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Specific adsorbents for myasthenia gravis autoantibodies using mutants of the muscle nicotinic acetylcholine receptor extracellular domains

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ABSTRACT

Myasthenia gravis (MG) is usually caused by antibodies against the muscle acetylcholine receptor (AChR). Plasmapheresis and immunoadsorption are often used to treat non-responsive patients. Antigen-specific immunoadsorption would remove only the pathogenic autoantibodies reducing side-effects. We expressed AChR extracellular domain mutants for use as specific adsorbents, and characterized them. Antigenicity and capacity for autoantibody binding were improved compared to the wild-type proteins. Adsorption appeared to be fast, as high plasma flow-rates could be applied. The bound autoantibodies were eluted repeatedly, allowing column reuse, without compromise in efficiency. Overall, the adsorbents were specific, efficient and suitable for use in therapy.

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 $(\alpha1)_2\beta1\gamma\delta$ for fetal (or denervated) and $(\alpha1)_2\beta1\varepsilon\delta$ for adult muscles (Tsetlin et al., 2011; Changeux, 2012). 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 of the AChR subunits (ECDs), while more than half of the autoantibodies are directed against the so-called main immunogenic region (MIR) (Tzartos and Lindstrom, 1980; Luo et al., 2009). The pathogenic ability of AChR antibodies has been demonstrated by the development of experimental MG when injected into rats, as well as by the clinical improvement of patients following plasmapheresis (Lindstrom et al., 1976; Newsom-Davis et al., 1979; Gomez et al., 2010).

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, 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. However, this results in the removal of all other plasma components, the replacement of which increases the risk for allergic and transfusion-related adverse effects.

Immunoadsorption poses as an improvement to plasmapheresis, as it involves the removal only of the immunoglobulins from the patient's circulation. Specifically, the plasma is passed through a suitable matrix which binds only the immunoglobulins, allowing the return of the rest of the plasma to the patient. Matrixes commonly used 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 immunoglobulins are removed indiscriminately, and costly replacements are usually administered.

An attractive alternative would be the selective removal of the pathogenic autoantibodies specifically (antigen-specific immunoadsorption). To this end, the use of the AChR ECDs as adsorbents would be advantageous, as they carry the pathogenically relevant epitopes. In the past

Abbreviations: MG, myasthenia gravis; AChR, nicotinic acetylcholine receptor; ECD, extracellular domain; MIR, main immunogenic region; AChBP, acetylcholine binding protein; α-Btx, α-bungarotoxin.

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we have shown that Sepharose-immobilized human AChR ECDs expressed in yeast or bacteria could successfully immunoadsorb AChR antibodies from MG patient sera (Lagoumintzis et al., 2014). The generation of ECD mutants expressed in yeast was required in order to achieve the expression yield and purity levels required for large-scale use (Lazaridis et al., 2014). In the same study, the construction of ECD concatamers was also described, which could have advantages over single ECDs as adsorbents (e.g. formation of inter-subunit epitopes).

Herein, we proceeded to characterize the properties of the mutant ECDs and the concatamers as adsorbents, such as their binding efficiency, capacity for autoantibody binding, speed of adsorption, recyclability and stability, in order to evaluate their therapeutic potential. We show the adsorbents to have improved binding efficiencies and capacities, and be able to maintain these features at high flow rates. Overall, our findings strongly support the feasibility of using these proteins as specific and efficient adsorbents for the development of an aphaeresis method for the treatment of MG.

2. Materials and methods

2.1. Serum and plasma samples

All serum samples are taken from our MG serum bank at the Hellenic Pasteur Institute. Sera 1–24 were selected based on their seropositivity for AChR and on their subunit specificity in order to compare the wild type and mutant AChR ECDs. Sera 25–58 were randomly selected based only on their seropositivity for AChR antibodies and were used for screening the adsorbents and the determination of efficiency. Sera MuSK1 and MuSK2 were randomly selected among the MuSK antibody positive sera of the serum bank, and used for the determination of specificity. Patients' plasmas, used for large scale experiments, were acquired from patients undergoing plasmapheresis at the Alexandra Hospital in Athens, with informed consent. Only plasmas seropositive for AChR antibodies were used; no other information was used as inclusion criterion for the serum or plasma samples.

