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Scale up and safety parameters of antigen specific immunoadsorption of human anti-acetylcholine receptor antibodies

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ABSTRACT

Myasthenia gravis is an autoimmune disease usually caused by autoantibodies against the muscle nicotinic acetylcholine receptor (nAChR). Current treatments are not specific, and thus often cause side effects. Here, we elaborate on our previous findings on antigen specific immunoadsorption towards scaling up the method as well as testing whole blood apheresis. The average percent of plasma or whole blood immunoadsorption was up to 79.5% ± 2.9. Moreover, neither pyrogens were co-administered nor did complement activation occur after immunoadsorption. Thus, antigen-specific apheresis of anti-AChR autoantibodies seems a safe and effective treatment for myasthenia gravis that can be scaled up for clinical testing.

1. Introduction

Myasthenia gravis (MG) is an autoimmune disease usually characterized by a reduced number of functional nicotinic acetylcholine receptors (nAChR), located in the postsynaptic voluntary muscle endplate membrane. The muscle AChR, a five-subunit transmembrane ligand-gated cation channel with the stoichiometry $\alpha 2\beta\gamma\delta$ at immature and denervated or damaged endplate, and $\alpha 2\beta\varepsilon\delta$ at mature normal endplate, is the target for the pathogenic anti-AChR autoantibodies (auto-Abs) in MG, whereas auto-Abs against functional proteins different than AChR may be present in myasthenic patients with no detectable circulating AChR auto-Abs (Hoch et al., 2001; Zhang et al., 2012). Each AChR subunit consists of an N-terminal extracellular domain (ECD) containing the characteristic 13-residue long Cys-loop of the ligand-gated ion channel superfamily, four membrane spanning α-helices, a large cytoplasmic loop, and a small extracellular C-terminal tail (Karlin, 2002). All the antigenic epitopes are located on the ECDs of the AChR while more than half of the auto-Abs 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 (Tzartos et al., 1998).

MG is clinically characterized by fluctuating weakness and fatigue. Although both T- and B-lymphocytes are involved in the autoimmune response to AChR, the specific effectors' arm of the immune response comprises solely of AChR auto-Abs that are highly disease specific and are detected in approximately 85% of patients with generalized MG (Dalakas, 2006).

At present, the first-line symptomatic treatment for MG is the use of anticholinesterase drugs. Immunomodulatory treatment, such as thymectomy, is used for early-onset and thymoma MG, whereas immunosuppressants, such as glucocorticoids, are used for almost all patients with MG (McDaneld et al., 2010). Plasmapheresis is mainly used as an efficient method in patients with severe MG symptoms or in myasthenic crisis, whereas administration of intravenous Ig (IVIg) is used in similar circumstances to plasmapheresis, and although it has only minor side-effects, it is also non-specific.

Plasmapheresis is a process by which a major part of the plasma of the patient, including components believed to cause or aggravate the disease, is removed from the whole blood. The cellular blood components are then combined with replacement plasma components or an inert substitute and returned to the patient. Although plasmapheresis seems an efficient therapeutic option for MG, its high cost, due to the need to replace serum proteins, and especially the concomitant removal of non pathogenic/protective Abs and/or other important molecules, are serious drawbacks (Yeh and Chiu, 2000).

Abbreviations: Ab, antibody; AChR, acetylcholine receptor; BSA, bovine serum albumin; a-Btx, α-bungarotoxin; CNBr, cyanogen bromide; ECD, extracellular domain; i.v, intravenous; IVIg, intravenous immunoglobulin; IPT, in vitro pyrogen test; LAL, limulus amoebocyte lysate; LPS, lipopolysaccharide; MG, myasthenia gravis; NHS, normal human serum; RIPA, radioimmunoprecipitation assay.

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Due to the above disadvantages, there is a demand for the development of MG-specific therapeutic approaches. These approaches are expected to offer several clinical advantages, including the avoidance of the concomitant removal of non pathogenic/protective Abs and/or other important molecules, and the absence of the need for replacement fluids, therefore reducing their side effects (e.g., allergy, transmission of infections). One such approach which we are trying to develop is the selective extracorporeal removal of only the pathogenic agents from the patients' plasma (Lagoumintzis et al., 2010). The development of an antigen-specific approach for the selective removal of anti-AChR auto-Abs from MG patients' sera has been previously attempted as an alternative to plasmapheresis by a few groups using mainly three approaches. Two of these consisted of the use of columns bearing a) a peptide corresponding to amino acids 183–200 of the *Torpedo* or human AChR α-subunit (Takamori and Maruta, 2001) or b) the ECD of the *Torpedo* AChR α-subunit expressed in *Escherichia coli* as a fusion protein with the maltose binding protein immobilized on amylase-resin (Guo et al., 2005). However, the majority of anti-AChR Abs are directed against several epitopes other than the α183–200 peptide, whereas, in the vast majority of MG patients, few auto-Abs bind to *Torpedo* AChR. We are developing a third approach, the antigen-specific Ab-apheresis, using recombinant ECDs of human muscle AChR subunits immobilized on CNBr-activated Sepharose beads as specific and efficient immunoadsorbents (Zisimopoulou et al., 2008a, 2008b).

So far, we have successfully used the yeast (*Pichia pastoris*) and the bacterial (*E. coli*) expression systems to produce individual recombinant AChR ECDs, which, when immobilized on Sepharose beads, can adsorb a high percentage of MG auto-Abs. In addition, the combination of more than one ECD on a single immunoadsorbent led to increased depletion of anti-AChR Abs, often in an additive manner, and, in some cases, to near-complete clearance (Zisimopoulou et al., 2008a).

