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Specific removal of autoantibodies by extracorporeal immunoadsorption ameliorates experimental autoimmune myasthenia gravis

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ABSTRACT

Myasthenia gravis (MG) is caused by autoantibodies, the majority of which target the muscle acetylcholine receptor (AChR). Plasmapheresis and IgG-immunoadsorption are useful therapy options, but are highly non-specific. Antigen-specific immunoadsorption would remove only the pathogenic autoantibodies, reducing the possibility of side effects while maximizing the benefit. We have extensively characterized such adsorbents, but *in vivo* studies are missing. We used rats with experimental autoimmune MG to perform antigen-specific immunoadsorptions over three weeks, regularly monitoring symptoms and autoantibody titers. Immunoadsorption was effective, resulting in a marked autoantibody titer decrease while the immunoadsorbed, but not the mock-treated, animals showed a dramatic symptom improvement. Overall, the procedure was found to be efficient, suggesting the subsequent initiation of clinical trials.

1. Introduction

Myasthenia gravis (MG) is an antibody mediated autoimmune disease targeting the neuromuscular junction (NMJ) of skeletal muscles. The pathophysiology of MG has been described in detail, making it a prototype autoimmune disease (Verschuuren et al., 2013). In the majority of MG patients (~85%) the autoantibodies target the muscle nicotinic acetylcholine receptor (AChR) (Meriggioli and Sanders, 2009).

The AChR is composed of five homologous subunits, with the stoichiometry $(\alpha1)_2\beta1\gamma\delta$ in fetal (or denervated) and $(\alpha1)_2\beta1\varepsilon\delta$ in adult muscles, which form an ion pore (Changeux, 2012; Tsetlin et al., 2011). Each subunit has an N-terminal extracellular domain (ECD), 4 transmembrane domains (M1-M4), an intracellular domain (ICD) between the M3 and M4, and a small extracellular tail after M4 (Unwin, 2005). Most of the antigenic epitopes involved in MG are located on the extracellular domains (ECDs) of the AChR subunits, while more than half of the autoantibodies are directed against the so-called main immunogenic region (MIR), a group of overlapping epitopes located on the ECD of the α1 subunit, whose central core lies between amino acids 67–76 (Luo et al., 2009; Tzartos et al., 1998; Tzartos and Lindstrom, 1980). Antibodies against intracellular or transmembrane epitopes are probably not clinically important since these epitopes are not accessible in undamaged muscle membranes. The pathogenicity of AChR antibodies has been demonstrated by the causation of passive transfer experimental MG when they are injected into rats, as well as by the clinical improvement of patients following plasmapheresis (Gomez et al., 2010; Lindstrom et al., 1976b; Newsom-Davis et al., 1979).

The most common treatment approaches for MG include cholinesterase inhibitors, immunosuppressives, thymectomy, intravenous immunoglobulin (IVIG) and plasmapheresis (Sanders and Evoli, 2010). However, these are largely non-specific and thus may be accompanied by a variety of side effects. Plasmapheresis is an attractive solution for patients who are refractory to other therapies or when an immediate effect is needed (*e.g.* during myasthenic crises or for pre-operative treatment) as it provides a fast, albeit temporary, improvement of MG symptoms (Gilhus et al., 2011). It consists of removing part of the patient's plasma, thus reducing the autoantibody titer. Unfortunately, this results in the removal of all other plasma components (fluids, plasma proteins, etc), the replacement of which increases the risk for allergic and transfusion-related adverse effects.

IgG-immunoadsorption poses as an improvement to plasmapheresis, since it involves the removal of only the immunoglobulins from the patient's circulation. Specifically, the plasma is passed through a suitable matrix which only binds the immunoglobulins, allowing the return of the rest of the plasma to the patient. Matrixes commonly used

Abbreviations: MG, myasthenia gravis; EAMG, experimental autoimmune MG; AChR, nicotinic acetylcholine receptor; ECD, extracellular domain; MIR, main immunogenic region

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are sheep anti-human IgG or protein A immobilized onto sepharose (Berta et al., 1994; Matic et al., 2001; Ptak, 2004). This results in a significantly reduced need for replacement fluids, but still removes all immunoglobulins indiscriminately, and thus costly replacements are usually administered.

An attractive alternative would be the selective removal of the pathogenic autoantibodies (antigen-specific immunoadsorption). This could be accomplished by using the target autoantigens immobilized onto a matrix. To this end, the use of the AChR ECDs would be advantageous, as they carry the pathogenically relevant epitopes, while being expressed as soluble proteins. In the past we have shown that sepharose-immobilized human AChR subunit ECDs expressed in yeast or bacteria could successfully immunoadsorb AChR antibodies from MG patient sera *in vitro* (Kostelidou et al., 2007; Lagoumintzis et al., 2014; Psaridi-Linardaki et al., 2005; Zisimopoulou et al., 2008). The generation of ECD mutants, which have had their cys-loop exchanged for that of the homologous acetylcholine binding protein (AChBP) from the snail *Lymnaea stagnalis*, led to the expression yields and purity levels required for large-scale use (Lazaridis et al., 2014). These proteins were also shown to retain the antibody binding efficiencies of their wild type counterparts, and showed very satisfactory characteristics *in vitro*, supporting their use as specific AChR antibody adsorbents (Lazaridis et al., 2015).

