Journal of Neuroimmunology 320 (2018) 64–75

Contents lists available at ScienceDirect

Journal of Neuroimmunology

journal homepage: www.elsevier.com/locate/jneuroim

Heterogeneity of auto-antibodies against nAChR in myasthenic serum and their pathogenic roles in experimental autoimmune myasthenia gravis

Ryuichi Nakamura[a,d,*], Tomohiro Makino[b], Takeshi Hanada[b], Maki Terakawa[c], Kazuhiro Nagahira[c], Jyoji Yamate[d], Hirokazu Shiraishi[e], Masakatsu Motomura[f]

[a] Regenerative Medicine Field, Asubio Pharma Co., LTD, Kobe 650-0047, Japan
[b] Drug Discovery Technology Function, Asubio Pharma Co., LTD, Kobe 650-0047, Japan
[c] Immunology & Inflammatory Disease Field, Asubio Pharma Co., LTD, Kobe 650-0047, Japan
[d] Laboratory of Veterinary Pathology, Osaka Prefecture University, Osaka 598-8531, Japan
[e] Department of Neurology and Strokology, Nagasaki University Hospital, Nagasaki 852-8501, Japan
[f] Medical Engineering Course, Department of Engineering, The Faculty of Engineering, Nagasaki Institute of Applied Science, Nagasaki 851-0193, Japan

ABSTRACT

Many myasthenia gravis (MG) patients have auto-antibodies against the nicotinic acetylcholine receptor (nAChR), and monoclonal antibodies against the main immunogenic region (MIR) of nAChR can induce experimental autoimmune MG (EAMG). We investigated whether Fab fragment of MIR antibody (Fab35) could block the pathogenicity of polyclonal antibodies. Fab35 partially inhibited nAChR downmodulation, blocked EAMG serum-induced binding of polyclonal antibodies and complement deposition *in vitro*. Moreover, Fab35 did not ameliorate the EAMG serum-induced EAMG phenotype in rats. These results suggested that the EAMG serum possessed several different pathogenic antibodies that might be sufficient to induce the EAMG phenotype.

1. Introduction

Myasthenia gravis (MG), an autoimmune disease, is characterized by fluctuating muscle weakness and abnormal fatigability (Berrih-Aknin and Le Panse 2014; Binks et al. 2016). Around 85% of MG patients have auto-antibodies (auto-Abs) against the nicotinic acetylcholine receptor (anti-nAChR Abs), and these auto-Abs are thought to induce disability of neuromuscular junctions (NMJs) in skeletal muscle. The pathogenic mechanism of MG induced by anti-nAChR auto-Abs is initiated by binding of these Abs to the receptor, which can trigger complement-mediated postsynaptic membrane damage, downmodulation of nAChRs and depletion of its surface pools, or antagonize receptor function in NMJs (De Baets and Stassen 2002; Huijbers et al. 2014).

nAChR in muscle consists of a heteropentamer (two α-subunits and one each of a β-subunit, δ-subunit, and either a γ-subunit [embryonic type] or ε-subunit [adult type]) organized around a central pore in the membrane (Albuquerque et al. 2009; Unwin 2005). In the experimental autoimmune myasthenia gravis (EAMG) rodent model, induced by nAChR immunization, many anti-nAChR Abs are raised in EAMG serum against an epitope on the N-terminal region of the nAChR α-subunit called the main immunogenic region (MIR) (Luo and Lindstrom 2012). The α-subunit sequence 66–76, the MIR loop, is crucial to the MIR; the antigenicity and myasthenogenicity of the MIR depend greatly on the native conformation (Luo et al. 2009; Tzartos et al. 1987). Furthermore, rat mAb35 and mAb195, known as mAbs against MIR, can solely induce EAMG, which indicates that auto-Abs against MIR are a major contributor to MG pathology (Papanastasiou et al. 2000; Tzartos et al., 1987).

