Anti-acetylcholine receptor autoantibodies in myasthenia gravis : pathogenicity and specificity related to their structure

Anti-Acetylcholine Receptor Autoantibodies in Myasthenia Gravis

Pathogenicity and specificity related to their structure

In de drukkosten van het proefschrift werd bijgedragen door de Internationale Stichting Alzheimer Onderzoek

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PROEFSCHRIFT

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Contents

Abbreviations

α-BT	α-bungarotoxin
AChR	acetylcholine receptor
CDR	complementarity determining region
CFA	Complete Freund's Adjuvant
CH	heavy chain constant region
CL	light chain constant region
D gene	diversity gene
EAMG	experimental autoimmune myasthenia gravis
ELISA	enzyme-linked immunosorbent assay
Fab	antigen binding fragment
HRP	horseradish peroxidase
IFA	Incomplete Freund's Adjuvant
IMAC	immobilized metal affinity chromatography
JH	heavy chain joining gene
Jκ	κ chain joining gene
mAbs	monoclonal antibodies
MG	myasthenia gravis
MIR	main immunogenic region
PBS	phosphate buffered saline
PCR	polymerase chain reaction
RIA	radioimmunoassay
ScFv	single chain variable fragment
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
VH	heavy chain variable region
VL	light chain variable region

Introduction

Myasthenia gravis

Myasthenia gravis (MG) is an organ-specific autoimmune disease mediated by autoantibodies directed against acetylcholine receptor (AChR) on postsynaptic membrane in neuromuscular junction[1, 2]. The first description of patients with MG-like symptoms was made by an English physician Thomas Willis in 1672[3] and the name myasthenia gravis was introduced in 1895 by Jolly. However, the autoimmune nature of MG had not been proposed until 1960s when Simpson and other authors noticed that high frequency of thymus abnormalities appeared in MG patients, components in sera of MG patients could damage nerve and muscle tissues, and serum complement levels were lower in MG patients[4-6]. In 1973, Patrick and Lindstrom first demonstrated the autoimmune nature of MG in experimental rabbits immunized with AChR isolated from electric eel resulting in muscular weakness which resembled human MG[7]. Since then much information about the pathogenetic mechanisms has been revealed[8].

The clinical signs and symptoms in MG result from loss of functional AChR which is entirely antibody-mediated[9]. Anti-AChR antibodies are detectable in approximately 85% - 90% of the MG patients, whereas the other seronegative MG patients (approximately 10% - 15%) may caused by antibodies against other membrane proteins in the synapse including muscle-specific receptor tyrosine kinase (MuSK) (A. Vincent, personal communication) or by a non-immunoglobulin factor[10]. Thymic hyperplasia and thymomas are found in 75% of the MG patients[11]. Further study on MG thymus shows that the thymus contains components necessary for production of autoantibodies against AChR[12-15].

The prevalence of MG is 60-70 per million and annually 2.5-9.8 per million[16]. Many MG patients develop generalized MG and approximately 15% of the MG patients remains ocular MG (only ocular muscle weakness). Presently, there is no specific immunosuppressive therapy for MG. Although the prolonged nonspecific immunosuppressive therapies currently used in treatment of MG can control MG to some extent and reduce its mortality[17], there are still some significant side effects.

Acetylcholine receptor

Muscle-type AChR in neuromuscular junction is a complex transmembrane glycoprotein of molecular weight 290 KD, consisting of five subunits in a stochiometry $\alpha_2\beta\gamma\delta$[18-20] in fetal muscle and $\alpha_2\beta\varepsilon\delta$ in adult muscle. Each subunit has 4 transmembrane segments (M1-M4)[21]. The M1-M3 are key structure for the ionchannel formation[22-24]. The extracellular domain, formed by the N-terminal 210 amino acids and followed by M1-M3 contains the acetylcholine (ACh) binding site[25, 26], which is formed at the interfaces between α and γ or ε subunits and between α and δ subunits[27, 28]. The intracellular domain forms a loop between M3 and M4. The homology between Torpedo and mammalian AChR is about 80% for the α-subunits and 55% for the other subunits[29-32], and the homology between equivalent subunits from mammalian species is 97% for α-subunits and 90% for the other subunits[33]. Therefore, Torpedo and other electric rays and eels provide a rich source of AChR for characterization of receptor and for induction of an experimental autoimmune myasthenia gravis (EAMG) model of MG in mammals[7].

A characteristic feature of the AChR is the presence of a main immunogenic region (MIR) to which more than half of the autoantibodies in MG patients or EAMG rats are directed[34-37]. The MIR is located on the α-subunit at residues 67-76[38-40], and its immunodominant epitopes are mainly formed by residues 68-71[41]. The MIR easily accessible in vivo to antibodies probably depends on its localization on extracellular surface and an unusual conformation[42]. The binding site of α-

bungarotoxin(α-BT) is demonstrated at residues 189-199 of α-subunit[43, 44] which overlaps the ACh binding site.

Experimental autoimmune myasthenia gravis

The first experimental animal model of MG was discovered in 1973 by Patrick and Lindstrom in rabbits which were immunized with purified AChR from electric eel and developed muscle weakness[7]. EAMG, closely resembling MG in both immunological and clinical signs can be induced in mice[45], rats and guinea pigs[8], and monkeys[46] by active immunization of purified AChR or passive transfer of anti-AChR antibodies. In EAMG induced by injection of AChR incorporated in Complete Freund Adjuvant(CFA), animals develop clinical signs 4-6 weeks after immunization with peak of anti-AChR antibodies in sera[47]. AChR from different species of animals or synthetic and recombinant AChR[48-52] are able to induce EAMG. In EAMG induced by injection of anti-AChR antibodies[45, 53-55], animals develop similar clinical signs 12-48 h after injection[56].

Pathogenesis in MG and EAMG

Several lines of study have demonstrated that MG and EAMG are entirely mediated by antibody. Mice receiving repeated injection of IgG purified from MG patient sera develop EAMG[45, 57]. Mothers with MG can passively transfer MG to their babies[58, 59]. Treatment by plasmaphoresis to remove antibody is beneficial for MG patients[60-62]. The AChR inactivation and degradation which impair signal transmission in the neuromuscular junction leading to muscle weakness are finally shared by a common pathway of antibody-mediated autoimmune attack to muscle AChR. The possible mechanisms in MG and EAMG are as follows.

1. Antigenic modulation. The binding of anti-AChR antibodies to AChR by cross-linking of adjacent AChR molecules resulting in accelerating AChR internalization is called antigenic modulation. Antibodies directed against the MIR are especially effective

in modulating the AChR[63]. The MIR is angled outward from the central axis of the AChR. A single bivalent anti-MIR antibody can not cross-link the two α-subunits within an AChR, however, it can position the cross-linking of adjacent AChR into aggregates[64], causing an increase in the rate of internalization and degeneration of AChR[36, 37, 65]. Antigenic modulation is independent of complement in vitro[65, 66].

2. Complement-mediated lysis. The binding of anti-AChR antibodies to AChR can also trigger the complement cascade, resulting in formation of membrane attack complex (MAC) which leads to focal lysis of postsynaptic membrane[55]. The severe muscular weakness or death seen in EAMG model of passive transfer of anti-AChR antibodies is likely caused by the fact that the effects of anti-AChR antibodies binding to AChR are amplified by C3 component of complement, which attracts phagocytes to mediate an antibody- and complement-dependent cell-mediated cytotoxic reaction[55, 67].

3. Inactivation of AChR. Less importantly, the binding of anti-AChR antibodies to AChR can block ACh from binding to AChR[68-72] or inhibit ion channel function[73].

Immunological therapy

The ideal immunosuppressive therapy for MG would be to eliminate the autoimmune response to AChR without affecting the immune response to other antigens. Unfortunately, no specific immunosuppressive therapy is currently applied in MG. Nonspecific immunosuppressive therapies such as immunosuppressants[74-77], thymectomy[78, 79] and plasmaphoresis [60-62, 80, 81] can control symptoms of MG and greatly reduce its mortality, however, side effects related to these therapeutic methods are also seen.

Several specific immunosuppressive therapies have been tested in EAMG based on the pathogenesis of MG. Anti-idiotypic antibodies to a mAb directed against AChR can prevent the induction of EAMG caused by the idiotype mAb[82]. Administration of AChR orally[83-85] or nasally[86-88] can suppress the immune response mediated by AChR specific T and B cells and prevent or inhibit clinical signs of EAMG. Since most

of the antibodies to AChR in MG and EAMG are directed against MIR and thus pathogenic, development of a therapeutic agent which can bind to MIR, just blocking the binding of anti-MIR antibodies to AChR, but does not have the activities to induce antigenic modulation or complement reaction has been the focus of many researches in MG. Fab fragment or single chain variable fragment (ScFv) of antibody are good candidates for this aim. Fab isolated from MG patient thymus[89, 90] and from rat anti-MIR mAbs[91, 92] or ScFv constructed from rat anti-MIR mAbs[37, 93, 94] can prevent anti-MIR mAbs or MG patient sera from binding to AChR or from inducing antigenic modulation in cultured TE671 cell line.

Mouse EAMG induced by human anti-AChR antibodies

The importance of human immunoglobulins in the pathogenesis of MG was first demonstrated in mice, which developed clinical signs of MG upon multiple injections of MG serum, by Toyka et al in 1975[45]. Another earlier study, in which MG patient thymus tissue or its single-cell suspensions was transplanted into severe combined immunodeficiency (SCID) mice, showed that human anti-AChR antibodies were produced in mouse sera, and also found at muscle end-plates, but no clinical signs of muscular weakness were observed[95]. This model was used to determine the role of T-helper cells in the pathogenesis of MG[96]. Mice have strain-dependent susceptibilities to EAMG, although anti-AChR antibody titers in sera and fine specificities are comparable[97, 98]. Mice transgenic for different HLA genes also have different susceptibilities to EAMG[99]. Since the mice transgenic for human immunoglobulin loci were established[100], it is possible to develop a new transgenic mouse EAMG model. The use of human genes and molecules in animal EAMG clearly matches the model more closely to MG.

Aim of the study

One of the key questions in understanding the pathogenesis of MG and EAMG induced by pathogenic anti-AChR autoantibodies is the antibody structure, which will be beneficial to the development of a potentially specific therapy for MG. Therefore, the first part of this thesis (Chapter 1 to Chapter 3) was focussed on the production and characterization of anti-AChR mAbs made from mice immunized with AChR, and the nucleotide and amino acid sequences of variable regions of the mAbs in relation with their pathogenicity and specificity. The second part of this thesis (Chapter 4) was concentrated on the construction and characterization of an human ScFv derived from an anti-MIR autoantibody isolated from a MG patient. The ScFv might be useful in specific immunotherapy of MG after appropriate genetic improvement in stability and affinity based on the understanding of antibody structure described in Chapter 2 and Chapter 3. The third part of this thesis (Chapter 5) was emphasized on the development of a new transgenic mouse EAMG model producing human immunoglobulins. This model is expected to be the first step towards an ideal EAMG model of mice transgenic for human immunoglobulin and HLA loci, and a source of human anti-AChR antibodies.

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Chapter 1

Production and characteristics of anti-acetylcholine receptor monoclonal antibodies in mice

Abstract

A mouse model of myasthenia gravis (MG), experimental autoimmune myasthenia gravis (EAMG), was used to produce monoclonal antibodies (mAbs) directed against the autoantigen, acetylcholine receptor (AChR). C57bl/6 and Balb/c mice were immunized with purified Torpedo AChR (tAChR) and human AChR (hAChR), and boosted 3 and 5 weeks after primary immunization. The mice were sacrificed 3 days after the last injection and cells from lymphnodes were fused with mouse myeloma cell line SP2/O-Ag14 or NS1. Hybridomas were initially screened for reactivity to AChR by ELISA. Most of the anti-AChR mAbs were found to be IgG1 and IgG2b as determined by a mouse isotyping kit, and some of them were cross-reactive with mouse and rat AChR as showed in radioimmunoassay (RIA) using mouse and rat muscle crude extracts as antigens. Determination of fine specificity of the anti-AChR mAbs binding to AChR using competitive ELISA or RIA showed that four different groups of the mAbs were identified: anti-main immunogenic region (MIR) mAbs (rat anti-MIR mAbs 35 as reference competitor), anti-α-bungarotoxin (α-BT) binding site mAbs (α-BT as reference competitor), anti-extracellular epitope mAbs and anti-intracellular epitope mAbs

Introduction

Myasthenia gravis (MG) is an autoimmune disease mediated by autoantibodies directed against acetylcholine receptor (AChR)[1]. Anti-AChR antibodies binding to AChR on postsynaptic membrane in neuromuscular junction result in AChR loss either by cross-linking of adjacent receptor molecules thereby accelerating the AChR turnover rate[2,3] or by activation of complement cascade leading to focal lysis of the muscle membrane[4,5]. The binding of anti-AChR antibodies may also result in functional inactivation of the AChR by either interfering with acetylcholine binding[6-10] or inhibiting ion channel function[11]. The animal model of MG, experimental autoimmune myasthenia gravis (EAMG), closely resembling human MG in both immunological and clinical signs, can be induced by active immunization with purified AChR or passive transfer of anti-AChR antibodies[12,13].

The AChR is a well characterized autoantigen[14]. The pathogenic antibodies are mainly found to be those that are directed against a region on the α-subunit of AChR, which includes the amino acid sequence 67-76, termed main immunogenic region (MIR)[15]. However, blocking antibodies have been demonstrated in sera of MG patients by inhibition of α-bungarotoxin (α-BT) binding to AChR in muscle culture or TE671 cells[16,17]. Therefore, the contribution of individual pathogenic anti-AChR antibodies to AChR loss or inhibition of AChR function is important for understanding the pathogenesis of MG or EAMG.

The anti-Torpedo AChR (tAChR) monoclonal antibodies (mAbs) were originally produced by Dr Y. Graus et al[18], and anti-human AChR (hAChR) mAbs by Dr P. Whiting et al[19]. Since this work formed the basis of the research in Chapter 2 and 3, we describe here the main techniques used in production and characterization of the mAbs in order to further investigate the pathogenicity of mAbs in relation to their structure.

Materials and methods

Animals

C57bl/6 and Balb/c mice of 8 weeks old (about 20 g body weight) were purchased from Charles River Wiga GmbH, Frankfurt, Germany and maintained under special pathogen free conditions.

Antigens

TAChR, extracted from electric organ of Torpedo californica (Pacific Biomarine, California, USA) and purified by affinity chromatography on Naja naja siamensis toxin (Miami Serpentarium, Florida, USA) linked to Sepharose-4B (Pharmacia LKB, Woerden, The Netherlands) according to a procedure described by Lindstrom et al[20], and hAChR, obtained from amputated limbs of a patient without vascular or neurological disease and purified as above, were used as antigens to immunize mice.

Production of hybridomas

Mice were hypodermically immunized with 15 μg tAChR (diluted with PBS) in Complete Freund Adjuvant (CFA) at base of the tail, and boosted 3 and 5 weeks after primary immunization with 15 μg tAChR in Incomplete Freund Adjuvant (IFA). The mice were sacrificed 3 days after the last injection. Lymphocytes from lymphnodes paraaortal, inguinal and poplitial were fused with the mouse myeloma cell line SP2/O-Ag14 according to Kohler and Milstein[21]. For the production of anti-hAChR mAbs, 12 pmol hAChR in CFA was used in primary immunization, 16 pmol hAChR in IFA in the first booster immunization and 40 pmol hAChR in PBS only in the second booster immunization. The fusion was performed with mouse myeloma cell line NS1.

Screening of mAbs

MAbs were initially screened for reactivity to tAChR by ELISA. Briefly, ELISA plates were coated with 5 μg/ml tAChR in 10 mM sodium bicarbonate buffer (pH 9.5) for 1 h at 37°C (50 μl/well). The plates, after washed 3 times with distilled water containing 0.5% Tween-20 (dH_2O/Tw), were blocked with PBS containing 0.5% bovine serum albumin (BSA) and 0.5% Tween-20 for 15 min at room temperature. Hybridoma culture supernatant (100 μl) were added to each well and the plates were incubated at 4°C overnight on a rocking platform. After washing 5 times with dH_2O/Tw, the plates were incubated with rabbit anti-mouse immunoglobulin coupled with horseradish peroxidase (HRP) (Dako ITK diagnostics, Uithoorn, The Netherlands) for 1 h at room temperature on a rocker. After washing the colormetric reaction was developed for 10 min at room temperature by addition of substrate solution of tetramethylbenzidine (TMB) (100 μg/ml TMB, 0.01% H_2O_2, 110 mM sodium acetate, pH 5.5) and stopped by adding 50 μl of 2 M H_2SO_4. The optical density (OD) of individual wells was measured at 450 nm using Titertek Twinreader (Amstelstad, Amsterdam, The Netherlands).

Cell lines secreting anti-hAChR mAbs were determined by radioimmunoassay (RIA) as described below using hAChR.

Isotype of mAbs

The isotypes of mAbs were determined by an ELISA using a mouse isotyping kit (Holland Biotechnology, Leiden, The Netherlands) according to the manufacturer's instructions.

Fine specificity of anti-AChR mAbs

MAbs were screened for reactivity to the MIR on AChR by a competitive inhibition ELISA using mAb 35[22] (a kind gift of Dr S. J. Tzartos, Institute Pasteur Hellenique, Athens, Greece) as a reference anti-MIR antibody. Briefly, 96-well ELISA plates were coated with 50 μl of 5 μg/ml purified tAChR as above and incubated for 1 h at 37°C with

50 µl of protein G purified anti-AChR mAb with increasing concentration (0.001-10 µg/ml). Subsequently, plates were incubated for 1 h at 37°C with 50 µl of mAb 35 coupled with HRP (0.04 µg/ml, 50% of maximum binding to AChR predetermined). The colormetric reaction was developed as described above. The results are expressed as percentage inhibition of mAb 35-HRP binding to AChR and calculated as follows: [(average OD_{450} of duplicate wells with mAb 35-HRP alone – average OD_{450} of duplicate wells in which mAb 35-HRP was tested in the presence of anti-AChR mAb) / average OD_{450} of duplicate wells with mAb 35-HRP alone] x 100. The mAbs with more than 50% inhibition of mAb 35-HRP to AChR were considered as anti-MIR antibodies[23].

MAbs were also tested for their ability to inhibit the binding of ^{125}I-α-BT to AChR by an assay as follows: ELISA plates were coated with 50 µl of 5 µg/ml purified tAChR as described above and incubated with 100 µl of hybridoma supernatant at room temperature overnight. The supernatant was removed by aspiration and plates were subsequently incubated with a limiting concentration of ^{125}I-α-BT (2 pmol/ml) for 2 h at room temperature. Plates were washed and radioactivity was counted in a gamma counter. The results are expressed as percentage inhibition of ^{125}I-α-BT binding to AChR and calculated as follows: [(average cpm of duplicate wells with ^{125}I-α-BT alone - average cpm of duplicate wells with ^{125}I-α-BT in the presence of anti-AChR mAb) / average cpm of duplicate wells with ^{125}I-α-BT alone] x 100.

MAbs were also investigated for binding to extracellular and intracellular epitopes on AChR rich memberane vesicles. ELISA plates were coated with purified mAb (25 µg/ml) for 1 h at 37 °C, and subsequently incubated with 150 µl AChR rich memberane vesicles (80 fmol AChR), isolated from electric organ of Torpedo californica as previously described[20], labeled with ^{125}I-α-BT at 4 °C overnight. After washing the bound radioactivity was counted as above.

MAbs which failed to inhibit the binding of mAb 35 to MIR or binding of ^{125}I-α-BT to AChR but were directed against the extracellular epitopes were further determined for recognizing subunits of AChR by Western blotting. Subunits of AChR were separated by SDS-PAGE and transferred onto nitrocellulose filter. The binding of mAbs to subunits was visualized by adding rabbit anti-mouse immunoglobulin coupled with HRP and

substrate diaminobenzidine tetrahydrochloride (DAB). The molecular weight standard was stained with ponceau[24].

Cross-reaction with mouse, rat and human AChR

MAbs were tested for cross-reactivity to mouse AChR (mAChR), rat AChR (rAChR) and hAChR by RIA. Briefly, 200 µl of mAChR (isolated from mouse muscle) or rAChR (isolated from rat muscle) or hAChR extracts were labeled with ^{125}I-α-BT (2 pmol ^{125}I-α-BT / pmol AChR) for 4 h at 4°C, and subsequently incubated with 200 µl hybridoma culture supernatant at 4°C overnight. The complex of mAbs and AChR were precipitated by adding rabbit anti-mouse immunoglobulin and bound radioactivity was counted as described above.

Passive transfer of anti-AChR mAbs

The potential of anti-MIR mAbs to induce clinical signs of EAMG was determined by passive transfer of anti-MIR mAbs to 8-10 week-old female Lewis rats (for mAbs A7, A24 , A42, A43, D6 and G10) or 8 week-old female C57bl/6 mice (for mAb A60)[24]. Passive transfer EAMG was induced by i. p. injection of 20 fold concentrated culture supernatant of anti-MIR mAbs in final volume of 5 ml. Control rats were injected with 5 ml PBS. Animals were killed 48 h after injection of the mAbs. Each group represents five animals. The severity of EAMG was established by a strength duration test performed according to Lennon et al[13]. Clinical signs of EAMG were expressed as 0 (no obvious abnormalities), + (no abnormalities before testing, but decreased strength at the end), ++ (clinical signs before testing, i. e. head down, hunched posture, weak grip) and +++ (severe clinical signs before testing, no grip, moribund).

Loss of AChR was confirmed by measurement of the muscle AChR concentration in whole carcasses according to Lindstrom et al[12], with minor modification[25]. Briefly, frozen rat (or mouse) muscle was homogenized in a Warring blendor at 4°C for 4 x 30 sec in 250 ml (150 ml for mouse) PBS containing 10 mM EDTA, 10 mM NaN_3, 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and 2% iodoacetamide. After centrifugation at 12 000 rpm (Beckman rotor JA14) at 4°C for 30 min, the pellet was resuspended in 15

ml (10 ml for mouse) of 2% Triton X-100 in buffer above and centrifuged again. An aliquot of 250 µl supernatant (crude extract of muscle AChR) was labeled with 2 nM ^{125}I-α-BT and incubated with excess rat anti-AChR IgG at 4°C overnight. The complex of rat anti-AChR IgG-AChR-^{125}I-α-BT was precipitated by addition of goat anti-rat serum. AChR concentration was expressed as pmoles ^{125}I-α-BT precipitated and percentage of AChR loss was calculated as follows: 1 – [(average AChR concentration of experimental group / average AChR concentration of control group) x 100].

Results

Isotypes of mAbs

Most of the mAbs were IgG1 and IgG2b and of κ type of light chain (see Table 1).

