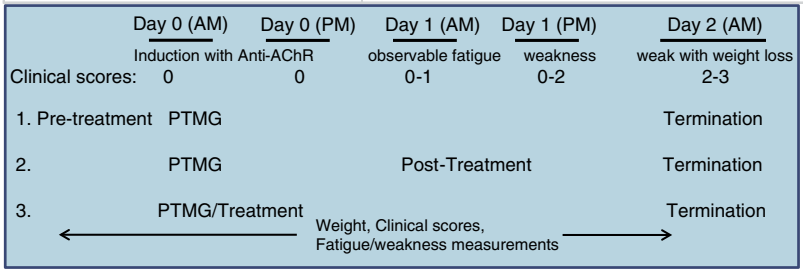
Therapeutic strategies using the PTMG model

Passive transfer of antibodies has also been used for therapeutic development (Lagoumintzis et al., 2010) (Fig. 1). Due to the 48–72 hour experimental timeframe, the PTMG model can function to determine dose–response and offer go-forward information to active immunization/EAMG experiments which require longer experimental periods. Therapeutics that target antibody turnover have shown efficacy in proof-of-concept studies. The increased turnover of antibodies has been facilitated by the use of proteolytic enzymes or antibodies to FcRn (Poulas et al., 2000; Liu et al., 2007). RNA aptamers (Hwang et al., 2003) and antibodies to denatured AChR (Krolick et al., 1996) have been shown effective in inhibiting binding of MIR antibodies. In AChR-specific PTMG mouse models, monovalent Fab fragments have been demonstrated to protect the AChR against the action of intact pathogenic antibodies (Toyka et al., 1980; Barchan et al., 1998; Papanastasiou et al., 2000). Complement depletion by cobra venom

A

Experimental design	Example
Animal species	Rat
Animal number	Power analysis based on clinical score
Randomization of animals	Stratify by weight and randomly assign to group
Number of groups and controls	Treatment response group
Antibody type	mAb35
Dose of antibody	20pmol/100g
Method of injection	Intraperitoneal
Analysis of Data	ANOVA

B



Schematic of PTMG experimental design. A. To properly plan a PTMG study, each aspect of the experiment must be determined prior to initiation. The table above provides some of these aspects with examples. B. The figure demonstrates the potential treatment schematic and course of clinical scores of PTMG induced animals. The pre-treatment would occur prior to initiation of PTMG. The treatment can occur after weakness is observed (24hrs.). Or, the treatment and initiation of PTMG can occur at the same time.

Fig. 1. Schematic of PTMG experimental design. A. To properly plan a PTMG study, each aspect of the experiment must be determined prior to initiation. The table above provides some of these aspects with examples. B. The figure demonstrates the potential treatment schematic and course of clinical scores of PTMG induced animals. The pre-treatment would occur prior to initiation of PTMG. The treatment can occur after weakness is observed (24 h). Or, the treatment and initiation of PTMG can occur at the same time.

factor demonstrated abatement of PTMG (Lennon et al., 1978). Therapeutic targets focused on complement demonstrated efficacy with inhibition of complement activity by antibody binding or protein interference (Biesecker and Gomez, 1989; Zhou et al., 2007; Soltys et al., 2009). Passively transferred IgG4 subclass AChR antibodies, which are incapable of activating complement and are functionally monovalent, effectively competed with pathogenic IgG1 subclass AChR antibodies and decreased disease severity in a rhesus monkey model of PTMG (van der Neut Kolfschoten et al., 2007).

