The pathophysiology of MuSK myasthenia gravis

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CHAPTER 1

General introduction

Adapted from: Pathogenic immune mechanisms at the neuromuscular synapse: the role of specific antibody-binding epitopes in myasthenia gravis

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ABSTRACT

Myasthenia gravis (MG) is a heterogeneous disease hallmarked by fatigable muscle weakness. Auto-antibodies against three different postsynaptic antigens and one presynaptic antigen at the neuromuscular junction are known to cause myasthenic syndromes. Diagnosing the antigen involved in the autoimmune response is essential as treatment responsiveness varies between myasthenic syndromes. Moreover, the mechanisms by which the auto-antibodies cause muscle weakness varies from antigenic modulation and complement mediated membrane damage, to inhibition of endogenous ligand binding and blocking of essential protein-protein interactions. These mechanisms are related to the auto-antibody titre, specific epitopes on the target proteins and IgG auto-antibody subclass. These characteristics guide the development of more specific treatment strategies for each of the myasthenic syndromes.

We review here the role of specific auto-antibody-binding epitopes in the different myasthenic syndromes, their possible relevance to the pathophysiology of the disease, and potential implications of epitope mapping knowledge for new therapeutic strategies.

INTRODUCTION

Myasthenia gravis (MG) is one of the best-characterized autoimmune diseases and is a direct consequence of autoimmunity at the neuromuscular junction (Fig. 1). To date, this disease has been associated with antibodies against four postsynaptic proteins at the neuromuscular synapse, including the acetylcholine (ACh) receptor (AChR), muscle-specific kinase (MuSK), low-density lipoprotein receptor-related protein 4 (LRP4) and agrin. Autoimmunity against the presynaptic Cav2.1 voltage-gated calcium channels (VGCCs) causes a distinct myasthenic syndrome, Lambert–Eaton myasthenic syndrome (LEMS). There are multiple mechanisms by which these serum auto-antibodies may interfere with the function of these proteins, including (i) complement-mediated destruction of the membrane, (ii) antigenic modulation by crosslinking of the target antigen, (iii) competition at ligand-binding sites, and (iv) steric hindrance that inhibits conformational changes or interactions with associated molecules.

These mechanisms are not equally important for the pathophysiology of all forms of MG and depend on the epitope specificity of the auto-antibody and its immunoglobulin subclass, as will be discussed below. An overview of these myasthenic syndromes is shown in Table 1.

MG associated with antibodies against AChR (AChR MG)

Although the existence of acetylcholine receptor (AChR) auto-antibodies had been postulated for many years, it was not until the mid-1970s that Lindstrom, Lennon et al. demonstrated their presence in 87% of MG patients (1,2,3). MG caused by AChR auto-antibodies is characterized by fluctuating muscle weakness, often starting with extraocular muscle weakness progressing in a craniocaudal direction. Epidemiological analysis of patients with AChR MG revealed a bimodal distribution with a female predominance in early-onset MG (at 20– 40 years of age) (4). Late-onset MG (above the age of 60 years of age) is more common in men, and there is a higher rate of association with thymoma in this age group.

Several lines of evidence suggest thymic involvement in the aetiology of AChR MG: 10% of patients have a thymoma (5), thymic lymphocytes can spontaneously produce AChR auto-antibodies (6), thymus epithelium expresses AChRs, and some patients benefit from thymectomy (7,8). There are indications of dysfunction of the autoimmune regulator protein and the interferon signalling pathway causing a disturbance of the quantitative expression of the AChR in the thymus, thereby lowering the threshold for AChR autoimmunity (9). However, the exact role of the thymus in the pathogenesis of AChR MG is not yet understood.

Pathogenesis of AChR MG

The pathogenesis of AChR MG is directly linked to AChR auto-antibodies of the IgG1 and IgG3 subtypes (10,11). These antibodies induce myasthenia through

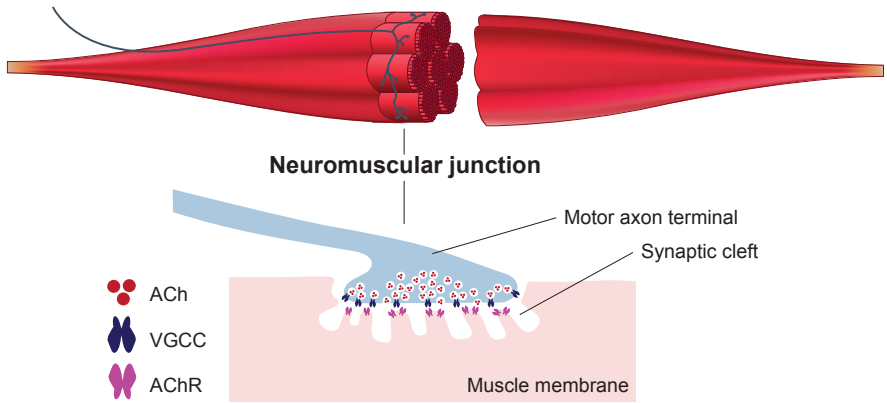

Figure 1. Schematic representation of the neuromuscular junction and its localization within the muscle. Neuromuscular junctions are located in the central part of the muscle. Each muscle fibre possesses one neuromuscular junction. The detailed image of the neuromuscular junction shows the presynaptic motor nerve terminal, where upon depolarization the voltage-gated calcium channels (VGCCs) open. This results in Ca^{2+} influx that activates the release of acetylcholine (ACh) from synaptic vesicles through exocytosis. The postsynaptic apparatus contains ACh receptors (AChRs) which are ligand-gated ion channels that open in response to ACh binding. The resulting ion fluxes depolarize the membrane which leads to a cascade of events that eventually cause muscle fibre contraction.