Fine specificity of anti-AChR mAbs

MAbs A7, A24, A26 and A60 were able to inhibit more than 50% binding of reference anti-MIR mAb 35 to AChR, indicating that they were directed against MIR on AChR (see Table 2). Another experiment showed that mAb D6 could inhibit the binding of mAb 35 to hAChR by up to 81%, and inhibit the binding of mAb G10 by 76%, indicating that both D6 and G10 are also anti-MIR or -overlapping MIR mAbs.

Table 1 Characteristics of anti-AChR mAbs

mAb	strain	isotype	specificity				cross-reaction			
			MIR	α-BT	extra	intra	tAChR	mAChR	rAChR	hAChR
A7	C57bl/6	IgG2a	+		+		+	+	+	
A9	C57bl/6	IgG1			+(γ / δ)		+			
A18	C57bl/6	IgG1			+(γ / δ)		+	+		
A24	C57bl/6	IgM	+		+		+	+	+	
A26	C57bl/6	IgG2b	+		+		+			
A42	Balb/c	IgG1			+(γ / δ)		+	+	+	
A43	Balb/c	IgG1			+(γ / δ)		+	+	+	
A49	C57bl/6	IgG2a				+	+	+	+	
A60	C57bl/6	IgG2b	+		+		+	+		
A62	C57bl/6	IgG2b		+	+		+			
A64	C57bl/6	IgG1		+	+		+			
A65	C57bl/6	IgG2b		+	+		+			
D6	Balb/c	IgG2a	+		+(α3-181)				+	+
G10	Balb/c	IgG1	+		+(α3-181)				+	+

MIR: the main immunogenic region located on the α-subunit (α67-76) of AChR. α-BT: α-bungarotoxin binding site on the α-subunit (α189-195) of AChR. extra: epitopes located on the extracellular surface of AChR. intra: epitopes located on the cytoplasmic surface of AChR accessible only on solubilized AChR. tAChR: AChR from electric organs of Torpedo californica. mAChR: AChR from mouse muscle. rAChR: AChR from rat muscle. hAChR: AChR from human muscle. γ / δ: γ and δ subunits of AChR. α3-181: binding to recombinant α-subunit amino acids 3-181 of hAChR determined by Jacobson et al[26].

Table 2 Concentrations of anti-MIR mAbs needed for 50% inhibition of mAb 35 binding to AChR (IC_{50})

mAb	IC_{50} (μg/ml)
A7	2
A24	1.5
A26	1
A60	0.4
mAb 35	0.35

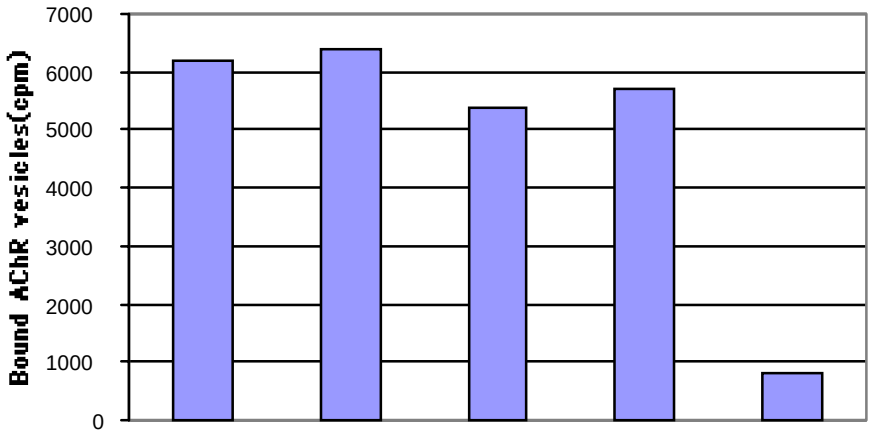
MAbs A62, A64 and A65 were able to block the binding of ^{125}I-α-BT to AChR for more than 50% suggesting that they recognized the epitopes overlapping the α-BT binding sites (see Table 3).

Table 3 Concentrations of anti-α-BT binding site mAbs needed for 50% inhibition of ^{125}I-α-BT binding to AChR (IC_{50})

mAb	IC_{50} (μg/ml)
A62	0.3
A64	0.25
A65	8.5

MAbs A9, A18, A42 and A43 were unable to inhibit either the binding of mAb 35 to AChR or the binding of ^{125}I-α-BT to AChR. However, they recognized an extracellular epitope of tAChR as showed in Figure 1. Further determination by Western blotting revealed that they could bind to γ / δ subunits of AChR (data not shown). However, mAb A49 bound to solubilized but not membrane incorporated AChR (see Figure 1).

A



B

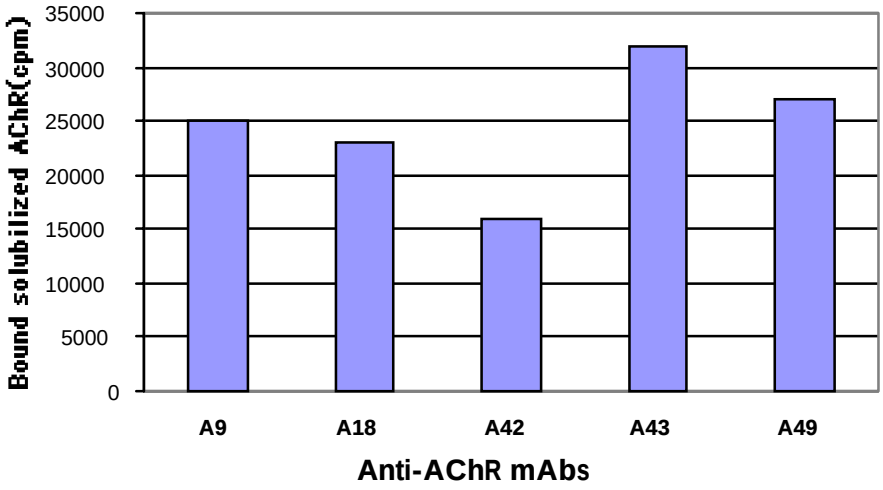


Figure 1 Binding of anti-AChR mAbs to extracellular and intracellular epitopes on AChR. Anti-AChR mAbs were tested for binding to AChR vesicles in which the receptor is embedded in the membrane and only extracellular epitopes are exposed (A) or binding to solubilized AChR on which intracellular epitopes are only accessible for antibodies in vitro (B).

Cross-reaction with mAChR, rAChR and hAChR

The cross-reactivity of anti-tAChR mAbs to AChR of other species was tested by RIA. Some of the mAbs cross-reacted with mAChR, other were able to bind to rAChR (see Table 1).

Pathogenicity of anti-AChR mAbs

Anti-AChR mAbs were tested for in vivo pathogenicity in rats or mice. The animals with EAMG showed AChR loss ranging from 29% ± 14% to 47% ± 15% (see Table 4).

Table 4 Pathogenicity of anti-AChR mAbs

mAb	AChR loss (mean ± SD)	EAMG
PBS	0 ± 7.5	0
A7	38.4 ± 7.2	+++
A24	13.2 ± 14.1	0
A42	0.1 ± 7.0	0
A43	4.0 ± 12.5	0
A60	48.2 ± 13.0	0
D6	47.0 ± 15.0	+++
G10	29.0 ± 14.0	++

Anti-AChR mAbs were investigated for in vivo pathogenicity by passive transfer to rats or mice (mAb A60). AChR loss at 48 h after injection of mAbs is expressed as a percentage of the AChR concentration of PBS-injected animals measured in whole carcass. Clinical signs of EAMG were evaluated as: +++, severe clinical signs; ++, clinical signs; 0, no obvious abnormalities.

Discussion

MAbs were obtained from hybridomas using pooled lymphnode cells from C57bl/6 and Balb/c mice immunized three times with AChR. Hybridomas were initially screened for binding to AChR by ELISA. The mAbs reactive with AChR were further determined on their isotypes by ELISA, their fine specificity by competitive ELISA or RIA, and their ability to cross-react with mAChR, rAChR and hAChR by RIA. The determination of isotypes of the mAbs will be useful in evaluation of pathogenicity of the mAbs in induction of EAMG and in design of primers used in PCR amplification of mAb variable region gene segments for subsequently sequencing.

It has been known that a major part of anti-AChR antibodies in MG and EAMG in rats immunized with intact AChR are directed against the MIR located on the α-subunit at residues 67-76[27,28]. Anti-MIR antibodies are very potent in accelerating AChR degradation by antigenic modulation[29] and are able to induce EAMG in animals by passive transfer[30]. Mouse mAbs against the MIR were distinguished from mAbs to other extracellular epitopes on AChR by competitive inhibition with reference anti-MIR mAb 35[31]. Varying amounts of these mAbs were needed to inhibit mAb 35 to bind to AChR for 50%, indicating that these mAbs show varying affinities for MIR or recognize epitopes partially overlapping the MIR, since mAbs with fine specificity for epitopes separated by only 7 residues can be distinguished using this competitive inhibition technique[23]. Since pathogenic anti-AChR antibodies were exclusively found among the anti-MIR antibodies, therefore, this group of antibodies will be used in analysis of the pathogenicity related with their molecular sequences of variable region genes (see Chapter 2). The α-BT binding site, including the cholinergic binding site, is located on the α-subunit (α189-195) distinct from the MIR[7]. MAbs A62, A64 and A65 were isolated that competitively inhibited the binding of α-BT to the AChR. MAbs A64 and A65 were able to inhibit α-BT binding almost completely, whereas mAb A62 inhibited α-BT biding to a maximum of 50%. MAbs completely inhibiting α-BT are probably directed to epitopes similar for both α-BT binding sites on each AChR molecule, whereas

mAbs partially inhibiting α-BT are directed to epitopes unique to each of the two binding sites [7-9].

The AChR is a transmembrane glycoprotein with extracellular epitopes accessible for antibody in vivo and intracellular epitopes only accessible on solubilized AChR in vitro. Since anti-AChR mAbs are generated against solubilized AChR both antibodies against extracellular and intracellular epitopes will arise. MAbs binding to extracellular epitopes can be distinguished from mAbs to intracellular epitopes by binding to AChR-rich membrane vesicles. MAbs A9, A18, A42 and A43 which failed to inhibit the binding of mAb 35 or α-BT to AChR, are directed against an extracellular epitope and γ / δ subunits confirmed further by Western blotting, whereas mAb A49 recognized only the intracellular epitopes.

Since the AChR is an evolutionary well conserved molecule[32, 33], the homology between Torpedo and mammalian AChR is about 80% for the α-subunit and 55% for the other subunits[34-37], and the amount of AChR in electric organs of Torpedo californica and other electric rays and eels is 100000 as high as that in mammalian skeletal muscle per kilogram muscle weight, therefore, tAChR provides a rich source of AChR used in mammalian study. Furthermore, since mAbs cross-reactive with mammalian AChR could be pathogenic in vivo, they could be used to induce EAMG as a model to investigate the pathogenesis of MG as well as to analyse the structure of pathogenic anti-AChR antibodies in relation to their pathogenicity.

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Chapter 2

Pathogenicity and sequence analysis of monoclonal antibodies directed against the main immunogenic region of acetylcholine receptor

Abstract

Binding of autoantibodies directed against acetylcholine receptor (AChR) to AChR results in AChR loss leading to muscular weakness in myasthenia gravis (MG) and its animal model, experimental autoimmune myasthenia gravis (EAMG). The role of different anti-AChR antibody sequences and specificities in pathogenesis of EAMG was investigated by sequencing a panel of 6 anti-AChR monoclonal antibodies (mAbs) against main immunogenic region (MIR). The EAMG was induced in rats or mice by passive transfer of the mAbs and evaluated by clinical signs and AChR loss. The variable regions of heavy and light chains of anti-MIR mAbs were sequenced. Comparison of the sequences at nucleotide and amino acid levels between the mAbs showed that they utilized a similar VH gene derived from mouse PC7183 germline family with high homology in complementarity determining region (CDR) 1 and 2. The large diversity found in heavy chain CDR3 of the anti-MIR mAbs may contribute to the difference in pathogenicity of the mAbs.

Introduction

Myasthenia gravis (MG) is an organ-specific autoimmune disease mediated by autoantibodies directed against acetylcholine receptor (AChR) on postsynaptic membrane in neuromuscular junction[1, 2]. Muscle-type AChR in neuromuscular junction is a complex transmembrane glycoprotein of molecular weight 290 KD, consisting of five subunits in a stochiometry $\alpha_2\beta\gamma\delta$[3-5]. A characteristic feature of the AChR is the presence of main immunogenic region (MIR) to which more than half of the autoantibodies in MG patients or experimental MG rats are directed[6-8]. The MIR is located on the α-subunit at residues 67-76[9-11] and easily accessible in vivo to antibodies.

The model of MG, experimental autoimmune myasthenia gravis (EAMG) can be induced in animals by immunization of purified AChR or passive transfer of anti-AChR antibodies[12-15]. Pathogenic anti-AChR monoclonal antibodies (mAbs) were found exclusively among the mAbs directed against MIR. Binding of anti-AChR antibodies to AChR causes cross-linking of adjacent AChR molecules resulting in accelerating AChR internalization[16] or activate complement cascade leading to focal lysis of postsynaptic membrane[15]. The lack of functional AChR leads to muscular weakness and paralysis of skeletal muscle.

The actual contribution of individual antibodies to the antibody-mediated AChR loss is an important question in the pathogenesis of MG. Further structural and functional analysis of pathogenic anti-AChR antibody specificity may contribute to the understanding of the pathogenic mechanisms. In the study presented here, a panel of 6 anti-MIR mAbs was investigated by sequencing the variable regions (V) of heavy (H) and light (L) chains of the mAbs[17].

Materials and methods

Anti-AChR mAbs

Hybridomas were made according to Kohler and Milstein[18] from C57bl/6 and Balb/c mice immunized with Torpedo AChR (tAChR), purified from electric organ of Torpedo californica (Pacific Biomarine, California, USA) by affinity chromatography on Naja naja siamensis toxin (Miami Serpentaricem, Florida, USA) linked to Sepharose-4B (Pharmacia LKB, Woerden, The Netherlands), and with human AChR (hAChR), purified from amputated limbs of a patient without vascular or neurological disease as above. MAbs were initially screened for reactivity to AChR by ELISA as described previously in Chapter 1.

Anti-MIR mAbs

MAbs A7, A24, A26, A60 and D6 were determined on their fine specificity to bind to MIR on AChR by a competitive inhibition ELISA using mAb 35[19] (a kind gift of Dr S. J. Tzartos, Institute Pasteur Hellenique, Athens, Greece) as a reference anti-MIR antibody. The mAbs with more than 50% inhibition of mAb 35 to AChR were considered as anti-MIR antibodies[20]. MAb G10 was tested on its specificity to bind hAChR in a competitive inhibition between mAbs G10 and D6 (see Chapter 1).

Pathogenicity of anti-MIR mAbs

The potential of anti-MIR mAbs to induce clinical signs of EAMG was determined by passive transfer of anti-MIR mAbs to 8-10 week-old female Lewis rats[21] (for mAbs A7, A24 , D6 and G10) or 8 week-old female C57bl/6 mice (for mAb A60). Passive transfer EAMG was induced by i. p. injection of 20 fold concentrated culture supernatant of anti-MIR mAbs in final volume of 5 ml. Control rats were injected with 5 ml PBS. Animals were killed 48 h after injection of the mAbs. Each group represents five animals. The severity of EAMG was established by a strength duration test performed according

to Lennon et al[22]. Clinical signs of EAMG were expressed as 0 (no obvious abnormalities), + (no abnormalities before testing, but decreased strength at the end), ++ (clinical signs before testing, i. e. head down, hunched posture, weak grip) and +++ (severe clinical signs before testing, no grip, moribund).

Loss of AChR was confirmed by measurement of the muscle AChR concentration in whole carcasses according to Lindstrom et al[23], with minor modification[24]. Briefly, frozen rat (or mouse) muscle was homogenized in a Warring blendor at 4°C for 4 x 30 sec in 250 ml (150 ml for mouse) PBS containing 10 mM EDTA, 10 mM NaN_3, 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and 2% iodoacetamide. After centrifugation at 12 000 rpm (Beckman rotor JA14) at 4°C for 30 min, the pellet was resuspended in 15 ml (10 ml for mouse) of 2% Triton X-100 in buffer above and centrifuged again. An aliquot of 250 µl supernatant (crude extract of muscle AChR) was labeled with 2 nM ^{125}I-α-bungarotoxin (α-BT) and incubated with excess rat anti-AChR IgG at 4°C overnight. The complex of rat anti-AChR IgG-AChR-^{125}I-α-BT was precipitated by addition of goat anti-rat serum. AChR concentration was expressed as pmoles ^{125}I-α-BT precipitated and percentage of AChR loss was calculated as follows: 1 – [(average AChR concentration of experimental group / average AChR concentration of control group) x 100].

Amplification of variable region genes of mAbs

Total RNA was isolated from 1-5 x 10^6 hybridoma cells secreting the mAbs using RNAzol (Cinna/Biotecx Laboratories Inc, Houston TX, USA). Oligo dT primed first strand cDNA was synthesized using AMV reverse transcriptase (Promega,USA) and used as a template for PCR amplification. The upstream primers used for H and L chain V region sequence amplification are based on a consensus sequence of the first 8 codons of the VH and VL domains according to Orlandi et al[25] and Kabat et al[26] with minor modification. The downstream primer for the VH is complementary to codons 120-126 within the CH1 domain of all IgG subclasses. The downstream primer for the VL is complementary to codons 114-122 of the Cκ domain. The complete sequences of primers are: VH upstream: 5' gg gaattc (gc)ag gt(gc) (ac)a(ag) ct(gt) cag (gc)ag tc(at) gg, CH1 downstream: 5' gg ggatcc agg ggc cag tgg ata ga(tc) ag, VL upstream: 5' cct gaattc gac

(ag) tt gt(gt) (ac)t(gc) acc ca(ag) (at)ct cc, Cκ downstream: 5' cct ggatcc act gga tgg tgg gaa gat gga tac. The sequences underlined are Eco R1 and Bam H1 recognition sites to facilitate the subsequent cloning. The PCR was run for 30 cycles with denaturation at 94 °C for 2 min, annealing at 55 °C for 1 min, and extension at 72 °C for 1.5 min in each cycle on DNA Thermal Cycler 480 (Perkin Elmer Cetus).

Sequencing of mAbs

The PCR products of VH and VL were digested with Eco R1 and Bam H1 and purified from 1% agarose gel with Geneclean kit (BIO 101 Inc, La Jolla, CA, USA), and ligated onto plasmid Bluescript M13 SK (Stratagene, La Jolla, CA, USA). The recombinant plasmids were transferred into E coli DH5α and analysed for an insert of the right size by digestion with Eco R1 and Bam H1. The dideoxy chain termination sequencing procedure was performed according to Sanger et al[27] using the Bluescript KS and SK primer sites and a T7 sequencing kit (Pharmacia, Woerden The Netherlands). Several independent clones and at least three different colonies of each H chain and L chain of each anti-AChR mAb were sequenced to confirm the determined VH and VL sequences.

Sequence comparison

Mutual homology of sequences at the nucleotide level between anti-AChR mAbs was determined both for the overall VH and VL regions and for the complementarity determining regions (CDRs) using the program DNAsis. Homology between anti-AChR mAbs and other antibodies in EMBL Data Library was performed by FASTA computer search. The sequences of VH and VL of mAbs A7, A24, A26, A60, D6 and G10 have been accepted by the EMBL Data Library as AC No (VH and VL): A7: X80962 and X80961, A24: X80972 and X80971, A26: X80970 and X80969, A60: X80954 and X80953, D6: X80942 and X80941, G10: X80940 and X80939.

Results

Characteristics of anti-MIR mAbs

The isotype, specificity and cross-reactivity of anti-MIR mAbs A7, A24, A26, A60, D6 and G10 were summarized in Table 1.

Table 1 Characteristics of anti-AChR mAbs

mAb	isotype	specificity[a]	AChR[b]	EAMG[c]
A7	IgG2a	MIR	TMR	+++
A24	IgM	MIR	TMR	0
A26	IgG2b	MIR	T	nd
A60	IgG2b	MIR	TM	0
D6	IgG2a	MIR	HR	+++
G10	IgG1	MIR	HR	++

[a] MIR: main immunogenic region. MAbs compete for binding to AChR with anti-MIR mAb 35[19] in ELISA.

[b] Cross-reactivity of mAbs with AChR from Torpedo (T), mouse (M), rat (R) and human (H) in RIA. Torpedo AChR was used as initial immunogen for A7, A24, A26 and A60, and human AChR for D6 and G10.

[c] Pathogenicity of mAbs to induce EAMG in Lewis rats or C57bl/6 mice (A60). Clinical signs were evaluated 48 h after injection of mAbs. +++: severe clinical signs, ++: clinical signs, 0: no obvious abnormalities, nd: not determined.

Pathogenicity of anti-MIR mAbs

MAbs A7, A24, A26, D6 and G10 were tested for capacity to induce EAMG in rats and mice. Rats receiving A7 already developed grade +++ muscular weakness 18 h after injection (Table 1). At this time point mAb A7-injected rats showed 38.4% ± 7.2% AChR loss (Figure 1). All other animals were killed 48 h after injection. At this time point, animals with EAMG showed AChR loss ranging from 29% ± 14% to 47% ± 15%. Rats receiving mAb A24 did not develop significant clinical signs of EAMG or AChR loss. MAb A60 induced highly significant AChR loss but no clinical signs of EAMG when injected in mice (Table 1 and Figure 1).

