

# Irrelevance of anti-nAChR cytoplasmic loop antibody in the degree of myasthenia gravis

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**Abstract.** Anti-nicotinic acetylcholine receptor (nAChR) antibody in myasthenia gravis (MG) usually refers to that against the extracellular domain (ECD) of nAChR. However, growing evidence has indicated that there also exists the nAChR antibody against the nAChR cytoplasmic loop (CL) in patients with MG. Some studies have demonstrated that the anti-CL antibody may play a protective role in animal models of experimental autoimmune MG. However, to date, limited or no information is available as to whether anti-CL antibody plays a beneficial role in patients with MG. In this, we examined the levels of anti-CL and anti-ECD antibodies in the sera of 76 patients with MG from the MG sample bank of our department, including 29 generalized MG (gMG) and 47 ocular MG (OMG) cases, as well as 40 healthy controls by radioimmunoassay. We also analyzed the correlation between anti-CL antibody levels and the degree of severity in patients with MG. Our results revealed that among the 76 patients with MG, 61 serum samples (80.3%) were positive for anti-ECD nAChR antibody, among which 38 (38/61, 62.3%) were positive for anti-CL antibody. The levels of anti-ECD antibodies in gMG were higher than those

in OMG; however, the levels of anti-CL antibodies in gMG were comparable with those in OMG. No clear correlation was observed between the anti-ECD antibody levels and those of anti-CL antibody. Taken together, our findings provide the first clinical evidence of the possibly different role of anti-CL antibody in patients with MG compared to its role in animal models of MG. Anti-CL antibody may not exert a beneficial effect in patients with MG, but may play an as yet undetermined role in the development of MG.

## Introduction

Myasthenia gravis (MG) is a typical autoimmune disease caused by antibodies against the muscle type nicotinic acetylcholine receptor (nAChR) of the neuromuscular junction. Anti-nAChR antibodies reduce the number of effective nAChRs at the endplate, causing the failure of synaptic electrical-chemical coupling, resulting in muscle weakness and fatigability (1,2). Anti-nAChR antibodies are detectable in approximately 90% of myasthenic patient sera (3).

nAChR, from fish electric organs and vertebrate skeletal muscles, is a transmembrane glycoprotein composed of 5 homologous subunits organized into a cation channel in the order of  $\alpha 1 \gamma \alpha 1 \delta \beta 1$  in denervated skeletal muscle, and  $\alpha 1 \epsilon \alpha 1 \delta \beta 1$  in innervated skeletal muscle (4). Each subunit in nAChR consists of 2 parts: the extracellular domain (ECD) and the cytoplasmic loop (CL). Agonists, such as acetylcholine and competitive antagonists, such as  $\alpha$ -bungarotoxin ( $\alpha$ -BTX) (5), bind to the two  $\alpha$  subunits and regulate the ion channel. Anti-nAChR antibodies do not bind to the Ach binding site of nAChR, and antibody-nAChR complexes extracted from muscle do bind <sup>125</sup>I $\alpha$ -BTX. Therefore, the concentration of anti-nAChR antibodies in sera can be determined in terms of the <sup>125</sup>I $\alpha$ -BTX-binding site (6).

In the sera from patients with MG, most anti-nAChR antibodies bind to a small region on the extracellular side of the  $\alpha 1$  subunit, the main immunogenic region (MIR) (7-9). Most of the known anti-nAChR antibodies recognize the extracellular side of nAChR (10). However, a growing body of evidence has indicated that anti-nAChR CL antibodies also exist in the sera of patients with MG (11-13). It has been reported that approximately 17% of patients with MG are anti-CL antibody-positive (14). At present, relatively little is known regarding the

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**Abbreviations:**  $\alpha$ -BTX,  $\alpha$ -bungarotoxin; BCR, B-cell antigen receptor; BME,  $\beta$ -mercaptoethanol; CL, cytoplasmic loop; EAMG, experimental autoimmune myasthenia gravis; ECD, extracellular domain; EOMG, early-onset myasthenia gravis; gMG, generalized myasthenia gravis; IPTG, isopropyl  $\beta$ -D-1-thiogalactopyranoside; IRB, institutional review board; LOMG, late-onset myasthenia gravis; MG, myasthenia gravis; MoAb, monoclonal antibody; nAChR, the nicotinic acetylcholine receptor; OMG, ocular myasthenia gravis; SD, standard deviation

**Key words:** myasthenia gravis, nicotinic acetylcholine receptor, autoantibody, cytoplasmic loop, extracellular domain, radioimmunoassay

association between anti-CL antibodies and the clinical profile of MG.

Recently, several studies have demonstrated that anti-CL antibody may play a protective role in animal models of experimental autoimmune MG (EAMG), in which they discovered a novel approach to the specific immunosuppression of EAMG with a therapeutic vaccine consisting of bacterially-expressed human nAChR CL, which has the potential to specifically suppress MG without the danger of causing exacerbation (11-13,15). However, to date, information about the constitutive effects of anti-CL antibody on the process of MG and its relevance to the degree of MG is lacking. In our retrospective study, we measured the levels of anti-CL antibody in the serum samples of 76 patients with MG, and analyzed its association with the clinical profile and anti-ECD muscle nAChR titers.

## Materials and methods

**Human subjects.** The sera from 76 patients with MG were collected from the MG sample bank of the Department of Neurology, Tangdu Hospital, Xi'an, China. All samples in the MG sample bank were diagnosed according to the diagnostic criteria. The following criteria were used to diagnose MG (16,17): i) acquired weakness of skeletal muscles, including cranial muscles; ii) fluctuation in muscle strength; iii) 10% decrease in compound muscle action potential amplitude in response to stimulation at 3-5 Hz and increased jitter on single-fiber electromyography; and iv) positive response to a cholinesterase inhibitor. Exclusion criteria were pregnancy. A total of 40 healthy subjects were recruited as the controls and were matched with the patients with MG in age, sex and demography. According to the principles expressed in the Helsinki Declaration and to the ethics committee-approved protocols, all subjects provided written and signed informed consent prior to enrollment. Blood sample collections from human subjects for detailed immunological assessments were approved by the Ethics Committee of the Fourth Military Medical University. All patients and healthy controls provided written informed consent.

