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Experimental autoimmune myasthenia gravis in the mouse

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Abstract

Myasthenia gravis (MG) is a T cell-dependent antibody-mediated autoimmune neuromuscular disease. Antibodies to the nicotinic acetylcholine receptor (AChR) destroy the AChR, thus leading to defective neuromuscular transmission of electrical impulse and to muscle weakness. This unit is a practical guide to the induction and evaluation of experimental autoimmune myasthenia gravis (EAMG) in the mouse, the animal model for MG. Protocols are provided for the extraction and purification of AChR from the electric organs of *Torpedo californica*, or eel. The purified receptor is used as an immunogen to induce autoimmunity to AChR, thus causing EAMG. The defect in neuromuscular transmission can also be measured quantitatively by electromyography. In addition, EAMG is frequently characterized by the presence of serum antibodies to AChR, which are measured by radioimmunoassay and by a marked antibody-mediated reduction in the number of muscle AChRs. AChR extracted from mouse muscle is used in measuring serum antibody levels and for quantifying muscle AChR content. Another hallmark of the disease is complement and IgG deposits located at the neuromuscular junction, which can be visualized by immunofluorescence techniques.

Keywords

myasthenia gravis; experimental autoimmune myasthenia gravis; acetylcholine receptor; neuromuscular junction

UNIT TITLE AND UNIT INTRODUCTION

Myasthenia gravis (MG) is a T cell–dependent antibody-mediated autoimmune neuromuscular disease. Antibodies to the nicotinic acetylcholine receptor (AChR) destroy the AChR, thus leading to defective neuromuscular transmission of electrical impulse and to muscle weakness.

This unit is a practical guide to the induction and evaluation of experimental autoimmune myasthenia gravis (EAMG) in the mouse (see Basic Protocol), the animal model for MG. EAMG is most frequently detected by clinical evaluation of muscle weakness of the mouse and the ability of neostigmine (an acetylcholinesterase inhibitor) to reverse the manifestations of disease. AChR can be extracted from the electric organs of *Torpedo californica*, or eel, and affinity purified (see Support Protocol 1) to be used as an immunogen to induce autoimmunity to AChR, thus causing EAMG. Purification requires a neurotoxin 3–agarose affinity column (see Support Protocol 2). The defect in neuromuscular transmission can also be measured quantitatively by electromyography (see Support

Protocol 3). In addition, EAMG is frequently characterized by the presence of antibodies to AChR, which can be measured by radioimmunoassay (see Support Protocol 4), and by a marked antibody-mediated reduction in the number of muscle AChRs (see Support Protocol 5). AChR extracted from mouse muscle (see Support Protocol 6) is used in measuring serum antibody levels and for quantifying muscle AChR content. Another hallmark of the disease is complement and IgG deposits located at the neuromuscular junction, which can be visualized by immunofluorescence techniques. Muscle C3 content can also be quantified by an ELISA based method (see Support Protocol 7).

BASIC PROTOCOL

INDUCING AND EVALUATING EAMG

The nicotinic acetylcholine receptor (AChR) is purified from the electric organs of *Torpedo californica* (see Support Protocols 1 and 2), emulsified in complete Freund's adjuvant (CFA), and used to induce experimental autoimmune myasthenia gravis (EAMG). Clinical signs of myasthenia gravis (MG) usually become evident days to weeks after the second immunization, although some mice (~10%) will develop signs 20 to 30 days after the first immunization. To increase the incidence and severity of disease a third immunization with AChR is required. Mice that demonstrate muscle weakness are injected with neo-stigmine, an acetylcholinesterase inhibitor, to test for temporary improvement in muscle strength. EAMG can be evaluated quantitatively by electromyography (see Support Protocol 3), by a radioimmunoassay for antibodies to AChR (see Support Protocol 4), by measuring muscle AChR (see Support Protocol 5) and complement and IgG deposits located at the neuromuscular junction, visualized by immunofluorescence techniques (see Support Protocol 7).

Materials

- 200 µg purified *Torpedo californica* AChR (see Support Protocol 1)
- PBS
- Complete Freund's adjuvant (CFA; Difco)
- 50 mg/ml sodium pentobarbital (Nembutal; Abbott Labs)
- H₂O, sterile
- Ten 8 to 10-week-old C57BL6/J mice (male or female), marked for identification
- 70% (v/v) alcohol
- 300 µl/ml neostigmine bromide in PBS
- 120 µg/ml atropine sulfate in PBS
- Test tubes, sterile
- BD Yale 10 ml and 5 ml glass syringes (VWR)
- Microemulsifying needle connector (Fisher Scientific)
- 1-ml plastic syringe with slip tip (Becton Dickinson Labware)
- Heating pad
- 25-G needles (Becton Dickinson Labware)
- 5 × 5-cm gauze sponges

Additional reagents and equipment for anesthetizing mice, collecting blood, evaluating EAMG by electromyography (see Support Protocol 3), by quantifying anti-AChR antibodies by radioimmunoassay (see Support Protocol 4), and/or by measuring muscle AChR content (see Support Protocol 5) and evaluation of muscle IgG and complement content (see Support Protocol 7).

Prepare immunogen—About 20 µg of purified AChR is used per mouse. 200 µl of emulsion of AChR, PBS and CFA is used per mouse. The steps below are for preparing immunogen for 10 mice. Prepare immunogen for 1 or 2 extra mice for dead space in syringe/needle or any unexpected loss during preparation or immunization.

1. Dilute 200 µg purified *Torpedo californica* AChR in 1 ml PBS in a sterile test tube.
2. Aspirate 1 ml AChR solution into one 2-ml glass syringe and connect the syringe to one end of a microemulsifying needle connector. Expel the air.
3. Mix CFA vigorously with a pipet tip (avoiding bubble) and aspirate 1 ml CFA into a second 2-ml glass syringe. Expel the air and connect the syringe to the other end of the connector. Make sure all connections are tight.
4. First, quickly push AChR solution into CFA-containing syringe through the metal connector. Then push the solution forward and backward from one syringe to the other for ~5 min or until the emulsion becomes thick and small air bubbles in the emulsion do not move up when syringes are kept vertical.
5. Wrap the syringes and connector with aluminum foil and cool 30 min at 4°C.

Cooling the emulsion makes it thicker.

Anesthetize and inject mice

- 6 Dilute 50 mg/ml sodium pentobarbital 1:10 in sterile water. Anesthetize marked 8- to 10-week-old mice. Using a 1-ml plastic syringe, inject 65 mg/kg sodium pentobarbital i.p. When the animals are asleep, it is better to place them on a warm heating pad to prevent hypothermia.
- 7 Mix emulsion through connector, remove one syringe (empty one) but not the connector. Connect a 1 ml plastic syringe (BD Tuberculin) and slowly transfer the emulsified AChR to the syringe and then attach a 26-G needle. Clean the injection sites with an alcohol-soaked gauze sponge. Inject 50 µl AChR emulsion subcutaneously in each of four sites (200 µl/mouse, 20 µg AChR).

For the first immunization, inject in the two hind foot-pads and the shoulders. For subsequent immunizations, inject in the shoulders (close to the previous immunization site) and thighs.

- 8 Put immunized mice back in the cages and observe until all of them wake up from anesthesia. Monitor the mice and evaluate EAMG

Monitor the mice and evaluate EAMG

- 9 Monitor mice for signs of muscle weakness by performing the paw-grip test at least once a week after first immunization and two- or three-times a week after second immunization.

A small percentage (~10%) of mice may develop muscle weakness between day 20 and day 30 after the first immunization.

- 10 After 30 days, immunize mice a second time (repeat steps 1 to 8).

- 11 Following the second immunization, monitor the mice daily for signs of EAMG, and collect blood from the tail vein once a week for immunoassays (see Support Protocols 4 and 5).

