Experimental autoimmune myasthenia gravis : target organ resistance and immunogenetics

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Experimental autoimmune myasthenia gravis: Target organ resistance and immunogenetics

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"A thousand miles journey begins with a single step."
Lao Tzu, 600 B.C.

Opgedragen aan mijn ouders
Voor Lucie

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ABBREVIATIONS

α-BT	: α-bungarotoxin
AChR	: Acetylcholine receptor
Anti-Id	: Anti-idiotype
CFA	: Complete Freund's adjuvant
CMAP	: Compound muscle action potential
CRI	: Cross-reactive idiotype
EAMG	: Experimental autoimmune myasthenia gravis
ELISA	: Enzyme linked immune sorbent assay
HRP	: Horse radish peroxidase
Id	: Idiotype
mAb	: Monoclonal antibody
MIR	: Main immunogenic region
MG	: Myasthenia gravis
PBS	: Phosphate buffered saline
RIA	: Radio immune assay
SPRIA	: Solid phase radio immune assay
V_H	: Heavy chain variable region
V_L	: Light chain variable region

Introduction

Myasthenia gravis (MG) is an organ-specific autoimmune disease that results from an antibody mediated assault on the muscle nicotinic acetylcholine receptor at the neuromuscular junction (1). Binding of antibodies to the acetylcholine receptor leads to loss of functional acetylcholine receptors, and impairs the neuromuscular signal transmission, resulting in muscular weakness. Although myasthenia gravis is a rare disease with an annual incidence of about 4 per million, it is one of the best studied autoimmune diseases. The disease is examplary for autoantibody mediated target tissue injury.
Our understanding of the pathogenesis of MG is considerably enhanced because of the molecular characterization of the acetylcholine receptor and the presence of a well characterized animal model: experimental autoimmune myasthenia gravis (EAMG) (2, 3). This experimental model is induced by immunization of rodents with purified acetylcholine receptor (AChR) or passive transfer of anti-acetylcholine receptor antibodies into these animals. The EAMG model resembles human MG in many respects (1). Although a great deal of information exists about the immunopathological mechanisms involved in acetylcholine receptor destruction, it is not known what aetiological factors determine the susceptibility for the disease.

The aim of this study was to investigate the factors responsible for the development of the disease in the EAMG model. In this model, the immunesystem can be triggered to produce an autoantibody response against the acetylcholine receptor. One line of approach was to investigate the properties of the anti-AChR antibody repertoire in relation to disease susceptibility. A second line of approach was to induce the effector phase of EAMG and study the immunological and physiological events at the neuromuscular junction; this junction is the target of the autoimmune response against the AChR.
Therefore, two main topics can be discerned in this thesis. First, susceptibility for induction of EAMG by a monoclonal anti-AChR antibody was found to be influenced by age. Age related resistance to EAMG provided a model in which immunological and physiological factors involved in the effector phase of the disease were studied in relation to disease susceptibility.
In the second part of this thesis the interest was focussed on characterization of the anti-AChR antibody repertoire elicited by immunization with AChR.
On the one hand the fine specificity of anti-AChR antibodies was studied in relation to disease susceptibility and on the other hand the diversity of anti-AChR antibodies was investigated by determining the genetic elements that code for these antibodies. The concept that autoantibodies are encoded by a limited number of genetic elements (4-6) was investigated for anti-AChR antibodies.

Chapter 1 is an introduction to the disease myasthenia gravis, its animal model and the target antigen(s) to which the autoimmune response is directed.
Chapter 2 describes the age related resistance to the induction of EAMG in rats. Aged rats, in contrast to young adult rats, were found to be resistant to the induction of EAMG by passive transfer of a monoclonal anti-AChR antibody. The mechanism(s) that underlie the resistance to disease are evaluated.
Chapter 3 introduces the second part of this thesis and reviews antibody structure and the genetic basis of antibody diversity.
Chapter 4 describes the isolation and characterization of anti-AChR receptor monoclonal antibodies from two mouse strains that differ in their susceptibility for the chronic form of EAMG. In addition, the fine specificities of circulating anti-AChR antibodies from high and low susceptible mice were determined at several time points after induction of chronic EAMG.
Chapter 5 describes the diversity in genes encoding the heavy chain variable region of anti-AChR antibodies isolated from high and low susceptible mice.
Chapter 6 characterizes the heavy chain variable region gene families in the rat and the genetic diversity of well defined rat anti-AChR monoclonal antibodies against defined antigenic regions on the acetylcholine receptor.

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Chapter 1

Myasthenia gravis: an autoimmune response against the acetylcholine receptor

Myasthenia gravis: an autoimmune response against the acetylcholine receptor

THE ACETYLCHOLINE RECEPTOR

Introduction

In 1936 Dale et al demonstrated that stimulated nerve-muscle preparations produced acetylcholine, and that this substance caused contractions in muscle preparations (1). The notion of a specific acetylcholine receptor protein was next postulated by Nachmansohn in 1952 (2). Discovery of α-neurotoxins, in particular α-bungarotoxin from the venom of *Bungarus multicintus* made it possible to define the nature of the receptor (3). Isolation of the receptor from the electric organs of *Torpedo californica* was first achieved in the early seventies by several investigators using α-toxins (4-7). Patrick and Lindstrom further characterized the AChR which led to the observation that myasthenia gravis was caused by an autoimmune response against the acetylcholine receptor (8). In the next decade monoclonal antibodies were used to define important pathogenic epitopes, and the use of molecular biology has revealed much information about the structure and function of the AChR.

Structure and function

The transmission of impulses from nerve to muscle depends on release of acetylcholine from the motor nerve ending and subsequent interaction with the acetylcholine receptor (AChR). Binding of acetylcholine to the AChR opens the AChR ion channel, allowing Na^+ ions to enter and to depolarize the muscle membrane, leading to contraction of the muscle. AChRs are located in the postsynaptic membrane at the neuromuscular junction. The postsynaptic membrane has deep infoldings that increase the surface area up to 10 times the lengths of the presynaptic membrane (9, 10). The AChR is situated at the top of these folds at a high density of 8-10 thousend molecules per μm^2 (11, 12).
The amount of AChR in mammalian skeletal musle is about 10 μg/kg. The electric organs of *Torpedo californica* and other electric rays and eels however, provide a richer source of AChR. The electroplax tissue contains approximately 100 mg/kg AChR and therefore was the starting point for purification and characterization of

the receptor. Since the AChR is an evolutionairy well conserved molecule, much information obtained from these fish AChRs can be applied to mammalian skeletal muscle AChR (13, 14). Information about the structural and functional properties of the AChR has been obtained from sequence analysis of the genes that code for the AChR subunits. At present the AChR genes have been sequenced from Torpedo, calf, rat, mouse, chicken (15) and human (16-19). The homology between Torpedo and mammalian AChR (calf) is about 80% for the α-subunit and 55% for the other subunits (20-23).
The AChR of electric fish is a transmembrane protein composed of four different subunits in a stochiometry $\alpha\beta\alpha\gamma\delta$ (24-26). The apparent and calculated protein molecular weights of these subunits from Torpedo AChR are α: 38000 and 50116, β: 49000 and 53681, γ: 57000 and 56279 δ: 64000 and 57565 dalton (13). The AChR molecule is approximately 125 Å long of which the extracellular domain protrudes 55 Å above the membrane and the intracellular domain extends 15 Å below the membrane (14). From above AChRs are seen as 85 Å diameter ring-like structures protruding from the membrane (15, 16). The independent subunits have considerable aminoacid sequence homology as a consequence of their evolution from a common ancestor gene by gene duplication (13). Therefore, the structure and transmembrane orientation is similar between the different subunits. The extracellular domain is formed by the N-terminal 210 aminoacids which are predominantly hydrophylic and contain the acetylcholine binding site near the double cysteine residues at position 192-193 (29, 30). The transmembrane part of the subunits is formed by four hydrophobic transmembrane helices called M1 to M4 (13, 31-34). The cytoplasmic domain of each subunit is formed by a loop between the third and fourth transmembrane domains and contain an amphipathic helix (MA) composed of hydrophobic and hydrophylic residues. The transmembrane domains M1, M2, and M3 from each subunit participate in formation of the ion channel, in which the M2 domain of each subunit lines the pore (35-37).
The sequence homology between equivalent subunits from mammalian species is around 97% for the α-subunit and 90% for the other subunits with the highest homology found in the transmembrane domains M1, M2 and M3 (38).

The AChRs present in foetal (non-innervated) and denervated muscle are found at a low density of 500/μm^2 over most of the sarcolemmal surface (39, 40). Several differences between embryonic (non-junctional) and mature junctional AChR are observed. Non-junctional AChRs have a half-life of about 1 day versus 10 days for junctional AChR and have a lower conductance but a 4 times longer open time compared to junctional AChR (41), and show differences in immunological properties (42-45). The distinctive properties between these AChR types result from the presence of a γ subunit in embryonic AChR which is replaced by the ε subunit in junctional AChR (46-48). This subunit replacement takes place during the first postnatal week in rats (49) and around 33 weeks *in utero* in the human fetus (50).
The acetylcholine binding site is localized at the outer surface of the α-subunits (26), and 2 acetylcholine molecules must bind simultaneously to the 2 binding sites on each AChR to open the cation channel. The effect of acetylcholine is terminated through hydrolysis by acetylcholine esterase anchored in the basal lamina between nerve and muscle. Opening of the cation channel is effectuated in less than 100 μs

and persists for ≈1-2 ms in adult type AChR, followed by a non-conductance state of ≈50 ms during which the AChR is non-sensitive to the following activation by acetylcholine (51-54).

Antigenic structure of AChR in MG and EAMG

In myasthenia gravis the acetylcholine receptor is target of an autoimmune response. The symptoms of the disease are mediated entirely by anti-AChR antibodies since the disease can be passively transferred with IgG (55). Formation of autoantibodies requires AChR specific B lymphocytes as well as AChR specific T helper lymphocytes that stimulate the B cells to make anti-AChR antibodies (56-59). AChR specific B cells recognize mainly conformation dependent determinants of the native AChR, whereas T cells recognize 13-17 amino acid peptides processed and presented by the antigen presenting cell in association with the MHC class II antigen (60). Much effort has been expended on elucidating the antigenic determinants (epitopes) recognized by pathogenic anti-AChR antibodies as well as epitopes activating AChR specific T cells, which may provide clues about the pathogenesis and applications of immune intervention.

B cell epitopes

In order to characterize the B cell epitopes on the AChR, monoclonal antibodies were isolated from mice, rats and humans against the AChR from Torpedo, (61-68), electric eel (69), fetal calf (66), chicken (70) and human (43, 71-73). Several antigenic regions have been characterized on both extra- and intracellular parts of the AChR. A characteristic feature of the AChR is the presence of a main immunogenic region (MIR) to which many anti-AChR mAbs are directed (63, 69, 72). Anti-MIR mAbs can inhibit the binding of 50 % or more of the antibodies to AChR from the serum of MG patients (74, 75), or rats immunized with AChR (76). Antibodies directed to the MIR do not directly impair AChR function (77) but can cross-link adjacent AChR molecules causing AChR loss by increasing the rate of AChR internalization (78). Binding of antibodies to the MIR is highly dependent on the intact conformation of the AChR (76, 79-81). The MIR region is localized on the α-subunit and has been mapped to residues 67-76 by binding to synthetic peptides (82-84). The immunodominant epitope(s) are mainly formed by the residues 68-71 for which a hairpin like loop with a type I β-turn is predicted (85). Anti-MIR mAbs interact with different amino acids within the sequence 67-76 (85-87), but residues 68 and 71 are indispensable for binding of all anti-MIR mAbs (87-89).
Other antigenic regions on the AChR include the residues α128-142 (90) which forms a loop at the extracellular part of the α-subunit and elicit pathogenic anti-AChR antibodies (91). Antibodies to this region are found in most MG patients and around 50 % of total anti-AChR antibodies was found to be directed to this region (92).
Furthermore, antibodies against the acetylcholine/α-bungarotoxin binding site can be demonstrated in most MG patients, by inhibition of α-bungarotoxin binding to

the AChR (93). The binding of α-bungarotoxin and several cholinergic agonists has been confined to the synthetic peptides α160-216 (82), α173-204 (94), α181-200 (95), α185-196 (96), and α189-195 (97) of Torpedo AChR or α181-200 (98), 185-199 (99) of the human AChR.
In general antibody epitopes are dependent on the conformation and particular charge patterns of the native AChR molecule rather than linear sequences (100).

T cell epitopes

Antibodies mainly bind to conformation dependent determinants, whereas T cells frequently recognize epitopes on the linear sequence of a protein. T cell stimulatory epitopes are therefore not necessarilly the same as for antibody production (101). T cells can be activated by native AChR, denatured AChR or its subunits (57, 102, 103). Synthetic peptides corresponding to the primary sequence of AChR have been used to stimulate lymphocytes and select T cell lines or clones specific for immunodominant T cell epitopes on the AChR. Several peptide sequences have been identified that stimulate T lymphocytes from inbred mice and rats. The peptides α97-112 (104), α73-90 and α100-116 (103) of Torpedo AChR strongly stimulate T lymphocytes from Lewis rats whereas T lympocytes from C57bl/6 mice are stimulated by the sequences α195-212 and α259-271 from human AChR (105) and α146-162 from Torpedo AChR (106, 273). It was demonstrated that the T cell response of different animal strains was peptide specific and MHC class II restricted (103, 105). Furthermore, immunodominant T cell epitopes may not necessarily be similar in Torpedo and human (mammalian) AChR (104).
Several sequence regions of the human AChR α-subunit stimulating T lymphocytes from MG patients, have now been identified. Many of these T-cell stimulating epitopes are located within the extracellular part of the α-subunit and include: α1-14, α6-22, α18-34 (107), α48-62, α101-120 (108, 109), α85-142 (110), α146-162 (111) α169-181 (112), α162-216 (113) and α37-181 (114). Immunodominant T cell epitopes were also demonstrated between the transmembrane regions M2 and M3 (115), within the cytoplasmic domain (108, 109, 112), or at the C-terminus of the α-subunit (108, 109). T cell stimulatory epitopes on subunits other than the α-subunit were demonstrated on the δ121-290 sequence (116) and on the γ subunit which is only present in embryonic muscle (117). In summary, these results indicate that individual MG patient's T lymphocytes may recognize more than one antigenic determinant on the AChR subunits and that the T lymphocyte response vary considerable among individual patients with different HLA phenotypes (111, 113). Interindividual variablility of the T cell specificities can be partially explained by the fact that T helper cells recognize the AChR peptides in association with differing MHC class II molecules; the antigenic determinant binding to the MHC class II molecule (agretope) restricts the possible stimulatory epitopes for the T helper cells (118).
T cell epitopes are mostly different from B cell epitopes (81, 103, 119). Immunodominant T cell epitopes are predominantly derived from the α-subunit and are found on both extracellular and cytoplasmic domains of the AChR.

MYASTHENIA GRAVIS

The first description of the symptoms of myasthenia gravis goes back to the year 1672 in which Thomas Willis described a patient with a curious fluctuating weakness which he called 'paralysia spuria non habitualis'. Since then, additional cases were described by Hérard, Charcot, Erb, and Goldflam in the period 1868 to 1893. Jolly et al first described electromyographic abnormalities in MG patients and introduced the name myasthenia gravis pseudoparalytica. Since 1893, many hypotheses addressing the pathophysiology have been proposed including involvement of toxic or curariform substances, influencing the motor neurons or endplate, and abnormalities of the thymus (120). An important contribution to the understanding and treatment of myasthenia came from Mary Walker who noticed that the symptoms of MG resembled curare poisoning. Hence, she treated MG patients with physostigmine, an agonist for curare intoxication which resulted in a considerable improvement of the disease (121). From this time it was assumed that the distrurbance causing the symptoms was localized at the neuromuscular transmission.
The autoimmune nature of MG was first proposed in 1960 by Simpson. He suggested that in myasthenia gravis antibody to endplate protein may be formed, which would have the properties of an acetylcholine-competitive-blocking substance (122). In the same year Nastuk observed that MG sera were cytotoxic to muscle membranes (123). Ultrastructural evidence for a postsynaptic defect in MG was provided by Engel and Santa (124). The first experimental indication for the autoimmune nature of MG was provided by Patrick and Lindstrom who induced MG like symptoms in rabbits by immunizing with purified AChR (8). In the same year Fambrough et al showed a reduction in the number of junctional AChRs in biopsies of MG patients (125). Subsequently, antibodies against the acetylcholine receptor were detected in the sera of MG patients (93, 126). Since then, much information about the immunological and pathophysiological mechanisms has been compiled.
Several acquired and congenital myasthenic syndromes can be distinguished. Acquired MG is a postsynaptic disorder of neuromuscular transmission in which at least three disease entities can be distighuised: I. Idiopathic autoimmune MG, II. Transient MG induced by the drug D-penicillamine (127), and III. Transient neonatal MG induced by maternal anti-AChR antibodies (128). Another acquired myasthenic disorder is the Lambert-Eaton myasthenic syndrome in which autoantibodies against the presynaptic voltage gated calcium channels impair neuromuscular transmission (129).
Congenital MG has a non-immunological aetiology and includes several pre- and postsynaptic defects regarding acetylcholine (re)synthesis, packaging and mobilization, acetylcholine esterase deficiency, AChR deficiency or regulation of AChR density, AChR ligand affinity, and abnormal gating properties of the AChR (130, 131, 133).
Acquired myasthenia gravis is an autoimmune disease affecting neuromuscular transmission. Autoantibodies directed against the acetylcholine receptor at the postsynaptic membrane result in fluctuating weakness of voluntary muscle and excessive fatigue. The disease usually has a variable course depending in part on the magnitude of the autoimmune response, but also on fluctuations in AChR reserve and the synaptic quantal content. The symptoms may be confined to ocular

or bulbar muscles or may be generalized also involving limb and trunk muscles, in part depending on the exertion of the muscles concerned. The impaired neuromuscular transmission is electromyographically characterized by decrement of action potentials upon repetitive stimulation and an increased jitter, which is an increased variability in neuromuscular transmission in separate endplates (134). Symptoms of MG are temporarily relieved by acetylcholine esterase inhibitors or rest (121). A more persistant remission of severe cases can be obtained by thymectomy, plasmaphoresis, and immunosuppresion (120). Antibodies against human AChR are found in 85-90 % of MG patients with generalized symptoms and in 70 % of patients with ocular symptoms (126, 135). The majority of MG sera lacking IgG anti-AChR antibodies in conventional radio immuno assays contain IgM antibodies that impair AChR function *in vitro* (136).
MG has a prevalence of about one out of 20000, and is most frequently observed in women of the child bearing age or men over 40 years (120). Patients with MG have been divided into three groups based on thymic pathology, age of onset and associations with different HLA polymorphisms (137).

	subgroup I	subgroup II	subgroup III
thymic pathology	hyperplasia	thymoma	thymic atrophy
age of onset	<40	30-60	>40
HLA association	B8, DR3	—	B7, DR2
sex predominance (M:F)	1:3	1:1	2:1

Although not absolute, the association with these haplotypes suggests a strong role for immunogenetic factors in disease susceptibility. Analysis of a HLA-DR3 subregion revealed that MG patients expressing this region have a relative risk of 35 compared to controls (138). Furthermore, a region between HLA B8 and the TNF locus within the MHC region, was found to be present in all group I patients possibly containing genes that determine MG susceptibility (139) However, the obvious differences in thymic pathology and HLA associations suggests that other aetiological factors or predisposing factors must contribute to the triggering of the autoimmune response in MG (140).

Possible aetiologic factors in MG

Although MG is one of the best characterized autoimmune diseases it is not yet determined what initiates and sustains the autoimmune response to AChR. Environmental factors like viral infection have been proposed to trigger loss of tolerance to AChR (141). However, no virus could be isolated from MG thymuses (142, 143) and the incidence of serum antibodies against several viruses was not significantly different from matched controls (144). Molecular mimicry between bacterial or viral determinants and epitopes on the AChR was also proposed as an initial trigger (145-150). Sharing of epitopes was demonstrated between the human sequence

α160-167 and the herpes simplex virus glycoprotein (146, 148, 149), and partial sequence similarity was found between the MIR α67-76 and the polymerase polyprotein sequence of several human retroviruses (150). In addition, the MIR sequence is highly homologous to the U1 small nuclear ribonucleoprotein, which is a marker autoantigen for SLE and mixed connective tissue disease (150).
Another postulated mechanism triggering an autoimmune response is binding of a neoantigen to the acetylcholine receptor (151). The drug D-penicillamine induces a transient form of MG in which anti-AChR antibodies and AChR loss is observed (127). The disease is reversed in about 8 months after D-penicillamine is omitted (152). The antigenicity of the AChR may be altered by binding of penicillamine, resulting in a transient loss of tolerance (151). In both D-penicillamine MG and neonatal MG anti-AChR antibodies are present which induce but do not sustain the disease. The fact that remission from the antibody mediated attack of the neuromuscular junction occurs in these forms of MG indicates that the mere presence of antibody is not sufficient to account for development of MG; additional factors must be present to break tolerance completely, thus perpetuating the disease for many years. A clue may be found in the fact that MG is sometimes observed after bone marrow transplantation in humans (153, 154). A possible defect in T cell education (selection) in the thymus may underlie this phenomenon and may eventually lead to loss of tolerance to the highly immunogenic AChR. Interesting evidence for this hypothesis is provided by an animal model for cyclosporin induced graft versus host disease (155) in which rats are lethally irradiated, reconstituted with syngeneic bone marrow and treated with cyclosporin for 4 weeks. After withdrawal of cyclosporin treatment, animals develop several autoimmune phenomena including occasionally myasthenia gravis (156). The thymus may be 'leaky' during this treatment, and autoreactive clones probably escape from selection or suppression in the thymus (155).