three distinct mechanisms: complement-mediated postsynaptic membrane damage (12); cross-linking by bivalent IgG1 and IgG3 molecules (antigenic modulation), causing internalization of AChRs and depletion of its surface pools (13); and competition with ACh on binding sites of AChRs, preventing activation and opening of the ion channel (14). These effects hamper neuromuscular transmission and ultimately result in fatigable muscle weakness. The importance of blocking of the ACh-binding site is controversial. Several groups have described blocking antibodies in varying proportions of their cohorts and showed that they were heterogeneous in their subclass distribution (15,16,17,18). In one study, it was found that there was no correlation between the titre of these ligand-blocking antibodies and clinical weakness (19). Others found significant correlations between the degree of ligand-binding block and muscle weakness (14). ACh-binding site antibodies may play an important pathogenic role in some, but not all AChR MG patients.

In addition, it has been hypothesized that some auto-antibodies might physically block the ion channel pore of the AChR; however, this has not been supported by experimental evidence. In fact, in an elegant study in 1995, Beroukhim and Unwin showed that auto-antibodies against the main immunogenic region (MIR) do not block the ion channel, but are located at the outer side of the subunit and extend away from the channel pore (20).

Epitope mapping in AChR MG

The effector functions of the auto-antibodies are highly correlated with their IgG subclass and the epitopes they recognize. The 'muscle type' nicotinic AChR is

a transmembrane protein comprising five subunits with a stoichiometry of α2βγδ (an overview of the AChR structure and MIR localization is shown in Fig. 2). In the adult AChR, the γ subunit is replaced by the ε subunit. Auto-antibodies against the AChR recognize a large variety of epitopes on the receptor (21). Epitope mapping in 86 patients revealed that, depending on disease severity, more than half of the different AChR auto-antibodies bind to a distinct part of the AChR α-subunit, thus termed the MIR (21,22). Parts of the β and γ subunits adjacent to the MIR were also found to be immunogenic (23). Auto-antibodies against the ε subunit have been reported to cause acquired slow channel myasthenia, which is characterized by delayed closure of the AChR ion channel (24). A study amongst 102 patients with either ocular or generalized AChR MG showed that anti-MIR antibodies are significantly more common in the patients with generalized disease and that the titre and presence of these antibodies exclusively correlate with disease severity (25). The authors of this study therefore concluded that MIR auto-antibodies play a crucial role in the pathogenesis, can be useful in predicting disease severity and may form a promising target for future therapies (26, 27, 28).

The AChR MIR is composed of a set of (overlapping) epitopes mainly located around a loop of amino acids 66–76 on the α1 subunit (21, 26). Interaction between the α1 66–76 loop and the N- terminal α1 1–14 amino acids creates a three-dimensional structure that is essential for making the MIR even more myasthenogenic (29,30) The structural integrity of this epitope is required for most auto-antibodies (31). When antibodies bind to the MIR, they often prevent binding of other antibodies against the MIR to the entire region (21). Amino acids 68 and 71 are of particular importance for monoclonal antibody binding to the MIR. Mutating these residues resulted in almost complete loss of antibody binding (32). Immunization of rats with a chimera of the human α1-MIR and ACh-binding protein from Aplysia induced experimental autoimmune myasthenia gravis (EAMG), suggesting that the MIR sequence alone is enough to trigger antibodies that cause clinical myasthenia (33, 34). Surprisingly, the ACh-binding protein alone also induced EAMG in some rats. This is most likely to be due to sequence homology (20%) between the ACh-binding protein and the human AChR α1 subunit. Thus, in AChR-associated EAMG, antibodies against the human AChR MIR are sufficient to induce myasthenia, whilst structural integrity and the presence of additional epitopes of other AChR regions are enhancing factors. This is in agreement with observations in passive transfer MG rat models in which monoclonal antibodies against the MIR alone had less myasthenogenic potency than polyclonal serum antibodies obtained from rats with EAMG induced by active immunization (2,35). These results emphasize the important role of epitope spreading in the development of autoimmune diseases.

Epitope spreading in AChR MG

The epitope spreading hypothesis proposes that initial epitope targets of auto-antibodies do not remain fixed, but extend to other epitopes within the same protein and/or even to other, closely associated, proteins. This phenomenon has been

described for a wide variety of autoimmune diseases (reviewed by Vanderlugt and Miller (36)). In EAMG, epitope spreading may be responsible not only for maintaining but also for enhancing myasthenia by potentiating antigenic modulation and surface depletion of the receptors.