A significant number (30% to 60%) of adult male or female C57BL6/J mice will develop muscle weakness between day 3 and day 15 after a second immunization with emulsified AChR. If very few mice develop muscle weakness after the second immunization, give the animals a third injection of emulsified AChR (repeat steps 1 to 8).

- 12 Exercise the animals using the paw-grip test to accentuate any muscle weakness. Assign an EAMG score to each injected mouse based on the following scale: grade 0, normal muscle strength and no muscle weakness, even after exercise (20 to 30 consecutive paw grips to a steel grid cage top; see Fig. 1A); grade 1, normal at rest but weak after exercise, with chin on the floor and inability to raise head, hunched back, and reduced mobility; grade 2, weakness at rest (see Fig. 1B); and grade 3, moribund, dehydrated, and paralyzed (quadriplegic).

A mouse with grade 1 weakness can advance to grade 2 or 3 or stay at grade 1; a mouse with grade 2 can advance to grade 3. Sometimes acute MG (grade 3) can ensue within 24 hr. Usually a mouse with grade 3 MG will die within 24 to 48 hr, so once grade 3 weakness is observed, the mouse should be euthanized. Clinical EAMG should also be confirmed by electromyography (EMG; see Support Protocol 3).

Exercise accentuates muscle weakness of EAMG due to excessive use of acetylcholine.

- 13 For objective measurement of muscle strength, exercise the animals with 40 paw grips on cage top grid. Following exercise, make them grasp a grid attached to a dynamometer (Chatillon Digital Force Gauge, DFIS 2, Columbus Instruments, Columbus, OH). While pulling them by their tails, record the force required to make them lose their grip (Tuzun et al., 2006).

- 14 Dilute 300 µg/ml neostigmine bromide 10-fold in PBS and dilute 120 µg/ml atropine sulfate 10-fold in PBS. Mix equal volumes of the two solutions to give final concentrations of 15 µg/ml neostigmine bromide and 6 µg/ml atropine sulfate in PBS. Inject 50 µl i.p. into mice that show clinical evidence of EAMG. Observe the mouse for 30 min and note any improvement in EAMG grade (see Fig. 1C).

There is usually a dramatic improvement in muscle weakness within 10 to 20 min (e.g., grade 2 improves to grade 1 or grade 0), generally lasting 1 to 2 hr. This temporary improvement is due to the availability of more acetylcholine at the receptors because synthesis of cholinesterase, which degrades acetylcholine, is being inhibited by the anticholinesterase activity of neostigmine. Atropine is used to prevent the cholinergic side effects.

SUPPORT PROTOCOL 1

EXTRACTION AND AFFINITY PURIFICATION OF AChR

Electroplax tissue from *Torpedo californica* is used as a source of acetylcholine receptor (AChR) protein. In this protocol, milligram amounts of crude AChR-containing protein can be prepared from frozen commercially available tissue for subsequent affinity purification. Pooled tissue is homogenized then ultracentrifuged. The pellets are homogenized in detergent-containing buffer and centrifuged to yield supernatants containing crude AChR.

This crude preparation is then applied to a neurotoxin 3 affinity column (Cuatrecasas and Anfinsen, 1971; Lindstrom et al., 1981) to prepare AChR. Fractions rich with AChR (as determined by protein quantitation assay) are collected, pooled and further fractionated passing through the hydroxylapatite column. Highly pure AChR is then aliquoted and stored at -70°C for use as an immunogen. A crude extract of muscle AChR prepared from mouse carcass using neurotoxin 3 column (see Support Protocol 6) is used to measure antibody to mouse muscle AChR (see Support Protocol 4) and muscle AChR content (see Support Protocol 5).

Materials

Bio-Gel HT hydroxylapatite (Bio-Rad) 10 mM Tris buffer (see recipe)

250 g electroplax tissue from *Torpedo californica* (E-PLAX-F, Pacific Bio-Marine Labs), frozen

Homogenization buffer (see recipe), 4°C and room temperature 10% (v/v) Triton X-100 in homogenization buffer

Neurotoxin 3–agarose (see Support Protocol 2)

0.2% cholate buffer (see recipe)

NaCl/Triton buffer (see recipe)

1 M carbomylcholine buffer (see recipe)

Column wash buffer (see recipe)

Elution buffer (see recipe)

Protein assay kit (Bio-Rad)

70% ethanol

4°C cold room or chromatography refrigerator (Revco)

1.5 × 20–cm Econo columns for low-pressure chromatography (Bio-Rad)

Waring Blendor

38.5-ml polyallomer ultracentrifuge tubes with open top (Beckman)

Ultracentrifuge and 60 Ti rotor (Beckman) or equivalent

96-well microtiter plate

Microtiter plate reader

Additional reagents and equipment for column chromatography, determining protein concentration, SDS-PAGE, and Coomassie blue staining

NOTE: Perform steps 1 to 16 in a 4°C cold room or chromatography refrigerator.

Prepare neurotoxin 3-agarose (See Support protocol 2)

Prepare homogenate

1. Add 250 g frozen electroplax tissue from *Torpedo californica* to 2.5 vol room temperature homogenization buffer in the flask of a Waring Blendor. Homogenize first at slow speed and then at high speed for ~1 min or until the tissue is completely homogenized.

Normally 250 g frozen electroplax tissue should yield 10 to 20 mg purified AChR.

2. Pour the homogenate (about 30 ml) into 50-ml polyallomer high speed compatible centrifuge tubes. Centrifuge 30 min at $50,000 \times g$ (20,000 rpm in JA20 rotor, Beckman), 4°C. Discard the supernatant.
3. Homogenize the pellet in 3 vol of 4°C homogenization buffer. Add 1% Triton X-100 exactly (not less) and agitate overnight on a shaker at low speed at 4°C (24 hours but no more than 1 day).
4. Pour the extract into a 38.5-ml polyallomer ultracentrifuge tube. Centrifuge 30 min at $100,000 \times g$ (or 35,000 RPM, Ti60 rotor), 4°C. Collect the supernatant, which contains AChR protein, and store at 4°C.

React AChR with neurotoxin 3–agarose

- 5 Connect a plastic tubing from both ends of the column. Lower end tube will be contained in a reservoir. Pass about 500 ml of NaCl/Triton buffer through neurotoxin-3 column from the upper end tubing. Add the supernatant that contains AChR protein to the column.

Wash and elute AChR from the columns

- 6 Wash the toxin-affinity column with 200 ml of 0.2% cholate buffer until there is no absorbance at 280 nm (A_{280}) due to Triton buffer from the affinity column.
- 7 Add 150 ml of 1 M carbomylcholine buffer to the top of the column to elute AChR.
- 8 Collect about 20 of 10 ml fractions. Save and pool fractions rich with AChR as determined by Bradford Protein quantitation assay (Biorad).

Concentrate AChR through hydroxylapatite column

Prepare hydroxylapatite column

- 9 Mix 25 ml Bio-Gel HT hydroxylapatite beads with 100 ml of 10 mM Tris buffer, swirl gently, and let settle for 5 min.
- 10 Decant the superfines in the supernatant and the fines on top of the settled bed.
- 11 Repeat steps 1 and 2 until there are no superfines in the supernatant.
It usually takes four to five repetitions of these steps to remove the superfines.
- 12 Add an equal volume 10 mM Tris buffer, swirl gently, and pour into a 1.5×20 -cm Econo column. Allow 2 to 3 cm of beads to settle under gravity, then open the column outlet and allow the gel to pack under flow. When the bed is stable, pass at least two bed volumes of 10 mM Tris buffer through the column.
The hydroxylapatite column collects AChR as it is eluted from neurotoxin 3–agarose. Hydroxylapatite is a lattice composed of calcium, hydroxyl, and phosphate groups, so its surface presents a complex mosaic of charges. All proteins can bind to the column, but the mechanism of binding depends on the net charge of the protein. AChR is negatively charged, so the carboxyl group complexes with calcium sites on the column.
- 13 Load column with pooled AChR collected from neurotoxin-3 column. Once AChR is absorbed in the hydroxylapatite column, rinse the column with 500 ml HT column wash buffer (1.21 g Tris base, 29.2 g NaCl, 0.5 ml Triton X-100 per litre, pH to 7.5).