The role of the thymus in MG

Thymic abnormalities are frequently found in myasthenia gravis patients (157, 158), suggesting an important aetiological role for the thymus. Thymic hyperplasia is frequently found among patients under 40 years of age and thymectomy often leads to improvement of MG (159). The hyperplastic thymus is characterized by germinal centers and large T cell areas. Both anti-AChR antibody producing B cells (160) and AChR specific T cells (59) are present in hyperplastic thymuses. Myoid cells expressing embryonic type AChR are demonstrated in normal or MG thymic medulla (161, 162). Interaction of myoid cells with interdigitating cells may initiate an autoimmune response (163).
Thymomas are found in approximately 15 % of MG patients and are usually associated with a later age of onset of the disease (120). The AChR is not present in thymoma tissue (162, 164, 165). However, mRNA coding for the α-subunit could be amplified from thymoma epithelial cells using the polymerase chain reaction (166). Other proteins (45 kd and 153 kd) biochemically different from AChR but sharing an intracellular epitope with the AChR, were demonstrated on thymoma epithelium of MG patients (167). Thymomas associated with MG are selectively

enriched in AChR reactive T cells (168). However, synthesis of anti-AChR antibody does not occur in the thymoma tissue itself, but in adjacent hyperplastic tissue (169). The anti-AChR antibody production in the MG thymus correlates well with serum titer. The *in vitro* production of anti-AChR antibodies by periferal blood lymphocytes was shown to disappear after thymectomy (170). However, the serum anti-AChR titer falls to a varying extent after thymectomy (171, 172). Moreover, AChR responsive T lymphocytes can still be detected (173). These findings indicate that the thymus is not the only site of anti-AChR antibody production (171, 172); the majority of the antibody is probably produced in the bone marrow (174).

EXPERIMENTAL AUTOIMMUNE MYASTHENIA GRAVIS

An experimental model for myasthenia gravis was discovered fortuitously in 1973 by Patrick and Lindstrom characterizing the acetylcholine receptor from electric eel. Rabbits immunized with purified AChR developed muscular weakness characteristic for MG. As in MG, the clinical signs diminished after treatment with acetylcholine esterase inhibitor (8). Experimental Autoimmune Myasthenia Gravis (EAMG) can be induced in mice, rats, guinea pigs and monkeys (175-177) by immunization with AChR from the electric organs of Electrophorus electricus or Torpedo californica.
EAMG provides an excellent model for MG and is very similar to MG with respect to electrophysiological, biochemical and immunological parameters (178). The two major differences between MG and EAMG are the absence of thymic abnormalities (179) and the self-limiting nature of the autoimmune response in EAMG. Although EAMG provides only little information about the events initiating MG, it shows that the autoimmune response in MG is similar to that obtained upon immunization with native AChR.

Induction of acute and chronic EAMG

EAMG can be induced in laboratory animals by immunization with purified syngeneic AChR (180) fetal calf AChR (181) and AChR from the electric organs of Electrophorus electricus or Torpedo californica (180) incorporated in complete Freund's adjuvant (CFA). EAMG is commonly induced by immunization with Torpedo AChR. Although there is a 80 % sequence homology between Torpedo and mammalian AChR α-subunit (20), only about 2% of anti-Torpedo AChR antibodies cross-react with syngeneic AChR (182). However, native Torpedo AChR is highly immunogenic inducing autoantibody concentration typical for MG patients after immunization with 1 μg or more Torpedo AChR. The first clinical signs of EAMG are seen 4 to 6 weeks after immunization when autoantibody titers peak (183). Affected animals show a generalized weakness, are unable to support their weight, have a hunched back, abducted thighs and flexed digits. In the chronic stage animals have difficulties in swallowing and show excessive growth of the teeth due to impairment of the masticatory muscles. Animals die from respiratory insufficiency, malnutrition and dehydration (176, 184).

When rats are immunized with Torpedo AChR in CFA with additional Bordetella pertussis vaccin, a biphasic course of the disease is observed (176). The acute form is seen 8-10 days after immunization and is transient. During the acute phase muscle endplates are invaded by phagocytic cells intruding into the muscular junction probably leading to focal denervation (184, 185). Animals recover from acute EAMG upon disappearance of phagocytic cells from endplates and stay in remission until 4-6 weeks after immunization when the first signs of chronic EAMG arise. When additional Bordetella pertussis vaccin is omitted, only the chronic phase of EAMG is observed (178).
The great immunogenic potential of AChR was demonstrated in mice by repeated intraperitoneal immunization of membrane bound (186) or purified syngeneic AChR (187) from the mouse tumor cel line BC3H-1 without adjuvant. Immmunization of mice with Torpedo AChR without adjuvants is also possible though less effective (188).
EAMG can also be induced by the individual AChR subunits (189) or synthetic peptides corresponding to defined regions of the AChR. Repeated immunization with a synthetic peptide corresponding to residues 125-147 of the α-subunit from Torpedo (91) or human AChR (190, 191) induced T cell responses and antibodies to native AChR, capable of modulating AChR *in vitro*. Peptide immunized rats showed reduced miniature endplate potentials and loss of AChR characteristic for EAMG. Another peptide, corresponding to α-subunit residues 183-200 containing the cholinergic binding site, induced EAMG by immunopharmacological blockade of the acetylcholine binding site (192). Both modulating and blocking antibodies were found after immunization of rats with recombinant human α1-210 fusion protein produced in E. coli, which comprises the whole extracellular part of the α-subunit (193). Other groups however, did not find electrophysiological or biochemical evidence for EAMG in mice, rats and rabbits immunized with synthetic peptides or recombinant human α-subunit (194-197). This may be explained by the absence of the proper three-dimensional structure in short synthetic peptides, since antibody epitopes are highly conformation dependent and anti-peptide antibodies do not necessarily bind to the native AChR. Furthermore, peptides and bacterial fusion proteins are not glycosylated which may influence antibody binding.

Passive transfer EAMG

EAMG can be induced by passive transfer of polyclonal (198, 61) or monoclonal (199-201) anti-AChR antibodies indicating that the disease is entirely mediated by antibody. Similar clinical signs as in chronic EAMG are seen in passive transfer EAMG 12 to 48 hrs after injection of anti-AChR antibody (202). Animals usually die within 72 hours or recover after clearance of the antibody. Recovered rats were found to be resistant to another passive transfer up to 11 weeks after the initial injection (203). Passive transfer EAMG resembles the acute form of EAMG in that infiltrating mononuclear cells are observed around the end plate region (204).
A chronic form of EAMG can be induced in rats by repeated administration of anti-AChR monoclonal antibody for 9-12 weeks, resulting in AChR loss, ultrastruc-

tural simplification of endplates and decreased miniature endplate potentials, but no clinical weakness (205).

Disease susceptibility

Susceptibility to induction of EAMG and severity of disease symptoms may vary considerably among different species and between different strains of a species. Considerable diffences are found among inbred rat strains: Wistar Munich and Fischer rats consistently develop EAMG, Lewis and BN rats show intermediate susceptibility and Wistar Furth and Copenhagen rats are resistant to EAMG (206). Cross-breeding of different strains showed that disease susceptibilty is recessive and is linked to one or two genetic loci.
Mice are less susceptible for induction of EAMG than rats and development of muscular weakness is only seen after a second immunization with Torpedo AChR (207). The susceptibility to EAMG differs in genetically defined strains of mice (208, 175) and is determined by several loci including the major histocompatibility complex, immunoglobulin and complement alleles (209, 210). The I-A_β chain of the mouse MHC class II molecule was shown to play an important role in the T-cell response to Torpedo AChR (211). In addition, a mutation at the I-A_β chain in C57bl/6^{bm12} mice prevents in part EAMG *in vivo* (212). Several other physiological factors, which together determine the "safety factor" of neuromuscular transmission (130), influence the susceptibility for EAMG.

Pathology of acetylcholine receptor destruction

Clinical manifestations and disease severity of EAMG in animals can be diagnosed by several means including endurance tests to measure muscular weakness (175, 176), loss of body weight caused by the inability to eat or drink (179), or electromyographic evaluation of the neuromuscular transmission. An impaired neuromuscular signal transmission can be demonstrated by measuring the decline in the amplitude of the compound muscle action potential upon repetitive nerve stimulation (decrement) (213), or by stimulated single fiber electromyography measuring the variability of the time interval between stimulus artefact and the action potential of a single muscle fiber (jitter) (214, 215).
Induction of EAMG is accompanied by several pathological changes at the neuromuscular junction. Different pathology can be observed during the bi-phasic course of EAMG. During the acute phase of EAMG, between day 7 and 11, an inflammatory exsudate composed of mononuclear phagocytic cells and some neutrophils is seen around the endplate. Occasional muscle fibers show segmental necrosis and invasion of macrophages immediately adjacent to the endplate region. Ultrastructural evaluation of the neuromuscular junction reveal degeneration of the terminal expansions of the postsynaptic membrane and a widened synaptic space filled with debris. The nerve terminal remains intact but is separated from the muscle fibers by infiltrating macrophages which may cause local denervation. The length of the highly simplified postsynaptic membrane is decreased (184, 185). These pathologi-

cal changes observed during the acute phase of EAMG are also seen 24-72 hours after passive transfer of anti-AChR antibodies (179, 216). Remission from acute phase EAMG coincides with disappearance of the phagocytic cells and the reestablishment of close contact between nerve terminals and the regenerating postsynaptic region. After 3-4 weeks some animals relapse into the chronic phase of EAMG in which the degeneration of the postsynaptic membrane becomes more intense. A renewed invasion of macrophages is not seen (184, 185). This chronic stage in EAMG is characterized by loss of 50-70 % of the muscle AChR (180), and the morphological changes at the neuromuscular junction resemble those found in human MG (184, 185, 124). However, no equivalent for an acute phase in human MG has been demonstrated so far (128).

Pathogenic mechanisms

Several lines of evidence have clearly demonstrated that MG is an antibody mediated autoimmune disease. Passive transfer of IgG from MG patients to mice induces the clinical signs of MG including decrement and AChR loss (198, 217). Furthermore, transient neonatal MG can be observed in babies from myasthenic mothers and is dependent on circulating anti-AChR antibodies in the baby (218, 219). Removal of anti-AChR antibodies from the patient by plasmaphoresis (or immuneadsorbens) has been proven to be beneficial in the treatment of MG (220-222).
The degradation or inactivation of functional AChR by anti-AChR antibodies observed in MG is probably caused by several mechanisms. The major part of the anti-AChR antibodies do not directly inhibit AChR function but lead to loss of AChR.

Antigenic modulation:
First, anti-AChR antibodies may result in AChR loss by cross-linking of adjacent receptor molecules thereby accelerating the AChR turnover rate (223, 224). Under normal conditions AChR is turned over by endocytosis and intracellular proteolytic degradation within lysosomes. Cross-linking of AChR molecules is called antigenic modulation, and increases the degradation of AChR on cultured muscle cells *in vitro* by a factor 2 to 6 (223-228). Antigenic modulation is temperature and energy dependent but independent of complement (225, 229). Antigenic modulation by purified IgG from EAMG rats could be demonstrated *in vitro* using the mouse muscle cell line BC3H-1 or diaphragms removed from rats (230, 231). Antigenic modulation is dependent on divalent antibodies since monovalent antigen binding fragments (Fab) were ineffective (224, 230). Antibodies directed to the MIR or other extracellular epitopes are especially effective in modulating the AChR (232). In fact 68-80 % of the modulating capacity of MG sera can be blocked by the Fab fragment of an anti-MIR mAb (233, 234).
Several studies have investigated the modulating potential of MG sera using rat muscle culture (223, 224, 235-238), mouse BC3H-1 cells (228, 229, 239, 240), human myotubes (233) or the AChR expressing human medulloblastoma cell line TE671 (234). Modulating antibodies were found in 66-100 % of MG patients sera (233, 235, 237, 238). The modulating potential of MG sera was mainly dependent

on the anti-AChR antibody titer (233). A correlation between observed AChR degradation by MG sera and the severity of MG is a topic of debate. A correlation between the degrading potential and the clinical state of the patient was found by some investigators (235, 237, 238) but contradicted by others (233, 236). It was however clearly demonstrated in all studies that antibodies from patients with mild or severe MG symptoms may have the same modulating capacity, suggesting that additional factors determine the patient's susceptibility to the disease.

Complement mediated lysis:
Another major mechanism of AChR degradation is the complement mediated focal lysis of the postsynaptic membrane. Binding of antibody to the AChR can activate the classical complement pathway generating the anaphylotoxins C3a and C5a which trigger an inflammatory reaction and formation of the lytic membrane attack complex (MAC) by components C5b-C9 that form a pore in the cell membrane leading to focal lysis of the muscle membrane (241). Complement component C3 and the MAC have been demonstrated at the neuromuscular junction of MG patients (242, 243) and a linear correlation was found between the length of the postsynaptic membrane bound with C3 and the decrease in miniature endplate potential (242). Moreover, MG sera induce complement dependent myotoxicity *in vitro* in rat myotubes (244, 245). C3 containing immune complexes at the neuromuscular junction were also demonstrated in chronic (246) and passive transfer EAMG (216). In both MG and chronic EAMG complement contributes to AChR degradation by focal lysis of the postsynaptic membrane. In addition, the early complement components C3a and C5a together with C3b and C3bi may attract and bind phagocytic cells to the damaged endplate in acute or passive transfer EAMG. The important contribution of complement mediated degradation to the pathogenesis of the disease was demonstrated in complement deficient or complement depleted animals. C4 deficient guinea pigs or C5 deficient mice immunized with AChR had significant less AChR loss and failed to develop clinical signs of chronic EAMG (210, 247). Similar, acute or passive transfer EAMG by mono- and polyclonal anti-AChR antibodies failed to develop in C4 deficient guinea pigs (248), or C3 depleted rats or mice (249, 55). Induction of passive transfer EAMG could also be prevented by pretreatment of rats with anti-complement C6 Fab antibody which inhibits the formation of the MAC (250). Anti-C6 treatment completely inhibited the accumulation of macrophages indicating that AChR degradation is dependent on focal lysis of the postsynaptic membrane and antigenic modulation of AChRs rather than the action of macrophages (250).

Functional inactivation of AChR:
The binding of anti-AChR antibodies may also result in functional inactivation of the AChR by interfering with either acetylcholine binding or impairment of the ion channel function. Isolation of monoclonal antibodies from AChR immunized animals demonstrates the existance of antibodies blocking the cholinergic binding sites (62, 64, 65, 68, 251) or inhibiting ion channel function (252). MG sera were demonstrated to reduce the MEPP under non-degrading conditions, indicating the presence of antibodies that directly block AChR function (253-255). Blocking antibodies have been demonstrated in MG sera by inhibition of α-bungarotoxin binding to AChR in

muscle culture or TE671 cells (93, 237, 256-263). The number of MG patients having blocking antibodies varied from 48-98 %, depending on the assay system used (237, 259-261). The relevance of blocking antibodies *in vivo* was shown by the protective effect of some MG sera against the lethal toxicity of α-bungarotoxin in mice (262). The amount of blocking antibody in MG patients may vary from 0-33 % of total anti-AChR antibodies (258). The blocking capacity of MG sera was often not related to the severity of the disease in generalized MG. Blocking antibodies are absent from sera of asymptomatic or ocular MG patients (260, 261).
Antibodies interfering with the ion channel function of AChR were demonstrated among MG and EAMG anti-AChR antibodies which reduced the agonist-induced Na^+ influx through the AChR in TE671 cells (264, 265, 136). The action of blocking antibodies may be important especially when the AChR number is reduced.

The cellular immune response to AChR

Several experiments in the EAMG model demonstrated T cell mediated immunity against AChR. A short and transient delayed type hypersensitivity reaction to AChR was observed 3 days after immunization (56). EAMG can be transferred to naive recipients by lymphnode cells (56). However, passive transfer of antibody alone is sufficient to induce all the features of MG. In addition, lymphocytes from EAMG animals have no direct cytotoxic effect on muscle cells *in vitro* (266). Therefore, antibodies against the AChR clearly mediate the effector phase of myasthenia gravis. However, the production of anti-AChR antibodies is T cell dependent. This was demonstrated *in vivo* in rats depleted by X-irradiation and thymectomy and subsequently reconstituted with B cells alone, which were unable to mount an immune response against the AChR, thus preventing the induction of EAMG (56). Similarly, anti-AChR antibody production by lymphnode cells from EAMG rats *in vitro* was completely prevented when T cells were selectively depleted (57). The AChR specific T cells express the helper phenotype and proliferation is MHC class II restricted (268).
Infiltration of macrophages was observed during the acute phase of EAMG or after passive transfer of anti-AChR antibody (184, 185, 204). In contrast, these cells are not observed in chronic EAMG (246). An equivalent of this acute phase has never been demonstrated in human MG, and may not be detected during early development of the disease (178). The role of inflammation in human myasthenia gravis is still controversial. Inflammatory infiltrates are only rarely seen in intercostal and limb muscle biopsies (124, 9, 269, 270), whereas others demonstrated mononuclear cells at the endplate regions in triceps- (271) or anconeus muscle (272). Whether these cells actively contribute to the disease process, or function as phagocytic cells that sequester the remnants of the destructed postsynaptic membrane, remains to be determined.

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Chapter 2

Age related resistance to experimental autoimmune myasthenia gravis in rats

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Age related resistance to experimental autoimmune myasthenia gravis in rats

ABSTRACT

The influence of age on the induction of experimental autoimmune myasthenia gravis (EAMG) was investigated. Immunization with acetylcholine receptor (AChR) or injection of varying amounts of anti-AChR mAb 35 into young adult (10-12 wk) BN rats induced severe signs of EAMG including weight loss and decrement of muscle action potential, whereas aged BN rats (120-130 wk) did not show any clinical or electrophysiological signs of EAMG. Serum anti-AChR mAb titers were not significantly different in young and aged rats up to 24 hrs after administration of mAb. No significant AChR loss was demonstrated in aged rats, whereas similar treated young rats showed extensive AChR loss. In contrast to young rats, no degradation of the postsynaptic membrane could be demonstrated by electron microscopy in aged rats. Complement component C3 could be demonstrated at the neuromuscular junction in both young and aged mAb treated rats. However, infiltrating macrophages and necrotic muscle fibers were only seen in young rats. These results suggest that the postsynaptic membrane in aged rats is resistant to autoantibody attack. AChR degradation by antigenic modulation may be less efficient in aged rats as a result of altered AChR density and distribution or rigidity of the postsynaptic membrane. Age related resistance in the EAMG model can provide more information about the factors that determine the severity of myasthenia gravis (MG). Manipulation of AChR density or lipid compostion of the postsynaptic membrane may be of therapeutic interest in MG.

INTRODUCTION

Myasthenia gravis is an autoimmune disease in which antibodies to the acetylcholine receptor result in a defective neuromuscular transmission. Clinical symptoms characteristic for myasthenia gravis can be induced in experimental animals by immunization with AChR (1) or passive transfer of polyclonal (2, 3) or monoclonal anti-AChR antibodies (4-7). Passive transfer of anti-AChR antibody brings about muscular weakness and excessive fatigue reaching a maximum at 48 hrs after injection, and disappears after one week. Three main pathogenic mechanisms contribute to the degradation of AChRs at the neuromuscular junction (NMJ). I Anti-AChR antibodies cross-link AChRs resulting in an increased internalization of AChRs by antigenic modulation (8). II Activation of complement results in destruction of the postsynaptic membrane by focal lysis (9). Furthermore, in acute and passive transfer EAMG, complement components attract macrophages that sequester remnants of the postsynaptic membrane and cause disruption of the NMJ (10). III Inactivation of AChR function by interference of antibody with ligand binding (10, 11) or ion channel function (12). These pathogenic mechanisms lead to AChR loss and simplification of the highly folded postsynaptic membrane.
This study investigates the influence of age on the induction of experimental autoimmune myasthenia gravis in the rat by immunization with Torpedo AChR or passive transfer of anti-AChR mAb 35 to young adult and aged BN rats. Aged rats in contrast to young rats appeared to be resistant to induction of both chronic and passive transfer EAMG.
Several parameters were studied in order to explain the observed resistance to induction of passive transfer EAMG. Resistance to induction of EAMG in aged rats could not be attributed to differences in antibody uptake or clearance. Complement was activated at motor endplates of both young and aged rats, but segmental muscle necrosis and infiltration of macrophages at motor endplates could only be demonstrated in severly affected young rats. These results suggest that complement mediated lysis of the postsynaptic membrane is not sufficient to induce EAMG and antigenic modulation of AChR may be less effective in aged rats as a consequence of age dependend changes in AChR density or fluidity of the postsynaptic membrane.

MATERIAL AND METHODS

Animals
Inbred 10-12 week old male and female Brown Norway rats were obtained from the the breeding colonies of the Department of Experimental Animal Services of the University of Limburg or from TNO, Rijswijk, The Netherlands. All animals were bred under specific pathogen free conditions.

Induction of chronic experimental autoimmune myasthenia gravis
Rats were immunized at the base of the tail with 10 µg/100 gr bodyweight Torpedo AChR emulsified in CFA. Rats were boosted with 10 µg/100 gr Torpedo AChR in IFA 6 weeks after primary immunization.

Induction of passive transfer experimental autoimmune myasthenia gravis
Rats were injected intraperitoneal with ascitic fluid or culture supernatant containing rat anti-AChR mAb 35 (a kind gift of Dr. S.J. Tzartos, Institute Pasteur Helenique, Athens, Greece). Control rats were injected with an equal amount of normal rat serum. MAb 35 is a rat mAb directed to the main immunogenic region on the α-subunit of the AChR (7). Rats were injected with a mAb 35 dose having a AChR binding capacity of 20 or 60 pmol rat AChR/100 grams bodyweight.

Determination of anti-rat AChR antibody titers
Antibody titers against rat AChR (rAChR) were measured by immunoprecipitation using ^{125}I-α-bungarotoxin (^{125}I-α-BT) labeled rAChR according to Lindstrom et al (13) with minor modifications. Briefly, a crude extract of denervated rat muscle AChR (approximately 0.6 nmol/l) was labeled with $2x10^{-9}$ M ^{125}I-α-BT for 4 hrs at 4 °C. A dilution range of serum samples (5 µl) were incubated with 200 µl labeled rAChR overnight at 4°C. Antibody-AChR complexes were precipitated by adding excess goat-anti-rat IgG antibodies. After 4 hrs at 4 °C, samples were centrifuged for 3 min in an Eppendorf centrifuge and subsequently washed once with 1 ml PBS buffer containing 0.5 % Triton-X100 (Sigma, Brunschwig chemie b.v., Amsterdam, The Netherlands) and 0.02 % NaN_3 (PBS/TX). Radioactivity was counted in a gamma counter (Compugamma, Pharmacia LKB, Woerden, The Netherlands). Values of ^{125}I-α-BT-rAChR pelleted in the presence of normal rat serum were considered background and substracted from the assay values. The antibody titers were expressed as moles of ^{125}I-α-BT binding sites per liter.

