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Standardization of the experimental autoimmune myasthenia gravis (EAMG) model by immunization of rats with *Torpedo californica* acetylcholine receptors — Recommendations for methods and experimental designs

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ABSTRACT

Myasthenia gravis (MG) with antibodies against the acetylcholine receptor (AChR) is characterized by a chronic, fatigable weakness of voluntary muscles. The production of autoantibodies involves the dysregulation of T cells which provide the environment for the development of autoreactive B cells. The symptoms are caused by destruction of the postsynaptic membrane and degradation of the AChR by IgG autoantibodies, predominantly of the G1 and G3 subclasses. Active immunization of animals with AChR from mammalian muscles, AChR from *Torpedo* or *Electrophorus* electric organs, and recombinant or synthetic AChR fragments generates a chronic model of MG, termed experimental autoimmune myasthenia gravis (EAMG). This model covers cellular mechanisms involved in the immune response against the AChR, e.g. antigen presentation, T cell-help and regulation, B cell selection and differentiation into plasma cells. Our aim is to define standard operation procedures and recommendations for the rat EAMG model using purified AChR from the *Torpedo californica* electric organ, in order to facilitate more rapid translation of preclinical proof of concept or efficacy studies into clinical trials and, ultimately, clinical practice.

Introduction

The serendipitous observation that immunization of rabbits with purified acetylcholine receptors (AChRs) led to MG-like symptoms has provided the basis for understanding the cause of myasthenia gravis (MG) and the mechanisms involved in its pathology (Patrick and Lindstrom, 1973). In this seminal work, experimental autoimmune MG (EAMG) was induced in rabbits by immunization with AChR from the electric organ of electric eels (*Electrophorus electricus*) in complete Freund's adjuvant (Patrick and Lindstrom, 1973). The immunization resulted in the production of antibodies to the *Electrophorus* AChR, binding of cross-reactive antibodies to the muscle AChR, and the subsequent paralysis and eventual death of the animals. EAMG has contributed to pre-clinical assessment and therapeutic discovery. Many variations of this animal model have been used since the 1970s. These later experiments included different amounts and sources of AChR, recipient species (see Table 1), sites for antigen injection (foot pads, base of the tail, hip and shoulder regions), and adjuvants [e.g. Titermax, incomplete Freund's adjuvant (IFA, based on mineral oil/water), complete Freund's adjuvant (CFA, IFA with additional heat killed *Mycobacterium tuberculosis*) or CFA with additional *Bordetella pertussis* toxin]. In each case, the animals mount an active immune response against the injected antigen; however only a small subset of the produced antibodies (~1%) cross-reacts with the animals' own muscle AChR (see Fig. 1) and this subset is responsible for the disease. Typically, muscle weakness occurs within 30–50 days after immunization. The EAMG model has been used extensively to analyze various aspects of MG pathology, and also experimental therapies to ameliorate MG (see Table 2). The chosen experimental parameters and procedures affect the disease time course, incidence and severity. EAMG scores can be increased using a susceptible strain, young animals, high amounts of AChR, a potent adjuvant and multiple injection sites for

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Table 1
AChR sources and species for EAMG induction.

Source of AChR	Recipient animal	Reference
Torpedo californica (electric organ)	Rat (*Rattus norvegicus*)	Lennon et al. (1978)
	Mouse (*Mus musculus*)	Berman and Patrick (1980)
	Pig (*Sus scrofa domesticus*)	De Haes et al. (2003)
	Rhesus monkey (*Macaca mulatta*)	Tarrab-Hazdai et al. (1975)
	Frog (*Rana ripiens*)	Nastuk et al. (1979)
	Guinea pig (*Cavia porcellus*)	Lennon et al. (1975)
Torpedo marmorata (electric organ)	Rat (*Rattus norvegicus*)	Elfman et al. (1983)
	Rabbit (*Oryctolagus cuniculus*)	Barkas and Simpson (1982)
Electrophorus electricus (electric organ)	Rabbit (*Oryctolagus cuniculus*)	Patrick and Lindstrom (1973)
	Rat (*Rattus norvegicus*)	Lennon et al. (1975)
	Guinea pig (*Cavia porcellus*)	Lennon et al. (1975)
Rat AChR (syngeneic muscle)	Rat (*Rattus norvegicus*)	Lindstrom et al. (1976)
Cat (denervated muscle)	Rabbit (*Oryctolagus cuniculus*)	Dolly et al. (1983)
Chicken (denervated muscle)	Rabbit (*Oryctolagus cuniculus*)	Dolly et al. (1983)
Human AChR (denervated muscle)	Rat (*Rattus norvegicus*)	Lennon et al. (1991)
1–210 sequence of the human AChR-α1 subunit (*Escherichia coli*)	Rat (*Rattus norvegicus*)	Lennon et al. (1991)
97–116 sequence of the rat AChR-α1 subunit (synthetic)	Lewis Rat (*Rattus norvegicus*)	Baggi et al. (2004)
Chimeric Aplysia ACh-binding protein (AChBP)/human muscle AChR	Lewis Rat (*Rattus norvegicus*)	Luo and Lindstrom (2012)