**Preparation of human muscle native nAChR.** Human skeletal muscle was obtained from the amputated leg of a diabetic patient treated for gangrene as the nAChR source. We followed the protocols described in the studies by Lindstrom *et al* (6) and Chang *et al* (18) and made necessary changes. The muscle was chopped into small sections of 10-15 g, flash-frozen in liquid nitrogen and stored at -80°C. Muscle was homogenized with a pre-chilled electrical blender for 1-2 min at 4°C in 300 ml of 0.1 M NaCl, 0.01 M phosphate buffer (pH 7.0). Following centrifugation at  $9 \times 10^3$  rpm for 20 min, the supernatant was discarded. Homogenization and centrifugation were repeated with the pellet. The pellets were extracted in 160 ml 2% Triton X-100, 0.1 M NaCl, 20 mM EDTA, 0.01 M phosphate buffer (pH 7.0), 0.01 M  $\text{NaN}_3$  by homogenizing for 20 sec, followed by gentle shaking for 1 h at 4°C. Following centrifugation at  $10^5$  g/4°C for 30 min, the resulting supernatant was suctioned using a syringe, pooled, glycerol was added to 10%, mixed well, and stored as 1 ml aliquot at -80°C.

## Preparation of recombinant human muscle nAChR CL

**Plasmid and bacterial strain.** The plasmid vector pET16b and the prokaryotic expression host strain *E. coli* BL21 (DE3) were purchased from Novagen, Darmstadt, Germany.

**Human muscle nAChR CL expression vector construction.** DNA sequences encoding the CL of the human muscle nAChR subunits,  $\alpha 1$  (residues 301-374),  $\beta 1$  (residues 312-412), and  $\delta$  (residues 315-416), were codon-optimized to *E. Coli* preferred codons and synthesized (Genscript Co., Nanjing, China). Wild-type CL from human muscle nAChR  $\alpha 1$  subunits lacked tyrosine residue, the key amino acid for iodination labeling. Therefore, 2 additional tyrosine residues were incorporated into the C-terminal of AChR $\alpha 1$  CL by PCR to generate the coding sequence. For the wild-type  $\beta 1$  and  $\delta$  subunits, which already contain the tyrosine residues, there was no need to change the synthesized coding sequences. These sequences were inserted into the *NdeI* and *BamHI* sites of pET16b (Novagen), and transformed into expression host BL21 (DE3) (Novagen). Recombinant clones were checked for insertion by restriction digestion and sequencing (Genscript Co.).

**Expression and purification of proteins corresponding to the nAChR CL.** Recombinant nAChR CL was generated from the BL21 (DE3) cells harboring the expression plasmids at an OD<sub>600</sub> of 4.0 using a homemade fermentor. This was followed by induction with 1 mM isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG) for 4 h at 37°C. The cell pellets were collected by centrifugation. For extraction of recombinant proteins, cell pellets were solubilized in 50 mM phosphate buffer (pH 8.0), 6 M guanidine hydrochloride, 5 mM imidazole, sheared with an electric blender to reduce the stickiness of the solution and centrifuged at  $28,000 \times g$  at 4°C for 30 min. The supernatant was then passed through a 5-ml Histrap FF column (GE Healthcare, Piscataway, NJ, USA). Bound protein was eluted by 50 mM phosphate buffer (pH 8.0), 6 M guanidine hydrochloride, 500 mM imidazole. Eluted fractions containing protein peaks were pooled, dialyzed against 0.1% SDS, 10 mM EDTA, and then loaded to several preparative SDS-PAGE gels. Protein bands were visualized by staining with 0.1 M  $\text{CuSO}_4$  solution and the band corresponding to the recombinant CL were excised. Proteins within the bands were electroeluted, dialyzed to remove residue  $\text{Cu}^{2+}$  ion, concentrated by ultrafiltration (Amicon Ultra 3K filters) and stored at -20°C.

## Iodination labeling of $\alpha$ -BTX and recombinant nAChR CL.

The recombinant nAChR CL proteins and  $\alpha$ -BTX were labeled with  $^{125}\text{I}$  using the Chloramine T method as previously described by Lindstrom *et al* (9) and Greenwood *et al* (19). Briefly, 2  $\mu\text{Ci}$  of  $^{125}\text{I}$  (Carrier free, Chengdu Gaotong Isotope Co., Ltd., Chengdu, China) was mixed with 12  $\mu\text{l}$  of 0.5 M sodium phosphate buffer (pH 7.0) and 70  $\mu\text{g}$  (approximately 7 nmol) of recombinant nAChR CL protein or  $\alpha$ -BTX, and then mixed with 2  $\mu\text{l}$  of 1.6 M chloramine T. The mixture was then incubated on ice for 25 min with occasional shaking and then loaded to a PD-10 Desalting column (GE Healthcare). Protein was eluted using phosphate sodium buffer containing 0.1% SDS for recombinant nAChR CL or phosphate sodium buffer for  $\alpha$ -BTX. Fractions of 0.3 ml were collected and monitored for radioactivity. Fractions containing radioactivity were pooled and the final volume was adjusted with phosphate buffer saline to yield a protein concentration of  $2 \times 10^{-6}$  M.