Determination of antibody clearance
The clearance of antibody from the circulation of young and aged rats was determined using ^{131}I labeled IgG (^{131}I-IgG). Three days prior to injection of ^{131}I-IgG, the drinking water supplied to the rats was supplemented with 0.1 % w/v KI to prevent ^{131}I-IgG uptake in the thyroid glands. ^{131}I-IgG was injected intravenously into the tail vein and blood samples were taken 0.25, 5, 24, 48 and 72 hrs after administration. The clearance of ^{131}I-IgG was expressed as the percentage of remaining radioactivity at several points in time after administration. The amount of ^{131}I-IgG measured 15 min after i.v. administration was considered 100 %.

Quantitation of AChR and antibody-AChR complexes in muscle
The concentration of AChR and antibody-AChR complexes was determined in whole carcasses according to Lindstrom et al (13) with modifications as previously described (14). The AChR concentration in muscle was expressed as pmol AChR/100 gr muscle. Antibody-AChR complexes were expressed as the % of the total AChR concentration.

Electromyographic evaluation
The decrement of the compound muscle action potential (CMAP) was determined in the gluteus medius muscle 48 hrs after administration of mAb 35. Rats were anaesthetized with 800 µl/kg ketamine (Nimatek). The body temperature during measurements was monitored with a thermal probe and the skin temperature was maintained at 33-35 °C by means of an infrared heating lamp (DISA, Copenhagen,

Denmark). Nerve stimulation and recording of CMAP was performed using monopolar teflon coated steel needles with a 0.8 mm bare tip. The recording electrodes were placed subcutaneously, one over the belly and the other in the distal tendon region of the gluteal muscle. The CMAP was recorded in response to supramaximal stimuli applied through needle electrodes to the superior gluteal nerve. In a pilot study in myasthenic rats we found decrement to be more pronounced with stimulation at 10 Hz than at 3 or 5 Hz, Therefore, 10 Hz stimulation was used in the present study. During each test 7 stimuli of 0.2 ms were given. The results of triplicate series were averaged and a positive decremental response was defined as an amplitude decrease of more than 10 % of the fifth response compared to the first response (15, 16, 17). Measurements were performed with a Viking EMG machine (Nicolet Biomedical Instruments, Madison, WI, U.S.A.).

Electron microscopy of neuromuscular junction
Muscle biopsies taken from young and aged rats 48 hrs after administration of mAb 35 were fixed in Periodate-Lysine-Paraformaldehyde for 90 min at 4°C. Cryosections were fixed in 1% osmium tetroxide for 60 min at room temperature (R.T.), dehydrated and embeded in epon 812. Ultrathin sections from regions with endplates were contrasted with uranyl acetate and lead citrate. Endplates were viewed using a Zeis 109 electron microscope.

Localization of C3
Deposition of complement at endplates was determined in cryosections of muscle biopsies taken from young and aged rats 48 hrs after administration of 20 pmol/100 gr bodyweight mAb 35 or normal rat serum. Cryosections were aceton fixed for 10 min at 4°C and air dried for 5 min. After 3 washes with PBS, the sections were preincubated with PBS containing 2% bovine serum albumin for 15 min and subsequently incubated with polyclonal rabbit-anti-rat C3 antibodies together with rhodamine labeled α-BT (Molecular Probes, Eugene, OR USA.) for 1 hr at R.T.. After 3 washes with PBS, sections were incubated with FITC conjugated swine-anti-rabbit antibodies for 1 hr to visualize bound anti-C3 antibodies.

Detection of macrophages
Muscle sections were stained for macrophages using mAb ED 1 (18). Bound ED 1 was visualized using FITC labeled goat-anti-mouse Ig (Cappel, Organon Technika, Boxtel, The Netherlands). AChR was localized in the same section by co-incubation with rhodamine labeled α-BT.

Statistical analysis
The students T-test and the Wilcoxon rank test were used for statistical analysis.

RESULTS

Induction of chronic EAMG in young and aged rats

To investigate the influence of age on the development of EAMG we induced EAMG in young adult (10-12 wk) and aged (120-130 wk) old rats by immunization with Torpedo AChR. Ten weeks after primary immunization both young and aged rats developed a similar antibody titer against rAChR (table I). At this point in time 9 out of 10 young rats showed muscular weakness and showed a mean AChR loss of 57±2 %. In contrast, aged rats showed no muscular weakness or AChR loss (table I).

Table I: Aged rats are resistant to induction of chronic EAMG

Rat group	Age	N	Ab titer[a] nmol/l	[AChR][b] pmol/100 gr muscle	AChR loss[c] %	AChR-Ab[d] %
young	12 wk	10	23 ± 4	24 ± 1	57 ± 2	37 ± 4
aged	125 wk	10	26 ± 9	52 ± 5	1 ± 10	32 ± 6

The mean antibody titer against rAChR, the total AChR concentration and the fraction of AChR complexed with antibody was determined 10 weeks after initial immunization with Torpedo AChR. Rats were immunized with 10 µg Torpedo AChR/100 gr bodyweight. [a] Antibody titer against rAChR (mean±SEM) 10 weeks after immunization. [b] The AChR concentration determined in whole carcasses and expressed as pmol AChR/100 gr muscle to compare animals of different bodyweight. [c] AChR loss is expressed as percentage of the AChR concentration in unmanipulated control rats. [d] The amount of AChR complexed with antibody is expressed as the % of the remaining amount of AChR.

Induction of passive transfer EAMG in young and aged rats

It was verified whether this resistance to induction of chronic EAMG was also observed when EAMG is induced by passive transfer of anti-AChR antibody. Therefore, passive transfer EAMG was induced in (10-12 wk) and aged (120-130 wk) BN rats by injection with anti-AChR mAb 35. Administration of 20 pmol mAb 35/100 gr bodyweight to young rats, resulted in clinical manifest EAMG characterized by severe muscular weakness and paralysis at 48 hrs after administration. Affected rats were unable to eat or drink and rapidly losed weight due to dehydration. Young rats showed extreme weight loss compared to control rats treated with normal serum (fig. 1). In contrast, aged rats treated with 20 pmol mAb 35/100 gr clearly showed no signs of EAMG and a marginal weight loss comparable to control rats. Even when aged rats were treated with 60 pmol mAb 35/100 gr mAb 35, no clinical signs were present whereas young rats, treated with the same mAb 35 dose, died within 24 hrs of respiratory failure. Again young rats showed extreme weight loss whereas aged rats were comparable to control young or aged rats (fig. 1).

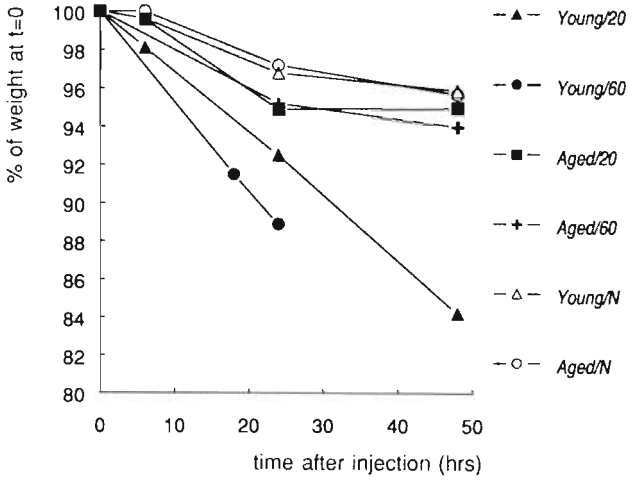

Figure 1: Weight loss as measure for EAMG severity.
The relative weight loss was determined at several points in time after injection of mAb 35. Each point represents the mean weight as % of the weight at the start of the experiment. Young/20: young rats injected with 20 pmol mAb 35/100 gr bodyweight. Young/60: young rats injected with 60 pmol mAb 35/100 gr. Young/N: young rats treated with normal rat serum. Aged/20: aged rats treated with 20 pmol mAb 35/100 gr. Aged/60: aged rats injected with 60 pmol mAb 35/100 gr. Aged/N: aged rats treated with normal rat serum.

Electromyographic evaluation of neuromuscular transmission

The decrement of the compound muscle action potential (CMAP) was determined to confirm the observed resistance to the induction of passive transfer EAMG in aged opposed to young rats. The decrement in young and aged rats was determined 48 hrs after administration of mAb 35. An example of a typical recording from a myasthenic rat is shown in fig. 2A. The decrement in CMAP of aged rats (n=5) injected with 60 pmol/100 gr was always within the normal range of less than 10 % (fig. 2B). In contrast, young rats (n=4) injected with 20 pmol/100 gr all showed decrement of CMAP ranging from 28 to 75 % (fig. 2B).

Anti-rAChR antibody titers during passive transfer EAMG

The titer of mAb 35 during the development of passive transfer EAMG was determined at different points in time after intraperitoneal administration of 20 pmol mAb 35/100 gr bodyweight. The mean antibody titer against rAChR at 6 and 24 hrs after mAb 35 treatment was not significantly different between young (n=4) and aged rats (n=3). However, at 48 hrs after administration the antibody titer against rAChR declines more rapidly in young rats and is significant lower than in aged rats ($p<0.05$) (fig. 3). The antibody clearance of ^{131}I-IgG in young and aged rats was measured to exclude that differences in antibody clearance determined the induce-

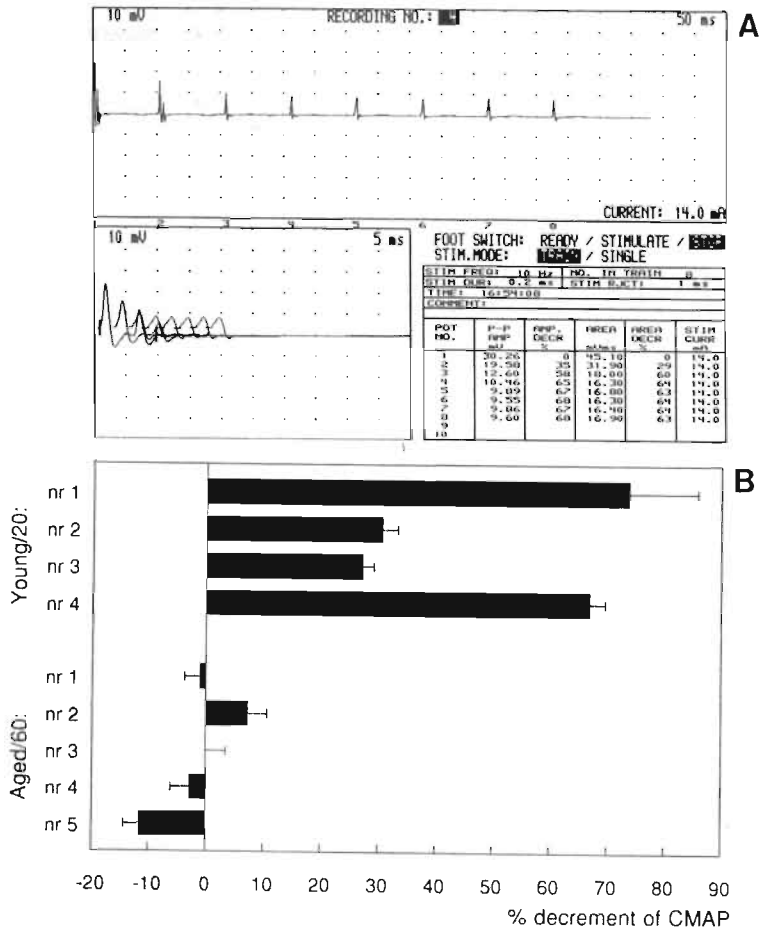

Figure 2: Neuromuscular signal transmission is unaffected in aged rats after passive transfer of mAb 35. *The compound muscle action potential (CMAP) at 10 Hz stimulation was determined 48 hrs after injection of mAb 35. (A) Example of an electromyogram from the Young/20 group, 48 hrs after passive transfer. (B) The decrement of the CMAP at the fifth stimulus is shown for individual rats. Bars represent the decrement of the CMAP (mean ± SD) from three measurements.*

ment of passive transfer EAMG in young and aged rats. The mean antibody clearance in young (n=3) and aged (n=3) animals at 5, 24, 48 and 72 hrs after administration was comparable (fig. 4).

AChR loss and antibody complexed AChR

The concentrations of total AChR and antibody complexed AChR were quantitated in young and aged rats 48 hrs after injection of mAb 35 or NRS. The AChR concentrations in the muscles of control young (n=15) and aged (n=10) rats were 55±11 and 52±11 pmol/100 gr muscle (mean±SD) respectively. Young rats injected

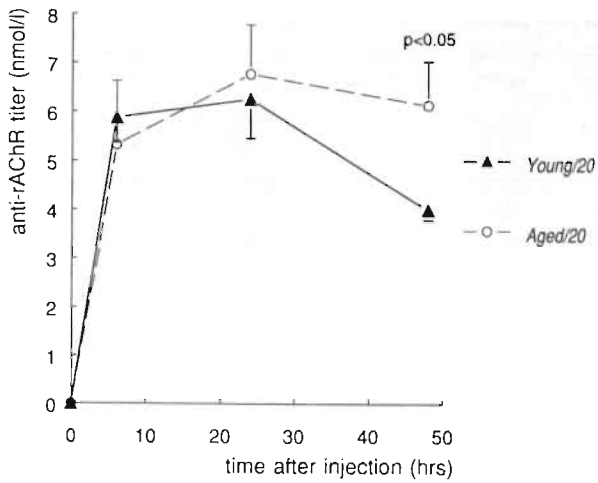

Figure 3: Kinetics of anti-rAChR antibody titer during passive transfer of mAb 35.
The mAb 35 titer against rAChR of Young/20 and Aged/20 rats was determined at 6, 24, and 48 hrs after injection. Values represent the anti-rAChR titer of each group (mean ± SD). Statistical differences between mean mAb 35 titers are indicated.

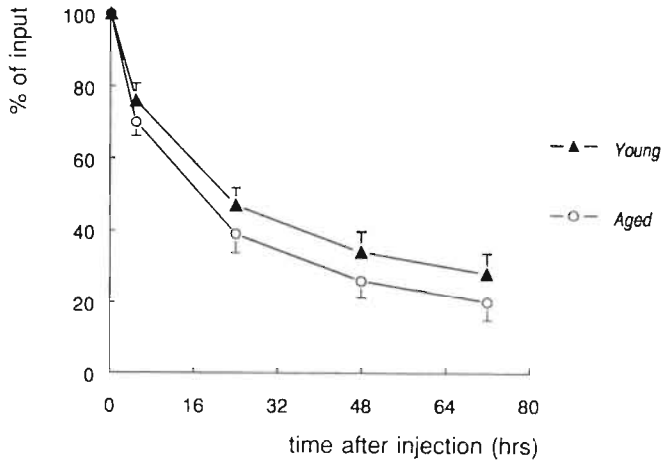

Figure 4: The immunoglobulin clearance in young and aged rats is similar.
The clearance of ^{131}I-IgG was determined in young and aged BN rats. ^{131}I-IgG was injected in the tailvein of young (n=3) and aged (n=3) rats and the clearance of ^{131}I-IgG was determined 0.25, 5, 24, 48, and 72 hrs after injection. Antibody clearance is expressed as percentage of the input of ^{131}I-IgG injected at the start of the experiment. Values represent mean ± SD.

with 20 pmol mAb 35/100 gr showed 59±10 % (mean±SD) AChR loss whereas similar treated aged rats showed no significant AChR loss (-1±12 %), 48 hrs after mAb 35 administration (table II). Even aged rats injected with 60 pmol mAb 35/100 gr showed only 10±1 % AChR loss at 48 hrs after administration. In contrast, young rats injected with the same dose died within 24 hrs after administration at which point in time already 44±8 % of the receptor was lost (table II). In both young and

Table II: AChR loss after passive transfer of mAb 35.

Rat group	Age	N	mAb 35 dose pmol/100 gr body weight	[AChR][a] pmol/100 gr muscle	AChR loss[b] %	AChR-Ab[c] %
young/N	10 wk	15	—	55 ± 11	—	—
young/20	10 wk	4	20	23 ± 5	59 ± 10	16 ± 1
young/60[d]	10 wk	6	60	29 ± 4	44 ± 8	23 ± 3
aged/N	130 wk	10	—	52 ± 11	—	—
aged/20	125 wk	3	20	52 ± 6	- 1 ± 12	11 ± 1
aged/60	125 wk	5	60	47 ± 16	10 ± 1	22 ± 2

The AChR concentration and the fraction of AChR complexed with mAb 35 was determined 48 hrs after injection of mAb 35. [a] *The AChR concentration is expressed as pmol AChR/100 gr muscle in order to compare animals of different bodyweight.* [b] *AChR loss is expressed as percentage of the AChR concentration in unmanipulated control rats.* [c] *The amount of AChR complexed with antibody is expressed as the % of the remaining amount of AChR.* [d] *The AChR concentrations in young rats injected with 60 pmol mAb 35/100 gr were determined at the timepoint of death due to paralysis (approximately 22-24 hrs after passive transfer).*

aged rats part of the remaining AChR was complexed with mAb 35. In young rats, 16±1 and 23±3 % of the AChRs were complexed with mAb 35 when treated with 20 or 60 pmol mAb 35/100gr respectively. In aged rats, 11±1 and 22±2 % of the AChRs were complexed when treated with 20 or 60 pmol mAb 35/100 gr

Morphology of neuromuscular junction

The pathogenic effect of mAb 35 on the neuromuscular junction was verified by electron microscopy of muscle biopsies taken from mAb 35 treated rats. The postsynaptic membrane in young rats injected with 20 pmol mAb 35/100 gr was highly simplified or almost completely destroyed. The synaptic cleft was filled with debris and a nerve terminal was separated from the underlying postsynaptic membrane by an infiltrating macrophage (fig. 5A). The neuromuscular junction of similar treated aged rats appeared morphologically intact although some degenerative changes were present. The nerve terminals were closely aposed to the postsynaptic membrane and the junctional folds are well preserved (fig. 5B).

Complement deposition and macrophage infiltration

The role of complement in the development of passive transfer EAMG in young versus aged rats was assessed by immunohistochemical staining of the C3 complement component in cryosections of muscle biopsies taken 48 hrs after administration of mAb 35. Depositions of C3 were found in the muscles of young mAb

Figure 5: Ultrastructural morphology of endplates after passive transfer of mAb 35.
(A) *Two neuromuscular junctions on one muscle fiber of an aged rat 48 hrs after injection with 20 pmol mAb 35/100 gr bodyweight. Degenerative changes are present near a nucleus in between two postsynaptic areas. The postsynaptic junctional folds are well preserved. Open arrow: see insert* (A') *Detail of junctional folds and synaptic vesicles in the nerve terminal.*
(B) *Neuromuscular junctions on one muscle fiber of a young rat 48 hrs after injection with 20 pmol mAb 35/100 gr. The postsynaptic part of the junctions are almost completely destroyed, though some folds can still be discerned. M: infiltrating macrophage. Open arrow: see insert* (B') *This detail shows synaptic vesicles in the preserved nerve terminal and complete degeneration of the postsynaptic part of the junction. Bars represents 1 μm.*

35 treated rats (fig. 6A), which coincided with the localization of AChR at the endplate using α-BT-rhodamine (fig. 6B). C3 depositions at the endplate were also demonstrated in EAMG resistant aged rats (fig. 6E and F). No C3 at the endplates could be demonstrated in young (fig. 6C and D) or aged (fig. 6G and H) control rats. Infiltration of activated macrophages in the vicinity of endplates and necrotic muscle fibers was observed in muscle biopsies of young mAb 35 injected rats (fig. 7A and B). In aged rats however, no macrophage infiltration was seen nor necrosis of muscle fibers (fig. 7C and D). Muscle sections from young or aged control rats were free of activated ED 1 positive macrophages (not shown).

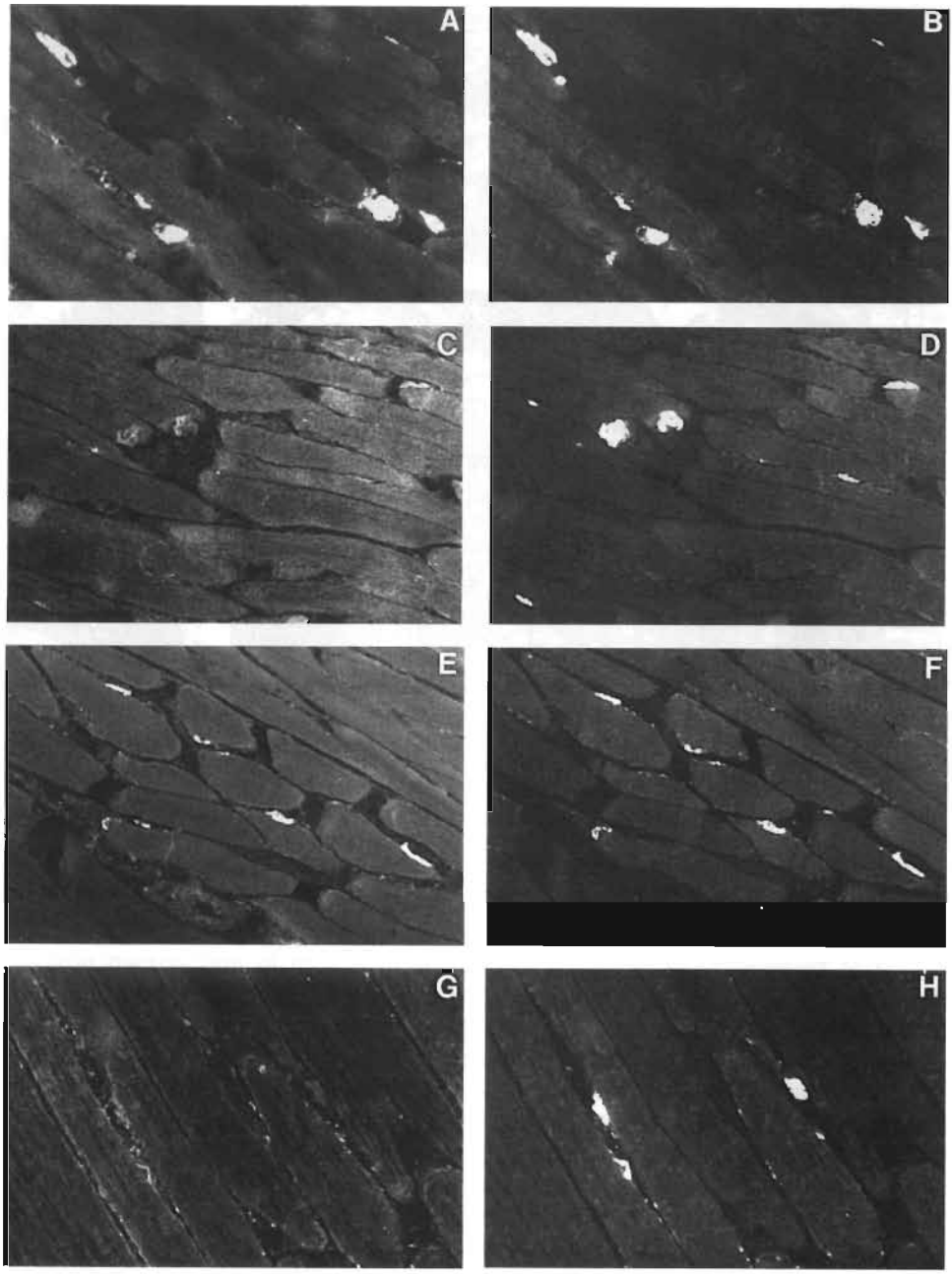

Figure 6: Localization of C3 at the endplate.
AChRs and C3 deposits were visualized by two color fluoresence in the same muscle section obtained from biopsies taken at 48 hrs after passive transfer. Figures (A), (C), (E), and (G): muscle sections stained for C3. Figures (B), (D), (F), and (H): the same muscle sections as in A, C, E, and G stained for AChR. Figures (A) and (B) are representative for Young/20 rats. (C) and (D): Young/N rats, (E) and (F): Aged/20 rats, (G) and (H): Aged/N rats.