- 14 Connect the hydroxylapatite column to a fraction collector and elute AChR with elution buffer (Sodium phosphate buffer, 152mM, pH7.5). Collect 20 fractions of 8 to 10 ml each.

Analyze the fractions

- 15 Using the protein assay kit, identify fractions that contain protein. Dilute the Bio-Rad protein assay reagent 1:5 in water and filter. Add 190 μ l of this solution to each well of a 96-well microtiter plate, then add 10 μ l of each fraction or protein standard. Read the absorbance at 595 nm with a microtiter plate reader against an elution buffer blank.
- 16 Collect and pool fractions based on A₅₉₅. Determine the protein concentration of the pooled fraction. Add glycerol to 10% (v/v) final concentration and aliquot to cryoprotective vials. Slowly freeze the vials in a -70°C freezer in 70% ethanol or in a styrofoam container. Store vials at -70°C .
- 17 Wash toxin column with 1 liter of each acetate buffer, tris buffer, pH 7.5 and NaCl/Triton buffer, pH 8. Store at 4°C for future use.

Column can be stored 1 to 2 years and used 4 to 5 times.

Assay by SDS-PAGE

- 18 Analyze 10 to 20 μ l purified AChR by SDS-PAGE on a 7.5% acrylamide gel.
- 19 Stain the gel with Coomassie blue.

The gel should show four bands, at 40, 50, 60, and 65 kD (Fig. 2). Each new AChR preparation should be compared with a sample of previously purified AChR.

The biological activity of AChR, the number of α -bungarotoxin-binding sites, can be determined using Support Protocol 5.

In vivo test for toxicity: Twenty to 30 μ g of purified AChR is injected in two mice and observed for a week to check any symptoms of toxicity.

SUPPORT PROTOCOL 2

PREPARATION OF NEUROTOXIN 3-AGAROSE

Neurotoxin 3 of *Naja naja siamensis* was used to prepare affinity columns for purification of acetylcholine receptor (AChR) because it binds AChR in a reversible manner in vitro (Karlsson et al., 1971). The quantities used in this protocol provide enough neurotoxin 3-agarose for purifying 10 to 15 mg of AChR. Purified neurotoxin 3 from *Naja naja kaouthia* (Sigma) is a suitable substitute for *Naja naja siamensis* neurotoxin 3 for affinity purification.

Materials

- 30 to 50 mg neurotoxin 3 of *Naja naja kaouthia* (Sigma)
- Coupling buffer: 0.1 M sodium carbonate buffer (pH 8.3; see recipe)/0.5 M NaCl
- 7.5g CNBr-activated cross-linked agarose (Sigma)
- 1 mM HCl
- 0.2 M glycine, pH 8.0
- 0.1 M acetate buffer (pH 4.0; see recipe)/0.5 M NaCl

NaCl/Triton buffer (see recipe)

0.2% cholate buffer (see recipe)

Sintered-glass filter with G3 porosity (VWR)

End-over-end rotator or equivalent

CAUTION: Neurotoxin 3 is extremely toxic ($LD_{50} < 500 \mu\text{g/kg}$). This toxin blocks postsynaptic transmission in skeletal muscles without depressing acetylcholine release from the motor nerve endings. It binds to nicotinic AChR on the postsynaptic membrane of the neuromuscular junction, thus preventing binding of acetylcholine. In experimental animals, the toxin causes death by respiratory paralysis, with violent spasms during the final stages of asphyxia. The binding is irreversible in vivo, and therefore precautions should be taken when working with it. Always wear examination gloves and mask and take other precautions appropriate for handling neurotoxin.

1. Dissolve 30 to 50 mg neurotoxin 3 from *Naja naja kaouthia* in coupling buffer to a final concentration of 5 to 10 mg/ml.
2. Swell 10 g freeze-dried CNBr-activated cross-linked agarose in 30 ml 1 mM HCl for 15 min.

3. Wash the swollen agarose on a sintered-glass filter with G3 porosity, using 100 ml of 1 mM HCl per gram of dry gel, added in several aliquots.

Washing with HCl preserves the activity of the reactive groups, which hydrolyze at high pH.

4. Wash the gel with 50 ml of coupling buffer (5 ml per gram dry gel). Transfer immediately to neurotoxin 3 solution. Mix toxin solution with gel suspension in end-over-end or similar rotator 2 hr at room temperature or overnight at 4°C.

Maximum coupling occurs at this concentration. This stage should be completed without delay because reactive groups on the gel hydrolyze at the coupling buffer pH of 8.3. A gel/buffer (swelled) ratio of 1:2 gives a suitable suspension for coupling.

Gentle stirring methods should be used. Do not use vortex or magnetic stirrers because they usually cause fragmentation of the gel beads.

5. Determine the amount of toxin bound to the CNBr-activated agarose indirectly by measuring the A_{280} , the toxin concentration in the eluate, to ensure that the coupling reaction is satisfactory.

Neurotoxin 3 at a concentration of 1.0 mg/ml has an A_{280} of 1.06. The resulting gel should then contain 0.5 μmoles of bound toxin per gram of wet gel.

6. Add 50 ml of 0.2 M glycine, pH 8, to the gel and let stand overnight at 4°C to block residual active groups.

The residual active groups that remain on the gel after coupling can also be hydrolyzed by letting the gel stand overnight at 4°C in mild alkaline pH or for 2 hr in 0.1 M Tris Cl, pH 8, at room temperature. Alternatively, these groups can be blocked by adding an excess of small primary amines (e.g., ethanolamine, glutamic acid, or glycine) and incubating overnight. Incubating the gel in 0.2 M glycine, pH 8, is most effective in minimizing nonspecific adsorption.

7. Remove excess uncoupled neurotoxin 3 by washing the adsorbent first with 4 to 5 volumes of coupling buffer, then with 0.1 M acetate buffer (pH 4)/0.5 M NaCl, and finally with coupling buffer.

This procedure ensures that no free neurotoxin 3 remains bound ionically to the immobilized neurotoxin 3. The wash cycle of low pH/high pH is essential because protein desorption occurs only when the pH is changed, but pH changes do not cause loss of covalently bound protein.

8. Wash the adsorbent with NaCl/Triton buffer and with 0.2% cholate buffer. Store the suspension at 4° to 8°C in the presence of a bacteriostatic agent (e.g., 1 mM sodium azide in PBS).
9. Pour neurotoxin 3–agarose suspension into a fresh 1.5 × 20–cm Econo column. Pour the mixture down the sides of the column and allow the buffer to run out of the column as the mixture is poured to pack the beads. Leave a few milliliters of buffer covering the surface of column and store the column at 4°C clipped at the bottom.

Neurotoxin 3 coupled to CNBr-activated cross-linked agarose is usually more stable to denaturation than the protein in free solution. Properly maintained, it can be reused for years.

The coupled neurotoxin 3–agarose is now ready to be used to absorb AChR in suspension (see Support Protocol 1, step 5), or it may be used to prepare an affinity column for large-scale purifications.

SUPPORT PROTOCOL 3

EVALUATING EAMG USING ELECTROMYOGRAPHY

Electromyography (EMG) is an objective, quantitative measurement of muscle weakness; it is used to demonstrate decremental responses to repetitive nerve stimulation. This test is used to diagnose myasthenia gravis (MG) in humans. In EMG, electric currents are delivered to the nerves at a rate of three per second, and action potentials are recorded from an electrode inserted into the respective muscle.