Methodology of the PTMG model

The PTMG model is developed by the injection of AChR-specific antibodies directly into the animal. The method of injecting the antibody is intravenous (iv) or intraperitoneal (ip) to assure full delivery of the maximal calculated dose and rapid equilibration with extracellular fluid. Injection sites should be cleaned prior to injection and observed for irritation. A brief isoflurane anesthesia is useful for both iv and for ip injections for the following reasons: 1) reduction in both pain and stress during injection, 2) increased precision of injection since anesthesia renders the animal immobile, 3) elimination of manual restraint of the animal, thus freeing the hands of the experimenter and 4) reduction of tension on the abdominal skin. By manually lifting the abdominal skin of the animal, the attached muscles and peritoneum are slightly elevated and IP injections can be performed easily, while minimizing the risk of injecting antibody into an organ or of causing injury with the needle tip. To perform intravenous injections, tails should be warmed to produce vasodilation. A small gauge needle is placed bevel side up into the observed vein and the injection should occur slowly to ensure proper placement and to avoid cardiovascular failure. Resistance or blebbing of the tissue indicates an improper placement. Animals should be monitored for signs of distress during the first six hours which would demonstrate too rapid disease progression and require euthanasia before a treatment effect could be observed. For reproducibility and consistency of results, documentation of experimental design must be comprehensive. Weight and clinical scores (or other side effects related to model or therapeutic) that occur during disease progression should be noted.

Animal care for short term study

Due to the limited time of the study, the animals should be monitored repetitively throughout the 48 h after injection. To limit the stress on the animals the following procedures are recommended. Only one or two personnel should handle the animals throughout the experiment. Cage change should take place 2–3 days before the initiation of experiment. Cages should be equipped with enriched environment supplies, nesting material, and a housing unit. Grip strength measurements, cage lifts, and mesh/hanging test (see below for protocol) should be initiated prior to study to have animals become familiar with the exercise. If any animal becomes clinically weak all cages should be supplied with aqua gel and soft food placed on the floor of the cage to ensure accessibility. All care given to animals should be documented.

Animal species and antibody source for inducing passive transfer myasthenia gravis

The PTMG model can use various animals for induction and, as mentioned above, multiple sources of antibody. We will discuss the use of rats, mice and rhesus monkeys for induction. The antibody that is used in pre-clinical assessment of a therapeutic should be able to bind the AChR and induce complement in the relevant animal species. To standardize the model, antibody availability to all researchers is important for reproducibility of the results and comparisons between therapeutics. To elicit PTMG, the antibodies will need to bind with high affinity to the recipient AChR and induce complement activating ability. Therapeutic experiments which focus on inhibition of antibody levels or competitive binding as the primary outcome measure may not require a complement activating antibody. The antibodies chosen should also be available to the scientific community for use. All information of antibody, source, dose, and method of injection should be documented.

Lewis rats

Female Lewis rats have been used successfully to induce PTMG. They have the advantage over mice by presenting with clearly distinguishable disease symptoms (tremor, hunched posture with head down, and respiratory distress/apnea). Nevertheless, variation of results can occur due to age and weight of the animals at onset of the experiment, source and dose of the antibody used in the passive transfer, and route of injection. These specifics will be discussed below.

The age and weight of the Lewis female rats used for passive transfer vary considerably in the literature. Differences in weight (reported from 100 to 200 g) would determine the effective extracellular concentration of the autoantibody which is a primary determinant of the pathophysiological outcome for synaptic transmission. The percent acute loss of body weight would vary based on the animal's initial weight. Larger rats would be more practical to use in a pre-clinical experiment to ensure that measurements other than survival are to be used. The age of the rat also influences the severity of the disease; younger rats (12 weeks) are more susceptible than older animals (120–130 weeks) (Graus et al., 1993). We recommend the use of female Lewis rats (10–12 weeks of age) for initial studies. A subsequent study should be done with age appropriate males.

Proposed procedure for induction of PTMG in rats

1. Determine experimental design: number of female Lewis rats (10–12 weeks of age) needed for statistical significance, groupings, outcome measurements, and method of data analysis (Fig. 1).
2. Initiate grip strength, mesh/hang or cage lift protocol to ensure that animal is familiar with technique prior to PTMG induction.
3. For induction of PTMG, anesthetize animal with 2.5% isoflurane in air. To verify anesthetic depth, pinch footpad of animal for reaction.
4. The animal is placed on the surgical table with isoflurane flow. (Note: if anesthesia is not being used to sedate animal, animal restraint is required. Check with your institutional veterinarian for proper restraint holds.)
5. Site of injection (abdomen or tail) is cleaned with ethanol, a 25 gauge needle in inserted at site and the monoclonal antibody is injected (monoclonal Ab3: 27 pmol/gm; monoclonal Ab35: 20 pmol/100 gm)
6. The animal is allowed to recover on a heated pad. Once the animal is ambulatory, it is returned to the cage.
7. Animals are observed every 12 h for clinical signs of weakness (see Outcome measurement/Clinical score).
8. The study is terminated at 48–72 h after induction; except earlier terminations should occur when clinical scores reach 3 or 15% weight loss is observed.

