Journal of Neuroimmunology 189 (2007) 111 – 117

Journal of Neuroimmunology

www.elsevier.com/locate/jneuroim

Isolation and functional characterization of anti-acetylcholine receptor subunit-specific autoantibodies from myasthenic patients: Receptor loss in cell culture

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Received 23 April 2007; received in revised form 4 June 2007; accepted 5 June 2007

Abstract

The muscle nicotinic acetylcholine receptor (nAChR) is the major autoantigen in the autoimmune disease myasthenia gravis (MG), in which autoantibodies bind to, and cause loss of, nAChRs. Antibody-mediated nAChR loss is caused by the action of complement and by the acceleration of nAChR internalization caused by antibody-induced cross-linking of nAChR molecules (antigenic modulation). To obtain an insight into the role of the various anti-nAChR antibody specificities in MG, we have studied nAChR antigenic modulation caused by isolated anti-subunit autoantibodies. Autoantibodies against the nAChR α or β subunits were isolated from four MG sera by affinity chromatography on columns carrying immobilized recombinant extracellular domains of human nAChR expressed in the yeast *Pichia pastoris*. The isolated anti-α and anti-β autoantibodies, as well as untreated MG sera, induced nAChR antigenic modulation in TE671 cells. Partially antibody-depleted sera exhibited reduced modulating activity, whereas a serum completely depleted of anti-nAChR antibodies exhibited no nAChR modulation. Interestingly, the anti-α autoantibodies were, on average, ~4.3 times more effective than the anti-β autoantibodies. The present work supports the notion that anti-nAChR autoantibodies may be the sole nAChR-reducing factor in anti-nAChR antibody-seropositive MG, and that anti-α-subunit autoantibodies are the dominant pathogenic autoantibody specificity.

Keywords: Acetylcholine receptor; Antigenic modulation; Autoantibodies; Extracellular domains; Myasthenia gravis

1. Introduction

The nicotinic acetylcholine receptor (nAChR), a transmembrane glycoprotein with a molecular weight of about 290 kDa located on the postsynaptic membrane of the neuromuscular junction, is composed of five homologous subunits in the stoichiometry of $a_2\beta\varepsilon\delta$ (adult form) or $\alpha_2\beta\gamma\delta$ (fetal form) (Lindstrom, 2000). The α subunits carry the two acetylcholine binding sites, which regulate the opening of the cation channel (Karlin, 1993).

The nAChR is the main autoantigen in the autoimmune disease myasthenia gravis (MG), a T cell-dependent antibody-mediated disease characterized by skeletal muscle weakness and fatigability (Vincent et al., 2001). Anti-nAChR autoantibodies are present in the majority (85–90%) of MG patients with generalized symptoms (Drachman, 1994; Lindstrom, 2000), while some of the remaining 10–15% patients have antibodies against the muscle-specific tyrosine kinase (Hoch et al., 2001; Vincent et al., 2003). nAChR-specific antibodies cause loss of functional nAChRs at the neuromuscular junction by cross-linking the nAChRs, thereby resulting in increased internalization and degradation of nAChRs (antigenic modulation) and complement-mediated destruction of the postsynaptic membrane. They may also block the binding of acetylcholine to

Abbreviations: nAChR, nicotinic acetylcholine receptor; MG, myasthenia gravis; MIR, main immunogenic region; cpm, counts per minute; ECD, extracellular domain; RIA, radioimmunoassay; NHS, normal human serum; α-Bgtx, α-bungarotoxin.

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doi:10.1016/j.jneuroim.2007.06.014

the nAChR, leading to insufficient neuromuscular transmission (Drachman et al., 1980; Tzartos et al., 1998).

Although experimental MG can be caused by immunization with purified nAChR or by injection of single anti-nAChR monoclonal antibodies (mAbs), thus proving the pathogenic role of experimental anti-nAChR antibodies in the model disease, the pathogenic role of anti-nAChR autoantibodies from patients' sera has only been demonstrated indirectly by the clinical improvement that follows plasma exchange and by the passive transfer of the disease in laboratory animals injected with patients' sera or IgG (Toyka et al., 1975, 1977; Newsom-Davis et al., 1978; Mossman et al., 1988; De Baets and Stassen, 2002). However, there is no good correlation between the serum anti-nAChR antibody titer and disease severity in patients or any direct proof that the pathogenic factors in MG sera are indeed the anti-nAChR antibodies and only these.

nAChR-specific autoantibodies are heterogeneous in terms of epitope specificity (Tzartos and Lindstrom, 1980; Heidenreich et al., 1988; Tzartos et al., 1998). A major fraction of nAChR-specific autoantibodies competes for binding to the nAChR with mAbs directed against an extracellular region of the α subunit containing amino acid residues 67–76, named the main immunogenic region (MIR) (Tzartos et al., 1998). mAbs directed against the MIR can cause passive transfer of experimental MG in animals (Tzartos et al., 1987). While the α subunit appears to contain a large fraction of the immunodominant epitopes, antigenic determinants on other nAChR subunits may also contribute to the pathogenesis of MG (Wang et al., 1998; Jacobson et al., 1999; Ragheb et al., 2005).