Additional Materials (also see Basic Protocol)

Styrofoam board

Band-Aids

Electromyography machine (e.g., Cadwell 7400, Cadwell Labs)

Electrodes (ground, stimulating, reference, and negative)

Gel

Additional reagents and equipment for anesthetizing animals (UNIT 1.4)

1. Dilute 50 mg/ml sodium pentobarbital 1:10 in sterile water. In a clean, well-ventilated, draft-free room, inject mouse with 60 mg/kg sodium pentobarbital.
2. Place the mouse on its back on a styrofoam board and secure the limbs to the board using Band-Aids. Connect the electrodes of an electromyography machine. Place a ground electrode with gel on the chest of mouse and secure it in place by a strap pinned to either side of the board (see Fig. 3A). Insert a stimulating (positive) electrode into the sciatic notch to stimulate the sciatic nerve and a negative electrode subcutaneously into the abdominal wall. Insert a recording electrode into the gastrocnemius muscle of the same leg and a reference electrode into the tendon of the gastrocnemius muscle.

The stimulating and negative electrodes deliver electrical stimulations; the recording and reference electrodes record the compound muscle action potentials from the gastrocnemius muscle.

3. Stimulate the sciatic nerve with a set of eight 3-Hz supramaximal stimuli and simultaneously record the evoked compound muscle action responses.
4. Evaluate the response to repeated stimulation by comparing the first (or second) evoked response with the fourth (or fifth).

If comparison of the fourth (fifth) evoked action potential with the first (second) shows a decrement of $\geq 10\%$ in amplitude, there is a significant positive decremental response to supramaximal stimulation (see Fig. 3B and C).

Because of the consumption of acetylcholine and the decreased numbers of acetylcholine receptors (AChR) on the postsynaptic membrane in patients with MG or mice with EAMG, a reduction in the amplitude of the evoked muscle action potential (decremental response) can be observed after repeated stimulation. This decremental response to repetitive nerve stimulation is diagnostic for MG in humans and for EAMG in mice (Christadoss et al., 1986).

SUPPORT PROTOCOL 4

EVALUATING EAMG BY ASSAYING FOR ANTI-AChR ANTIBODIES

Myasthenia gravis (MG) is primarily an antibody-mediated disease, so measuring serum levels of anti-acetylcholine receptor (AChR) antibodies by radioimmunoassay (Lindstrom et al., 1981; Christadoss and Dauphinee, 1986) can be used to evaluate mice with experimental autoimmune MG (EAMG). Because the antibodies that are pathogenic in EAMG are cross-reactive with mouse muscle AChR, it is appropriate to measure antibody to mouse muscle AChR. This assay can also be used to measure antibodies to *Torpedo californica* AChR to determine that a humoral response to *Torpedo californica* AChR has been established; however, the presence of such antibodies is irrelevant to disease pathogenesis. AChR-antibodies of different isotypes (IgM, IgG, IgG1 and IgG2b) can be measured by radioimmunoassay or ELISA. Since complement activation is an essential component of EAMG pathogenesis, the complement fixing IgG2b isotype is presumably more preferentially involved in neuromuscular junction destruction and therefore it is more important to measure this isotype. Also, in some mouse strains and in mice receiving certain immunomodulating treatments, AChR immunization might alter the serum levels of some Ig isotypes, while levels of the other isotypes may remain relatively unaffected (Yang et al., 2005; Tuzun et al., 2004). Therefore, for a thorough examination of the AChR-antibody response, evaluation of the major Ig isotypes is required.

MEASUREMENT OF AChR-ANTIBODIES BY RADIOIMMUNOASSAY (IgG)

Materials

Mouse muscle AChR prepared from normal C57BL/6/J mice (see Support Protocol 6)
 0.5% (v/v) Triton X-100 buffer (see recipe), 4°C
 100 μ Ci [125 I] α -bungarotoxin in 0.2 ml (240 Ci/mmol; Perkin Elmer)
 Serum from experimental mice
 Normal mouse serum
 Polyclonal rabbit anti-mouse Ig
 12 $\times$ 75-mm borosilicate glass tubes

Sorvall centrifuge and H-1000B rotor (or equivalent)

γ counter (Beckman)

1. Mix 0.1 ml mouse muscle AChR prepared from normal C57BL6/J mice ($\sim 1 \times 10^{-9}$ M bungarotoxin binding sites in 100 μ l) and 0.9 ml of 4°C 0.5% Triton X-100 buffer containing 0.1 mCi ($\sim 2 \times 10^{-9}$ M) [125 I] α -bungarotoxin in a 12 $\times$ 75-mm borosilicate glass tube. Incubate 3 to 4 hr at 4°C.

Although a single labeling reaction will provide sufficient labeled AChR for reproducible results in experienced hands, labeling should be performed in duplicate or triplicate.

2. To 1 ml labeled normal muscle AChR, add 1 μ l serum from experimental (e.g., EAMG) mouse and 9 μ l normal mouse serum (as carrier). For controls, add 10 μ l normal mouse serum. Mix well and incubate overnight at 4°C.
3. Add 0.1 ml polyclonal rabbit anti-mouse Ig serum (titered against anti-AChR serum from immunized mice) and incubate 4 hr at room temperature.

The amount of rabbit anti-mouse Ig should be enough to precipitate all of the immunoglobulin.

4. Centrifuge sample 10 min at 3000 $\times$ g (3800 rpm in H-1000B rotor), 4°C.
5. Aspirate the supernatant and wash the pellet with 0.5 ml of 0.5% Triton X-100 buffer.

Be careful to avoid pellet loss.

6. Centrifuge sample 10 min at 3000 $\times$ g, 4°C. Aspirate the supernatant.
7. Count the pellet (immunoprecipitate) in a γ counter.
8. Calculate the number of α -bungarotoxin-binding sites precipitated as a measure of the amount of serum anti-AChR antibodies.

The number of α -bungarotoxin-binding sites precipitated is calculated by dividing the dpm specifically bound by serum from the EAMG animal by the specific activity of the α -bungarotoxin used to label the AChR.

For example, for an EAMG (experimental) sample with a mean of 1132 cpm bound and normal mouse serum (controls) with a mean of 100 cpm bound, specific binding is 1132 – 100 cpm, or 1032 cpm. To convert this value to dpm, divide by 0.5 (the counting efficiency) to get 2064 dpm, which is equivalent to 9.3836×10^{-10} Ci bound (based on 2.2×10^{12} dpm/Ci). Dividing by the specific activity of the α -bungarotoxin used to label the AChR (240 Ci/mmol) and correcting for the sample size (1 μ l = 10^{-6} liter) gives 3.9×10^{-7} mmol/liter or 3.9×10^{-9} M α -bungarotoxin-binding sites precipitated per liter of serum.

MEASUREMENT OF AChR-ANTIBODY ISOTYPES BY ELISA (IgM, IgG, IgG1, IgG2b)

Materials

Mouse muscle AChR prepared from normal C57BL6/J mice (see Support Protocol 6)

96-well microtiter plate (e.g. Dynatech Immulon 2; Dynatech Labs)

Multichannel pipette

Microtiter plate reader

Carbonate/bicarbonate buffer, pH 9.6 for ELISA (see recipe)

Washing buffer for ELISA (see recipe)

Dilution buffer for ELISA (see recipe)

Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgM, IgG, IgG1, IgG2b (e.g. Caltag Laboratories, San Francisco, CA)

Ready-to-use 3,3',5,5'-TetraMethylBenzidine (TMB) single solution chromogen/substrate (e.g. Invitrogen)

Stop solution (1 N hydrochloric acid)

1. Coat a 96-well ELISA plate with 1 µg/ml of mouse muscle AChR diluted in carbonate/bicarbonate buffer, pH 9.6.
2. Incubate overnight at 4°C.
3. Next day, aspirate each well and wash 3 times with 100 µl of washing buffer per well by a multichannel pipette.
4. Block the plates with 2% bovine serum albumin in PBS at room temperature for 30 minutes.
5. Add mouse serum samples diluted 1/1000 in dilution buffer and incubate at 37°C for 90 minutes.