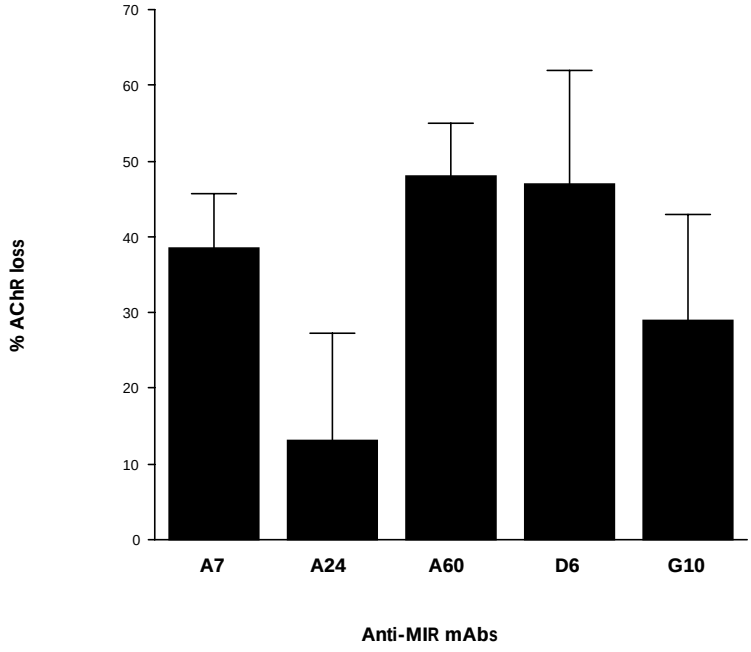


Figure 1 AChR loss after passive transfer of mAbs. AChR concentration was determined 48 h after injection of mAbs in RIA and expressed as pmoles ^{125}I-α-bungarotoxin precipitated. AChR loss is expressed as percentage of AChR concentration in unmanipulated animals

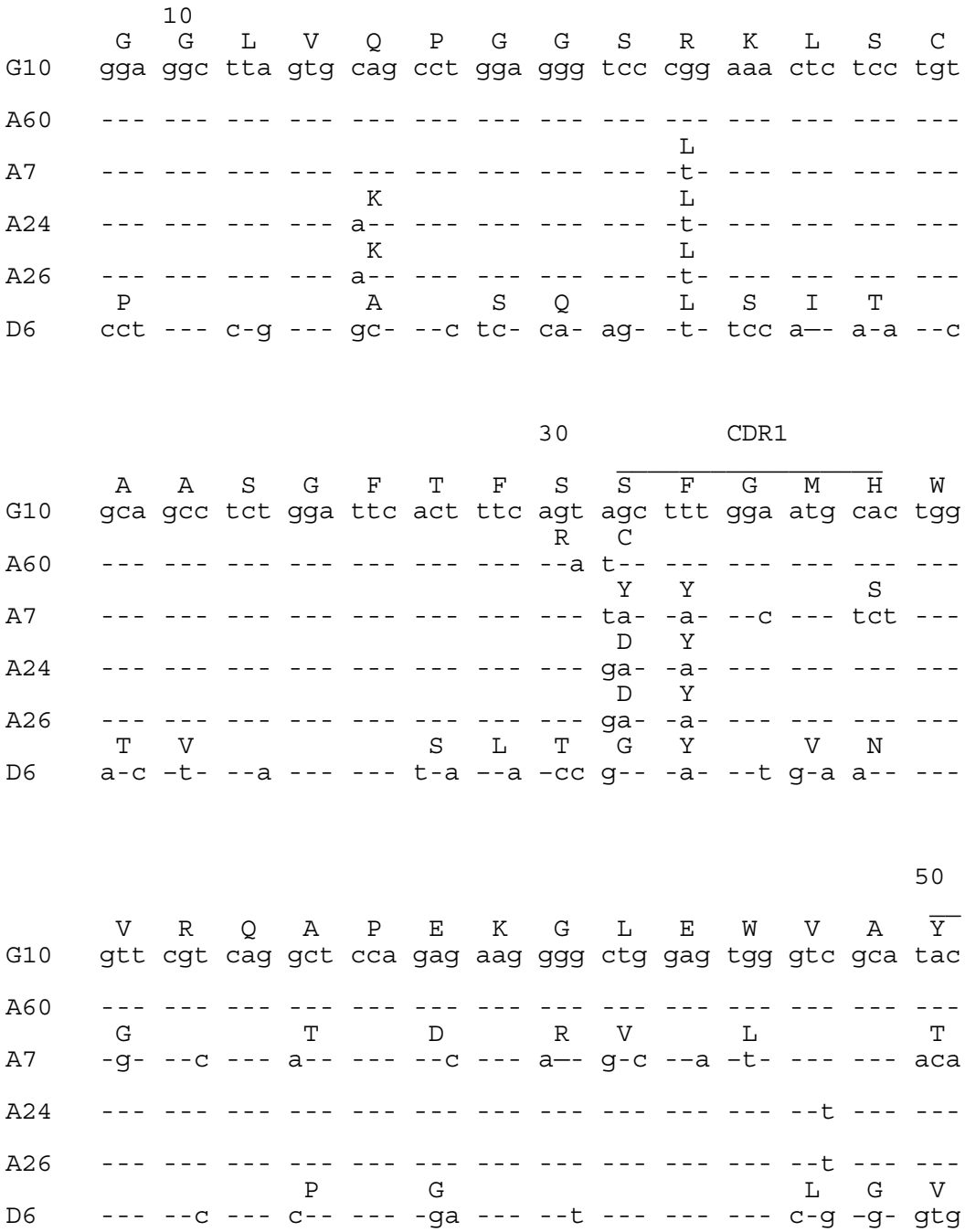
Gene utilization of anti-MIR mAbs

The H chain variable region gene of mAb A7 is 345 bp in length (the first 24 bp in VH upstream primer not included) encoding 115 amino acids and determining the codons 9-113 of VH (Figure 2A). The mAb A7 uses a VH gene belonging to mouse germline family PC7183. The homology between mAb A7 and germline gene DFL 16.2 VH sequence[28] is 93.7%. The A7 VH gene is joined with D and JH gene belonging to JH4 segment[29]. MAbs A24 and A26 use almost the same VH gene which is also derived from mouse germline family PC7183. The homology between mAbs A24 and A26 and germline gene VH61-1P[30] is 95.2% and 95.6% respectively. MAb A60 also uses mouse germline family PC7183 but with a 42 bp deletion in framework 3 region. MAb G10 is 97.4% homologous to the MOPC21 germline VH gene[31] which belongs to PC7183 family. All the five mAbs above utilize exact mouse JH4 segment (100% homology)[29]. However, mAb D6 uses a VH gene derived from mouse Q52 germline family (EMBL Data Library, AC No X14515), and joined with D and JH3 segment[29].

The L chain variable region gene of mAb A7 is 306 bp in length (the first 24 bp in VL upstream primer not included), encoding 102 amino acids and determining the codons 9-107 of VL (Figure 2B). The VL of mAb A7 is a member of the Vκ3 subgroup and derived from mouse Vκ 21E germline gene (EMBL Data Library, AC No L08207). The A7 VL is associated with Jκ belonging to Jκ2 segment[32]. MAbs A24 and A26 utilize different Vκ genes which are members of mouse Vκ3 and Vκ1 subgroups respectively. However, they both use the same Jκ2 gene[32]. Mab A60 uses almost the same L chain as mAbs A7 and A24 do. The VL genes used by mAbs G10 and D6 are members of mouse Vκ1 and Vκ5 subgroups[26] respectively and both associated with Jκ1 segment[32].

The H and L chain genes encoding six anti-MIR mAbs are summarized in Table 2.

A


10
G G L V Q P G G S R K L S C
G10 gga ggc tta gtg cag cct gga ggg tcc cgg aaa ctc tcc tgt
A60 --- --- --- --- --- --- --- --- --- --- --- --- --- ---
L
A7 --- --- --- --- --- --- --- --- --- -t- --- --- --- ---
K L
A24 --- --- --- --- a-- --- --- --- --- -t- --- --- --- ---
K L
A26 --- --- --- --- a-- --- --- --- --- -t- --- --- --- ---
P A S Q L S I T
D6 cct --- c-g --- gc- --c tc- ca- ag- -t- tcc a-- a-a --c
30 CDR1
A A S G F T F S S F G M H W
G10 gca gcc tct gga ttc act ttc agt agc ttt gga atg cac tgg
R C
A60 --- --- --- --- --- --- --- --a t-- --- --- --- --- ---
Y Y S
A7 --- --- --- --- --- --- --- --- ta- -a- --c --- tct ---
D Y
A24 --- --- --- --- --- --- --- --- ga- -a- --- --- --- ---
D Y
A26 --- --- --- --- --- --- --- --- ga- -a- --- --- --- ---
T V S L T G Y V N
D6 a-c -t- --a --- --- t-a --a -cc g-- -a- --t g-a a-- ---
50
V R Q A P E K G L E W V A Y
G10 gtt cgt cag gct cca gag aag ggg ctg gag tgg gtc gca tac
A60 --- --- --- --- --- --- --- --- --- --- --- --- --- ---
G T D R V L T
A7 -g- --c --- a-- --- --c --- a-- g-c --a -t- --- --- aca
A24 --- --- --- --- --- --- --- --- --- --- --- --t --- ---
A26 --- --- --- --- --- --- --- --- --- --- --- --t --- ---
P G L G V
D6 --- --c --- c-- --- -ga --- --t --- --- --- c-g -g- gtg

CDR2

	I	S	S	G	S	S	T	L	H	Y	A	D	T	V
G10	att	agt	agt	ggc	agt	agt	acc	ctc	cac	tat	gca	gac	aca	gtg
A60	---	---	---	---	---	---	---	---	---	---	---	---	---	---
		T		N	G	G	S	T	F		P		R	
A7	---	-c-	---	aat	g-a	g--	-g-	ac-	ttt	---	c--	---	-gg	---
		M				R			I	Y				
A24	---	--g	---	---	---	--a	---	---	a--	t--	---	---	---	---
		M				R			I	Y				
A26	---	--g	---	---	---	--a	---	---	a--	t--	---	---	---	---
		W			D	G	S	T	D		K	S	A	L
D6	--a	t-g		--t	ga-	g-a	-g-	aca	g--	---	aa-	tca	g-t	c-c

70

	K	G	R	F	T	I	S	R	D	N	P	K	N	T
G10	aag	ggc	cga	ttc	acc	atc	tcc	aga	gac	aat	ccc	aag	aac	acc
						T								
A60	---	---	---	---	---	-c-	---	---	---	---	---	---	---	---
											A			
A7	---	---	---	---	---	---	---	---	---	---	g--	---	---	---
											A			
A24	---	---	---	---	---	---	---	---	---	---	g--	---	---	---
											A			
A26	---	---	---	---	---	---	---	---	---	---	g--	---	---	---
		S		L	S						S		R	Q
D6	--a	tc-	a--	c-g	-g-	---	ag-	--g	---	--c	t--	---	-ga	caa

	L	Y	L	Q	M	R	S	L	K	S	E	D	T	A
G10	ctg	tac	ctg	caa	atg	aga	agt	ctg	aag	tct	gag	gac	acg	gcc
		F												
A60	---	-t-	---	---	---									
A7	---	---	---	---	---	---	---	---	---	---	---	---	---	---
		L				T	G		R					
A24	---	ct-	---	---	---	-cc	g--	---	-g-	---	---	---	---	---
		L				T			R					
A26	---	ct-	---	---	---	-cc	---	---	-g-	---	---	---	---	---
	V	F		K		N			Q	T	D			
D6	g-t	-t-	t-a	a--	---	-ac	---	---	c-a	a--	--t	---	--a	---

```
          90                                                  CDR3
                                                  ____________________________________
       M   Y   Y   C   V   R   D   D   S   G   D   S   P   Y
G10    atg tat tac tgt gtg aga gat gat tcg ggg gat agc cca tat
                           K   L   P       L   C   Y
A60                        -a- cta ccc --a cta tgc tat

A7     --- --- --- --- --- --- --- --- --- --- --- --- --- ---
                       A   T   R   K   E   P   L   R   G
A24    --- --- --- --- -ca -cg agg a-g gaa cca cta c-a ggc
                       A   T   R   K   E   P   L   R   G
A26    --- --- --- --- -ca -cg agg a-g gaa cca cta c-a ggc
        R              A       G   G   Y   R
D6     -g- --- --- --- -cc --- -gg -gc -ac a--


                                                                  110
       ____________________________
       Y   Y   A   M   D   Y   W   G   Q   G   T   S   V   T
G10    tac tat gct atg gac tac tgg ggt caa gga acc tca gtc acc

A60                    --- --- --- --- --- --- --- --- --- ---

A7     --- --- --- --- --- --- --- --- --- --- --- --- --- ---

A24        --- --- --- --- --- --- --- --- --- --- --- --- ---

A26        --- --- --- --- --- --- --- --- --- --- --- --- ---
                           F                       L
D6             --- --- --- -t- --- --- --- --g --t ctg --- --t


        V   S   S
G10     gtc tcc tca

A60     --- --- ---

A7      --- --- ---

A24     --- --- ---

A26     --- --- ---
                A
D6      --- --t g--
```

B

```
          10
        A   S   L   A   V   S   L   G   Q   R   A   T   I   S
A7    gct tcc tta gct gta tct ctg ggg cag agg gcc acc atc tca

A24   --- --- --- --- --- --- --- --- --- --- --- --- --- ---

A60   --- --- --- --- --- --- --- --- --- --- --- --- --- ---
        S                       V       E   K   V       M
A26   t-c --- c-- --- --g --a g-t --a g-- -a- -tt --t --g agc
        K       M   S   T       V       D       V   S       T
G10   aaa --- a-g t-c ac- --a g-a --a g-c --- -t- -g- --- a-c
                S   A       V       E   T   V           T
D6    --c --- c-- t-- -c- --- g-- --a g-a -ct -t- --- --- a--
```

```
                                                                30
                                                CDR1
        Y   R   A   S   K   S   V   S   T                   S   G   Y
A7    tac agg gcc agc aaa agt gtc agt aca                 tct ggc tat

A24   --- --- --- --- --- --- --- --- ---                 --- --- ---

A60   --- --- --- --- --- --- --- --- ---                 --- --- ---
        C   K   S       Q       L   L   Y   S   N   N   Q   K
A26   -g- -a- t-- --t c-g --c c-t tta tat agt aac aa- caa a-g
        C   K           Q                           N   V   R
G10   -g- -a- --- --t c-g                         aa- -tt cg-
        C               E                           N   I   N
D6    -gt c-a --a --t g-g                         aa- att a-c
```

```
        S   Y   M   H   W   N   Q   Q   K   P   G   Q   P   P
A7    agt tat atg cac tgg aac caa cag aaa cca gga cag cca ccc
                                        R
A24   --- --- --- --- --- --- --- --- -g- --- --- --- --- ---

A60   --- --- --- --- --- --- --- --- --- --- --- --- --- ---
        N       L   A       Y                           S
A26   -ac --c t-- gc- --- t-- --g --- --- --- --g --- t-t --t
        T   A   V   A       Y                           S
G10   -c- gc- g-t gc- --- t-t --- --- --- --t --g --- t-t --t
                L   A       Y               Q       K   S
D6    --- --c t-a gca --- t-t --g --- --- -ag --- a-a t-t --t
```

```
                                50
                                                CDR2
                                ____________________
       R   L   L   I   Y   L   V   S   N   L   E   S   G   V
A7   aga ctc ctc atc tat ctt gta tcc aac cta gaa tct ggg gtc

A24  --- --- --- --- --- --- --- --- --- --- --- --- --- ---

A60  --- --- --- --- --- --- --- --- --- --- --- --- --- ---
       K                   W   A       T   R
A26  -a- --g --g --t --c tgg -c- --- -ct agg --- --- --- ---
       K   A                   A           R   H   T
G10  -a- gca --g --t --c t-g -c- --- --- -gg c-c a-- --a ---
       Q   F       V       N   A   K   T       A   E
D6   cag t-- --g g-- --- aa- -c- aaa -c- t-- -c- gaa --t --a



                                                    70
       P   A   R   F   S   G   S   G   S   G   T   D   F   T
A7   cct gcc agg ttc agt ggc agt ggg tct ggg aca gac ttc acc

A24  --- --- --- --- --- --- --- --- --- --- --- --- --- ---

A60  --- --- --- --- --- --- --- --- --- --- --- --- --- ---
           D           T                                   K
A26  --- -at c-c --- -ca --- --- --a --- --- --- --t --- -aa
           D           T
G10  --- -at c-c --- -ca --- --- --a --- --- --- --t --- --t
           S                                       Q       S
D6   --a t-a --- --- --- --- --- --a --a --c --- c-g --t t-t



       L   N   I   H   P   V   E   E   E   D   A   A   T   Y
A7   ctc aac atc cat cct gtg gag gag gag gat gct gca acc tat

A24  --- --- --- --- --- --- --- --- --- --- --- --- --- ---

A60  --- --- --- --- --- --- --- --- --- --- --- --- --- ---
           T       S   S       K   A           L       V
A26  --- -c- --- agc ag- --- a-- -ct --a --c ctg --- gtt ---
           T       S   N       Q   S           L       D
G10  --- -c- --t agc aa- --- c-a tct --a --c ctg --- gat ---
           K       N   S   L   Q   P           F   G
D6   --g --g --- a-c agc c—- c—- cct --a --- tt- -gg --t ---
```

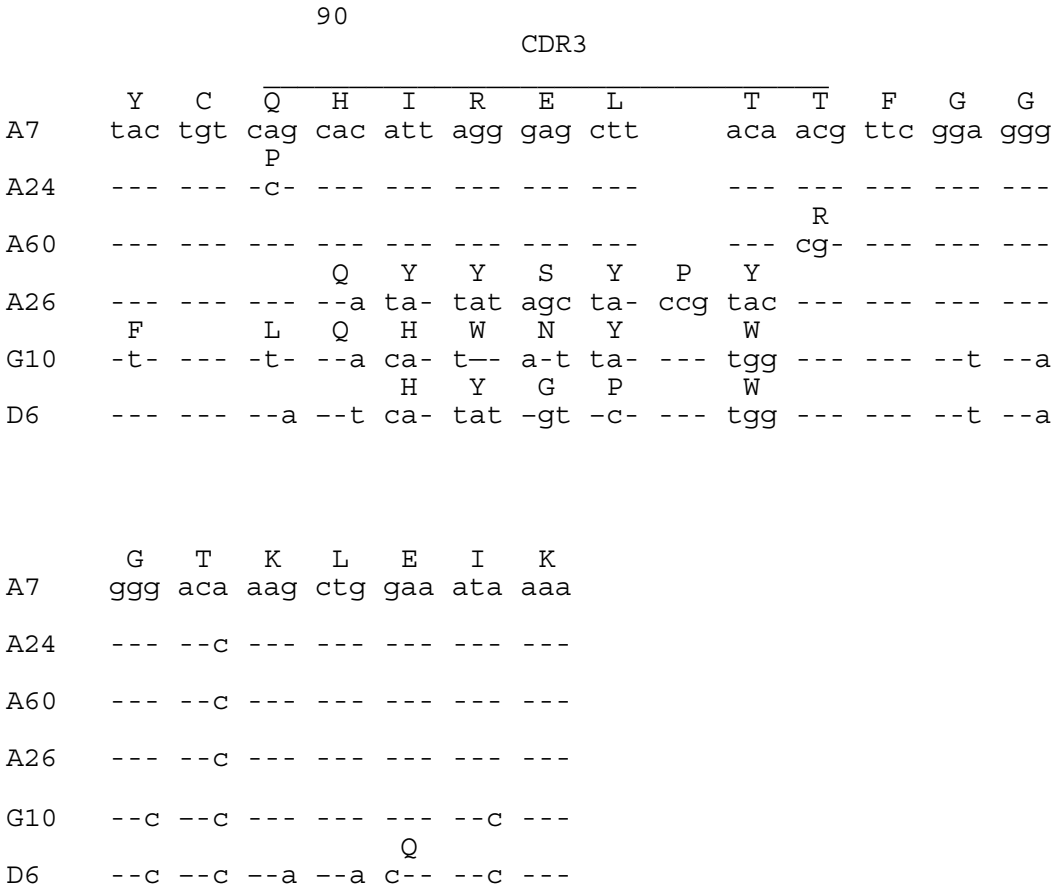


Figure 2 Sequence comparison of anti-MIR mAbs. Sequences of anti-MIR mAbs are compared for the heavy chain (A) and light chain (B). The complete nucleotide sequences and deduced amino acid sequences are aligned starting from codon 9 as numbered according to Kabat et al[26]. Sequence identity of anti-MIR mAbs with mAb G10 (heavy chain) or with mAb A7 (light chain) is indicated by dashes. Complementarity determining regions (CDRs) are also indicated.

Table 2 Gene utilization of anti-MIR mAbs

	A7	A24	A26	A60	G10	D6
H chain						
VH family	PC7183	PC7183	PC7183	PC7183	PC7183	Q52
JH segment	4	4	4	4	4	3
L chain						
Vκ subgroup	3	3	1	3	1	5
Jκ segment	2	2	2	2	1	1

The gene utilization was determined according to the following: VH family: EMBL Data Library, JH segment: Sakano et al[29], Vκ subgroup: Kabat et al[26], Jκ segment: Sakano et al[32].

Sequence homology between anti-MIR mAbs

The overall homology among H chain variable region genes of anti-MIR mAbs derived from the same mouse germline family PC7183 is 80.4% - 99.7% with a highest homology of 99.7% between mAbs A24 and A26 (only 1 nucleotide difference in framework 3). For the L chain variable region genes, a 98.7% homology is seen among mAbs A7, A24 and A60, a 77.1% between mAbs A26 and G10, and a 61.2% - 64.8% among others (Table 3).

Table 3 Overall homology of nucleotide sequences of variable regions of anti-MIR mAbs

mAb	heavy chain					light chain				
	A24	A26	A60	G10	D6	A24	A26	A60	G10	D6
A7	81.7	82.0	80.4	89.3	59.7	98.7	64.7	98.7	62.9	61.6
A24		99.7	89.1	88.2	60.4		64.8	98.7	63.5	61.6
A26			89.1	88.5	60.7			64.7	77.1	64.6
A60				92.6	57.1				62.9	61.2
G10					62.0					63.6

Sequence homology is calculated between the complete overlapping VH-D-JH region and shown as percentage of homology

The sequence comparison of H chain CDRs at amino acid level shows an identical CDRs between mAbs A24 and A26, an identical CDR2 between mAbs A60 and G10, an identical CDR3 between mAbs A7 and G10. The homology is 80% in CDR1 among A7, A24 (A26), A60 and G10, 76.5% in CDR2 between A24 (A26) and A60 (G10) (35.3% – 41.7% for other antibodies) and 41.7% in CDR3 between A7 (G10) and A24 (A26) which is 2 amino acids shorter than A7 (G10) in CDR3. MAbs A60 and D6 have only 8 amino acids in CDR3. In contrast, the H chain of mAb D6 shows only 46.6% - 60.0% homology in CDR1, 41.6% - 60.0% in CDR2, and 50.0% - 60.0% in CDR3 with mAbs A7, A60 and G10. The L chain are identical in CDR1 and 2 and only 1 amino acid different in CDR3 among A7, A24 and A60. Although mAbs A24 and A26 share identical H chain CDRs, mAb A26, which does not cross-react with mammalian AChR, is encoded by a different L chain sequence with only 36.6%, 57.1% and 39.1% homology to A24 in CDR1, 2 and 3 respectively. MAbs G10 and D6 also have different L chain CDRs compared with A7, A24 and A60.

Discussion

One of the key questions in the pathogenesis of MG is the contribution of individual antibody specificity to AChR destruction. In this study, a panel of 6 anti-AChR mAbs with fine specificity to MIR was analysed for structural properties in relation to their potential to cause AChR loss and muscular weakness in passive transfer EAMG model. The anti-MIR mAbs cross-reactive to mouse AChR (mAChR) or rat AChR (rAChR) were tested for in vivo pathogenicity. Rats were used instead of mice for the mAbs which could cross-react with both rAChR and mAChR since mice are more resistant to induction of EAMG than rats[33]. MAb A7, D6 and G10 could induce AChR loss by up to 29% - 47% and severe EAMG demonstrating that they are potent pathogenic anti-MIR mAbs. The mAbs A42 and A43, directed against an extracellular epitope (γ / δ) of AChR and cross-reactive to mAChR and rAChR failed to induce EAMG in rats (see Chapter 1). This finding is in concordance with previous studies in which pathogenicity of anti-MIR mAbs was demonstrated using rat anti-AChR mAbs[15, 34].

