Towards a specific therapy for myasthenia gravis : a quest for armor against autoantibody attack

Citation for published version (APA):

Stassen, M. H. W. (2003). *Towards a specific therapy for myasthenia gravis : a quest for armor against autoantibody attack*. [Doctoral Thesis, Maastricht University]. Datawyse / Universitaire Pers Maastricht. <https://doi.org/10.26481/dis.20031204ms>

Document status and date:

Published: 01/01/2003

DOI:

[10.26481/dis.20031204ms](https://doi.org/10.26481/dis.20031204ms)

Document Version:

Publisher's PDF, also known as Version of record

Please check the document version of this publication:

- A submitted manuscript is the version of the article upon submission and before peer-review. There can be important differences between the submitted version and the official published version of record. People interested in the research are advised to contact the author for the final version of the publication, or visit the DOI to the publisher's website.
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Towards a specific therapy for myasthenia gravis

A quest for armor against autoantibody attack

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Omslagfoto: Henk Veldman, Universiteit Utrecht
Uitgeverij: Universitaire Pers Maastricht, Maastricht
ISBN 90-5278-403-5

Towards a specific therapy for myasthenia gravis

A quest for armor against autoantibody attack

Proefschrift

ter verkrijging van de graad van doctor
aan de Universiteit Maastricht,
op gezag van de Rector Magnificus,
prof. dr. A.C. Nieuwenhuijzen Kruseman,
volgens het besluit van het College van Decanen,
in het openbaar te verdedigen
op donderdag 4 december 2003 om 14:00 uur

door

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“What’s the point of all this hoax?
Is it the chicken and the egg time? Are we just yolks?
Or, perhaps, we’re just one of God’s little jokes [...]
In this life, what is our fate?
Is there Heaven and Hell? Do we reincarnate?
Is mankind evolving, or is it too late? [...]
While the scientists say
We’re just simply spiralling coils of self-replicating DNA.”

— Eric Idle, *The meaning of life*

“Een overwinning kan worden voorzien.
Ze kan echter niet worden geforceerd.”

— Sun-tzu, *De kunst van het oorlogvoeren*

Preface

The human body is protected by the immune system against viruses, bacteria and parasites, but also against malignant tumors that invade healthy tissue. For an adequate immune response, the immune system has to recognize invaders, i.e. it has to differentiate between (healthy) self and foreign. Antibodies and T cell receptors (TCRs) are the proteins that facilitate the identification of foreign antigens, and upon recognition trigger a humoral or cellular immune response, respectively. A failure of the immune system to be tolerant to self can lead to the unwanted situation where TCRs and antibodies bind to self substances, resulting in an autoimmune disease.

Myasthenia gravis (MG) is a prototype autoimmune disease, since its antibody-mediated pathogenesis is restricted to one target, the postsynaptic membrane of the neuromuscular junction (NMJ). The muscular nicotinic acetylcholine receptor (AChR) is the major autoantigen located at the NMJ. Autoantibodies directed to the AChR lower the number of functional AChRs, leading to an impaired neuromuscular signal transduction, which results in a fatigable skeletal muscle weakness (reviewed in **Chapter 1**).

MG patients can be successfully treated for their muscle weakness, but most treatments have side effects. Several experimental approaches for a specific immunotherapy of MG have been investigated by others including induction of tolerance by administering AChR-derived peptides, anti-idiotypic antibodies, peptides homologue to AChR that block anti-AChR antibodies and genetically engineered antigen-presenting cells that, as a kind of “guided missile”, destroy AChR-specific T cells. The model used for these experiments is experimental autoimmune MG (EAMG), an animal model that closely resembles MG of patients.

This thesis describes two experimental approaches that could lead to a specific MG therapy. First, the AChR could be protected against degradation by blocking the binding of anti-AChR antibodies by therapeutic antibodies. It is known that rat anti-AChR monoclonal antibodies can block the pathogenic binding of autoantibodies to the AChR. However, therapeutic antibodies should be of human origin to prevent immunologic reactions. The human origin and the AChR specificity of the potential therapeutic antibodies were obtained by isolating antibodies from either transgenic EAMG mice (**Chapter 2**) or MG patients (**Chapters 3, 4 and 5**). Modifying these cloned anti-AChR antibodies by genetically truncating them to antigen-binding fragments (F_{ab} s) or single-chain variable fragments (scFvs) eliminated their pathogenic characteristics (**Chapters 3 and 4**). However, a full-size antibody is preferred, considering the low serum half-life of scFvs and F_{ab} s. In order to construct a more metabolically stable protective antibody, a molecule with the characteristics of F_{ab} (monovalent for AChR and no effector functions typical for MG autoantibodies) and IgG (high serum half-life) was

designed. In a first step towards the production of this therapeutic antibody, a human IgG1 was reconstructed from an anti-AChR F_{ab} and one of its effector functions deleted (**Chapter 5**).

Finally, the postsynaptic membrane of the NMJ could be protected against loss of AChR by overexpression of rapsyn, an AChR clustering and anchoring protein (**Chapter 6**). In congenital myasthenic syndrome (CMS) patients, mutations in the rapsyn gene can be found. These mutations lower the functional level of rapsyn in the patients' muscles. In some animals resistant to the induction of EAMG, increased levels of rapsyn are found. These findings suggest a role of the level of rapsyn in the susceptibility to (EA)MG. If the level of rapsyn correlates with the susceptibility, modifying the level *in vivo* could be the basis of a new therapy.

Chapter 1 gives a detailed introduction to MG autoantibodies. Muscle autoantigens, diagnostic value of autoantibody assays, immunopathogenesis, etiology hypotheses and usages of EAMG models are reviewed.

A new transgenic mouse EAMG model is described in **Chapter 2**. The so-called HuMAb-Mouse is transgenic for human immunoglobulin loci, thereby giving rise to human anti-AChR antibodies upon immunization. This not only models MG more closely, but could also be a source of human antibodies that are the basis of therapeutic antibodies.

The previously cloned human anti-AChR F_{ab} 637 is a potential therapeutic agent, since it protects against AChR loss *in vitro*. In order to test the *in vivo* effects of the primate-specific anti-AChR F_{ab} 637, large quantities are needed for a monkey EAMG model. **Chapter 3** assessed the large-scale production of F_{ab} 637 in yeast, while **Chapters 4** and **5** describe alternative recombinant variants of F_{ab} 637. In **Chapter 4**, a scFv variant of F_{ab} 637, scFv637, was constructed and characterized. It was found that the bacterially produced scFv637 could serve as an alternative to F_{ab} 637, since it was able to compete for binding to AChR with MG sera. In **Chapter 5**, a human MG autoantibody, IgG1-637, was reconstructed from F_{ab} 637, by genetically fusing the latter's heavy chain to the human $\gamma 1 F_c$. IgG1-637, produced by a chinese hamster ovary cell line, was shown to be pathogenic in a monkey EAMG model, as predicted. IgG1-637 could serve as the basis of a therapeutic full-size antibody, which is a good alternative to F_{ab} 637 and scFv637, considering the metabolic stabilities.

In **Chapter 6**, a second therapeutic strategy for MG is studied. In order to determine if increasing the level of rapsyn is sufficient to induce resistance to EAMG, rapsyn was overexpressed in the muscles of rats and subsequently EAMG induced. Rapsyn was overexpressed by a non-viral gene transfer technique, *in vivo* electroporation. This rapsyn gene therapy was shown to have a protective effect on the induction of EAMG. The finding that the level of rapsyn indeed correlates with the susceptibility to EAMG could be useful in a therapeutic strategy to protect against loss of AChR from inside the muscle fibers. Besides MG patients, such a therapy could also benefit CMS patients.

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Chapter 1

review

The role of antibodies in myasthenia gravis

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published in the
Journal of the Neurological Sciences 202 (2002) pp. 5-11

Abstract

Myasthenia gravis is an autoimmune disease associated with antibodies directed to the postsynaptic acetylcholine receptor. These antibodies reduce the number of receptors. Autoantibodies against AChR and other muscle antigens can be used for the diagnosis of myasthenia gravis and related disorders. The origin and the role of these antibodies in the disease are discussed. Experimental autoimmune myasthenia gravis, an experimental model closely mimicking the disease, has provided answers to many questions about the role of antibodies, complement macrophages and AChR anchor proteins. Genetically modified anti-AChR antibodies may also be used in the future to treat myasthenia.

Introduction

Myasthenia gravis is an autoimmune disease associated with antibodies directed to the postsynaptic nicotinic acetylcholine receptor (AChR) at the neuromuscular junction. These antibodies reduce the number of AChR, which leads to muscle weakness. This weakness is prominent in muscles that are used frequently and repetitively, for example, the eye muscles, and results in ptosis and double vision. Lennon and Carnegie [1] proposed the concept that anti-AChR antibodies accounted for the clinical symptoms of MG. Antibodies were found to block receptor function or cause local damage to the tissues. They applied this concept to MS, MG, and autoimmune endocrine diseases. Antigens of microorganisms that resemble membrane receptors appeared to trigger the autoimmune disease. Overt autoimmune diseases only occurred in genetically predisposed individuals [1].

Since then, the role of antibodies against membrane receptors including calcium channels, glutamate receptors and potassium channels has been demonstrated in the pathogenesis of many other neurological diseases. Table 1 lists the diseases in which anti-AChR antibodies are involved. Channelopathies can also be genetic in origin. The clinical presentation of both genetic and autoimmune types is similar. However, the former usually presents soon after birth while the latter develop later in life.

Table 1: Autoimmune diseases with antibodies against AChRs

Disease	AChR	Main symptoms [reference]	Congenital syndrome
myasthenia gravis	$\alpha 1$ subunit (MIR)	muscular weakness [2]	yes
arthrogryposis multiplex congenita	γ subunit	arthrogryposis [3]	yes
acquired slow channel disease	ϵ subunit	muscular weakness [4]	yes
Guillain-Barré syndrome	functional assay	muscle weakness [5]	no
autonomic neuropathy	neuronal AChR	orthostatic hypertension [6]	yes

The players: AChR and antibodies

AChR is a pentameric transmembrane protein composed of five subunits in a stoichiometry of $\alpha 1_2, \beta 1, \gamma, \delta$ (Figure 1). The $\alpha 1$ subunit displays a highly immunogenic epitope at the top of the extracellular part, called the main immunogenic region (MIR), towards which the majority of the autoantibodies detected in MG and EAMG sera are directed [7]. Antibodies against the embryonal γ subunits directly inhibit AChR function and are responsible for the autoimmune type of arthrogryposis multiplex congenita [3]. In adults, the receptor γ subunit is replaced by the ϵ subunit. Antibodies against the ϵ subunits are responsible for delayed closure of the ion channel. This is called myasthenic acquired slow channel syndrome, which is clinically indistinguishable from classic MG [4]. On nerve trunk

stimulation, however, a double compound muscle action potential is seen after a single stimulus.

Antibodies are encoded by about 400 immunoglobulin genes. DNA rearrangements in B cells generate an immense diversity (10^{14} different antibody molecules per individual), even before the encounter of antigens. Subsequently, class switch and somatic mutation of the Ig variable region occurs. Autoantibodies are not structurally different from antibodies against microbial or other antigens.

Figure 1

The muscular AChR is a pentameric transmembrane protein composed of five subunits in a stoichiometry of $\alpha_2\beta\gamma\delta$. The α subunit displays a highly immunogenic epitope at the top of the extracellular part, called the main immunogenic region (MIR), towards which the majority of the autoantibodies detected in MG and EAMG sera are directed. The right panel depicts the α subunit containing the MIR (67-76) and the acetylcholine (ACh)-binding site (185-199).

Diagnostic usage of autoantibodies against muscle antigens

Reproducible measurements of serum anti-AChR antibodies can be made by means of a radioimmunoassay using solubilized human muscle AChR labeled with ^{125}I - α -bungarotoxin. Fetal AChR extracted from a more readily available rhabdomyosarcoma cell line (TE671) or adult type receptor from a genetically modified TE676 cell line (CN21) can be used. The sensitivity of the RIA using TE671 cells is >1.5 and >1 nM for the RIA using CN21 cells. For titers >2 nM both TE671 and muscle AChR correlate well [8,9]. So, for sensitive diagnostic use, human denervated muscle AChR obtained from ischemic amputated legs is to be used (IBL radioimmunoassay information <http://www.ibl.com>), sensitivity >0.25 nM. The UK external quality assessment service (Sheffield, UK <http://www.uknequas.org.uk>) reported that 2/3 of the European laboratories use human muscle AChR as antigen and 1/3 cell line AChR.

In generalized early or late onset MG, 90% of the patients have circulating anti-AChR antibodies. MG patients with a thymoma are all anti-AChR antibody-positive. In purely ocular MG, only 50% of the patients are antibody-positive. Titers are highly variable among MG

patients and correlate weakly with the clinical disease severity [10-13], although in individual patients the titer correlates well with the clinical score. Therefore, several measurements are useful in the follow-up to treatment or to prevent clinical relapse of the disease. A titer decrease of 50% almost always coincides with a substantial clinical improvement [14].

Finally, anti-AChR antibody measurements are the cornerstone of the confirmatory tests for the diagnosis along with EMG/single-fiber EMG (SFEMG) and perhaps the neostigmine test.

The range of myasthenic antibodies also includes antibodies against other muscular cytosolic proteins, such as actin, myosin, a actinin, titin, rapsyn, ryanodin receptor and muscle specific kinase (MuSK). Of this series of antigens, titin and MuSK are important in the diagnosis of paraneoplastic-associated MG and seronegative MG, respectively. More than 95% of the thymoma patients have antibodies against striated muscle antigen, i.e. titin, a very large structural cytoplasmic protein that spans from the M line to the Z line and is associated with myosin [15]. The presence of antistriated muscle antibodies in patients younger than 40 years at disease onset suggests the presence of a thymoma and a more severe disease. In patients older than 40 years, this test is of less diagnostic importance for the presence of a thymoma, since 75% of the MG patients over 60 years old have antistriated muscle antibodies [16]. Combined testing for antibodies against titin and ryanodin receptors, voltage-activated calcium release channels located in the sarcoplasmic reticulum, yields the highest sensitivity and specificity for the detection of a thymoma in MG irrespective of age [15].

The origin of anti-muscle antigens and their relation to the pathogenesis of a thymoma can best be explained by the presence of a cross-reactive antigen (probably neurofilament M) in the thymus (cross-reacting with anti-AChR at the neuromuscular junction) [17]. This means that, in an effort to eliminate the thymoma, the immune system induces cross-reactive antibodies with AChR and thus myasthenia. In non-thymoma MG patients, antistriated antibodies might be induced secondary to the local muscle damage by anti-AChR antibodies, exposing cytoplasmic proteins including titin and the ryanodin receptor.

A third class of recently discovered antibodies in MG is directed against MuSK. These antibodies can be detected in 70% of the so-called seronegative (i.e. no detectable anti-AChR antibodies) generalized MG patients but not in seropositive MG patients or thymoma MG patients. MuSK functions as a receptor for agrin secreted by the nerve terminal, which induces AChR clustering. These clusters at the top of the junctional postsynaptic folds are located closely to the presynaptic active zones providing an optimal interaction between the acetylcholine quantum and AChRs. Serum anti-MuSK antibodies interfere with the formation of these clusters in vitro. It is not yet clear how these antibodies induce MG in an adult neuromuscular junction that already contains AChR clusters [18].

Immunopathogenesis of myasthenia gravis

The muscular weakness in MG is a consequence of a reduced number of functional receptors on the postsynaptic membrane. Antibodies that are mainly directed against the α subunit are able to cross-link AChR, thus stimulating the normal internalization and degradation process. Membrane fragments containing AChR are internalized by endocytosis and degraded by lysosomal enzymes. The half-life of AChR is reduced from 7 to 2 days. This process is called antigenic modulation. There is a compensatory increase of AChR synthesis as measured by increased mRNA levels for the α receptor subunit [19,20].

A second important mechanism that is responsible for the neuromuscular defect is complement-mediated focal lysis of the postsynaptic membrane. By binding to AChR, antibodies trigger a complement cascade resulting in a focal destruction of the postsynaptic membrane by the membrane attack complex. However, no lysis of the entire muscle fiber occurs, because this is a very large syncytium that is resistant to degradation processes. The damage to the postsynaptic membrane is only partially restored, resulting in the reduction of the number of postsynaptic folds. The complement-mediated lysis is also essential for antigenic modulation, because in complement-deficient animals, no experimental autoimmune myasthenia gravis (EAMG) can be induced in spite of the presence of abundant antibodies at the endplate. Complement-mediated attack probably removes the cytoskeletal restraints of AChR in the membrane, thus facilitating AChR endocytosis [21,22].

Thirdly, antibodies against the acetylcholine-binding site or the ion channel may inhibit AChR function. Also, monoclonal antibodies against the acetylcholine-binding site inhibit ion channel function. Injection of anti-acetylcholine site antibodies into experimental animals (newly hatched chickens) induced an acute curare-like paralysis which differs from EAMG induced by anti-AChR antibodies [23]. In MG sera, low titers of anti-AChR-binding site antibodies are detectable but their functional importance is not so clear. However, in the serum of mothers with a baby suffering from arthrogryposis multiplex congenita, antibodies against the γ subunit of the fetal AChR interfere with ion channel function [3] of the baby's AChR. The mother has no muscular weakness, because she expresses only adult type AChR with the ϵ subunit instead of the fetal γ subunit.