Do not add sera to at least 3 AChR-coated wells (blank wells)

6. Aspirate each well and wash 4 times with 100 µl of washing buffer per well by a multichannel pipette.
7. Add 100 µl of HRP-conjugated goat anti-mouse IgM, IgG, IgG1 or IgG2b diluted 1:500 in dilution buffer and incubate at 37°C for 90 minutes.
8. Aspirate each well and wash 4 times with 100 µl of washing buffer per well by a multichannel pipette.
9. Add 100 µl of TMB solution to each well and incubate for 30 minutes at room temperature.

TMB and peroxide react to produce a blue byproduct (maximum absorbance at 605 nm).

10. Add 100 µl of stop solution to each well.

Addition of stop solution changes the color to yellow.

11. Determine the optical density (OD) at 450 nm using a microplate reader within 30 minutes.
12. Calculate the average OD value of the blank wells. Subtract this average value from the OD values of wells incubated with sera. Express the result for each mouse sample by taking the average of the subtracted values for 3 wells containing serum samples from the same mouse.

SUPPORT PROTOCOL 5

EVALUATING EAMG BY MEASURING MUSCLE AChR CONTENT

The primary pathology in myasthenia gravis (MG) and experimental autoimmune MG (EAMG) is a significant loss of muscle acetylcholine receptors (AChR) due to antibody-mediated attack. The extent of pathology can be measured biochemically using the following radioimmunoassay to quantify the amount of mouse muscle AChR.

Materials

Muscle extract from EAMG and control mice (see Support Protocol 6)

0.5% Triton X-100 buffer (see recipe)

0.05 M benzoquinonium dibromide (Tocris Bioscience)

100 μ Ci [125 I] α -bungarotoxin (240 Ci/mmol; Perkin Elmer)

Mouse anti-AChR serum collected from immunized mice (see Basic Protocol)

Polyclonal rabbit anti-mouse Ig

1.5 ml microfuge tubes

Sorvall centrifuge and H-1000B rotor (or equivalent)

γ counter (Beckman)

1. For each mouse muscle extract, set up two reactions with or without benzoquinonium in 1.5 ml microfuge tubes as follows:

Tube 1:

0.5 ml 0.5% Triton X-100 buffer

100 μ l mouse muscle extract from experimental or control mouse

20 μ l benzoquinonium (0.001 M final)

0.1 μ Ci 125 I- α -bungarotoxin.

Tube 2:

0.5 ml 0.5% Triton X-100 buffer

100 μ l muscle extract from experimental or control mouse 0.1 μ Ci [125 I] α -bungarotoxin.

Incubate both tubes 4 hr at 4°C.

The biological activity of Torpedo californica AChR can be determined by adding 1 to 10 μ l purified Torpedo californica AChR instead of mouse muscle extract.

Benzoquinonium protects acetylcholine-binding sites.

2. Add 10 μ l mouse anti-AChR serum (previously obtained from EAMG mice) and incubate overnight at 4°C.
3. Add 100 μ l rabbit anti-mouse Ig and incubate 4 hr at room temperature.
4. Centrifuge the sample 10 min at 3000 $\times$ g (3800 rpm in H-1000B rotor), 4°C.
5. Aspirate the supernatant and wash the pellet with 0.5 ml of 0.5% Triton X-100 buffer.

Be careful to avoid pellet loss.

6. Centrifuge the sample 10 min at 3000 $\times$ g, 4°C. Aspirate and discard the supernatant.
7. Count the pellet in a γ counter.
8. Calculate the moles of α -bungarotoxin-binding sites per mouse.

The moles of α -bungarotoxin binding sites per mouse are calculated by dividing the Ci specifically precipitated by the extract from an EAMG or control mouse (cpm

precipitated in the absence of benzoquinonium – cpm precipitated in the presence of benzoquinonium, converted to Curies) by the specific activity of the α -bungarotoxin and correcting for the volume of the sample used for the assay.

For example, using the data in Table 1, specific counts bound for the experimental mouse is $6768 - 1285 = 5483$ cpm or 10,966 dpm or 4.98×10^{-9} Ci; for the control mouse it is $11,509 - 1300 = 10,209$ cpm or 20,418 dpm or 9.28×10^{-9} Ci. Dividing by the specific activity of the α -bungarotoxin (240 Ci/mmol) and correcting for the total volume of extract (13 ml) gives 2.69×10^{-12} moles α -bungarotoxin-binding sites for the experimental mouse and 5.027×10^{-12} moles for the control mouse.

- 9 Calculate the percent loss of AChR as follows:

$$\left(\frac{1 - \text{moles } \alpha\text{bungarotoxin binding sites for the experimental mouse}}{\text{moles } \alpha\text{bungarotoxin binding sites for the control mouse}} \right) \times 100$$

For the data in this example, this is $(1 - 2.69 \times 10^{-12} / 5.027 \times 10^{-12}) \times 100 = 46.49\%$ loss.

SUPPORT PROTOCOL 6

PREPARATION OF MOUSE MUSCLE AChR

Mouse muscle is used to prepare extracts that contain acetylcholine receptors (AChR) for determining the level of serum anti-AChR antibodies (see Support Protocol 4) or for quantifying the number of AChR in muscle from experimental or control animals (see Support Protocol 5).

Materials

Normal uninjected (control), or EAMG C57BL6/J (experimental) mouse

Homogenization buffer (see recipe), 4°C

Triton X-100

Glycerol

Blender with miniflask (e.g., Waring) 15- and 50-ml centrifuge tubes

Beckman centrifuge and JA-20 rotor (or equivalent) Spatula or glass rod

Reciprocal shaker

38.5-ml ultracentrifuge tubes Ultracentrifuge

4°C cold room or chromatography refrigerator

5-ml cryovials

Additional reagents and equipment for euthanizing mice

Prepare homogenate

1. Euthanize the C57BL6/J mouse and decapitate it. Remove the skin and eviscerate the carcass. Weigh the carcass.

The carcass can be used immediately to prepare AChR or may be frozen at -70°C and extracted later.

2. Cut carcass into small pieces and place in the miniflask of a blender.

3. Add 4 ml of 4°C homogenization buffer per gram of carcass (e.g., 32 ml for an 8 g carcass). Blend 1 min at low speed and 1 min at high speed until there are no large pieces of carcass.
4. Add the homogenized extract to a 50-ml centrifuge tube.
Use a separate tube for each mouse extract.
5. Centrifuge 30 min at $17,000 \times g$ (15,000 rpm in JA-20 rotor), 4°C.
6. Discard the supernatant. Remove the pellet with a spatula or glass rod and place in the blender.

Prepare AChR

- 7 Add 4°C homogenization buffer (a volume equal to the initial tissue weight, 8 ml) and blend it 1 min more at high speed.
- 8 Put the extract in a 50-ml centrifuge tube and add 0.1 vol Triton X-100 (~1.2 ml). Mix gently using a spatula or glass rod.
- 9 Shake the extract in the 50-ml centrifuge tube at low speed on a reciprocal shaker for 3 to 4 hr at 4°C.
- 10 Transfer extract to a high speed compatible centrifuge tube. Centrifuge the extract 30 min at $17,000 \times g$, 4°C.
- 11 Transfer the supernatant to 38.5-ml ultracentrifuge tube and centrifuge 30 min at $50,000 \times g$, 4°C.
- 12 Aspirate the creamy lipid layer on top of the sample and discard. Pour remaining supernatant into a 15-ml test tube. Discard the pellet. Add glycerol to the extract to a final concentration of 10% (v/v) and mix well by vortexing at high speed. Divide the extract, which contains AChR, into aliquots in 5-ml cryovials and store at -70°C .