For AChR MG, epitope spreading has been described in several EAMG animal models (34, 37,38,39). Vincent et al. (37) immunized rabbits with a mixture of synthetic peptides including amino acids 138–199 of the human α1 AChR subunit. This resulted in a broad immune response against epitopes outside of this region of the rabbit AChR (including the MIR). A more recent study demonstrated a similar effect in rats immunized with parts of the extracellular domain of the AChR α subunit. After 3 weeks, auto-antibodies against the cytoplasmic domain could be detected and they seemed to correlate with the onset of clinical weakness in the animals (39). Investigating epitope spreading in myasthenic humans is difficult as there is usually a delay between the onset of the disease and the first visit to the clinic. However, in addition to the MIR antibodies, auto-antibodies against the cytoplasmic domain of the α1 AChR subunit have been detected in sera from MG patients, suggesting that epitope spreading occurs in humans as well (40).

Besides enhancing cross-linking and subsequent endocytosis of AChRs, the epitope spreading phenomenon may also facilitate complement activation because it eventually induces the build-up of a high density of antigen–antibody complexes at the postsynaptic membrane (41,42). Epitope spreading can also expand the immune response beyond the initial antigen. For AChR MG, an array of other antigenic targets are known (i.e. titin, ryanodine receptor 1 and 2, Kv 1.4 α-subunit, actin, α-actinin, tropomyosin, myosin, filamin, vinculin, rapsyn and HSP-70) (43,44,45,46,47,48). There is no evidence that these auto-antibodies induce MG by themselves, that is, without the presence of AChR antibodies. However, they are associated with a more severe clinical progression. One hypothesis is that these antibodies develop as secondary responses to muscle fibre destruction, exposing intracellular muscle proteins to an inflammatory environment, thereby facilitating the breakage of immunotolerance. Alternatively, these antibodies are often only found in the context of a thymoma, suggesting that they might result from an anti-tumour response rather than an anti-muscle response.

Various causes of epitope spreading have been suggested. It might be a useful ability of the immune system to enhance the response towards invading pathogens, such as bacteria or viruses. Another possible explanation is the opportunity for the immune system to deviate the pathogenic response into a protective immune response (49). Activating immunity against other nonpathogenic epitopes can divert the immune response away from the pathogenic response and dilute its effects (50). This theory has been applied for the development of more specific therapies for MG (see below). Understanding the basis of epitope spreading will be of importance for further development of specific treatments.

Role of IgG subclass in AChR MG

Human serum contains four different IgG subclasses, each with specific characteristics. AChR MG is caused by auto-antibodies of the IgG1 and IgG3 subclass (10,11). These auto-antibodies have high affinity for Fc receptors on immune cells and are also potent complement activators, in contrast to IgG2 and IgG4. Complement-mediated pathology in MG is strongly suggested by the deposition of complement factors at neuromuscular junctions of both humans with AChR MG and animals with EAMG (51,52). Furthermore, complement consumption increases during exacerbations of the disease; rats that are deficient in complement components C3 and C5 are protected against experimental MG after both active and passive immunization protocols, and susceptibility to EAMG is increased in mice deficient in complement inhibitor decay accelerating factor (53,54).

The other main pathological mechanism in AChR MG, antigenic modulation, is directly linked to the functional bivalency of IgG1 and IgG3 and their ability to bind two antigen molecules. Drachman and colleagues showed both *in vitro* and *in vivo* that AChR antibodies cause loss of postsynaptic AChRs (13,55). However, monovalent Fab fragments (produced from these antibodies by papain digestion) failed to produce such AChR depletion. Crosslinking of these Fab fragments restored their AChR-reducing ability. These results demonstrated that AChR auto-antibodies deplete surface AChRs through crosslinking and internalization. Therefore, the specific characteristics of the IgG subclasses involved are key determinants in the pathophysiology of AChR MG.

Therapeutic strategies evolved from epitope mapping in AChR MG

Currently, symptomatic treatment of AChR MG mainly consists of acetylcholinesterase (AChE) inhibition. Furthermore, the immune system is often (non-specifically) suppressed by plasmapheresis, intravenous administration of immunoglobulins and/or immunosuppressive drugs, or thymectomy. For successful treatment, these therapies often need to be continued for several years to prevent return of the symptoms. Specific treatment, which ideally would block the production and/or effects of only the pathogenic auto-antibodies in one therapeutic session, is lacking. Epitope mapping has provided clues to the development of more specific treatment options; some of the approaches are discussed below.

Several groups demonstrated that nasal and oral tolerization with either recombinant fragments of the AChR, synthetic peptide sequences or native AChRs can prevent the onset of EAMG in animals (56,57,58,59,60,61). Active EAMG could be modestly inhibited in rats by oral administration of recombinant α1 subunit extracellular domain (62). However, there are concerns about the risk of intensifying the autoimmune response rather than suppressing it (63, 64). Luo et al. (50) developed an immunotherapy to divert the immune response away from the AChR MIR in EAMG by administering a mixture of cytoplasmic domains of human AChR subunits. This type of epitope spreading successfully suppressed ongoing EAMG in rats by shifting