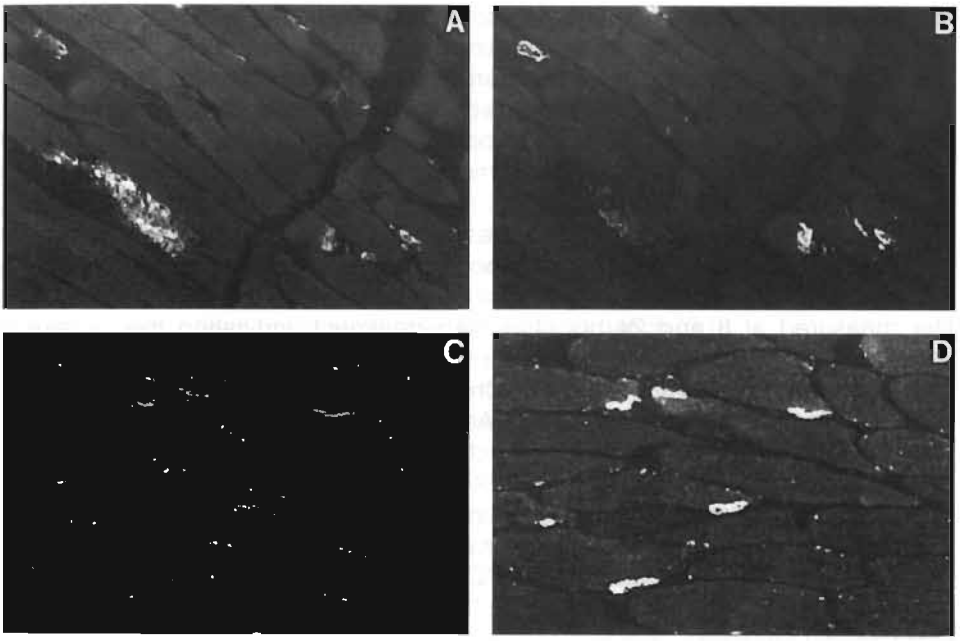

Figure 7: Macrophage infiltration at the endplates after passive transfer of mAb 35.
AChR and infiltrating macrophages were visualized by two color fluoresence in the same muscle section, obtained from biopsies taken at 48 hrs after passive transfer. (A) Muscle sections of young mAb 35 injected rats show massive infiltration of macrophages at the motor endplate and in necrotic muscle fibers. (B) The same muscle section stained for AChR with rhodaminated α-BT. (C) Macrophages could not be demonstrated at endplates of mAb treated aged rats. (D) The same muscle section stained for AChR.

DISCUSSION

This present study established the influence of age on the induction of EAMG in the rat. Aged rats were found to be resistant to induction of EAMG by immunization with Torpedo AChR or passive transfer of anti-AChR antibody. The highly reproducible passive transfer EAMG model was used to further investigate this age related resistance to induction of EAMG. Injection of young adult BN rats with monoclonal anti-AChR antibody induced signs of EAMG including severe weight loss, muscle weakness, decrementing electromyographic responses, and ultrastructural alterations of the motor endplate. Similarly injected aged rats remained unaffected and showed no electromyographic or ultrastructural abnormalities at the motor endplate. Aged rats remained resistant even after treatment with a mAb 35 dose exceeding the total AChR content in the muscles. This difference in disease susceptibility between young and aged rats paralleled the magnitude of AChR loss; aged, mAb 35 injected rats showed no loss of AChR whereas young rats showed extensive AChR loss. Depositions of C3 at the neuromuscular junction could be demonstrated in both young and aged mAb treated rats, however, infiltration of macrophages and segmental necrosis of muscle fibers was only seen in young rats.

Apparently, no degradation of AChRs occurred in aged rats injected with anti-AChR mAb since no AChR loss or decremental responses could be demonstrated, indicating that neuromuscular signal transmission was unaffected. Several mechanisms could explain the resistance against induction of EAMG in aged rats; differences in antibody uptake or clearance, accessability of the AChR for antibody, activation of complement, or antigenic modulation of AChR could account for age related resistance to EAMG.

A prerequisite for comparison of passive transfer EAMG in young and aged rats is a similar anti-rAChR antibody titer in both rat groups after injection of mAb 35. Administration of mAb 35 on weight basis resulted in a similar anti-rAChR antibody titer measured at 6 and 24 hrs after mAb treatment, indicating that uptake of intraperitoneal injected mAb 35 into the circulation was equally efficient in young and aged rats. However, the anti-rAChR titer declined more rapidly in young compared to aged rats at 48 hrs after mAb administration. The observed difference in anti-rAChR titer could not be explained by an age related difference in antibody clearance; a comparable clearance of ^{131}I-IgG from the circulation was measured in both age groups. The rapid decline in the mAb 35 titer in young compared to aged rats could be the result of an increased AChR turnover by antigenic modulation (8, 19); after internalization of mAb-AChR complexes, AChRs are resynthesized and again capable of binding mAb resulting in a higher mAb expenditure.

Complement mediated focal lysis is one of the pathogenic mechanisms leading to AChR loss. In both young and aged animals deposits of C3 were demonstrated in the same distribution as AChRs at the neuromuscular junctions. This indicates that the injected mAb was bound to AChRs in both young and aged rats, and the lack of AChR degradation as seen in aged rats could not be explained by the inaccessibility of the AChR for mAb. Determination of C3 at the endplates furthermore indicates that complement could be activated in both age groups excluding a complement deficiency in aged rats. Complement activation via the classical pathway (20) is one of the pathogenic mechanisms initiating AChR degradation; complement components were demonstrated at the motor endplate in MG (21, 22) and EAMG (23, 24). The importance of complement mediated degradation of AChRs in passive transfer EAMG was demonstrated by C3 depletion with cobra-venom factor (9, 25), or inhibition of membrane attack complex formation with anti-C6 Fab (26), preventing induction of passive transfer EAMG in rats. Furthermore, induction of EAMG could not be induced in C4 deficient guinea pigs (20) or C5 deficient mice (27).

In addition to C3 deposits, infiltrating macrophages were found in the vicinity of endplates and in necrotic muscle fibers of young mAb treated rats. This cellular inflammatory reaction was not found in mAb treated aged rats despite binding of C3 at the endplates. This suggests that the early complement components C3a and C5a were not sufficient to attract mononuclear cells and macrophages to the motor endplate of aged rats. Absence of infiltrating macrophages was also demonstrated during inhibition of passive transfer EAMG by treatment with anti-C6 Fab (26), indicating that early complement components were not sufficient to trigger macrophage infiltration. Infiltrating macrophages may therefore be a secondary phenomenon in passive transfer EAMG subsequent to massive tissue destruction by the complement mediated focal lysis in concomitance with antibody mediated in-

creased AChR turnover. Infiltrating macrophages were not found in chronic EAMG (28). Since induction of chronic EAMG in aged rats also revealed resistance to induction of the disease it was not likely that the absence of infiltrating macrophages was the only factor that determined EAMG resistance.
The resistance to induction of EAMG in aged rats resembled the refractory state observed in rats which recovered from an initial episode of passive transfer EAMG. When these animals were challenged a second time with anti-AChR mAb they remained clinically and electromyographically normal and showed marginal AChR loss (29). This refractory state was characterized by the absence of infiltrating macrophages. It was suggested that AChR concentration or density might be beneath a critical level to initiate further AChR degradation (30).
Beside complement mediated focal lysis, AChR loss is also caused by an increased AChR turnover due to antibody cross-linking. Anti-AChR mAb 35 used to induce passive transfer EAMG is capable of increasing AChR turnover *in vitro* and protect antigenic modulation of AChR by MG sera (31). The relative contribution of these two pathogenic mechanisms to the development of MG is unknown. These pathogenic mechanisms may not function exclusively, and both mechanisms probably combine to bring about the disease. Complement mediated focal lysis by itself could be insufficient to induce the disease in aged rats and antigenic modulation may be impaired or absent. An increased AChR synthesis rate protected against increased AChR loss by antigenic modulation (32). However, similar AChR half-lives were found in 70 v.s. 844 day old rats (33) and a difference in AChR synthesis between young and aged rats was therefore not likely to explain the resistance to EAMG. The density of AChRs in aged rats may be different from that of young animals, and beneath a critical AChR density at which increased AChR turnover by antibody cross-linking can no longer contribute to AChR degradation. A marked decrease of AChRs per junction has been observed at the endplates of aging rats (33). Moreover, the length of the postsynaptic membrane increases with aging (34), whereas the total AChR concentration in muscle of young and aged rats was found to be comparable. The AChR molecules are 8 nm in diameter and clustered with very high density at the NMJ; the center to center distance is approximately 10 nm between two adjacent molecules (35). Cross-linking by antibody is only possible if AChR molecules are in close proximity, at which antibody is able to span the distance between two AChR molecules. The maximum span between the two binding sites of an antibody is approximately 12 nm (36). Complement activation however, could also be initiated at lower AChR densities. The physical distance between adjacent Fc parts in order to bind C1q can be as large as 35 nm (37).
Taken together, these results suggest that resistance of aged rats against induction of EAMG was related to changes at the NMJ rather than immunological factors. MG is also less frequently observed in humans over 60 years, and the incidence of purely ocular symptoms is relatively higher in patients over the age of 40 (38).
The AChR density and organization may be different in aged rats. A different lipid composition of the postsynaptic membrane may influence AChR organization (39). Furthermore, the membrane rigidity increases with aging (40) and could diminish AChR turnover by antigenic modulation. Age related resistance in the EAMG model can provide more information about the factors that determine the severity of MG.

Manipulation of AChR density or the lipid composition of the postsynaptic membrane may be of therapeutic interest in MG.

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Chapter 3

The genetic basis of antibody diversity

The genetic basis of antibody diversity

Introduction

An important feature of the immune system is its ability to recognize and interact with any foreign antigen in a very specific fashion. This potential repertoire may include specificities that interact with self components that emerge in autoimmune disease. Autoimmune disease may arise from loss of suppression of pathogenic specificities within the normal repertoire, or result from abnormalities in the generation of the T and B cell receptor repertoire. The autoimmune disease myasthenia gravis is mediated by pathogenic antibodies against the acetylcholine receptor. However, what initiates this humoral autoimmune response is yet unknown. Insight into the generation of these antibodies could provide important aetiological information about the disease.

It was demonstrated in a previous study that various anti-AChR mAbs share antigenic determinants on their variable region (cross-reactive idiotopes). The cross-reactive idiotopes were expressed on both rat and mouse anti-AChR antibodies (1). Cross-reactive idiotopes can have a role in regulation of the immune response by interaction with anti-idiotypes; administration of a low dose of polyclonal affinity purified anti-idiotype 65 antibodies to neonatal rats resulted in an increased antibody response to both Torpedo and rat AChR after immunization with AChR at adult age (104).

Expression of a cross-reactive idiotype (CRI) by anti-AChR antibodies suggested that these antibodies may be structurally related and could be encoded by related genetic elements. If anti-AChR antibodies consist of a group of related antibodies it could be possible that anti-AChR antibodies arise from one or a few clones due to clonal expansion. To address this question we investigated the diversity of genetic elements that are used to encode the heavy chain region of anti-AChR mAbs.

To study the genetic diversity of anti-AChR antibodies it is useful to have insight in the structural elements that determine antibody diversity. This chapter gives an outline of the genetic elements encoding the variable region of antibodies which are very well characterized in the mouse. Furthermore, an overview of the genetic diversity of (auto)antibodies from normal and autoimmune mice strains is given, and the structural basis for expression of a CRI will be discussed.

Immunoglobulin structure

Immunoglobulin (Ig) molecules are composed of two identical heavy chains and two light chains with a molecular weight of 50000 and 25000 dalton respectively (2, 3). Each chain consists of a variable part (V_H and V_L) involved in antigen recognition, and a constant part (C_H and C_L). The constant part of the heavy chain (C_H) defines the isotype of the immunoglobulin molecule and mediates biological functions including complement fixation and activation, and binding to phagocytic cells. In mice, eight isotypes (IgM, IgD IgG3, IgG1, IgG2b, IgG2a, IgE, and IgA) have been identified all having different biological functions (4).
The variable region of Ig heavy (V_H) and light (V_L) chains consist of a framework of relatively conserved amino acids interrupted by three highly variable regions. The three hypervariable- or complementary determining regions (CDR) of each heavy and light chain together form the antigen binding site (5, 6).

Generation of antibody diversity

The Ig molecule is encoded by three unlinked clusters of genes: the light chain can be encoded by two separate gene clusters whereas the heavy chain is encoded by a single cluster of heavy chain genes. In the mouse, the λ and κ genes are localized on chromosomes 16 and 6 respectively, and the heavy chain genes on chromosome 12 (10, 11, 12). The majority of the light chains in the mouse is encoded by κ light chain genes and only about 5 % by λ light chain genes. The κ light chain can be encoded by approximately 300 variable, 4 joining and 1 constant gene segment (7, 8), whereas the λ light chain can be encoded by 2 variable, 4 joining and 4 constant gene segments (9).
The gene that encodes the variable part of the heavy chain is assembled from three separate germline gene segments; a variable (V_H), diversity (D), and a joining (J_H) gene segment (13). The variable part of the light chain gene is encoded by a variable (V_L), and joining (J_H) gene segment (fig. 1). Two of the CDRs are encoded by the V_H or V_L genes and the third CDR is encoded by the V-D-J junctional region in heavy chains and the V-J junctional region in light chains.
The variable region of the heavy chain can be coded by 200-1000 V_H, 13 D, and 4 J_H gene segments (14). The diversity of antibodies is generated at several levels:

I Multiple distinct germline gene segments encode unique V, D or J amino acid sequences
II Different V_H, D and J_H or V_L and J_L genes can recombine to form many different complete variable region genes
III Imprecise joining of gene segments resulting in deletion or *de novo* addition of nucleotides generating additional diversity in CDR 3
IV Differential combining of complete heavy- and light chains
V Somatic mutation can modify the coding capacity of complete assembled variable region genes

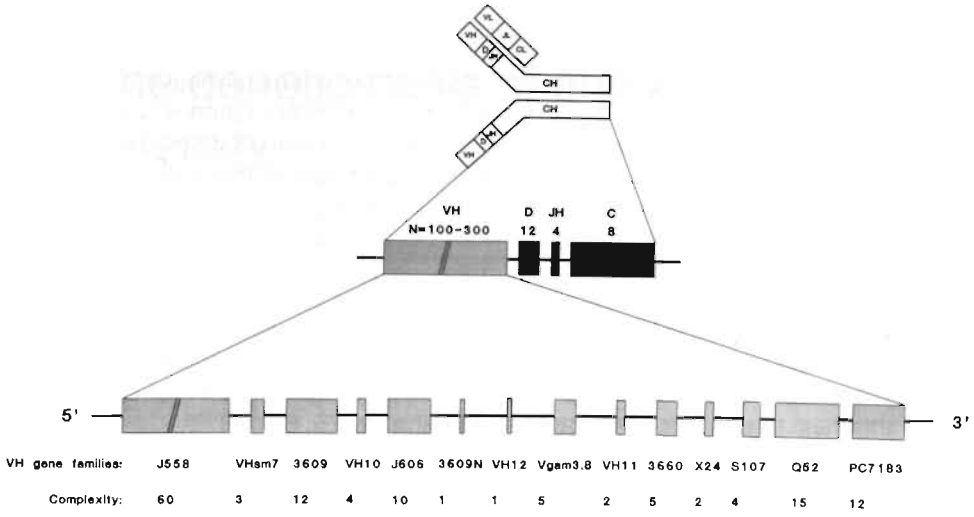

Figure 1: Overview of the VH gene families
The immunoglobulin heavy chain is composed of 4 different elements encoding the variable (V_H), diversity (D), joining (J_H) and constant (C) region. The V_H genes are currently grouped in 14 V_H gene families that comprise V_H genes with a sequence homology of 80 % or more. The sequence homology between members of different families is less than 70 %. The presumed localization of the V_H gene families relative to the diversity region is shown. The complexity of a V_H gene family represents the estimated number of V_H genes belonging to that family

The combined effect of these mechanisms is estimated to generate 10^9 different antigen specificities.

The mouse IgV_H gene locus

The 200-1000 V_H genes are the largest group of gene fragments that contribute to the formation of the heavy chain variable region and are responsible for a significant portion of the potential antibody repertoire. Murine heavy chain V_H gene segments were originally divided into three major subgroups on the basis of protein sequences (15). However, when V_H genes were compared on nucleotide sequence homology, they could be divided in 7 different V_H gene families (16, 17). Now 14 different V_H gene families are discriminated (17-22). V_H gene segments showing a mutual homology of 80 % or more are considered to belong to the same V_H gene family while V_H gene segments that show less than 70 % sequence homology are considered to be from different families (16, 17). The size or complexity of a V_H gene family was estimated by counting the number of germ-line DNA fragments on a Southern blot hybridized with a probe representative for a V_H gene family. This approach was based on the observation that individual murine V_H gene segments are located with 10 to 20 kb intervals on the chromosomal DNA (23, 24). Therefore, when genomic DNA is cut with restriction enzyme Eco RI generating fragments ranging from 1 to 20 kb, each fragment should contain a single V_H gene. The total

number of hybridizing fragments gives a minimum estimate of the number of sequences that are more than 80 % homologous to the V_H probe used (17, 24). Using this approach the total number of V_H gene sequences is estimated to be 200 (17), but may be underestimated and be as large as 1000 or more (25, 26). This underestimation may be due to the fact that V_H gene sequences are more closely spaced resulting in multiple V_H sequences on one restriction fragment and the existence of restriction fragments of equal size that co-migrate during gel electroforesis and can not be discriminated as separate bands (26, 27-29). However, the number of unique V_H gene sequences encoding functional and disctinct antibody specificities is probably less than the total number of hybridizing V_H gene segments. The total number of hybridizing V_H gene segments contains a considerable number of V_H pseudogenes incapable of coding a functional V_H gene product and the existence of multiple copies of identical V_H gene sequences (25, 28, 29-31). The size of the total available V_H gene repertoire was determined in normal splenic hybridomas or B-cell colonies and suggested to be less than 300 (32) and the frequency of occurence of V_H gene segments from a particular family in normal B cells is in good agreement with the complexity of that family determined by "band counting" in a Southern blot (31, 33, 34).
Murine V_H gene families are generally grouped as clusters of related V_H genes (23, 24, 26, 35, 36). The V_H gene families have been mapped relative to the constant region genes using deletion mapping. Deletion mapping is based on the fact that rearrangement of a V_H gene to the D and J_H genes results in the deletion of DNA originally separating the V_H gene from the D-J_H-C_H region (37). In B cells that have rearranged V_H genes on both chromosomes, all V_H gene segments lying downstream (or 3') from the used V_H gene segments are deleted and can no longer be detected in a Southern blot. Using this technique it was possible to map all V_H gene families relative to each other (21, 22, 38-43). The relative organization and complexity of each V_H gene family is depicted in figure 1. In general each V_H gene family is clustered although the 3660, Vgam3.8 and S107 V_H gene families are dispersed into two or three subregions. The J558 and 3609 V_H gene families are partially interspersed whereas members of the Q52 and PC7183 V_H gene families are completely interspersed at the 3' end of the IgV_H locus (30, 40, 41).

V_H gene utilization by the normal B cell repertoire

Selection of V_H genes from the different V_H gene families was found to be highly restricted early in B cell development and eventually becomes random during maturation of the B cell repertoire. B cells isolated from mouse fetal liver and neonatal spleen early in ontogeny preferentially rearrange V_H genes from the most D-proximal V_H gene family PC7183 (35, 36, 44-46). Within 5-7 days after birth the V_H gene repertoire is randomized such that expression of V_H genes correlates with the size (or complexity) of the V_H gene family from which the V_H gene is derived (45, 46). Individual V_H gene segments expressed in LPS stimulated (33, 34, 47, 48-50) or unstimulated (50, 51) B cells were found to be distributed stochastically over the different V_H gene families which suggests that all individual V_H genes are represented at equal frequencies in the available and actual adult B cell repertoires

respectively. In contrast, The V_κ gene family expression in LPS stimulated B lymphocytes was not proportional to the complexity of the V_κ gene families (52). The underlying mechanism of this "normalization" of V_H gene family utilization during B cell development is unknown, but several factors including intrinsic genetical properties governing rearrangement and transcription of V_H genes (53), interactions with accessory cells (54) or stimulation by exogenous antigens (55) contribute to this process.