SUPPORT PROTOCOL 7

EVALUATING IgG CONTENT OF MUSCLE SAMPLES

Another major pathological finding in myasthenia gravis (MG) and experimental autoimmune MG (EAMG) is the presence of IgG and complement deposits at the neuromuscular junction. These deposits can be visualized by a direct immunofluorescence test (Tuzun et al., 2003). After AChR immunization, C3 levels are elevated in the muscle tissue (see Support Protocol 8). This elevation can be demonstrated quantitatively using an ELISA-based method (Poussin et al., 2002).

DETECTION OF NEUROMUSCULAR JUNCTION IgG AND COMPLEMENT DEPOSITS

Materials

Normal non-immunized (control) and EAMG C57BL6/J (experimental) mice
 Additional reagents and equipment for euthanizing mice
 Insulated container
 Liquid nitrogen
 Screw-top eppendorf tubes
 Long forceps

-70°C freezer
Cryomold
Tissue freezing medium
Cryostat
Small brushes
Coated, precleaned slides (*will attract tissue sections electrostatically*)
Coverslips
Paper towels, gauze sponges
Humidity chamber
Hydrophobic marker
Glass Coplin staining jars with plastic screw caps (for 5 slides)
PBS
Blocking solution (5% normal goat serum in PBS)
Tetramethylrhodamine-conjugated α -BTx solution
(e.g. Molecular Probes, Eugene, OR; recommended dilution, 1:500)
Fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG
(e.g. Southern Biotech; recommended dilution, 1:200)
FITC-conjugated anti-mouse C3
(e.g. ICN/Cappel; recommended dilution, 1:200)
FITC-conjugated anti-human MAC
(e.g. anti-human C9, Cedarlane; recommended dilution, 1:200)
(crossreacts with mouse MAC since human and mouse MAC components are highly homologous)
Acetone
Fluorescent mounting media
Fluorescence microscope

Prepare the muscle sample

1. Fill an insulated container with liquid nitrogen (enough to cover an eppendorf tube).
2. Euthanize mouse and remove the skin.
3. Dissect the limb muscle, place it in a screw-top eppendorf tube and snap freeze the muscle sample by placing the eppendorf tube in liquid nitrogen for 10 seconds.
4. With the long forceps, take the eppendorf out of the liquid nitrogen and store at -70°C until making frozen sections.

Obtain frozen sections

- 5 Remove the muscle sample from the -70°C freezer and place it in a cryomold filled with tissue freezing medium. Make sure sample is covered with tissue freezing medium.
- 6 Place the cryomold in the cryostat immediately and thus adjust muscle sample to temperature in the cryostat (e.g. -20°C).
- 7 Cut sections 7 - 10 μm thick. If necessary, flatten the sections with a small brush.
- 8 Touch section with slide to pick up tissue section.

Ten sections from one muscle specimen might give a good idea about the intensity of deposits. Muscle sections can be kept at -70°C until assayed. Avoid keeping the sections for more than 1 month at -70°C for best results.

Perform direct immunofluorescent antibody test

- 9 Take frozen sections out of freezer and let the white frost dry out at room temperature for about 1 hour.
- 10 Meanwhile, place wet towels in the humidity chamber and keep the lid closed all through the assay.
- 11 Prepare the blocking solution (5% normal goat serum in PBS), tetramethylrhodamine-conjugated α -BTx solution (in PBS) and FITC-conjugated antibody solutions (in blocking solution). Protect α -BTx and antibody solutions from light.

Optimal working dilutions should be determined experimentally by the investigator. Recommended dilutions are given in the materials section.

- 12 Circle tissue sections with hydrophobic marker and label slides with a pencil.
- 13 Fill one Coplin jar with 100% chilled acetone (-20°C) and three Coplin jars with PBS. Use fresh PBS for each wash.
- 14 Fix the sections with chilled acetone for 10 min at room temperature.
- 15 Wash 3×5 min with PBS at room temperature.

Do not allow sections to dry for the remaining procedure.

- 16 Drain slides and wipe dry with gauze the back and the edges of the front of the slides. Do not allow sections to dry.
- 17 Place the slide in the humidity chamber, add 100 μl of blocking solution on the section and incubate for 30 min at room temperature.
- 18 Drain slides and wipe dry with gauze. Do not wash.
- 19 Add 100 μl of tetramethylrhodamine-conjugated α -BTx solution and incubate for 1 hour at room temperature in the humidity chamber.
- 20 Repeat steps 15 and 16.
- 21 Add 100 μl of FITC-conjugated anti-mouse IgG, C3 or MAC antibody solutions (different antibodies for different sections) and incubate for 1 hour at room temperature in the humidity chamber.
- 22 Repeat steps 15 and 16.

- 23** Mount with fluorescent mounting media and coverslip. Use fluorescent mounting media with DAPI if counterstaining is desired. View the sections under a fluorescence microscope.

This method provides the colocalization of α -BTx binding sites (in red color) and IgG or complement deposits (in green color) and thus demonstrates the presence of neuromuscular junction deposits, the hallmark of EAMG and MG pathology (Fig. 4). Since the neuromuscular junction has a characteristic appearance (Fig. 4), it is sufficient to use the muscle section of a non-immunized mouse as a negative control. Non-immunized mice should only display α -BTx binding sites (in red color) and should not display any IgG or complement deposits. Nevertheless, a FITC-conjugated isotype control may be used, if desired.

SUPPORT PROTOCOL 8

DETECTION OF C3 CONCENTRATION IN MUSCLE EXTRACT

As noted in Support Protocol 7, following AChR immunization, C3 levels are elevated in the muscle tissue. This can be demonstrated quantitatively using an ELISA-based method (Poussin et al., 2002) described presented in this protocol.

Materials

- Muscle extract from EAMG and control mice (see Support Protocol 6)
 - 96-well microtiter plate (e.g. Dynatech Immulon 2; Dynatech Labs)
 - Multichannel pipette
 - Microtiter plate reader
 - Carbonate/bicarbonate buffer, pH 9.6 for ELISA (see recipe)
 - Washing buffer for ELISA (see recipe)
 - Dilution buffer for ELISA (see recipe)
 - HRP-conjugated goat anti-mouse C3 (e.g. ICN Biomedicals/Cappel)
 - Ready-to-use 3,3',5,5'-TetraMethylBenzidine (TMB) single solution chromogen/substrate (e.g. Invitrogen)
 - Stop solution (1 N hydrochloric acid)
- 1.** Dilute mouse muscle extracts from individual mice 1/10 in carbonate/bicarbonate buffer, pH 9.6.
 - 2.** Add 100 μ l of each diluted muscle extract to coat a 96-well ELISA plate.
Coat 3 wells with a diluted extract from each mouse.
Leave at least 3 wells uncoated (blank wells).
 - 3.** Incubate the samples overnight at 4°C.
 - 4.** Next day, aspirate each well and wash 3 times with 100 μ l of washing buffer per well by a multichannel pipette.
 - 5.** Add 100 μ l of HRP-conjugated goat anti-mouse C3, diluted in 1:500 in dilution buffer and incubate 90 minutes at room temperature.
 - 6.** Aspirate each well and wash 5 times with 100 μ l of washing buffer per well by a multichannel pipette.

7. Add 100 µl of TMB solution to each well and incubate for 30 min. at room temperature.
TMB and peroxide react to produce a blue byproduct (maximum absorbance at 605 nm).
8. Add 100 µl of stop solution to each well.
Addition of stop solution changes the color to yellow.
9. Determine the OD at 450 nm using a microplate reader within 30 min.
10. Calculate the average OD value of the blank wells. Subtract this average value from the OD values of wells containing muscle extracts. Express the result for each mouse sample by taking the average of the subtracted values for 3 wells containing extracts from the same mouse.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps.