The comparison of the variable region nucleotide sequences between anti-MIR mAbs reveals that highly homologous (80.4% - 99.7%) VH-D-JH sequences are found in this group of mAbs. Most of the mAbs (5/6) tested in this study use a similar VH gene derived from mouse germline family PC7183 joined with an unmutated JH4 segment. The nucleotide substitutions seen in the VH gene utilized by this group of mAbs may result from somatic mutation driven by antigen AChR. MAbs A7, A24 and A60 also share a similar L chain (98.7% homology among them). However, the L chain used by mAbs A7, A24 and A26 is also frequently shared by anti-DNA antibodies, which are commonly observed in MG patients[35], but non of the anti-MIR mAbs can bind to ss or dsDNA (tested by Dr R. Smeenk, Central Laboratory of Blood Transfusion, Amsterdam, The Netherlands) indicating that the H chain plays a more important role in the specificity of the mAbs.

The structural features of anti-MIR mAbs in relation to their pathogenicity were investigated by sequence comparison in the CDRs. Pathogenic anti-MIR mAbs A7 and G10 share an identical D-JH4 sequence encoding CDR3, whereas G10 and A60 differ in one amino acid in CDR1 and share an identical CDR2. The CDR3 is considered most

important in epitope binding, and sharing of an identical CDR3 in A7 and G10 suggests selection for binding to the same determinant of the MIR. Pathogenic mAb D6 shows large sequence diversity in both H and L chains compared with mAbs A7 and G10. MAb D6 probably recognizes different determinants or conformation of the MIR since it is a conformation-dependent region on the extracellular tip of α-subunit[36]. Even though the MIR is a 10 amino acid epitopes, it elicits several different antibody specificities. Similarly, sequence analysis of mAbs directed against residues 97-107 of tabacco mosaic virus revealed a wide range of different region sequences for both H and L chains[37].

In conclusion, sequence analysis of anti-MIR of AChR mAbs shows that the VH genes utilized are related to PC7183 germline family. H chain CDR3 may mainly contribute to the pathogenicity of the mAbs.

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Chapter 3

Epitope mapping and sequence analysis of monoclonal antibodies directed against the α-bungarotoxin binding site of acetylcholine receptor

Abstract

Myasthenia gravis (MG) is an antibody-mediated autoimmune disease. The binding of antibodies to acetylcholine receptor (AChR) results in AChR loss and leads to muscular weakness. The pathogenic anti-AChR antibodies are mainly found to be those that are directed against the main immunogenic region (MIR) on α-subunit (α67–76) of AChR. However, the antibodies to the α-bungarotoxin (α-BT) binding site on α-subunit (α189–199) of AChR may contribute to the clinical signs in some MG patients. In the present study, three anti-α-BT binding site monoclonal antibodies (mAbs) were investigated on their epitope specificity and their nucleotide and deduced amino acid sequences of variable regions. The three mAbs recognized the same epitope on AChR as showed in a competitive inhibition assay between individual mAbs. The sequence analysis of the mAbs showed that they all utilized the same VH gene derived from mouse Q52 germline family, but different VL genes, indicating that the heavy chain play more important role in binding to AChR. Comparison of sequences between the anti-α-BT binding site mAbs and the pathogenic anti-MIR mAb revealed that a large diversity was observed in both overall sequences and complementarity determining regions (CDRs).

Introduction

The major characteristic of myasthenia gravis (MG), an autoimmune disease, is the production of autoantibodies directed against the acetylcholine receptor (AChR) on postsynaptic membrane in neuromuscular junction[1-3]. The binding of anti-AChR antibodies to AChR results in AChR loss and leads to fatigability and weakness of skeletal muscle[4]. MG can be induced in animal model, the experimental autoimmune myasthenia gravis (EAMG), by immunization of purified AChR or passive transfer of anti-AChR antibodies[5-8]. The main mechanisms of pathogenesis of MG and EAMG are that the cross-linking of adjacent AChR molecules by anti-AChR antibodies causes an increase in the rate of internalization and degeneration of AChR[9-11], or that the binding of anti-AChR antibodies to AChR activate complement cascade leading to focal lysis of postsynaptic membrane[8]. However, the blocking anti-AChR antibodies by binding to acetylcholine (ACh) or α-bungarotoxin (α-BT) binding site[12-16] may contribute to the pathogenesis in some cases of MG.

The AChR is well characterized autoantigen[17]. The binding site of α-BT is demonstrated at residues 189–199 of α-subunit[18, 19] which overlaps the ACh binding site. Although about 65% of the antibodies in the sera of MG patients are directed against the main immunogenic region (MIR, α67–76)[20-22], blocking antibodies may make up 0–33% of the total anti-AChR antibodies in MG patient sera[23]. Therefore, the knowledge of specificity and molecular structure of blocking antibodies is also a key question in understanding the pathogenesis of MG. In this study, a panel of 3 anti-α-BT binding site monoclonal antibodies (mAbs) made from mice immunized with purified Torpedo AChR (tAChR) were investigated on their epitope mapping and on their variable region sequences amplified from hybridoma cDNA. The sequences of anti-α-BT binding site mAbs were compared with pathogenic anti-MIR mAb[24].

Materials and methods

Production of anti-AChR mAbs

Hybridomas were made from C57bl/6 mice immunized with tAChR purified from electric organ of Torpedo californica (Pacific Biomarine, California, USA) by affinity chromatography on Naja naja siamensis toxin (Miami Serpentaricem, Florida, USA) linked to Sepharose-4B (Pharmacia LKB, Woerden, The Netherlands) according to Kohler and Milstein[25]. MAbs were initially screened for reactivity to AChR by ELISA as described previously in Chapter 1.

Specificity of anti-α-BT binding site mAbs

MAbs A62, A64, and A65 were tested for their ability to inhibit the binding of ^{125}I-α-BT to AChR as previously described in Chapter 1. The mAbs with more than 50% inhibition of ^{125}I-α-BT to AChR were considered as anti-α-BT binding site mAbs.

Epitope mapping of anti-α-BT binding site mAbs

The epitope mapping of anti-α-BT binding site mAbs was performed by competitive inhibition between individual anti-α-BT binding site mAbs for binding to tAChR according to Roberts et al[26]. Briefly, 96-well microtiter plates were coated with 50 µl of 5 µg/ml tAChR for 1 h at 37 °C, preincubated with PBS containing 0.5% BSA and 0.5% Tween-20, and incubated with 50 µl of anti-α-BT binding site mAbs (0.08–10 µg/ml) overnight at 4 °C. Subsequently, 50 µl of ^{125}I-anti-α-BT binding site mAbs, previously determined to give 50% of the maximum binding to tAChR, was added without washing and incubated for 1 h at room temperature. Plates were washed, and bound radioactivity was counted. The percentage of inhibition between ^{125}I-anti-α-BT binding site mAbs and unlabeled anti-α-BT binding site mAbs was calculated as follows: 100 x [(average cpm of duplicate wells with ^{125}I-anti-α-BT binding site mAb alone -

average cpm of duplicate wells with ^{125}I-anti-α-BT binding site mAb in the presence of unlabeled putative inhibitor anti-α-BT binding site mAb) / average cpm of duplicate wells with ^{125}I-anti-α-BT binding site mAb alone]. Inhibition of ^{125}I-anti-α-BT binding site mAb by control monoclonal and polyclonal antibodies never exceeded 15%, therefore, 0-25% inhibition was considered nonspecific.

Cloning and sequencing of anti-α-BT binding site mAbs

Total RNA was isolated from 1-5 x 10^6 hybridoma cells secreting the mAbs using RNAzol (Cinna/Biotecx Laboratories Inc, Houston TX, USA). Oligo dT primed first strand cDNA was synthesized using AMV reverse transcriptase (Promega,USA) and used as a template for PCR amplification. The upstream primers used for heavy (H) and light (L) chain variable region (V) sequence amplification are based on a consensus sequence of the first 8 codons of the VH and VL domains according to Orlandi et al[27] and Kabat et al[28] with minor modification. The downstream primer for the VH is complementary to codons 120-126 within the CH1 domain of all IgG subclasses. The downstream primer for the VL is complementary to codons 114-122 of the Cκ domain. The complete sequences of primers are: VH upstream: 5' gg gaattc (gc)ag gt(gc) (ac)a(ag) ct(gt) cag (gc)ag tc(at) gg, CH1 downstream: 5' gg ggatcc agg ggc cag tgg ata ga(tc) ag, VL upstream: 5' cct gaattc gac (ag) tt gt(gt) (ac)t(gc) acc ca(ag) (at)ct cc, Cκ downstream: 5' cct ggatcc act gga tgg tgg gaa gat gga tac. The sequences underlined are Eco R1 and Bam H1 recognition sites to facilitate the subsequent cloning. The PCR was run for 30 cycles with denaturation at 94 °C for 2 min, annealing at 55 °C for 1 min, and extension at 72 °C for 1.5 min in each cycle on DNA Thermal Cycler 480 (Perkin Elmer Cetus). The PCR products of VH and VL were digested with Eco R1 and Bam H1 and purified from 1% agarose gel with Geneclean kit (BIO 101 Inc, La Jolla, CA, USA), and ligated onto plasmid Bluescript M13 SK (Stratagene, La Jolla, CA, USA). The recombinant plasmids were transferred into E coli DH5α and analysed for an insert of the right size by digestion with Eco R1 and Bam H1. The dideoxy chain termination sequencing procedure was performed according to Sanger et al[29] using the Bluescript KS and SK primer sites and a T7 sequencing kit (Pharmacia, Woerden The Netherlands). Several independent clones

and at least three different colonies of each H chain and L chain of each anti-AChR mAb were sequenced to confirm the determined VH and VL sequences.

Mutual homology of sequences at the nucleotide level between anti-AChR mAbs was determined both for the overall VH and VL regions and for the complementarity determining regions (CDRs) using the program DNAsis. Homology between anti-AChR mAbs and other antibodies in EMBL Data Library was performed by FASTA computer search. The sequences of VH and VL of mAbs A62, A64, and A65 have been accepted by the EMBL Data Library as AC No (VH and VL): A62: X80952 and X80951, A64: X80948 and X80947, and A65: X80946 and X80945.

Results

Characteristics of anti-α-BT binding site mAbs

The isotype, specificity and cross-reactivity of anti-α-BT binding site mAbs A62, A64 and A65 were summarized in Table 1.

Table 1 Characteristics of anti-α-BT binding site mAbs

mAb	isotype	specificity[a]	AChR[b]
A62	IgG2b	α-BT binding site	T
A64	IgG1	α-BT binding site	T
A65	IgG2b	α-BT binding site	T

[a] MAbs able to block the binding of α-bungarotoxin to AChR.

[b] Reactivity of mAbs with AChR from Torpedo (T).

Epitope specificity of anti-α-BT binding site mAbs

Mutual inhibition experiments between anti-α-BT binding site mAbs elicited against tAChR demonstrated that mAbs A62 and A65 recognized the same or overlapping epitopes. MAb A64 probably recognized the same as mAbs A62 and A65 but with higher affinity (Figure 1).

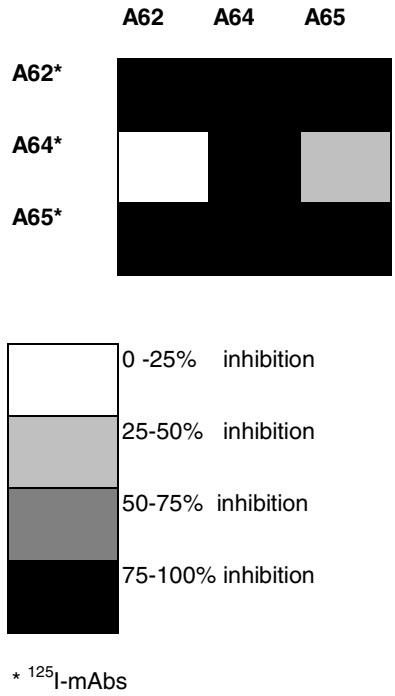


Figure 1 Epitope mapping of anti-α-BT binding site mAbs. Epitope specificity of anti-α-BT binding site mAbs was defined by mutual inhibition between mAbs for binding to AChR. The binding of ^{125}I-mAb (50% of maximum binding) was inhibited with 0.08-10 μg/ml of unlabeled competitor mAb. The results in this figure represent the percentage of inhibition at 2 μg/ml unlabeled competitor.

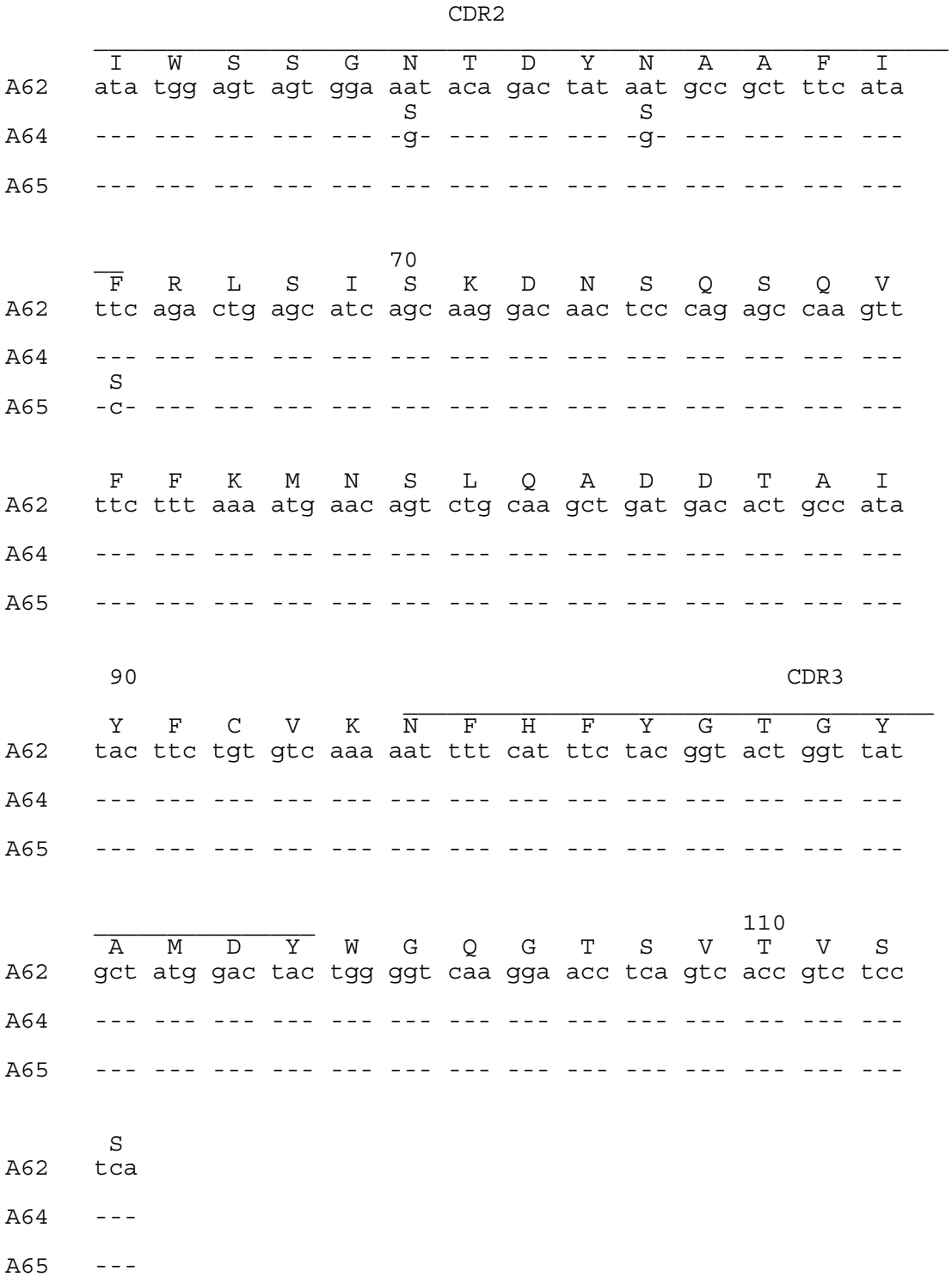
Genetic features of anti-α-BT binding site mAbs

All H chain variable region genes of anti-α-BT binding site mAbs A62, A64 and A65 are 339 bp in length (the first 24 bp in VH upstream primer not included), encoding 113 amino acids and determining the codons 9-113 of VH (Figure 2A). All the three mAbs use a VH gene derived from mouse germline family Q52 (EMBL Data Library, AC No M24271), associated with D and JH4[30].

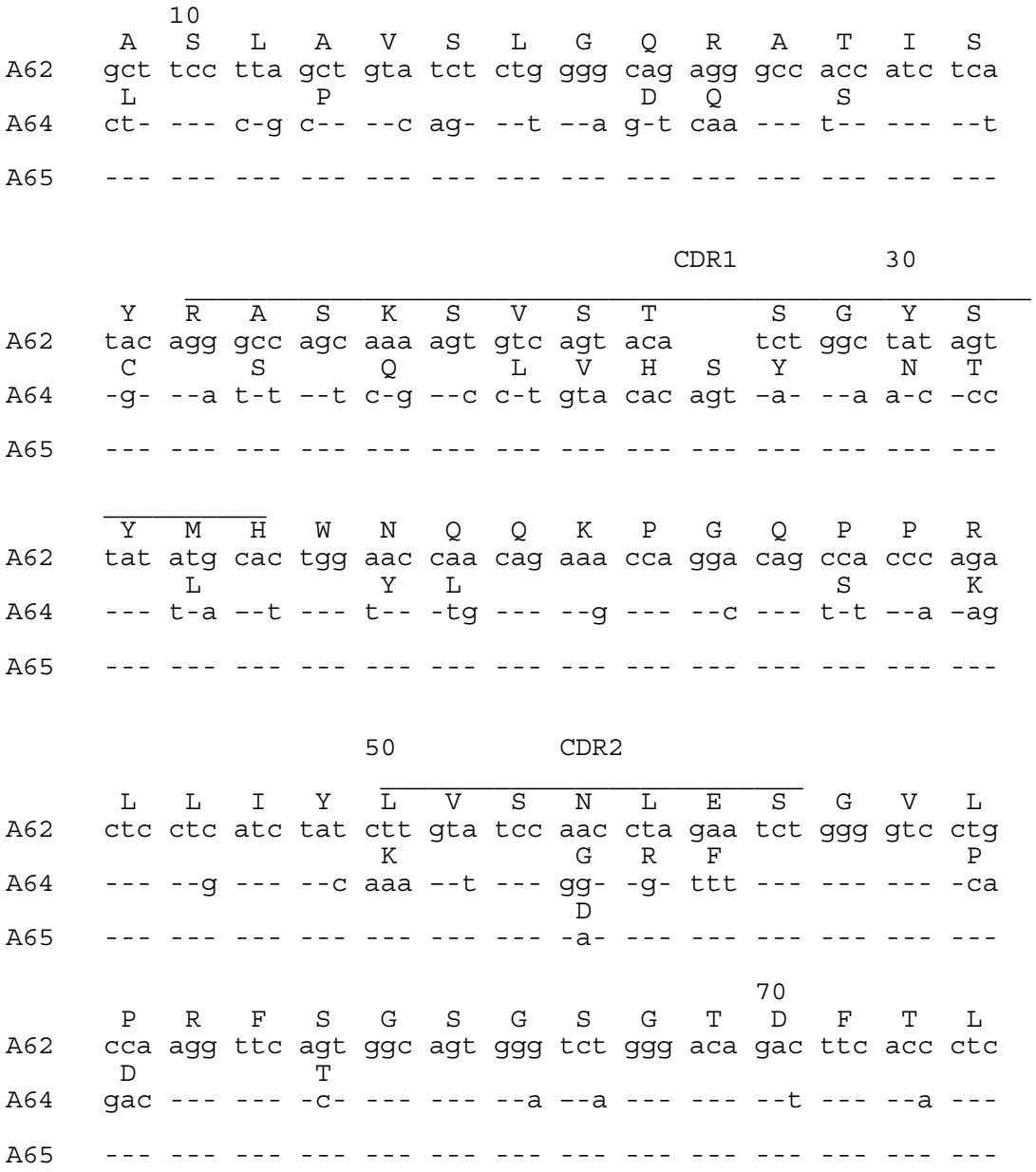
The L chain variable region gene of anti-α-BT binding site mAb A62 is 306 bp in length (the first 24 bp in VL upstream primer not included), encoding 102 amino acids and determining the codons 9-107 of VL (Figure 2B). MAb A62 uses a VL gene being a member of mouse subgroup Vκ3 joined with Jκ1 segment[31]. The L chain variable region gene of mAbs A64 and A65 have extra 2 amino acids with one in CDR1 and one in CDR3 as compared with mAb A62. The VL gene used by mAbs A64 and A65 is a member of mouse Vκ2 subgroup and also associated with Jκ1 segment.