In human MG pathology, the subtypes of auto-Abs generated against nAChR are mainly the IgG1 and IgG3 subclass (Huijbers et al., 2014; Liu et al. 2011; Rodgaard et al. 1987); human IgG1, 2, and 3 reported to have complement activation potential(Gomez et al. 2010; Vidarsson et al. 2014), and IgGs derived from MG patients also have the potential to induce EAMG by passive transfer to rat (Buschman et al. 1987), which indicates that complement-dependent cytotoxicity contributes to human MG pathology. The serum titers of nAChR Abs against MIR, determined by the mAb35 inhibition assay, tend to correlate with

Abbreviations: Ab, antibody; α-Btx, alpha-Bungarotoxin; CDC, complement dependent cytotoxicity; cpm, count per minute; C_{max}, maximum plasma concentration; EAMG, experimental autoimmune myasthenia gravis; EDL, extensor digitorum longus; Fab, Fab fragment of monoclonal antibody; FCM, Flow cytometry; mAb, monoclonal antibody; MFI, mean fluorescence intensity; MG, myasthenia gravis; MIR, main immunogenic region; nAChR, nicotinic acetylcholine receptor; NMJs, neuromuscular junctions; ROI, region of interest; T_{max}, maximum plasma concentration arrival time; $T_{1/2}$, Plasma concentration half-life

* Corresponding author at: Rare Disease Laboratories, Cell Therapy Discovery Group, Daiichi Sankyo Co., LTD, Tokyo 140-8710, Japan.
E-mail address: nakamura.ryuichi.a7@daiichisankyo.co.jp (R. Nakamura).

https://doi.org/10.1016/j.jneuroim.2018.04.014
Received 20 February 2018; Received in revised form 23 April 2018; Accepted 23 April 2018

disease severity in patients with MG (Masuda et al. 2012), suggesting the importance of MIR Abs. Furthermore, the pathogenicity of serum in the EAMG model could be eliminated by depletion of auto-Abs (Kordas et al. 2014).

In a previous report the Fab fragment of MIR Ab, mAb195, prevented the induction of EAMG caused by mAb35 and mAb195 in a rat model (Papanastasiou et al., 2000). In addition, it has also been reported that the IgG4 form of MIR Ab, mAb637-IgG4, or its single-armed Ab could also alleviate the pathogenicity of EAMG caused by the IgG1 form (Graus et al. 1997; Li et al. 2014; Losen et al. 2017). However, the therapeutic effect against polyclonal pathogenic auto-Abs remains to be elucidated. As the proof of concept study, we have performed *in vivo* study for the first time to determine whether the single Fab form of MIR Ab could block the pathogenicity of EAMG polyclonal Abs. We found that blocking nAChR with a single MIR Ab was not sufficient to protect against the pathogenicity of EAMG, suggesting that EAMG serum possess several different pathogenic mAbs that recognize other region of nAChR than MIR.

2. Material and methods

2.1. Preparation of EAMG serum

All animal experiments were approved by the Committee on Animal Experimentation at Asubio Pharma Co., Ltd. Animals were obtained from Charles River Laboratories Japan and treated in accordance with the Guide for Animal Experimentation.

A chronic EAMG model was prepared in accordance with previously described methods (Losen et al. 2015). After AChR was extracted from the electric organ of *Torpedo californica* (tAChR), eight-week-old female LEW/CrlCrlj rats were immunized with the emulsion of 40–80 μg tAChR antigen and complete Freund's adjuvant (CFA) including 1 mg/mL *M. tuberculosis* (Sigma-Aldrich) by intracutaneous injection at the root of the tail and boosted 2 weeks later with the same amount of the emulsion. Disease score was graded twice a week as follows: grade 0, no symptom; grade 1, weakness after exercise; grade 2, weakness without exercise; grade 3, no ability to grip, respiratory distress; grade 4, moribund/death. Sera were obtained and pooled from 18 rats exhibited more than grade 2 in disease score from 28 to 64 days after first immunization.

2.2. Cell culture

The human rhabdomyosarcoma cell lines TE671, which expresses human nAChR on its surface (Franciotta et al. 1999; Luther et al. 1989), and DB40, transfected ε-subunit genes to TE671 for the stable expression of fetal and adult nAChRs (Beeson et al. 1996; Keefe et al. 2009; Lozier et al. 2015), were purchased from the American Type Culture Collection (ATCC) and ISIS Innovation Ltd., Oxford, respectively. These cell lines were cultured at 37 °C and 5% CO_2, with RPMI 1640 + 10% FBS or DMEM +10% FBS+ 0.5 mg/mL of G418, respectively, in T75 flasks.