Acetate buffer, pH 4.0, 0.1 M

13.6 g sodium acetate triphosphate (0.1 M final)
29.22 g NaCl (0.5 M final)
900 ml H₂O
Adjust pH to 4.0 with acetic acid
Add H₂O to 1 liter
Store up to 30 days at 4°C

Carbomylcholine buffer, 1 M

182.6 g carbomylcholine (1 M final)
1.21 g Trizma (10 mM final)
0.065 g NaN₃ (1 mM final)
1 ml Triton X-100 (0.1% final) H₂O to 1 liter
Store up to 30 days at 4°C

CAUTION: *Sodium azide (NaN₃) is poisonous; wear gloves.*

Carbonate/bicarbonate buffer, pH 9.6 for ELISA

0.795 g Na₂CO₃
1.465 g NaHCO₃
0.1 g NaN₃
H₂O to 500 ml
Adjust pH to 9.6 if necessary

CAUTION: *Sodium azide (NaN₃) is poisonous; wear gloves.*

Cholate buffer, 0.2%

2 g sodium cholate (0.2 % final)

0.65 g NaN_3 (10 mM final)
10 mM sodium phosphate buffer (see recipe) to 1 liter
Adjust pH to 7.5
Store up to 30 days at 4°C

CAUTION: *Sodium azide (NaN_3) is poisonous; wear gloves.*

Column wash buffer

1.21 g Tris base (10 mM final)
0.065 g NaN_3 (1 mM final)
1 ml Triton X-100 (0.1% final) H_2O to 1 liter
Store up to 30 days at 4°C

CAUTION: *Sodium azide (NaN_3) is poisonous; wear gloves.*

Dilution buffer for ELISA

0.5 ml Tween 20
PBS to 1 liter
Store up to 30 days at 4°C

Elution buffer

ml Triton X-100 (0.1% final)
0.065 g NaN_3 (1 mM final)
mM sodium phosphate buffer, pH 7.5 (see recipe) to 1 liter
Store up to 30 days at 4°C

CAUTION: *Sodium azide (NaN_3) is poisonous; wear gloves.*

Homogenization buffer

5.8 g NaCl (0.1 M final)
0.65 g NaN_3 (10 mM final)
2.922 g EDTA (0.01 M final)
3.804 g EGTA (0.01 M final)
1.849 g iodacetamide (0.01 M final)
0.179 g phenylmethane sulfonyl fluoride (PMSF; 1 mM final)
100 ml 10 mM sodium phosphate buffer (see recipe; 1 mM final)
Adjust pH to 7.5 using NaOH
Add H_2O to 1 liter
Store up to 30 days at 4°C

CAUTION: *Sodium azide (NaN_3) is poisonous; wear gloves.*

NaCl/Triton buffer

29.2 g NaCl (0.5 M final)
5 ml Triton X-100 (0.5% final) PBS to 1 liter
Store up to 30 days at 4°C

Sodium carbonate buffer, pH 8.3, 0.1 M

1.36 g sodium carbonate (0.01 M final)
7.35 sodium bicarbonate (0.09 M final)
950 ml H₂O
Adjust pH to 8.3 with 1 M HCl if necessary
Add H₂O to liter
Store up to 30 days at 4°C

Sodium phosphate buffer, pH 7.5, 10 mM

0.193 g NaH₂PO₄ (monobasic)
0.511 g Na₂HPO₄ (dibasic)
500 ml H₂O
Adjust pH to 7.5, if necessary
Store up to 30 days at 4°C

Sodium phosphate buffer, pH 7.5, 152 mM

14.6 g Na₂HPO₄ (dibasic; 0.102 M final)
6.9 g NaH₂PO₄ (monobasic; 0.05 M final)
0.066 g NaN₃ (1 mM final)
1 ml Triton X-100 (0.001% final)
H₂O to 1 liter
Adjust pH to 7.5, if necessary
Store up to 30 days at 4°C

CAUTION: *Sodium azide (NaN₃) is poisonous; wear gloves.*

Tris buffer, 10 mM, pH 7.5

1.21 g Tris base (10 mM final)
1 ml Triton X-100 (0.1% final)
0.065 g NaN₃ (1 mM final)
H₂O to 1 liter
Store up to 30 days at 4°C

CAUTION: *Sodium azide (NaN₃) is poisonous; wear gloves.*

Triton X-100 buffer, 0.5%

5 ml Triton X-100 (0.5% final)

0.065 g NaN_3 (1 mM final)

PBS to 1 liter

Store up to 30 days at 4°C

CAUTION: *Sodium azide (NaN_3) is poisonous; wear gloves.*

Washing buffer for ELISA

9 g NaCl

0.5 ml Tween 20

H_2O to 1 liter

Store up to 30 days at 4°C

COMMENTARY**Background information**

Murine experimental autoimmune myasthenia gravis (EAMG) mimics human MG both in its clinical and immunopathogenic aspects (see Table 2). EAMG provides an excellent model for dissecting the cellular and molecular mechanisms of autoimmune disease pathogenesis and designing specific therapies. Because the autoantigen, the acetylcholine receptor (AChR), has been characterized biochemically and molecularly, EAMG serves as a prototype for other autoimmune diseases, especially antibody-mediated autoimmune diseases.

MG is a classical autoantibody-mediated autoimmune disease. The autoantibodies against AChR destroy AChR on the postsynaptic membrane and cause the muscle weakness seen in MG (Drachman, 1994; Vincent and Rothwell, 2004) and EAMG (Christadoss, 1989; Christadoss et al., 2000). Production of antibodies to AChR is dependent on T cells in human MG and murine EAMG (Hohlfeld et al., 1984; Christadoss and Dauphinee, 1986; Christadoss et al., 2000). MG is linked to HLA/DQ β -chain polymorphism (Bell et al., 1986), and major histocompatibility complex (MHC) class II molecules play a crucial role in the development of EAMG. Either a mutation in MHC class II β -chain gene or an MHC class II gene disruption prevents development of EAMG (Christadoss et al., 1985; Kaul et al., 1994). Anti-CD4 antibody or anti-Ia antibody treatment can either prevent or induce remission of EAMG (Waldor et al., 1983; Christadoss and Dauphinee, 1986).

One of the T cell epitopes situated on the α chain of the AChR molecule, α 146-162, has been shown to be one of the pathogenic T cell epitopes involved in the development of EAMG in C57BL/6/J mice (Shenoy et al., 1993, 1994). T lymphocytes reactive to the 146-162 peptide appear to help B cells produce pathogenic anti-AChR antibodies. T cell proliferation in response to AChR and dominant AChR α -chain peptides has been documented in vitro (Shenoy et al., 1993). However, this parameter is not used to screen mice for EAMG. In both human MG and mouse EAMG, multiple T cell receptor V β genes have been utilized (Melms et al., 1993; Wu et al., 1995).

In keeping with the crucial role of T cells in EAMG pathogenesis, development of EAMG can be prevented with lymphocyte suppressing reagents such as a chemotherapeutic agent called daunomycin or interferon α (Christadoss et al., 1991; Shenoy et al., 1995; Deng et al., 1996). Further, oral tolerance to AChR can suppress the development of EAMG (Okumura

et al., 1996). Anti-AChR idiotypic antibody therapy or treatment with a monoclonal antibody to a complementary peptide for the main immunogenic region of AChR ameliorates development of EAMG (Aguis and Richman, 1986; Arafa et al., 1996).

Various cytokines (e.g. IL-1, IL-6, IL-12, IFN- γ , TNF- α) play important roles in T and B cell proliferation and differentiation and thus are remarkably involved in EAMG pathogenesis. The inhibition of these cytokines by such reagents as IL-1 receptor antagonist and recombinant human tumor necrosis factor receptor Fc protein prevent or treat established EAMG (Conti-Fine et al., 2008; Deng et al., 2002; Goluszko et al., 2002; Poussin et al., 2002; Yang et al., 2005). Moreover, in a prospective pilot trial, Etanercept (TNF inhibitor) treatment has ameliorated the muscle weakness of patients with MG (Rowin et al., 2004).