C57Bl/6J mouse

The mouse offers the possibility to control the expression of the proteins of the NMJ based on genetic engineering (expression levels, specific mutants, transgenic animals). For example, to determine the potential efficacy of a therapeutic that inhibits complement, a mouse engineered to be absent of complement regulators (Lin et al., 2002; Kusner et al., 2013) would provide a suitable environment for the therapeutic to show function.

The mouse PTMG model has been used to test for pathogenic antibodies in serum from EAMG animals (Dedhia et al., 1998; Zhang et al., 1999) and MG serum (Mossman et al., 1988; Losavio et al., 1989;

Mundlos et al., 1990; Burges et al., 1994). To elicit PTMG in mice, mAb3 (Lennon and Lambert, 1981) has been used successfully (Lin et al., 2002; Kaminski et al., 2004, 2006; Morgan et al., 2006; Kusner et al., 2013, 2014).

Proposed procedure for induction of PTMG in mice

The above protocol for rats can be used to induce PTMG in mice ((monoclonal Ab3: 91 pmol/gm).

When the pathogenicity of IgG from MG patients is examined, or the polyclonal nature of IgGs is desired, PTMG can be induced by transfer of IgGs isolated from the serum of human donors. To induce PTMG with IgG from MG patients, multiple daily injections of 10–60 mg/day for up to 10 days may be required for the mouse to demonstrate weakness. In this case, on the first day the mice are also injected *i.p.* with 300 μg/g of cyclophosphamide, to prevent the formation of antibodies against the human IgGs (Toyka et al., 1975; Mossman et al., 1988).

Rhesus monkey

For the development of novel MG therapies, the use of non-human primates can be justified only if a close homologue to humans is an essential element of the pre-clinical assessment. For example, certain complement inhibitors are only effective against human and primate, but not rodent complement factors. PTMG has been performed in rhesus monkeys using either polyclonal MG patient IgG or the human AChR mAb IgG1 637 ('t Hart et al., 2005). Muscle weakness was transient, lasting 1–4 days, depending on the dose. This is different from the corresponding non-human primate EAMG model (Tarrab-Hazdai et al., 1975; Toro-Goyco et al., 1986) which is progressive and may be fatal. Examples of therapies that can be tested in the rhesus PTMG model are competitor IgG4 antibodies (van der Neut Kolfschoten et al., 2007) or complement inhibitors. If available, genetically engineered rodents, expressing for example human complement factors or human AChR subunits are in many aspects preferable alternatives to non-human primate models since the underlying physiological neuromuscular and immunological mechanisms are highly homologous in mammals.

Source of the antibody by hybridoma technology in the PTMG model

The following monoclonal antibodies generated by hybridomas technology from EAMG rats are commonly used in rat PTMG studies. Monoclonal Ab35 (Tzartos et al., 1981) was effectively used in various studies to produce a rat PTMG model (Tzartos et al., 1987; Hoedemaekers et al., 1997, 1998; Papanastasiou et al., 2000; Poulas et al., 2000; Reyes-Reyna et al., 2002; Garcia et al., 2003; Garcia and Krolick, 2004; De Haes et al., 2005; Losen et al., 2005; Krolick, 2006; Hepburn et al., 2007; Liu et al., 2007). Monoclonal Ab198 (Tzartos et al., 1983) also produced weakness in the rat (Hwang et al., 2003). mAb3 (Lennon and Lambert, 1981) showed extensive complement mediated damage to the NMJ of rats (Zhou et al., 2007, 2014; Ruff and Lennon, 2008; Soltys et al., 2009; Kusner et al., 2014).

