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In vivo adsorption of autoantibodies in myasthenia gravis using Nanodisc-incorporated acetylcholine receptor

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ABSTRACT

Autoantibodies directed against the skeletal muscle acetylcholine receptor (AChR) play a critical role in the pathogenesis of the autoimmune disease, myasthenia gravis (MG). The pathogenic importance of anti-AChR antibodies is substantiated clinically by the often dramatic clinical improvement that follows removal of circulating antibodies utilizing extracorporeal plasma exchange (PE). Unfortunately, the effects of PE are non-specific as immunoglobulins (IgG) and other plasma proteins are removed in addition to anti-AChR IgG. In this study, we have successfully incorporated the AChR protein purified from *Torpedo californicus* into a Nanodisc (ND) membrane scaffold protein/phospholipid structure. We go on to demonstrate the effectiveness of this ND–AChR complex, administered intravenously, in the *in vivo* down-modulation of anti-AChR antibodies and subsequent amelioration of clinical disease in the experimental murine model of MG. These results provide proof-of-principle for the *in vivo* antigen-specific reduction of pathogenic anti-AChR antibodies utilizing ND–AChR particles. Further development of this strategy may provide an effective, antigen-specific, and readily accessible acute therapy for exacerbating MG or myasthenic crisis.

Introduction

Autoimmune myasthenia gravis (MG) is a T-cell dependent, antibody-mediated disease in which autoantibodies directed against the skeletal muscle nicotinic acetylcholine receptor (AChR) impair neuromuscular transmission resulting in a loss in the number of functional AChRs at the motor endplate (Hoedemaekers et al., 1997; Vincent et al., 2001; Meriggioli and Sanders, 2009). The AChR is a ligand-gated ion channel receptor transmembrane protein formed by five subunits (α_2, β, γ (ε), and δ), with the α-subunit constituting the main immunogen involved in the majority of cases of MG (Lindstrom, 2000). The important role of the antibody-mediated attack on the AChR, resulting in destruction of the muscle endplate region is well established (Engel et al., 1977; Sahashi et al., 1978). An experimental animal model of MG, experimental autoimmune myasthenia gravis (EAMG), is induced in mice by immunization with AChR purified from the electric organs of the electric ray, *Torpedo californicus* (Berman and Patrick, 1980). In both MG and EAMG, anti-AChR antibodies bind to the AChR at the neuromuscular junction, activate complement, and accelerate AChR destruction, culminating in neuromuscular transmission failure and fatigable muscle weakness. The majority of pathogenic anti-AChR antibodies are directed against the main immunogenic region of the α subunit (core amino acids 67–76 and 125–147) separate from the acetylcholine binding sites, and the binding of anti-AChR autoantibodies is highly conformation-dependent (Luo et al., 2009).

An important intervention in treating MG, particularly when a quick therapeutic response is desirable, is extracorporeal plasmapheresis or plasma exchange (PE). PE has been successfully used to treat severe exacerbations of MG, and often produces rapid improvement in myasthenic weakness associated with reductions in the titer of anti-AChR-Abs and immunoglobulin (IgG) levels (Dau et al., 1977; Chiu et al., 2000; Gajdos et al., 1997). However, this method removes normal plasma components as well as IgG, and removes IgG non-specifically rather than anti-AChR IgG selectively. In addition to the removal of factors and immunoglobulins of potential pathogenic significance, nonspecific immunoglobulin depletion may have adverse affects on MG, possibly removing regulatory antibodies (Jambou et al., 2003), leading to increased synthesis of new pathogenic anti-AChR antibodies. Although very effective in inducing clinical improvement, the general usefulness of PE is also limited by its restriction to major medical centers and the frequent need for large-bore venous catheters. Infections and thrombotic complications

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related to venous access occur (Gajdos et al., 1997; Seybold, 1987). PE can also reduce coagulation factors, particularly with repeated treatments, leading to bleeding tendencies (Seybold, 1987).

Nanodiscs (ND) are soluble, nanoscale phospholipid bilayers which can self-assemble and incorporate membrane proteins for biophysical, enzymatic or structural investigations (Borch and Hamann, 2009; Nath et al., 2007). The ND consists of a non-covalent assembly of a phospholipid bilayer surrounded by an annulus composed of two copies of the amphipathic membrane scaffold protein (MSP) (Denisov et al., 2004). A trans-membrane protein inserted in a Nanodisc is thus surrounded by a lipid bilayer providing an environment that approximates its native state. The ND system provides a novel platform that has been utilized mainly for the purpose of understanding membrane protein function. Recently however Nanodisc incorporated Hemagglutinin vaccine has been shown to illicit a protective immune response in an animal model, demonstrating the potential of Nanodiscs as a vaccine platform (Bhattacharya et al., 2010). Membrane associated proteins, such as the AChR, are particularly suited for ND incorporation, potentially allowing for other *in vivo* delivery applications in addition to vaccines. We investigated a novel application of this technology, hypothesizing that AChR incorporated Nanodiscs (ND–AChR) could function as effective "autoantibody traps" for antigen-specific, adsorption of pathogenic anti-AChR antibodies in MG. Accordingly, we have successfully incorporated the AChR protein purified from *T. californicus* into the ND MSP/phospholipid structure, and report the effects of intravenous administration of ND–AChRs on disease severity and levels of anti-AChR antibodies in EAMG.