The origin of autoantibodies against muscle antigens

A large number of young-onset myasthenia gravis patients have a hyperplastic thymic medulla containing anti-AChR-producing B cells and plasma cells, sensitized T cells and antigen-presenting cells, and AChR-expressing myoid cells. This suggests that the primary sensitization against muscle antigen in MG takes place in the thymus. This intrathymic

pathogenesis model was proposed by Wekerle and Ketelsen [24] in 1977. The most convincing experiment supporting this hypothesis is the transplantation of thymic hyperplastic tissue into mice with severe combined immunodeficiency (SCID) (immunologically cripple mice not able to produce mature T/B cells and therefore not rejecting the thymic graft). The transplantation resulted in a prolonged production of anti-AChR antibodies and AChR loss in the recipients [25]. Other investigators believe that the sensitization of T and B cells occurs in the peripheral lymphoid system. A small proportion of lymphocytes recirculate to the thymus [26], where restimulation occurs. Microbial antigens expressing cross-reactive epitopes can stimulate AChR-reactive T and B cells through a mechanism called molecular mimicry in genetically susceptible individuals [27]. Alteration in AChR expression or structure might trigger the immune response. Analogous to the glutamate type receptor subunit 3 (Glu 3), the target of the immune response in Rasmussen encephalitis, AChR contains an internal N-linked glucosylation site (Asn 141) [28]. If these sites are not glucosylated, autoantigenic peptides might be generated after cleavage with granzyme B, a serine protein released by activated immune cells [29]. In MG, an association is found between the AChR α subunit gene (CHRNA), DQA1 * 101 and the DR3 haplotype, suggesting a three-gene disease model associating two HLA and one AChR α gene in MG [30].

Myasthenia gravis associated with thymoma is a paraneoplastic disease, in which proliferating epithelial cells attract large numbers of immature T cells [31]. These epithelial cells may express AChR or AChR-related epitopes, titin or ryanodine receptor-like epitopes but not the intact proteins [32-34]. However, the majority of these thymomas lack activation markers. A minute fraction of activated T cells leave the thymus and are found in the peripheral blood lymphocytes [35] and activate B cells. Indeed, all thymoma patients have circulating anti-AChR antibodies and anti-titin antibodies, and, to a lesser extent, antibodies against the ryanodin receptor, which implicates the autoimmune nature of thymoma-associated MG. Unlike the transplantation of the thymus with follicular hyperplasia in SCID mice that induces high titers of anti-AChR antibodies, thymoma explants never cause anti-AChR antibody production nor EAMG [36].

Marx *et al.* suggest that aberrant expression of neurofilaments expressing AChR or titin-related epitopes induces a false positive selection of immature T cells leaving the thymus. These T cells are activated in the periphery where they are stimulated to maintain a continuous antibody production even after removal of the thymoma. Local destruction of postsynaptic membrane provides a continuous source of autoantigens for the stimulation of autoreactive T and B cells.

Experimental autoimmune myasthenia gravis: comparison of EAMG and MG

An experimental animal model closely mimicking the disease has provided answers to many questions about the role of anti-AChR antibodies in MG. We will address these questions below.

MG can be reproducibly induced by injecting anti-AChR antibodies into experimental animals. This passively transferred disease occurs within 6 h to a few days after a single injection of monoclonal antibodies against AChR. Patients' sera or polyclonal antibodies can be used, but this model is not as reproducible and needs repeated injections.

Active immunization with AChR from different species or subunits or peptides derived from AChR induces a chronic EAMG model with symptoms starting from 30 days after immunization. Both acute and chronic EAMG resemble MG.

Animals have muscular weakness after repetitive use of the muscles. The defective neuromuscular transmission is demonstrated by repetitive neurostimulation. This causes a decremental response by repetitive nerve stimulation showing increased jitters and blocking. Serum antibodies against AChR are mainly directed against the MIR, and anti-MIR mAbs are very effective inducing EAMG. As little as 40 pmol of antibody 35 can induce severe MG in a rat expressing about 70 pmol of AChR in muscles. So, no higher antibody excess to AChR is needed to downmodulate AChR. A compensatory increase in AChR mRNA at the synapse can be measured [37].

Are pathogenic antibodies structurally related?

Both in MG and EAMG, two thirds of serum antibodies are directed to the MIR on the α subunit. Apparently in contrast to the restricted specificity of anti-AChR antibodies, the origin of serum [38-40] and endplate antibody is polyclonal, indicating that a large population of plasma cells produce anti-AChR antibodies. Sequence analysis of monoclonal antibodies immortalized from B cells of EAMG animals reveals a high number of somatic mutations. These findings indicate that the immune response is antigen-driven and not the result of some accidental mutation. Among pathogenic antibodies a high sequence homology (>90%) has been found, but none of these antibodies were identical [41].

To investigate the individual contributions of anti-human AChR responses in MG, we dissected the immune response into monoclonal antibodies. A phage-display technique was used to immortalize the anti-AChR secreting clones from the thymus of myasthenic patients. Four human monoclonal antibodies were isolated by us and showed to bind human AChR more accurately against the main immunogenic region. The antibodies were able to inhibit the binding of human MG sera to human AChR up to 80%. They were also able to protect against AChR loss by antigenic modulation induced by MG sera, making them potential therapeutic tools for patients with myasthenic crisis. In total, 18 F_{ab} s were

isolated by three different groups. Of the eight published amino acid sequences, all eight were different [41-43].

From the published sequences for antibodies from EAMG animals and MG patients, it can be concluded that pathogenic antibodies are not structurally related. All anti-MIR antibodies recognizing a small decapeptide α 67-76 [44] at the top of the external side of a subunits are structurally different. Indeed, antibodies in general recognize pentapeptides, which means that they can recognize at least five partially overlapping epitopes by their linear amino acid structure. Moreover, the MIR, being highly conformationally dependent, expresses additional epitopes that can be recognized by their respective antibodies. The role of antibodies against non-MIR epitopes, i.e. other subunits and cytoplasmic domains in the pathogenesis of myasthenia, has not been proven since only monoclonal antibodies against the MIR are capable of inducing EAMG.

What is the role of complement in EAMG/MG?

Both in MG and EAMG endplates, the lytic membrane attack complex (MAC) is found at the neuromuscular junction [45,46], suggesting the role of complement in the focal lysis of the postsynaptic membrane. Complement can be activated both by anti-AChR and antibodies against striational antigens [47]. Complement levels are decreased during exacerbation of MG, suggesting increased complement consumption. Complement-mediated focal lysis of the postsynaptic membrane removes the restraint on AChR, thus facilitating AChR internalization. Muscle cells are able to upregulate the synthesis of complement regulatory proteins (CD55, CD59 and vitronectin) as a defensive mechanism against complement-mediated damage [46].

Animals that are deficient of certain proteins for the complement cascade including C3 or C5 do not produce MAC that parallels their resistance against EAMG [21]. Treatments including those with anabolic steroids that increase complement levels also increase the susceptibility to acute EAMG [48]. In view of the important role of complement in the disease and the beneficial effect of complement inhibitors in EAMG, these inhibitors or non-complement fixing anti-MIR antibodies could be used as therapeutic agents to rapidly reverse a myasthenic crisis.

The role of macrophages in EAMG

In response to the antibody-mediated attack at the neuromuscular junction and the release of complement components including C3a and C5a, macrophages migrate to the endplate region. The question is whether these macrophages are a primary cause of AChR loss in EAMG. Therefore, we depleted macrophages in rats by means of irradiation. After induction of EAMG with anti-MIR monoclonal antibody 35, muscular weakness developed to a similar extent in macrophage depleted animals as in control animals. AChR and complement depositions were similar in both groups. However, no infiltrating macrophages were seen in irradiated

animals. This macrophage depletion does not interfere with the induction of acute EAMG by injection of monoclonal antibody 35. The macrophage infiltration seen in the non-depleted animals may be secondary to the focal damage at the endplate [49]. In MG, macrophage infiltrations are present in muscles to a lesser extent, but mostly not in the vicinity of endplates [50]. None of the inflammatory cells penetrate the muscle fiber near the endplate. In contrast, Maselli *et al.* [51] observed some macrophages in the endplate region. Based on the results of EAMG studies, however, it seems unlikely that macrophages play a role in the endplate damage, because endplate-bound anti-AChR antibodies and complement depositions suffice to induce AChR loss.

Resistance against the induction of EAMG

Aged rats, contrary to young rats, are resistant to the induction of passive transfer and EAMG induced by active immunization. An injection of a complement-fixing anti-MIR antibody 35 in aged animals does not induce muscular weakness nor clinical signs of EAMG in these old animals. This resistance could not be explained by deficient antibody uptake, increased antibody clearance, inaccessibility of AChRs for antibodies in aged animals, absence of infiltrating macrophages, nor by deficient complement activation. Therefore, it is most likely that the resistance resides at the level of the postsynaptic membrane, more specifically by resistance of the AChR molecules to antibody-mediated degradation. Semiquantitative analysis of the several proteins of the postsynaptic membrane in aged rats revealed an increase in the content of some of the anchor proteins including rapsyn, the most important AChR anchor protein. These results suggest that the susceptibility and clinical course of EAMG and possible MG is not only determined by the immune attack towards the neuromuscular junction, but also by the rigidity and the number of anchor proteins of AChR in the postsynaptic membrane [37].

In this context, we are trying to increase the concentration of rapsyn in the neuromuscular fiber by injecting a rapsyn expression system into muscular fibers by means of electroporation [52].

The experiments in age resistant animals could explain the absence of a correlation between anti-AChR titer and disease severity. So, procedures aimed at increasing the number of AChR anchor proteins might protect the endplate against the antibody-mediated attack. Electrotransfer of DNA encoding for rapsyn into the levator palpebrae or orbicularis oculi muscles might result in a long-term overexpression of AChR at the endplate.

Therapeutic antibodies as a treatment of MG

Antibodies against the extracellular epitopes of the α subunit are capable of cross-linking receptors, thus facilitating AChR internalization and degradation. Monovalent F_{ab} fragments of anti-AChR antibodies are able to interfere with this process both *in vitro* [41,53] and *in vivo* [54].

In order to increase the half-life of the F_{ab} molecules, we cloned its chain into an IgG molecule which has only one antigen-binding site and lacking

the C1q-binding sites that are necessary for complement activation. This molecule will be tested for its capacity to inhibit a passive transfer EAMG using MG patient serum.

In summary, the experimental model has been very useful to elucidate the central role of antibodies in autoimmune MG. Pathogenic antibodies binding mainly to the MIR are not structurally related. Binding of complement proteins including C1q to anti-AChR antibodies is essential to activate the complement cascade and remove the restraints of AChR molecules in the postsynaptic membrane, thus facilitating AChR internalization. The use of complement inhibitors is beneficial in EAMG and may also be tried in MG exacerbations. Macrophages are not necessary for the antibody-mediated damage of the neuromuscular junction but are infiltrating to remove the damaged fibers. Serendipitously, we found that rats older than 1 year (for a life span of 27 months) are resistant against the induction of EAMG. The mechanisms of resistance reside in the postsynaptic membrane that contains more AChR anchor proteins. Based on these findings in EAMG, new therapeutical measures could be developed including complement inhibitors, non-pathogenic anti-AChR competitor antibodies, and gene therapy aimed at overexpression of AChR anchor proteins.

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Chapter 2

Experimental autoimmune myasthenia gravis in mice expressing human immunoglobulin loci

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published in the
Journal of Neuroimmunology 135 (2003) pp. 56-61

Abstract

Antibodies (Abs) specifically directed against the muscular acetylcholine receptor (AChR) mediate the pathogenesis of myasthenia gravis (MG). The animal model experimental autoimmune MG (EAMG) can be induced by passive transfer or by active immunization of anti-AChR Abs. We report a new EAMG mouse model that generates human anti-AChR Abs upon immunization with *Torpedo* AChR (tAChR). Mice transgenic for human μ , $\gamma 1$, and κ germ line genes (HuMAb-Mice) were immunized with tAChR. Serum titers of anti-tAChR Abs were in the nanomolar range, and anti-rodent AChR Abs were in picomolar range. Some HuMAb-Mice had signs of muscle weakness, clearly indicating their susceptibility to EAMG. Human Ab-mouse AChR complexes were found at the neuromuscular junction, while AChR loss was up to 65%. Spleen and lymph nodes were used for producing hybridomas. Of the anti-tAChR monoclonal Ab-producing hybridomas, 2% had cross-reactivity with rodent AChR and non with human AChR. Immunization with a fusion protein, Trx-H α 1-210, displaying the human main immunogenic region did not result in EAMG or the generation of human anti-human AChR monoclonal Abs. These experiments show that the HuMAb-Mouse represents a suitable model to generate and 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 cause 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 endplates, EAMG was not induced [10]. Mice have strain-dependent susceptibilities to EAMG, although anti-AChR Ab serum titers and fine specificities are comparable [11-13]. Mice transgenic for different HLA genes have different susceptibilities to EAMG, implying an important role of these human genes in the pathogenesis of MG [14]. 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, using a mouse strain that is transgenic for human Ig loci [15,16]. 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. Furthermore, we examined the possibility to generate human anti-human AChR mAbs.

Materials and methods

Immunization

HuMAB-Mice transgenic for human μ , $\gamma 1$, and κ germ line genes, while production of endogenous immunoglobulins was inactivated [15,16], were immunized at the base of the tail with 15 μ g tAChR in CFA and boosted twice with the same dose of tAChR or 500 μ g Trx-Ha1-210 [17] in IFA. Control HuMAB-Mice were immunized with CFA only.

Clinical scoring

The severity of clinical signs of disease in EAMG was scored by measuring muscular weakness. The animals' muscle strength was assessed by their ability to grasp and lift repeatedly a 300 g rack from the table, while suspended manually by the base of the tail for 30 seconds. Clinical scoring was based on the presence of tremor, hunched posture, muscle strength and fatigability. Disease severity was expressed as: 0, no obvious abnormalities; +, no abnormalities before testing, but reduced strength at the end; ++, clinical signs present before testing, i.e. tremor, head down, hunched posture, weak grip; +++, severe clinical signs present before testing, no grip, moribund [8,18].

Hybridoma fusion and screening

Lymph node and spleen cells were isolated and fused with SP2/0 cells for production of hybridomas. Culture supernatants of hybridoma clones were screened for anti-tAChR mAb production using an ELISA. Microtiter plates were coated with 50 μ l of 5 μ g/ml tAChR in a 10 mM bicarbonate buffer, pH 9.5, for 1 h at 37°C. After washing three times with water containing 0.5% Tween 20 (wash buffer), wells were blocked with 200 μ l PBS containing 0.5% BSA and 0.5% Tween 20 (incubation buffer) for 15'. 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 F_c Abs in incubation buffer for 1 h. After washing, 50 μ l of alkaline phosphatase substrate solution (Bio-Rad Laboratories, Hercules, CA, USA) was added. The staining reaction was developed for 30' and OD measured at 450 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 by a RIA, using ¹²⁵I- α -bungarotoxin (¹²⁵I- α -BT) labeled AChR [1]. AChR – either purified tAChR, crude extract of denervated rat muscle, or crude extract of hAChR-producing cell line TE671 – was labeled with 2 nM ¹²⁵I- α -BT for 4 h at 4°C. 5 μ l of serum or 100 μ l of hybridoma culture supernatant was incubated with 200 μ l ¹²⁵I- α -BT-labeled AChR overnight at 4°C. Ab-AChR complexes were precipitated by incubating with excess goat anti-human Ig Abs for 4 h at 4°C. After washing with PBS containing 0.5% Triton-X100 and 0.02% NaN₃, radioactivity was counted in a γ counter. Ab titers were expressed as moles of α -BT sites per liter. hAChR-specific FACS analysis was performed with permeabilized TE 671 cells and FITC-conjugated rabbit anti-human IgG F(ab)₂ (1/100; Dako, Glostrup, Denmark), according to the Cytotfix/Cytoperm Plus Kit manual (PharMingen, San Diego, CA, USA).

Quantification of AChR in muscle

To quantify the AChR loss in EAMG mice, the concentration of AChR in muscle was determined as previously described [19,20]. In short, mouse

carcasses were minced and homogenized in 150 ml of 10 mM sodium phosphate pH 7.5, 100 mM NaN₃, 10 mM iodoacetamide and 1 mM PMSF. Homogenate was centrifuged at 12.000 rpm, 4°C for 30', and pellet was resuspended in 10 ml of 10 mM sodium phosphate pH 7.5, 100 mM NaN₃, 10 mM iodoacetamide, 1 mM PMSF and 2% Triton-X100. Extraction was performed for 1 h at 4°C on a shaker. After centrifugation at 12.000 rpm, 4°C for 30', the volume of the supernatant was measured and aliquots of 1 ml were labeled with ¹²⁵I- α -BT, with or without 1 mM acetylcholine and neostigmine-bromide. 1 μ l of pooled high-titer rat anti-AChR serum and normal rat serum were added, and triplicates of 250 μ l mixture (with or without acetylcholine and neostigmine-bromide) were incubated overnight at 4°C. Subsequently, 100 μ l goat anti-rat Ig Abs was added and incubated for 4 h at 4°C. After centrifugation for 3', pellet was washed once with PBS counting 0.5% Triton-X100 and 0.02% NaN₃ and counted in γ counter. AChR concentrations were compared with CFA-immunized HuMAb-Mice.

Immunohistochemical staining of muscle

Muscle biopsies from HuMAb-Mice were frozen in 2-methylbutane, cut at 6 μ m and fixated with acetone for 10' at 4°C, and air-dried for 5'. After washing three times with PBS, sections were pre-incubated with PBS containing 2% bovine serum albumin for 15', and subsequently stained with 1/100-diluted goat anti-human IgG (Cappel, ICN Pharmaceuticals, Aurora, OH, USA) for 45'. After washing with PBS, sections were 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', in order to co-localize human anti-AChR Abs with mouse AChR.

Results

Table 1: Clinical EAMG signs of Torpedo AChR-immunized HuMAb-Mice

Clinical score	0	+	++	+++
Number of mice	9	3	-	-

Clinical scoring: 0, no obvious abnormalities; +, no abnormalities before testing, but reduced strength at the end; ++, clinical signs present before testing, i.e. tremor, head down, hunched posture, weak grip; +++, severe clinical signs present before testing, no grip, moribund.

Induction of EAMG

HuMAb-Mice were immunized with tAChR to determine their susceptibility to EAMG. After the second boost, three of the twelve animals had a weak grip, while the other nine mice showed no clinical signs of EAMG (Table 1). 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%. 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

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 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. Cryosections of *Torpedo* AChR-immunized (a, b) and non-immunized (c, d) mouse muscles were double stained with goat anti-human IgG and FITC-conjugated rabbit anti-goat Ig Abs (a, c) and rhodamine-conjugated α -bungarotoxin (b, d), co-localizing the AChRs and human antibodies in *Torpedo* AChR-immunized mice.