Purification of antibodies from hybridomas

The antibodies are produced from culture hybridomas and collected in a serum-free medium. Purification of the antibodies requires use of a column, either a DEAE-sepharose column (Poulas et al., 2000) or hydroxyapatite columns (Reyes-Reyna et al., 2002; Garcia et al., 2003). However, if the hybridoma requires serum for survival, the use of Protein G sepharose (Liu et al., 2007) or Protein A sepharose (Chamberlain-Banoub et al., 2006) should be used to extract the immunoglobulin. The final preparation is dialyzed against Ringer's buffer (140 mM NaCl, 5.4 mM KCl, 1 mM $CaCl_2$, 2.4 mM $NaHCO_3$, pH 7.4) (Poulas et al., 2000). After purification, the purity of mAb should be assessed by SDS-PAGE and quantified by immunoprecipitation of ^{125}I-α-bungarotoxin-ligated muscle AChR corresponding to the investigated recipient species (Tzartos and Lindstrom, 1980; Lindstrom et al., 1981).

Preparation of IgG from human serum

To induce PTMG, the autoantibodies from the human serum need to cross-react with the AChR of the animal. Therefore, the resistance to respond to human IgG may be due to the inability of polyclonal autoantibodies to recognize their target. The sera must first be checked for cross-reactivity with the AChR of the injected species. Then, total IgGs or subclasses can be isolated from serum and plasma samples by means of ammonium sulphate precipitation or affinity columns. Due to the variability of human IgG cross-reactivity and the autoantibody titers, a small scale experiment should be performed initially, in order to determine the amount of IgG required to yield a clear phenotype, but is not lethal for the animals.

The properties of AChR-specific antibodies can be designed using recombinant antibody engineering. The sequences of a few AChR specific antibodies have been published (Graus et al., 1995, 1997; Farrar et al., 1997; Matthews et al., 2002; Fostieri et al., 2005; Vrolix et al., 2014) and these can be used to construct corresponding antibody expression vectors for heterologous production.

Statistical assessments

The experimental design and method of data analysis should be determined prior to the initiation of the experiment. Personnel involved in the assessment of the animals should be blinded to which group or treatment the animals are assigned. Requirement for specific number of animals per group will be determined by power analysis based of several outcome measurements. At least one measurements of the longitudinal study (clinical score, grip strength and weight of animals) should be used to determine group size. Animals should be randomly assigned to groups. When treatment occurs after observable weakness, first stratify the animals from weakest to strongest. Next, randomize each graded weakness into assigned group. The stratification ensures that each weakness level is properly represented per grouping. For pre-treatment and treatment that occurs at time of induction, the same strategy should be applied to weight so each group contains representation of the weight spectrum of the animals on the study. Outcome measurements based on AChR content, electromyography (EMG), and immunohistological markers could also be used in the assessment of statistical relevance. To determine statistical significance of the outcome measurements, statistical tests should be defined prior to initiation of the PTMG study.

Outcome measurements

Several outcome measurements can be used to describe or quantitate the severity of the induced symptoms. The animal weight, clinical score, grip strength, muscle AChR content, electromyography and visualization of the NMJ are among the most widely used and are described in more detail below. Outcome measurements should be chosen based on the therapeutic that is tested; for example, histology of the NMJ by hematoxylin and eosin stain is used to demonstrate inflammatory cellular infiltrates, while electron microscopy of the NMJ can verify complement-mediated damage to the endplate, and ELISA or RIA can be used to determine the amount of circulating AChR antibody injected into the animal. Clinical scores, that demonstrate health of the animals during the disease induction and therapeutic process, are required outcome measurement for pre-clinical assessments. During the planning stage of an experiment, outcome measurements should be decided based on therapeutic involvement.

Clinical score

The clinical score of the animal is a necessary outcome measurement of PTMG. Clinical scores should be taken every 12 h or less if the animals demonstrate severe weakness. Dose of the antibody should be based on its ability to demonstrate clear weakness in the animals by 48 h, without inducing immobility or death. A percent of survival endpoint should not be used since careful dosing of antibodies allows clear-cut and reproducible generation of moderate disease. To determine clinical score of the animal, observation of the activity of animal in the cage should be noted prior to handling. Animals should be allowed to move freely in a secured area to assess movement, ability to stand on hindlimbs, and appearance when sedate. To classify animals, handler should exercise animals that do not show weakness to determine fatigable state (see below for cage lift, grip strength and mesh/hang). Clinical scores for animals are as follows: 0, no weakness; 1, fatigable or weakness is only observed after exercise; 2, clinical signs of weakness present before exercise, hunched posture, or head down; 3, severe clinical signs of weakness: no ability to grip, hindlimb paralysis, respiratory distress/apnea, weight loss > 15%, immobility or moribund; and 4, death. The individual scoring of animals and times during the day that scoring is performed should be kept constant for the entire PTMG experiment.