V_H gene utilization by autoantibodies

A key question in the understanding of antibody mediated autoimmune disease is whether autoantibodies are a separate entity within the whole B cell population. Autoreactive B cells may be normal constituents of the immune system which remain unstimulated or suppressed under normal physiologic conditions. Alternatively, pathogenic autoantibodies may arise from abnormalities in the generation of the B cell repertoire and anti-self antibodies may be encoded by unique V_H gene segments. It was suggested that murine autoantibodies of various specificities are encoded by a restricted number of V_H genes in particular V_H genes from the V_H gene families Q52 and PC7183 which are most proximal to the diversity region genes (56, 57). Moreover, B cell hybridomas selected for expression of V_H genes from the PC7183 family produce autoantibodies at a high frequency (58). V_H genes

Table I: Overview of V_H gene family utilization of autoantibodies from autoimmune prone mice.

mouse strain	stimulus[a]	mAb specificity[b]	V_H gene family utilization[c]									Nr.[d]	Ref.
			J558	3609	J606	Vgam3.8	3660	X24	S107	Q52	7183		
MRL lpr/lpr	-	RF	0	0	0	0	9	0	0	0	0	9	67
MRL lpr/lpr	-	RF	32	0	0	0	1	0	0	0	0	35	68
MRL lpr/lpr	-	RF	3	0	0	0	0	0	0	0	0	3	70
MRL lpr/lpr	-	DNA	7	0	0	0	0	0	1	2	3	13	56
MRL lpr/lpr NZB, CBA, 129SV	-	RF, DNA, Sm, BrMRBC	7	0	0	0	0	0	1	1	10	19	57
C57bl/6 me^v/me^v	LPS	Several auto-Ag	8	0	0	0	1	0	0	2	0	12	71
C57bl/6 me^v/me^v	-	Several auto-Ag	5	0	2	0	0	3	3	0	2	17	72
NZB	-	mouse RBC	6	1	1	0	0	0	0	0	0	8	73
NZBxNZW	-	DNA	15	1	0	0	0	0	1	1	3	23	74
SM/J	LPS	Thymocytes	7	4	0	1	0	0	0	2	0	14	74
MRL lpr/lpr	-	Several auto-Ag	70	0	3	0	1	0	5	15	8	119	103
		total	160	6	6	1	12	3	11	23	26	272	
		%	59	2	2	0	4	1	4	8	10	-	

Spontaneous or lipopolysaccharide (LPS) stimulated autoreactive hybridomas were isolated from autoimmune prone mice and tested for V_H gene family utilization by Northern blot- or DNA sequence-analysis. The number of hybridomas positive for each V_H gene family is shown. [a]B cells were either unstimulated (-), or LPS stimulated. [b]Autoantigen specificity: RF; rheumatoid factor, Sm; Smith antigen, BrMRBC; Bromelain treated mouse red blood cells. [c]Nine of the 14 known V_H gene families arranged in presumed chromosomal order relative to the D-region. [d]Total number of hybridomas tested for V_H gene family utilization.

Table II: Overview of V_H gene family utilization of autoantibodies from normal non-autoimmune mice

mouse strain	stimulus[a]	mAb specificity[b]	V_H gene family utilization[c]									Nr.[d]	Ref.
			J558	3609	J606	Vgam3.8	3660	X24	S107	Q52	7183		
Balb/c, CBA BDA/1	→	Several auto-Ag	10	0	0	0	0	0	0	4	10	24	57
Balb/c	insulin	insulin	2	0	0	2	0	0	0	0	0	43	79
	-	tissue antigens	7	2	0	0	0	0	0	3	1	13	76
C57bl/6xDBA/2	DBA/2 T cells	kidney	3	0	0	0	0	0	0	0	1	4	80
C57bl/6	LPS	several auto-Ag	2	0	0	2	0	0	0	0	0	8	71
DBA1, B10p	collagen	collagen II	6	0	0	0	2	1	0	1	2	13	81
CBA/J	Tg	thyroglobulin	6	0	0	0	0	0	0	2	1	10	82
?	LPS, ?	RF	6	0	1	0	0	0	0	0	1	8	77
BDF/1	DBA/2 T cells	tissue antigens	47	0	1	0	1	2	0	3	1	56	83
A/J	LPS + dextran	several auto-Ag	16	0	2	0	1	0	0	5	2	26	78
		total	105	2	4	4	4	3	0	18	19	166	
		%	63	1	2	2	2	2	0	11	11	-	

Autoreactive hybridomas from non-autoimmune mice were tested for V_H gene family utilization by Northern blot- or DNA sequence- analysis. [a] B cells were either unstimulated (-), polyclonally stimulated by lipopolysaccharide, or induced by active immunization. [b] Autoantigen specificity: Tg; thyroglobulin, RF; rheumatoid factor. [c] Nine of the 14 known V_H gene families arranged in presumed chromosomal order relative to the D-region. [d] Total number of hybridomas tested for V_H gene family utilization

from these D-region proximal V_H gene families are predominantly rearranged early in B cell development (35, 36, 59). During this stage in the ontogeny, B cells are mostly multireactive and frequently display specificity for several autoantigens (60). In addition, ly-1$^+$ (CD5$^+$) B cells which phenotypically resemble early B cells frequently have autoantigen specificity (61, 62). These results suggested that autoantibodies are encoded by a limited set of V_H genes which may be related to a defective control of V_H gene expression (63). Autoantibody producing B cells may escape from this primary B cell pool during shaping of the adult B cell repertoire. Since this observation many investigators have addressed the question whether autoantibodies show a biased V_H gene usage. To determine a possible bias in V_H gene usage from different V_H gene families, hybridomas with several autoantigen specificities were isolated from either autoimmune prone or normal mice.

V_H gene family utilization in autoimmune prone mice:
Autoimmune prone mice strains provide spontaneously arising models for autoimmune diseases like Coombs hemolytic anaemia (NZB), Systemic lupus erythrematosus (NZBxNZW), rheumatoid arthritis (MRL lpr/lpr). Furthermore, viable motheaten mice show severe autoimmune syndromes and nearly all of their B cells are of the Ly-1 lineage (64). The V_H gene family usage of autoreactive hybridomas from autoimmune prone animals are summarized in table I. Several studies reported a

D-proximal bias in V_H gene family utilization of autoantibodies from autoimmune prone mice (56, 57, 65, 66). Furthermore, a preferential usage of the 3660 (67) or the J558 (68) family was demonstrated for rheumatoid factors isolated from MRL lpr/lpr mice. However, it is clear that all other V_H genes from all tested V_H gene families can encode autoantibodies. Moreover, when the results of all these studies are compiled, the V_H gene family utilization follows a stochastic distribution over the V_H gene families and no bias or preferential use of certain V_H gene families is observed (fig. 2).
Taken together these results indicate that autoreactive B cells in these autoimmune prone animals are not likely the result of an impaired normalization of the initial D-proximal bias in V_H gene family utilization early in ontogeny. This notion is supported by studies that determine V_H gene family utilization among unstimulated B cells from autoimmune mice by direct quantative *in situ* hybridization. These studies in MRL lpr/lpr (51, 69) and "viable motheaten" mice (47) showed no D-proximal bias in V_H gene family utilization.

V_H gene family utilization in autoantibodies from normal mice:
Autoreactive hybridomas obtained from normal non-autoimmune mice, either natural occuring (75), experimentally induced by polyclonal stimulation (71, 77, 78), or active immunization (57, 79-83), were found to be encoded by V_H genes from most V_H gene families (table II). Monestier et al reported a D-proximal bias in V_H gene family usage among autoantibodies derived from different non-autoimmune mice strains, with various autoantigen specificity (57). However, when results from individual studies are compiled, the V_H gene utilization is distributed stochastically over the V_H gene families (fig. 2). These results indicate that a wide variety of V_H genes are found in autoantibodies. V_H genes encoding autoantibody specificities are probably not predisposed to a certain V_H gene family (44).

The structural basis for cross-reactive idiotypes

Idiotypes are antigenic markers of the variable region of immunoglobulins (Ig) that were originally detected by antisera (anti-idiotypes) obtained by immunization of animals with antibody (84). Individual antigenic determinants (idiotopes) on the variable region can be subdivided in private idiotopes that are unique for one or a few antibodies and public or cross-reactive idiotopes found on many different Ig molecules. When the interaction of anti-idiotype antibodies (that defines the idiotype) with the idiotype on an antibody is competed by antigen, the idiotype is located at or near the antigen binding site or paratope. The idiotype is associated with the framework of the antibody if the interaction of anti-idiotype with idiotype is not inhibited by antigen (86).
The fact that antibodies express cross-reactive idiotopes suggests that these antibodies are encoded by closely related variable region genes. Most detailed information about the structural basis of cross-reactive idiotopes has been obtained from antibodies against defined small haptens. Sequence analysis of anti-hapten antibodies in several idiotype systems (PC, NP, DNP, GAT, ARS, OX) demonstrated that antibodies that express the same idiotype were encoded by one or a few V_H

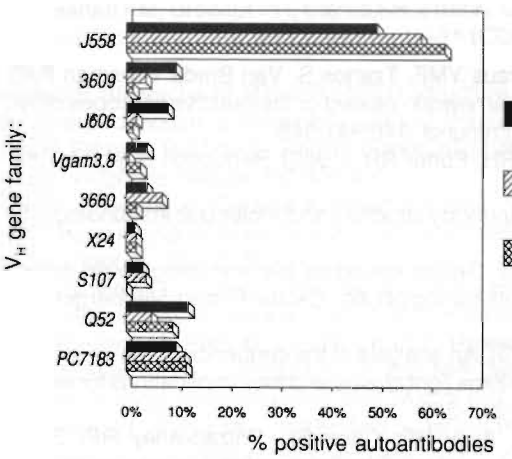

Figure 2: VH gene family usage of autoantibodies; accumulated data
Graphic representation of the V_H gene family usage of autoantibodies from autoimmune prone and normal mice. The V_H gene family usage is expressed as the percentage of mAbs positive for a given V_H gene family and was compared to a stochastic distribution based on the estimated size of the family. The V_H gene family utilization of autoantibodies from autoimmune prone mice represents the accumulated data of the studies presented in table I. The V_H gene family utilization of autoantibodies from normal mice represents the accumulated data of the studies presented in table II.

genes (24, 26, 27, 86-88) and in most cases a restricted number of light chains (88-92). A clear example of the structural basis of a cross-reactive idiotype was demonstrated for the T15 idiotope shared by some anti-phosphorylcholine (PC) antibodies (24). The V_H sequence of 10 out of 19 anti-PC antibodies was identical to one of the four germline genes (V1) of the S107 V_H gene family. The variable region of the other 9 anti-PC antibodies differed by 1-9 amino acids but were closely related to the V1 germline sequence. It was concluded that the T15 idiotype was encoded by a single S107 germline gene and that variant antibodies arose by somatic mutations.

Autoantibodies were found to be frequently idiotypically connected and express cross-reactive idiotopes (57, 58, 65, 93). A cross-reactive idiotype is often expressed by antibodies encoded by V_H genes from a single V_H gene family. Expression of a cross-reactive idiotype may be a marker for a V_H gene family or subfamily (93-96). Sharing of a cross-reactive idiotype was demonstrated on antibodies against a single antigen (93-96) or on antibodies against unrelated antigens (57, 58, 97). However, it has become apparent that antibodies can share a cross-reactive idiotype in spite of the fact that they are encoded by V_H genes from different V_H gene families (57, 58, 97, 98). The structural basis for such complex cross-reactive idiotypes among a heterogeneous group of antibodies is not well understood and could -in addition to similarities in V genes- be correlated with D and J gene usage or V_H-D or D-J_H junctional similarities (99-102).

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Chapter 4

Characterization of anti-acetylcholine receptor antibodies from mice differing in susceptibility for experimental autoimmune myasthenia gravis

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Characterization of anti-acetylcholine receptor antibodies from mice differing in susceptibility for experimental autoimmune myasthenia gravis

ABSTRACT

In the murine model for experimental autoimmune myasthenia gravis (EAMG) we investigated the relation between disease susceptibility and fine specificity of anti-AChR antibodies obtained from high susceptible C57bl/6 and low susceptible Balb/c mice after immunization with Torpedo AChR. Monoclonal anti-AChR antibodies with fine specificity for the main immunogenic region (MIR), the α-bungarotoxin/acetylcholine (α-BT) binding sites and other extra- and intracellular epitopes were isolated from both mouse strains. The relative frequencies of hybridomas cross-reactive with autologous mouse AChR, or directed against the MIR and the α-BT binding site were higher in C57bl/6 than in Balb/c mice. This observed difference in anti-AChR repertoire was evaluated in the polyclonal anti-AChR response of EAMG mice. However, the concentration of antibodies directed against the MIR and α-BT binding site in the sera of both mouse strains were found to be similar after secondary immunization. These results indicate that strain specific differences in disease susceptibility in murine EAMG are not related to differences in the overall concentration of antibodies against the MIR or α-BT binding site.

INTRODUCTION

In myasthenia gravis, the acetylcholine receptor is target of an autoimmune response (1). The nicotinic acetylcholine receptor involved in the signal transmission at the neuromuscular junction, is a transmembrane glycoprotein composed of five subunits in the stochiometry $\alpha_2\beta\varepsilon\delta$ (2). The extracellular domain of the α-subunits contain the cholinergic binding site (3) and a main immunogenic region (MIR) against which a major part of the anti-AChR antibodies in MG and EAMG is directed (4, 5). Anti-AChR antibodies decrease or inactivate functional AChR resulting in an impaired neuromuscular transmission. In MG, functional AChR can be inactivated by several mechanisms. Antibodies bound to AChR can activate complement leading to focal lysis of the postsynaptic membrane (6, 7). Anti-AChR antibodies cause accelerated receptor turnover by cross-linking AChRs, resulting in a diminished number of functional AChR molecules (8, 9). Furthermore, AChRs can be functionally inactivated by antibodies interfering with acetylcholine binding (10), or by impairment of the ion channel function of the AChR (11, 12). Differences in antibody fine specificities contributing to these pathogenic mechanisms may influence the severity of the disease since the total anti-AChR antibody titer in MG and EAMG does not closely correlate with the severity of the disease (44).
Differences in susceptibility for EAMG are found among different strains of mice (13, 14, 15) and rats (16). Susceptibility to EAMG in mice is controlled by multiple genes including the class II MHC, and genes that control complement activity (15). In addition, strain specific differences in the available anti-AChR antibody repertoire could contribute to disease susceptibility. In this report a possible relation between antibody fine specificity and disease susceptibility was investigated. Anti-AChR mAbs directed against the MIR, the α-BT binding site and other extra- and intracellular epitopes were isolated from both high susceptible C57bl/6 and low susceptible Balb/c mice. The relative frequencies of mAbs against well defined epitopes on AChR in the clonable anti-AChR repertoire of both mice strains were determined.
MAbs cross-reactive with mouse AChR (mAChR), or directed against the MIR and the α-BT binding site were more frequently found in EAMG susceptible C57bl/6 mice whereas mAbs against an intracellular epitope were more frequently found in Balb/c mice. Possible differences in the available anti-AChR antibody repertoire were also evaluated in IgG obtained from EAMG and control mice. C57bl/6 and Balb/c mice showed similar concentrations of antibodies against the MIR and the α-BT binding site, in contrast with differences in the relative frequencies of these specificities found in the clonable repertoire. These results indicate that disease susceptibility in murine EAMG is not determined by major differences in the anti-AChR repertoire.

MATERIAL AND METHODS

Animals and antigens
8 wk old C57bl/6 and Balb/c mice were purchased from Charles River Wiga GmbH, Frankfurt, FRG and were maintained under specific pathogen free conditions. AChR from electric organ of Torpedo californica (Pacific Biomarine, California, USA) was

purified by affinity chromatography on Naja naja siamensis toxin (Miami Serpentarium, Florida, USA) linked to Sepharose-4B (Pharmacia LKB, Woerden, The Netherlands) (17).

Production and screening of hybridomas

Mice were immunized with 15 µg purified tAChR in CFA. Animals were boosted 3 and 5 weeks after primary immunization with 15 µg AChR in IFA. Animals were sacrificed three days after the last booster injection and cells of para-aortal, inguinal and poplitial lymphnodes were fused with the mouse tumor cell line SP2/0-Ag14 according to Köhler and Milstein (18). Hybridomas were initially screened for reactivity to tAChR by an ELISA.

96 wells polystyrene microtiter plates (Greiner, Alphen a/d Rijn, The Netherlands) were coated with 5 µg/ml AChR or KLH in 10 mM sodiumbicarbonate buffer pH=9.5 for 1 hr at 37 °C (50 µl/well). After washing three times with H_2O containing 0.5 % Tween-20 (H_2O/Tw), plates were incubated for 15 min with PBS containing 0.5 % BSA and 0.5 % Tween-20 (PBSA/Tw) to occupy remaining bindingsites. Plates were incubated with 100 µl of hybridoma culture supernatant for 18 hr on a rocking platform. After washing with H_2O/Tw the wells were incubated for 1 hr at room-temperature (R.T.). with rabbit-anti-mouse Ig coupled to HRP (Dako ITK diagnostics, Uithoorn, The Netherlands) on a rocking platform. After washing with H_2O/Tw the colorimetric reaction was performed by adding 100 µl of 110 mM sodiumacetate buffer, pH 5.5, containing 100 µg/ml TMB and 0.01 % H_2O_2. The colorreaction was stopped after 10 minutes by adding 50 µl 4n H_2SO_4. The optical density was measured at 450 nm using a Titertek Twinreader (Amstelstad, Amsterdam, The Netherlands).

Fine specificity of anti-AChR mAbs

MAbs were screened for binding to extra- and intracellular located epitopes on AChR rich membrane vesicles (liposomes). Flexible PVC 96 wells ELISA plates (Flow ICN, Amsterdam, The Netherlands) were coated with protein G purified mAb (25 µg/ml) for 1 hr at 37 °C. Plates were washed and preincubated as described above. Coated plates were incubated with 150 µl AChR rich membrane vesicles (80 femtomoles AChR) labeled with ^{125}I-α-BT for 16 hrs at 4 °C. Membrane vesicles rich in AChR were isolated from electric organ of Torpedo californica as described previously (17). After washing the plates with PBS buffer containing 0.5 % Triton X-100 (Sigma/Brunschwig chemie, Amsterdam, The Netherlands) and 0.02 % NaN_3 (PBS/TX), bound radioactivity was counted in a gamma counter.

MAbs were screened for reactivity to the MIR of the AChR by a competitive inhibition ELISA using mAb 35 as a reference anti-MIR antibody. Briefly, 96 wells ELISA plates were coated with 5 µg/ml purified AChR (50 µl) and incubated for 1 hr with 50 µl anti-AChR mAb with increasing concentration (0.001-10 µg/ml). Subsequently, plates were incubated for 1 hr with 50 µl HRP conjugated mAb 35 (0.04 µg/ml, 50 % of maximum binding to AChR) without washing and bound mAb 35-HRP was measured. The results are expressed as percentage inhibition of mAb 35-HRP binding and calculated as follows:

[(average A_{450} of duplicate wells with mAb 35-HRP alone - average A_{450} of duplicate wells in which mAb 35-HRP was tested in the presence of putative anti-MIR mAb)/average A_{450} of duplicate wells with mAb 35-HRP alone]x100.

MAbs were considered to be directed to the MIR when binding of mAb 35-HRP to AChR was inhibited by more than 50 % (21).
MAbs were also tested for there ability to inhibit the binding of ^{125}I-α-BT to AChR by the following assay: 96 wells flexible ELISA plates were coated with Torpedo AChR (5 µg/ml) and incubated with 100 µl hybridoma supernatant or purified mAb at R.T. for 16 hrs. After incubation the supernatant was removed by aspiration and plates were subsequently incubated with a limiting concentration of ^{125}I-α-BT (2 picomoles/ml) for 2 hrs at R.T.. Plates were washed and bound radioactivity counted as described above. Results are expressed as percentage inhibition of ^{125}I-α-BT binding to AChR. The percentage inhibition was calculated as follows:

[(average cpm of duplicate wells with ^{125}I-α-BT alone - average cpm of duplicate wells with ^{125}I-α-BT in the presence of putative inhibitor mAb)/average cpm of duplicate wells with ^{125}I-α-BT alone]x100.

Cross-reaction with mAChR
Anti-AChR mAbs were screened for cross-reactivity to mAChR by a liquid phase precipitation radio immunoassay (RIA) (19), or solid phase radio immunoassay (SPRIA) in which binding of ^{125}I-α-BT labeled mAChR to solid phase bound mAbs was tested. MAbs that failed to precipitate mAChR in the RIA were purified from hybridoma supernatant, coated to ELISA plates and tested for ^{125}I-α-BT-mAChR binding; mAbs purified on protein G Sepharose-4B were coated on 96 wells PVC ELISA plates (25 µg/ml). Plates were subsequently incubated overnight at 4 °C with 150 µl of ^{125}I-α-BT-mAChR (0.6 nM). After incubation plates were washed and bound radioactivity counted as described above. MAbs against KLH and thyroglobulin (22) were used as negative controls.
In addition mAbs cross-reactive with mAChR were tested for binding to neuromuscular junctions in cryosections of mouse extensor digitorum longum muscle by immunofluorescence (20). Briefly, cryosections fixed for 10 min in icecold aceton were incubated with 25 µl hybridoma supernatant or purified mAb (20-100 µg/ml) for 1 hr. Bound antibody was visualized by fluorescence microscopy using FITC-conjugated goat-F(ab)$_2$-anti mouse Ig (Cappel, Organon Technika, Boxtel, The Netherlands).

Antibody isotype
The isotypes of monoclonal antibodies were determined using a mouse mAb isotyping kit (Holland Biotechnology, Leiden, The Netherlands).

Passive transfer of anti-AChR mAb
Eight week old C57bl/6 mice were injected intraperitoneal with 1ml/20 gr body-weight mAb 60 ascitic fluid having a mAChR binding capacity of 45 pmol/ml. Control animals were injected with PBS. Animals were killed at 48 hrs after injection for determination of AChR concentration.

Induction of chronic EAMG
EAMG was induced in mice by immunization with 15 μg tAChR emulsified in CFA at the base of the tail and both footpads and boosted with 15 μg AChR in IFA after 31 days.

Anti-mAChR antibody titer
Antibody titers against mAChR were measured by immunoprecipitation using ^{125}I-α-BT labeled mAChR according to Lindstrom et al (19) with minor modifications. A crude extract of denervated mouse muscle AChR (approximately 0.6 nanomoles mAChR/l) was labeled with $2x10^{-9}$ M ^{125}I-α-BT for 4 hrs at 4 °C. Diluted serum samples (5 μl) were incubated with 200 μl ^{125}I-α-BT-mAChR overnight at 4°C. Antibody-AChR complexes were precipitated by adding excess goat-anti-mouse Ig antibodies. After 4 hrs at 4 °C samples were centrifuged for 3 min in an Eppendorf centrifuge and subsequently washed once with 1 ml PBS buffer containing 0.5 % Triton-X100 (Sigma, Brunschwig chemie b.v., Amsterdam, The Netherlands) and 0.02 % NaN_3 (PBS/TX). Radioactivity was counted in a gamma counter (Compu-gamma, Pharmacia LKB, Woerden, The Netherlands). Values of ^{125}I-α-BT-mAChR pelleted in the presence of normal mouse serum were considered background and subtracted from the assay values. The antibody titers were expressed as moles of ^{125}I-α-BT binding sites per liter.