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 3

AChR loss in *Torpedo* AChR-immunized HuMAb-Mice. Total AChR concentrations of mouse muscles were determined by immunoprecipitation, using ^{125}I - α -bungarotoxin, and compared to control animals ($p < 0.05$).

Human anti-tAChR mAbs

Spleens and lymph nodes were isolated for the production of anti-AChR-specific hybridoma cells. 433 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 (Table 2). 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. But no hybridoma mAb could bind hAChR with high specificity.

Table 2: Specificities of mAbs produced by *Torpedo* AChR-immunized HuMAb-Mouse hybridomas

Specificity	Number of mAbs (%)
anti- <i>Torpedo</i> 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.

Human anti-hAChR mAbs

In order to generate human anti-hAChR mAbs, HuMAb-Mice were immunized with a fusion protein, Trx-H α 1-210, displaying the human MIR. Trx-H α 1-210 consists of the first 210 amino acids of the hAChR α -subunit (H α 1-210) fused to thioredoxin (Trx), giving H α 1-210 a more

native conformation [17]. The conformation of the antigen is important, considering that many pathogenic anti-MIR mAbs don't cross-react with H α 1-210. Five animals were immunized with tAChR and boosted twice with Trx-H α 1-210. Sera were anti-hAChR Ab positive, as shown by FACS analysis (Figure 4). No clinical signs of EAMG were observed. Hybridomas were produced as before and selected on Trx-H α 1-210 specificity by ELISA, yielding 20 anti-Trx-H α 1-210 mAb-producing cell lines. The mAbs were screened for binding to unfused H α 1-210 and Trx by ELISA, showing that none of these anti-Trx-H α 1-210 mAbs binds to the denatured form of H α 1-210 or to Trx. But, a hAChR-specific RIA of the mAbs was also negative. These results (Table 3) suggest that all the mAbs are directed against a non-AChR-specific epitope region, unique for the fusion protein.

Figure 4
FACS analysis of Trx-H α 1-210-boosted HuMAb-Mouse sera. TE 671 cells were incubated with human anti-AChR IgG1-637 (b), pre-immunization sera (1/25; c, e), post-immunization sera (1/25; d, f) or nothing (a).

Table 3: Specificities of mAbs produced by Trx-H α 1-210-boosted HuMAb-Mouse hybridomas

Specificity	Number of mAbs (%)
anti-Trx-H α 1-210	20 (100)
anti-H α 1-210	0 (0)
anti-Trx	0 (0)
anti-human AChR	0 (0)

Cross-reactivity of human anti-Trx-H α 1-210 AChR mAbs was determined by ELISA for H α 1-210 and Trx, and by RIA for human AChR.

Discussion

Mice transgenic for unrearranged human Ig loci make it possible to set up a new mouse EAMG model, in which human anti-AChR Abs are generated upon active immunization. In the study presented here, HuMAb-Mice, which produce human IgG1 κ and IgM κ molecules, were immunized with tAChR extracted from *Torpedo californica*, in order to determine their susceptibility towards EAMG. After the second booster injection of tAChR, the anti-tAChR and -rAChR Ab titers were determined and the clinical signs of EAMG examined to evaluate if a successful EAMG model had been established. Furthermore, the generation of anti-hAChR mAb-producing hybridoma cells was assessed.

It was found that serum titers of Abs against tAChR were in the nanomolar range. The anti-rAChR Ab serum titer, ranging from 0.2 to 4.0% of that to tAChR, was comparable to the findings in other mouse strains [12]. The clinical signs of muscular weakness, a key symbol of EAMG, were successfully induced in some of the HuMAb-Mice, directly indicating that EAMG was established. This was further demonstrated by the deposits of human anti-mAChR Abs at the neuromuscular junction of muscle sections and by muscle AChR loss up to 65%. Human anti-AChR Abs could bind to the postsynaptic membrane, and reduce the amount of mAChR, which impairs the neuromuscular transmission. In contrast, the MG thymus tissue-transplantation model, the only other model in which human Abs are produced in a mouse, 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 with specificity to 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 [13], and failed to find anti-hAChR mAbs from tAChR-immunized mice.

Human anti-human MIR mAbs could be used as a source for potential therapeutic Ab fragments, like F_{ab} [21,22]. Since the tAChR immunization did not generate a set of high-affinity human anti-hAChR mAbs, the HuMAb-Mice were immunized with the fusion protein Trx-H α 1-210. Non of the mice developed clinical EAMG, although it has been shown that EAMG can be established in rats by immunization with high doses of Trx-H α 1-210 [17]. Due to the low immunogenicity of Trx-H α 1-210 compared to tAChR, we only produced 20 anti-Trx-H α 1-210 hybridomas. Moreover, all these hybridomas were specific for an epitope unique for the fusion protein, so no human anti-hAChR mAb could be cloned.

These experiments show that the HuMAb-Mouse represents a suitable model to study the effects of human anti-AChR Abs *in vivo*. Although we were unable to clone human anti-hAChR mAbs, the HuMAb-Mouse is a promising source of these Abs.

Acknowledgement

This work was supported by a grant from L'Association Française contre les Myopathies.

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Chapter 3

*Expression of a human anti-acetylcholine receptor F_{ab} in *Pichia pastoris**

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Abstract

The human anti-acetylcholine receptor (anti-AChR) antigen-binding fragment (F_{ab}) 637 blocks the binding of anti-AChR autoantibodies of myasthenia gravis patients, thereby preventing the autoantibody-mediated increased degradation of AChRs *in vitro*. To produce large amounts of F_{ab} 637 for *in vivo* protection studies, we assessed the possibility to express F_{ab} 637 in *Pichia pastoris*. Plasmids containing the coding sequences of the light chain and the heavy-chain fragment (F_d) of F_{ab} 637, under the control of the alcohol oxidase 1 promoter, were electroporated into *P. pastoris*. The light chain was secreted into the culture medium, but the F_d was not produced, although its mRNA was transcribed. A single-chain variable fragment (scF_v) variant of F_{ab} 637 was also not produced, indicating that failure of expression might be based on the sequence of the heavy-chain variable domain (V_H).

Introduction

The major autoantigen in myasthenia gravis (MG) is the muscular nicotinic acetylcholine receptor (AChR), an ion channel at the neuromuscular junction (NMJ). Autoantibodies directed to the AChR cause disturbed neurotransmission, either by hindrance of acetylcholine binding or by decrease of the number of functional AChRs. Two mechanisms that lead to this loss of AChR are anti-AChR-activated complement reaction, leading to focal lysis of the muscle membrane, and cross-linking of receptors by antibodies that are capable of binding to two AChRs simultaneously, resulting in an increased rate of internalization and degradation of the receptor (antigenic modulation) [reviewed in 1].

The human antigen-binding fragment (F_{ab}) 637, which is highly specific for the human main immunogenic region (MIR) of the AChR, is a potential *in vivo* protective agent against pathogenic MG autoantibodies [2]. Its monovalent binding capacity and lack of the crystallizable fragment (F_c) prevent antigenic modulation and complement activation, respectively. *In vitro* experiments have shown that F_{ab} 637 blocks antigenic modulation by MG patient sera, without interfering with ion channel function. F_{ab} 637 shows only cross-reactivity with primate AChRs. For *in vivo* protective studies in monkeys, large amounts of F_{ab} 637 are needed. The current bacterial expression system, used to select anti-AChR F_{ab} s from phage-display libraries derived from MG patients' thymuses, is inefficient for producing large quantities of F_{ab} 637. Cultures of the original F_{ab} 637-producing *Escherichia coli* strain have a yield of a few $\mu\text{g/l}$ in a shake flask, while large-scale fermentation had even lower yields [unpublished data].

Yeast is a popular host for heterologous and recombinant protein production, because of its eukaryotic post-translational modification mechanisms, ease of manipulation, and inexpensive culturing. The methylotrophic yeast *Pichia pastoris* has shown to be a suitable host for large-scale production of functional single-chain variable fragments (scFvs), diabodies, scFv- F_c fusion proteins, F_{ab} and full-size antibodies [3-10]. The yield of purified antibody fragments in *P. pastoris* have been reported to be as high as 250 mg/l [6]. We tried to produce F_{ab} 637 in *P. pastoris* by similar methods, using the methanol-inducible promoter of the alcohol oxidase 1 gene (AOX1) [reviewed in 11,12].

Materials and methods

Construction of expression plasmids

The coding sequences of the heavy-chain fragment (F_d 637) and the λ light chain (λ 637) of F_{ab} 637 were PCR cloned from its original pComb3H plasmid [2]. Primers used for cloning F_d 637 (annealing sequences underlined): 5'-gcg gaa ttc gga gac ttg gtc aag cct gga gg-3' and 5'-cgc aag ctt atc tag acc ttt gtc aca aga ttg ggg-3'. Primers used for cloning λ 637: 5'-gcg gaa ttc cac tct gtg tcg gag tct ccg gg-3' and 5'-gcg aag ctt cgc ggc cgc tta tga aca ttc tgt

agg-3'. Amplicons were subcloned into pUC18 (Pharmacia Biotech) with *EcoRI* and *HindIII*, and verified by sequencing. Subsequently, F_d637 was cloned into pPICZα (Invitrogen, Breda, The Netherlands) by *EcoRI* and *XbaI* (pPICZα-F_d637), and λ637 into pPIC9K (Invitrogen) with *EcoRI* and *NotI* (pPIC9K-λ637). A plasmid containing both antibody chain expression cassettes, pPICF_{ab}637, was constructed by fusion of pPICZα-F_d637 and pPIC9K-λ637 by *BglII* cloning.

Figure 1

Expression constructs for antibody chain production in *P. pastoris*. The coding sequences of the F_d (F_d637) and the λ light chain (λ637) of F_{ab}637 were fused to the N-terminal signal sequence (SS) of *Saccharomyces cerevisiae* α-factor and placed under the control of the AOX1 promoter (P_{AOX1}) and AOX1 transcription termination (TT), either separately (a-c) or together in one cassette (d). F_d637 was also fused to a C-terminal c-myc and His₆ tag. KEX2 was used as a proteolytic cleavage site for the single-peptide construct (d), and is also present at the 3' end of SS. Plasmids were based either on pPICZα, containing the zeocin selection marker (a, d), on pPIC9K, containing the *HIS4* selection marker (b) or fusion of both (c).

The two chains are encoded as a single peptide in the plasmid pPICF_{ab}637KEX2. During or after translation, the chains should be separated by KEX2, whose proteolytic cleavage site is introduced between the two chains [13]. Therefore, λ637 was PCR cloned from its original plasmid using the backward primer 5'-gcg aag ctt cgc ggc cgc tga aca ttc

tgt agg ggc-3', eliminating the original stop codon, and F₆₃₇ by the forward primer 5'-g gaa ttc gcg gcc gca cga atg gat aaa agg gag gct gga gac ttg gtc aag cct gga gg-3' (KEX2 site double underlined, Glu-Ala repeat dotted underlined), introducing the KEX2 cleavage site upstream of F₆₃₇, and subsequently cloned into pPICZα.

A plasmid (pPICscF₆₃₇) expressing a single-chain variable fragment (scF_v) variant of F₆₃₇ was constructed by subcloning the variable domains from the original pComb3H in pHEN4II for introducing a linker between them. Primers used for V_H and V_L cloning, respectively: 5'-cat gcc atg gcc gaa ttc gga gac ttg gtc aag cct gga gg-3', 5'-cgc aag ctt ggt gac cca cga tga gac agt gac cag ggt gc-3', 5'-gcg gaa ttc ggc gcg cct cac tct gtg tgc gag tct ccg gg-3' and 5'-gcg aag ctt cgc ggc cgc tac gac ggt cag ctt ggt ccc tc-3'. Subsequently, the scF₆₃₇ cistron was cloned in pPICZα by EcoRI and NotI.

Transformation of *P. pastoris*

Prior to transformation, 10 μg plasmid was linearized with either SacI, which favors the integration into the *aox1* locus, or Sall, which favors the integration into the histidinol dehydrogenase locus (*his4*) of *P. pastoris*. The *HIS4* deficient *P. pastoris* strain GS115 was electroporated, according to the manual of the EasySelect *Pichia* Expression Kit (Invitrogen), with linearized DNA and plated on YPD (1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) dextrose) + 500 μg/ml zeocin (Invitrogen)) or histidine-free MM (1.34% (w/v) yeast nitrogen base, 4x10⁻⁵% (w/v) biotin, 0.5% (v/v) methanol)) selection plates at 30°C.

Screening of transformants

Antibody chain production was induced by methanol on replica plates. A nitrocellulose membrane was placed on top of the colonies during first 24 h of expression. Membrane was incubated with biotin-conjugated goat anti-human λ light chain (Sigma-Aldrich, Steinheim, Germany), biotin-conjugated goat anti-human γ heavy chain (Sigma-Aldrich) or sheep anti-human F_d (The Binding Site, Birmingham, UK), subsequently with alkaline phosphatase (AP)-streptavidin or AP-conjugated rabbit anti-sheep F_c (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) and stained with 1-Step NBT/BCIP (Pierce, Rockford, IL, USA).

Small-scale expression

Individual colonies were inoculated in 10 ml BMGY medium (1% (w/v) yeast extract, 2% (w/v) peptone, 1.34% (w/v) yeast nitrogen base, 4x10⁻⁵% (w/v) biotin, 1% (v/v) glycerol, 100 mM potassium phosphate pH 6.0) in a 50-ml Falcon tube. After incubation at 30°C, 250 rpm for 24 h, the cells were collected by centrifugation (2,000 g, 5 min, RT) and resuspended in 5 ml BMMY medium (BMGY with 4% (v/v) methanol replacing glycerol). The cells were incubated at 30°C, 250 rpm for 24 h and the culture supernatant was separated from the cells by centrifugation. Expression levels were determined by SDS-PAGE and western blotting, using the aforementioned staining.

Northern blotting

For the detection of $F_{ab}637$ mRNA in transfected cells, total RNA was blotted on Hybond N+ membrane (Amersham Pharmacia Biotech, Roosendaal, The Netherlands) after electrophoresis. A probe was synthesized, using pPICZ α - $F_{ab}637$ as template, and labeled using the PCR DIG Probe Synthesis Kit (Roche, Almere, The Netherlands) according to the manufacturer's manual. The membrane was blocked and washed using the Dig Wash and Block Buffer Set (Roche) and probe detected with an anti-digoxigenin HRP conjugate (Roche) according to the manufacturer's protocol.

Results

Figure 2

Analysis of $F_{ab}637$ accumulation in *Pichia pastoris*. 30 μ l of culture supernatant from different *P. pastoris* clones were separated by SDS-PAGE and analysed by immunoblot. Expression of the λ light chain of anti-hAChR $F_{ab}637$ was detected with anti-human λ light chain antibody. Yeast clones 1-6 are transformed with the light chain 637 expression cassette and are *HIS4*⁺; clones 1, 3 and 4 show expression of light chain 637, mainly as a dimer or proteolytic fragment of a dimer (monomer is not visible). Untransformed *P. pastoris* strain GS115 and transgenic clones with a control single-chain variable fragment (scF_{ab}) or $F_{ab}637$ were used as negative controls.

The coding sequences of the heavy-chain fragment (F_{ab}) and the λ light chain (λ) of $F_{ab}637$ were placed separately into two expression cassettes, containing the zeocin (pPICZ α) or *HIS4* selection marker (pPIC9K), respectively (Figure 1a and b). Expression of both chains was placed under control of the *AOX1* promoter, directed to the secretion pathway by the α -factor signal sequence. In addition, the $F_{ab}637$ in pPICZ α contained a C-terminal His₆ and c-myc tag for detection and purification.

P. pastoris strain GS115 (*HIS4* deficient) was transformed with pPIC9K- $\lambda 637$ (Figure 1b). Integration into the host genome was directed either into the *aox1* or *his4* locus, resulting in transformation efficiencies of 3% and 75%, respectively. In both cases, $\lambda 637$ was secreted mainly as a light-chain homodimer (Figure 2). This corresponds to the human *in vivo* situation,

where overproduced light chains form Bence Jones proteins. The secretion of λ 637 was effective, since no product could be found in cell lysate. The expression level in shake flask was 10 μ g/ml.

Subsequently, a λ 637-producing clone was transformed with pPICZ α -F $_d$ 637 (Figure 1a), directed to *aox1* integration. The transformation efficiency was low, only a few zeocin-resistant clones were generated. After induction, all clones produced λ 637 but accumulation of F $_d$ 637 was below the detection limit of the immunoblot, using anti-His $_6$, anti-c-myc, anti- γ chain and anti- λ chain antibodies for detection (data not shown).

To increase the number of transfected clones, *P. pastoris* was transformed with pPICF $_{ab}$ 637 (Figure 1c) and integrated in the host's *his4* locus. Although a high number of zeocin-resistant clones was obtained which all produced λ 637, F $_d$ 637 production could not be detected in any of these clones. However, northern blot analysis revealed that the mRNA of F $_d$ 637 was transcribed in these clones (Figure 3), indicating the functionality of the F $_d$ 637 expression cassette.

Figure 3
Northern blot analysis of anti-AChR F $_{ab}$ 637 expression in *Pichia pastoris*. Steady state transcript levels of induced (I) and non induced (NI) *P. pastoris* clones, transformed with the expression cassettes of only the λ light chain (λ) or both chains of F $_{ab}$ 637 (Fd+ λ) were analyzed. After a 48-hours induction period, total RNA was isolated from the cells and blotted. The blots were probed with labeled DNA from the full-length sequences of the chains.

Finally we transformed *P. pastoris* with the expression construct pPICF $_{ab}$ 637KEX2 (Figure 1d), encoding the two antibody chains as a single peptide. λ 637 was produced while F $_d$ 637 was not detectable, indicating that pre-pro- λ 637-F $_d$ 637 was at least partially translated (data not shown).