immunization. However, the disadvantages of a severe EAMG model are increased animal suffering, animal deaths, and an unrealistically stringent assessment of a therapeutic intervention. A mild EAMG model would be ineffective to demonstrate a beneficial effect of an experimental therapy, since little room exists for improvement of neuromuscular transmission. Below, the influence of various experimental parameters on the EAMG model is summarized and recommendations are offered for obtaining a robust and well-balanced EAMG model.

Animal care, safety and regulatory aspects

The use of the EAMG model is limited by ethical, environmental and safety regulations. The myasthenic muscle weakness itself constitutes an intrinsic discomfort and therefore the use of the EAMG model implies some degree of animal suffering that is unavoidable. Additional discomfort arises from stress while handling, anesthesia and injections. These aspects must be balanced against the expected benefit of new insights into the function of the neuromuscular junction, disease pathology or treatment efficacy of experimental drugs. We recommend that researchers planning to use the EAMG model seek advice from groups that have expertise in using it in order to reduce animal numbers and suffering to a minimum. Such an external review can be used for the application to institutional ethical boards which is in most countries required by law and also a prerequisite for publication in most journals. To minimize stress, the animals must be handled by experienced personnel. Lower stress was observed in rats that were caged with enrichment, such as, nestling, variety of objects and tunnels (Moncek et al., 2004).

Many reagents that interact with the proteins of the neuromuscular junction, and in particular with the AChR or the acetylcholine esterase (AChE) are highly toxic; e.g. alpha bungarotoxin, alpha cobratoxin, benzoquinonium, curare, sarin and neostigmine. Additionally, alpha bungarotoxin is frequently used in a ^{125}I radiolabeled form and any accidental physical contact might result in accumulation of ^{125}I in the thyroid gland. Careful planning of experiments, personal protection and working in dedicated laboratories reduce the risk to an acceptable level. Some of the reagents that are needed for realizing the EAMG model or for analyzing outcome measures involve wild living animals. These include the alpha toxin from the Indian cobra (*Naja naja*), the alpha bungarotoxin of the Taiwan banded krait (*Bungarus multicinctus*) and the AChR of the pacific electric ray (*Torpedo californica*). Import and export of these species, their tissues and proteins are in many countries restricted by national laws and/or need special permits of authorities. In many cases, however, it is possible to obtain access to the abovementioned purified proteins through collaborating research groups.

General animal care and housing

All care given to animals should be documented. To limit the stress and discomfort of the animals the following procedures are recommended. The number of personnel that handle the animals throughout the experiment should be kept to a minimum. A maximum of 2 researchers should be involved in immunizing the rats and assessing the clinical feature of EAMG. An inverted day–night cycle is advisable in order to perform the experimental procedures during the awake phase of the animals and avoid sleep-deprivation. The time of day that therapeutic drugs are administered and clinical scoring is performed should be kept constant. 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. We recommend social housing of young female Lewis rats (weight <300 g) in the cages with a floor area of ≥ 800 cm^2 and a height of >17.5 cm, with 3 animals per cage (National Research Council (U.S.) Committee for the Update of the Guide for the Care and Use of Laboratory Animals. et al., 2011). If any animal becomes clinically weak (grade 2 or grade 3, see section 'Clinical scoring' below) all the cages should be supplied with water gel (e.g. HydroGel® or AQUA-JEL®) and soft food should be placed on the bottom of the cage. The same type of food should be administered to control animals and EAMG animals. Otherwise, the diet type should be kept constant throughout the study. Reporting the food vendor in published studies is recommended. Overgrown teeth can impair eating, ultimately causing starvation, and thus should be trimmed. Animals should be housed in specific pathogen free conditions. A health report including tested pathogens, and analytical methodology should be available (Kunstyr and Nicklas, 2000). When by accident some infection does occur, but disease symptoms are mild (e.g. a rotavirus infection resulting in diarrhea or staphylococcus infection at the immunization site), we suggest that the experiment can be continued under the following provisions: Animals should be treated as necessary and all infections, treatments and symptoms of each animal should be clearly documented in any resulting publication. If a suitable alternative exists, anti-inflammatory agents should be avoided due to potential obstruction with EAMG development (see also immunization section below).

Source and amount of AChR

The natural abundance of AChR in the electric organs of different fish species, such as *E. electricus*, *Torpedo californica* or *Torpedo marmorata*, confers an important practical advantage for generating sufficient amounts of purified AChR for the EAMG model. Other sources of AChR have been used successfully in various rat EAMG models (see Table 1),