**Radioimmunoassay of anti-native nAChR and anti-CL antibodies from the sera of patients with MG.** Serum anti-nAChR antibodies were measured using radioimmunoprecipitation (20) by  $^{125}\text{I}$ -BTX-labeled native nAChR,  $^{125}\text{I}$ -labeled nAChR $\alpha$ 1-CL,  $^{125}\text{I}$ -labeled nAChR $\beta$ 1-CL, and  $^{125}\text{I}$ -labeled nAChR $\delta$ -CL. For the measurement of antibodies against nAChR-CL, 500  $\mu\text{l}$  of diluted  $^{125}\text{I}$ -labeled nAChR CL protein (final concentration, approximately  $10^{-9}\text{ M}$ ) in 0.5% Triton X-100, 0.005% SDS, were incubated with 10  $\mu\text{l}$  of MG patient serum, followed by the addition of 10  $\mu\text{l}$  of rabbit anti-human antibody for 2 h. For the measurement of antibodies against native human nAChR,  $^{125}\text{I}$ -labeled- $\alpha$ -BTX was mixed with 1 ml of human skeletal muscle extract to a final concentration of  $10^{-9}\text{ M}$ , followed by the addition of 10  $\mu\text{l}$  of rabbit anti-human antibody for 2 h. The immune complex was precipitated by centrifugation at 13,000 rpm/4°C/5 min, and the pellets washed twice with PBS (pH7.2) containing 0.5% Triton X-100. The radioactivity was measured using a  $\gamma$ -counter (CNNC Xi'an Nuclear Instrument Factory, Xi'an, China). Background radioactivity was measured without patient serum.

**Statistical analysis.** The antibody concentrations of anti-native nAChR and anti-CL are expressed in nM. The data were analyzed using SPSS 17.0 software. Continuous variables are listed as the means  $\pm$  standard deviation (SD) and were analyzed using the Student's t-test. The differences in sex, MG type according to onset age, MG type and onset MG type were analyzed using Chi-square tests, and the analysis of antibody of ECD and CL were tested by linear regression analysis. A two-tailed P-value of  $<0.05$  was considered to indicate a statistically significant difference.

## Results

**Expression and purification of three CLs from human muscle nAChR in the  $\alpha$ 1,  $\beta$ 1 and  $\delta$  subunits.** We generated 3 target proteins with a gradient of molecular masses at approximately 15 kDa (Fig. 1A). We optimized the purification of CLs from nAChR proteins with recombinant protein fractions (Fig. 1B).

**Patient data.** A total of 76 patients were clinically diagnosed with MG. The patient characteristics are shown in Table I and summarized in Table II. There were 46 (60.5%) males and 30 (39.5%) females. The median age at diagnosis was 45 years (range, 2-79 years). A total of 11 patients (14.5%) had thymoma; 2 patients (2.6%) had hyperthyroidism and 2 patients (2.6%) had rheumatoid arthritis. In the present study, 29 patients (38.2%) were clinically classified as ocular MG (OMG) and 47 patients (61.8%) were classified as generalized MG (gMG). In total, 42 patients (55.3%) were diagnosed with OMG initially and 13 (30.1%) of these 42 patients then developed gMG. A total of 34 (44.8%) patients were initially diagnosed with gMG.

**Anti-CL antibody levels in patients with different types of MG.** In total, 61 (80.3%) were native nAChR antibody-positive. In addition, 29 (38.2%), 27 (35.5%) and 25 samples (32.9%) were positive for antibodies to  $\alpha$ 1CL,  $\beta$ 1CL and  $\delta$ CL, respectively. The results revealed that 50% (38 of the patients with MG) had antibodies to at least one of the three CL domains of nAChR (Table II). We found that 30.3% (23 of the patients with MG) had antibodies to more than one of the three CL domains

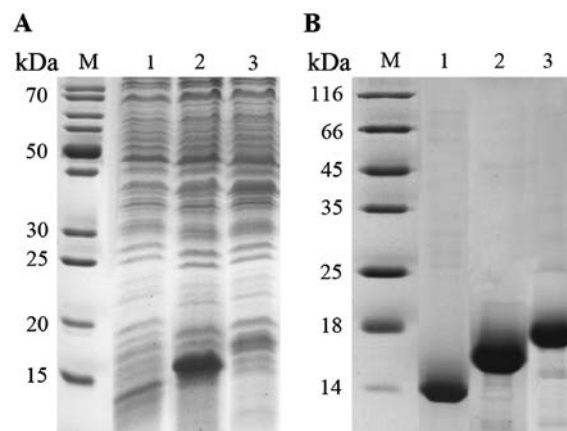


Figure 1. Expression and purification of CLs of  $\alpha$ 1,  $\beta$ 1 and  $\delta$  subunits in nAChR. (A) SDS-PAGE of recombinant CLs of  $\alpha$ 1,  $\beta$ 1 and  $\delta$  subunits in nAChR before purification. Lane M, marker; lane 1, CLs of  $\alpha$ 1 subunit; lane 2, CLs of  $\beta$ 1 subunit; lane 3, CLs of  $\delta$  subunit. (B) SDS-PAGE of recombinant CLs of  $\alpha$ 1,  $\beta$ 1 and  $\delta$  subunit in nAChR after purification. Lane M, marker; lane 1, CLs of  $\alpha$ 1 subunit; lane 2, CLs of  $\beta$ 1 subunit; lane 3, CLs of  $\delta$  subunit.

of nAChR. Of these, 20 were positive for all three CL antibodies. The results also revealed that 38 (38/61, 62.3%) were positive for anti-CL antibody among the 61 anti-ECD antibody-positive patients. These results suggested that approximately half of the patients with MG were anti-CL antibody-positive.

The levels of anti- $\alpha$ 1CL antibodies in the patients with OMG were higher compared with those of the control subjects ( $P<0.01$ ). The gMG rates were also significantly higher compared with the controls ( $P<0.001$ ); however, there was no difference in these levels between the patients with OMG and gMG (Fig. 2A). The results of  $\beta$ 1 and  $\delta$  subunits displayed a similar pattern (Fig. 2B and C). However, the levels of anti-native nAChR antibodies in gMG were higher than those in OMG (Fig. 2D). We measured absorption to the ECD of nAChR as follows: the absorption of  $^{125}\text{I}$ -toxin-labeled native nAChR minus the absorption of  $^{125}\text{I}$ -labeled CL titers. The levels of anti-ECD antibodies in gMG were also higher than those in OMG (Fig. 2E). Taken together, our findings indicated that anti-CL antibodies in the patients with MG were higher than those in the controls, particularly in the patients with gMG; however, the levels of anti-CL antibodies did not differ between the patients with OMG and gMG.