To further support the hypothesis of a post-transcriptional expression problem of the F $_{ab}$, expression studies with a plasmid encoding for a scF $_v$ variant of F $_{ab}$ 637, scF $_v$ 637 [14], were performed. Accumulation of scF $_v$ 637 in *P. pastoris* was not detectable by immunoblot, using anti-c-myc and -His $_6$ antibodies for detection. This result suggests that the heavy-chain variable domain (V $_H$) and not constant domain 1 (C $_H$ 1) of F $_d$ 637 is responsible for the nonexistence of the heavy-chain fragment.

Discussion

The F_{ab} 637 coding sequences were cloned into different expression plasmids for production of F_{ab} in *P. pastoris*. The production of its light chain λ 637 by *P. pastoris* (100 μ g/ml) was higher than in the original bacterial expression systems (60 μ g/l). We assume that the expression could be further improved, e.g. by multi-copy insertions in the host's genome and optimizing nutritional and physical culturing parameters [12]. In contrast to λ 637, no detectable levels of F_d 637 were found, although its mRNA was transcribed.

F_d 637 as well as the scF_v derivative of F_{ab} 637 [14], were not produced in *P. pastoris*, although they are produced by *E. coli* and F_d 637 genetically fused to the human γ 1 F_c is expressed by chinese hamster ovary cells, with a yield of 55 mg/l purified IgG1 [15]. Recently, Lange *et al.* [9] showed that functional F_{ab} (atrazine specific) can be produced in *P. pastoris* with a yield of 40 mg/l pure F_{ab} . They also used a single pPICZ α -based plasmid with the expression of each chain controlled by a separate AOX1 promoter, indicating that this setup can be successfully used for F_{ab} expression in *P. pastoris*. But, Lange *et al.* only C-terminally fused a His₆ tag to the heavy chain, while λ 637 was His₆ and F_d 637 c-myc and His₆ tagged. The only differences between our anti-AChR F_{ab} and the atrazine-specific F_{ab} are the V_Hs and the c-myc tag. Therefore, the expression problem is either caused by the sequence of V_H637 or the c-myc tag. In recombinant antibodies, the c-myc tag is frequently used, also for the successful production in *P. pastoris* [16,17]. Sequence-depending levels of expression are found with the production of scF_vs, both in *P. pastoris* [unpublished results] and in *E. coli* [18]. The F_d 637 nucleotide or amino acid sequence could have caused translational or proteolytic problems.

In future experiments we will perform modification of the F_d 637 sequence. This includes the adaptation of the codon usage to *P. pastoris* and the exchange of certain amino acids of the framework regions that are responsible for the stability of the F_d fragment. The generated data will provide new insights on the correlation between recombinant protein sequence and accumulation.

Acknowledgements

We would like to thank Nicole Raven for her help and fruitful discussions. This work was supported by a grant from L'Association Française contre les Myopathies.

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Chapter 4

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

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published in
Autoimmunity 35 (2002) pp. 125-133

Abstract

Pathogenic anti-acetylcholine receptor (AChR) antibodies in myasthenia gravis (MG) and the corresponding animal model, experimental autoimmune myasthenia gravis (EAMG), principally recognize the main immunogenic region (MIR) of the AChR. Bivalent anti-MIR antibodies binding to the α -subunits of AChR result in AChR loss by antigenic modulation and complement activation. Monovalent F_{ab} and single-chain variable fragments (scF_v) of pathogenic anti-AChR antibodies can interfere with AChR binding of the pathogenic antibodies. In the present study, scF_{v637} was constructed from its parental F_{ab637} , previously isolated from a thymus-derived phage display library with specificity toward anti-MIR of human AChR (hAChR), by PCR amplification. Bacterial produced scF_{v637} was able to bind to hAChR in standard precipitation radioimmunoassay (RIA). ScF_{v637} also bound to monkey AChR *in situ* on monkey neuromuscular junctions as showed in immunohistochemical staining. Furthermore, scF_{v637} was capable of inhibiting the binding of its intact IgG637 and anti-MIR mAb35 binding to hAChR up to 32.9% and 73.0% respectively demonstrated in a competitive ELISA, and of MG patient sera from up to 45.5% in a competitive RIA. Therefore, scF_{v637} , easier for manipulation in improvement of affinity and stability compared with its parental F_{ab637} , may serve as an alternative candidate for specific immunotherapy in MG.

Introduction

In the prototype autoimmune anti-receptor disease, myasthenia gravis (MG), the binding of autoantibodies to the acetylcholine receptor (AChR) results in AChR loss and leads to fatigue 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 with purified AChR or by passive transfer of polyclonal or monoclonal antibodies (mAbs) against AChR [4-6], which is a well characterized autoantigen [7]. Muscle-type AChR in neuromuscular junction is a complex transmembrane glycoprotein and consists of five subunits: $\alpha_2\beta\gamma\delta$ [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 to antibodies *in vivo*.

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, a mechanism in which the binding of anti-AChR antibodies to AChR by cross-linking of adjacent AChR molecules results in accelerated AChR internalization [19]. 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 MG research. F_{ab} and single-chain variable fragment (scF_v) of antibodies derived from anti-MIR antibodies can bind to AChR, blocking the binding of anti-MIR antibodies to AChR, but do not have the activity to induce antigenic modulation or the complement reaction. F_{ab} isolated from MG patient thymus [27,28] and from rat anti-MIR mAbs [29,30] or scF_v constructed from rat anti-MIR mAbs [31-33] 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, a human scF_{637} directed against the MIR of human AChR (hAChR) was constructed from its parental F_{ab637} , isolated from thymus-derived phage display library of a MG patient earlier in our laboratory [27]. ScF_{637} will not possess immunogenicity for application in MG patients, considering its human origin, and easily undergoes genetic manipulation to improve its stability and affinity.

Material and methods

Construction of scF_v637

V_H cDNA of scF_v637 was amplified from pComb3H-F_{ab}637 [27] by PCR using V_H upstream primer 5' aa tctaga ccatg gcc gag gtg cag ctg ctg gag tct ggg 3' (the sequences underlined are the recognition sites of endonucleases *Xba*I and *Nco*I, respectively) encoding the first 8 amino acids of the V_H, and V_H downstream primer 5' at gaattc ctcga gga tga gac agt gac cag gg 3' (underlined are *Eco*RI and *Xho*I sites, respectively) complementary to the nucleotides of amino acids 107-113. The V_L cDNA of scF_v637 was amplified using V_L upstream primer 5' aa tctaga gtgca ccc gag ctc act cag ccc 3' (underlined are *Xba*I and *Apa*LI sites, respectively) encoding the first 7 amino acids of the V_L, and V_L downstream primer 5' at gaattc gcggccgc tag gag ggt cag ctt gg 3' (underlined are *Eco*RI and *Not*I 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 V_H and V_L cDNA fragments were purified with QIAquick PCR Purification Kit (Qiagen GmbH, Hilden, Germany). The purified V_H cDNA fragments were digested with *Nco*I and *Xho*I, and 1% agarose gel-purified with GeneClean Kit II (Bio 101 Inc, La Jolla, CA, USA), and cloned into the *Nco*I and *Xho*I site of vector pHEN2 (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* DH5 α and isolated and purified with Wizard Plus SV Minipreps (Promega, USA). Subsequently, the purified V_L cDNA fragments were digested with *Apa*LI/*Not*I, and cloned into the pHEN2-VH as above to construct a complete scF_v637 gene segment.

Production of soluble scF_v637

E. coli HB2151 transformed with pHEN2-scF_v637 were grown from a single colony overnight at 37 °C in a 5 ml 2x TY medium containing 100 μ g/ml ampicillin and 1% glucose, and subsequently diluted in 500 ml 2x TY medium with 100 μ g/ml ampicillin and 0.1% glucose. The culture was further incubated at 37 °C to an OD_{600nm} of 0.6. ScF_v637 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 x *g* for 10 min at 4 °C.

The periplasmic fraction containing scF_v637 was prepared with sodium borate [34]. The bacterial pellet was resuspended in 5 ml ice-cold sodium borate solution (200 mM sodium borate, 160 mM NaCl, 1 mM EDTA, pH 8.0). After incubation on ice for 20 min, the bacterial suspension was centrifuged at 15 000 x *g* for 10 min at 4 °C. The supernatant represented

the periplasmic fraction. *E. coli* HB2151 which do not bear pHEN2-scF_v637 or bear pHEN2 only were used as control.

Detection of scF_v637

The production of soluble scF_v637 was detected with nitrocellulose dot blot [35]. 5 µl of periplasmic fraction or culture supernatant of scF_v637 was loaded on nitrocellulose filter (0.45 µm, Bio-Rad Laboratories, Hercules, CA, USA). 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 (ATCC clone number CRL-1729) 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 the anti-c-myc mAb to scF_v637 was detected by adding rabbit anti-mouse IgG conjugated with HRP (1/2000, Pierce, Rockford, IL, USA). The HRP activity was visualized with DAB.

Purification of scF_v637

The purification of scF_v637 was performed with immobilized metal affinity chromatography (IMAC) using Ni-NTA agarose (Qiagen GmbH, Hilden, Germany). Periplasmic fraction of *E. coli* HB2151 was dialysed against lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, 3 mM imidazole, pH 8.0), and loaded on Ni-NTA resin column. After washing with wash buffer (50 mM NaH₂PO₄, 300 mM NaCl, 20 mM imidazole, pH 8.0), the column was eluted with elution buffer (50 mM NaH₂PO₄, 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 scF_v637 by western blotting using mouse anti-c-myc mAb.

Determination of scF_v637 specificity

The specificity of scF_v637 binding to AChR was determined with precipitation RIA using crude hAChR extract, isolated from TE671 cells [36], as antigen. Briefly, 200 µl of periplasmic fraction of scF_v637 were incubated overnight at 4 °C with 200 µl of crude hAChR extract (0.3 nM, preincubated with 2 pmol ¹²⁵I-α-BT/pmol AChR for 4 h 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-scF_v637-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 scF_v637 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 (10 pM) isolated from the electric organ of *Torpedo californica* (Pacific Biomarine, California, USA).

Immunohistochemical staining of scF_v637

Binding of scF_v637 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, fixed 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}, Molecular Probes Europe BV, Leiden, The Netherlands) 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 scF_v637 and IgG637 (recombinant full-size IgG1 variant of F_{ab}637) and rat anti-MIR mAb35 [37], and in a competitive RIA between scF_v637 and MG patient sera. ELISA plates were coated with 50 μ l mAb153 (15 μ g/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 μ l hAChR (0.3 nM) extracted from TE671 cells, and after washing, further incubated with periplasmic fraction of scF_v637 (0.0032 – 50 μ l) overnight at 4 °C. For inhibition of mAb35, 50 μ l mAb35 conjugated with HRP (IC₅₀ = 0.9 μ g/ml, predetermined in ELISA) was added and incubated for 1 h at 37 °C without washing, and HRP reaction was developed using TMB (Sigma, St. Louis, MO, USA). For inhibition of IgG637, 50 μ l IgG637 (IC₅₀ = 80 ng/ml, predetermined in ELISA) was added and incubated for 1 h at 37 °C without washing. Subsequently 50 μ l goat anti-human IgG (F_c specific) coupled with alkaline phosphatase (dilution 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 pNPP. In RIA, 50 μ l hAChR (0.3 nM) labeled with ¹²⁵I- α -BT at 4 °C for 4 h was pre-incubated with 250 μ l of periplasmic fraction of scF_v637 overnight at 4 °C, subsequently 10 μ l of MG patient sera (70% of the maximum binding to hAChR) was added. After an additional 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

Construction of scF_v637

V_H and V_L cDNAs of scF_v637 were PCR amplified from its parental F_{ab}637, isolated from thymus-derived phage display library of a MG patient, and subsequently cloned into vector pHEN2. The generated scF_v637 is 762 bp long and its V_H and V_L sequences are identical to those of F_{ab}637 at amino acid level although there are two nucleotide differences in primer-encoded sequences of the V_H (Figure 1). The original nucleotide sequences of F_{ab}637 were described earlier [27].

	VH upstream primer	
	1 2 3 4 5 6 7 8 9	30
FR1	E V Q L L E S G G D L V K P G G S L R L S C A A S G F K S T	
scFv637	gag gtg cag ctg ctg aag tct ggg	
Fab637	--- --- --c --- --- ---	
	31 35	
CDR1	D Y Y M S	
	36 49	
FR2	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 94	
FR3	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 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	
FR4	W G R G T L V T V S S	
scFv637	acc ctg gtc act gtc tca tcc	
Fab637	--- --- --- --- --- --- ---	

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

		<u>VL upstream primer</u>								
		1	2	3	4	5	6	7	8	23
FR1		A	P	E	L	T	Q	P	H S V S E S P G K T V T I S C T	
	scFv637	gca	ccc	gag	ctc	act	cag	ccc		
	Fab637	---	---	---	---	---	---	---		
		24		27a				34		
CDR1		R	S	S	G	S	I	A	S	N Y V Q
		35							49	
FR2		W	Y	Q	Q	R	F	G	S	S P T T V I Y
		50				56				
CDR2		E	D	N	Q	R	F	S		
		57						72a b		88
FR3		G	V	P	D	R	F	S	G	S I D S S N H A S L F I C G L E T E R E A D Y Y C
		89				95a b	97			
CDR3		Q	S	Y	D	S	N	S	G	G R V
		<u>VL downstream primer</u>								
		98	102	103	104	105	106	107		
FR4		F	G	G	G	T	K	L	T	V L
	scFv637		acc	aag	ctg	acc	gtc	cta		
	Fab837		---	---	---	---	---	---		

Amino acid sequences of heavy (H) and light (L) chains of scF₆₃₇. The primers used both in PCR amplification of V_H and V_L genes and for sequencing of scF₆₃₇ are also indicated as nucleotide sequences, and compared with its parental F₆₃₇. The codon "ctc" at amino acid position 5 in the V_H of F₆₃₇ was changed into "ctg" in scF₆₃₇ to remove XhoI recognition site (ctcgag) without alterations of the corresponding amino acids. The codon change "tcg" → "tcc" from F₆₃₇ to scF₆₃₇ at amino acid position 113 of the V_H was created in V_H downstream primer to match the XhoI restriction sequence without introduction of an extra amino acid between the V_H and the linker. The sequence identity of F₆₃₇ with scF₆₃₇ in primers is indicated by dashes. The amino acid position is numbered according to Kabat *et al.* [39]. FR: framework region; CDR: complementarity determining region; •: missing amino acid.

Expression and purification of soluble scF₆₃₇

The phagemid pHEN2-scF₆₃₇ construct was transformed into non-suppressor *E. coli* HB2151 for production of soluble scFv₆₃₇. The soluble scF₆₃₇ accumulated in the bacterial periplasm but not in culture medium of bacteria after induction of IPTG for 3 h at 30 °C. However, no scF₆₃₇ production was detectable in either periplasm or culture supernatant of control bacteria or bacteria without IPTG induction by nitrocellulose dot blot assay with anti-c-myc antibody (result not shown).

SDS-PAGE and western blotting analysis of fractions from IMAC purification showed that the apparent molecular weight of scF₆₃₇ was approximately 36 kDa although it was calculated to be 30.4 kDa from the amino acid sequence (Figure 2).

Figure 2

SDS-PAGE and western blotting analysis of fractions from IMAC purification of scF₆₃₇ carrying c-myc and His₆ tags. Two identical gels were run in parallel. Protein bands were detected by Coomassie staining (A) and the scF₆₃₇ 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 weight marker.

Antigen binding activity of scF_v637

The binding activity of scF_v637 to hAChR extracted from TE671 cells was tested using a precipitation RIA. ScF_v637 produced in periplasm of different bacterial clones were able to bind hAChR but not rAChR or tAChR. Whereas, no binding activity was found in bacterial culture medium and in control bacteria, expressing the non-specific scF_v637=8aa [unpublished data], which lacks the first eight amino acids of framework 1 of V_H and V_L (Figure 3).

ScF_v637 cross-reacted with primate AChR at neuromuscular junction of monkey intercostal muscle section. ScF_v637 bound to endplate regions (Figure 4, A and C) which were colocalized in the same slides with α-BT^{Rh} (Figure 4, B and D), demonstrating the *in situ* binding capacity of scF_v637.

Figure 3

Binding activities of scF_v637 to AChRs. ¹²⁵I-α-BT-AChR-scF_v637 complex was precipitated by addition of mouse anti-c-myc mAb and goat anti-mouse IgG in standard precipitation RIA. hAChR: human AChR; rAChR: rat AChR; tAChR: *Torpedo* AChR; P: periplasmic fraction; S: culture supernatant; scF_v637=8aa: scF_v637 without the first 8 amino acids of V_H and V_L, resulting in loss of specificity; HB2151: *E. coli* HB2151 control; numbers represent different bacterial colonies.

Protective capacity of scF_v637 to hAChR

The inhibition of scF_v637 to anti-MIR antibodies or MG patient sera for binding to hAChR was determined in a competitive ELISA and RIA. ScF_v637 expressed in the periplasmic fraction of *E. coli* HB2151 inhibited the binding of anti-MIR mAb35 to hAChR up to 73.0% and that of IgG637 up to 32.9%, due to the affinity difference between the two F_{ab}637 variants (Figure 5A). It also inhibited the binding of MG patient sera to hAChR from

up to 45.5% (Figure 5B), indicating its protective capacity towards a broad range of polyclonal anti-AChR antibodies.

Figure 4

In situ binding of scF₆₃₇ to AChR at neuromuscular junction of monkey intercostal muscle section. The binding of scF₆₃₇ to AChR at endplate was demonstrated by immunohistochemical staining. Mouse anti-c-myc mAb and goat anti-mouse IgG conjugated with FITC were used for visualization. Rhodamine-labeled α -BT (α -BT^{rh}) was used to colocalize the same endplates. A and C: scF₆₃₇ from 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: scF₆₃₇ without the first 8 amino acids of V_H and V_L (scF₆₃₇=8aa) failed to bind to AChR at endplates; F: colocalization of AChR in the same endplates as in E. Magnification: x 200.

Discussion

Pathogenic anti-AChR antibodies are mainly those that are directed against the MIR and make up most part of the total anti-AChR antibodies in both MG and EAMG. However, F_{ab} or scF_v 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 the F_c. F_{ab} or scF_v 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].