In this paper, we extend our previous findings with *E. coli*-expressed ECDs, and present solid experimental evidence for the successful scaling-up of the antigen-specific Ab-apheresis method towards its safe clinical application. In this context, aspects such as column capacity, immunoadsortpion speed, complement activation and pyrogen contamination, or clinical parameters such as whole blood immunoadsorption, are delineated in order to set the path for a more specific, safe and efficient MG therapy.

2. Materials & methods

2.1. Human muscle nAChR ECD constructs

The cDNA constructs encoding the entire N-terminal extracellular segment of the human muscle nAChR subunits, namely α (1–222), β (1–219), γ (1–225) and ε (1–220), were produced by amplification of the corresponding sequences of the full-length subunit cDNA using PCR. The detailed cloning, expression and purification protocols of the recombinant *E. coli*-ECDs used in this study have been previously described (Zisimopoulou et al., 2008a).

2.2. Immobilization of ECDs on CNBr-Sepharose beads

Immobilization of recombinant ECDs on Sepharose beads was performed as previously described (Zisimopoulou et al., 2008a), with minor modifications in terms of scaling-up the protocol (where needed). In brief, 5 mg of each ECD were immobilized separately on 1.5 g of CNBr-activated Sepharose 4B (Pharmacia-Biotech). The protein in denaturing buffer (8 M urea, pH 8.3, containing 0.15 M NaCl) was incubated with CNBr-activated Sepharose for 1 h at room temperature; then any remaining active groups on the Sepharose were blocked by incubation for 2 h at room temperature in 0.1 M Tris–HCl buffer pH 8.0, and the beads were washed with at least three cycles of 0.1 M acetate buffer, pH 4.0, containing 0.5 M NaCl, followed by 0.1 M Tris–HCl buffer, pH 8.0, containing 0.5 M NaCl. The Sepharose-protein conjugate was stored in 8 M urea, pH 7.4, containing 0.05% sodium azide, until further use. In parallel, 5 mg of BSA was immobilized on 1.5 g of CNBr-activated Sepharose under the same conditions as a control. For the immunoadsorption experiments, columns were extensively washed with PBS prior to use, in order to dilute the chaotropic agent (i.e. urea).

2.3. MG plasma — Ab titer and anti-AChR subunit specificity

Plasma samples from 4 MG patients (MG1–MG4) of moderate to high titer were collected during therapeutic plasmapheresis procedure at the Department of Clinical Therapeutics, Renal Unit, Alexandra Hospital, Athens, Greece. The plasma donors were selected based on previous small scale experiments with MG sera, so as they have relatively high titers and large percentages of their anti-AChR antibodies bind to the AChR ECDs. In addition, normal human plasma (NHP) from apparently healthy individuals was obtained from the Blood Transfusion Centre, University Hospital, Rio, Patras, Greece. Written consensus was taken from all donors participating in this study. All patients' samples were tested for Ab binding to human muscle AChR by radioimmunoprecipitation assay (RIPA) according to the manufacturer's instructions (see below). Anti-AChR subunit specificity of MG sera was determined by small scale immunoadsorption of anti-AChR auto-Abs by immobilized ECDs as previously described (Zisimopoulou et al., 2008a). In brief, 120 μl of the Sepharose-ECD suspension (containing 10 μg of *E. coli*-expressed ECD) or Sepharose-BSA suspension was incubated for 2 h at 4 °C with 40 μl of PBS, 0.2% BSA containing 20 fmol of anti-AChR Abs, followed by centrifugation. The supernatants from the immunoadsorption assay containing unbound anti-AChR Abs were tested by RIPA.

2.4. Scaling up the immunoadsorption of anti-AChR auto-Abs

Immunoadsorption experiments were performed as previously described (Zisimopoulou et al., 2008a), with minor modifications in terms of scaling-up the volume of the Sepharose column and the concentration of the ECDs. Briefly, MG plasma sample was passed through a mixture of all the 5 Sepharose-ECDs. BSA-Sepharose beads were used in parallel under identical conditions as a negative control. The reduction in the amount of free Abs represents the fraction of auto-Abs in the treated plasma reactive with the combined-ECDs used. In the present experiments, a 25 ml column containing 5 mg of each of the *E. coli*-expressed ECDs (i.e. 25 mg of all five ECDs) was used to imunoadsorb ~1.5 l of MG plasma. Sample flow rates were tested at ~4–12 ml/min without peristaltic pump. Results are expressed as % of immunoadsorption.

2.5. Radioimmunoprecipitation assay

MG plasma samples used in this study or fractions collected from the immunoadsorption containing any unbound anti-AChR Abs were tested using a commercial diagnostic AChR auto-Ab RIPA kit according to the manufacturer's instructions (RSR Ltd, UK). Briefly, up to 5 μl plasma sample was incubated for 2 h at room temperature with 25 μl (~35,000 cpm) of ^{125}I-AChR solution, and the ^{125}I-AChR-Ab complexes were precipitated by incubation for 2 h at 4 °C with 25 μl of anti-human IgG sera. The precipitated radioactivity was measured in a γ-counter (2470 WIZARD^2 TM). The percentage immunoadsorption was estimated using the equation $100 \times \{[\Delta cpm\ BSA] - [\Delta cpm\ ECD]\} / [\Delta cpm\ BSA]$, where Δcpm is the cpm of I^{125}-α-Btx-labeled AChR precipitated by an MG serum minus that precipitated by a control normal human serum (NHS), and Δcpm BSA and Δcpm ECD are the Δcpm for samples preincubated with immobilized BSA or ECD, respectively.