**Association between anti-CL antibody and anti-native nAChR antibody in patients with MG.** We tested sera from 76 patients with MG for antibodies directed against native nAChR and the CLs of nAChR by immunoprecipitation with  $^{125}\text{I}$ -BTX-labeled nAChR and  $^{125}\text{I}$ -labeled CLs. Three antibodies against CLs in the  $\alpha$ 1,  $\beta$ 1 and  $\delta$  subunits of nAChR were arranged in order of the increasing concentration of anti-native nAChR antibodies (Fig. 3A). In total, 11 patients (14.5%) had thymoma (Table II). Approximately half of these were anti-CL-negative. The other half of the patients with thymoma, even though they were anti-CL antibody-positive, the levels of anti-CL antibody were  $<2\text{ nM}$  (Fig. 3A).

We classified the patients with MG into 4 groups according to the concentration of anti-native nAChR antibody as  $<0.6$ , 0.6-6.6, 6.6-66, or  $>66\text{ nM}$  (Fig. 3B). We compared the positive rates of anti-CL antibodies among the 4 groups. We

Table I. Patient characteristics.

No.	Age (years)	Sex	Disease duration (months)	Maximum MGFA class	Thymus change	Other autoimmune disease	AChR titer (nmol/l)	All previous therapies at blood draw
1	46	M	9	II	Thymoma	None	38.08	PB, TS
2	9	M	36	II	None	None	6.98	PB
3	46	M	180	II	None	Hyperthyroidism	1.03	PB
4	17	M	0.3	I	n.a.	None	0.19	None
5	13	M	120	I	None	None	178.04	PB, Pred
6	35	M	84	II	None	None	0.17	PB, Pred, MMF
7	12	M	1	II	None	None	0.56	PB
8	38	F	4	II	None	None	45.48	PB
9	18	F	2	II	Thymoma	None	166.74	PB, Pred, TS
10	54	M	2	II	None	None	75.16	PB
11	22	F	2	I	None	None	135.24	None
12	31	F	8	II	None	None	60.50	PB, Pred, CYC
13	51	F	3	II	None	Hypothyroidism	33.78	PB
14	55	F	2	II	Thymoma	None	31.48	PB, TS
15	50	M	84	II	None	None	289.84	PB, Pred
16	67	M	36	II	None	None	29.08	PB, Pred, CYC
17	2.1	M	2	I	None	None	0.68	PB
18	27	F	24	II	None	None	26.58	PB, Pred, AZA
19	53	F	324	I	None	None	1.34	PB, Pred, MMF
20	55	F	6	II	None	RA	120.24	PB
21	60	M	6	II	None	None	115.70	n.a.
22	54	M	2	II	Thymoma	None	8.18	PB
23	23	F	12	II	None	None	378.14	PB, Pred
24	55	M	2	I	None	None	1.19	None
25	66	F	8	II	Thymoma	None	1.44	PB, TS
26	53	F	36	II	None	RA	125.24	PB, Pred
27	47	M	3	I	None	None	323.24	PB
28	57	M	24	I	None	None	324.64	PB
29	51	M	3	II	None	None	4.35	n.a.
30	30	M	11	I	None	None	0.90	n.a.
31	51	M	0.6	I	n.a.	None	87.63	PB
32	39	F	360	II	None	None	21.18	PB
33	67	M	72	II	None	None	80.96	n.a.
34	46	F	3	II	None	None	1.76	PB
35	26	F	12	II	None	None	506.84	PB
36	23	M	36	I	None	None	0.47	PB, Pred, AZA
37	16	M	2	I	None	None	1.70	None
38	50	F	0.3	II	n.a.	None	23.98	None
39	13	F	60	II	None	None	91.76	PB, Pred
40	71	M	0.6	II	n.a.	None	201.64	None
41	39	M	3	II	None	None	54.10	None
42	13	M	132	I	None	None	68.02	PB, Pred, AZA
43	17	M	36	II	None	None	16.28	PB
44	32	F	180	I	None	None	105.07	PB, CYC, IVIg
45	50	M	24	II	None	None	51.94	PB
46	61	M	480	III	None	None	2.01	PB, Pred, CYC
47	61	F	84	II	None	None	11.38	n.a.
48	47	M	60	II	Thymoma	None	61.57	PB, TS
49	9	M	8	II	Thymoma	None	0.44	PB, TS
50	28	M	264	II	None	None	145.44	PB, Pred, AZA, IVIg
51	47	M	60	III	Thymoma	None	483.84	PB, Pred, AZA, IVIg, TS

Table I. Continued.

No.	Age (years)	Sex	Disease duration (months)	Maximum MGFA class	Thymus change	Other autoimmune disease	AChR titer (nmol/l)	All previous therapies at blood draw
52	55	M	4	II	None	None	0.58	None
53	55	M	10	I	None	None	397.14	None
54	3	M	0.6	I	n.a.	None	0.50	None
55	65	M	0.3	II	None	None	0.52	None
56	64	M	2	II	None	None	359.54	None
57	34	F	120	II	Thymoma	None	4.33	PB, Pred, AZA, TS
58	59	F	9	I	None	None	0.07	PB
59	50	M	4	II	Thymoma	None	9.58	PB, TS
60	17	M	24	I	None	None	458.84	PB, Pred
61	24	F	8	II	None	None	57.50	PB
62	24	F	8	II	None	None	156.24	PB
63	79	F	24	II	Thymoma	None	437.64	PB, Pred, AZA, IVIg, TS
64	45	M	60	I	Thymoma	None	226.94	PB, Pred, TS
65	24	F	8	II	None	None	42.98	PB
66	51	F	24	II	None	None	18.28	None
67	24	F	0.1	II	None	None	189.64	None
68	10	F	36	II	None	None	6.33	PB, Pred, AZA
69	2.6	M	1	I	None	None	13.48	PB
70	31	M	156	I	None	None	39.98	n.a.
71	11	M	96	I	None	None	35.98	PB
72	27	M	1	I	None	None	214.04	None
73	27	F	0.6	II	n.a.	None	3.27	PB, Pred, AZA
74	51	F	10	II	None	None	3.66	PB
75	67	M	12	II	None	None	7.35	PB, Pred, CYC
76	42	F	24	I	None	None	48.78	None