Table 1. Summary of the features of myasthenia subtypes

	AChR MG	MuSK MG
Percentage of myasthenic patients	85%	8%
Muscle weakness distribution pattern	Ocular at onset, progressing to generalized proximal weakness in a craniocaudal direction	Generalized, often with weakness in bulbar and respiratory muscles
Tumour	Thymoma in 10% of patients	–
Responsiveness to AChE inhibitors	+++	–
HLA association	A1-B8-DR3 in early-onset MG	DR14-DQ5
IgG subclass	IgG1 and IgG3	IgG4
Target antigen	AChR	MuSK
MIR	α-subunit: amino acids 66–67	N-terminal Ig-like 1 domain
Additional epitopes within the main antigen	α-subunit β-subunit γ-subunit ε-subunit	Cysteine-rich domain Ig-like 2 domain
Epitope spreading outside of the main antigen	Antibodies against other proteins include: RyR1, RyR2, titin, actin, actinin, myosin, filamin, vinculin, rapsyn, HSP-70,	Additional antibodies against the AChRs or LRP4
Structural epitope	Yes	Yes
Pathogenic mechanisms:		
Complement mediated	+++	–
Antigenic modulation	+++	++
Ligand binding site competition	+ (with ACh)	+++ (with ColQ and LRP4)
Steric hindrance	–	Not known

the auto-antibody specificity towards the cytoplasmic domain. These antibodies are considered harmless, because they cannot access and bind to the AChR *in vivo*. There was also a slight shift in AChR auto-antibody subclass from less IgG2b (inflammatory in rats) to an increase in IgG1 (anti-inflammatory in rats), although this could not solely account for the marked decrease in myasthenic symptoms. This approach is considered safer as no potentially pathological significant epitopes are introduced.

Another epitope-specific approach was explored by Araga and colleagues. They used complementary peptides to generate an anti-idiotype antibody response. These anti-idiotype antibodies were able to neutralize two monoclonal pathogenic MIR AChR antibodies *in vitro* and prevented the onset of EAMG *in vivo* (65, 66). Similar results were also reported after immunization with a peptide that was developed to inhibit AChR reactive T cells by an anti-idiotype approach (67).

LRP4 MG	LEMS
5%	2%
Generalized, sometimes with bulbar muscle weakness	Proximal leg weakness at onset, progressing to generalized proximal weakness in caudocranial direction
–	Small cell lung cancer in 50–60% of patients
++	+
Not known	B8-DR3 (in early onset patients without tumour)
IgG1	Probably IgG1
LRP4	$Ca_v2.1$ (P/Q-type) VGCC
Not known	α1-subunit
Not known	β subunit
Additional antibodies against MuSK, AChR or VGCC	Antibodies against other proteins including synaptotagmin, m1 AChR, SOX proteins and ERC1
Not known	Probably both structural and linear epitopes
Not known	–
Not known	++
++ (with agrin)	–
Not known	Not known

Furthermore, specific removal of pathogenic AChR auto-antibodies has been attempted with affinity-based apheresis. This approach would modify and improve the current practice of total plasmapheresis, as it does not concomitantly remove other, potentially important, plasma components. Two expression systems (*Escherichia coli* and *Pichia pastoris*) have been used to produce the ectodomains of all human AChR subunits which were then immobilized on a column resin for immunoadsorption of the pathogenic antibodies from MG sera (68,69, 70). In a preliminary experiment using one EAMG rabbit, such selective apheresis prevented a further increase in AChR antibody titre (71).

Finally, another (non-epitope-related) therapeutic approach would be to force the immune system to shift to an IgG4 immune response. IgG4 is considered functionally monovalent and is unable to activate complement (72). *In vitro* it has been shown