A

```
          10
       P   G   L   V   Q   P   S   Q   S   L   S   I   T   C
A62  cct ggc cta gtg cag ccc tca cag agc ctg tcc atc acc tgc

A64  --- --- --- --- --- --- --- --- --- --- --- --- --- ---

A65  --- --- --- --- --- --- --- --- --- --c --- --- --- ---

                                30          CDR1
                                            ____________
       T   V   S   G   F   S   L   T   S   Y   G   I   H   W
A62  aca gtc tct ggt ttc tca tta act agc tat ggt ata cac tgg

A64  --- --- --- --- --- --- --- --- --- --- --- --- --- ---

A65  --- --- --- --- --- --- --- --- --- --- --- --- --- ---

                                                        50
                                                        __
       V   R   Q   P   P   G   K   G   L   E   W   L   G   V
A62  gtt cgc cag cct cca gga aag ggt ctg gaa tgg ctg gga gtg

A64  --- --- --- --- --- --- --- --- --- --- --- --- --- ---

A65  --- --- --- --- --- --- --- --- --- --- --- --- --- ---
```


CDR2
I W S S G N T D Y N A A F I
A62 ata tgg agt agt gga aat aca gac tat aat gcc gct ttc ata
S S
A64 --- --- --- --- --- -g- --- --- --- -g- --- --- --- ---
A65 --- --- --- --- --- --- --- --- --- --- --- --- --- ---
70
F R L S I S K D N S Q S Q V
A62 ttc aga ctg agc atc agc aag gac aac tcc cag agc caa gtt
A64 --- --- --- --- --- --- --- --- --- --- --- --- --- ---
S
A65 -c- --- --- --- --- --- --- --- --- --- --- --- --- ---
F F K M N S L Q A D D T A I
A62 ttc ttt aaa atg aac agt ctg caa gct gat gac act gcc ata
A64 --- --- --- --- --- --- --- --- --- --- --- --- --- ---
A65 --- --- --- --- --- --- --- --- --- --- --- --- --- ---
90
CDR3
Y F C V K N F H F Y G T G Y
A62 tac ttc tgt gtc aaa aat ttt cat ttc tac ggt act ggt tat
A64 --- --- --- --- --- --- --- --- --- --- --- --- --- ---
A65 --- --- --- --- --- --- --- --- --- --- --- --- --- ---
110
A M D Y W G Q G T S V T V S
A62 gct atg gac tac tgg ggt caa gga acc tca gtc acc gtc tcc
A64 --- --- --- --- --- --- --- --- --- --- --- --- --- ---
A65 --- --- --- --- --- --- --- --- --- --- --- --- --- ---
S
A62 tca
A64 ---
A65 ---

B


10
A S L A V S L G Q R A T I S
A62 gct tcc tta gct gta tct ctg ggg cag agg gcc acc atc tca
L P D Q S
A64 ct- --- c-g c-- --c ag- --t --a g-t caa --- t-- --- --t
A65 --- --- --- --- --- --- --- --- --- --- --- --- --- ---
CDR1 30
Y R A S K S V S T S G Y S
A62 tac agg gcc agc aaa agt gtc agt aca tct ggc tat agt
C S Q L V H S Y N T
A64 -g- --a t-t --t c-g --c c-t gta cac agt -a- --a a-c -cc
A65 --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Y M H W N Q Q K P G Q P P R
A62 tat atg cac tgg aac caa cag aaa cca gga cag cca ccc aga
L Y L S K
A64 --- t-a --t --- t-- -tg --- --g --- --c --- t-t --a -ag
A65 --- --- --- --- --- --- --- --- --- --- --- --- --- ---
50 CDR2
L L I Y L V S N L E S G V L
A62 ctc ctc atc tat ctt gta tcc aac cta gaa tct ggg gtc ctg
K G R F P
A64 --- --g --- --c aaa --t --- gg- -g- ttt --- --- --- -ca
D
A65 --- --- --- --- --- --- --- -a- --- --- --- --- --- ---
70
P R F S G S G S G T D F T L
A62 cca agg ttc agt ggc agt ggg tct ggg aca gac ttc acc ctc
D T
A64 gac --- --- -c- --- --- --a --a --- --- --t --- --a ---
A65 --- --- --- --- --- --- --- --- --- --- --- --- --- ---

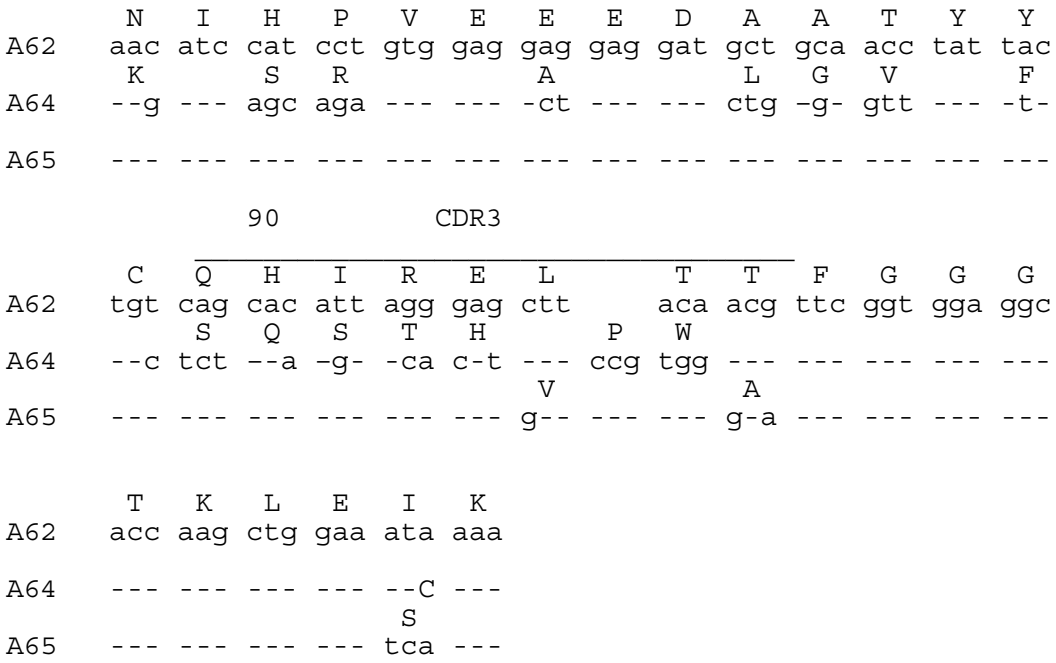


Figure 2 Sequence comparison of anti-α-BT binding site mAbs. Sequences of anti-α-BT binding site mAbs are compared for the heavy chain (A) and light chain (B). The complete nucleotide sequences and deduced amino acid sequences are aligned starting from codon 9 as numbered according to Kabat et al[28]. Sequence identity of anti-α-BT binding site mAbs with mAb A62 or with A64 (A65 was compared with A64 in light chain) is indicated by dashes. Complementarity determining regions (CDRs) are also indicated.

Discussion

The clinical signs and symptoms in MG result from loss of functional AChR which is entirely antibody-mediated[4]. The pathogenic anti-AChR antibodies are mainly found to be those that are directed against a region on the α-subunit of AChR (α67–76), MIR[32]. However, antibodies to the cholinergic and neurotoxin binding sites may contribute to the clinical signs in some MG patients[33, 34]. Therefore, the understanding of contribution of individual anti-α-BT binding site antibodies to AChR loss is also important in the pathogenesis of MG. Unfortunately, since the anti-α-BT binding site mAbs obtained

from mice immunized with tAChR were not able to cross-react with mammalian AChR (rat or mouse AChR), we could not investigate the pathogenicity of the mAbs in vivo by passive transfer of the mAbs into rats or mice. However, the epitope mapping and the comparison of sequences among the anti-α-BT binding site mAbs and between the mAbs and pathogenic anti-MIR mAb revealed some important features.

The H chain sequence of variable region of mAb A62 shows a 99.4% homology at the nucleotide level as compared with mAb A64. Two nucleotide difference are identified. Both appear in CDR2 and lead to amino acid changes (in codons 56 and 60, Asn and Asn in A62, whereas Ser and Ser in A64). MAbs A62 and A65 almost share an identical H chain at the amino acid level with only one amino acid substitution in CDR2 (a Phe in A62 and a Ser in A65 in codon 65). The overall sequence homology of H chain between mAbs A64 and A65 is 98.8% with 3 amino acid differences in CDR2. The L chain sequence of variable region of mAb A62 shows a 65.2% and 64.3% homology respectively at the nucleotide level as compared with mAbs A64 and A65, since they are derived from different mouse subgroup genes. However, the homology of L chain between mAbs 64 and A65 is 97.8% with 7 nucleotide substitutions. One occurs in codon 53 within CDR2 and leads to amino acid change (Gly in A64 and Asp in A65). Three appear in 2 codons of CDR3 (codons 94 and 97, Leu and Thr in A64, whereas Val and Ala in A65).

The epitope mapping of the anti-α-BT binding site mAbs shows that mAbs A62 and A65 recognize the same epitope. The analysis of gene utilization of the mAbs indicates that the H chain of A62 and A65 are derived from the same mouse germline family. The substitutions in CDR2 between the mAbs A62 and A65 (Phe→Ser) did not lead to specificity change. By contrast, the L chains of both mAbs A62 and A65 belong to different mouse subgroup and express only 46.7%, 57.1% and 47.8% homology at nucleotide level in CDR1, 2 and 3 respectively, suggesting that the H chain play more important role in binding to AChR. MAbs A64 and A65 are likely to be derived from the same germline gene but undergone limited somatic mutation driven by antigen AChR. The conserved substitutions in CDR2 of H chain between A64 and A65 probably did not influence the antibody specificity. However, the substitutions in CDR2 and CDR3 of L chain seem to determine the higher affinity of mAb A64 to AChR.

Alignment of sequence of the anti-α-BT binding site mAbs with that of pathogenic anti-MIR mAb A7 (see Chapter 2) reveals a large sequence diversity. MAb A7 shows only a 63.7%, 64.0% and 63.4% overall sequence homology in H chains and a 97.0%, 64.7% and 64.1% in L chains with mAbs A62, A64 and A65 respectively. Furthermore, the homology in CDRs of both H and L chains is all below 40% at amino acid level (except A62 L chain). Despite the highly homologous L chains used by mAbs A7 and A62, the quite diverse H chains determine the different specificities of the mAbs.

In conclusion, sequence analysis of anti-α-BT binding site mAbs shows that the VH genes utilized are related to Q52 germline family, and a large sequence diversity is found between the anti-α-BT binding site mAbs and the pathogenic anti-MIR mAb.

Acknowledgements

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Chapter 4

Construction and characterization of a single chain antibody fragment derived from thymus of a patient with myasthenia gravis

Abstract

Pathogenic anti-acetylcholine receptor (AChR) antibodies in myasthenia gravis (MG) and its animal model experimental autoimmune myasthenia gravis (EAMG) are mainly those that are directed against the main immunogenic region (MIR) of AChR. Bivalent anti-MIR antibodies binding to α-subunits of AChR result in AChR loss by antigenic modulation and activation of complement. Monovalent Fab and single chain variable fragment (ScFv) of pathogenic anti-AChR antibodies can prevent the pathogenic antibodies from binding to AChR. In the present study, a ScFv637 was constructed from its parental Fab637, previously isolated from thymus-derived phage display library with specificity of anti-MIR of human AChR (hAChR), by PCR amplification. PCR products of VH and VL genes of Fab637 were assembled into vector phagemid pHEN2 containing a GS rich linker and a c-myc tag and a 6x His tag at C-terminus for specific detection and for efficient purification. The recombinant pHEN2-ScFv637 construct was transformed into E coli HB2151 for soluble production of ScFv after induction with IPTG. ScFv637 was efficiently produced in periplasmic fraction but not in culture supernatant of bacteria as detected by nitrocellulose dot blot using mouse anti-c-myc monoclonal antibody (mAb). ScFv637 was able to bind to hAChR in standard precipitation radioimmunoassay (RIA). ScFv637 could also bind to monkey AChR in situ on monkey neuromuscular junction as showed in immunohistochemical staining. Furthermore, ScFv637 was capable of inhibiting the binding of its intact IgG637 and anti-MIR mAb35 to hAChR up to 32.9% and 73.0% respectively demonstrated in a competitive ELISA, and of MG patient sera from 27.8% to 45.5% in a competitive RIA. Therefore, ScFv637, easier for manipulation in improvement of affinity and stability compared with its parental Fab637, may serve as an alternative candidate for specific immunotherapy in MG.

Introduction

In the autoimmune disease myasthenia gravis (MG), the binding of autoantibodies to acetylcholine receptor (AChR) results in AChR loss and leads to fatigability and weakness of skeletal muscle[1-3]. Experimental autoimmune myasthenia gravis (EAMG), an animal model that closely resembles human MG, can be induced by active immunization of purified AChR or by passive transfer of polyclonal or monoclonal antibodies (mAbs) against AChR[4-6]. The AChR is well characterized autoantigen[7]. Muscle-type AChR in neuromuscular junction is a complex transmembrane glycoprotein and consists of five subunits of $\alpha_2\beta\gamma\delta$[8-10]. The AChR contains a characteristic region, termed the main immunogenic region (MIR) on the α-subunit at residues 67-76[11-13] which is easily accessible in vivo to antibodies.

More than half of the autoantibodies in MG patients or EAMG rats were found to be directed against the MIR[14-16] and pathogenic mAbs were found exclusively among mAbs against MIR[6, 17, 18]. Anti-MIR antibodies are especially effective in antigenic modulation[19], a mechanism in which the binding of anti-AChR antibodies to AChR by cross-linking of adjacent AChR molecules results in accelerating AChR internalization. Furthermore, the binding of anti-MIR antibodies to AChR can also trigger the complement cascade leading to focal lysis of postsynaptic membrane[6].

The ideal immunosuppressive therapies for MG would be to eliminate the autoimmune response to AChR without affecting the immune response to other antigens. Several specific immunosuppressive therapies have been tested in EAMG based on the pathogenesis of MG including anti-idiotypic antibodies[20] and administration of AChR orally[21-23] or nasally[24-26]. Meanwhile, the development of a bioimmunological agent for specific treatment of MG patients has been the focus of many researches in MG. Fab and single chain variable fragment (ScFv) of antibodies derived from anti-MIR antibodies can bind to AChR, blocking the binding of anti-MIR antibodies to AChR, but does not have the activity to induce antigenic modulation or complement reaction. Fab isolated from MG patient thymus[27, 28] and from rat anti-MIR mAbs[29, 30] or ScFv constructed from rat anti-MIR mAbs[31-33] could prevent anti-MIR mAbs or MG patient

sera from binding to AChR or from inducing antigenic modulation in a cultured TE671 cell line.

In this study, an human ScFv637 directed against the MIR of human AChR (hAChR) was constructed from its parental Fab637, isolated from thymus-derived phage display library of a MG patient earlier in our laboratory[27]. ScFv637 derived from human does not possess immunogenicity for application in MG patients and easily undergoes genetic manipulation to improve its stability and affinity.

Materials and methods

Anti-MIR Fab637

Fab637 was isolated from the thymus of a patient with MG by phage display library using vector phagemid pComb3H, and panned on hAChR extracted from TE671 cell culture[34]. The reconstructed phagemid DNA was transformed into E coli XL1-blue for production of soluble Fab637 after last round of panning. Fab637 was able to bind hAChR in precipitation radioimmunoassay (RIA) and solid phase RIA (SPRIA)[27].

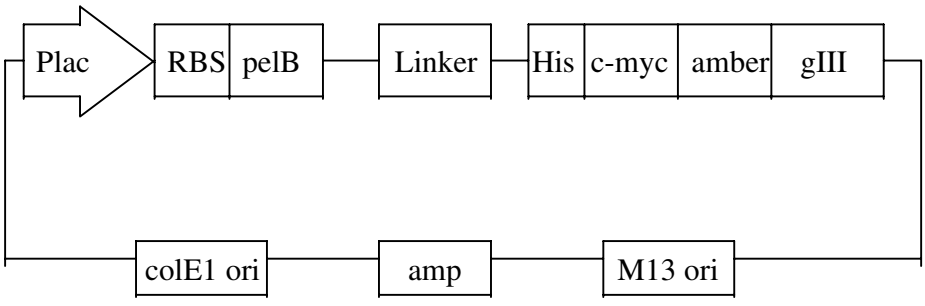
Construction of ScFv637

VH gene of ScFv637 was amplified from pComb3H-Fab637 by PCR using VH upstream primer 5' aa tctaga ccatg gcc gag gtg cag ctg ctg gag tct ggg (the sequences underlined are the recognition sites of endonucleases XbaI and NcoI respectively) encoding the first 8 amino acids of VH region, and VH downstream primer 5' at gaattc ctcga gga tga gac agt gac cag gg (underlined are EcoRI and XhoI sites respectively) complementary to the gene of amino acids 107-113. The VL gene of ScFv637 was amplified using VL upstream primer 5' aa tctaga gtgca ccc gag ctc act cag ccc (underlined are XbaI and ApaLI sites respectively) encoding the first 7 amino acids of VL region, and VL downstream primer 5' at gaattc gcggccgc tag gac ggt cag ctt gg (underlined are EcoRI and NotI sites respectively) complementary to the gene of amino acids 102-107. The PCR

was performed for 35 cycles with denaturation at 95 °C for 1 min, annealing at 50 °C for 1 min and extension at 72 °C for 2 min using high fidelity enzyme mix (Roche Diagnostics GmbH, Mannheim, Germany).

The VH and VL gene fragments were purified with QIAquick PCR Purification Kit (QIAGEN GmbH, Hilden, Germany). The purified VH gene fragments were first digested with NcoI and XhoI and 1% agarose gel-purified with Geneclean Kit II (Bio 101 Inc, La Jolla, CA, USA), and cloned onto NcoI and XhoI site of vector pHEN2 (Figure 1; a generous gift of Dr G. Winter, Center for Protein Engineering, MRC Center, University of Cambridge, UK). The recombinant phagemid was transformed into competent E coli DH5α and isolated and purified with Wizard Plus SV Minipreps (Promega, USA). The right insert was confirmed by digestion with the same endonucleases NcoI/XhoI. Subsequently, the purified VL gene fragments were digested with ApaLI/NotI, and cloned onto the pHEN2-VH as above to construct a complete ScFv637 gene segment. The ScFv637 was then transformed into E coli DH5α and cloned and purified with QIAGEN Plasmid Midi Kit (QIAGEN GmbH, Hilden, Germany). The recombinant phagemid was digested with NcoI/XhoI to confirm the right size of VH insert, with ApaLI/NotI to confirm the VL insert, and with NcoI/NotI to confirm the ScFv insert.

A



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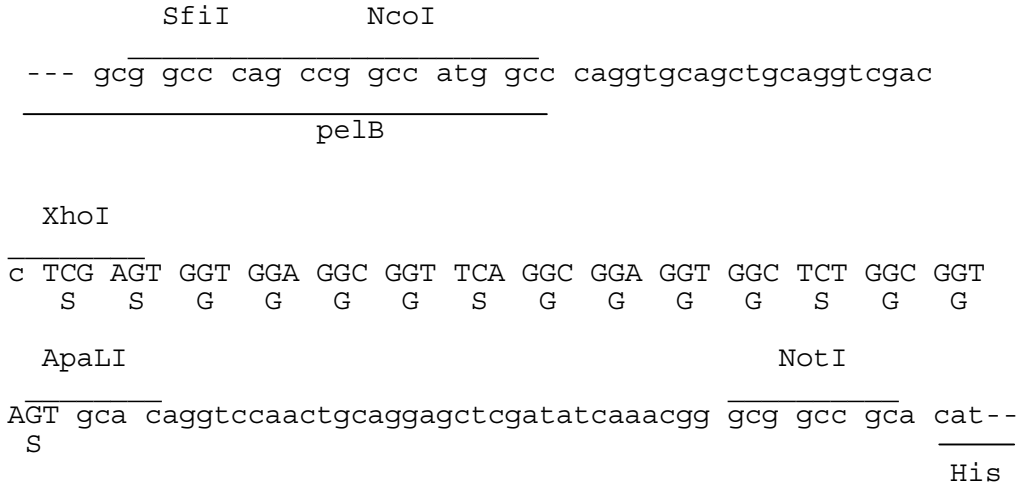

Figure 1 Phagemid vector pHEN2 used for construction of single chain antibody fragment (ScFv). A: Structure of pHEN2. B: Sequence of cloning sites of pHEN2. Sequences underlined are part of pelB leader or 6x His tag. Nucleotide sequence of linker is depicted in capital letters.

DNA sequencing

The sequencing reaction of purified pHEN2-ScFv637 was performed with dye terminator cycle sequencing method using Terminator Ready Reaction Mix (Perkin Elmer, ABI Prism, USA) in PCR at the conditions of denaturation at 96 °C for 30 sec, annealing at 50 °C for 15 sec, and extension at 60 °C for 4 min. The primers VH upstream, VH downstream and VL upstream used in PCR amplification of VH and VL gene fragments above were used as sequencing primers. The PCR was run for 26 cycles in a total volume of 20 µl. The PCR products were purified with Edge Gel Filtration Cartriges (Edge BioSystems, USA) and sequenced on ABI Prism 310 Genetic Analyzer (PE BioSystems, USA).

Production of soluble ScFv637

The purified pHEN2-ScFv637 from E coli DH5α was transformed into calcium chloride-treated competent E coli HB2151 for production of soluble ScFv637. The transformant E coli HB2151 were grown from a single colony overnight at 37 °C in a 5 ml 2x TY medium containing 100 μg/ml ampicillin and 1% glucose, and subsequently diluted in 500 ml 2x TY medium with 100 μg/ml ampicillin and 0.1% glucose. The culture was further incubated at 37 °C to an OD_{600nm} of 0.6. The expression was induced by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) at final concentration of 1 mM. After an additional 3 h of incubation at 30 °C with shaking, the bacteria were harvested by centrifugation at 10 000 xg for 10 min at 4 °C.

The periplasmic fraction of ScFv637 was prepared with sodium borate[35]. The bacterial pellet was resuspended in 5 ml ice-cold sodium borate solution (200 mM sodium borate, 160 mM sodium chloride, 1 mM EDTA, pH 8.0). After incubation on ice for 20 min, the bacterial suspension was centrifuged at 15 000 xg for 10 min at 4 °C. The supernatant represented the periplasmic fraction. E coli HB2151 which do not bear pHEN2-ScFv637 or bear pHEN2 only were used as control.

Detection of ScFv637

The production of soluble ScFv637 was detected with nitrocellulose dot blot[36]. A 5 μl of periplasmic fraction or culture supernatant of ScFv637 was loaded on nitrocellulose filter (0.45 μm, Bio Rad). Non-specific binding sites on the filter were blocked for 1 h at room temperature in PBS containing 2% dried skimmed milk, and subsequently incubated with 300 ng/ml of mouse anti-c-myc mAb (a kind gift of Dr R. Fischer, Institute of Biology I, RWTH Aachen, Germany) for 1 h at room temperature with gentle shaking. After washing 3 times with PBS containing 0.05% Tween 20 and 2 times with PBS alone, the binding of ScFv637 to anti-c-myc mAb was detected by adding rabbit anti- mouse IgG conjugated with HRP (1/2000, Pierce). The HRP activity was visualized with diaminobenzidine tetrahydrochloride (DAB).

Purification of ScFv637

The purification of ScFv637 was performed with immobilized metal affinity chromatography (IMAC) using nickel-nitrolotriacetic acid (Ni-NTA) agarose (QIAGEN, Westburg bv, Leusden, The Netherlands). Periplasmic fraction of E coli HB2151 made with sodium borate solution was dialysed against lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 3 mM imidazole, pH 8.0), and loaded on Ni-NTA resin column. After washing with wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 8.0), the column was eluted with elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8.0). The collected elution fractions were analysed by 12% SDS-PAGE. Protein bands were detected by Coomassie staining and ScFv637 by Western blotting using mouse anti-c-myc mAb.

Determination of ScFv637 specificity

The specificity of ScFv637 binding to AChR was determined with precipitation RIA using crude hAChR extract as antigen. Briefly, 200 µl of periplasmic fraction of ScFv637 were incubated with 200 µl of crude hAChR extract (preincubated with 2 pmol ^{125}I-α-BT/pmol AChR for 4 h at 4 °C) overnight at 4 °C, and further incubated with 100 µl mouse anti-c-myc mAb (10 µg/ml) for additional 2 h at 4 °C. The complex of hAChR-ScFv637-anti-mouse c-myc mAb was precipitated by addition of goat anti-mouse IgG and bound activity was counted in γ counter.