M, male; F, female; n.a., not available; RA, rheumatoid arthritis; PB, pyridostigmine bromide; TS, thymuslectomy; Pred, prednisone/prednisone; MMF, mycophenolate; CYC, cyclophosphamide; AZA, azathioprine; IVIg, intravenous immunoglobulin.

found that no patients were anti-CL antibody-positive in the sera titer <0.6 nM group. In the group with sera titers between 0.6 and 6.6 nM, anti-CL positivity was 20%. The anti-CL antibody positive rates in the sera titer group between 6.6 and 66 nM group was 50%, and 40% in the >66 nM group. We randomly selected samples from the 4 groups to further analyze the consistency in the 3 anti-CL antibodies. We found that the 3 anti-CL antibodies exhibited similar results, particularly in the low titer of anti-CL antibodies in patient nos. 6 and 52 (Fig. 3C).

We examined the correlation of antibodies directed against the CLs of nAChR and native nAChR. The results revealed no correlation between antibodies to CLs and antibodies to native nAChR (Fig. 4A-C). Likewise, antibodies to CLs of nAChR were not associated with antibodies to ECD of nAChR (Fig. 4D-F).

*No relevance of anti-CL antibody with sex, age and MG type in patients with MG.* We assumed the sample was anti-CL antibody-positive if any of the 3 anti-CL antibody levels (from subunits  $\alpha 1$ ,  $\beta 1$  and  $\delta$ ) was >0.6 nM (21). Out of the 29 patients with OMG, 14 (48%) were anti-CL-positive; of the 47 patients

with gMG, 24 (51%) were anti-CL-positive. However, there was no significant difference in anti-CL positivity between the patients with OMG and gMG. According to the age at onset, MG was classified into early-onset MG (EOMG) and late-onset MG (LOMG). We found no statistically significant difference in the anti-CL antibody positivity rates between the patients with EOMG and LOMG (Table III). Apart from the frequency of positive antibody, the levels of anti-CLs, anti-native nAChR and anti-ECD antibodies were compared between sexes, the type of MG according to the onset age and the onset MG type. The results revealed no differences in the level of anti-CL antibodies between sexes, the type of MG according to the age at onset and the type of MG at onset. The results were comparable with those of the anti-native nAChR antibody and anti-ECD antibody (data not shown).

## Discussion

In the present study, we successfully expressed and purified the  $\alpha 1$ ,  $\beta 1$  and  $\delta$  subunits of CL in nAChR, which are common in both fetal and adult nAChR. We detected anti-CL antibodies

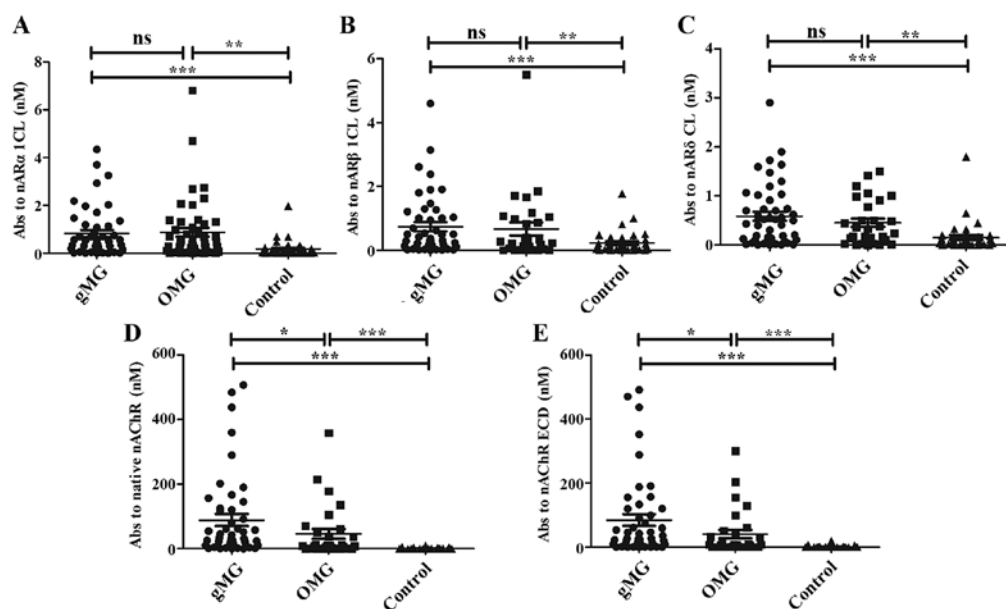


Figure 2. Levels of nAChR antibody compared between different MG types. Antibodies directed against CLs in  $\alpha 1$ ,  $\beta 1$  and  $\delta$  subunits and against native and ECD in nAChR compared in the controls and in patients with OMG and gMG. All serum was tested 3 times; each data point represents the mean of individual subjects. Horizontal lines represent the group median. ns, no significance ( $P>0.05$ ); \* $P<0.05$ ; \*\* $P<0.01$ ; \*\*\* $P<0.001$ . nAChR, nicotinic acetylcholine receptor; MG, myasthenia gravis; CL, cytoplasmic loop; ECD, extracellular domain; OMG, ocular myasthenia gravis; gMG, generalized myasthenia gravis.