Figure 5
 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 by ELISA. hAChR was captured by bound mAb153 and incubated with various amounts of periplasmic fractions 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 ¹²⁵I- α -BT was preincubated with 250 μ l of Ni-NTA-purified 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.

F_{ab}637 is a MG thymus-derived anti-MIR antibody fragment [27]. It prevents MG serum from binding to hAChR and protects AChR from loss induced by MG serum. Since F_{ab}637 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 F_{ab}637 by transfecting it into chinese hamster ovary cells for mammalian expression is in progress). We have constructed a scF_v derived from F_{ab}637. The aim of this study is to try an alternative possibility using antibody fragment for the specific immunosuppressive therapy of MG. Since a scF_v is smaller than a F_{ab}, it could penetrate the target area better. The change in biodistribution compared to the half-life difference between scF_v and F_{ab} could be beneficial. Furthermore, the V_H and V_L cDNAs of scF_v are located in the same cistron, this property makes it easier for manipulation of genetic improvement in its affinity and stability.

ScF_v637 was constructed from V_H and V_L cDNAs of F_{ab}637 by PCR amplification and cloning into the vector pHEN2, which contains GS (Gly and Ser) rich linker and c-myc and His₆ tags for specific detection and efficient purification. The V_H upstream primer was designed according to the first 8 codons of F_{ab}637's V_H. However, the first 6 codons used in F_{ab}637 was artificially designed in vector pComb3H, and codons 5 and 6 (ctc gag) was used as XhoI restriction site for original cloning of V_H. Since vector pHEN2 contains the same XhoI restriction site for cloning and we prefer the V_H-linker-V_L orientation construct of scF_v, 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 F_{ab}637 to scF_v at amino acid position 113 of V_H was created in V_H downstream primer to match the XhoI site without introduction of an extra amino acid between V_H and linker. For the V_L cloning, codon "gca" (encoding Ala) in ApaLI restriction sequence was designed in V_L upstream primer as first codon of V_L of scF_v637 and "ccc" (encoding Pro) as second codon to match both ApaLI restriction site and annealing to template in pComb3H.

The specificity of bacterial produced scF_v637 binding to hAChR was determined in RIA in which scF_v637 first bound to AChR and then to the c-myc tag in the complex of scF_v637-AChR was recognized by mouse anti-c-myc mAb. However, interestingly, when a solid-phase RIA (SPRIA) in which the c-myc tag of scF_v637 was captured first by fixed mouse anti-c-myc mAb was utilized to determine the specificity of scF_v637 binding to hAChR, no binding activity of hAChR to the scF_v637-mAb complex could be found (data not shown). In contrast, its parental F_{ab}637 could bind AChR in SPRIA suggesting that the binding of anti-c-myc mAb to the C-terminal c-myc tag of scF_v637 may interfere with the binding of scF_v637 to AChR. A similar result was described in a previous study of Goel *et al.* [40], who found that the C-terminal position of His₆ tag partially covered the antigen-binding site and affected the binding properties of scF_v 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 helpful to determine the effects of an N-terminal c-myc tag on scF_v637 specificity since

the effects of affinity tags on scF_v specificity depend on the primary sequences and conformation of the scF_v. It was also reported that scF_v exhibited higher cross-reactivity with analogs of the antigen originally used for production of its parental mAb [49]. Nevertheless, scF_v637, just like F_{ab}637, could specifically bind to AChR *in situ* at monkey neuromuscular junction but did not cross-react with rAChR or tAChR.

Interestingly, scF_v637=8aa, which lacks the first eight amino acids of framework regions (FRs) 1 of V_H and V_L compared to scF_v637, is a good negative control, since it has no specificity towards AChR (Figures 3 and 4). Li *et al.* [50] found that sequence changes at the N-terminus of both V domains introduced by PCR may seriously affect antigen binding. 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 scF_v637 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 F_{ab}637. Presently, we are trying to prepare a conjugate by linking the scF_v, without tags, with human serum albumin (HSA) or polyethyleneglycol (PEG) to increase its half-life and alter its biodistribution.

Acknowledgements

We would like to thank Drs G. Winter, M. Jonker and S. Tzartos for kindly supplying pHEN2, monkey muscle, mAb35, and mAb153. We are also grateful to Dr B. Machiels for excellent laboratory assistance and helpful discussion. This work was supported by grants from Prinses Beatrix Fonds (to Dr M. De Baets) and National Foundation for Natural Science of China (to F. Meng).

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Chapter 5

Characterization of a human IgG1 reconstructed from an anti-acetylcholine receptor F_{ab}

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Abstract

In myasthenia gravis (MG), autoantibodies against the acetylcholine receptor (AChR) lower the number of AChRs, leading to an impaired neuromuscular signal transmission. Previously, we have cloned an anti-AChR F_{ab} , F_{ab} 637, from an MG patient's thymus. F_{ab} 637 protects against loss of AChRs by blocking the binding of pathogenic MG autoantibodies *in vitro*. In order to test the primate-specific anti-AChR F_{ab} 637 *in vivo*, large amounts of this F_{ab} are needed in a monkey experimental autoimmune MG (EAMG) model. But, the current bacterial expression system has a low yield. Therefore, we genetically fused the F_{ab} 637's F_d to the F_c of human IgG1 and transfected chinese hamster ovary (CHO) cells with a plasmid encoding for IgG1-637. CHO cells produced 55 mg/l protein G-purified IgG1-637. IgG1-637 specifically recognized the human $\alpha 1$ subunit of AChR in its native conformation, like its parental F_{ab} . Three daily doses of 1.7 or 5 mg IgG1-637 per kg body weight induced muscle weakness in rhesus monkeys, indicating the reconstruction of a myasthenia gravis autoantibody. IgG1-637 could be used for the enzymatic production of the potential therapeutic F_{ab} 637 or could be used as the genetic basis of a therapeutic IgG-like molecule, which has an increased serum half-life compared to F_{ab} .

Introduction

In myasthenia gravis (MG), binding of autoantibodies to the acetylcholine receptor (AChR) at the neuromuscular junction leads to fatigable weakness of skeletal muscles. The nicotinic, muscle type AChR is an ion channel, formed by five subunits: $\alpha 1$, γ (replaced by ϵ in adult), $\alpha 1$, δ and $\beta 1$. The extracellular domain of the $\alpha 1$ subunit contains the acetylcholine (ACh)-binding site and a main immunogenic region (MIR), to which a major part of the pathogenic MG antibodies is directed [1,2]. Anti-AChR antibodies cause impaired neuromuscular transmission by lowering the number of functional AChRs. Three mechanisms account for the functional AChR loss. Anti-AChR antibodies can crosslink AChR, leading to an increased internalization and degradation of AChR (antigenic modulation) [3,4]. Antibodies can activate complement upon binding to the AChR, leading to focal lysis of the postsynaptic membrane [5,6]. To a lesser extent, the autoantibodies can functionally inhibit the AChR by interfering with ACh binding [7] or ion channel function [8]. Anti-MIR antibodies make up more than half of the autoantibodies in MG patients or experimental autoimmune MG (EAMG) rats [1,9,10], and are especially effective in antigenic modulation [11].

Currently, there is no specific immune therapy for MG. Several specific immunotherapy approaches have been tested, like anti-idiotypic antibodies [12,13], a peptide that competes with a pathogenic mAb for binding with AChR [14], induction of tolerance by administration of AChR [15] or AChR-derived synthetic peptides [16], and elimination of AChR-specific T cells by genetically engineered antigen-presenting cells that act as "guided missiles" [17]. MG and EAMG antibody-derived F_{ab} s have been shown to protect against AChR loss in rodent EAMG models [18,19]. F_{ab} s block the binding of pathogenic antibodies and, because of their monovalent antigen-binding capacity and their lack of F_c , do not induce AChR loss themselves. A few human anti-human AChR F_{ab} s have been cloned and their ability to block MG serum antibodies explored in the last years [20-23]. But, F_{ab} s are not ideal blockers due to their rapid serum clearance.

An MG patient-derived anti-MIR F_{ab} , F_{ab} 637, protects against loss of AChR by MG sera *in vitro*, without interference with ion channel function [21]. To test F_{ab} 637 *in vivo*, a monkey EAMG model is essential, because it only shows crossreactivity with primate AChR. To date, only chronic monkey EAMG, induced by immunization with *Torpedo californica* AChR, has been reported in the literature [24,25]. But, transfer of 1 g/kg purified IgG from MG patients to monkeys, like to mice [26], induces EAMG as well [Heiniger and Toyka, personal communications].

The current bacterial expression system of F_{ab} 637 has a too low yield and production in the yeast *Pichia pastoris* was not successful [27]. Besides, the serum half-life of F_{ab} is low, requiring even a larger amount of F_{ab} 637 for *in vivo* experiments. Fusing F_{ab} s to polyethylene glycol or albumin only increases their serum half-life by 15-17 times to about 1 day [28,29], while

IgG1 has a half-life of 21 days [30]. Sanna *et al.* [31] genetically linked an F_d to an F_c and expressed the full-size heavy chain (HC) and light chain (LC) in chinese hamster ovary (CHO) cells.

In this study, we reconstructed a fully human $\gamma 1$ immunoglobulin variant of $F_{ab}637$ (IgG1-637) by genetically fusing its F_d to an F_c . The specificity of IgG1-637 was compared to its parental F_{ab} and its predicted pathogenicity was tested in rhesus monkeys. As an alternative to shielding the AChR with enzymatically produced $F_{ab}637$ from IgG1-637, we designed a recombinant IgG1-637 with monovalent AChR specificity and no complement-activating capacity, because of its expected higher serum half-life. The K322A mutation in the C1q-binding site has been reported to abolish or at least reduce complement-dependent cytotoxicity (CDC) of human IgG1 and -3 [32-34]. Besides mutations in amino acid residues, changes in the glycosylation affect complement activation. Production of a chimeric mouse-human IgG1 in the cell line CHO-Lec1 results in an antibody with truncated glycosylation, which abolishes CDC [35]. Potential non-complement-activating variants of IgG1-637 were tested *in vitro*.

Methods

Cloning

The mammalian expression plasmid of the IgG1 isotype of $F_{ab}637$, pIgG1-637, was constructed by *Xba* I and *Sac* I cloning of the LC and *Xho* I and *Bst*E II cloning of the F_d from the bacterial expression plasmid of $F_{ab}637$, a pComb3 plasmid [21], into the same pIgG1 vector. In pIgG1-637, the expression of each chain was regulated by q human cytomegalovirus promoter/enhancer (hCMV P/E) element, the secretion of the HC by a mouse HC leader peptide, the secretion of the LC by a mouse κ leader peptide and F_d637 was fused to the human $\gamma 1 F_c$.

The K322A mutation was introduced using the oligonucleotide G GAG TAC AAG TGC GCG GTC TCC AAC AAA GCC C and the oligonucleotide with the complementary sequence with the QuickChange XL Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA), according to the manufacturer's manual. The sequence PLKP in the complementary determining region (CDR) 3 of the HC was deleted using the oligonucleotide GGA ATT TTT CGA GGG CTA GAG TAC TAC TTC G and the oligonucleotide with the complementary sequence.

Cell culture

CHO-K1 (ATCC Number CCL-61) was cultured in HAM's F12 (Gibco/Invitrogen, Breda, The Netherlands) and the *N*-acetylglucosaminyltransferase I-deficient CHO-Lec1 (ATCC Number CRL-1735) in MEM Eagle, alpha modification (Sigma-Aldrich, Zwijndrecht, The Netherlands), both supplemented with 10% fetal bovine serum (Bodinco, Alkmaar, The Netherlands). Cells were transfected with Lipofectin (Gibco/Invitrogen, Breda, The Netherlands), according to the

manufacturer's manual. Transfected cells were adapted to suspension culture.

Radioimmunoassay

The specificity of CHO-produced antibody was determined by RIA [36], using human (prepared from the human TE671 rhabdomyosarcoma cell line [37]), *Torpedo* (prepared from *Torpedo californica*) or hybrid (human $\alpha 1$ subunit and *Torpedo* β , γ and δ subunits [38]) AChR, marked with ^{125}I -labeled α -bungarotoxin. For anti-human AChR titer measurements, the Acetylcholine Receptor Autoantibody RRA Kit (IBL, Hamburg, Germany) was used.

Flow cytometry analysis

The recombinant antibodies were tested for their binding of AChR expressed on TE671 or MITC cells [39]. Trypsinized cells were incubated with sample antibody and subsequently with FITC-conjugated goat anti-human Ig. Signal was amplified by EAS Kit (Flow-Amp Systems, Cleveland, OH, USA). Paraformaldehyde solution (2%) was added to the cell suspension to permeabilize cells for cytoplasmic AChR detection. Flow cytometry was performed on a FACSCalibur apparatus (Becton Dickinson Immunocytometry Systems, Mountain View, CA, USA).

Complement binding

Functional activation of the classical pathway by the recombinant antibodies was assessed by ELISA [40]. The human antibodies were coated in Nunc Maxisorb plates (Nunc, Roskilde, Denmark) in 100 μl coating buffer (100 mM $\text{Na}_2\text{CO}_3/\text{NaHCO}_3$, pH 9.6) for 2 h at 37°C. Residual binding sites were blocked by incubation with PBS containing 1% BSA for 1 h at 37°C. Normal human serum (diluted 1/50 or 1/100) was used as the source of complement. Complement binding was assessed using dig-conjugated anti-human C1q (mAb 2214) and anti-human C3 (RFK22), followed by detection of mAb binding using HRP-conjugated sheep anti-dig F_{ab} (Roche Molecular Biochemicals, Mannheim, Germany).

EAMG in monkey

EAMG was induced in monkeys by passive transfer of IgG from MG patient plasma. Pooled plasma was clotted by 25 mM CaCl_2 and supernatant diluted with 1 volume of phosphate buffered saline (PBS). IgG was precipitated by adding drop-wise 1 volume of saturated ammonium sulphate (SAS) pH 7, resuspended in water and dialyzed against PBS. Solution of 10 g/l IgG contained 78 nM anti-human AChR IgG. EAMG was induced in monkeys by 3 daily injections of 0.8 g IgG per kg body weight. Animal experiments were performed at the Biomedical Primate Research Center (Rijswijk, The Netherlands) with permission of the Committee on Animal Welfare (DEC), according to Dutch governmental rules.

Electromyography

Decrement of compound muscle action potential (CMAP) was measured in the flexi digiti quinti. Monkeys were anesthetized with ketamine. For stimulation and recording, two small monopolar needle electrodes were placed subcutaneously. To detect a decremental response, 10 repetitive stimuli of 0.2 ms were given at 3, 5 and 10 Hz with an EMG system (Nicolet Biomedical Inc., Madison, WI, USA). The test was considered positive when the average of three consecutive measurements of the same muscle showed a decrement of at least 10% of both the amplitude and the area of the negative (upward) peak of the CMAP.

Results

Construction of expression plasmids

In order to produce a full-size human IgG1 variant of the previously cloned F_{ab} 637, its F_d and LC coding sequences were cloned into the mammalian expression plasmid pIgG1. The F_d 637 was genetically fused to the human $\gamma 1 F_c$, resulting in a full-size human heavy chain (HC) cistron. So, pIgG1-637 contained both the HC and LC cistron, each under the control of an hCMV P/E element.

To produce an IgG1-637 variant that does not activate complement, a mutation was introduced in the C1q binding site. Lysine 322 was mutated to alanine by site-directed mutagenesis of its codon, resulting in pIgG1-637/K322A. Interestingly, by chance also another mutant was created, pIgG1-637 Δ C1q, which had six insertions of the K322A oligonucleotides downstream the mutated codon, resulting in a tandem of seven repeats.

In order to produce a non-specific antibody that has the same LC as IgG1-637, the CDR3 of the HC was mutated. The amino acid sequence PLKP in the CDR3 was deleted by site-directed mutagenesis, resulting in pIgG1-637/CDR3 Δ PLKP.

Antibody production by CHO cells

In order to produce large amounts of IgG1-637, CHO cells were transfected with pIgG1-637 by lipofectin. Monoclonal stable transfected cells were obtained by limiting dilution and auxotroph-based selection. The highest producer, as determined by human Ig-specific ELISA (data not shown), was selected for large-scale production. A 10-liter hollow fiber culture yielded 558 mg protein G-purified IgG1-637.

IgG1-637 Δ C1q was similarly produced in CHO-K1. A 10-liter culture of a monoclonal pIgG1-637 Δ C1q-transfected CHO-K1 cell line yielded 135 mg protein G-purified IgG1-637 Δ C1q. To create an alternative to IgG1-637 Δ C1q and IgG1-637/K322A, for abolishing the complement-activating capacity of IgG1-637, the glycosylation of IgG1-637 was changed. CHO-Lec1 cells were transfected with pIgG1-637 and IgG1-637/Lec1 was isolated from culture by protein G. IgG1-637/CDR3 Δ PLKP was produced by CHO-K1 in culture flasks and not purified.

Figure 1

Specificities of the human anti-AChR mAb IgG1-637. Specificities of non- and Alexa488-conjugated IgG1-637 were determined by RIA with extracts of human muscle AChR-expressing cell lines TE671 and CN21, by RIA with purified *Torpedo* AChR, by RIA with a hybrid receptor of human $\alpha 1$ and *Torpedo* β , γ and δ subunits, and by ELISA with extracellular domain peptide human $\alpha 1$ -210.

IgG1-637 specifically binds the human AChR $\alpha 1$ subunit

To determine if the epitope specificity of IgG1-637 was the same as its parental F_{ab}637, several RIAs were performed (Figure 1). IgG1-637 was able to precipitate human AChR and a human-*Torpedo* hybrid receptor, which contained the human $\alpha 1$ subunit and the *Torpedo* β , γ and δ subunits, while *Torpedo* AChR was not precipitated. In contrast, IgG1-637/CDR3 Δ PLKP did not precipitate human AChR, indicating the necessity of PLKP in the CDR3 for the specificity (data not shown). The human $\alpha 1$ -210 peptide was not bound by IgG1-637, indicating the specificity of the recombinant Ab for the native conformation of the $\alpha 1$ subunit. The anti-human AChR Ab titer, as determined by a diagnostic kit, of a 1 mg IgG1-637/ml preparation was 8.4 mmol, calculated with the kit's standard of pooled MG patient antibodies. These data indicated that the specificity was maintained after reconstruction, i.e. IgG1-637 specifically bound the native human AChR $\alpha 1$ subunit.