In this study, we proceeded to address the *in vivo* efficacy of the novel aphaeresis method. To this end we used an experimental autoimmune MG (EAMG) model, induced by immunization of rats with the human AChR ECDs, recently developed by our group (Lazaridis et al., 2017). We found that extracorporeal immunoadsorption in symptomatic rats using sepharose-ECD adsorbents, resulted not only in the marked reduction of the AChR antibody titers, but also importantly, in the fast improvement of their clinical symptoms. Several immunoadsorption sessions were performed on the same animals, without any adverse effects supporting the safety of the procedure. Overall, our findings strongly support the therapeutic potential of the AChR ECDs as specific and efficient adsorbents for the development of an aphaeresis approach for the treatment of MG patients.

2. Materials and methods

2.1. Experimental animals

6–7 week old female Lewis rats were obtained from the animal breeding unit of the Department of Animal Models for Biomedical Research of the Hellenic Pasteur Institute. They were maintained in the rodent unit of the Department. All experiments described were approved by the Institute Ethics Board and conducted according to the regulations and guidelines for animal care (EU Directive 2010/63/EU for animal experiments).

2.2. Induction and clinical evaluation of EAMG

Before immunization the rats were anaesthetized with 2% isoflurane supplemented with oxygen. They were immunized once in the hind footpads with 160 μg of human α and β ECD mix, or PBS for controls, in CFA (Becton, Dickinson and Company, New Jersey, USA) supplemented with 2 mg/ml inactivated *Mycobacterium tuberculosis* H37RA (Becton, Dickinson and Company), in a final volume of 250 μl.

The rats were monitored once a week for the first 4 weeks after immunization and every other day thereafter. After treatment initiation the animals were monitored daily. Each monitoring session consisted of weighing of the rats and observation on a flat bench before and after exercise (repetitive grasping of a rack for 30 s) for clinical scoring. EAMG clinical scores were evaluated as follows: 0: normal strength, no symptoms; 1: symptoms observed only after exercise due to fatigue (decreased activity, tremor, lowered head, hunched posture); 2: the previous symptoms present before exercise; 3: very severe symptoms at rest, hind limb paralysis, moribund; 4: death. For animals that had to be euthanized, the final values obtained (weight and EAMG score) were used for the remaining time points during the analyses described in Section 3.3 and Fig. 4.

2.3. Construction of the adsorbents

Mutants of the human AChR ECDs with their cys-loop exchanged for that of the acetylcholine binding protein from *Lymnaea stagnalis*, were used as adsorbents and for all rat immunizations. The ECDs were expressed in the yeast *Pichia pastoris* and purified by metal affinity chromatography followed by size exclusion chromatography as previously described (Lazaridis et al., 2014). The ECDs were immobilized onto CNBr-activated sepharose beads (GE Healthcare), according to the manufacturer's instructions, using 5 mg of each α and β ECDs per 1 ml sepharose. Sepharose beads loaded with 10 mg BSA per 1 ml sepharose were used as controls.

For the *in vitro* small scale immunoadsorptions, 10 μl sepharose beads containing 1 μg protein (ECD or BSA) were mixed with a serum volume corresponding to 20 fmol AChR antibodies and incubated for 2 h at 4 °C. Following, the supernatant was assayed in duplicate for the presence of unbound autoantibodies using radioimmunoprecipitation assay (RIPA) as described below. The percentage of immunoadsorption was calculated as the percent reduction of autoantibodies in the supernatant of the ECD-loaded beads compared to the BSA-loaded beads.

For the construction of the columns used in the animal experiments, 1 ml of sepharose-ECD or sepharose-BSA was immobilized into a 5-ml syringe, by means of nylon net filters with 40 μm pore diameter (Millipore); the filter pores allowed the passage of blood cells but not of the sepharose beads. The columns were stored in citrate buffer (11 mM $Na_3C_6H_5O_7$, 40 mM $C_2H_3NaO_2$, 83 mM NaCl, 8 mM Na_2HPO_4 and 2 mM KH_2PO_4, pH 7) at 4 °C. They were washed with 50 ml saline before being used.

2.4. Femoral vein catheterization and immunoadsorption

The rats were anaesthetized with a mixture of ketamine (9 mg/100 g) and xylazine (1.25 mg/100 g). The femoral vein of the right hind limb was exposed and tied at the distal end using a 5.0 silk thread. A small incision was made and a silicone catheter (diameter 0.94 mm) (Scientific Commodities, USA) filled with heparinized saline was gently inserted 6 cm from the incision site. The catheter was secured in place with two additional sutures. The free end of the catheter was passed through a subcutaneous tunnel towards the back of the rat and exerted through an incision at the level of the shoulder blades. The catheter was secured in place, leaving a short length (~1 cm) protruding from the back and capped with a 23G plug. Routine flushing with heparinized saline preserved the catheter's patency for several weeks after the procedure.

For the immunoadsorption procedure, the rats were anaesthetized with isoflurane, and the column-syringe containing the sepharose beads was connected to the silicone catheter. Using the syringe plunger, blood was drawn at a speed of ~3 ml/min into the syringe up to a total volume of 1.5 ml and then immediately returned to the rat's circulation, each time passing through the beads. This was repeated up to 10 times for every immunoadsorption session. Small blood samples (100 μl) were taken before and 15 min after the completion of the session, the serum was isolated and stored at −20 °C. In some cases smaller samples (50 μl) were taken during the session directly from the column to test for red blood cell damage.

The rats received three immunoadsorptions per week over a period of three weeks (*i.e.* a total of 9 treatments unless euthanized sooner), at the end of which all surviving animals were euthanized, and final serum and muscle samples were collected.

The day the treatments were initiated for each rat is considered Day 0 for data synchronization; this is also the reference point for their body