2.2. Construction of the adsorbents

Mutants of the AChR ECDs with their cys-loop replaced with that of the acetylcholine binding protein from *Lymnaea stagnalis*, as well as mutant ECDs linked in tandem to form multimeric concatamers (α1–β1 and β1–α1 dimers or β1–δ–α1–γ–α1 and β1–δ–α1–ε–α1 pentamers) were used as adsorbents. All the adsorbents were expressed in the yeast *Pichia pastoris*, secreted as soluble proteins and purified by metal affinity chromatography followed by size exclusion chromatography as previously described (Lazaridis et al., 2014). The monomeric and concatameric ECDs were immobilized onto CNBr-activated Sepharose beads (GE Healthcare), according to the manufacturer's instructions, using 2 mg ECD per 1 g Sepharose, while Sepharose beads loaded with BSA were used as controls. Binding took place for 16 h at 4 °C in 0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.3, and then any unoccupied binding sites were blocked with 1 M ethanolamine in 0.1 M $NaHCO_3$, pH 8.3. The beads were stored in PBS, 0.05% sodium azide. For the comparison of the concatamers with the mixture of individual ECDs, the total amount of adsorbent was kept equal, maintaining the relative ratio of the different subunits.

2.3. Determination of immunoadsorption efficiency

Sepharose beads containing 1 μg protein (ECD or BSA) were mixed with a serum volume corresponding to 20 fmol anti-AChR antibodies. The total volume was made up to 160 μl with PBS and incubated for 2 h at 4 °C. Following, 40 μl of the supernatant was assayed in duplicate for the presence of unbound autoantibodies using a radio immunoprecipitation (RIPA) kit (RSR Ltd., UK). 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.

2.4. Determination of adsorbent capacity for autoantibodies

To calculate the maximum amount of antibodies able to bind to a given quantity of adsorbent, Sepharose beads containing 0.025 mg of ECD adsorbent were packed into a glass column (Biorad), and increasing volumes of 3 plasmas of known autoantibody titer (titers: 100 nM, 618 nM and 3200 nM) were passed through at a rate of 0.35 ml/min. The flowthrough fractions were tested for anti-AChR by RIPA. The total amount of antibodies bound (expressed as nmol autoantibody bound/mg adsorbent) was calculated based on the total plasma volume required to pass through the column before the beads were saturated with autoantibodies, and taking into account the efficiency of adsorption for each plasma used.

2.5. Assessment of adsorbent stability

The ECDs were labeled with ^{125}I using the chloramine T method as previously described (Psaridi-Linardaki et al., 2005), and immobilized onto Sepharose beads. To mimic their use for immunoadsorption, a small volume of beads (10 μl, containing ~50,000 cpm) was mixed with 240 μl of plasma or PBS and incubated at 37 °C for 2 h. Alternatively, to assess the adsorbent resistance to hydrolysis during exposure to low pH, the beads were incubated with 240 μl of 0.1 M glycine pH 2.5 or PBS for 60 min at RT. In both cases, the supernatants were removed, and the beads and supernatants were measured in a 1470 Wizard γ-counter (Perkin Elmer, USA). The counts of the supernatants are given as the percentage of the total counts of the beads.

2.6. Adsorbent regeneration

Sepharose beads with immobilized ECD adsorbent were packed into a glass column. A volume of plasma exceeding the adsorbent capacity was passed through the column and the flowthrough fractions were tested for anti-AChR with RIPA to verify the column capacity. Next the beads were treated with 0.1 M glycine pH 2.5 to elute the bound antibodies, and then washed with PBS. The immunoadsorption-elution cycles were repeated several times, and each time the capacity of the adsorbent was calculated. During each cycle, the glycine fractions were immediately neutralized with the addition of 1/5 of the volume of 1.5 M Tris pH 8.8, and assayed for anti-AChR with RIPA.

2.7. High flow-rate immunoadsorption

12 ml of Sepharose beads containing 6 mg ECD adsorbent were packed into a glass column (4.5 cm diameter). Using a peristaltic pump, MG plasma was passed through the column at different rates as indicated (4, 12, 25 and 50 ml/min). The volume of plasma passed was varied at each flow rate, so as to have a minimum of 15 min continuous flow. A FRAC-100 fraction collector was used to fractionate the flow-through, and the fractions (1 ml) were assayed by RIPA for anti-AChR.

3. Results

3.1. Investigation of the binding efficiency of the mutant ECDs and concatamers for MG antibodies

The expression of the mutant AChR ECDs has been described previously (Lazaridis et al., 2014). In short, the cys-loop of the α1, β1, δ and ε AChR subunit ECDs was replaced by that of the acetylcholine binding protein (AChBP), a soluble homologue, to create the α1-, β1-, δ- and ε-*BPloop* mutants respectively. Furthermore, the glycosylation site of the α1 ECD at Asn141 was re-introduced to reconstitute glycosylation