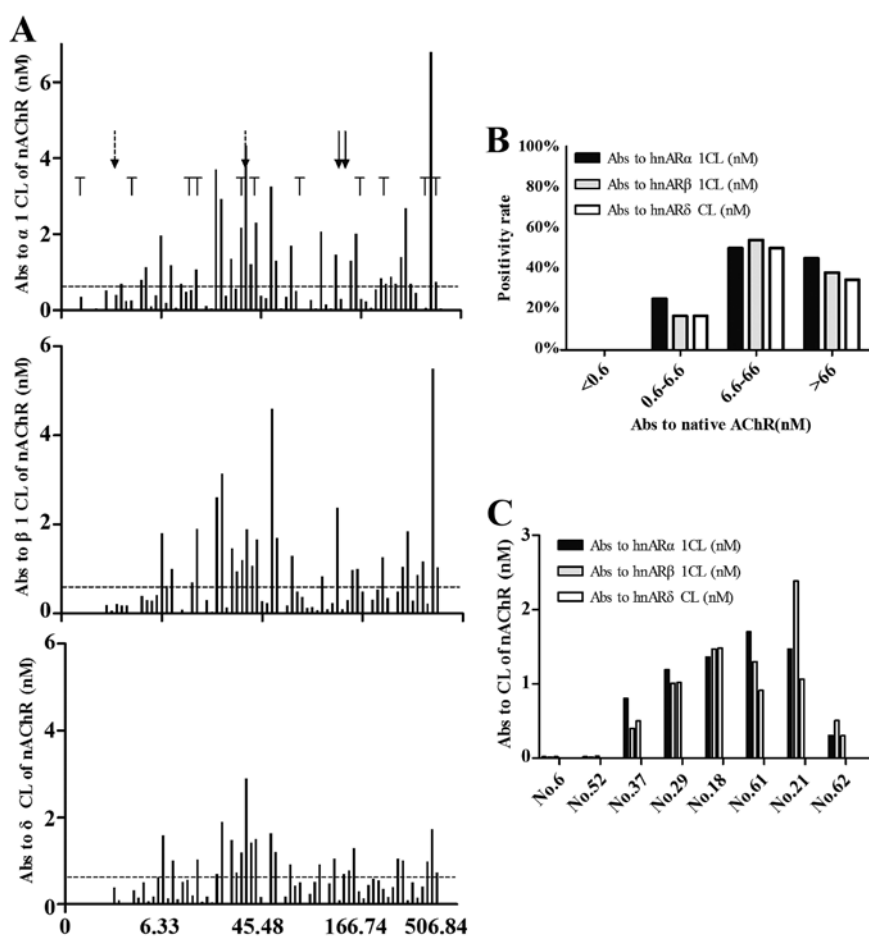


Figure 3. Radioimmunoassay detected antibodies directed against 3 nAChR subunits ( $\alpha$ ,  $\beta$ ,  $\delta$ ) CL from sera of patients with MG.  $^{125}\text{I}$ -labeled AChR $\alpha 1$ CL,  $^{125}\text{I}$ -labeled AChR $\beta 1$ CL and  $^{125}\text{I}$ -labeled AChR $\delta$ CL were incubated with  $10\ \mu\text{l}$  of one of 76 MG patient sera, the Ab-bound labeled AChRCL was immunoprecipitated using rabbit anti-human immunoglobulin sera. (A) The sera are arranged in order of increasing concentration (nM); the 20th, 40th, 60th and 76th sera are indicated. The horizontal dotted lines indicate the concentration of the antibodies in sera  $\geq 0.6$  nM. 'T' marks indicate sera from 12 patients with MG with thymoma. Arrows with solid and dashed line indicate patients with MG with hyperthyroidism and RA, respectively. (B) The positive rate of anti-CL antibodies are displayed in 4 groups according to the sera titer of anti-native nAChR. We assume that any type of anti-CL antibody  $>0.6$  nM was antibody positive. (C) We selected 8 patients randomly from the 76 patients with MG, and compared the coherence of 3 types of anti-CL antibodies. nAChR, nicotinic acetylcholine receptor; CL, cytoplasmic loop; MG, myasthenia gravis.

Table II. Demographic and clinical characteristics of the 76 patients with MG.

Variable	Value (no. of patients, %)
Sex	
Male	46 (60.5)
Female	30 (39.5)
MG type according to age at onset	
EOMG	51 (67.1)
LOMG	25 (32.9)
MG type	
OMG	29 (38.2)
gMG	47 (61.8)
MG type at onset	
OMG	42 (55.3)
gMG	34 (44.8)
With thymoma	11 (14.5)
With hyperthyroidism	2 (2.6)
With RA	2 (2.6)
Native AChR Abs positivity	61 (80.3)
CL Abs positivity	38 (50.0)
α1CL Abs positivity	29 (38.2)
β1CL Abs positivity	27 (35.5)
δCL Abs positivity	25 (32.9)
More than one CL Abs positivity	23 (30.3)
All three CL Abs positivity	20 (26.3)

MG, myasthenia gravis; EOMG, early-onset myasthenia gravis; LOMG, late-onset myasthenia gravis; OMG, ocular myasthenia gravis; gMG, generalized myasthenia gravis; RA, rheumatoid arthritis; ECD Ab positive, antibodies to extracellular domain of human nicotinic acetylcholine receptor  $\geq 0.6$  nM; CL Ab positive, any of three kinds of antibodies against to cytoplasmic loop  $\geq 0.6$  nM; α1CL Ab, antibodies to cytoplasmic loop in α1 subunit  $\geq 0.6$  nM; β1CL Ab, antibodies to cytoplasmic loop in β1 subunit  $\geq 0.6$  nM; δCL Ab, antibodies to cytoplasmic loop in δ subunit  $\geq 0.6$  nM.

in 76 patients with MG and found that the levels of any of the 3 anti-CL antibodies were significantly higher in the patients with MG compared with the controls.

It is important to address the significance of the existence of antibodies directed against CL of nAChR. It is important to determine whether these antibodies directed against CL of nAChR play a functional role in MG. Several studies have reported that anti-CL antibody of nAChR plays a protective role in the development of EAMG (11-13). For example, Luo *et al* demonstrated a therapeutic vaccine consisting of bacterially-expressed human muscle nAChR cytoplasmic domains for the antigen-specific immunosuppression of MG to be safe, robust and long-lasting (15). Several studies have confirmed the existence of anti-CL of nAChR antibodies in both patients with MG and EAMG (22-24). However, the mechanisms involved are still unknown. It has been suggested that B cells are suppressed by the secreting antibody, supporting the inhibitory receptor FcγRIIB on the surface of B cells (25,26).