Measures of weakness and fatigability

A grip meter can be used to test for forelimb strength in rats and mice, specifically when clinical scores are in the range of 0–2 (Kusner et al., 2013). The animals are suspended by their tails by an experienced handler and front paws are allowed to grip on a bar or mesh connected to a sensitive force gauge. A pull consist of the handler tugging on the animal's tail to free the animal's grip on the bar. The tug should not be forceful as to dissuade the animal from gripping the bar. The animals should be exercised by repetitive grips (15–20 grips) and force generation of the first five pulls should be documented. The rodents are then tested for fatigability by five pulls after exercise. The force generation is documented and compared with the pre-exercise force generation. Animal position on the bar/mesh should be kept constant for each pull. The handler and time of day that protocol is performed should be kept constant for the entire PTMG experiment to avoid altering the pull force.

Rats' muscle strength can also be assessed by their ability to grasp and lift repeatedly a rack or cage lid from the table, while suspended manually by the base of the tail for 30 s (Verschuuren et al., 1990). The exercise will determine if the rat can grip and has strength to lift the lid. Repeated cage lifts will determine the fatigability of the animal. The appearance of the animal before and after exercise should be noted.

The mesh/hanging test can be used with mice. For the hanging test, mice are placed at the center of a frame holding a wire mesh, and the frame inverted over soft bedding (Kaminski et al., 2006). The time until they release their grip is measured (maximum hanging time 10 min). This is a reliable and observer-independent test of fatigue. The animal weight, learnt behavior, and stress can alter the results. If the animal release is not due to fatigue (i.e. animal jumps from the wire mesh), the animal should be immediately retested. If the mouse attempts to climb over the edges of the frame, they should be replaced and inverted as mentioned above. Handlers and time of day that any protocol is performed should be kept constant for the entire PTMG experiment.

Electromyography

Decrement of compound muscle action potential (CMAP) is generally measured in the tibialis anterior or the gastrocnemius muscles of PTMG animals 24–48 h after antibody transfer. Rats are anaesthetized with 60 mg/kg sodium pentobarbital or with 2.5% isoflurane in air. The animal must be kept warm (skin temperature between 35 and 37 °C) by means of an infrared heating lamp or a heat pad, but do not overheat (risk of myasthenic decompensation). For stimulation, two small monopolar needle electrodes are used. The cathode is inserted near the peroneal nerve at the level of the knee and the anode more proximal and lateral (at a distance of 3–4 mm in the rat). For recording, a third monopolar needle electrode is inserted subcutaneously over the tibialis anterior muscle. A ring electrode distally around the relevant hind leg or a subcutaneous needle electrode at the distal tendon serves as a reference, and the animal is grounded by a ring electrode around the tail. Movement artefacts must be avoided. Stimulation and recording can be performed with EMG systems that are also used in clinical practice. To detect a decrementing response, a series of 8–10 supramaximal stimuli are given at 3 Hz with a stimulus duration of 0.2 ms. The test is considered positive for decrement when both the amplitude and the area of the negative peak of the CMAP show a decrease of at least 10% (Kimura, 2001). To demonstrate reproducibility, at least three recordings are made of all investigated muscles.

In case only subclinical disease is present, the impairment of neuromuscular transmission can be quantified in a terminal experiment by combining decrement measurements with intraperitoneal curare challenge (for rats of ~200 g: 20 μg/ml at a rate of 0.33 μg curare/minute). In this case, the elapsed time until decrement is observed (an equivalent of the cumulative curare dose) is a measure for the muscle weakness (Gomez et al., 2011). If the diaphragm of the animals is severely affected by PTMG, curare infusion might result in respiratory failure before decrement is observed in the tibialis anterior or gastrocnemius muscles. This can be avoided by mechanical ventilation of the animal under anesthesia. Animals should be sacrificed under anesthesia since the long-term effect of curare prevents recovery of the animals.