Antibody specificity in EAMG sera
Antibodies directed to the MIR and the α-BT binding site in the sera of EAMG mice were determined using a competitive inhibition ELISA. Sera obtained from C57bl/6 and Balb/c mice were pooled and IgG was purified by affinity chromatography on protein G sepharose (Pharmacia LKB, Woerden, The Netherlands). EAMG or controle IgG (0.0005-50 μg/ml) was used to inhibit the binding of reference anti-MIR mAb 35 or α-BT as described above. Results are expressed as percentage inhibition of mAb 35-HRP or ^{125}I-α-BT binding to AChR.

Quantitation of AChR in muscle
The concentration of AChR was determined in whole carcasses according to Lindstrom et al (19) with modifications as previously described (43). The AChR concentration in muscle was expressed as pmol AChR/100 gr muscleweight.

RESULTS

Anti-AChR mAbs from C57bl/6 and Balb/c mice

Monoclonal antibodies directed against AChR, were obtained from three separate fusions using pooled lymphnode cells from C57bl/6 (n=3) and Balb/c (n=3) mice immunized three times with AChR from Torpedo californica. Hybridomas were screened for binding to tAChR. Anti-AChR mAbs were of the κ-light chain type and IgG_1 (35%), IgG_{2a} (13%), IgG_{2b} (26%), IgA (6%) and IgM (9%) isotypes. All characteristics of anti-tAChR mAbs are summarized in table I.

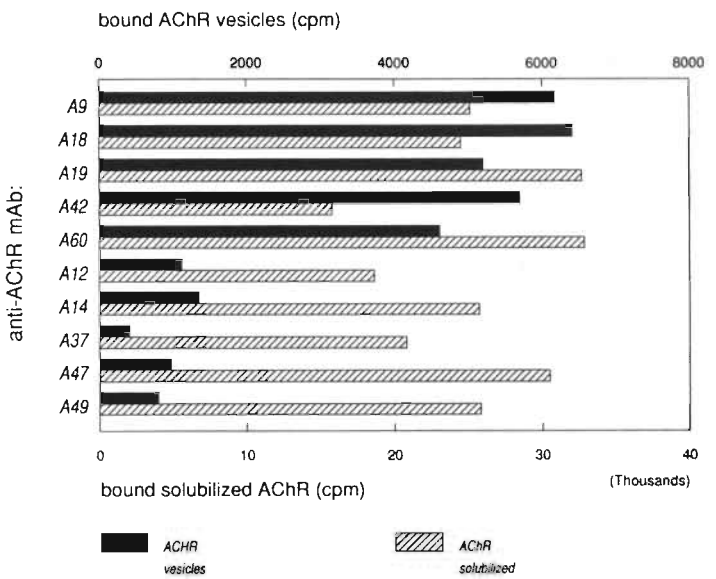

Figure 1: Membrane bound AChR only binds to mAbs against extracellular epitopes.
Anti-AChR mAbs were tested for binding to solubilized AChR or AChR vesicles in which the receptor is embedded in the membrane and only extracellular epitopes are exposed. The striped bars represent the binding of solubilized AChR to solidphase mAbs. The solid bars represent the binding of AChR vesicles to mAbs.

MAbs against extra- and intracellular epitopes

The AChR is a transmembrane glycoprotein with extracellular epitopes accessible for antibody *in vivo* and intracellular epitopes only accessible on solubilized AChR *in vitro*. Since anti-AChR mAbs are generated against solubilized AChR both antibodies against extra- and intracellular epitopes will arise. MAbs binding to extracellular epitopes can be discriminated from mAbs to intracellular epitopes by binding to AChR rich membrane vesicles. Thirty-seven out of 65 mAbs bound to both membrane incorporated and solubilized AChR, whereas 12 out of 65 mAbs bound to solubilized but not membrane incorporated AChR. Binding of representative mAbs to solubilized or membrane bound tAChR are shown in figure 1.

MAbs directed to the main immunogenic region

In both MG and EAMG it was reported that the major part of pathogenic antibodies is directed against the MIR; a 10 amino acid epitope on the α-subunit of the AChR. Seventeen out of 65 mAbs were directed against the MIR as was determined by competitive inhibition with reference rat anti-MIR mAb 35. Representative inhibition curves and the concentrations of anti-MIR mAbs needed for 50 % inhibition (I_{50}) are shown in figure 2.

Table I: Characteristics of anti-AChR mAbs.

C57bl/6		Specificity					
mAb	Isotype	tAChR[a]	mAChR[b]	MIR[c]	α-BT[d]	extra[e] cell.	intra[f] cell.
A2	IgM	•					
A6	IgG2a	•					•
A7	IgG2a	•	•	•		•	
A9	IgG1	•	•	•		•	
A10	IgG2b	•					
A11	IgM	•	•	•		•	
A13	IgG2a	•				•	
A14	IgG2a	•	•				•
A15	IgG2b	•					
A18	IgG1	•	•			•	
A19	IgG1	•	•			•	
A20	IgA	•					
A21	IgG1	•				•	
A22	IgG2a	•	•	•		•	
A23	IgA	•					
A24	IgM	•	•			•	
A25	IgG1	•		•		•	
A26	IgG2b	•		•		•	
A27	N.D.	•				•	
A28	IgM	•					
A29	IgG2b	•		•		•	
A32	IgG2a	•				•	
A46	IgG1	•					•
A49	IgG2a	•	•				•
A50	IgG2b	•					
A53	IgG2b	•				•	
A54	IgG1	•				•	
A55	IgG1	•				•	
A56	IgG2b	•					
A57	IgG1	•					
A58	IgG1	•	•		•	•	
A59	IgG2b	•				•	
A60	IgG2b	•	•	•		•	
A61	N.D.	•				•	
A62	IgG2b	•			•	•	
A63	IgG2b	•			•	•	
A64	IgG1	•			•	•	
A65	IgG2b	•			•	•	
		38	11	8	5	25	4

Balb/c		Specificity					
mAb	Isotype	tAChR[a]	mAChR[b]	MIR[c]	α-BT[d]	extra[e] cell.	intra[f] cell.
A1	IgG1	•		•		•	
A3	IgM	•		•		•	
A4	IgG2a	•				•	
A5	IgA	•					
A8	IgG1	•			•	•	
A12	IgG1	•	•				•
A16	IgG1	•					
A17	IgA	•					
A30	IgG1	•					•
A31	IgG1	•					
A33	IgG2a	•					
A34	IgG2b	•		•		•	
A35	IgG2b	•		•		•	
A36	IgG1	•		•		•	
A37	IgG1	•	•				•
A38	IgG2b	•		•		•	
A39	IgG2b	•		•		•	
A40	IgG1	•		•		•	
A41	IgG1	•		•		•	
A42	IgG1	•	•				
A43	IgG1	•	•			•	
A44	IgG2b	•					•
A45	IgM	•					•
A47	IgG2a	•	•				•
A48	IgG2a	•					•
A51	IgG2b	•					
A52	IgG2	•					
		27	5	9	1	12	7

Isotype and specificity of mAbs directed to AChR raised in EAMG high susceptible C57bl/6 and low susceptible Balb/c mice. [a] *All mAbs are specific for AChR of Torpedo californica.* [b] *MAbs cross-reactive with solubilized AChR from denervated mouse muscle.* [c] *MIR: the main immunogenic region located on the α-subunit (α67-76) of AChR.* [d] *α-BT: the α-bungarotoxin binding site on the α-subunit (α189-195) of AChR.* [e] *Extracell.: epitopes located on the extracellular surface of AChR.* [f] *Intracell.: epitopes located on the cytoplasmic surface of AChR only accessible on solubilized receptor.*

MAbs directed to the α-bungarotoxin binding site

Six anti-AChR mAbs were directed to the binding site for α-BT which coincides with the binding site for acetylcholine. Four mAbs (A8, A58, A64 and A65) were able to block the binding of ^{125}I-α-BT to AChR for more than 80 % suggesting that these

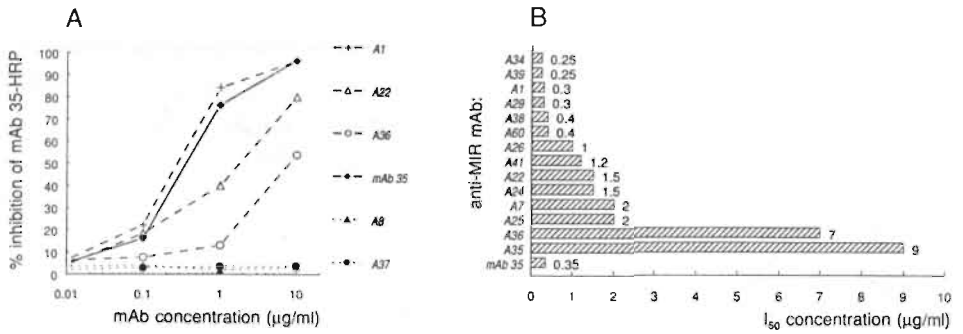

Figure 2: Competitive inhibition of reference anti-MIR mAb 35 binding to AChR by anti-AChR mAbs.
(A) Representative inhibition curves are shown in which the binding of HRP conjugated mAb 35 to AChR was inhibited by mouse anti-MIR mAbs. MAbs which inhibited the binding of mAb 35 by more than 50 % were considered to be directed to the MIR (42). Non-MIR mAbs A8 (directed to the α-BT binding site on the α-subunit) and A37 (directed to a cytoplasmic epitope on the α-subunit) did not show any inhibition of mAb 35 binding.
(B) Differences in affinity or fine specificity of anti-MIR mAbs are represented by the concentrations of anti-AChR mAbs needed for 50 % inhibition of mAb 35-HRP binding to AChR (I_{50}).

mAbs recognized both α-BT binding sites, whereas mAb A62 and A63 only inhibited ^{125}I-α-BT binding around 50 %, suggesting that these mAbs only recognized one of the two α-BT binding sites on each AChR molecule (fig. 3).

Cross-reaction with mAChR

The cross-reaction of mAbs against tAChR with mAChR was determined either by direct binding of ^{125}I-α-BT-mAChR to solidphase mAb (SPRIA) or precipitation of ^{125}I-α-BT-mAChR (RIA). Sixteen out of 65 anti-tAChR mAbs showed cross-reactivity with mAChR using the SPRIA. Representative examples of the binding of ^{125}I-α-BT-mAChR and ^{125}I-α-BT-tAChR to solid phase mAbs are shown in figure 4. The cross-reactive mAbs A42, A43, A49, and A60 could also precipitate ^{125}I-α-BT-mAChR using the RIA. All mAbs cross-reactive with mAChR as determined by SPRIA, were also tested for binding to neuromuscular endplates in cryosections of mouse muscle. Twelve of the 16 cross-reactive mAbs bound to endplates (not shown).

In vivo pathogenicity

To determine the pathogenicity *in vivo*, mAb A60 was passively transferred to naive C57bl/6 mice to induce the effector phase of EAMG. Mice injected with mAb A60 did not show muscle weakness or weight loss at 48 hrs after injection. However, mAb A60 injected mice showed a mean AChR concentration of 17±6 pmol/100 gr compared to 35±10 pmol/100 gr muscle (mean±SEM) in control mice.

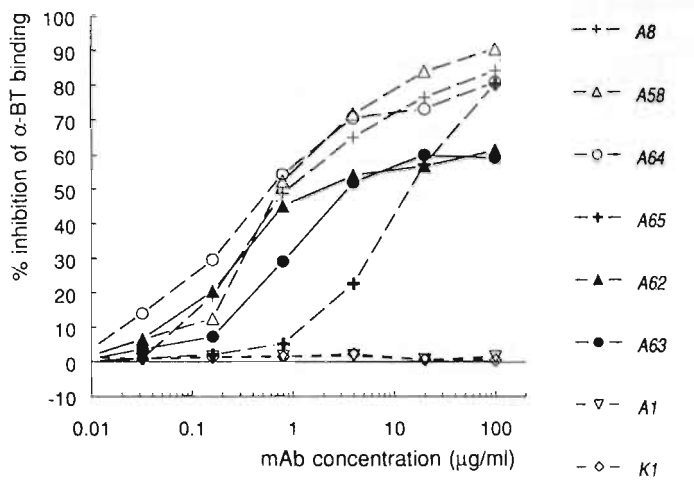

Figure 3: Anti-AChR mAbs directed against the α-BT binding site competitively inhibit the binding of ^{125}I-α-BT to AChR.
The binding of a limiting concentration of ^{125}I-α-BT to AChR was tested in the presence of putative anti-α-BT binding site antibodies. Results are expressed as percentage inhibition of α-BT binding in the absence of antibody. Anti-MIR mAb A1 and anti-KLH mAb K1 are not capable of inhibiting α-BT binding to AChR.

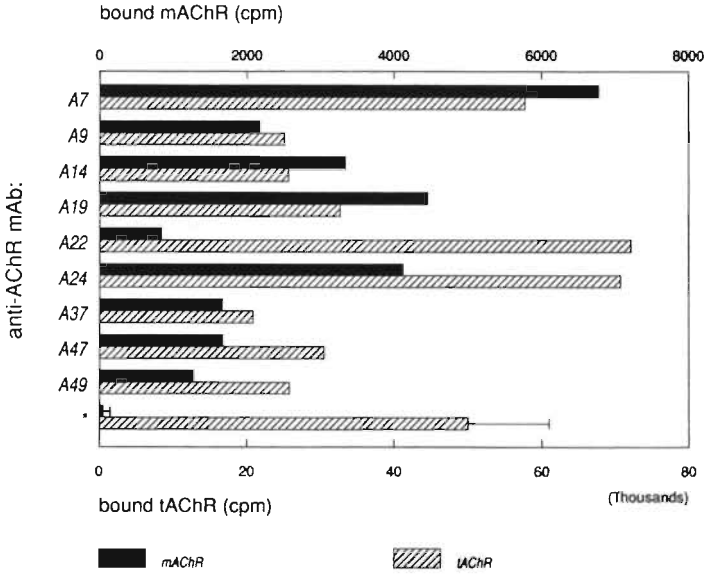

Figure 4: Cross-reactive mAbs detected by solid phase radio immunoassay.
Solid phase anti-AChR mAbs were tested for binding to Torpedo or mouse AChR. The solid bars represent the binding of mAChR to solid phase mAb. The striped bars represent the binding of t AChR to mAbs.
The asteriks denotes the mean binding of all anti-AChR mAbs considered non cross-reactive.

Table II: Frequencies of anti-AChR specificities in the clonable repertoire of C57bl/6 and Balb/c mice

	C57bl/6		Balb/c	
Specificity[a]	%	n[b]	%	n
mAChR	26	(308)	14	(209)
MIR	20	(182)	17	(151)
α-BT	4	(308)	1	(190)
α371-378	1	(360)	5	(330)

The relative frequencies of clonable B cells from C57bl/6 and Balb/c mice were determined among initial clones. [a] *B cell clones were isolated which were cross-reactive with mAChR, directed to the MIR, α-BT binding site and an intracellular epitope on the α-subunit of AChR (α371-378). The relative frequency of a certain specificity was expressed as the percentage of clones positive of the total number tested.* [b] *The total number of tested clones is shown in parenthesis.*

Frequencies of antibody specificities from C57bl/6 and Balb/c mice

The relative frequencies of certain specificities obtained from the clonable anti-AChR antibody repertoire of C57bl/6 and Balb/c mice were compared. The occurance of hybridomas cross-reactive with mAChR and antibodies against the MIR, α-BT binding site, and an intracellular epitope (α371-378) among primary hybridomas was expressed as the percentage of the number of tested hybridomas. Hybridomas cross-reactive with mAChR were more frequently isolated from C57bl/6 (26 %) compared to Balb/c mice (14 %) (table II). Hybridomas specific for the MIR and the α-BT binding site were found in 20 % and 4 % respectively of the C57bl/6 hybridomas compared to 17 % and 1 % in Balb/c derived hybridomas. However, hybridomas with specificity for an intracellular epitope are more frequently found in Balb/c (5%) than C57bl/6 (1%) mice.

Induction of chronic EAMG in C57bl/6 and Balb/c mice

EAMG was induced in C57bl/6 (n=8) and Balb/c (n=8) mice. Four out of 8 C57bl/6 mice developed rapidly progressive EAMG after the second immunization. Sick animals had typical signs of myasthenia including muscle weakness, tremor, and an inability to adduct the hindlegs. Affected mice showed a rapid loss of weight due to dehydration and usually died within 2 days after onset of the disease (fig. 5A). In contrast, only 1 Balb/c mouse showed weightloss (fig. 5C) and died from severe EAMG. Unaffected C57bl/6 or Balb/c mice showed no weightloss compared to PBS immunized control mice (fig. 5B and 5D). All mice immunized with tAChR developed antibodies against mAChR (fig. 6A and 6B). However, no significant difference in mean anti-mAChR antibody titer between C57bl/6 and Balb/c mice was seen. A broad range of anti-mAChR titers was found in both sick and healthy mice. The

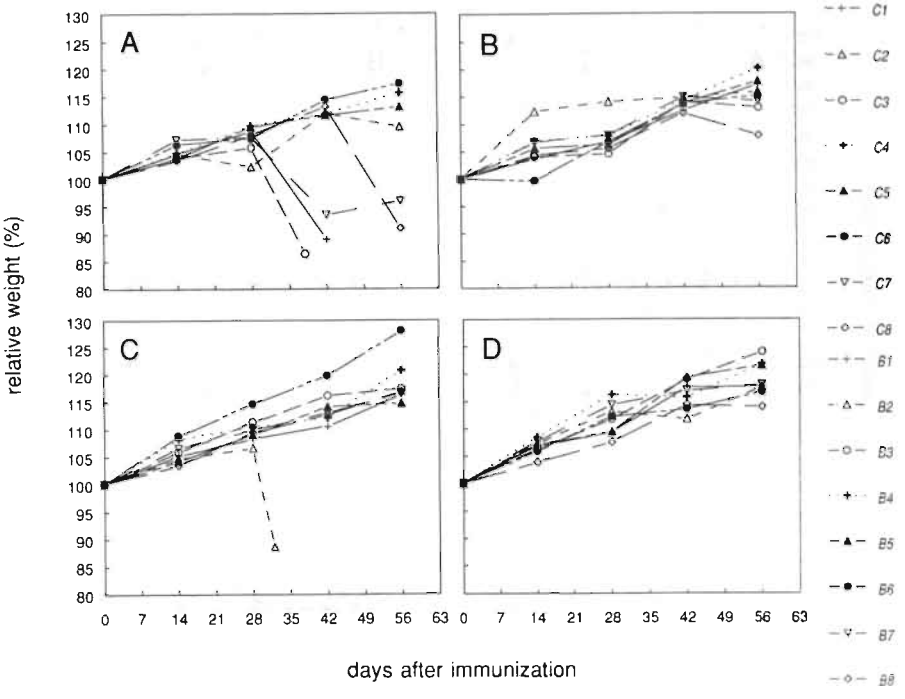

Figure 5: Weightloss as parameter of disease severity.
The weightloss of C57bl/6 and Balb/c mice was monitored at 2 week intervals after immunization with AChR or PBS. Animals with severe EAMG showed a dramatic weight loss due to paralysis. Bodyweight was expressed as percentage of the weight at day 0. C1-C8: C57bl/6 nr 1-8. B1-B8: Balb/c nr 1-8. (A) C57bl/6 mice immunized with AChR. (B) C57bl/6 mice immunized with PBS. (C) Balb/c immunized with AChR. (D) Balb/c mice immunized with PBS.

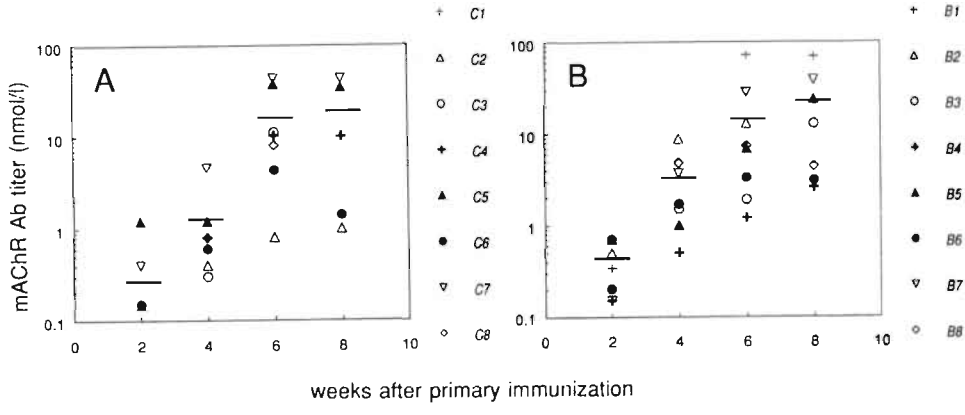

Figure 6: C57bl/6 and Balb/c mice develop similar mean antibody titers against autologous AChR.
The antibody titer against mAChR was determined at 2 week intervals after immunization with tAChR. The mean anti-mAChR titer is represented by the horizontal bars. (A) The anti-mAChR titers of C57bl/6 mice. C1-C8: C57bl/6 nr 1-8. (B) The anti-mAChR titers of Balb/c mice. B1-B8: Balb/c nr 1-8.