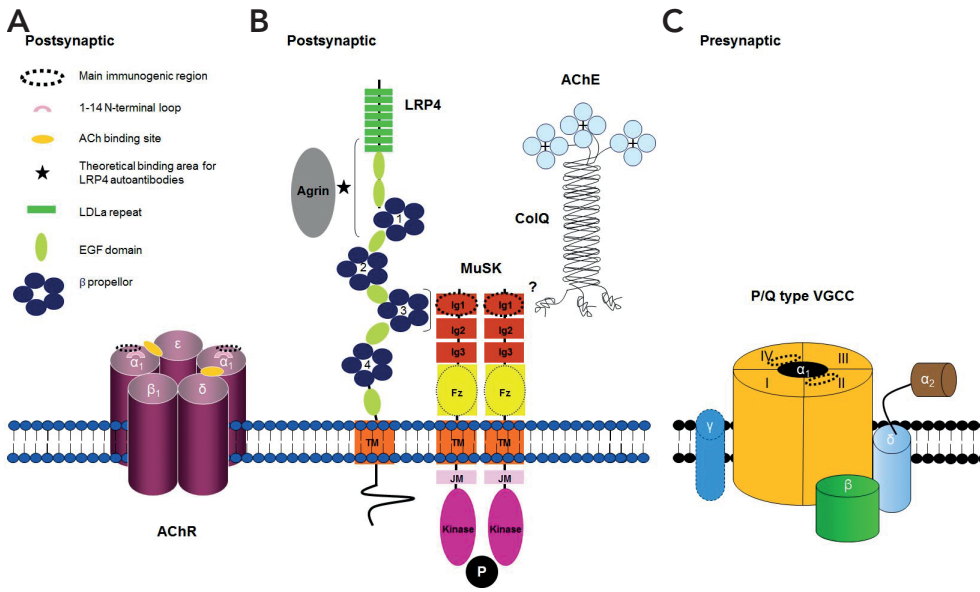

Figure 2. Overview of the antigenic targets in myasthenia gravis (MG) and Lambert–Eaton myasthenic syndrome (LEMS). The acetylcholine receptors (AChRs) are located in the postsynaptic membrane of the neuromuscular junction and each consists of five subunits. The main immunogenic region (MIR) for auto-antibodies in AChR MG is located on the outer side of the extracellular part of the α subunits of the AChR. Muscle-specific kinase (MuSK) and low-density lipoprotein receptor-related protein 4 (LRP4) are also transmembrane proteins located on the postsynaptic muscle membrane. Once agrin binds to LRP4, the Ig-like 1 domain and part of the Ig-like 2 domain of MuSK interact with LRP4. This stimulates the dimerization of MuSK and the activation of its kinase domain, essential for AChR clustering and maintenance of the neuromuscular junction. MuSK also interacts with collagen Q (ColQ) [which anchors acetylcholinesterase (AChE) in the synaptic cleft], although the precise domains involved in this interaction are unknown. The MIR of MuSK is located in the first Ig-like domain. However, antibodies directed against other epitopes have also been described. Presynaptically, auto-antibodies against the Cav2.1 type (P-/Q-type) voltage-gated calcium channel (VGCC) cause LEMS. This channel protein complex consists of one pore-forming main subunit (α1), which has four repeating domains (I–IV), and several associated subunits. Only the α1 subunit and the α2 part of the α2δ subunit protrude into the extracellular space and are directly accessible for auto-antibodies *in vivo*. MIRs are contained in the extracellular parts of the α1 domains II and IV. Fz, Frizzled-like domain; TM, transmembrane domain; JM, juxtamembrane domain; LDLa, low-density lipoprotein-like domain class A; EGF, epidermal growth factor-like domain.

that Fab fragments from AChR antibodies can protect the AChR against antigenic modulation and internalization by patients' auto-antibodies (13). Interestingly, an experimental MG model in monkeys with cloned human IgG1 or IgG4 AChR antibodies revealed that the IgG4 antibodies could not induce myasthenia *in vivo*, in contrast to the IgG1 antibodies. Furthermore, administration of these IgG4 antibodies protected the animals from MG when they were subsequently challenged with the IgG1 antibodies (72).

MG associated with antibodies against MuSK (MuSK MG)

In 2001, auto-antibodies against MuSK, a postsynaptic neuromuscular junction transmembrane protein involved in AChR clustering, were discovered (73). MuSK MG is typically observed at an earlier age than when the AChR is involved, is more common in women and is characterized by the involvement of bulbar weakness and atrophy (74). It has been reported that 46% of patients experience respiratory crisis at some point in their lives (74). Moreover, MuSK MG is strongly associated with HLA-DR14-DQ5 (75, 76). In contrast to patients with AChR MG, in those with MuSK MG ocular weakness is less common in the chronic phase of disease, there is no association with thymoma and the response to treatment with AChE inhibitors is generally not effective. In fact, symptoms may worsen in some cases following this treatment (77, 78). In general, patients are treated with prednisone and other immunosuppressive drugs, similar to AChR MG. Recently, it was shown that rituximab, a B-cell-inhibiting monoclonal antibody, might be an effective drug (79).

Pathogenesis of MuSK MG

The involvement of IgG4 auto-antibodies in MuSK MG was suggested by the high prevalence of IgG4 MuSK antibodies in patients' serum and a correlation between IgG4 auto-antibody levels and disease severity (80, 81). Indeed, we have recently demonstrated that IgG4 antibodies alone from MuSK MG patients are able to induce myasthenia in mice (**Chapter 2**). In addition, we showed in this study (82), performed in NOD/SCID mice which lack a functional innate and adaptive immune system, that the IgG4 auto-antibodies against MuSK directly induce the disease. This is in contrast to AChR MG, which, as discussed above, is caused by IgG1 and IgG3 auto-antibodies that cause complement-mediated membrane destruction and cross-linking and internalization of the AChRs. How MuSK auto-antibodies induce pathology was largely unknown.

IgG4 antibodies are unable to activate complement and have a low affinity for Fc receptors on immune cells (72, 83). It is likely that the mechanism by which the auto-antibodies induce pathology directly depends on the epitope to which these antibodies bind. The extracellular domain of MuSK comprises three Ig-like domains and a Frizzled-like domain (an overview of the structure of MuSK and its main immunogenic regions is shown in Fig. 2). Epitope mapping revealed that most auto-antibodies recognize epitopes within the first two extracellular Ig-like domains (79, 84, **Chapter 4**). In one study, 30% of the patients had additional reactivity against the cysteine-rich domain (in the Frizzled-like domain) (85). The cysteine-rich domain has been implicated as a Wnt receptor and signalling through this pathway was shown to be essential for AChR clustering (86,87,88). The pathology seen in patients with antibodies against the cysteine-rich domain might therefore be due to interference with this pathway. Most patients however have auto-antibodies against the Ig-like