The components of the common (e.g. C3 and C5) and classical (e.g. C1q and C4) complement pathways have also crucial roles in EAMG induction. Acquired or inherited deficiency of these complement factors renders mice highly resistant to EAMG without reducing serum anti-AChR antibody levels, suggesting that destruction of the neuromuscular junction in EAMG is dependent on the activation of the classical complement pathway by anti-AChR antibodies (Tuzun et al., 2006; Tuzun et al., 2003; Tuzun et al., 2008). Treatment and prevention of EAMG by intraperitoneal administration of C1q inhibiting antibodies further support the significance of the classical complement pathway in EAMG pathogenesis (Tuzun et al., 2008).

EAMG can also be induced in rat. For rats, use 100 μ g AChR in CFA for immunization. There are two distinct episodes of clinical disease (Lennon et al., 1975). The first peak is usually acute in onset, appearing between days 7 and 12, and it is often transient, with apparent full recovery by days 11 to 15. The second peak usually begins between days 26 and 35 and involves development of progressive weakness. In the rat model one immunization is usually sufficient to induce EAMG, but in the mouse model, one to two additional immunizations of AChR in CFA are needed.

Critical Parameters and Troubleshooting

As with many other mouse models for autoimmune disease, the strain used to study EAMG is critical. Strains of H-2^b haplotype (e.g., C57BL6/J, C57BL10/J, and C57L) are susceptible to the development of EAMG, but strains of H-2^{k, p} haplotypes are relatively resistant (Christadoss, 1989).

The quality of the emulsion and the route of injection are very critical for disease induction. The aqueous AChR solution should be added to the complete Freund's adjuvant (CFA) first (water into oil). The first several transfers between the syringes should be done quickly to make a good water-in-oil emulsion, and the emulsion should be stored at 4°C to reduce the heat generated during emulsification and to make the emulsion thicker. There are two ways to check the emulsion. One way is to observe whether small air bubbles in the emulsion are fixed in position. The other way is to express a small drop of emulsion through a 25-G needle onto the surface of cold water in a beaker. The first several drops may spread out, but later drops should remain intact. Because subcutaneous injection is more immunogenic than other routes of injection, the fine needle should be properly positioned and the needle should be clearly visible beneath the skin before the emulsion is injected at each site.

Clinical signs of MG should appear 3 to 10 days after the second immunization. Approximately 10% of mice may develop signs of disease as early as 25 days after the primary immunization. If the incidence of disease is low, a third immunization with AChR

may be administered 30 days after the second immunization. EAMG of at least clinical grade 1 should be elicited.

After immunization, especially before and after the second immunization, mice should be observed carefully on a daily basis and evaluated for muscle weakness. It takes a considerable amount of time to evaluate disease. If an abscess appears at the site of immunization, anesthetize the animal and surgically drain the abscess. After the procedure, apply antibiotic to the site to prevent infection and check the animals daily for any recurrence. Consult a veterinarian if the animals show signs of infection or illness. Animals that become severely ill should be euthanized, as should all animals at the termination of the experiment.

Mice should be evaluated for muscle weakness, and the extent of disease should be determined by electromyography (EMG, the repetitive nerve stimulation test; see Support Protocol 3) and by quantifying serum anti-AChR antibody level (see Support Protocol 4) and muscle AChR loss (see Support Protocol 5). Demonstration of a change in response to the repetitive nerve stimulation test is diagnostic and specific for MG and EAMG. Although EMG can be performed easily, initially it may be necessary to collaborate or consult with an electrophysiologist to set up the assay. More sophisticated tests for muscle weakness, such as those that measure miniature end plate potential (MEPP), are usually invasive, difficult to perform, and not practical.

Measuring the serum level of antibody to muscle AChR is crucial, because that is the target of the autoimmune response in EAMG, due to cross-reactive antibody generated against *Torpedo californica* AChR. Measurement of serum antibody to *Torpedo californica* AChR is not necessary, because these antibodies are not the pathogenic antibodies.

Anticipated Results

Thirty to seventy percent of immunized animals should develop muscle weakness of at least grade 1. The decremental response to repeated nerve stimulation should be >10%. Serum levels of anti-AChR antibodies should range from 0 to 6 mM α -bungarotoxin-binding sites per liter of serum. Approximately 10% to 20% of mice immunized with AChR in CFA may not generate antibodies to mouse muscle AChR (i.e., they will be seronegative). Loss of muscle AChR should range from 20% to 70%. IgG and complement deposits are detected in all AChR-immunized mice but not in CFA-immunized or non-immunized mice.

Time Considerations

Two to three months are required to complete an experiment—enough to induce EAMG and maintain the mice with clinical disease for a month in order to measure various parameters.

To extract and purify AChR by affinity column chromatography requires 7 to 10 days. It takes 15 to 30 min to prepare an AChR/complete Freund's adjuvant emulsion, and 40 to 60 min to immunize ten mice. Electromyography for ten mice requires 60 to 90 min. Measuring serum levels of anti-AChR antibodies or AChR loss requires 2 days. Preparing mouse muscle AChR requires 1 day.

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Figure 1. Clinical evaluation of experimental autoimmune myasthenia gravis. (A) Paw grips exercise on cage top steel grids. (B) Posture of an animal with grade 1 or grade 2 weakness. (C) Posture of a normal mouse or a mouse with grade 1 to grade 2 weakness after treatment with neostigmine.

Figure 2.
Coomassie blue-stained SDS-PAGE gel showing four acetylcholine receptor polypeptides (α subunit is duplicated).

Figure 3. Evaluation of experimental autoimmune myasthenia gravis using electromyography. (A) Photograph showing the positions of electrodes for electromyography. (B) Electromyogram showing no significant decremental response to repetitive nerve stimulation in a normal mouse. (C) Electromyogram showing significant (>10%) decremental response to repetitive nerve stimulation in an EAMG mouse (compare amplitude of fourth or fifth action potential with that of the first action potential).

Figure 4.

Acetylcholine receptor (AChR) immunization induces IgG and complement deposition at the neuromuscular junctions (NMJ). At termination, proximal forelimb muscle samples are obtained from mice, snap frozen and stained with rhodamine-conjugated bungarotoxin (BTx), which binds AChR at the NMJ, as well as FITC-conjugated antibodies specific for IgG, complement factor C3 or the membrane attack complex (MAC) (photographs of representative frozen sections are shown, magnification for all, x100).

Table 1

Data from an experiment to quantify mouse muscle acetylcholine receptors.

Mouse	cpm	
	+ benzoquinonium	– benzoquinonium
Experimental	1,285	6,768
Control	1,300	11,509

Table 2Clinical and immunopathological similarities between MG and EAMG^a

Characteristic	MG	EAMG
Muscle weakness and temporary improvement with anti-cholinesterase	Yes	Yes
EMG	Decrement	Decrement
MEPP	Reduction	Reduction
Autoantigen	AChR (~90%)	AChR
Genetic predisposition (Genetic control)	MHC class II (HLA-DQ β chain polymorphism)	MHC class II I-A ^b (β chain)
Pathology	End plate AChR loss	End plate AChR loss
Cause of pathology	Anti-AChR antibody Complement activation	Anti-AChR antibody Complement activation
In vitro lymphocyte response	Class II restricted	Class II restricted
T cell epitopes	α 146-162 α 182-198	α 146-162 α 182-198
Anti-CD4 therapy	Benefit	Benefit

^a Abbreviations: AChR, acetylcholine receptor; EAMG, experimental autoimmune myasthenia gravis; EMG, electromyography; MEPP, miniature end plate potential; MG, myasthenia gravis; MHC, major histocompatibility complex.