The cross-reactions of ScFv637 to rat AChR (rAChR) or Torpedo AChR (tAChR) were also tested by RIA using 200 µl of crude rAChR extracted from rat muscle or 200 µl of purified tAChR (0.25 µg/ml) isolated from electric organ of Torpedo californica (Pacific Biomarine, California, USA).

Immunohistochemical staining of ScFv637

Binding of ScFv637 to AChR in situ at the neuromuscular junction was verified by immunohistochemical staining on monkey Macaca fascicularis intercostal muscle section

(a kind gift from Dr M. Jonker, Biomedical Primate Research Center, Rijswijk, The Netherlands). Slides, fixated with cold acetone for 10 min at 4 °C, were preincubated with PBS containing 2% BSA, and subsequently incubated with 1/10 diluted periplasmic fraction of bacteria for 1 h at room temperature. After washing 3 times with PBS with 5 min for each time, slides were incubated with mouse anti-c-myc mAb together with rhodamine-labeled α-BT (α-BT^{Rh}, Sigma) for 1 h at room temperature. The binding activity was visualized by addition of goat anti-mouse IgG conjugated with FITC.

Protection of hAChR from binding of anti-MIR antibodies and MG patient sera

The protective ability to hAChR was measured in a competitive ELISA between ScFv637 and its intact IgG637 and rat anti-MIR mAb35[37], and in a competitive RIA between ScFv637 and MG patient sera. ELISA plates were coated with 50 µl mAb153 (15 µg/ml), a rat anti-AChR mAb directed against a cytoplasmic epitope[38], for 1 h at 37 °C, then blocked with PBS containing 3% BSA and 0.05% Tween 20. The plates were incubated overnight at 4 °C with 50 µl hAChR (0.3 nmol/L) extracted from TE671 cells, and after washing, further incubated with periplasmic fraction of ScFv637 (0.0032 – 50 µl) overnight at 4 °C. For inhibition of mAb35, 50 µl mAb35 conjugated with HRP (IC_{50} = 0.9 µg/ml, predetermined in ELISA) was added and incubated for 1 h at 37 °C without washing, and HRP reaction was developed with tetramethylbenzidine (TMB). For inhibition of IgG637, 50 µl IgG637 (IC_{50} = 80 ng/ml, predetermined in ELISA) was added and incubated for 1 h at 37 °C without washing. Subsequently 50 µl goat anti-human IgG (Fc specific) coupled with alkaline phosphatase (AP, 1/250, preabsorbed with 1% normal rat serum at 37 °C for 1 h) was added and incubated for 1 h at 37 °C, and the color reaction was visualized with p-nitrophenyphosphate (pNPP). In RIA, 50 µl hAChR (0.3 nmol/L) labeled with ^{125}I-α-BT at 4 °C for 4 h was preincubated with 250 µl of periplasmic fraction of ScFv637 overnight at 4 °C, subsequently 10 µl of MG patient sera (70% of the maximum binding to hAChR) was added. After an aditional 3 h incubation at 4 °C, the complex was precipitated by addition of goat anti-human immunoglobulin serum, and the radioactivity was counted.

The result was expressed as percentage of inhibition of binding of anti-MIR antibodies or MG patient sera.

Results

Cloning of ScFv637 gene

VH and VL genes of ScFv637 were amplified by PCR from parental Fab637 isolated from thymus-derived phage display library of a MG patient, and subsequently cloned into vector pHEN2. The reconstructed phagemid pHEN2-ScFv637 was transformed into E coli DH5α. After isolation and purification, pHEN2-ScFv637 was digested for the right size of insert of VH, VL and ScFv with the same endonucleases used in cloning, and checked in 1% agarose gel electrophoresis. The VH, VL and ScFv should be 378 bp, 339 bp and 762 bp in length respectively according to design (Figure 2).

Sequencing of ScFv637 construct

The complete amino acid sequence of ScFv637 was expressed in Figure 3. The VH and VL sequences of ScFv637 are identical to those of its parental Fab637 at amino acid level although there are two nucleotide differences in primer-encoded sequences of VH fragment. The original nucleotide sequences of Fab637 were described earlier[27].

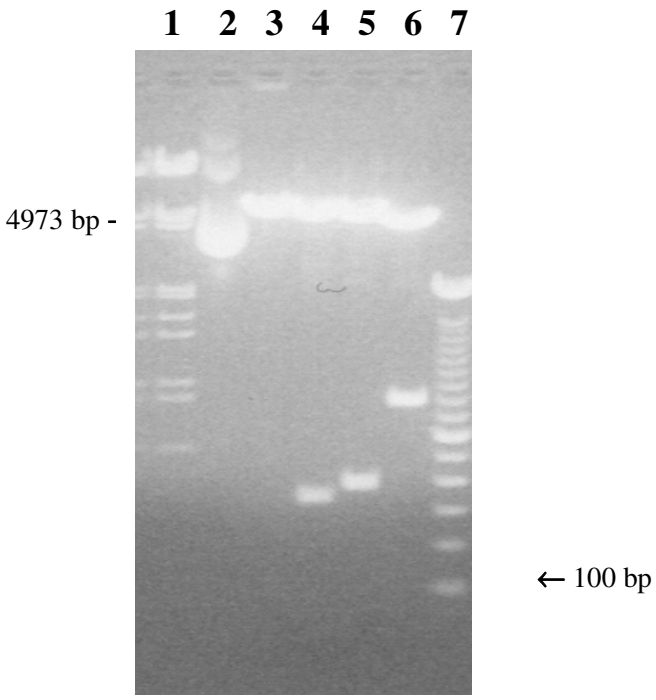

Figure 2 Analysis of ScFv637 construct in 1% agarose gel electrophoresis. The VH and VL genes of ScFv637 were cloned into phagemid pHEN2 from parental Fab637. After transformation in E coli DH5α, the recombinant pHEN2-ScFv637 were digested with ApaLI/NotI for VL (lane 4), NcoI/XhoI for VH (lane 5), and NcoI/NotI for ScFv637 (lane 6). Lanes 2 and 3 are undigested ScFv637 and ScFv637 digested with XhoI alone respectively. Lanes 1 and 7 are λ DNA marker digested with EcoRI/HindIII and 100 bp ladder respectively.

VH

```
                    VH upstream primer
                   ___________________________
                   1   2   3   4   5   6   7   8
                   E   V   Q   L   L   E   S   G
      ScFv637 gag gtg cag ctg ctg gag tct ggg
FRW1
      Fab637  --- --- --- --- --c --- --- ---

                   9                                                  30
                   G D L V K P G G S L R L S C A A S G F K S T

                   31      35
CDR1               D Y Y M S
```

```
            36                          49
FRW2        W V R Q A P G R G L E W V S

            50    52a                         65
CDR2        F I • S  G R V F T N Y T A S V R G

            66                                  82a b c
FRW3        R F T V F R E D D N T S V Y L Q M S   R L R V E D T
                  94
            A V Y Y C A R

            95          100a b c d e f g h i j   102
CDR3        L R G I F R G    P L K P L E Y Y F D L

                        VH downstream primer
                        _____________________________
            103       107 108 109 110 111 112 113
            W  G R G  T   L   V   T   V   S   S
      ScFv637         acc ctg gtc act gtc tca tcc
FRW4
      Fab637          --- --- --- --- --- --- --g


            1                           15
Linker      S S G G G G S G G G G S G G G S


VL                  VL upstream primer
                    _____________________
             1   2   3   4   5   6   7
             A   P   E   L   T   Q   P
      ScFv637 gca ccc gag ctc act cag ccc
FRW1
      Fab637          --- --- --- --- ---

            8                               23
            H S V S E S P G K T V T I S C T

            24      27a             34
CDR1        R S S G S  I A S N Y V Q

            35                          49
FRW2        W Y Q Q R P G S S P T T V I Y

            50          56
CDR2        E D N Q R P S

            57                                72a b
FRW3        G V P D R F S G S I D S S S N S A   S L T I S G L K
                        88
            T E D E A D Y Y C
```

```
                89            95a b   97
CDR3            Q S Y D S N S G   G R V

                          VL downstream primer
                          ____________________
                98        102 103 104 105 106 107
                F G G G    T   K   L   T   V   L
      ScFv637             acc aag ctg acc gtc cta
FRW4
      Fab637              --- --- --- --- --- ---
```

Figure 3 Amino acid sequences of heavy (H) and light (L) chains of ScFv637. The primers used both in PCR amplification of VH and VL genes and for sequencing of ScFv637 are also indicated as nucleotide sequences, and compared with its parental Fab637. The codon "ctc" at amino acid position 5 in VH of Fab637 was changed into "ctg" in ScFv637 to remove XhoI recognition site (ctcgag) without substitution of amino acid. The codon "tcg"→"tcc" from Fab637 to ScFv637 at amino acid position 113 of VH was created in VH downstream primer to match the XhoI restriction sequence without introduction of an extra amino acid between VH and linker. The sequence identity of Fab637 with ScFv637 in primers is indicated by dashes. The amino acid position is numbered according to Kabat et al[39]. FRW: framework; CDR: complementarity determining region; •: missing of amino acid.

Expression and purification of soluble ScFv637

The construct of ScFv637 was transformed into non-suppressor E coli HB2151 for production of soluble ScFv637. The production was detected from both periplasmic fraction and culture supernatant of bacteria by nitrocellulose dot blot assay. The soluble ScFv637 was produced in periplasm but not in culture medium of bacteria after induction of IPTG for 3 h at 30 °C. However, no production was detectable in either periplasm or culture supernatant of control bacteria or bacteria without induction of IPTG (result not shown).

SDS-PAGE and Western blottiong analysis of fractions from IMAC purification showed that the apparent molecular weight of ScFv637 was approximately 36 kDa although it was calculated to be 30.4 kDa from the amino acid sequence (Figure 4).

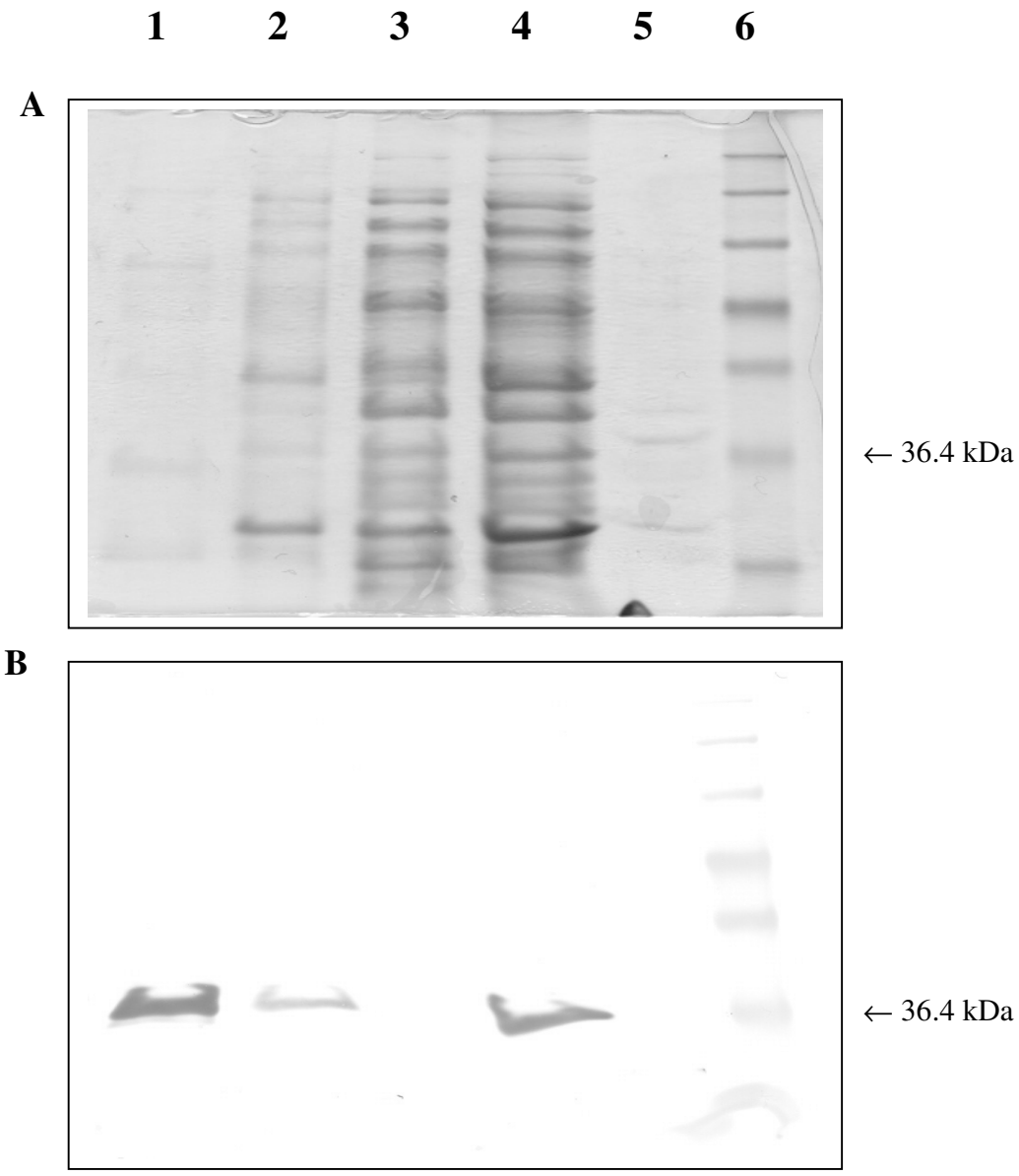

Figure 4 SDS-PAGE and Western blotting analysis of fractions from IMAC purification of ScFv637 carrying c-myc and 6x His tags. Two identical gels were run in parallel. Protein bands were detected by Coomassie staining (A) and the ScFv637 by Western blotting using mouse anti-c-myc mAb and followed by rabbit anti-mouse IgG conjugated with HRP and substrate DAB (B). Lanes: 1, eluate with 250 mM imidazole; 2, washing fraction with 20 mM imidazole; 3, flow-through; 4, unpurified periplasmic fraction; 5, periplasmic fraction from E coli HB2151 as negative control; 6, molecular standards.

Antigen binding activity of ScFv637

The binding activity of ScFv637 to hAChR extracted from TE671 cells was tested in a precipitation RIA. ScFv637 produced in periplasm of different colonies of bacteria determined in nitrocellulose dot blot were able to bind hAChR but not rAChR or tAChR. Whereas, no binding activity was found in bacterial culture medium and in control bacteria (Figure 5).

ScFv637 could also cross-react with monkey AChR at neuromuscular junction of monkey intercostal muscle section. ScFv637 bound to endplate regions (Figure 6, A and C) which were colocalized in the same slides with α-BT^{Rh} (Figure 6, B and D).

Protective capacity of ScFv637 to hAChR

The inhibition of ScFv637 to anti-MIR antibodies or MG patient sera for binding to hAChR was determined in a competitive ELISA or RIA. ScFv637 expressed in periplasmic fraction of E coli HB2151 inhibited the binding of anti-MIR mAb35 to hAChR up to 73.0%, whereas it inhibited the binding of its intact IgG637 only up to 32.9% (Figure 7A), probably due to its low afinity and the very high affinity of IgG637 to hAChR (unpublished data). ScFv637 could also inhibit the binding of MG patient sera to hAChR from 27.8% to 45.5% (Figure 7B), clearly indicating its protective capacity towards a broad range of polyclonal anti-AChR antibodies.

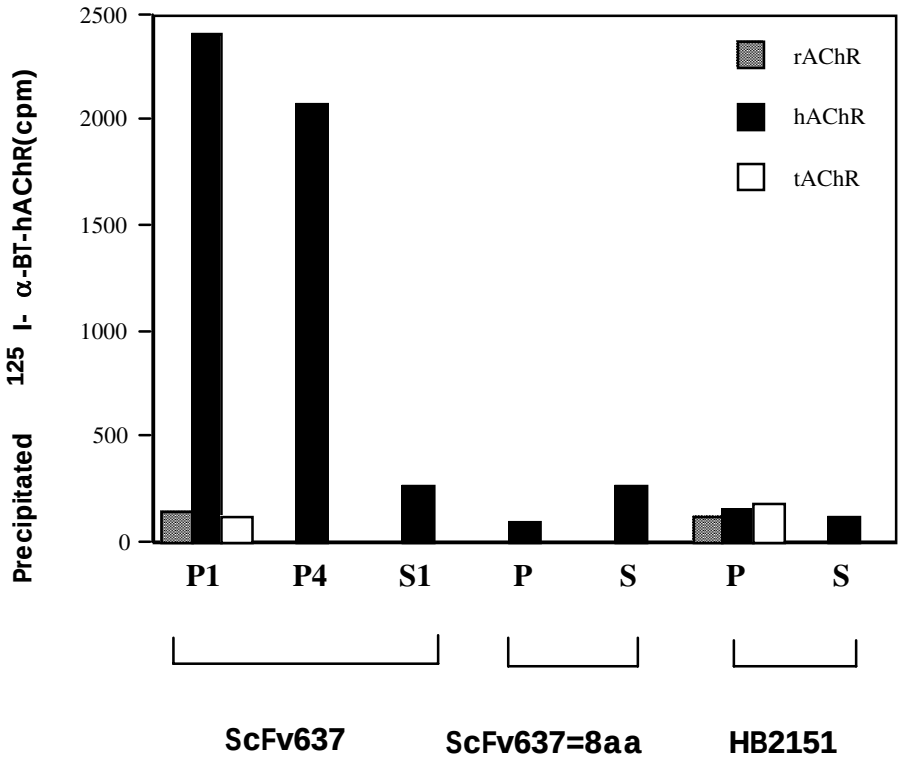

Figure 5 Binding activities of ScFv637 to AChR. ^{125}I-α-BT-AChR-ScFv637 was precipitated by addition of mouse anti-c-myc mAb and goat anti-mouse IgG in standard precipitation RIA. hAChR: human AChR isolated from TE671 cells; rAChR: rat AChR isolated from rat muscle; tAChR: Torpedo AChR isolated from electric organ of Torpedo californica; P: periplasmic fraction; S: culture supernatant; ScFv637=8aa: ScFv637 without the first 8 amino acids of VH and VL; HB2151: control bacterium E coli HB2151; Numbers represent different bacterial colonies.

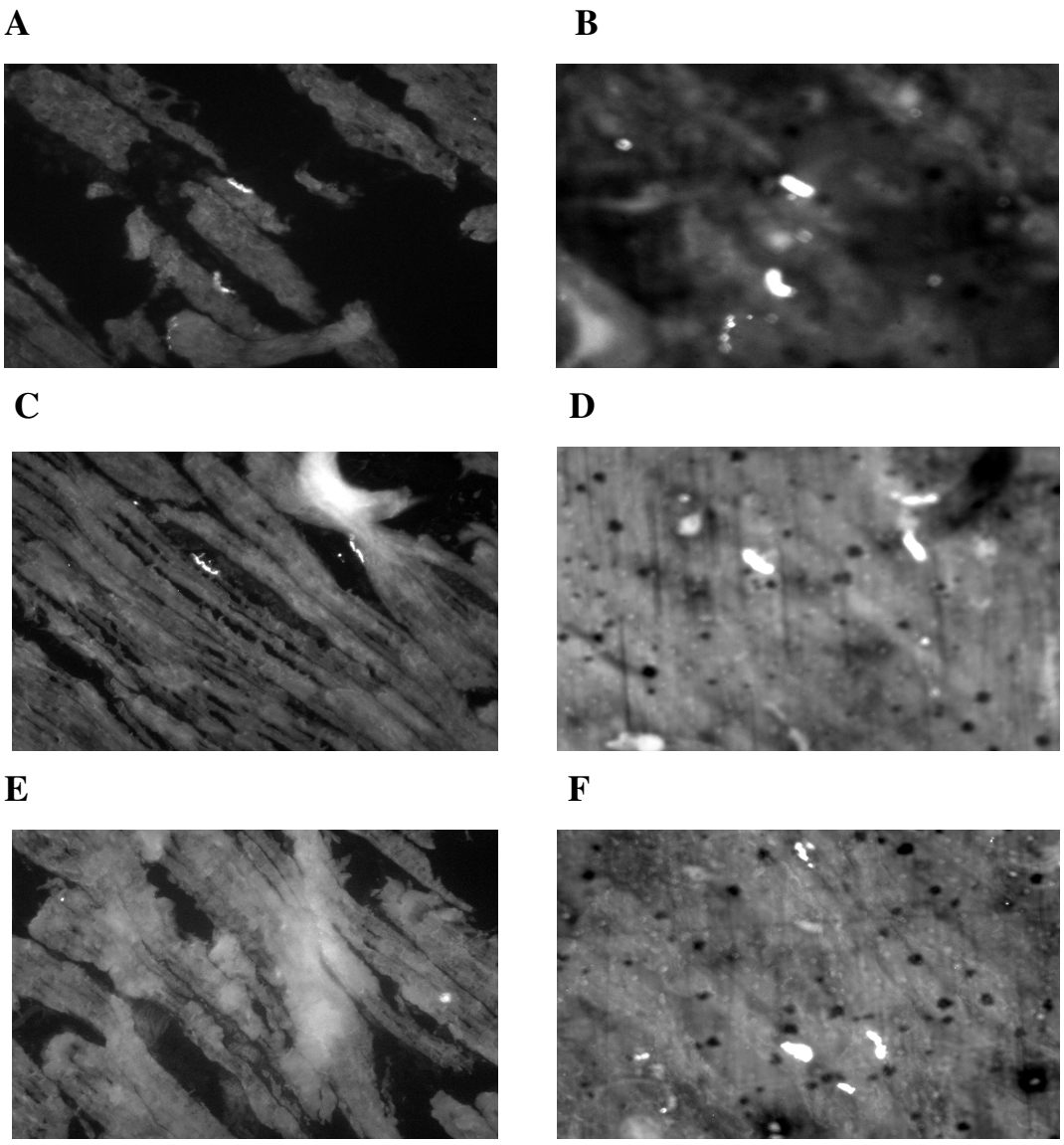

Figure 6 Binding of ScFv637 to AChR in situ at neuromuscular junction of monkey intercostal muscle section. The binding of ScFv637 to AChR at endplate was demonstrated by immunohistochemical staining. Mouse anti-c-myc mAb was used as second antibody and goat anti-mouse IgG conjugated with FITC as for visualization. Rhodamine-labeled α-BT (α-BT^{Rh}) was used to colocalize the same endplates. A and C: ScFv637 colonies 1 and 4 bound to AChR at endplates; B and D: Colocalization of AChR in the same endplates as in A and C respectively; E: ScFv637 without the first 8 amino acids of VH and VL (ScFv637=8aa) failed to bind to AChR at endplates; F: Colocalization of AChR in the same endplates as in E. Magnification: x 200.