Figure 2

FACS analysis of human anti-AChR IgG1-637. The human fetal muscle AChR-expressing cell line TE671 was incubated with IgG1-637 and stained with goat anti-human^{119C} Ig (A) and signal amplified (B) or cells permeabilized (C). The human fetal and adult muscle AChR-expressing cell line MITC was incubated with IgG1-637 and stained with goat anti-human^{119C} Ig (D).

IgG1-637 FACS analysis

FACS analysis of IgG1-637 on TE671 cells gave a half log-scale shift after amplification (Figure 2). This indicates that IgG1-637 is able to bind the membrane-bound human AChR.

IgG1-637ΔC1q does not activate complement

To measure the complement activation capacities of IgG1-637 and its variants IgG1-637ΔC1q and IgG1-637/Lec1, their C1q and C3 binding were measured. IgG1-637 bound C1q and C3 in a concentration depended way (Figure 3), as predicted considering its IgG1 isotype. But, IgG1-637/Lec1 also bound C1q and C3, indicating that the changed glycosylation only slightly influenced the complement activation. Interestingly, IgG1-637ΔC1q did not bind C1q or C3.

IgG1-637 is pathogenic

The transfer of 0.8 g/kg MG IgG per dose in two monkeys resulted in clinical signs in one monkey, while both monkeys showed decrement of the CMAP (Table 1). To determine if IgG1-637 is pathogenic, five monkeys were injected with three daily doses of 0.5, 1, 1.7 or 5 mg/kg. The monkeys which received the doses of 1.7 and 5 mg/kg showed clinical signs of EAMG, indicating the pathogenic characteristic of IgG1-637.

Figure 3

Complement binding by IgG1-637 and its variants. The complement binding characteristics of IgG1-637 and its variants IgG1-637/Lec1, with truncated glycosylation, and IgG1-637 Δ C1q, with altered C1q-binding site, were determined by ELISAs.

Discussion

In order to produce a completely human, full-size IgG1 isotype, the MG thymus-derived anti-AChR F_{ab} 637 was genetically fused to the F_c of human IgG1. The F_d and LC coding sequences of F_{ab} 637 were cloned into expression plasmid pIgG1, creating two hCMV promoter-driven, full-length chain cistrons. CHO cells transfected with pIgG1-637 yielded 56 mg/l of protein G-purified IgG1-637. RIA, FACS and immunohistochemistry data showed that IgG1-637 specifically bound the native primate α 1 subunit, indicating the maintained specificity of F_{ab} 637. The pathogenicity of IgG1-637 was shown

in a monkey EAMG model, confirming the reconstruction of an MG antibody. IgG1-637/CDR3 Δ PLKP was shown to be non-specific for AChR, while IgG1-637 Δ C1q did not activate complement.

Table 1: Passive transfer EAMG in monkeys

monkey [amount/kg/day]	clinical signs of EAMG	decrement of CMAP
0.5 mg IgG1-637	-	-
1.0 mg IgG1-637	-	+
1.7 mg IgG1-637	+	+
1.7 mg IgG1-637	+	+
5.0 mg IgG1-637	+	+
0.8 g MG IgG	-	+
0.8 g MG IgG	+	+

Rhesus monkeys were intravenously injected for three successive days with an amount of IgG per kg body weight. Clinical signs (muscle weakness) were seen within one week after the first injection (+) or not at all (-), and decrement of the compound muscle action potential (CMAP) was determined (+, at least 10% decrement).

In an earlier study, the V_H and V_L of F_{ab} 637 were cloned in a scF_v-expression plasmid [41]. The bacterial produced scF_v637 binds specifically to primate AChR, like the parentally F_{ab} 637 and IgG1-637. Only a few human monoclonal anti-AChR antibodies have been cloned by hybridoma technology before the reconstructed IgG1-637 [42-45]. IgG1-637 recognized the membrane-bound AChR, which is consistent with other FACS data we obtained in a different set-up earlier [46]. CHO-produced IgG1-637 had a high yield, compared to the bacterial production yields of F_{ab} 637 and scF_v637. Moreover, the IgG1-637 yield was higher than similar IgG1 or F_{ab} production in CHO by others [31,47-49]. The high yield makes plgG1-637-transfected CHO a good source for a standard monoclonal human MG antibody, which could be used as a reference in diagnostic assays. Production could be further increased by, e.g. supertransfection of the clone by plgG1-637.

IgG1-637 is pathogenic itself, but it could serve as a source of enzymatic F_{ab} 637 production. The therapeutic effect of F_{ab} 637 could then be tested in the passive transfer monkey EAMG model. But considering the low serum half-life of F_{ab} , an IgG1-637-based molecule could be a alternative. Such a therapeutic antibody should not be able to activate complement and should be monovalent for AChR. IgG1-637 Δ C1q does not activate complement, which is required for the establishment of clinical signs [6,50,51]. Deletion of four amino acids (PLKP) in the CDR3 of the HC makes IgG1-637 non-specific. A "bispecific" immunoglobulin (bsIg) with one anti-AChR arm, one non-specific arm and both F_c s with a disrupted C1q-binding site could be a potential therapeutic design (Figure 4).

The pathogenic reconstructed MG antibody IgG1-637 could serve as a reference human monoclonal antibody in diagnostic assays, as a source for the enzymatic production of F_{ab}637, which is protective *in vitro*, or could serve as the genetic basis of a therapeutic bslg.

Figure 4
Design of a potential therapeutic, “bispecific” immunoglobulin derived from IgG1-637, bslgG637. One antibody arm is specific for the MIR of the human AChR, while the other arm is non-specific, thereby shielding the AChR without causing antigenic modulation. The C1q-binding sites are mutated to prevent focal lysis of the postsynaptic membrane. “Knobs-into-holes” engineering [52] favors the formation of bslg over homomeric byproducts. The His₆ tag can be used for purification and detection.

Acknowledgements

We would like to thank Ms M. Pilar Martínez-Martínez for her discussions. This work was supported by grants from the Quality of Life Program of the European Union, Prinses Beatrix Fonds and L'Association Française contre les Myopathies.

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Chapter 6

Overexpression of rapsyn in muscles prevents experimental autoimmune myasthenia gravis

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Abstract

The target organ of myasthenia gravis (MG) is the postsynaptic membrane of the neuromuscular junction (NMJ) with the acetylcholine receptor (AChR) as the primary autoantigen. Susceptibility to experimental autoimmune MG (EAMG) decreases with age in Brown Norway (BN) and Lewis rats due to an intrinsic resistance of the postsynaptic membrane of old animals to the antibody-mediated loss of AChR at the NMJ. Previously, we found that the level of rapsyn, an AChR clustering and anchoring protein, at the NMJ is higher in old BN rats. In the present study, we investigated effects of rapsyn overexpression in EAMG susceptible rats. Gene transfer of rapsyn into rat muscles by means of *in vivo* electroporation resulted in rapsyn overexpression. Transfected muscle fibers had extrasynaptic membrane rapsyn clusters. The level of AChR was increased in muscles two weeks after electroporation. In EAMG rats, rapsyn-overexpressing muscles showed no loss of AChR, in contrast to control muscles. Repetitive nerve stimulation showed no decrement of the compound muscle action potential of the rapsyn-overexpressing muscles, in contrast to sham-treated muscles of the same EAMG animals. Electron microscopic examination showed that endplates of rapsyn-overexpressing muscles of EAMG rats had a normal morphology of the postsynaptic folds, while endplates of sham-treated muscles of the same animals had damaged folds. These data suggest an important role for rapsyn in the susceptibility to EAMG.

Introduction

In myasthenia gravis (MG) the target organ is the postsynaptic membrane of the neuromuscular junction (NMJ). The muscular nicotinic acetylcholine receptor (AChR) is the primary autoantigen in eight out of ten MG patients, while in anti-AChR antibody-negative patients the muscle-specific kinase (MuSK) has recently been found to be an important antigen [1]. Anti-AChR antibodies cause functional loss of AChR at the motor endplate, either by: crosslinking the receptors, leading to increased internalization (antigenic modulation); activation of complement, leading to focal lysis of the postsynaptic membrane, which also reduces the folding of this membrane; or by inhibiting the AChR's ion channel function [reviewed in 2]. Functional AChR loss impairs neuromuscular signal transmission, resulting in skeletal muscle weakness.

Rapsyn, a 43-kD cytoplasmic protein, clusters and anchors AChRs and is essential for the formation of the postsynaptic apparatus at the NMJ. In innervated muscle fibers, AChR and rapsyn are clustered in high density at the motor endplate. Pre-assembled AChR-rapsyn complexes, which are already present in post-Golgi vesicles [3], are linked to the cytoskeleton and are clustered upon induction by agrin, a neuronal released synaptogenic protein of which the receptor complex includes MuSK [4,5]. In MG patients, point mutations in the rapsyn gene (*RAPSN*), which hinder the recruitment of AChR, and mutations in the *RAPSN* promoter, which decrease the level of rapsyn expression, lead to congenital myasthenic syndromes (CMS) [6,7]. Mice deficient in rapsyn die at birth because the postsynaptic specialization of the NMJ, including its cytoskeleton, has failed to develop [8]. Agrin-induced clustering of AChRs can be restored in cultured rapsyn-deficient myotubes by introducing a rapsyn expression plasmid [9]. Overexpression of rapsyn in *in vitro* models, however, reduces the size of AChR clusters dramatically, independent of agrin treatment, and alters the intracellular trafficking of AChR [9-11]. In cell lines, cotransfection of rapsyn and AChR expression plasmids increases the half-life of AChR [12,13]. Rapsyn also reduces antigenic modulation in transfected fibroblasts when incubated with the anti-AChR monoclonal antibody (mAb) 35 [12].

Experimental autoimmune MG (EAMG) is an animal model which closely resembles clinical MG. EAMG can be induced passively by transfer of MG patient sera or anti-AChR mAbs, or can be induced actively by immunization with AChR. Brown Norway (BN) rats have an age-related resistance to the induction of passive and chronic EAMG [14]. In contrast to young BN rats, transfer of anti-AChR antibodies does not result in AChR loss or muscular weakness in aged BN rats. This resistance could not be explained by age differences in the immune response including macrophage infiltration, antibodies and complement activation or by compensatory mechanisms such as increased AChR expression [15-17]. The postsynaptic NMJ membrane is intrinsically resistant to antibody-mediated degradation in aged animals [18]. Rapsyn, as well as S-laminin, is found to

be upregulated in aged BN rats as compared to young BN rats, suggesting a role for rapsyn in making the MG target organ resistant [18].

In the present study, the effects of rapsyn overexpression on AChR level and on EAMG induction in EAMG susceptible Lewis rats were examined. To achieve this, we transfected the tibialis anterior muscle fibers of rats with a rapsyn expression plasmid. Gene transfer into the muscles was performed by *in vivo* electroporation (electropermeabilization), an efficient, non-viral technique [19].

Methods

Construction of expression plasmid

The mouse rapsyn cDNA [13] was cloned into the CMV promoter-driven mammalian expression vector pcDNA1.1/Amp (Invitrogen, Breda, The Netherlands) by *EcoR* I digestion and ligation, resulting in the plasmid pcDNA-rapsyn. The pcDNA-rapsyn plasmid was prepared for electropermeabilization with the Qiagen Midiprep kit, according to the manufacturer's manual, and finally dissolved in 0.9% NaCl at a concentration of 2 µg DNA/µl.

Animals

11-week-old female Lewis rats were obtained from the Department of Experimental Animal Services, University of Maastricht, The Netherlands, with permission of the Committee on Animal Welfare (DEC), according to Dutch governmental rules.

Electropermeabilization

Prior to electropermeabilization, rats were anesthetized with inhalational Halothane and hind legs were shaved. One tibialis anterior was injected with 75 µl pcDNA-rapsyn solution and the other with 75 µl 0.9% NaCl. 5 min after the injection, the leg was placed between caliper electrodes (BTX, San Diego, CA, USA) with conductive gel and 8 pulses of 200 V/cm, 20 ms, 1 Hz (reversal of polarity after first 4 pulses) were given by an Electro Square Porator ECM 830 (BTX, San Diego, CA, USA) [19].

Induction of EAMG

Rats were intraperitoneally injected with 20 pmol mAb35 per 100 g body weight [15] at 2 weeks after electropermeabilization with pcDNA-rapsyn. After another 48 hours, the rats were clinically scored.

Clinical scoring

The severity of clinical signs of disease in EAMG was scored by measuring muscular weakness. The animals' muscle strength was assessed by their ability to grasp and lift repeatedly a 300 g rack from the table, while suspended manually by the base of the tail for 30 seconds. Clinical scoring was based on the presence of tremor, hunched posture, muscle strength

and fatigability. Disease severity was expressed as: 0, no obvious abnormalities; +, no abnormalities before testing, but reduced strength at the end; ++, clinical signs present before testing, i.e. tremor, head down, hunched posture, weak grip; +++, severe clinical signs present before testing, no grip, moribund [20,21].

Histochemical staining

Isolated tibialis anterior was frozen in melting isopentane and cryosectioned. Rapsyn was stained by incubating with mouse anti-rapsyn mAb 1234 (dilution 1/1000; a kind gift of Prof. Dr S.C. Froehner) in phosphate-buffered saline with 2% bovine serum albumin (PBSA) for 45 min and subsequently washed and incubated with Alexa488-conjugated goat anti-mouse Ig (dilution 1/100; Molecular Probes, Leiden, The Netherlands) in PBSA for 45 min. AChR was visualized by incubating with Alexa594-conjugated α -bungarotoxin (dilution 1/300; Molecular Probes, Leiden, The Netherlands) in PBSA for 45 min.

Measurement of AChR level

The AChR concentrations of isolated tibialis anterior muscles were performed as described previously [22,23], with the following modifications. Muscles were minced and homogenized with an ultra-turrax in 10 ml Buffer A (PBS, 10 mM EDTA, 10 mM NaN_3 , 10 mM iodoacetamide, 1 mM PMSF) for 3 times 30 s at 4°C. Homogenate was centrifuged (22100 g, 30 min) and the resulting pellet resuspended in 2.5 ml Buffer B (Buffer A with additional 0.5% Triton X-100). Extraction was performed for 1 h at 4°C on a platform shaker. After centrifugation (22100 g, 30 min), aliquots of 1 ml were labeled with 1 μl [^{125}I -Tyr 54] α -bungarotoxin (74 TBq/mmol, Amersham Pharmacia Biotech Benelux, The Netherlands) with or without 1 mM acetylcholine and neostigmine bromide overnight at 4°C. Immunoprecipitated triplets were counted in a γ -counter.

Electromyography (EMG)

Decrement of compound muscle action potential (CMAP) was measured in the tibialis anterior muscle 48 hours after mAb35 transfer. Rats were anesthetized with 60 mg/kg sodium pentobarbital. For stimulation, two small monopolar needle electrodes were used. The cathode was inserted near the peroneal nerve at the level of the knee and the anode more proximal and lateral at a distance of 3 to 4 mm. For recording, a third monopolar needle electrode was inserted subcutaneously over the tibialis anterior muscle. A ring electrode distally around the relevant hind leg served as a reference and the animal was grounded by a ring electrode around the tail. Stimulation and recording were performed with the EMG system Viking IV (Nicolet Biomedical Inc., Madison, WI, USA). To detect a decremental response, series of 8 supramaximal stimuli were given at 3 Hz. Stimulus duration was 0.2 ms. The test was considered positive for decrement when both the amplitude and the area of the negative (upward) peak on the CMAP showed a decrease of at least 10% [24]. To demonstrate

reproducibility, at least 3 recordings were made of all investigated muscles. During the measurements, skin temperature was kept between 35 and 37°C by means of an infrared heating lamp (DISA, Copenhagen, Denmark). All EMG studies were performed by the same investigator (F.S.).

Electron microscopy (EM)

Rapsyn-treated and control tibialis anterior muscles from three EAMG rats were fixed isometrically in 2% glutaraldehyde in 10 mM CaCl₂, 0.1 M cacodylate buffer pH=7.3, postfixed in 1% osmiumtetroxide in the same buffer, dehydrated through a graded acetone series and embedded in epoxy resin (Glycid ether 100, Serva, Heidelberg, Germany). Endplates were located in toluidine blue stained semi-thin sections from the central region of each muscle. Ultra-thin sections from selected areas were contrasted with uranyl acetate and lead citrate and viewed with Jeol 1200 EX electron microscope. All endplates were photographed up to a total of at least two per muscle per animal.

Figure 1

Immunohistochemical staining of rapsyn-treated muscles. Cryosections of muscles were stained with mouse anti-rapsyn mAb and Alexa488-conjugated goat anti-mouse Ig. Rapsyn-treated muscles (a, c, d) contained rapsyn clusters outside the postsynaptic membrane, in contrast to non-treated muscle (b) where rapsyn was only located at the NMJ. (a) A typical staining of rapsyn-treated muscle; (b) non-treated muscle; (c) variable rapsyn expression in transfected fibers; (d) three transfected fibers in a longitudinal section.

Results

Electropermeabilization led to rapsyn overexpression in muscle fibers

To overexpress rapsyn *in vivo*, the left tibialis anterior muscle was electropermeabilized with pcDNA-rapsyn (rapsyn-treated muscle) and the right tibialis anterior muscle with 0.9% NaCl only as internal control. After two weeks, the muscles were isolated and cryosections were double stained for rapsyn and AChR. Rapsyn-treated muscles had transfected fibers containing non-postsynaptic rapsyn clusters (Figure 1a) whereas fibers from control muscles had no detectable rapsyn outside the postsynaptic membrane (Figure 1b). Transfected muscle fibers had variable levels of rapsyn overexpression, with some showing a high level of expression leading to rapsyn-specific staining of the complete cytoplasm (Figure 1c) while others only had a few rapsyn clusters (Figure 1d). Most transfected muscle fibers however, showed a large number of rapsyn clusters equally distributed throughout the muscle fiber (Figure 1a).