1 domain of MuSK. The Ig-like 1 domain has two essential functions. First, the external face of this domain mediates association between MuSK and LRP4, which, when bound to neuronal agrin, strengthens the interaction between MuSK and LRP4 and activates downstream signalling leading to AChR clustering. Secondly, the opposite medial side of the Ig-like 1 domain mediates MuSK dimerization (89, 90). We hypothesize that auto-antibodies against MuSK can induce myasthenia through three potential mechanisms: (i) antigenic modulation and internalization of surface MuSK, (ii) inhibition of MuSK dimerization and/or (iii) interference with MuSK binding part- ners (**Chapter 3**). Any of these mechanisms would cause defects in the maintenance of postsynaptic AChR clustering, thereby impairing neuromuscular transmission and thus causing clinical myasthenia. Indeed, loss of surface MuSK and AChRs was observed both *in vitro* and *in vivo* in passive transfer experiments (91).

The effects of MuSK antibodies on the interaction of MuSK with its binding partners have partly been addressed. Several proteins [e.g. collagen Q (ColQ), biglycan and LRP4] are known to interact with the extracellular domain of MuSK (92,93,94,95). ColQ is able to bind to MuSK when expressed by non-permeabilized COS cells, and it has been suggested that it may be involved in MuSK and AChE localization (93). However, the direct interaction between MuSK and ColQ could not be reproduced in another study (92). Nevertheless, the findings of a recent study indicated that MuSK auto-antibodies can prevent the interaction between MuSK and ColQ [96]. Passive transfer of patient IgG reduced neuromuscular junction ColQ and AChR levels. The authors of this study suggested that myasthenia is induced by a loss of ColQ–MuSK interaction and subsequent loss of AChR clustering (96). However, the same authors have recently shown that MuSK auto-antibodies induce myasthenia in *ColQ* deficient mice, thus suggesting that loss of MuSK-ColQ interaction is not the key pathomechanism in MuSK MG (97). Furthermore, they excluded effects of MuSK auto-antibodies on MuSK–LRP4 interaction in a plate-binding assay. However, agrin, which has been shown to be essential for appropriate association between MuSK and LRP4 (89), was not included in the assay. A possible effect of MuSK auto-antibodies may therefore have been overlooked.

Muscle-specific kinase mutant and knockout mouse models have shown that MuSK is essential for neuromuscular junction formation and maintenance (98, 99). In AChR MG patients and mouse models, the loss of AChR clusters is compensated by upregulation of presynaptic ACh release, via retrograde signalling at individual neuromuscular junctions (100). MuSK MG passive transfer (82,101) and active immunization experiments (102) in mice as well as patient muscle biopsy studies (103,104) have shown that this compensatory upregulation is missing in MuSK MG neuromuscular junctions. This might suggest that the retrograde signalling is regulated by the N-terminal Ig-like domains of MuSK or the interactions that occur at this site. Recently, Yumoto et al. (105) established that LRP4 is a bi-directional signalling molecule. LRP4 is able to bind outgrowing motor axons and induces

presynaptic differentiation independent of MuSK. In parallel, LRP4 is involved in transducing the neuronal agrin signal to stabilize postsynaptic AChR clustering. Thus, interference with patient MuSK auto-antibodies of MuSK–LRP4 interaction might explain the lack of presynaptic compensatory response to the decreased level of AChRs.

Epitope spreading in MuSK MG

The presence of multiple epitopes in MuSK MG suggests that epitope spreading occurs in this disease (**chapter 4**). Furthermore, it has been reported that some patients have auto-antibodies against multiple neuromuscular junction antigens including MuSK (106,107). Passive transfer MuSK MG mouse studies show that there is variation between patients' auto-antibodies in terms of disease potency (82, 108). This might also correlate with the exact epitopes for these auto-antibodies.

Therapeutic strategies from epitope mapping in MuSK MG

Although MuSK MG is an IgG4-mediated disease, many parallels can be drawn with AChR MG and LEMS. Both forms of auto-antibodies require structural epitopes, and their MIRs are located on the most protruding portion of their extracellular domains. Immune evasion therapies as described earlier might therefore be applicable for MuSK MG as well. The role of the IgG4 antibodies in this case is of particular interest. In one case study, it was reported that a patient underwent a class switch from IgG4 MuSK auto-antibodies to IgG1 MuSK auto-antibodies and went into stable remission (81). The loss of monovalent MuSK auto-antibodies might account for this improvement. However, in an active immunization EAMG rabbit model, both monovalent and divalent MuSK antibodies were able to interfere with proper MuSK functioning (109). It remains to be determined whether this is also true for human MuSK auto-antibodies. Drug-induced class switching for MuSK auto-antibodies might thus form a potential future therapy in other MuSK MG patients.

MG associated with antibodies against LRP4 (LRP4 MG)

Recently, LRP4 auto-antibodies were discovered in another subgroup of 'seronegative' MG patients (106,107,110). Antibodies of this type occur in an extremely variable proportion (3–92%) of 'seronegative' MG patients, which might depend on their ethnic origin or the selection criteria for inclusion. Four percent of the 574 samples tested were negative for AChR and MuSK antibodies but were positive for LRP4 antibodies. LRP4 antibodies were also found in 1% of this population where patients also had antibodies against either MuSK or VGCC. Most patients had a MuSK MG-like phenotype, experiencing generalized muscle weakness often combined with bulbar weakness (106,110). Moreover, no association with thymoma was found (106). As in AChR MG, patients with LRP4 MG in general respond well to AChE inhibitors.