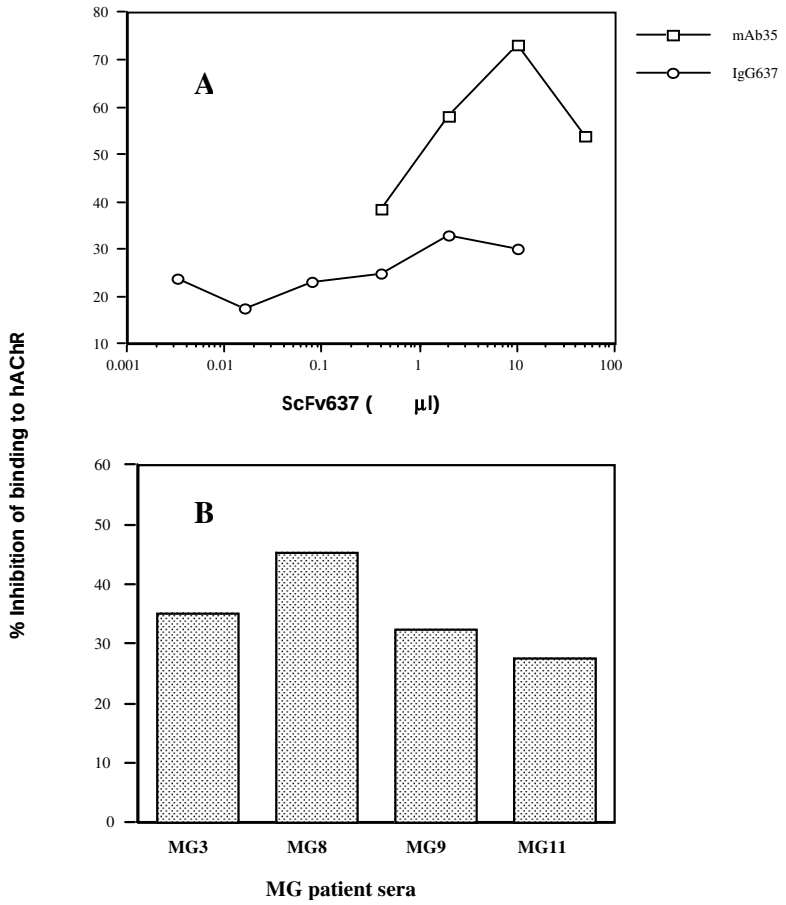

Figure 7 Inhibition of binding of anti-MIR antibodies and MG patient sera to hAChR by ScFv637. A: ScFv637 was tested for the ability to inhibit the binding of its intact IgG637 and rat anti-MIR mAb35 to hAChR in an ELISA. HAChR was captured by fixed mAb153 and incubated with various amounts of periplasmic fraction of ScFv637. The competition was determined at 50% of the maximum binding of IgG637 or mAb35 to hAChR. B: Capacity of ScFv637 to protect hAChR from binding of MG patient sera was tested in a RIA. HAChR labeled with ^{125}I-α-BT was preincubated with 250 µl of periplasmic fraction of ScFv637, then incubated with MG patient serum (70% of the maximum binding to hAChR) and precipitated with goat anti-human immunoglobulin serum. The result was expressed as percentage of inhibition of binding of anti-MIR antibodies or MG patient sera to hAChR.

Discussion

Pathogenic anti-AChR antibodies are mainly those that are directed against MIR due to the bivalent nature of whole molecule of antibody and make up most part of the total anti-AChR antibodies in both MG and EAMG. However, Fab or ScFv fragments can not themselves cross-link AChR to cause antigenic modulation since they are monovalent and can not activate the complement cascade to cause focal lysis of postsynaptic membrane since they lack Fc fragment. Fab or ScFv derived from anti-MIR antibodies could block the binding of MG patient sera or their parental anti-MIR antibodies to AChR[27, 33], and protect the AChR from antigenic modulation induced by MG patient sera or anti-MIR mAbs in cultured TE671 cells[27, 29, 31, 32].

Fab637 is a MG thymus-derived anti-MIR fragment[27]. It prevents MG serum from binding to hAChR and protects AChR from loss induced by MG serum. Since Fab637 can bind monkey AChR in situ on monkey intercostal muscle section, it is ideal to test its in vivo protective ability in monkey before clinical application in MG patient (a large-scale production of Fab637 by transfecting it into chinese hamster ovary cells for mammalian expression is in progress). We have constructed a ScFv637 derived from its parental Fab637. The aim of this study is to try an alternative possibility using antibody fragment for the specific immunosuppressive therapy of MG. Furthermore, since ScFv is smaller than Fab, and VH and VL genes of ScFv are located in the same cistron, this property makes it easier for manipulation of genetic improvement in its affinity and stability.

ScFv637 was constructed from VH and VL genes of Fab637 by PCR amplification on vector pHEN2 which contains a GS (Gly and Ser) rich linker, and a c-myc tag and a 6x His tag for specific detection and efficient purification. The VH upstream primer was designed according to the first 8 codons of Fab637 VH fragment. However, the first 6 codons used in Fab637 was artificially designed in vector pComb3H, and codons 5 and 6 (ctc gag) was used as XhoI restriction site for original cloning of VH. Since vector pHEN2 contains the same XhoI restriction site for cloning and we prefer the VH-linker-VL orientation construct of ScFv, therefore, “ctc” in codon 5 was changed into “ctg” to remove the XhoI restriction site without substitution of amino acid. Similarly,

codon “tcg”→”tcc” from Fab637 to ScFv at amino acid position 113 of VH was created in VH downstream primer to match the XhoI site without introduction of an extra amino acid between VH and linker. For the VL cloning, codon “gca” (encoding Ala) in ApaLI restriction sequence was designed in VL upstream primer as first codon of VL of ScFv637 and “ccc” (encoding Pro) as second codon to match both ApaLI restriction site and annealing to template in pComb3H.

The reconstructed pHEN2-ScFv637 was transformed into non-suppressor E coli HB2151 for soluble ScFv production which was detected in nitrocellulose dot blot using mouse anti-c-myc mAb. The specificity of ScFv637 binding to hAChR was determined in RIA in which ScFv637 first bound to AChR and then c-myc tag in the complex of ScFv637-AChR was recognized by mouse anti-c-myc mAb. However, interestingly, when SPRIA, in which c-myc tag of ScFv637 was captured first by fixed mouse anti-c-myc mAb and then ScFv637 in the complex of mAb-ScFv637 bound to AChR, was utilized to determine the specificity of ScFv637 binding to hAChR, no binding activity could be found (data not shown). In contrast, its parental Fab637 could bind AChR in SPRIA suggesting that the binding of anti-c-myc mAb to c-myc tag (C-terminus) of ScFv637 may interfere with the binding of ScFv637 to AChR. A similar result was described in a previous study of Goel et al[40] who found that the C-terminal position of His tag partially covered the antigen binding site and affected the binding properties of ScFv construct in SPRIA. Although several studies showed that the affinity tags of different types had no significant effects on the activity of native proteins[41-48], it will be nice to determine the effects of the N-terminal c-myc tag of ScFv637 on its specificity since the effects of affinity tags on specificity of ScFv depend on the primary sequences and conformation of the ScFv. It was also reported that ScFv exhibited higher cross-reactivity with analogs of the antigen originally used for production of its parental mAb[49]. Nevertheless, ScFv637, just like its parental Fab637, could specifically bind to AChR in situ at monkey neuromuscular junction but did not cross-react with rAChR or tAChR.

We also construct a ScFv from Fab637 without the first 8 amino acids in framework 1 of both VH and VL (ScFv637=8aa). ScFv637=8aa was efficiently produced in both periplasmic fraction and culture supernatant of bacteria as detected by nitrocellulose dot blot, however no specificity of ScFv637=8aa binding to hAChR was

observed in SPRIA or RIA (Figure 5). Furthermore ScFv637=8aa was not able to bind AChR at endplate of monkey muscle section either (Figure 6, E and F). Li et al[50] also found that the sequence changes of the V region N-terminus introduced by PCR may seriously affect antigen binding but not the expression of antibody. Several other groups noted the same phenomena[51-54]. Since the N-terminus of both V domains can be contiguous with the complementarity determining region (CDR) surface [55] they would influence the structure of the combining site and the antigen binding characteristics of antibodies.

Competition experiment showed that ScFv637 was able to protect hAChR from the binding of anti-MIR antibodies and MG patient sera, which makes it an alternative candidate for specific immunosuppressive therapy compared with its parental Fab637. Presently we are trying to make a conjugate by linking it with human serum albumin (HSA) or polyethylenoglycol (PEG) to increase its half-life.

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We would like to thank Drs G. Winter, R. Fischer, M. Jonker and S. Tzartos for kindly supplying pHEN2, anti-c-myc mAb, monkey muscle and mAb35, mAb153 respectively. We are also grateful to Dr B. Machiels for excellent laboratory assistance and helpful discussion. This work was supported by a grant from Het Prinses Beatrix Fonds.

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Chapter 5

Experimental autoimmune myasthenia gravis in mice expressing human immunoglobulin loci (HuMAb-Mice)

Abstract

Antibodies (Abs) specifically directed against the autoantigen, the muscle acetylcholine receptor (AChR), mediate the pathogenesis of myasthenia gravis (MG). The animal model experimental autoimmune MG (EAMG) can be induced by passive transfer or active generation of anti-AChR Abs by immunization. We report a new EAMG mouse model that generates human anti-AChR Abs. Mice transgenic for human μ, γ1, and κ germ line genes (HuMAb-Mice) were immunized with Torpedo AChR (tAChR). Serum titers of anti-tAChR Abs were in the nanomolar range, and anti-rodent AChR Abs were in picomolar range. Human Ab-mouse AChR complexes were found at the neuromuscular junction, while AChR loss was up to 65%. Some HuMAb-Mice had signs of muscle weakness, clearly indicating their susceptibility to EAMG. Spleen and lymph nodes were used for producing hybridomas. From the anti-tAChR monoclonal Ab-producing hybridomas 2% had cross-reactivity with rodent AChR. These experiments show that the HuMAb-Mouse represents a suitable model to study the effects of human anti-AChR Abs in vivo.

Introduction

Autoantibodies in the organ-specific autoimmune disease myasthenia gravis (MG) are directed mainly to the acetylcholine receptor (AChR)[1]. Anti-AChR antibodies (Abs) cause a disturbed neuromuscular transmission, either by decrease of AChR concentration or, to a lesser extent, functional inhibition of the ion channel[2, 3]. Two mechanisms that lead to this receptor loss are anti-AChR Ab-activated complement reaction, resulting in focal lysis of the membrane[4, 5], and cross-linking of receptors by Abs that are capable of binding to two AChRs simultaneously, leading to an increased rate of internalization of the receptor (antigenic modulation)[6, 7].

Experimental autoimmune MG (EAMG) is the animal model that resembles MG closely. Immunization of animals with Torpedo AChR (tAChR), isolated from the ray Torpedo californica, gives rise to anti-tAChR Abs, of which a small percentage cross-reacts with autologous AChR and causes EAMG[1, 8]. Passive transfer of MG patient's immunoglobulins (Igs) in mice also induces EAMG, indicating the importance of these human Igs in the pathogenesis of MG[9]. In another study that used patient material, MG thymus tissue was transplanted into severe combined immunodeficiency (SCID) mice, resulting in the production of human anti-AChR Ab titers, but although human Abs were found at the muscle end-plates, EAMG was not induced[10]. Mice have strain-dependent susceptibilities to EAMG, although anti-AChR Ab serum titers and fine specificities are comparable[11, 12]. Mice transgenic for different HLA genes have different susceptibilities to EAMG, implying an important role of these human genes in the pathogenesis of MG[13]. The use of human genes and molecules in EAMG clearly matches the model more closely to MG.

In this study, we demonstrate a new EAMG mouse model. In 1994, Lonberg et al[14] established a mouse strain that was transgenic for human Ig loci, while expression of endogenous Igs was disabled. Upon immunization, this so-called HuMAb-Mouse is a source of antigen-specific human monoclonal Ab (mAb)-producing lymphocytes. In order to determine if the HuMAb-Mouse is susceptible to EAMG, we immunized the mouse with tAChR.

Materials and methods

Immunization

HuMAb-Mice (n = 12) transgenic for human μ, γ1, and κ germ line genes, while production of endogenous immunoglobulins was inactivated[14, 15], were immunized at the base of the tail with 15 μg tAChR in CFA and boosted twice with the same dose of tAChR in IFA. Control HuMAb-Mice were immunized with only CFA.

Hybridoma fusion and screening

Para-aortal lymph node and spleen cells were isolated from all the 12 mice and fused separately with SP2/0 cells for production of hybridomas, according to Köhler and Milstein[16]. Culture supernatants of hybridoma clones were screened for anti-tAChR mAb production using an ELISA. Microtiter plates were coated with 50 μl of 5 μg/ml tAChR in a 10 mM bicarbonate buffer, pH 9.5, for 1 h at 37ºC. After washing three times with water containing 0.5% Tween 20 (wash buffer), wells were blocked with 200 μl PBS containing 0.5% BSA and 0.5% Tween 20 (incubation buffer) for 15 min. Subsequently, plates were incubated with a appropriate dilution of hybridoma culture supernatant in incubation buffer for 1 h at room temperature. After washing, plates were incubated with 1/500 alkaline phosphatase-conjugated goat anti-human IgG (Fc specific) Abs in incubation buffer for 1 h. After washing, 50 μl of alkaline phosphatase substrate solution (Bio-Rad Laboratories, Hercules, CA, USA) was added. The colormetric reaction was developed for 30 min and OD measured at 405 nm.

Anti-AChR Ab serum titer and mAb cross-reactivity

Ab titers against tAChR, human AChR (hAChR) and rat AChR (rAChR) and cross-reactivity of mAbs were determined from 9 mouse sera (no sera were obtained from the other 3 animals, wich died after cardiac puncture), by a RIA using ^{125}I-α-bungarotoxin (^{125}I-α-BT) labeled AChR[17]. AChR – either purified tAChR, crude extract of

denervated rat muscle, or crude extract of hAChR-producing cell line TE671 – was labeled with 2 nM ^{125}I-α-BT for 4 h at 4ºC. A 5 µl of serum or 100 µl of hybridoma culture supernatant was incubated with 200 µl ^{125}I-α-BT-labeled AChR overnight at 4ºC. Ab-AChR complexes were precipitated by incubating with excess goat anti-human Ig Abs for 4 h at 4ºC. After washing with PBS containing 0.5% Triton-X100 and 0.02% NaN_3, radioactivity was counted in a γ counter. Ab titers were expressed as moles of α-BT sites per liter.

Quantification of AChR in muscle

To quantify the AChR loss in EAMG mice, the concentration of AChR in muscle was determined as previously described[17, 18]. In short, mouse carcasses were minced and homogenized in 150 ml of 10 mM sodium phosphate (pH 7.5), 100 mM NaN_3, 10 mM iodoacetamide and 1 mM PMSF. Homogenate was centrifuged at 12 000 rpm, 4ºC for 30 min, and pellet was resuspended in the same buffer above but with 2% Triton-X100. Extraction was performed for 1 h at 4ºC on a shaker. After centrifugation at 12 000 rpm, 4ºC for 30 min, the volume of the supernatant was measured and aliquots of 1 ml were labeled with ^{125}I-α-BT, with or without 1 mM acetylcholine and neostigmine-bromide. One µl of pooled high-titer rat anti-AChR serum and normal rat serum were added, and triplicates of 250 µl mixture (with or without acetylcholine and neostigmine-bromide) were incubated overnight at 4ºC. Subsequently, 100 µl goat anti-rat Ig Abs was added and incubated for 4 h at 4ºC. After centrifugation for 3 min, pellet was washed once with PBS containing 0.5% Triton-X100 and 0.02% NaN_3 and counted in γ counter. AChR concentrations were compared with control animals.

Immunohistochemical staining of muscle

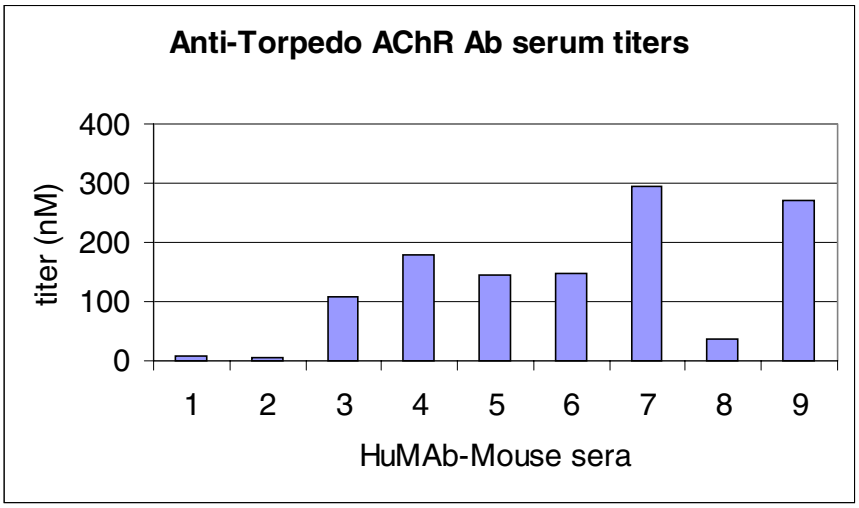
Muscle biopsies from HuMAb-Mice were frozen in 2-methylbutane, cut at 6 µm and fixated with acetone for 10 min at 4ºC, and air-dried for 5 min. After washing three times with PBS, sections were pre-incubated with PBS containing 2% BSA for 15 min, and subsequently incubated with 1/100-diluted goat anti-human IgG (Cappel, ICN Pharmaceuticals, Aurora, OH, USA) for 45 min. After washing with PBS, sections were

further incubated with 1/50 FITC-conjugated rabbit anti-goat Ig Abs (Cappel, ICN Biochemicals, Eschwege, Germany) together with 1/250 rhodamine-conjugated α-BT (Molecular Probes, Eugene, OR, USA) for 45 min, in order to co-localize human anti-AChR Abs with mouse AChR.

Results

Induction of EAMG

HuMAb-Mice were immunized with tAChR to determine their susceptibility to EAMG. After the second booster, a few animals showed mild clinical signs of EAMG (grade +, according to Lennon et al[8]). The mice had Ab titers of anti-tAChR ranging from 7 to 296 nM and of anti-rodent AChR ranging from 155 to 539 pM (Figure 1), indicating a successful immunization with tAChR in all mice. The anti-rAChR/anti-tAChR Ab serum titer ratio was between 0.2% and 4.0%. Staining of AChR at the neuromuscular junction in muscle sections with rhodamine-conjugated α-BT was co-localized with human Abs, visualized with goat anti-human IgG and FITC-conjugated rabbit anti-goat Ig Abs (Figure 2). To determine the damage that was inflicted by the human anti-mouse AChR (mAChR) Abs, the AChR loss in muscles was measured. The AChR contents in the tAChR-immunized mouse muscles were 0 to 65% lower, compared to control mice (Figure 3). These data clearly demonstrate that HuMAb-Mice were susceptible to EAMG.



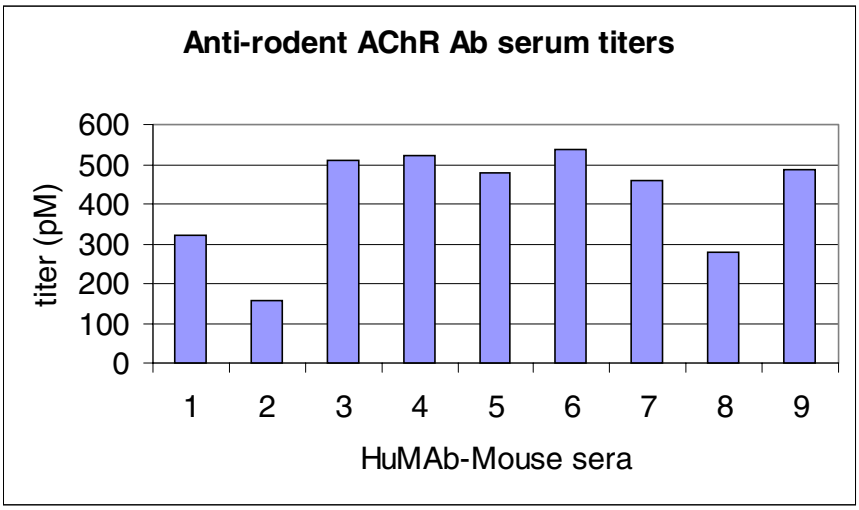


Figure 1 Human anti-AChR Ab serum titers in Torpedo AChR-immunized HuMAb-Mice. Serum titers (moles α-bungarotoxin binding sites per liter serum) were measured in 9 mice, by a RIA using ^{125}I-α-bungarotoxin-labeled Torpedo or rat AChRs.

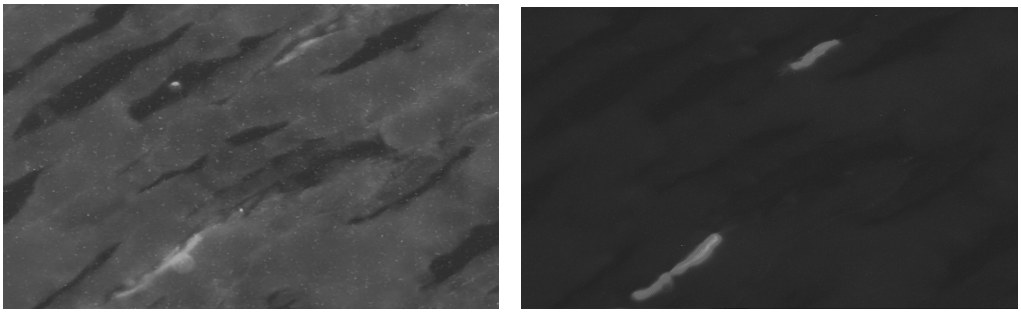


Figure 2 Human Ab-mouse AChR complexes at the neuromuscular junction of Torpedo AChR-immunized HuMAb-Mice. A cryosection of mouse muscle was double stained with goat anti-human IgG and FITC-conjugated rabbit anti-goat Ig Abs (left panel) and rhodamine-conjugated α-bungarotoxin (right panel), co-localizing the AChRs and human antibodies.