Materials and methods

Purification of T. californicus AChR

AChR was purified from the electric organs of *T. californicus* by affinity chromatography using a conjugate of neurotoxin coupled to agarose, as previously described (Wu et al., 2001; Sheng et al., 2006). Purity of the isolated product was tested by SDS-PAGE. Intact AChR complex was obtained in mg quantities by extraction with Triton X-100 and subsequent chromatographic separation. The purified AChR was used for incorporation into Nanodiscs as well as to induce EAMG.

Protein isolation and purification and assembly of ND–AChR

Details of the production and purification of the membrane scaffold protein (MSP) component and the assembly of ND–AChR are available on-line. Briefly, assembly conditions for Nanodiscs were performed as described previously (Bayburt et al., 2007). Purified AChR (0.35 mg/ml) obtained from *T. californicus* membranes (Wu et al., 2001) was incubated with sodium cholate solubilized phospholipids 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) and membrane scaffold proteins (MSP1E3D1, 32.6 kDa) in the presence of 0.1% Triton X-100 while incubating at 4 °C. *Torpedo* ND–AChR assemblies were formed by a self-assembly reaction initiated by the removal of detergent using hydrophobic Bio-Beads (Denisov et al., 2004; Bayburt and Sligar, 2003).

Purification of ND–AChR

The ND–AChR assembly mixture was batch adsorbed with Ni-NTA resin equilibrated with 20 mM Tris–HCl (pH 7.4), 100 mM NaCl. The column was washed with 50 mM imidazole and ND and ND–AChR particles were eluted with 0.4 M imidazole. Ni-column eluate was dialyzed overnight at 4 °C against low-ionic strength 20 mM Tris–HCl buffer (pH 7.4), 20 mM NaCl and the solution was passed over an affinity resin comprised of the acetylcholine analog 4,7,10-trioxa-1,13-carboxyethyl-2-trimethylaminetridecanediaamine (TDAC) (Tierney et al., 2004). The column was extensively washed with 10 column volumes of low-salt buffer to eliminate empty NDs, and ND–AChR was eluted with a step gradient of 50–150 mM NaCl to enrich the sample for ND–AChR. The 50 and 100 mM fractions containing AChR were concentrated (Amicon Ultra 15, 10,000 MWCO), passed through a 0.2 μm filter and separated by SEC.

ND–AChR quantification

AChR content and ND content (as determined by concentration of MSP) were measured by ELISA. Briefly, 96 well ELISA plates were coated with varying amounts of AChR and MSP in 0.1 M carbonate buffer (pH 9.6) by overnight incubation at 4 °C. Plates were blocked (25 °C for 2 h) with PBS containing Tween 20 (0.05% v/v) and BSA (3% w/v). After a thorough washing with PBS-Tween 20 (0.05% v/v) the plates were further incubated with anti-AChR antibodies (1:5000, from EAMG mouse sera) or anti-His tag antibodies (1:5000, BD 6xHistag Ab). After further washing, samples were incubated (25 °C for 1 h) separately with HRP anti-mouse IgG (1:5000) (for AChR), and HRP anti-mouse IgG2b (1:5000) (for MSP). Samples were then incubated with TMB substrate solution in the dark for 15 min. The reaction was stopped by adding 2 M H_2SO_4 and the colorimetric change was measured as the optical density at 450 nm (OD_{450}) using a microplate reader (Bio-Rad, Model 550). Concentration dependent standard curves were constructed for known quantities of AChR and MSP from the respective OD_{450} values, and AChR and MSP concentrations of the unknown ND–AChR mixtures were then calculated from the corresponding OD_{450} values by extrapolation. The concentration of AChR in the pooled sample used for treatment was determined to be 7.5 μg/100 μL; for MSP 15 μg/100 μL. The volume administered was 200 μL, based on an estimate of a safe volume for injection in the mouse.

Induction and clinical scoring of EAMG

Animal experiments were carried out under a protocol approved by the Animal Care and Use Committee of the University of Illinois at Chicago. Eight-week old female C57BL6/J mice were immunized with 40 μg of *Torpedo* AChR emulsified in CFA, 200ul subcutaneously, and received two "boosts" with 20 μg of AChR in IFA in 200 μl volume injected in the flanks and tail base every 30 days. Mice were observed and scored every other day. For clinical examination, mice were evaluated for myasthenic weakness and assigned clinical scores as previously described (Sheng et al., 2006; Sheng et al., 2008). Briefly, mice were observed on a flat platform for a total of 2 min. They were then exercised by gently dragging them suspended by the base of the tail across a cage top grid repeatedly (20–30 times) as they attempted to grip the grid. They were then placed on a flat platform for 2 min and again observed for signs of EAMG. Clinical muscle weakness was graded as follows: grade 0, mouse with normal posture, muscle strength, and mobility at baseline and after exercise; grade 1, normal at rest but with muscle weakness characteristically shown by a hunchback posture, restricted mobility, and difficulty in raising the head after exercise; grade 2, grade 1 symptoms without exercise during observation period; grade 3, dehydrated and moribund with grade 2 weakness; and grade 4, dead. The evaluator was blinded to treatment status for all clinical evaluations.

Administration of ND–AChR

After initial priming and two booster immunizations, the mice were divided into four groups of 10 mice per group, consisting of equal numbers of mice with various disease severities in each group. Mice received (1) ND–AChR, (2) "bare AChR" (7.5 μg), (3) empty ND (15 μg), or (4) PBS in a total volume of 200 μL via tail vein injection on 5 consecutive days, followed by 2 days of "rest", and then five additional days of treatment. The day of treatment initiation was designated "day