Rapsyn overexpression increased AChR level

In order to analyze the effect of rapsyn overexpression on the level of AChR in muscles, we measured the concentration of AChR using a RIA two weeks after electropermeabilization. The rapsyn-treated muscles (n=5) had a 42% increase in AChR concentration as compared to control muscles (n=5) of same animals (Figure 2).

Figure 2
Effect of rapsyn overexpression on AChR level in non-EAMG rats and mAb35-induced EAMG rats. The AChR concentration of rapsyn-treated and non-treated tibialis anterior muscles of the same rats, with or without mAb35 transfer 14 days after electropermeabilization, were measured by a RIA.

Rapsyn overexpression protected against AChR loss in EAMG rats

To determine if *in vivo* rapsyn overexpression conveyed protective effects on the AChR level in rats suffering from EAMG, we induced EAMG in rats (n=5) two weeks after rapsyn gene transfer. Within 48 hours, all mAb35-transferred animals had clear clinical signs of EAMG (score of + or ++). The AChR loss in sham-treated muscles of EAMG rats was 39% while the rapsyn-treated muscles of EAMG rats showed no AChR loss at all (Figure 2).

Rapsyn overexpression prevented decrement of CMAP in EAMG rats

The non-rapsyn-treated legs of EAMG rats, which had a clinical score of ++, showed a mean amplitude decrement of 19.6% and a mean area decrement of 17.6% (n=5), confirming the presence of abnormal neuromuscular transmission (Figure 3). In contrast, the rapsyn-treated legs of the same five EAMG animals showed no decrement, illustrating the protective effect of rapsyn overexpression on EAMG induction.

Figure 3

Effect of rapsyn overexpression on decrement of the compound muscle action potential in EAMG rats. Recording of the rapsyn-overexpressing tibialis anterior muscle (left panel) and of the non-electropermeabilized muscle (right panel) of the same EAMG rat.

Rapsyn overexpression prevented the destruction of postsynaptic folds in EAMG rats

The structure of the motor endplates was examined to determine the effect of rapsyn overexpression on the postsynaptic folds in EAMG rats. In control muscles of EAMG rats (n=3), all examined endplates (14 out of 14) had morphological abnormalities. The postsynaptic folds were strongly reduced (Figure 4b, d). In rapsyn-treated muscles of the same EAMG animals, only one endplate with an abnormal morphology (1 out of 14) was found (Figure 4a, c). The data suggest a protective effect of rapsyn overexpression on the mAb35-mediated destruction of postsynaptic folds.

Figure 4
 Electron microscopic examination of the postsynaptic folds of motor endplates in EAMG rats. Typical neuromuscular junctions of a rapsyn-treated (A and enlargement of folds in C) or a sham-treated muscle (B and enlargement of folds in D). Bars are 1 μ m.

Discussion

We have shown that overexpression of rapsyn prevents the induction of EAMG in rat muscles electroporably treated with rapsyn cDNA. Antigenic modulation, focal lysis of the postsynaptic membrane and decrement of muscle action potential were absent in rapsyn-treated muscles of EAMG rats.

Transfected muscles fibers of the tibialis anterior muscles, which were electroporably treated with pcDNA-rapsyn, overexpressed rapsyn, as revealed by immunohistochemical staining. The level of rapsyn found in transfected fibers ranged from a few large rapsyn clusters per fiber, located at the non-postsynaptic membrane, to small rapsyn clusters densely distributed throughout a fiber's cytoplasm. The influence of the level of rapsyn expression on rapsyn cluster size, i.e. high expression decreases cluster size, in rapsyn-treated muscles was comparable to the effects of rapsyn overexpression *in vitro* reported by others [9,10]. Differences in level of rapsyn expression were probably due to the variation in number of pcDNA-rapsyn molecules taken up by fibers and variation in their nuclear distribution in a syncytium.

The amount of AChR in rapsyn-treated muscles was increased by 42% after two weeks, as measured by RIA of total muscle extracts. Marchand and coworkers [25] found a 50% increase in AChR at the cell surface when rapsyn was coexpressed in COS-7 cells. The increased AChR concentration we found in rapsyn-treated muscles was likely due to the metabolic stabilizing effects of rapsyn [12,13]. The 25% increased rapsyn level at the NMJ in aged BN rats, however, does not coincide with increased AChR or $\alpha 1$ subunit mRNA levels [14,18]. In CMS patients with mutations in the *RAPSN* promoter, a lower level of rapsyn at the NMJ coincides with a decreased level of AChR [7].

Rapsyn overexpression prevented AChR loss in EAMG rats. The AChR concentration of electroporably treated muscles was not decreased after EAMG induction two weeks after rapsyn treatment, while AChR loss was seen in sham-treated muscles of the same animals. AChR loss is also not seen in aged BN rats subjected to passive transfer of EAMG [14], which have altered levels of several postsynaptic proteins [18]. Apparently, AChR levels can be protected against antigenic modulation by overexpressing rapsyn alone.

Rapsyn treatment prevented muscle weakness in EAMG rats, which was confirmed by electrophysiological measurements. EMG of rapsyn-treated tibialis anterior muscles of rats suffering from EAMG showed no decrement of CMAP, while significant decrement was seen in non-treated muscles of the same animal. The high AChR level of rapsyn-treated muscles apparently restored normal neuromuscular transmission in EAMG rats.

Postsynaptic folds of rapsyn-treated muscles of EAMG rats were not destroyed, as shown by EM studies. The endplates of sham-treated muscles were all damaged, as expected. Engel and coworkers [26,27] found that 96% of the endplates have an abnormal morphology in the acute phase of

EAMG. Our data indicated that rapsyn overexpression prevents the antibody-mediated destruction of postsynaptic folds.

In cultured myotubes it has been found that rapsyn overexpression inhibits agrin-induced AChR clustering [9] and affects the distribution and size of the clusters [10,11], which would have a negative effect on neuromuscular transmission. In the present study, however, at two weeks after the rapsyn treatment, the rats moved normally, showed no EMG decrement and their postsynaptic membrane had a normal morphology. These data indicate that *in vivo*, rapsyn overexpression does not lead to a dysfunction of neuromuscular transmission. Rapsyn-overexpressing muscle fibers were innervated prior to induction of EAMG, which could explain the difference in the responsiveness to agrin of AChR-rapsyn clustering with cultured myotubes. A functional motor endplate, which already contains large AChR-rapsyn clusters, seems able to maintain these clusters after two weeks of rapsyn overexpression. Rapsyn overexpression impairs the formation of large AChR-rapsyn clusters, but apparently has little effect on their maintenance.

The protective effect of exogenous rapsyn overexpression on the induction of EAMG in Lewis rats is similar to the increased endogenous rapsyn expression in aged BN rats, indicating that rapsyn is an important factor in age-related EAMG resistance. Changes in endogenous rapsyn expression could determine the susceptibility of MG, irrespective of the anti-AChR antibody titer, while slight changes could influence the severity. Rapsyn treatment is potentially therapeutic for the treatment of selected muscles of MG patients, e.g. the eyelid muscles, and individuals with mutations in the rapsyn gene.

Acknowledgements

We would like to thank Dr J.T. Vilquin (Institut National de la Santé et de la Recherche Médicale U-582 Institut de Myologie, Groupe Hospitalier Pitie-Salpetriere, 75013 Paris, France) for the helpful discussions concerning electroporabilization. Furthermore, we wish to thank Ms M. Pilar Martínez-Martínez for her excellent laboratory assistance and discussions. This work was supported by grants from Prinses Beatrix Fonds and L'Association Française contre les Myopathies.

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Summery and general discussion

Chapter 1 reviews the role of autoantibodies in myasthenia gravis (MG). The majority of MG patients have autoantibodies against the muscular nicotinic acetylcholine receptor (AChR). Although anti-AChR antibodies are the main MG antibodies, patients can be seronegative for them or specificities for other muscle autoantigens can coincide. Besides electromyography (EMG) and single-fiber EMG (SFEMG), analysis of the autoantibodies of patient sera can diagnose MG and can also lead to the early detection of thymoma. The immunopathogenesis of seropositive MG has been studied in detail. Anti-AChR antibodies lower the number of functional AChRs at the motor endplate by: crosslinking of AChRs, which increases their internalization and degradation (antigenic modulation); complement activation, which causes focal lysis of the postsynaptic membrane; and functional inhibition of the AChR's ion-channel function. AChR loss leads to an impaired neuromuscular signal transmission, explaining the muscle weakness of the MG patients. The etiology of MG is not clear. However, the thymus plays an important role in MG, since hyperplastic thymic medullas or thymoma are found in many patients. Experimental autoimmune MG (EAMG) is an animal model that closely resembles MG. It can be induced by immunization with AChR or transfer of MG sera. EAMG is used to study MG and EAMG antibodies, the role of complement and macrophages, the resistance to the induction of the disease, and the treatment of EAMG.

Chapter 2 describes a new EAMG mouse model that generates human anti-AChR antibodies. Mice transgenic for human μ , $\gamma 1$, and κ germ line genes (HuMAb-Mice) were immunized with AChR from *Torpedo californica* (tAChR). Serum titers of anti-tAChR antibodies were in the nanomolar range, and anti-rodent AChR antibodies were in picomolar range. Some HuMAb-Mice had signs of muscle weakness, clearly indicating their susceptibility to EAMG. Human antibody-mouse AChR complexes were found at the neuromuscular junction, while AChR loss was up to 65%. Spleen and lymph nodes were used for producing hybridomas. Of the anti-tAChR monoclonal antibody (mAb)-producing hybridomas, 2% had cross-reactivity with rodent AChR and none with human AChR. Immunization with a fusion protein, Trx-H α 1-210, displaying the human main immunogenic region did not result in EAMG or the generation of human anti-human AChR mAbs.

In Chapter 3, a large-scale production method of the potential therapeutic F_{ab}637 was assessed. F_{ab}637 protects the AChR from antigenic modulation *in vitro*. To test the *in vivo* effects of F_{ab}637, which only crossreacts with primate AChR, large quantities of purified F_{ab} are needed for a monkey EAMG model. But, the current bacterial expression system is inefficient for this purpose. The methylotrophic yeast *Pichia pastoris* has

been successfully used by others to produce antibodies and antibody fragments. *P. pastoris* was transfected with plasmids encoding for the λ light chain (λ 637) and the heavy chain of F_{ab} 637 (F_d 637). Stable transfected cells produced about 100 mg λ 637 per liter, which was secreted as a homodimer. However, co- or supertransfection of F_d 637 plasmid did not result in the production of F_{ab} or F_d 637. Although F_d 637 was not produced in *P. pastoris*, its mRNA was transcribed. A plasmid encoding for a single-chain variable fragment (scF_v) variant of F_{ab} 637 did not express scF_v in *P. pastoris*.

In Chapter 4, a scF_v variant of F_{ab} 637, scF_v637, was constructed and characterized. Bacterially produced scF_v637 was able to bind to human and monkey AChR, like its parental F_{ab} 637. Furthermore, scF_v637 was capable of inhibiting the binding of its intact IgG1 isotype variant (IgG1-637) and anti-AChR mAb35 binding to human AChR up to 33 and 73%, respectively, and of MG patient sera up to 46%.

Chapter 5 describes a fully human γ 1 immunoglobulin that was reconstructed from F_{ab} 637, IgG1-637. The coding sequences of λ 637 and F_d 637 were cloned in one expression plasmid. The F_d 637 was genetically fused to the human γ 1 F_c , resulting in a complete IgG1 heavy-chain coding sequence. Monoclonal transfected chinese hamster ovary (CHO) cells produced up to 55 mg purified IgG1-637 per liter. IgG1-637 bound human and monkey AChR, indicating a maintained specificity after the reconstruction. The pathogenicity of the reconstructed MG autoantibody was tested in a monkey EAMG model. Three daily injections of 1.7 to 5 mg IgG1-637 per kg body weight resulted in clinical signs of EAMG in rhesus monkeys.

In Chapter 6, it is shown that overexpression of rapsyn prevents the induction of EAMG. In age-related resistance to EAMG, the postsynaptic membrane of old rats is resistant to antigenic modulation. The AChR-clustering and -anchoring protein rapsyn is upregulated in old Brown Norway (BN) rats. In order to test if rapsyn overexpression could transfer the resistance to susceptible rats, muscles were *in vivo* transfected with a rapsyn expression plasmid. Transfected muscle fibers showed, in contrast to non-transfected fibers, extra-synaptic clusters of rapsyn. The rapsyn overexpression increased the AChR level of the muscles by 42%. Two weeks after transfection, EAMG was passively transferred. Rapsyn overexpressing muscles of EAMG rats had no AChR loss, in contrast to control muscles of the same animals. Moreover, repetitive nerve stimulation of rapsyn-overexpressing muscles showed no decrement of the compound muscle action potential, in contrast to control muscles of the same EAMG rats. Electron microscopic examination of the motor endplates of EAMG rats showed a normal morphology of the postsynaptic folds in rapsyn-overexpressing muscles, while the folds were damaged in control muscles.

A specific therapy for MG would reduce the side effects of non-specific treatments. The hypothetical immunotherapy with a human anti-AChR antibody-derived protein could help treating patients with an acute MG phase [1]. MG patients can be treated by plasmapheresis during a

myasthenic crisis, thereby removing the autoantibodies from the circulation [2]. To prolong the remission effect of plasmapheresis, the MG patient's AChRs could be shielded against newly produced autoantibodies by a therapeutic antibody. A recombinant antibody with F_{ab}637's binding characteristic [3] and IgG4's bispecific and none-complement-activating characteristics [4] would combine the advantages of F_{ab} and IgG. The resulting IgG-like molecule could block the AChR for a long period (expected serum half-life of 21 days), compared to scF_v637 and F_{ab}637. IgG1-637 and its mutants IgG1-637ΔC1q, which does not activate complement, and IgG1-637/CDR3ΔPLKP, which is non-specific, could form the basis for the construction of a therapeutic antibody.

Gene therapy, on the other hand, is a potential long-lasting therapy for MG. Although overexpression of rapsyn prevents the induction of EAMG, the effect on ongoing EAMG has to be investigated. After an immune attack, the postsynaptic membrane is focally destroyed by the membrane attack complex. In contrast to the maintenance, formation of new AChR clusters could be impaired by a high overexpression of rapsyn. Considering the impact of slight changes in rapsyn expression in old BN rats [5] and in a subset of congenital myasthenic syndrome (CMS) patients [6,7] versus the rapsyn overexpression in cultured cells [8-10], a more moderate rapsyn expression induced by gene therapy is better for the formation of AChR clusters. An inducible, tissue-specific promoter instead of the currently used constitutive CMV promoter is an option to optimize the level of expression. The stability of long-term (over)expression of rapsyn in muscles has to be determined. The level of expression of luciferase is reported to be stable from day 3 to at least day 270 after electroporation [11]. Also, the long-term effects of rapsyn (over)expression have to be assessed, e.g. a possible anti-rapsyn response. If rapsyn gene therapy has a therapeutic effect, selected muscles could be *in vivo* electroporated, like the eyelid muscles.

The expression level of rapsyn plays an important role in the susceptibility to myasthenia. Down-regulation causes CMS, while up-regulation provides protection against MG. Slighter changes in the endogenous level of rapsyn could correlate with the severity of MG. Therefore, it might be interesting to determine the level of rapsyn in different subsets of MG patients.

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Samenvatting

Het menselijk lichaam wordt beschermd door het immuunsysteem tegen virussen, bacteriën en parasieten, maar ook tegen kwaadaardige tumoren die gezond weefsel binnendringen. Voor een adequate immuunrespons moet het immuunsysteem de binnendringers herkennen, met andere woorden het moet een onderscheid maken tussen (gezond) eigen en vreemd. Antilichamen en T-celreceptoren (TCR'en) zijn de eiwitten die de identificatie van lichaamsvreemde antigenen verzorgen en na herkenning een, respectievelijk, humorale of een cellulaire immuunrespons te weeg brengen. Falen van het immuunsysteem in het tolerant zijn voor eigen kan leiden tot de ongewenste situatie waarin TCR'en en antilichamen binden aan eigen onderdelen, wat resulteert in een auto-immuunziekte.

Myasthenia gravis (MG) is een prototypische auto-immuunziekte, omdat de antilichaam-gemedieerde pathogenese is beperkt tot één doelwit, namelijk de postsynaptische membraan van de neuromusculaire junctie (NMJ). De musculaire acetylcholinereceptor (AChR) is het belangrijkste autoantigen dat zich bevindt in de postsynaptische membraan. Autoantilichamen gericht tegen de AChR verlagen het aantal functionele AChR'en, wat resulteert in een vermoeibare zwakte van skeletspieren.

In hoofdstuk 1 wordt de rol van autoantilichamen in MG gereviewd. Het merendeel van de MG-patiënten heeft autoantilichamen tegen de AChR. Ondanks dat anti-AChR-antilichamen de belangrijkste MG-antilichamen zijn, kunnen MG-patiënten seronegatief voor deze antilichamen zijn of kunnen ze daarnaast ook antilichamen hebben die gericht zijn tegen andere spierantigenen. Voor de diagnose van MG kan naast elektromyografie (EMG) en *single-fiber* EMG (SFEMG) ook de analyse van autoantilichamen in patiëntensera worden gebruikt, dat tevens kan leiden tot de vroege diagnose van een thymoma. De immunopathogenese van seropositieve MG is gedetailleerd onderzocht. Anti-AChR-antilichamen verlagen de functionele concentratie aan AChR'en ter hoogte van de motoreindplaat door: het aaneenschakelen van AChR'en, wat resulteert in een verhoogde internalisatie en afbraak (antigene modulatie); complementactivering, wat resulteert in de focale lyse van de postsynaptische membraan; en functionele hindering van de functie van ionkanaal van de AChR. Het verlies aan AChR'en leidt tot een verslechterde neuromusculaire transmissie, wat de spierzwakte van MG-patiënten verklaart. De etiologie van MG is niet duidelijk. De thymus speelt echter een belangrijke rol in MG, gezien het feit dat thymoma en hyperplastische thymusmedulla's worden gevonden in veel MG-patiënten. Experimentele auto-immuun-MG (EAMG) is een proefdiermodel dat nauw overeenkomt met MG bij de mens. Het kan worden geïnduceerd door immunisering met AChR of transfusie met MG-sera. EAMG wordt gebruikt

ter bestudering van MG- en EAMG-antilichamen, de rol van complement en macrofagen, de resistentie tegen de inducering van de ziekte en de behandeling van MG.