Immunofluorescence analysis of neuromuscular junctions

The density of AChR, its associated proteins or deposition of complement factors on the postsynaptic membrane can be analyzed by immunofluorescence. As a reference, a presynaptic marker is used. Isolated muscles (e.g. tibialis anterior or diaphragm) of PTMG and control animals are frozen on melting isopentane. Cryosections of 10 μm are dried, fixed and blocked with PBSA (phosphate-buffered saline with 2% bovine serum albumin). Sections can then be incubated with primary antibodies against the vesicular acetylcholine transporter (VAChT) or the synaptic vesicles protein 2 (SV2) to localize the NMJ. To determine the deposition of complement, antibodies to C3, C9 or membrane attack complex (C5b-9) can be used. Subsequently the sections are incubated with fluorescent-conjugated α-bungarotoxin and the corresponding secondary antibodies. An excess of primary and secondary antibodies, and bungarotoxin should be used so these do not limit the staining intensity. All sections are stained and processed in parallel to avoid inter-assay variations. Importantly, since immunoglobulins are deposited on the NMJ in PTMG, the specificity of the secondary antibodies should be confirmed on sections of PTMG muscles without primary antibodies.

For quantitative analysis, pictures of muscle sections are taken using a fluorescent microscope with a digital camera and analysis software. The exposure time is set to a constant value for each channel ensuring that no saturation of the pictures occurs. Also all other microscope settings are maintained constant. Endplate areas are identified by presynaptic markers and the mean intensity of staining in each channel is measured in the corresponding area. The presynaptic marker can be used to normalize the expression of the postsynaptic proteins. Multiple NMJs should be assessed for staining intensity. All sections are stained and processed in parallel to avoid inter-assay variations (Losen et al., 2005).

Table 1
Use of PTMG to test therapeutics.

Strategy	Therapeutic	Species, strain	Antibody
Complement inhibition			
Kusner et al. (2014)	Single chain Fv of mAb35-DAF	Female Lewis rats	mAb3
Soltys et al. (2009)	C5 specific inhibitor	Female Lewis rats	mAb3
Hepburn et al. (2007)	C5 specific inhibitor	Female Lewis rats	mAb35
Zhou et al. (2007)	Anti-C5	Female Lewis rats	mAb3
Biesecker and Gomez (1989)	Anti-C6	Female Wistar Furth rats	mAb-AChR 371A
Antibody turnover			
Liu et al. (2007)	Anti-rat FcRn mAb	Female Lewis rats	mAb35
Poulas et al. (2000)	Papain	Female Lewis rats	mAb35
Competitor antibody			
Hwang et al. (2003)	RNA aptamer	Female Lewis rats	mAb198
Papanastasiou et al. (2000)	Fab fragment	Female Lewis rats	mAb195 and mAb35
Barchan et al. (1998)	Recombinant fragments AChR α	Female Lewis rats	mAb 198
Krolick et al. (1996)	Antibodies to denatured AChR	Female Lewis rats	mAb35
Buschman et al. (1987)	Alpha-fetoprotein	C57BL/6 mice	Human MG IgG
van der Neut Kolfschoten et al. (2007)	IgG4 637	Rhesus monkey	IgG1 637