Pathogenesis of LRP4 MG

Lipoprotein receptor-related protein 4 is a transmembrane protein of which the extracellular domain consists of eight LDLa domains, two EGF domains and four β-propellors which are each separated by one EGF-like repeat (an overview of the LRP4 protein structure is shown in Fig. 2). Epitope mapping has thus far not been performed for LRP4 MG. In one study, LRP4 auto-antibodies were classified as IgG1 subclass, which would suggest complement involvement (106); however, this has not yet been confirmed. Application of LRP4 antibodies in a solid-phase binding assay resulted in a loss of agrin–LRP4 interaction and in some cases reduced AChR clustering in a cellular system (106,110). Agrin is crucial for the LRP4–MuSK signalling cascade leading to AChR clustering (90). Therefore, interference in the interaction between agrin and LRP4 could result in myasthenia. Crystal structure analysis revealed that LRP4 and agrin can interact even if only the first β-propeller of LRP4 is present (111). However, in a solid-phase binding assay, the last two LDLa domains of LRP4 were shown to strengthen this interaction significantly (90). It is likely that the LRP4 auto-antibodies bind in this region to affect the agrin–LRP4 interaction (Fig. 2). It will be interesting to further pinpoint the binding epitope of the LRP4 antibodies and assess whether these auto-antibodies can also interfere with MuSK–LRP4 interaction and/or cause cross-linking and internalization of LRP4. Two patients with *LRP4* mutations and a progressive muscle weakness have been described. In these patients the mutations were located in the third beta-propeller domain and inhibited an effective binding with MuSK. (112) Moreover, compensatory upregulation of presynaptic ACh release, which may involve LRP4-mediated signalling, might also be affected by LRP4 antibodies.

Lambert-Eaton Myasthenic Syndrome (LEMS) with antibodies against P/Qtype VGCC

About 90% of patients with LEMS have auto-antibodies against presynaptic Cav2.1 (P-/Q-type) VGCCs (113,114). In about 50–60% of these patients, small cell lung cancer (SCLC) is detected (114,115,116). LEMS can occur at all ages and affects both men and women. A bimodal distribution is observed in patients with non-tumour-associated LEMS, as in those with MG, with a peak at about 35 years of age, predominantly in female patients. A second peak is observed above 60 years of age, with an equal sex distribution (117). In addition, above the age of 50 years, a larger peak of mostly male smokers with SCLC is seen (118,119). LEMS usually presents with proximal leg weakness and loss of tendon reflexes (115). Weakness progresses to other muscle groups with increasing disease severity; however, this occurs in a caudocranial direction in contrast to AChR MG (120). Furthermore, 80–90% of LEMS patients experience relatively mild symptoms of autonomic dysfunction which include dry mouth and eyes, erectile dysfunction, constipation and blurred vision (115,116,121).

Pathogenesis of LEMS

Antibodies against Cav2.1 VGCCs are presumed to be pathogenic in LEMS because the disease can be passively transferred into mice (113,114,122,123). Active immunization with parts of the α1 subunit also led to a LEMS phenotype in mice (124). Moreover, transfer of antibodies from an affected mother to child resulted in transient neonatal weakness (125).

The Cav2.1 VGCC is present in both SCLC cells and the motor nerve terminal (126). Freeze-fracture electron microscopy of mouse neuromuscular junction treated with purified IgG from LEMS patients shows a depletion and aggregation of active zone particles, which are presumed to represent VGCCs (127). The Cav2.1 VGCC is necessary for Ca^{2+} influx at these active zones, which enables release of ACh from the nerve terminal. In another passive transfer study, both LEMS IgG and divalent antibody fragments of LEMS patients caused a reduction in quantal content (i.e. the number of synaptic vesicles containing ACh exocytosed per nerve impulse) (128). Monovalent (Fab) fragments, however, did not affect neuromuscular transmission. The same effect of divalent but not monovalent antibody fragments was shown *in vitro* for Ca^{2+} flux in SCLC cells (128). The active zone particles are arranged in double parallel rows about 16–21 nm apart, enabling cross-linking. This suggests an important role for surface depletion of VGCCs through cross-linking and internalization as in AChR MG. Because IgG4 is considered functionally monovalent, the involvement of divalent antigen binding suggests that IgG1 and IgG3 subclass auto-antibodies might play a more important role than the IgG4 subclass antibodies.

In contrast to AChR MG, the effects of LEMS IgG seem to be complement independent, because passive transfer experiments in C5-deficient mice and after C3 depletion by cobra venom factor induced a LEMS phenotype (122, 129). Conductance of individual VGCCs remained intact after exposure to serum from LEMS patients, indicating that a competitive or direct blocking effect of antibodies is unlikely (130).