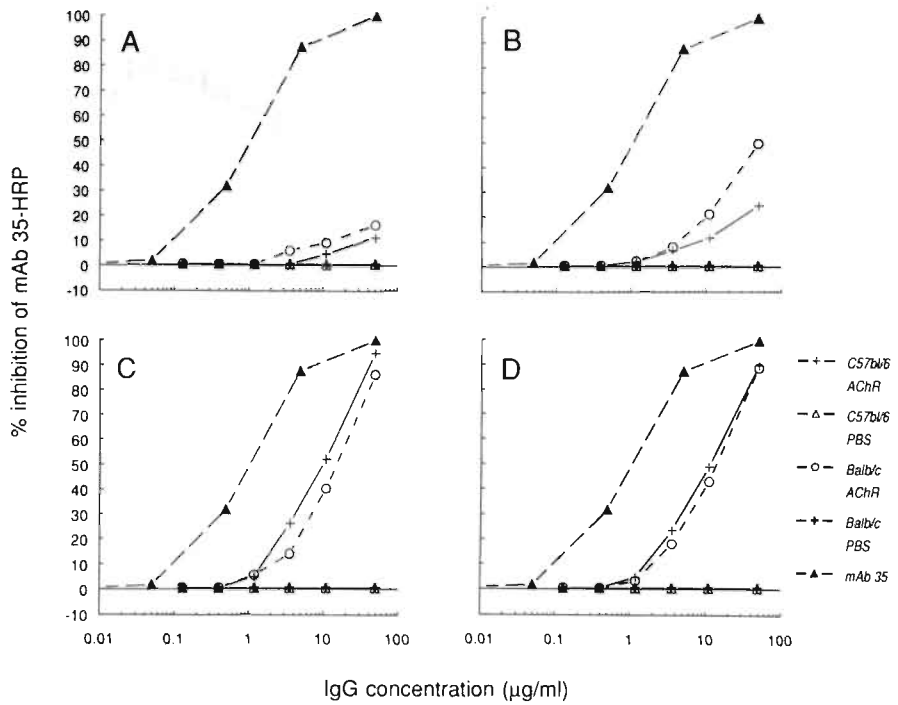

Figure 7: C57bl/6 and Balb/c mice have similar concentrations of antibodies directed against the MIR
Antibodies directed against the MIR were detected in IgG from EAMG and control mice by competitive inhibiton of reference anti-MIR mAb 35. (A) Inhibition of mAb 35-HRP 2 weeks after immunization. (B) 4 weeks. (C) 6 weeks. (D) 8 weeks after immunization.

anti-mAChR titer in sick C57bl/6 ranged from 0.8 to 43 nmol/l whereas the titer of mice without clinical signs of disease ranged from 1 to 34 nmol/l. No relation between anti-mAChR antibody titer and disease susceptibility could be demonstrated.

Antibody fine specificity in EAMG sera

The fraction of anti-AChR antibodies directed against the MIR and the α-BT binding site were determined in EAMG sera at several points in time after immunization with AChR. Two weeks after the primary immunization less than 0.1 % of total IgG from C57bl/6 or Balb/c was directed against the MIR (fig. 7A). The fraction of anti-MIR antibodies was increased 4 weeks after immunization. IgG from Balb/c mice showed a stronger inhibition of mAb 35 binding compared to IgG from C57bl/6 mice (fig. 7B). The higher inhibitory capacity of Balb/c compared to C57bl/6 IgG levelled out after the second immunization (fig. 7C). The inhibitory capacity of C57bl/6 and Balb/c IgG did not further increase 8 weeks after immunization and both mouse strains showed comparable inhibition of mAb 35 binding (fig. 7D). When

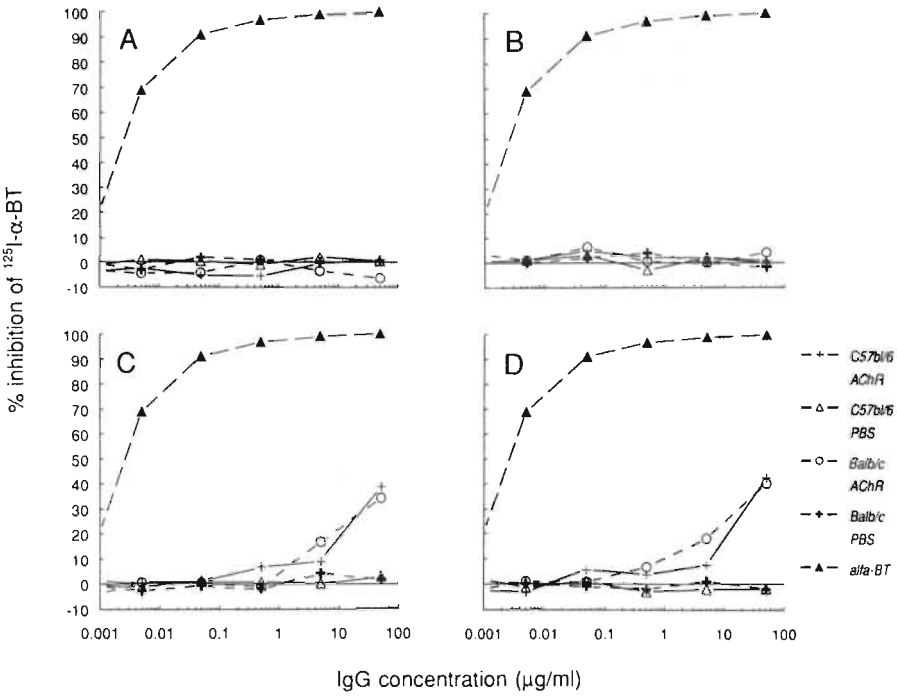

Figure 8: C57bl/6 and Balb/c mice have similar contrations of anti-site antibodies.
Antibodies directed against the α-bungarotoxin binding site were detected in IgG from EAMG and control mice by competitive inhibition of α-BT. (A) Inhibition of α-BT 2 weeks after immunization. (B) 4 weeks. (C) 6 weeks. (D) 8 weeks.

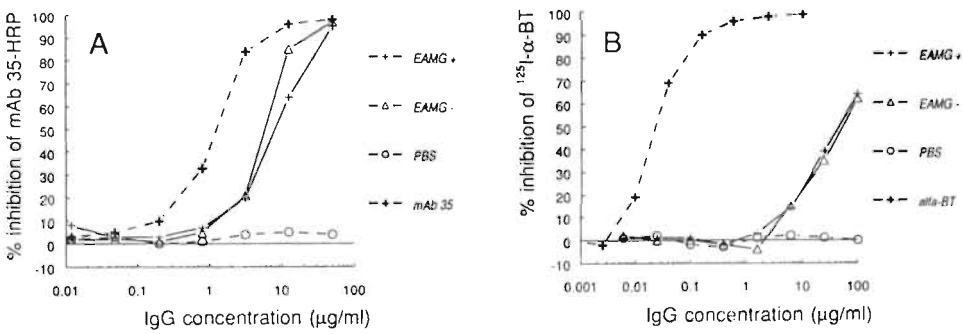

Figure 9: Antibody fine specificity for the MIR or α-BT binding site does not differ between paralyzed and non paralyzed C57bl/6 mice.
The fraction of antibodies against the MIR or the α-BT binding site were determined in IgG from paralyzed (EAMG +) and non-paralyzed (EAMG -) C57bl/6 mice by competitive inhibition 8 weeks after primary immunization. (A) Inhibition of mAb 35 binding. (B) Inhibition of α-BT binding.

I_{50} concentrations of mAb 35 and EAMG IgG were compared at this point in time, approximately 10 % of total IgG was directed against the MIR in both mouse strains. Antibodies against the α-BT binding site were not detected after the first immunization with AChR (fig. 8A+B). A similar fraction of total IgG from C57bl/6 or Balb/c mice was directed against the α-BT binding site 6 weeks after primary immunization (fig. 8C), which was not increased at 8 weeks (fig. 8D). At 8 weeks less than 0.5 % of total EAMG IgG was directed against the α-BT binding site.
When antibody fine specificities were compared between paralyzed and non-paralyzed C57bl/6 mice, no differences in the fractions of antibodies to the MIR or α-BT binding site were observed (fig. 9A+B).

DISCUSSION

Monoclonal antibodies against AChR were obtained from EAMG high susceptible C57bl/6 and low susceptible Balb/c mice. Antibodies directed to several epitopes including the MIR and α-BT binding site were isolated from both C57bl/6 and Balb/c mice. Among mAbs that bound AChR in membrane vesicles, several were directed against the MIR as determined by competitive inhibition with reference anti-MIR mAb 35 (26). Among mAbs that bound solubilized AChR but no AChR incorporated in vesicles, several were competitive with mAb 155 (5) against an intracellular epitope (α371-378) but none of these mAbs were competitive with mAb 35 against the extracellularly located MIR. These results are in agreement with electronmicroscopic imaging of gold labeled anti-AChR mAbs against extra- and intracellular epitopes which were demonstrated to bind to the exterior or interior surface of these AChR vesicles (27).
A major part of anti-AChR antibodies in MG and rats immunized with intact AChR are directed against the MIR localized on the α-subunit at residues 67-76 (4, 23). Anti-MIR mAbs are very potent in accelerating AChR degradation by antigenic modulation (28) and are able to induce EAMG in animals by passive transfer (29). Mouse mAbs against the MIR were distinguished from mAbs to other extracellular epitopes on AChR by competitive inhibition with reference anti-MIR mAb 35. Seventeen mAbs were competitive with anti-MIR mAb 35 and varying amounts of these mAbs were needed to inhibit mAb 35 for 50 %, indicating that these mAbs show variing affinities for the MIR or recognize epitopes partially overlapping the MIR. MAbs with fine specificity for epitopes separated by only seven residues can be distiguished using this competitive inhibition technique (21). Similarly, mouse mAbs against human AChR were also found to inhibit mAb 35 and are directed against two antigenic regions overlapping the MIR (24).
Six mAbs were isolated that competitively inhibit the binding of α-BT to the AChR. The α-BT binding site, including the cholinergic binding site, is located on the α-subunit (α189-195) distinct from the MIR (31). Four anti-α–BT binding site mAbs were able to inhibit α–BT binding almost completely whereas two mAbs inhibited α-BT binding to a maximum of 50 %. MAbs completely inhibiting α-BT are probably directed to epitopes similar for both α-BT binding sites on each AChR molecule, whereas mAbs partially inhibiting α-BT are directed to epitopes unique to each of the two binding sites (31, 32, 33). The mAbs A62 and A63 inhibited α-BT to a

maximum of 50 % and are probably directed to the same α-BT binding site because when mAbs were combined they still inhibited α-BT binding no more than 50 %. Furthermore, the crossreaction of anti-AChR mAbs with autologous AChR was determined since mAbs reacting with mAChR could be pathogenic *in vivo*. Sixteen out of 65 mAbs were cross-reactive with mAChR as determined by solid- or liquid phase radio immunoassay or binding to the motor endplates in muscle sections. Four of these mAbs precipitated mAChR using the standard liquid phase precipitation radio immunoassay (19). Precipitation of some mAbs may be less efficient because they were not recognized by the polyclonal goat-anti-mouse IgG used to precipitate mAb-AChR complexes.

Differences in disease susceptibility between C57bl/6 and Balb/c mice could reside from differences in the available anti-AChR antibody repertoire. Therefore, the frequency of different antibody specificities in the clonable B-cell repertoire was compared between C57bl/6 and Balb/c mice. Among initial hybridomas isolated from C57bl/6 mice, 26 % was cross-reactive with mAChR compared to 14 % Balb/c mice. Autoreactive clones may therefore be more abundant in susceptible mice. Furthermore, the frequency of clones against the MIR and α-BT binding site were higher in C57bl/6 compared to Balb/c mice whereas clones against an intracellular epitope were more frequently found in Balb/c mice. It was further investigated whether these differences in the frequency of available antibody specificities could also be demonstrated in the polyclonal anti-AChR response after induction of EAMG by immunization with tAChR.
C57bl/6 and Balb/c mice immunized with tAChR developed a similar mean serum anti-mAChR antibody titer which did not reflect the observed difference in the frequency of autoreactive hybridomas between C57bl/6 and Balb/c mice. The frequency of autoreactive hybridomas *in vitro* may not be representative for the *in vivo* activation of autoreactive clones.
Although the mean serum anti-mAChR antibody titers for each mouse strain were similar, large differences in titer were found between individual mice. Furthermore, the anti-mAChR titer did not correlate with appearance of clinical signs. The high variability in anti-mAChR antibody titer and absence of an obvious correlation between anti-mAChR antibody concentrations and development of EAMG is also found by other investigators (14, 15, 34, 35) and mirrors the lack of correlation between serum anti-AChR antibody concentration and the clinical state in MG (36, 37). This can be explained by the fact that part of the antibodies cross-reactive with mAChR may not be detected in the sera due to *in vivo* binding to the AChR. Moreover, antibodies against the α-BT binding site are not detected in the conventional RIA used for anti-AChR antibody determination. Furthermore, only a small part of total anti-AChR antibody pool may be relevant for the pathogenesis of the disease, and the major fraction of the detected anti-AChR antibody pool may consist of non-pathogenic antibodies which confuse the correlation between anti-AChR antibody titer and disease severity.
Thus, the absolute amount of antibody does not determine disease susceptibility. However, differences in antibody fine specificity may determine the outcome of the disease. Antibodies against the MIR of the AChR were demonstrated in pooled IgG from EAMG mice by competitive inhibition with reference anti-MIR mAb 35. After

secondary immunization approximately 10 % of total IgG was directed against the MIR in both mouse strains. The observed difference in frequency of clonable anti-MIR B-cells was not observed in the polyclonal anti-AChR response. No difference in the fraction of serum anti-MIR antibodies was found that could be related to susceptibility for EAMG. Antibodies against the MIR constitute a substantial part of the anti-AChR antibodies in MG (23, 24). However, no relation was found between antibody specificity and clinical state of the patient (23, 24). The fraction of antibodies against the MIR in MG patients remained stable even after decline of the total anti-AChR antibody titer and clinical improvement (24).
Similar fractions of total IgG were found to be directed against the α-BT binding site in C57bl/6 and Balb/c mice and therefore no relation was found between disease susceptibility and the fraction of site specific antibodies. Less than 0.5 % of the total EAMG IgG was directed to the α-BT binding site. Antibodies against the cholinergic binding site probably play a minor role in EAMG (38). Furthermore, the concentration of anti-site antibodies did not correlate with the severity of MG (39, 40).
Taken together, these results indicate that strain specific differences in disease susceptibility in murine EAMG are not related to differences in the overall concentration of antibodies against the MIR or α-BT binding site. Moreover, no differences in the fractions of antibodies against the MIR or α-BT binding site were found between paralyzed and non-paralyzed C57bl/6 mice. Our results are in agreement with a report of Berman and Patrick, in which accelerated AChR degradation by sera from paralyzed and nonparalyzed mice, was shown to be indistinguishable (14). However, other antigenic fine specificities which consitute only a minor part of the total anti-AChR antibody pool could be important in causing the disease. It was demonstrated that transfer of pooled EAMG sera from susceptible C57bl/6J mice to naive C3H/HeJ or C57bl/6J mice induced muscle weakness, whereas transfer of resistant C3H/HeJ EAMG sera were unable to induce weakness in both mice strains (41). Similar results were obtained with susceptible Lewis vs. resistant Wistar Furth rats (45). Antibodies that impair the ion channel (12, 42, 43) function of AChR may contribute to impairment of the neuromuscular signal transmission combined with AChR loss by complement mediated destruction and antigenic modulation. Probably multiple immunological (15) as well as physiological factors (44) determine susceptibility for EAMG.
Monoclonal anti-AChR antibodies from both mouse strains will be used to investigate possible differences in the genetic diversity of anti-AChR antibodies that could be related to disease susceptibility.

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Chapter 5

V_H gene family utilization of anti-acetylcholine receptor: Antibodies in murine experimental autoimmune myasthenia gravis

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V_H gene family utilization of anti-acetylcholine receptor: Antibodies in murine experimental autoimmune myasthenia gravis

ABSTRACT

In the mouse model for experimental autoimmune myasthenia gravis (EAMG) we determined the utilization of immunoglobulin heavy chain (V_H) gene families by anti-acetylcholine receptor (AChR) mAbs obtained from two mouse strains differing in their susceptibility for induction of EAMG by immunization with Torpedo AChR. Previously, a preferential usage of D-proximal V_H gene families in autoimmune diseases was suggested. To investigate a possible bias in V_H gene family usage in the EAMG model we determined the V_H gene family usage of 65 anti-AChR mAbs and 20 anti-KLH mAbs isolated from high susceptible C57bl/6 (H-2^d, IgH^b) and low susceptible Balb/c (H-2^b, IgH^a) mice by RNA slot-blot analysis, using probes for nine V_H gene families. Anti-AChR mAbs were found to be encoded by V_H genes from at least 6 different families. The V_H gene family usage of anti-AChR mAbs approached a stochastic distribution over the different V_H gene families, however significant deviations from this distribution were found in the J606 and Vgam3.8 families. The Vgam3.8 family was markedly overrepresented in mAbs directed to the α-bungarotoxin binding site. No correlation was found between V_H gene family usage and antibody specificity for the main immunogenic region of the AChR. Expression of cross-reactive idiotopes by anti-AChR mAbs was irrespective of the V_H gene family usage. Furthermore, strain dependent differences in susceptibility for EAMG are not reflected in an aberrant V_H gene family usage.
These findings indicate that anti-AChR mAbs can be encoded by V_H genes from most of the V_H gene families, and no bias in V_H gene family usage towards D-proximal V_H gene families could be demonstrated.

INTRODUCTION

Myasthenia gravis is an antibody mediated autoimmune disease in which antibodies to the AChR impair the neuromuscular transmission (1). The antibody dependence of MG was demonstrated by passive transfer of anti-AChR antibodies from myasthenic patients (2) or animals with EAMG (3, 4, 5). The clinical features of MG can be induced in experimental animals by immunization with AChR from Torpedo californica, eliciting antibodies that cross-react with autologous AChR. Antibodies directed to the AChR play a central role in the pathogenesis of MG and therefore insight in the genetic origin of these autoantibodies could give information about the pathogenic mechanisms of the disease. Many studies have examined the diversity of V_H genes used in autoantibodies derived from normal mice (6-10), from autoimmune prone animals (11-24) and experimentally induced autoantibodies (25-32).
The V_H genes in the mouse are currently classified into 12 V_H gene families comprising V_H genes with more than 80 % nucleotide sequence similarity (33-37). The V_H gene families are generally grouped in clusters which are localized on chromosome 12 adjacent to the D-region (38, 39).
Some of the studies analyzing the V_H gene family utilization of autoantibody producing hybridomas showed a preferential usage of the D-proximal Q52 and PC7183 families (27-29, 40), suggesting a biased utilization of D-proximal located V_H gene families in autoantibodies. Other reports concerning the V_H gene family usage in autoantibodies are in favor of a random V_H gene family utilization (7, 17, 21, 22, 32), which is proportional to the size of the V_H gene family (41).
D-proximal V_H gene families are preferentially used in the fetal and neonatal B cell repertoires (42), before antigenic selection can play a role (6). Furthermore, a high frequency of multi- and autoreactive hybridomas was found among this early B cell repertoire (43, 44). Autoantibodies could therefore arise from this multireactive repertoire and display a biased V_H gene family usage. To investigate a possible preferential usage of V_H genes in EAMG we determined the V_H gene family utilization of anti-AChR mAbs obtained from C57bl/6 and Balb/c mice immunized with AChR from Torpedo californica. It has been demonstrated that inbred mice strains differ in their susceptibility for the induction of EAMG; C57bl/6 mice ($H\text{-}2^d$, IgH^b) are susceptible, whereas Balb/c mice ($H\text{-}2^b$, IgH^a) are relatively resistant to the induction of disease (45, 46). In this study, the relationship between V_H gene family usage of anti-AChR mAbs and strain specific differences in disease susceptibility was analyzed. Furthermore, mAbs directed to the MIR (47) and the α-BT binding site (48) were selected and the V_H gene familiy usage was correlated with specificity for these well defined epitopes on AChR.
In a previous report we showed that rat anti-AChR mAbs express a cross-reactive idiotype (CRI) (49). In this report we extended this study to mouse anti-AChR mAbs and investigated if expression of CRI was correlated to a particular V_H gene family usage.

It could be demonstrated that anti-AChR mAbs were encoded by V_H genes from at least 6 different families and the V_H gene family usage approached a stochastic distribution over the different V_H gene families, except for the J606 and Vgam3.8

families which were significantly under- and overrepresented respectively. The Vgam3.8 family was overrepresented in anti-α-BT binding site mAbs, but expression of CRI was found to be irrespective of V_H gene family usage. Differences in disease susceptibility could not be explained by a deviant V_H gene family usage, except for anti-α-BT binding site mAbs obtained from C57bl/6 mice, which showed a overrepresentation of the Vgam3.8 family.

MATERIAL AND METHODS

Anti-AChR monoclonal antibodies
Monoclonal antibodies against Torpedo AChR were isolated from C57bl/6 and Balb/c mice. Induction and characterization of anti-AChR mAbs are previously described in chapter 2.

Analysis of cross-reactive idiotopes on mouse anti-AChR mAbs
A cross-reactive idiotype (CRI) specific for anti-AChR mAbs was defined by polyclonal affinity purified anti-idiotype antibodies (anti-Id) against rat mAbs as previously described (49). A polyclonal anti-Id against anti-thyroglobulin mAb 62 (50) was used as control anti-Id. Briefly, purified anti-AChR and control mAbs were coated to ELISA plates (5 µg/ml) and incubated with anti-Id6, 35 and 62 antibody (10 µg/ml). Bound anti-Id antibodies were detected by HRP conjugated swine-anti-rabbit Ig antibodies.

Preparation of RNA
Total cytoplasmic RNA of 10^8 viable hybridoma cells was prepared according to Maniatis et al (51), using 20 mM vanadyl ribonuclase inhibitor complex (New England Biolabs, Beverly MA, USA).
The concentration of RNA preparations was determined using a DNA dipstick kit (Invitrogen, San Diego CA, USA). The RNA samples were diluted in 20 x SSC to a range of 1, 0.5, 0.25 µg and immobilized on B85 nitrocellulose filters (Scheicher and Schuell, 's-Hertogenbosch, The Netherlands) using a Minifold II slot-blot manifold. Nitrocellulose filters were air dried and baked at 80 °C for 2 hours.

Hybridization and washing conditions
Filters were prehybridized for 4 hours at 42 °C in hybridization mix containing 50 % w/v formamide, 10 % w/v dextran sulfate, 1 M NaCl, 50 mM Tris-HCl (pH=8.0) 10 % v/v Denhardt's solution containing 2 % w/v Ficoll, 2 % w/v BSA, 2 % w/v polyvinylpyrrolidone and 100 µg/ml denatured salmon sperm DNA. Filters were hybridized with ^{32}P-labeled DNA probes (approximately $5x10^6$ cpm/ml) in hybridization mix for 16 hours at 42 °C and subsequently washed in 3 X SSC, 0.1 % w/v SDS, and twice in 1 X SSC, 0.1 % w/v SDS at 42 °C. Autoradiography was performed by a 24-72 hrs exposure to Kodak XAR-5 X-ray film using an intensifier screen (Du Pont) at -70 °C.