Electron microscopy

PTMG rats are anaesthetized with ketamine (100 mg/kg) and xylazine (15 mg/kg) and transcardially perfused with Tyrode solution (0.1 M) followed by fixation buffer (2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4). The recommended anesthesia for mice is ketamine (70–100 mg/kg), xylazine (10– 20 mg/kg) and acepromazine (2–3 mg/k). The tibialis anterior muscles are removed, post-fixed for 2 h, and sectioned on a vibratome at 1 mm. Sections are postfixed with 1% osmium tetroxide in 0.1 M phosphate buffer, pH 7.4, dehydrated through a graded ethanol series and embedded in epoxy resin. Endplates are located in toluidine blue-stained semi-thin sections from the central region of each muscle. Ultra-thin sections from selected areas are contrasted with uranyl acetate and lead citrate and viewed with a transmission electron microscope. At least five endplate regions are photographed from each muscle. Pictures are scanned for morphometric analysis using the ImageJ software. The key parameter to be analyzed for PTMG by morphometric analysis is the folding index, i.e. the ratio of the length of the postsynaptic membrane per length of the adjacent presynaptic membrane in each nerve bouton (Engel et al., 1976, 1979; Wood and Slater, 1997; Losen et al., 2005). Since infiltrating cells are commonly found at the NMJ in PTMG, the percentage nerve boutons that are displaced from the postsynaptic membrane by infiltrating leucocytes should also be included in the analysis.

Radioimmunoassay for measurement of AChRs

The AChR concentrations of isolated tibialis anterior muscles from PTMG animals are measured as described (Lindstrom et al., 1976; Verschuuren et al., 1992; Losen et al., 2005). Muscles are minced and homogenized at 4 °C in 10 ml Buffer A (PBS, 10 mM EDTA, 10 mM NaN_3, 10 mM iodoacetamide and 1 mM PMSF). Homogenates are centrifuged (22,100 *g*, 30 min) and the resulting pellets are resuspended in 2.5 ml Buffer B (Buffer A with additional 2% Triton X-100). Extraction is performed for 1 h at 4 °C on a reciprocal shaker. After centrifugation (22,100 *g*, 30 min), six aliquots of 250 μl are taken from the supernatant and incubated with ^{125}I-α-BT (~74 TBq/mmol, 12.5 μl/ml) followed by rat polyclonal anti-rat AChR (~150 μl/ml with a titer of ~200 nmol/l against rat AChR). As a negative control, three of the aliquots are supplemented with 1 mM acetylcholine and 1 mM neostigmine bromide. After overnight incubation at 4 °C, the immune complexes are precipitated with goat anti-rat Ig (100 μl polyclonal serum) for 4 h and centrifuged at 15,000 *g* for 5 min. The pellets are washed three times in PBS with 0.5% Triton X-100 and measured in a gamma-counter. Results are calculated in fmol ± standard deviation, and differences between concentrations are presented in percentage ± standard error of the means. When using polyclonal anti-AChR sera to induce PTMG there is a risk that some of the antibodies mask the α-BT binding sites resulting in underestimation of the AChR amounts.

Conclusion

The PTMG model is unique in that the antibodies induce a specific effect on the AChR in a short 48 hour window. Elimination of the antibodies or the complement-mediated damage ablates the effect. The model does have intrinsic limitations. The afferent arm of the autoimmune process is not involved; therefore, therapeutics that targets the lymphocytes, cytokine expression, or antigen recognition cannot be tested using PTMG. Secondly, the PTMG does not induce a chronic autoimmune disease and definitive testing of a therapeutic should also rely on the EAMG model: the narrow window of severe weakness during which the therapeutic can be tested on its ability to reverse weakness.

The PTMG has been used successfully in testing of therapeutics for complement inhibition, increased antibody turnover, and antibody competitive inhibition (Table 1). Future direction of therapeutics in these fields may prove beneficial and translate to an improved therapy for myasthenia gravis.

Acknowledgments

The authors' research was supported by the following funding agencies: the Prinses Beatrix Fonds (Project WAR08-12 to M.L.), the Netherlands Organization for Scientific Research (Veni Fellowship of 916.10.148 to ML), the Brain Foundation of the Netherlands (fellowship FS2008(1)-28 to ML), the Association Française contre les Myopathies (AFM trampoline grant 15853 to P.M.-M.); the Greek General Secretariat of Research and Technology (program "Thalis" project Autoimmunity and program "Aristeia" project Myasthenia no 1154 to S.T.), National Institute of Neurological Disorder and Stroke (grant no. R24EY014837 to LK) and the Muscular Dystrophy Association (grant 186874 to J.L.).

The conference for standardizing the PTMG model was supported by the Myasthenia Gravis Foundation of America and the National Institute of Neurological Disorders and Stroke.

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