In summary, VGCC auto-antibodies induce LEMS by antigenic modulation and surface depletion of the ion channels. Complement-mediated membrane destruction, steric hindrance and/or competition with the Ca^{2+} binding site do not seem to play important roles in the pathogenesis of LEMS.

Epitope mapping in LEMS

The VGCC is a heteromultimeric protein complex on the presynaptic motor nerve terminal membrane and consists of an α1 subunit and α2δ, β and possibly γ accessory subunits (Fig. 2). The α1 subunit is the ion conducting pore, whereas the other subunits modulate gating and serve as a membrane anchor (131). The α1 and a part of the α2δ subunit extend into the synaptic cleft. The α1 subunit contains four domains (I–IV), each having six transmembrane segments (S1–S6). The search for binding epitopes has mostly focused on this subunit, especially the extracellular S5–S6 linker regions between domains which are exposed extracellularly. Antibodies against synthetic peptides corresponding to the S5–S6

linker region of domain II and IV have been detected in 43–75% of LEMS patients (132,133). The presence of antibodies that recognized domain IV seemed to be more common in patients without associated tumour compared with patients with SCLC (134). Conflicting results have been reported with regard to a binding epitope in the domain III S5–S6 linker region, which is the domain with the highest Ca^{2+} affinity (132,133,135). In an active immunization model, six of ten rats immunized with this synthetic peptide showed muscle weakness and defective neuromuscular transmission, suggesting that it is likely to be immunogenic (124).

Another target for auto-antibodies is the presynaptic protein synaptotagmin I (136), which is present in both SCLC cells and at presynaptic active zones where it is involved in the release of ACh. Immunizing rats with segments of synaptotagmin I induced electrophysiological abnormalities reminiscent of presynaptic dysfunction as seen in LEMS.

Epitope spreading in LEMS

Antibodies against the β subunit of the VGCC have also been described in some LEMS patients. Because this subunit is only present intracellularly, these antibodies are probably secondary to immune-mediated damage (137,138). The presynaptic active zone protein ERC1/ELKS1 has recently been reported as an antigen in a VGCC-positive LEMS patient, which is also only present intracellularly and unlikely to be of pathogenic relevance (139). Epitopes in other associated proteins might be more relevant for modulating the disease process. Antibodies against the M1-type muscarinic AChR, for example, can be found in most patients with LEMS (140). This receptor type is implicated in presynaptic compensation for impairment of Ca^{2+} entry that is necessary for ACh release. Epitope spreading to this receptor later in the disease course could impair this compensation mechanism. Upregulation of other VGCC subtypes capable of ACh release seems to be another compensatory mechanism in LEMS patients (141). Some patients harbour auto-antibodies against the Cav2.2 (N-type) and Cav1 (L- type) VGCC subtypes although the significance of this immune response is uncertain (142,143).

Epitope spreading also occurs in the antitumor immune response against SCLC. About 65% of LEMS patients with associated SCLC have antibodies against the intracellular nuclear SOX1 protein compared with 22–36% of patients with SCLC alone (144,145). Because these antibodies are rare in LEMS patients without associated tumour, they can be used as a serological marker for SCLC-associated LEMS. No survival effect or specific patient characteristics were reported for SOX antibodies; thus, a pathogenic role is unlikely (144,146). The significance of these antibodies in anti-tumour immune responses and development of autoimmunity remains unclear.

SCOPE OF THE THESIS

Studies from a broad spectrum of antibody-mediated autoimmune diseases, including the above described neuromuscular autoimmune diseases have emphasized that the pathomechanism, course of disease and treatment response of antibody-mediated autoimmune diseases can depend on:

1. Auto-antibody titre

2. Auto-antibody subclass

3. Epitope(s) bound by the auto-antibodies

4. Structural integrity of the MIR

Understanding what role these factors play in the development of autoimmune disease not only gives insight in the pathomechanism of disease, but also highlights which treatment strategy is most likely to be beneficial for the patient. Identifying the main binding epitopes, pathomechanism of disease and establishing the presence of auto-antibodies against a MIR can subsequently be useful for predicting the course of the disease and may provide insights for the development of new therapies. For MuSK MG these aspects had as yet not been investigated in detail.

The aim of this thesis is to investigate the pathomechanism of MuSK MG and to characterize the auto-antibodies causing the disease.

The first part of this thesis will focus on the research question:

How do MuSK auto-antibodies cause myasthenia gravis?

Chapter 2 investigates the pathogenicity of the IgG subclass antibodies from MuSK MG patient plasmapheresis material. IgG4 and IgG1-3 fractions were affinity purified and subsequently used in passive transfer studies in NOD/SCID mice.

Chapter 3 studies the exact mechanism by which the IgG4 MuSK auto-antibodies cause myasthenia *in vitro*.

The second part of the thesis investigates:

How MuSK auto-antibodies characteristics contribute to disease course?

Chapter 4 investigates the role of epitope spreading in disease severity and treatment responsiveness in MuSK MG patients.

Chapter 5 uses our experience in detecting MuSK auto-antibodies to study their role in patients with amyotrophic lateral sclerosis.

The last part of the thesis, **Chapter 6**, focusses on the relevance of our findings for other IgG4-mediated autoimmune diseases and provides future directions for (antigen-specific) treatment strategies.

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