Table III. Clinical differences between patients who were CLAb-positive and those that were CL Ab-negative.

Variable (no. of patients)	CL Abs (n=38)		P-value
	Positive (%)	Negative (%)	
Sex			
Male (46)	23 (50)	23 (50)	1
Female (30)	15 (50)	15 (50)	
MG type according to age at onset			
EOMG (51)	27 (53)	24 (47)	0.46
LOMG (25)	11 (44)	14 (56)	
MG type			
OMG (29)	14 (48)	15 (52)	0.81
gMG (47)	24 (51)	23 (49)	
MG type at onset			
OMG (42)	19 (45)	23 (55)	0.36
gMG (34)	19 (56)	15 (44)	

MG, myasthenia gravis; EOMG, early-onset myasthenia gravis; LOMG, late-onset myasthenia gravis; OMG, ocular myasthenia gravis; gMG, generalized myasthenia gravis; CL Ab, antibodies to human nicotinic acetylcholine receptor cytoplasmic loop.

It is tempting to speculate that following the immunization of the CLs of nAChR, the levels of anti-CL antibodies increase and clinical symptoms are relieved. This may be due to the combination of the antigen-antibody complexes and inhibitory receptor FcγRIIB, which strongly inhibits specific B cells that produce anti-ECD antibodies (27) in patients with MG. Based on these data, we proposed a negative feed-back theoretical model to explain how the anti-CL loop protects against EAMG in animal models (Fig. 5). In this proposed mechanism, B cells specific to the ECD of the α subunit capture the nAChR, which is bound with an antibody to CL of the δ (Fig. 5A), or α (Fig. 5B) subunits, or the ECD of α subunit (Fig. 5C). The Fc portions of these antibodies bind to the FcγRIIB, an inhibitory receptor on the surface of the same B cell, leading to inactivation of those B cells.

However, even though the levels of anti-ECD antibody in patients with gMG were higher than those in patients with OMG, there were no differences observed in the levels of anti-CL antibodies between patients with OMG and gMG in our results. There was also no correlation between anti-CL and anti-ECD antibody. These findings suggest that anti-CL antibodies may be not beneficial antibodies in patients with MG.

In fact, EAMG differs from MG in that EAMG is triggered by immunizing animals with detergent-solubilized native nAChR, such as Triton X-100. Electron microscopy studies have indicated that these Triton X-100-solubilized nAChRs are embedded in tubular and globular structures formed by membrane lipids and detergent micelles (28,29). The CLs of nAChR were exposed to the immunological environment rather than protected by the cellular membranes, which can mediate the immune response at the initiation of EAMG. Although

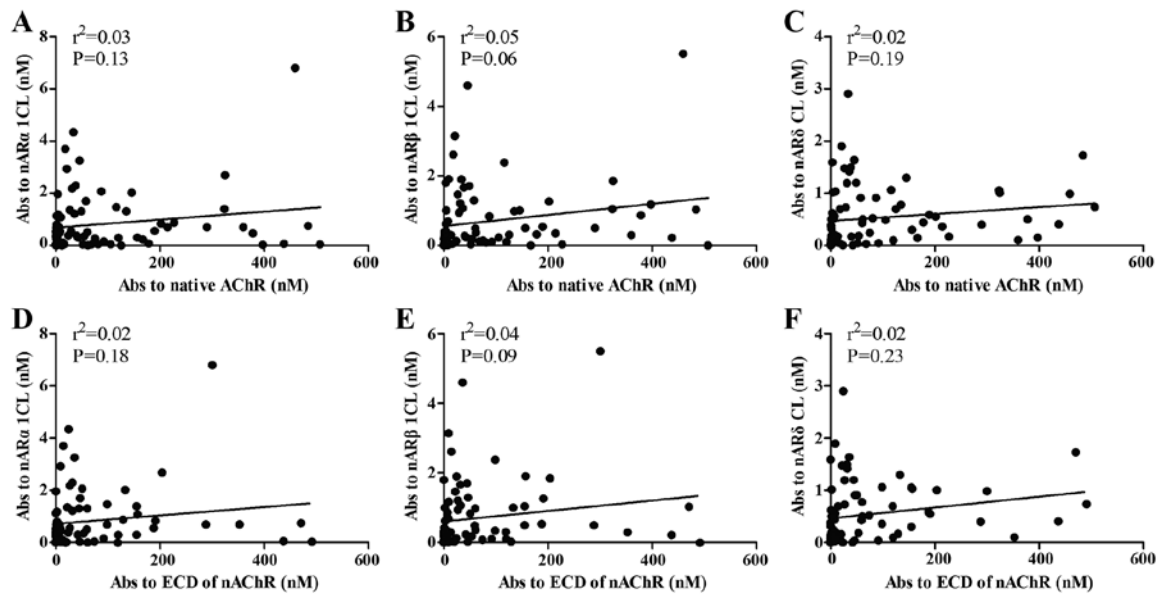


Figure 4. Correlations between anti-CL antibodies and anti-ECD antibodies. (A-C) The correlation between the concentration of antibodies to CL in  $\alpha 1$ ,  $\beta 1$  and  $\delta$  subunit of nAChR (nM) and antibodies to native nAChR. (D and E) The correlation between the concentration of antibodies to CL in  $\alpha 1$ ,  $\beta 1$  and  $\delta$  subunit of nAChR (nM) and antibodies to ECD of nAChR. The titers of antibodies to ECD of nAChR was converted according to the absorption of  $^{125}\text{I}$ -toxin-labeled native AChR minus the adsorption of  $^{125}\text{I}$ -labeled CL. (A-F) Each data point represents individual subjects. The oblique line is a line with linear regression. The regression coefficient and the statistical significance are shown as  $r^2$  and P-values. CL, cytoplasmic loop; ECD, extracellular domain; nAChR, nicotinic acetylcholine receptor.