Given the poor correlation between serum autoantibody titer and disease severity, it would be of great interest to examine the pathogenicity of different isolated subunit-specific autoantibody populations and any possible remaining activity of serum after elimination of anti-nAChR antibodies. As far as we are aware, antibodies against either whole nAChR or specific subunits have not been purified from patients' sera, and the activity of patients' anti-nAChR autoantibodies, free of other serum molecules, has not been studied.

The present work is based on our recently acquired ability to isolate subunit-specific anti-nAChR autoantibodies from patients' sera. Using recombinant proteins corresponding to extracellular domains (ECDs) of individual nAChR subunits as immunoadsorbents, we isolated autoantibodies which specifically bind to these subunits. We then used the well established TE671 human muscle cell line that expresses muscle-like nAChRs (Luther et al., 1989) to examine the *in vitro* functions of subunit-specific autoantibody populations through their ability to induce nAChR antigenic modulation.

Our results clearly demonstrated that autoantibodies against the α or β subunit can cause nAChR loss *via* antigenic modulation in a dose-dependent manner, the anti-α autoantibodies being much more effective than the anti-β autoantibodies. Furthermore, we showed that the autoantibody-depleted sera were much less effective, or were completely inactive, at causing nAChR loss, suggesting that the anti-nAChR autoantibodies in MG sera are the sole pathogenic factor in anti-nAChR antibody-seropositive MG.

2. Materials and methods

2.1. MG sera and evaluation of the autoantibody titer

MG serum samples used in our study were randomly selected from a relatively large number of high anti-nAChR titer sera, held in our lab for diagnostic purposes only. These sera had a high percentage of autoantibodies against the α and β nAChR subunits. MG1 and MG2 patients were males with generalized symptoms of the disease at the time of the serum sampling. The other two MG sera (MG3 and MG4) were females, with MG4 presenting generalized symptoms of the disease, and MG3 presenting ocular symptoms (blepharoptosis). Only patient MG3 was thymectomized. Since our lab is only responsible for the diagnostic evaluation of the patients' sera and not for the clinical follow-up of the patients, we do not have complete data about the severity and the clinical status (past or present) of these patients. Sera from healthy donors (normal human serum-NHS) were obtained from the Blood Transfusion Centre, University Hospital, Rio-Patras, Greece.

All patients' samples were tested for antibody binding to human muscle nAChR by a modification of the radioimmunoassay (RIA) described by Lindstrom et al. (1981). Briefly, 14 fmol of human muscle-type nAChR extracted from the CN21 cell line, which expresses both the ε and γ nAChR subunits at a ratio of approximately 2:1 (Beeson et al., 1996), was labeled with ^{125}I-α-Bgtx (50,000 cpm; about 60 fmol). After the addition of MG sera or isolated autoantibodies, the samples were incubated at 4 °C overnight, then the immune complexes formed were precipitated by the addition of goat anti-human gamma globulin antiserum (Lampire Biological Laboratories, Pipersville, PA, USA), incubation for 2 h at 4 °C, and centrifugation at 2500 *g* for 15 min at 4 °C. The precipitates were washed three times with phosphate-buffered saline (PBS), pH 7.4, 0.5% Triton-X100 and the radioactivity was measured in a γ-counter (Wallac 1275). Antibody titers (in nmol/l) were estimated as (cpm precipitated by the MG sample − cpm precipitated by NHS)/(cpm precipitated by high excess of a high titer MG sample which presumably precipitates all of the nAChR − cpm precipitated by NHS) × 14 fmol/MG sample volume in μl.

2.2. Expression and purification of recombinant proteins corresponding to extracellular nAChR subunits

Construction and expression of recombinant polypeptides corresponding to the ECDs of human nAChR (α-ECD, residues 1–210; β-ECD, residues 1–221; γ-ECD, residues 1–218; and ε-ECD, residues 1–219) were achieved using the eukaryotic yeast *Pichia pastoris* expression system described previously (Psaridi-Linardaki et al., 2002; Kostelidou et al., 2006), in which the proteins are secreted into the culture medium. The C-terminal of the α-ECD was fused to the c-myc epitope followed by a His6 tag (Psaridi-Linardaki et al., 2002), while all non-α-ECDs (β, γ, and ε) were flanked by an N-terminal FLAG epitope (DYKDDDDK) and a C-terminal His6 tag (Kostelidou et al., 2006). The recombinant ECDs were purified using Ni^{2+}-