2.3. Preparation of recombinant Abs

The detailed preparation of recombinant Abs used in this study was described previously (Makino et al. 2017). For the construction of a human chimeric rat anti-nAChR Ab (mAb35xich1), the VH/VL DNA sequence of mAb35 was amplified directly from cDNA derived from a mAb35 hybridoma (clone ATCC TIB-175™) using conventional methods. After this, the fragments were cloned into pSF-CMV-HuIGG1 HC/HuKappa LC (Sigma-Aldrich) carrying the human IgG1 and Igκ framework with an *Nco*I/*Xba*I restriction site. The purified paired plasmids IgH and IgL were then co-transfected in 30 mL or 150 mL of Expi293 cells (Thermo Fisher Scientific) according to the manufacturer's instructions. After five days of incubation, culture supernatant was harvested and clarified by centrifugation. Subsequently, Abs were purified and concentrated using HiTrap Protein G HP 5 mL (GE Healthcare) following the manufacturer's instructions. Fab fragments of mAb35xich1 Ab were obtained using a Pierce™ Fab Preparation Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Control Fab fragment of rat IgG was purchased from Rockland Immunochemicals Inc.

2.4. Flow cytometry (FCM)-based binding assay and competition assay

For the binding assay, DB40 and TE671 cells were detached from the culture flask using a non-enzymatic dissociation solution (Sigma-Aldrich Inc.,), and 5×10^4 cells/well were incubated with 50 μL of diluted culture supernatant. Serially diluted EAMG serum was added to V-shaped 96-well plates for 30 min on ice. After washing with PBS + 0.5% BSA + 2 mM EDTA (FCM buffer) twice, 100 μL of PE-conjugated goat anti-rat IgG (Fc) Ab (polyclonal, BioLegend Inc.,), PE-conjugated mouse anti-rat IgG1 Ab (clone MRG1-58, BioLegend Inc.,), PE-conjugated mouse anti-rat IgG2a Ab (clone MRG2a-83, BioLegend Inc.,), FITC-conjugated mouse anti-rat IgG2b Ab (clone MRG2b-85, BioLegend Inc.,), or FITC-conjugated mouse anti-rat IgM Ab (clone MRM-47, BioLegend Inc.,) diluted 1:100 in FCM buffer was then added and allowed to bind for another 30 min on ice. After a washing step, cells were resuspended in 100 μL of FCM buffer containing 7-AAD (BD Biosciences). The mean fluorescence signal of PE or FITC was collected after eliminating debris and dead cells based on scatter signals and 7-AAD fluorescence by FCM (MACSQuant Analyzers, Miltenyi Biotec), and the signal intensities of DB40 and TE671 were compared. Serially diluted control serum was used as a negative control.

The competitive inhibition assay was carried out similarly, except for the addition of a pre-incubation step; Fab35 or control Fab was held for 30 min on ice before adding the EAMG serum to be tested.

2.5. FCM-based nAChR modulation assay

Downmodulation of nAChRs on the surface of DB40 cells was monitored based on a previously described method (Lozier et al., 2015) with some modifications, particularly in detection. DB40 cells were plated at 5×10^5 cells per well in 200 μL in V-shaped 96-well plates. Serially diluted Fab35 or control Fab were added with the appropriate concentrations of EAMG serum. Cells were then incubated for 4 h at 37 °C and 5% CO_2. Fluorescein-labeled antagonists (α-Bungarotoxin conjugated with Alexa Fluor 488 [α-Btx-Alexa Fluor 488, Thermo Fisher Scientific]) were used to monitor the amount of nAChR on the surface of cells. After incubation, cells were labeled with α-Btx-Alexa Fluor 488 (2 μg/mL final concentration) for 30 min on ice and washed with FCM buffer. Data are presented as the mean fluorescence signal intensity of Alexa Fluor 488 after eliminating debris and dead cells based on scatter signals and 7-AAD fluorescence by FCM.

2.6. FCM-based complement-dependent cytotoxicity (CDC) assay

To measure CDC activity, DB40 cells were plated at 1×10^5 cells per well in 100 μL in V-shaped 96-well plates and incubated with appropriate concentrations of EAMG serum for 30 min on ice. Serially diluted Fab35 or control Fab were added 30 min prior to EAMG serum. After removal of supernatant, the cells were incubated with baby rabbit complement (CEDERLANE®) for 2 h at 37 °C and 5% CO_2. After a washing step, necrotic and apoptotic cells were detected by FCM with FITC Annexin V Apoptosis Detection Kit (BD Pharmingen™) (Ayuk et al. 2008; Daneshmanesh et al. 2012; Zent et al. 2008).

2.7. MIR Ab assay using radioimmuno-precipitation

Competitive binding activity toward rat nAChR against EAMG serum was determined for each human MIR Ab by radioimmuno-