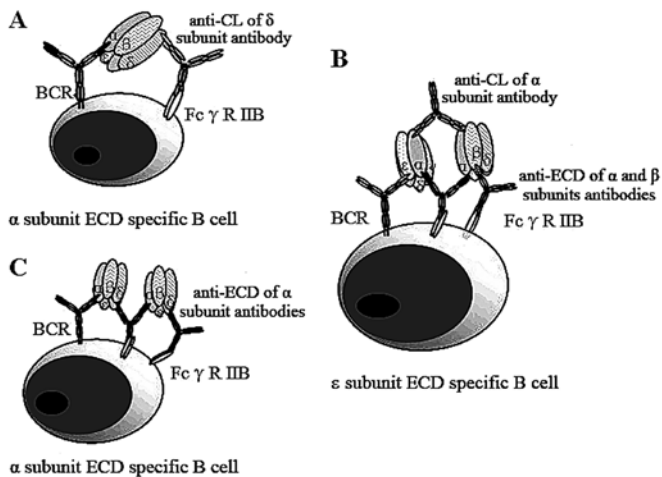


Figure 5. Proposed mechanism of inhibition of specific B cell by anti-CL of nAChR antibody. (A) Specific B cell to ECD of  $\alpha$  subunit captured an antigen of nAChR. The antigen nAChR was bound with an-anti-CL of  $\delta$  subunit antibody. The Fc portion of this antibody connected to the FcγRIIB inhibitory receptor on B cell and prevented this B cell from producing antibodies to ECD of  $\alpha$  subunit. (B) Specific B cell to ECD of  $\epsilon$  subunit captured nAChR, which cross linked another nAChR by antibody to the CL of subunit. The corresponding antibodies to ECD of the two nAChR connected to the FcγRIIB inhibitory receptor and prevented B cell from producing antibodies to ECD of  $\epsilon$  subunit. (C) Specific B cell to ECD of  $\alpha$  subunit captured a complex of nAChR- antibody Fc portions of these antibodies in the complex connected to the FcγRIIB inhibitory receptor on the B cell and prevented B cell from producing antibodies to ECD of  $\alpha$  subunit. nAChR, nicotinic acetylcholine receptor; CL, cytoplasmic loop; ECD, extracellular domain.

we can explain the protective effect of anti-CL antibodies on EMAM using a negative feed-back theory, it does not confirm that this mechanism exists in patients with MG. This means that anti-CL antibody may not serve as a negative feed-back bridge if the CL of nAChR that triggers the autoimmune response in MG patient is not exposed to the environment.

Since there was no beneficial effect of anti-CL antibody in patients with MG, it would be beneficial to determine how anti-CL antibody could possibly occur and what the role of anti-CL antibody is in patients with MG. CLs are not exposed on the cell surface in healthy humans; thus, no anti-CL antibodies are present. In patients with MG with anti-nAChR antibody, nAChR is destroyed through antigen-antibody reaction. The CL is exposed as the nAChR is destroyed. Therefore, anti-CL antibodies are likely produced as a result of pathological changes. However, the function of anti-CL antibody in patients with MG warrants further investigation.

In addition, we found that approximately 50% of MG sera contained antibodies directed against the CL of nAChR. This is significantly higher than findings from earlier experiments that measured the ability of a monoclonal antibody (mAb) to inhibit the binding of MG sera to the nAChR (30), and also measured the antibody of MG sera to inhibit the binding of a MoAb detected against the CL of the nAChR (14). Our results showed that approximately a quarter of patients with MG with thymoma were anti-CL antibody-positive. This is higher than the finding of a previous study that found that no patients with MG with thymoma were anti-CL antibody-positive (14). This can be explained by the following reasons: i) the experimental methods were different. Our radioimmunoassay was direct and more sensitive. Previous methods used competition experiments that indirectly detected antibodies, missing minor antibody populations in MG sera. Due to the specificity of the MoAb used, those experiments only detected the presence of antibodies against certain epitopes of CL of  $\alpha$  and  $\beta$  subunits. The detection of a narrow portion of anti-CL antibodies was widened by radioimmunoassay. ii) Patients with MG in different countries had different conditions. In our study, 14.5% of patients with MG had thymoma; however, only 5.3% had thymoma in the previous study (14). iii) Patients with



MG may have been treated with different medicines. Drugs, such as corticosteroids and immunosuppressive agents affect the immune system, particularly the expression of antibodies.

No patients in the sera titer <0.6 nM group were positive for anti-CL antibodies. A higher positivity rate was concentrated in the sections of sera titer between 6.6 and 66 nM, and in sera titer >66 nM. These results suggest that the anti-CL antibodies are prone to be distributed in MG with a higher sera titer. In 11 patients with thymoma-associated MG, 10 were anti-ECD positive, but only 4 patients were anti-CL-positive (data not shown). We thus questioned whether there was an association between these two antibodies. The number of tested thymoma sera in this study was too small to draw definitive conclusions.

It should be noted that the present study was retrospective. The majority of patients coming to our hospital had been diagnosed and treated in their local primary hospital. Thus, as a preliminary exploration, we included the ready-made sera from patients with MG. Currently, we are collecting the sera of patients with MG before and after treatment for further analysis. Our preliminary study revealed that the titers of anti-CL antibodies between patients with MG with and without immunosuppressive therapy exhibited no statistically significant difference (unpublished data). Therefore, we assumed that anti-CL antibodies may not be affected under the immunosuppressive therapy.

The limitation of the present study was that the sample size was not large enough. We aim to keep collecting the sera of patients with MG to enlarge the number of samples in future studies. Despite its preliminary character, our findings still provide the first clinical evidence that anti-CL antibodies may be not a beneficial antibody in patients with MG.

In conclusion, we found a phenomenon that there was no correlation between anti-nAChR CL and the severity of MG. Anti-CL antibody in patients with MG may not seem to be predictive of the severity of MG; however, additional studies are warranted to uncover the clinical implications of anti-CL antibody in patients with MG.

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