In hoofdstuk 2 wordt een nieuw EAMG-model beschreven dat humane anti-AChR-antilichamen opwekt in muizen. Muizen die transgeen zijn voor de humane μ -, γ 1- en κ -kiemlijnen (HuMAb-Muizen) werden geïmmuniseerd met AChR van de *Torpedo californica* (tAChR). Serumtiters van anti-tAChR-antilichamen bevonden zich in het nanomolaire bereik en anti-ratten-AChR-antilichamen bevonden zich in het picomolaire bereik. Sommige HuMAb-Muizen hadden klinische symptomen, wat hun vatbaarheid voor EAMG aantoonde. Complexen van humane antilichamen en muizen-AChR'en werden gevonden ter hoogte van de NMJ, terwijl het verlies aan AChR tot 65% bedroeg. Milt en lymfknoopen werden gebruikt om hybridoma's te maken. Van de anti-tAChR monoclonale antilichamen (mAb's) vertoonde 2% kruisreactiviteit met ratten-AChR en geen met humaan AChR. Immunisering met een fusie-eiwit, Trx-H α 1-210, dat het humane *main immunogenic region* (MIR) bevat, resulteerde niet in EAMG of in het opwekken van humaan anti-humaan AChR mAb's. Deze experimenten tonen aan dat de HuMAb-Muis een geschikt model is voor het opwekken en bestuderen van humane anti-AChR-antilichamen *in vivo*.

In hoofdstuk 3 werd een mogelijke grootschalige productiemethode voor het potentieel therapeutische F_{ab}637 onderzocht. F_{ab}637 beschermt de AChR tegen antigene modulatie *in vitro*. Om F_{ab}637, die enkel met primaat-AChR kruisreageert, *in vivo* te testen zijn grote hoeveelheden aan gezuiverd F_{ab} nodig voor een apen-EAMG-model. Echter, het huidige bacterieel expressiesysteem is niet toereikend voor dit doel. The methyloτροφische gist *Pichia pastoris* werd succesvol gebruikt door anderen voor de productie van antilichamen en antilichaamfragmenten. *P. pastoris* werd getransfecteerd met plasmiden die coderen voor de λ lichte keten (λ 637) en de zware keten van F_{ab}637 (F_d637). Stabiël getransfecteerde gistcellen produceerden circa 100 mg/l λ 637, dat werd uitgescheiden als homodimeer. Echter, co- of supertransfectie van het F_d637-plasmide resulteerde niet in de productie van F_{ab}- of F_d637. Alhoewel F_d637 niet werd geproduceerd door *P. pastoris*, werd het mRNA wel afgeschreven. Een plasmide dat codeerde voor een *single-chain variable fragment* (scF_v)-variant van F_{ab}637 produceerde geen scF_v in *P. pastoris*. Deze gegevens tonen aan dat het expressieprobleem van F_d637 mogelijk afhangt van de sequentie van het variabele domein van de zware keten.

In hoofdstuk 4 werd de scF_v-variant van F_{ab}637, scF_v637, geconstrueerd en gekarakteriseerd. Bacterieel geproduceerde scF_v637 was in staat om humaan en apen-AChR te binden, net als het oorspronkelijke F_{ab}637. Bovendien inhibeerde scF_v637 de binding van zijn intacte IgG1 isotype-variant (IgG1-637), anti-AChR mAb35 en MG-patiëntensera aan de humane AChR met, respectievelijk, maximaal 33, 73 en 46%. ScF_v637 is hiermee een alternatieve kandidaat voor immunotherapie van MG.

Hoofdstuk 5 beschrijft een volledig humaan γ 1 immunoglobuline dat werd gereconstrueerd uit een F_{ab}637, IgG1-637. De coderende sequenties

van λ_{637} en F_{d637} werden gecloneerd in één expressieplasmide. De F_{d637} werd genetisch gefuseerd met de humane $\gamma 1 F_c$, wat resulteerde in een coderende sequentie voor een complete IgG1-zware keten. Monoklonale, getransfecteerde *chinese hamster ovary* (CHO)-cellen produceerden 55 mg gezuiverd IgG1-637 per liter. IgG1-637 bond aan humaan en apen-AChR, wat de gehandhaafde specificiteit aantoont na reconstructie. De pathogeniteit van het gereconstrueerde MG-autoantilichaam werd getest in een apen-EAMG-model. Drie dagelijkse doses van 1,7 tot 5 mg IgG-637 per kg lichaamsgewicht resulteerde in klinische EAMG symptomen in de rhesus apen. IgG1-637 kan worden gebruikt voor de enzymatische productie van het potentieel therapeutische F_{ab637} of kan dienen als basis voor een therapeutisch IgG-achtig molecuul, dat een hogere serum halfwaardetijd heeft dan een F_{ab} .

In hoofdstuk 6 wordt aangetoond dat overexpressie van rapsyne de inductie van EAMG voorkomt. Bij de leeftijdsafhankelijke resistentie tegen EAMG is de postsynaptische membraan van oudere ratten resistent tegen antigene modulatie. Rapsyne, dat AChR'en klustert en verankert, is upgereguleerd in oude Brown Norway (BN)-ratten. Om te testen of rapsyne-overexpressie de resistentie kan overdragen op gevoelige ratten, werden de spieren *in vivo* geëlektroporeerd met een rapsyne-expressieplasmide. Getransfecteerde spiervezels hadden rapsyneclusters buiten de synaps, in tegenstelling tot niet-getransfecteerde vezels. De rapsyneoverexpressie verhoogde het AChR-niveau in de spieren met 42%. Twee weken na transfectie werd EAMG passief geïnduceerd. Spieren die rapsyne tot overexpressie brachten hadden geen verlies aan AChR, in tegenstelling tot controle spieren van dezelfde dieren. Bovendien lieten repetitieve zenuwstimulaties geen decrement zien van de actiepotentialen van behandelde spieren, terwijl controle spieren van dezelfde EAMG-ratten dit wel vertoonden. Elektronenmicroscopisch onderzoek van de motoreindplaten van EAMG-ratten toonde aan dat de morfologie van de postsynaptische vouwen normaal was in spieren die rapsyne tot overexpressie brengen, maar de vouwen waren beschadigd in controle spieren. Deze resultaten wijzen op een belangrijke rol voor rapsyne in de vatbaarheid voor EAMG.

Een specifieke therapie voor MG zou de bijwerkingen van aspecifieke behandelingen kunnen reduceren. De hypotetische immunotherapie met een eiwit dat is afgeleid van een humaan anti-AChR-antilichaam zou MG-patiënten kunnen helpen die in een myasthene crisis verkeren. MG-patiënten worden behandeld met plasmaferesis gedurende een myasthene crisis, zodat de circulerende autoantilichamen worden verwijderd. Om het remissie-effect van de plasmaferesis te verlengen, zouden therapeutische antilichamen de AChR kunnen afschermen tegen nieuw gevormde autoantilichamen. Een recombinant antilichaam met de bindingskarakteristieken van F_{ab637} en de bispecifieke en niet-complementactiverende karakteristiek van IgG4 zou de voordelen combineren van F_{ab} en IgG. Het resulterende IgG-achtige molecuul zou de AChR voor langere tijd kunnen afschermen dan scF_{v637} of F_{ab637} . IgG1-637

en zijn mutanten IgG1-637 Δ C1q, die geen complement activeert, en IgG1-637/CDR3 Δ PLKP, die aspecifiek is, kunnen de basis vormen voor de constructie van een therapeutisch antilichaam.

Gentherapie is daarentegen een potentiële therapie met een langdurige werking. Alhoewel de overexpressie van rapsyne de inductie van EAMG vóórkomt, moet het effect hiervan op reeds geïnduceerde EAMG nog worden onderzocht. Daarnaast moeten de effecten van langdurige overexpressie van rapsyne worden bepaald, zoals een mogelijke anti-rapsynerespons. Indien wordt aangetoond dat gentherapie met rapsyne-DNA niet alleen preventief maar ook therapeutisch is, zou een selecte groep spieren, bijvoorbeeld de ooglidspieren, kunnen worden behandeld met *in vivo* elektroporatie.

Het expressieniveau van rapsyne speelt een belangrijke rol in de vatbaarheid voor myasthenie. Het verlagen van het niveau veroorzaakt een congenitaal myastheen syndroom, terwijl het verhogen een bescherming geeft tegen MG. Kleinere veranderingen in het endogeen rapsyneniveau zouden kunnen correleren met de ernst van MG. Het zou daarom interessant zijn het rapsyneniveau te vergelijken tussen patiëntengroepen die verschillen in de mate van MG.

Dankwoord/Acknowledgements

Het is onmogelijk alleen wetenschappelijk onderzoek te doen. Ik wil dan ook een ieder die op welke wijze dan ook een positieve bijdrage heeft geleverd aan de tot stand koming van dit proefschrift van harte bedanken.

Dr. M.H. De Baets, beste Marc. Als neuro-immunoloog, met een grote kennis van myasthenia gravis, en als nieuwsgierige wetenschapper leidde je mijn onderzoek. Zeker als je een interessant artikel had gelezen over een nieuwe techniek, dan wou je meteen de mogelijke toepassing daarvan in het myasthenie-onderzoek verkennen. Zo ook met de gentherapie met rapsyne-DNA. Een project dat ik sceptisch benaderde, omdat ik niet kon geloven dat het tot overexpressie brengen van één eiwit van de spier het beoogde effect zou kunnen verwezenlijken.

De collegae van Marcs neuro-immunologiegroep (Neurim): *Diplombiologe* Mario Losen, dr. Pilar "Piluca" Martínez-Martínez, dr. ir. Barbie Machiels en onze alumnus prof. dr. Fanping Meng. Beste Barbie, toen mijn werkplek verplaatste van Aken naar Maastricht hebben we met z'n tweeën succesvol recombinant DNA-technieken en celkweek geïntroduceerd op de laboratoria van onderzoeksinstituut Hersenen & Gedrag (H&G). *Dear Fanping, I enjoyed our discussions, especially those about antibody therapy, which led to the concept of a bispecific IgG4-like molecule. It's a pity that the distance between your own department and Maastricht made contact difficult later on.* Beste Mario, jouw aanwezigheid sinds de laatste twee jaar van mijn promotie-onderzoek heeft vooral door jouw toewijding voor het werk – al mag best wat vaker iemand anders het licht op werk uit doen – en door je collegialiteit van Neurim eindelijk een echte groep gemaakt. *"It's alive, Igor!" Dear Piluca, your enthusiasm for our research during your own Spanish PhD project ensured a post-doc position in Neurim. Taken together with your friendship, you ensured a position as one of my paranympths.*

Bedanken voor het in mij gestelde vertrouwen wil ik mijn promotores, prof. dr. Jaap Troost en prof. dr. Harry Steinbusch, die als leider van divisie 3 van H&G de lastige taak had het jonge Neurim een plaats binnen zijn divisie te geven.

De overige medewerkers van divisie 3 van H&G wil ik ook bedanken voor hun bijdrage, waarvan de volgende bij naam, mede omdat ze me niet hebben laten bungelen aan H&G als een myasthene spier aan het perifeer zenuwstelsel. Ing. Marjanne Markerink-van Ittersum, dé analiste met hart voor de zaak. Dr. ir. Nicole Senden, voormalig mede-paranimf, bedankt voor je hulp. Ing. Hellen Steinbusch, ik doe liever *talkie talkie* tijdens het overhandigen van een exemplaar van dit proefschrift, om het maar in het Helleniaans uit te drukken. De secretaresses Lillian Swaen en Akke Spauwen, fijn dat ik altijd het secretariaat kon binnen vallen. Dra. Marjo van de Waarenburg, niet voor niets één van mijn paranimfen.

Prof. dr. Frank Spaans, altijd bereid om de “kleine patiëntjes” met kleine voltages te diagnostiseren na hun behandeling door mij met wat hogere stroompulsen.

Coworkers of the RWTH Aachen, Dr. Stephan “Joe” Hellwig and Dr. Stefan Schillberg. Dear Stefan, thank you for helping me to plan the production of our F_{ab} in yeast and your support, in spite that the heavy-chain fragment was a “difficult fragment (F_{H})”, as you call it. Dear Joe, thank you for introducing me to biotechnology during your own PhD project. I would also like to thank Nicole Raven, MSc, and Yvonne Helgers, who were a great help during the beginning of my yeast experiments. Prof. Dr. Rainer Fischer, thank you for your hospitality in your Institute of Molecular Biotechnology.

Alle mede-promovendi en studenten die mij in enige vorm hebben geholpen de afgelopen vijf jaar wil ik bedanken, waarvan de volgende in het bijzonder *I wish to thank all (fellow PhD) students who helped me in the last five years, a specially the following.* Drs. Ronald “Sjors” Deumens, bedankt voor de gesprekken over “normen- en waardendingen” onder het genot van een kop lunchsoep. Dr. ir. Simone Dorf Müller, jouw hulp als ondermeer *Dolmetscher* was onontbeerlijk gedurende mijn beginfase in Aken. Dr. Dieter Peschen, *it seems that single-chains are easier then whole antibodies.* Anouk Roemen, je humor en ongecompliceerd karakter maakte van het histologielab een onvergetelijk gezellige en toch bedrijvige werkplek (een bezige bij zoemt). *Andrea Scheepers, MSc, I really enjoyed your company. Many times you shouted in the lab: “Alle Männer sind Schweine! Außer dich, Moritz”. Danke. Monica Sica, MSc, come on!*

Dr. René Hoet, jouw begeleiding gedurende mijn stage in Nijmegen was de perfecte basis voor mijn promotie-onderzoek.

Dr. Patrick Starremans, we hebben zo’n beetje gedurende onze hele lifescience-carrière met elkaar gediscussieerd, van fundamenteel tot over de grens van sciencefact en sciencefiction. Helaas door onze promoties minder frequent, maar daarom niet minder. Ir. Jan-Willem Deumens, onze roots gaan terug tot onze kleutertijd.

Kees[†] en Riek Pauw. Oma, bedankt voor uw steun gedurende mijn gehele opleiding. Jammer dat opa, die ons eerder dit jaar op 91-jarige leeftijd heeft moeten verlaten, dit net niet meer heeft mogen meebeleven. Marinke van de Ven-Stassen, mijn biologisch geweten. Léon en Annie Stassen. Mam, pap, bedankt.

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Curriculum vitae

Maurice Stassen is geboren op 28 februari 1971 te Geleen. Hij is opgegroeid in Limbricht en behaalde in 1988 zijn mavo- en in 1990 zijn havo-diploma. In 1990 begon hij de opleiding chemische technologie van het hoger technisch onderwijs aan de voormalige Hogeschool Heerlen (nu Hogeschool Zuyd), maar veranderde zijn specialisatie in biochemie door in het propedeutisch jaar over te stappen naar de chemische laboratoriumopleiding van het hoger laboratoriumonderwijs aan dezelfde school. Na de achtmaandse afstudeerstage, die werd gelopen bij de vakgroep Pathologie van de voormalige Rijksuniversiteit Limburg (nu Universiteit Maastricht) onder leiding van dr. Ton de Goeij, behaalde hij in 1995 de titel van ingenieur. In datzelfde jaar startte hij zijn biologiestedie aan de Katholieke Universiteit Nijmegen (KUN). Vanaf december 1996 tot en met september 1997 werd de eerste doctoraalstage gelopen bij de vakgroep Biochemie van de KUN, onder leiding van prof. dr. Walter van Venrooij en dr. René Hoet. De tweede doctoraalstage werd gelopen vanaf november 1997 tot en met april 1998 bij de Target Discovery unit Oss van NV Organon, onder leiding van dr. Christine Boersma. Het doctoraal-examen in de medische biologie werd in 1998 behaald. In datzelfde jaar werd begonnen aan het promotie-onderzoek beschreven in dit proefschrift, binnen het onderzoeksinstituut Hersenen & Gedrag aan de Universiteit Maastricht, onder leiding van dr. Marc De Baets. Gedurende de eerste twintig maanden werd het promotie-onderzoek verricht op de laboratoria van het Institut für Molekulare Biotechnologie, Rheinisch-Westfälische Technische Hochschule Aachen, Duitsland, in samenwerking met dr. Stephan Hellwig en dr. Stefan Schillberg.

Maurice Stassen was born in Geleen, the Netherlands on the 28th of February 1971. He completed his secondary education in 1990 and started his study of biochemistry at the former college of technology "Hogeschool Heerlen" (now "Hogeschool Zuyd"), the Netherlands the same year. After his internship at the Department of Pathology of the former State University of Limburg (now University of Maastricht), the Netherlands under the supervision of Dr. Ton de Goeij, he took his BSc degree in 1995. In the same year, he started his study of medical biology at the Catholic University of Nijmegen, the Netherlands. For this study, his first internship was at the Department of Biochemistry, Catholic University of Nijmegen under the supervision of Prof. Dr. Walter van Venrooij and Dr. René Hoet from December 1996 till September 1997. After his second and final internship at the Target Discovery Unit Oss of NV Organon, the Netherlands under the supervision of Dr. Christine Boersma from November 1997 till April 1998, he took his MSc degree. In 1998, he became PhD student at the Research Institute of Brain and Behavior, University of Maastricht, the Netherlands under

the supervision of Assoc. Prof. Dr. Marc De Baets. The experiments carried out as a PhD student are described in this thesis. Part of these experiments was performed at the Institute of Molecular Biotechnology, the university of technology "RWTH Aachen", Germany in collaboration with Drs. Stephan Hellwig and Stefan Schillberg.