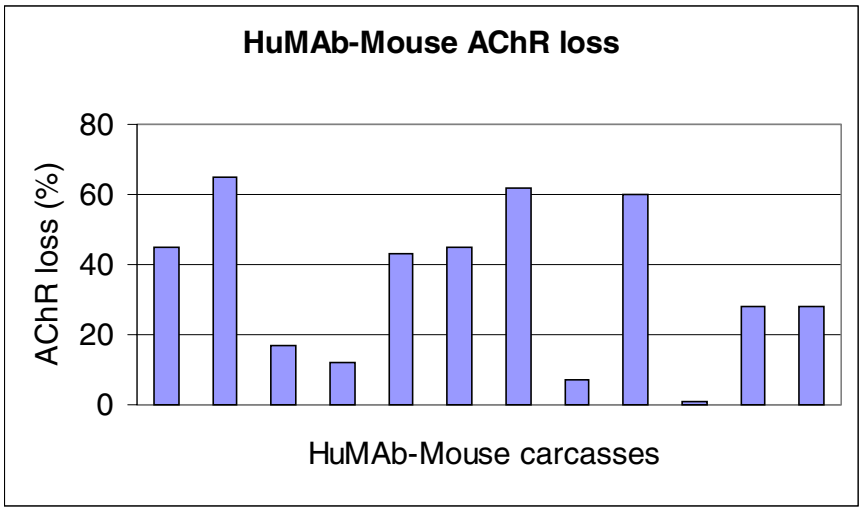


Figure 3 AChR loss in Torpedo AChR-immunized HuMAb-Mice. Total AChR concentrations of mouse muscles were determined in all 12 mice, by immunoprecipitation using ^{125}I-α-bungarotoxin, and compared to control animals. (Numbering of animals is not available.)

Human anti-AChR mAbs

Spleens and lymph nodes were isolated for the production of anti-AChR-specific hybridoma cells. Four hundred and thirty-three anti-tAChR mAb-producing clones were selected after initial screening of the hybridomas by ELISA. The number of hybridomas that had cross-reactivity with rodent AChR – those mAbs that were most likely pathogenic in the mice – was 7 (2%), as determined by a RIA. In order to explore the possibility of a human mAb cross-reacting with hAChR, that could be used for studying MG immunotherapy, a hAChR-specific RIA was performed. However, no mAb could bind hAChR with high specificity (Table 1).

Table 1 Specificities of mAbs produced by Torpedo AChR-immunized HuMAb-Mouse hybridomas

mAb	number (%)
anti-Torpedo AChR	433 (100)
anti-rodent AChR	7 (2)
anti-human AChR	0 (0)

Cross-reactivity of human anti-Torpedo AChR mAbs was determined by ^{125}I-α-bungarotoxin-labeled AChR immunoprecipitation.

Discussion

The establishment of mice transgenic for human Ig loci makes it possible to set up a new mouse EAMG model, in which the mice produce human anti-AChR Abs upon immunization with AChR from various species. In the study presented here, transgenic HuMAb-Mice, which produce human IgG1κ and IgMκ molecules, were immunized with tAChR extracted from Torpedo californica, a rich source of AChR commonly used in mammalian study of MG. After the second booster injection of tAChR, the anti-tAChR

and –rodent AChR Ab titers were determined, and the clinical signs of EAMG examined to evaluate if a successful EAMG model had been established.

It was found that serum titers of Abs against tAChR, an initial immunogen, were in the nanomolar range, similar to those found in MG patients[19], and approximately a hundred-fold higher than those observed in the MG thymus tissue-transplanted model[10], in which the original immunogen should be hAChR. The difference in the serum Ab titers between the C57bl/6 mice, immunized with tAChR[12] and the HuMAb-Mice may reflect the strain-dependent susceptibilities to the induction of EAMG. The anti-rAChR Ab serum titer, ranging from 0.2% to 4.0% of that to tAChR, is comparable to the findings in other mouse strains[11].

The clinical signs of muscular weakness, a key symbol of EAMG, were successfully induced in some of the HuMAb-Mice, directly indicating that the EAMG had been established. This was further demonstrated by the deposits of human anti-mAChR Abs at the neuromuscular junction of muscle sections, as detected by goat anti-human IgG, and by the muscle AChR loss up to 65%. Human anti-AChR Abs can bind to the postsynaptic membrane, and reduce the amount of mAChR, which interferes with the neuromuscular transmission. In contrast, the thymus tissue- or its single–cell suspensions-transplanted model is not accompanied by muscular weakness[10].

The Ab repertoire was analyzed by hybridoma technology. From tAChR-immunized HuMAb-Mice, hybridomas were made and initially screened for the reactivity to tAChR. Of those reactive with tAChR, 2% cross-reacted with rAChR and none with hAChR. This result is consistent with our previous study, in which we found a similar pattern of cross-reaction in the high-susceptible C57bl/6 and the low-susceptible Balb/c mice[12], and failed to find anti-hAChR mAbs from tAChR-immunized mice (see Chapter 1, Table 1). Since the tAChR immunization did not generate a set of high-affinity human anti-hAChR mAbs, we are planning to immunize HuMAb-Mice with a recombinant peptide of the hAChR with native conformation[20]. These human anti-hAChR Abs could be used, after modification or fragmentation, as treatment for exacerbation of MG.

Our HuMAb-Mouse EAMG model is the first model in which human Abs were actively induced in mice, permitting the study of human tAChR-induced Abs. This chronic model resembles MG more closely, and holds promise for the development of a

more appropiate mouse model with an entire human immune response, including human Igs, MHC and AChR genes.

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Summary and general discussion

Myasthenia gravis (MG) is an organ-specific autoimmune disease mediated by autoantibodies directed against acetylcholine receptor (AChR). The AChR is a well characterized transmembrane glycoprotein, consisting of five subunits $\alpha_2\beta\gamma\delta$ or $\alpha_2\beta\varepsilon\delta$. A cross-linking of an extracellular region, termed the main immunogenic region (MIR), of α-subunit in AChR at neuromuscular junction by bivalent antibody leads to accelerated internalization of AChR (antigenic modulation), or activation of complement (focal lysis), resulting in muscle weakness. Less importantly, antibodies binding to acetylcholine (ACh) binding site or α-bungarotoxin (α-BT) binding site directly interfere with the ion channel function. The animal model of MG, experimental autoimmune myasthenia gravis (EAMG), can be induced by active immunization with purified AChR or passive transfer of anti-AChR antibodies.

Anti-MIR antibodies are found to make up 60% of the total anti-AChR antibodies in MG patient sera or EAMG rat sera, thus are major pathogenic autoantibodies in pathogenesis of MG or EAMG. The contribution of individual pathogenic anti-AChR antibodies to the AChR loss is important for understanding the pathogenesis of MG or EAMG. Further structural and functional analysis of pathogenic and non-pathogenic anti-AChR antibodies may lead to the better understanding.

Single chain variable fragments (ScFv) of antibody are univalent, and can not themselves cross-link AChR and can not activate complement. However, ScFv derived from anti-MIR antibodies can bind to MIR on AChR, just protecting the MIR against the binding of pathogenic anti-MIR antibodies. ScFv derived from human does not possess immunogenicity for application in patients, and readily undergoes genetic manipulation in improvement of stability and affinity.

The establishment of mice transgenic for human immunoglobulin loci makes it possible to set up a new mouse EAMG model. This model is expected to be the first step towards an ideal EAMG model of mice transgenic for human immunoglobulin and HLA loci, and a source of human anti-AChR antibodies.

In **Chapter 1**, an EAMG mouse model was used to produce monoclonal antibodies (mAbs) directed against AChR. C57bl/6 and Balb/c mice were immunized with purified Torpedo AChR (tAChR) and human AChR (hAChR), and boosted 3 and 5 weeks after primary immunization. The mice were sacrificed 3 days after the last injection and cells from lymphnodes were fused with mouse myeloma cell line SP2/O-Ag14 or NS1. Hybridomas were initially screened for reactivity to AChR by ELISA. Most of the anti-AChR mAbs were found to be IgG1 and IgG2b as determined by a mouse isotyping kit, and some of them were cross-reactive with mouse and rat AChR as showed in radioimmunoassay (RIA) using mouse and rat muscle crude extracts as antigens. Determination of fine specificity of the anti-AChR mAbs binding to AChR using competitive ELISA or RIA showed that four different groups of the mAbs were identified: anti-MIR mAbs (rat anti-MIR mAbs 35 as reference competitor), anti-α-BT binding site mAbs (α-BT as reference competitor), anti-extracellular epitope mAbs and anti-intracellular epitope mAbs.

In **Chapter 2**, the role of different anti-AChR antibody sequences and specificities in pathogenesis of EAMG was investigated by sequencing a panel of 6 anti-MIR mAbs. The EAMG was induced in rats or mice by passive transfer of the mAbs and evaluated by clinical signs and AChR loss. The variable regions of heavy and light chains of anti-MIR mAbs were sequenced. Comparison of the sequences at nucleotide and amino acid levels between the mAbs showed that they utilized a similar VH gene derived from mouse PC7183 germline family with high homology in complementarity determining region (CDR) 1 and 2. The large diversity found in heavy chain CDR3 of the anti-MIR mAbs may contribute to the difference in pathogenicity of the mAbs.

In **Chapter 3**, a panel of 3 anti-α-BT binding site mAbs were investigated on their epitope specificity and their nucleotide and deduced amino acid sequences of variable

regions. The three mAbs recognized the same epitope on AChR as showed in a competitive inhibition assay between individual mAbs. The sequence analysis of the mAbs showed that they all utilized the same VH gene derived from mouse Q52 germline family, but different VL genes, indicating that the heavy chain play more important role in binding to AChR. Comparison of sequences between the anti-α-BT binding site mAbs and the pathogenic anti-MIR mAb revealed that a large diversity was observed in both overall sequences and CDRs.

In **Chapter 4**, an human ScFv637 was constructed from its parental Fab637, previously isolated from thymus-derived phage display library with specificity of anti-MIR of hAChR, by PCR amplification. PCR products of VH and VL genes of Fab637 were assembled onto vector phagemid pHEN2 containing GS rich linker and c-myc tag and 6x His tag at C-terminus for specific detection and for efficient purification. The recombinant pHEN2-ScFv637 construct was transformed into E coli HB2151 for soluble production of ScFv after induction with IPTG. ScFv637 was efficiently produced in periplasmic fraction but not in culture supernatant of bacteria as detected by nitrocellulose dot blot using mouse anti-cmyc mAb. ScFv637 was able to bind to hAChR in standard precipitation RIA. ScFv637 could also bind to monkey AChR in situ on monkey neuromuscular junction as showed in immunohistochemical staining. Furthermore, ScFv637 was capable of inhibiting the binding of its intact IgG637 and anti-MIR mAb35 to hAChR up to 32.9% and 73.0% respectively demonstrated in a competitive ELISA, and of MG patient sera from 27.8% to 45.5% in a competitive RIA. Therefore, ScFv637, easier for manipulation in improvement of affinity and stability compared with its parental Fab637, may serve as an alternative candidate for specific immunotherapy in MG.

In **Chapter 5**, a new EAMG mouse model, that generates human anti-AChR antibodies, was reported. Mice transgenic for human μ, γ1, and κ germ line genes (HuMAb-Mice) were immunized with tAChR. Serum titers of anti-tAChR antibodies were in the nanomolar range, and anti-rodent AChR antibodies were in picomolar range. Human antibody-mouse AChR complexes were found at the neuromuscular junction, while AChR loss was up to 65%. Some HuMAb-Mice had mild signs of muscle weakness,

clearly indicating their susceptibility to EAMG. Spleen and lymph nodes were used for producing hybridomas. From the anti-tAChR mAb-producing hybridomas 2% had cross-reactivity with rodent AChR. These experiments show that the HuMAb-Mouse represents a suitable model to study the effects of human anti-AChR antibodies in vivo.

The results from the first part of the study (**Chapters 1** to **3**) indicate that the pathogenic anti-AChR antibodies are exclusively among the mAbs which are directed against MIR, and most of anti-MIR mAbs utilize a VH gene derived from mouse PC7183 germline family. Furthermore, The CDR sequences are different between pathogenic and non-pathogenic anti-MIR mAbs, and between pathogenic anti-MIR mAbs and anti-α-BT binding site mAbs, and CDR3 of heavy chain may contribute to the pathogenicity of the mAbs. This conclusion will form the basis for the genetic manipulation of ScFv637, an alternative candidate for specific immunosuppressive therapy of MG described in the second part of the study (**Chapter 4**), in order to improve its affinity and stability. The experiment from the third part of the study (**Chapter 5**) demonstrates that mice transgenic for human immunoglobulin loci is a suitable model to investigate human anti-AChR antibodies in vivo, which holds promise for the development of an ideal mouse model with entire human immune response.

Samenvatting en algemene discussie

Myasthenia gravis (MG) is een orgaanspecifieke auto-immuunziekte die wordt gemedieerd door autoantilichamen gericht tegen de acetylcholinereceptor (AChR). De AChR is een goed gekarakteriseerd transmembraaneiwit, bestaande uit vijf subunits: $\alpha_2\beta\gamma\delta$ of $\alpha_2\beta\gamma\varepsilon$. Het verbinden van twee identieke epitopen van de zogenaamde *main immunogenic region* (MIR), gelegen op de α-subunit van de AChR, door bivalente antilichamen in de neuromusculaire junctie leidt tot versnelde internalisatie van de AChR (*antigenic modulation*), wat resulteert in spierzwakte. Complementactivering (focale lysis) en, in mindere mate, interferentie met de functie van het ionkanaal door binding van antilichamen aan de acetylcholine- of α-bungarotoxinebindingsplaats geven hetzelfde resultaat. Het diermodel van MG, experimentele auto-immuun MG (EAMG), kan worden geïnduceerd door actieve immunisering met gezuiverd AChR of door passieve overdracht met anti-AChR antilichamen.

Anti-MIR antilichamen beslaan tot 60% van het totaal aan anti-AChR antilichamen in MG-patiëntensera of EAMG-rattensera; ze zijn dus belangrijke pathogene autoantilichamen in de pathogenese van MG of EAMG. De bijdrage van individuele pathogene anti-AChR antilichamen aan het AChR-verlies is van belang voor het begrijpen van de pathogenese van MG of EAMG. Verdere structurele en functionele analyses van pathogene en niet-pathogene anti-AChR antilichamen kan leiden tot een beter inzicht.

Single-chain variabele fragmenten (scFv's) van antilichamen zijn univalent en kunnen geen AChR's crosslinken en complement activeren. Echter, scFv's die zijn afgeleid van anti-MIR antilichamen kunnen aan de MIR binden en daarmee de AChR afschermen tegen binding van pathogene anti-MIR antilichamen. ScFv's die zijn afgeleid van humane antilichamen zijn niet immunogeen voor toepassingen in patiënten en

kunnen gemakkelijk genetisch worden gemodificeerd voor het verbeteren van de stabiliteit en affiniteit.

De ontwikkeling van muizen die transgeen zijn voor humane immunoglobulinenloci heeft het mogelijk gemaakt om een nieuw muizen EAMG-model op te zetten. Dit model is waarschijnlijk de eerste stap naar een ideaal EAMG-model, van muizen transgeen voor humane immunoglobulinen- en HLA-loci, en daarnaast een bron voor humane anti-AChR antilichamen.

In **hoofdstuk 1** werd een EAMG-muizenmodel gebruikt voor de productie van monoklonale antilichamen (mAb's) gericht tegen AChR. C57b1/6 en Balb/c muizen werden geïmmuniseerd met gezuiverde *Torpedo* AChR (tAChR) en humane AChR (hAChR) en na drie en vijf weken geboost. De muizen werden drie dagen na de laatste injectie opgeofferd en de cellen van de lymfeknopen werden gefuseerd met muis myelomacellijn SP2/O-Ag14 of NS1. Hybridoma's werden onderzocht op reactiviteit tegen AChR met ELISA. De meeste anti-AChR mAb's waren van het IgG1- of IgG2b-isotype en enkele hadden kruisreactiviteit met muis en rat AChR, zoals aangetoond met een radio-immunoassay (RIA) met muis of rat spierextracten als antigeen. Middels een competitieve ELISA en RIA, werden de anti-AChR mAb's naar specificiteit ingedeeld: anti-MIR mAb's (met rat anti-MIR mAb 35 als referentiecompetitor), anti-α-bungarotoxinebindingsplaats mAb's (met α-bungarotoxine als referentiecompetitor), anti-extracellulair epitoop mAb's en anti-intracellulair mAb's.

In **hoofdstuk 2** werd de rol onderzocht van verschillende anti-AChR antilichaamsequenties en -specificiteiten in de pathogenese van EAMG, middels het bepalen van de sequentie van een panel van zes anti-MIR mAb's. EAMG werd geïnduceerd in ratten of muizen middels passieve overdracht met de mAb's en geëvalueerd op klinische kenmerken en AChR-verlies. De variabele regio's van de zware en lichte ketens van de anti-MIR mAb's werden gesequenced. Uit het vergelijken van de sequenties op nucleotide- en aminozuurniveau, bleek dat de mAb's eenzelfde VH-gen bezitten, uit de muis PC7183 kiemlijnfamilie, met hoge homologie in de *complement determining region* (CDR) 1 en 2. De grote diversiteit in de CDR3's van de zware ketens zou kunnen bijdragen aan de verschillen in pathogeniciteit van de mAb's.

In **hoofdstuk 3** werd een panel van drie anti-α-bungarotoxinebindingsplaats mAb's onderzocht op de epitoopspecificiteit en de nucleotidesequentie en de daarvan afgeleide aminozuursequentie van de variabele regio. De drie mAb's herkennen hetzelfde epitoop op de AChR, zoals aangetoond met een competitieve inhibitie-assay tussen de afzonderlijke mAb's. De sequentie-analyse van de mAb's wees uit dat de VH-genen afstamden van de muis Q52-kiemlijnfamilie, terwijl de VL-genen verschilden. Dit duidt er op dat de zware ketens een belangrijkere rol spelen in de binding aan AChR. Het vergelijken van de sequenties van anti-α-bungarotoxinebindingsplaats mAb's en de pathogene anti-MIR mAb duidde op een grote diversiteit in sequenties en CDR's in beide groepen.

In **hoofdstuk 4** werd het humane scFv637 geconstrueerd middels PCR-klonering uit Fab637, die eerder was geïsoleerd uit een faagdisplaybibliotheek met specificiteit voor de humane MIR. PCR-producten van de VH- en VL-genen van Fab637 werden gekloneerd in het vector faagmide pHEN2, die een GS-rijke linker en een C-terminale c-myc- en een 6xHis-tag heeft voor specifieke detectie en efficiënte zuivering. Het recombinante construct pHEN2-scFv637 werd getransformeerd in E. coli-stam HB2151 voor de productie van oplosbare scFv's, na inductie met IPTG. ScFv637 werd efficiënt geproduceerd in de periplasmatische fractie, maar niet in het kweeksupernatant, zoals aangetoond met een nitrocellulose dotblot met een muis anti-c-myc mAb. ScFv637 was in staan hAChR te precipiteren in een RIA. Daarnaast kon scFv637 ook aap AChR binden in situ, zoals immunohistochemisch aangetoond in aap neuromusculaire juncties. Bovendien had scFv637 de capaciteit om de binding van IgG637 en anti-MIR mAb35 aan hAChR te inhiberen met percentages tot respectievelijk 32,9 en 73,0 – aangetoond met competitieve ELISA – en om de binding van MG-patiëntensera te inhiberen met 27.8 tot 45.5% – competitieve RIA. Daarom is scFv637, ook gezien het gemakkelijker manipuleren ter verbetering van affiniteit en stabiliteit in vergelijking tot Fab637, een alternatieve kandidaat voor specifieke immunotherapie van MG.

In **hoofdstuk 5** werd een nieuw EAMG-muismodel beschreven, dat humane anti-AChR antilichamen genereert. Muizen transgeen voor μ-, γ1- en κ-kiemlijngenen (HuMAb-

Mice) werden geïmmuniseerd met tAChR. Serumtiters van anti-tAChR antilichamen waren in het nanomolaire bereik en de anti-knaagdier AChR antilichamen in de picomolaire bereik. Humaan antilichaam-muis AChR-complexen weren gevonden ter hoogte van de neuromusculaire junctie, terwijl AChR-verlies opliep tot 65%. Enkele HuMAb-Mice hadden lichte symptomen van spierzwakte, er op duidend dat ze vatbaar zijn voor EAMG. Milt en lymfeknopen werden gebruikt voor de productie van hybridoma's. Van de anti-tAChR mAb-producerende hybridoma's vertoonde 2% kruisreactiviteit met knaagdier AChR. Deze experimenten tonen aan dat de HuMAb-Mouse een geschikt model is voor het bestuderen van de effecten van humane anti-AChR antilichamen in vivo.

De resultaten van het eerste gedeelte van deze studie (**hoofdstuk 1** tot en met **3**) tonen aan dat de pathogene anti-AChR antilichamen uitsluitend voorkomen onder de mAb's die zijn gericht tegen de MIR en dat de meeste anti-MIR mAb's een VH-gen gebruiken dat afstamt van de muis PC7183-kiemlijnfamilie. Bovendien zijn de CDR-sequenties verschillend tussen pathogene en niet-pathogene anti-MIR mAb's en tussen pathogene anti-MIR mAb's en anti-α-bungarotoxinebindingsplaats mAb's; en de CDR3 van de zware keten kan bijdragen aan de pathogeniciteit van de mAb's. Deze conclusie vormt de basis voor de genetische modificatie ten behoeve van verbetering van de stabiliteit en affiniteit van scFv637, een alternatieve kandidaat voor de specifieke immunosuppressietherapie van MG, beschreven in het laatste gedeelte van dit onderzoek (**hoofdstuk 4**). De experimenten van het derde deel van de studie (**hoofdstuk 5**) tonen aan dat muizen die transgeen zijn voor humane immunoglobulinenloci geschikt zijn als model voor het onderzoeken van humane anti-AChR antilichamen in vivo. Dit is belovend in de ontwikkeling van een ideaal muismodel met een volledige humane immuunrespons.

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Curriculum vitae

Fanping Meng was born on December 16, 1958 in Linkou, Heilongjiang Province, P. R. China. From September 1979 to July 1984, he studied medicine as an undergraduate in Yanbian University College of Medicine, China and gained the Bachelor of Medicine degree. From September 1984 to July 1987, he was a postgraduate in the the Department of Microbiology and Immunology, Yanbian University College of Medicine, China and gained the Master of Medicine degree. Then he worked as an assistant and lecturer in the Department of Microbiology and Immunology, Yanbian University College of Medicine, China. From May 1992 to October 1994, he studied as a PhD student in the Department of Immunology (Dr Marc de Baets), and the Department of Psychiatry and Neuropsychology (Prof Harry Steinbusch and Prof Jellemer Jolles), University of Maastricht, The Netherlands. He went back to China in 1994 and was appointed as associate professor in 1995, and professor and head of the Department of Microbiology and Immunology, Yanbian University College of Medicine, China in 1998. From May 2000 to May 2001, he continued his study as a PhD student in the Department of Neurology, Institute of Brain and Behaviour, and the European Graduate School of Neuroscience (EURON), University of Maastricht, The Netherlands under the direction of Prof Jaap Troost, Prof Harry Steinbusch and Dr Marc de Baets.