DNA probes
DNA probes representative for the mouse V_H gene families J558, PC7183, Q52, J606, 3660, S107 and X24 used in this study are previously described (6). The V_H

23-9 probe (V_H 3609) is a 500 bp Bam HI-Pst I fragment containing a V_H sequence belonging to the 3609 family. The Vgam3.8 probe contains a 550 bp Pst 1-Eco R1 fragment of the sequence of Vgam3.8. These probes were gifts from Drs. D. Holmberg, J. Kearney, F. Alt, and D. Schulze.

Statistical methods

χ^2 test was applied to compare differences in V_H gene family utilization of mAbs or subgroups of mAbs. For comparison of subgroups of anti-AChR mAbs, the V_H gene families were devided into three groups according to their location relative to the D-region (39). Group I include the families J558, 3609 and J606 which are located most distal from the D-region, group II contains the Vgam3.8, 3660, X24 and S107 families situated between group I families and the D-region proximal families Q52 and PC7183 which comprise the group III V_H gene families.

RESULTS

Characteristics of mouse anti-AChR mAbs

Monoclonal antibodies (n=65) raised against AChR from Torpedo californica were obtained from fusions using pooled lymphnode cells from Balb/c (n=3) or C57bl/6 mice (n=3) immunized with AChR. Anti-AChR mAbs were specific for AChR and did not bind to KLH. Controle mAbs generated against KLH in both Balb/c and C57bl/6 mice were obtained from simultaneous performed fusions. The fine specificity and cross-reaction with mAChR were determined in Chapter 2. The characteristics of anti-AChR mAbs are summarized in table I.

V_H gene family utilization

The utilization of V_H gene families by 65 anti-AChR and 20 anti-KLH hybridomas was determined by hybridization of DNA probes representative for 9 of the 12 known mouse V_H gene families to total cytoplasmic RNA of hybridomas by slot-blot analysis. Hybridomas were assigned to a particular family if they gave a positive signal with only one of the tested V_H gene family probes under high stringency conditions. Three hybridomas hybridized with more than one V_H gene family probe (Q52 and PC7183) and were excluded from this study. Examples of positive hybridization signals representative for each V_H gene family are shown in figure 1. The V_H gene family usage by hybridomas specific for AChR or KLH are shown in figure 2A. Fifty-eight out of 65 (89 %) anti-AChR hybridomas used V_H genes from 6 V_H gene families (J558, 3609, Vgam3.8, 3660, Q52 and PC7183), 7 anti-AChR hybridomas (11 %) could not be assigned to one of the 9 tested V_H gene families at the stringency conditions used. The V_H gene family usage of anti-AChR and anti-KLH mAbs was compared to the expected distribution based on V_H gene family complexity (table II). The V_H gene family usage of anti-AChR mAbs approached a stochastic utilization of the different V_H gene families except for the J606 family

Table I: Characteristics of anti-AChR mAbs.

C57bl/6			Specificity					
mAb	V_H gene family	Isotype	tAChR[a]	mAChR[b]	MIR[c]	α-BT[d]	extra[e] cell.	intra[f] cell.
A2	J558	IgM	•					
A6	PC7183	IgG2a	•					•
A7	N.A.	IgG2a	•	•	•		•	
A9	J558	IgG1	•	•	•		•	
A10	J558	IgG2b	•					
A11	J558	IgM	•	•	•		•	
A13	N.A.	IgG2a	•				•	
A14	3609	IgG2a	•	•				•
A15	J558	IgG2b	•					
A18	J558	IgG1	•	•			•	
A19	J558	IgG1	•	•			•	
A20	3609	IgA	•					
A21	J558	IgG1	•				•	
A22	N.A.	IgG2a	•	•	•		•	
A23	J558	IgA	•					
A24	J558	IgM	•	•			•	
A25	J558	IgG1	•		•		•	
A26	J558	IgG2b	•		•		•	
A27	J558	N.D.	•				•	
A28	Q52	IgM	•					
A29	Vgam3.8	IgG2b	•		•		•	
A32	Q52	IgG2a	•				•	
A46	J558	IgG1	•					•
A49	J558	IgG2a	•	•				•
A50	N.A.	IgG2b	•					
A53	Vgam3.8	IgG2b	•				•	
A54	Vgam3.8	IgG1	•				•	
A55	J558	IgG1	•				•	
A56	J558	IgG2b	•					
A57	J558	IgG1	•					
A58	N.A.	IgG1	•	•		•	•	
A59	J558	IgG2b	•				•	
A60	J558	IgG2b	•	•	•		•	
A61	J558	N.D.	•				•	
A62	Vgam3.8	IgG2b	•			•	•	
A63	Vgam3.8	IgG2b	•			•	•	
A64	J558	IgG1	•			•	•	
A65	Vgam3.8	IgG2b	•			•	•	
			38	11	8	5	25	4

Balb/c			Specificity					
mAb	V_H gene family	Isotype	tAChR[a]	mAChR[b]	MIR[c]	α-BT[d]	extra[e] cell.	intra[f] cell.
A1	J558	IgG1	•		•		•	
A3	J558	IgM	•		•		•	
A4	PC7183	IgG2a	•				•	
A5	J558	IgA	•					
A8	3609	IgG1	•			•	•	
A12	PC7183	IgG1	•	•				•
A16	PC7183	IgG1	•					
A17	N.A.	IgA	•					
A30	Q52	IgG1	•					•
A31	Q52	IgG1	•					
A33	Q52	IgG2a	•					
A34	N.A.	IgG2b	•		•		•	
A35	3660	IgG2b	•		•		•	
A36	J558	IgG1	•		•		•	
A37	3660	IgG1	•	•				•
A38	3660	IgG2b	•		•		•	
A39	J558	IgG2b	•		•		•	
A40	J558	IgG1	•		•		•	
A41	J558	IgG1	•		•		•	
A42	J558	IgG1	•	•				
A43	J558	IgG1	•	•			•	
A44	J558	IgG2b	•					•
A45	J558	IgM	•					•
A47	J558	IgG2a	•	•				•
A48	J558	IgG2a	•					•
A51	PC7183	IgG2b	•					
A52	J558	IgG2	•					
			27	5	9	1	12	7

V_H gene family usage, isotype and specificity of mAbs directed to AChR raised in high susceptible C57bl/6 and low susceptible Balb/c mice. [a] *All mAbs are specific for AChR of Torpedo californica.* [b] *MAbs cross-reactive with solubilized AChR from denervated mouse muscle.* [c] *MIR: the main immunogenic region located on the α-subunit (α67-76) of AChR.* [d] *α-BT: the α-bungarotoxin binding site on the α-subunit (α189-195) of AChR.* [e] *Extracell.: epitopes located on the extracellular surface of AChR.* [f] *Intracell.: epitopes located on the cytoplasmic surface of AChR only accessible on solubilized receptor. N.A.: mAb could not be assigned to one of the 9 tested V_H gene families.*

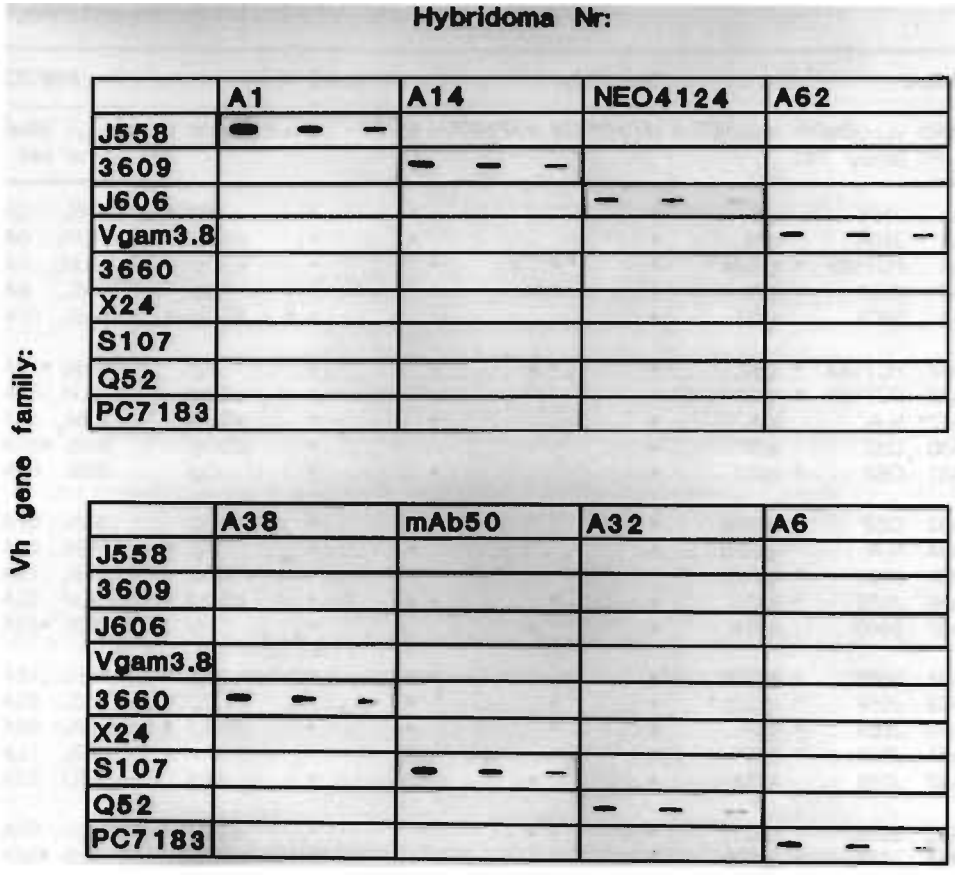

Figure 1: Representative examples of slot-blot hybridization of hybridomas to V_H gene family specific probes.
Aliquots of 1, 0.5 and 0.25 µg RNA were immobilized to nitrocellulose and hybridized to probes specific for nine V_H gene families. NEO4124 is a mAb isolated from a 5 day old nonimmunized Balb/c mouse (10) and mAb50 is a mAb against electric eel AChR.

which was significantly underrepresented ($p<0.01$) and the Vgam3.8 family which was overrepresented ($p<0.05$). The V_H gene family utilization of anti-AChR hybridomas showed no bias in utilization towards D-proximal V_H gene families.
An important subgroup of these anti-AChR mAbs are those cross-reacting with autologous mouse AChR which can cause EAMG. When the V_H gene family usage of this subgroup of potential pathogenic antibodies is considered, V_H genes from at least four different V_H gene families are used; J558 (10/16), 3609 (1/16), 3660 (1/16) and PC7183 (1/16). No significant difference in V_H gene family usage can be observed between mAbs crossreactive with mAChR (n=16) and mAbs specific for Torpedo AChR (n=49) ($\chi^2=1.8$ d.f.=2) (table III).

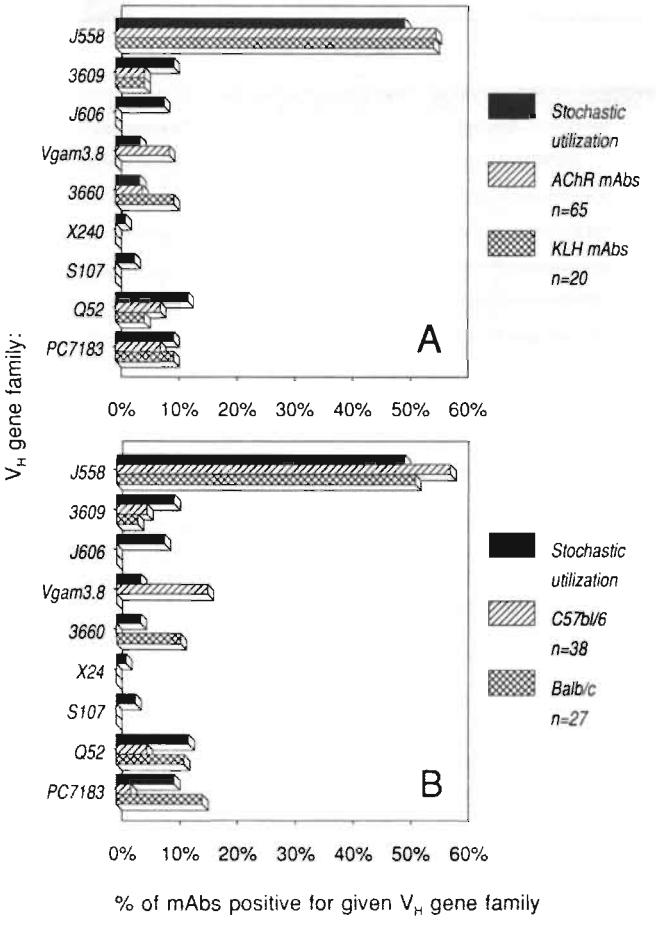

Figure 2: The V_H gene family distribution of anti-AChR and anti-KLH mAbs over nine V_H gene families. *The V_H family usage is expressed as the percentage of mAbs positive for a given V_H gene family and was compared to a stochastic distribution based on the estimated size of the family. Seven anti-AChR mAbs and 3 anti-KLH mAbs could not be assigned to one of the tested V_H gene families. (A) V_H gene family usage of anti-AChR v.s. anti-KLH mAbs. (B) V_H gene family usage of anti-AChR mAbs from high susceptible C57bl/6 and low susceptible Balb/c mice.*

V_H gene family usage in relation to fine specificity

In order to relate V_H gene family usage to antibody fine specificity, mAbs were selected with similar epitope specificity to see if they were encoded by related V_H genes. Therefore V_H gene family usage of mAbs against the MIR (α67-76) and the α-BT binding site (α189-195) were analyzed. Anti-MIR mAbs (n=17) were encoded by at least three different V_H gene families; J558 (11/17), Vgam3.8 (1/17) and 3660 (2/17). The observed V_H family gene usage among anti-MIR mAbs (n=17) and non-MIR mAbs (n=48) was not significant different (χ^2=3.7 d.f.=2) (table III).

Table II: The V_H gene family usage of anti-AChR and anti-KLH mAbs.

V_H family[a]	Complexity[b]	% of total V_H genes	tAChR mAbs using (%) V_H family[c]		KLH mAbs using (%) V_H family		Significance[d]
J558	60	48	36	(55)	11	(55)	N.S.
3609	12	10	3	(5)	1	(5)	N.S.
J606	10	8	0	(0)	0	(0)	p<0.01
Vgam3.8	5	4	6	(9)	0	(0)	p<0.05
3660	5	4	3	(5)	2	(10)	N.S.
X24	2	2	0	(0)	0	(0)	N.S.
S107	4	3	0	(0)	0	(0)	N.S.
Q52	15	12	5	(8)	1	(5)	N.S.
PC7183	12	10	5	(8)	2	(10)	N.S.
N.A.[e]			7	(11)	3	(15)	N.S.

[a] The name and the relative chromosomal order of the tested V_H gene families relative to the D-region (77). [b] The number of V_H genes in each family as determined by Brodeur and Riblet (37) [c] The number of mAbs using genes from a particular V_H gene family. [d] The distribution of V_H gene families among anti-AChR and anti-KLH mAbs was compared to the expected distribution based on V_H family complexity using the χ^2 test. [e] N.A.: Not assigned. RNA of these mAbs failed to hybridize to V_H family specific probes at used stringency conditions.

Table III: Comparison of V_H gene family usage of anti-AChR mAbs or subgroups of anti-AChR mAbs.

V_H group[a]:			I	II	III
			J558 3609 J606	3660 Vgam3.8 X24 S107	Q52 PC7183
Localization[b]:			D-region distal		D-region proximal
Complexity[c] (%):			82 (66%)	16 (13%)	27 (20%)
A	mAChR	(n=16)	11 (69%)	1 (6%)	1 (6%)
	tAChR	(n=49)	29 (59%)	8 (16%)	9 (18%)
B	MIR	(n=17)	11 (65%)	3 (18%)	0 (0%)
	non-MIR	(n=48)	28 (58%)	6 (13%)	10 (21%)
C	Balb/c	(n=27)	15 (56%)	3 (11%)	7 (26%)
	C57bl/6	(n=38)	24 (63%)	6 (16%)	3 (8%)

[a] V_H gene families were combined for statistical evaluation by the χ^2 test. Group I comprises V_H families located most distal from the D-region, group II V_H families are a group of small interspersed families located between the D-region distal Group I and D-region proximal group III V_H families Q52 and PC7183. [b] Relative localization of V_H gene families compared to the D-region. [c] The complexity represents the total estimated size of V_H gene families comprised in the combined V_H family groups.

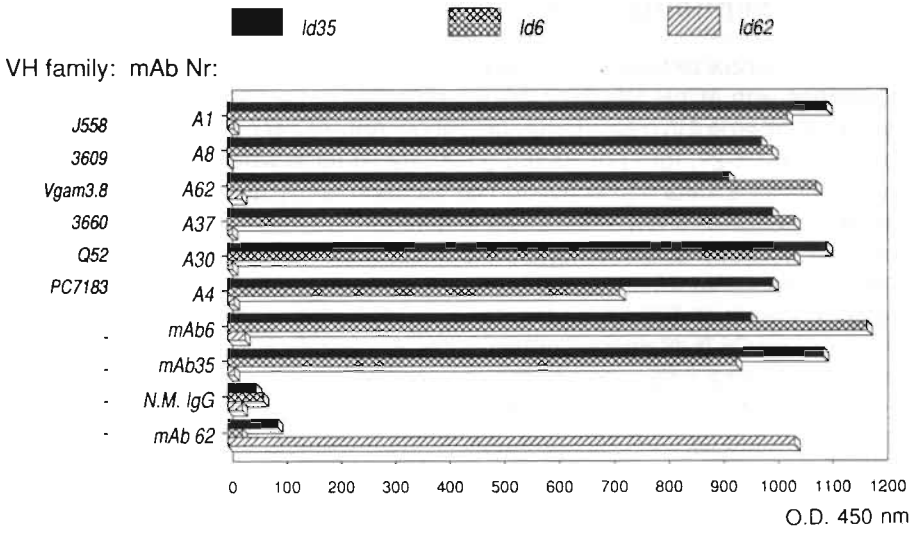

Figure 3: Anti-AChR mAbs encoded by V_H genes from six different families express a CRI.
Expression of a CRI by mouse anti-AChR mAbs encoded by different V_H gene families was determined using two affinity purified polyclonal anti-Ids. Id 6 and 35 are the CRIs expressed by rat anti-AChR mAbs 6 and 35 respectively. Id62 is a CRI expressed by anti-thyroglobulin mAb 62. Binding of anti-Ids to mouse anti-AChR mAbs was determined at 50 % of the maximum binding of anti-Id to its respective idiotype.

Monoclonal antibodies directed against the α-BT binding site (n=6) are encoded by at least 3 different V_H gene families; J558 (1/6), 3609 (1/6) and Vgam3.8 (3/6). Interesting however the Vgam3.8 family is overrepresented in anti-α-BT binding site mAbs (50 %).

V_H gene family usage in relation to idiotype expression

To investigate the relation between expression of CRI and utilization of a certain V_H gene family, mAbs representative for each V_H gene family were selected and expression of CRI was determined with affinity purified polyclonal anti-idiotypes (anti-Id) which are directed to the (cross-species) CRI determinants expressed by rat anti-AChR mAb 6 and 35. Both Id 6 and 35 are expressed on mouse anti-AChR mAbs but not on control antibodies. Control anti-Id62 specific for a CRI expressed by anti-thyroglobulin antibodies does not recognize CRI on any of the mouse anti-AChR mAbs (fig. 3). Anti-AChR mAbs expressing CRI are encoded by V_H genes from at least 6 different V_H gene families. Thus expression of CRI is irrespective of the V_H gene family used by the mAb.

EAMG susceptibilty in relation to V_H gene family usage

Mouse strains show differences in their susceptibility for the induction of EAMG by immunization with AChR. We therefore investigated if disease susceptibility can be related to an aberrant V_H gene family utilization. Anti-AChR mAbs were derived from either C57bl/6 (H-2^d, Igh^b) or Balb/c (H-2^b, IgH^a) mice which show high and low susceptibility for EAMG respectively. The V_H gene family utilization of C57bl/6 and Balb/c derived mAbs show strain specific differences which are shown in figure 2B. The V_H gene family utilization in Balb/c mice shows a relative bias towards the D-region proximal families Q52 and PC7183 compared to C57bl/6 mice. This is reflected in the ratio of J558 positive to Q52+PC7183 positive mAbs which is 2 for Balb/c and 7 for C57bl/6 derived mAbs. Some V_H gene families are exclusively used by anti-AChR mAbs originating from either one of the mouse strains. The 3660 V_H gene family is exclusively used by Balb/c anti-AChR mAbs whereas the Vgam3.8 family is only used by C57bl/6 anti-AChR mAbs. However, no significant difference could be observed, when V_H gene family utilization is compared between C57bl/6 and Balb/c derived mAbs (χ^2= 3.7 d.f.=2) (table III). Thus there is no indication for a biased or aberrant use of V_H gene families that would explain the difference in disease susceptibility.

DISCUSSION

We determined the V_H gene family utilization of anti-AChR mAbs derived from high susceptible C57bl/6 and low susceptible Balb/c mice, in which EAMG was induced by immunization with Torpedo AChR. V_H gene family usage was related to mAb fine specificity, expression of CRI and differences in disease susceptibility. Anti-AChR mAbs used V_H genes from 6 out of the 9 tested V_H gene families and V_H gene family utilization approached a stochastic distribution over the different families. No bias in V_H gene family usage towards D-region proximal families was observed among mAbs directed to Torpedo AChR or mAbs cross-reactive with mouse AChR. Our results are in agreement with other studies of V_H gene family usage by antigen induced autoantibodies that show a stochastic utilization of V_H genes from the various V_H gene families (30-31), which resembles the V_H gene family usage of the normal B cell repertoire (52, 53). Anti-AChR mAbs show no bias in V_H gene family usage towards D-proximal V_H gene families as was suggested for spontaneous and antigen induced autoantibodies (27-29). However, anti-AChR mAbs show a significant deviation from stochastic usage concerning the J606 and Vgam3.8 gene families. The overrepresentation of V_H genes from the Vgam3.8 family could be due to selection of related V_H genes in mAbs binding to a defined antigenic region (54), as was suggested by the fact that V_H genes from this small family are preferentially expressed (50 %) by mAbs directed to the α-BT binding site.

Subgroups of the anti-AChR mAbs used in this study are directed to well defined epitopes on the AChR. Antibodies against the MIR, a 10 amino acid epitope on the extracellular side of the α-subunit (47) can cause acute EAMG in experimental animals by increasing AChR turnover (3, 55). Anti-MIR mAbs are encoded by at

least three different V_H gene families and we found no restricted V_H family usage among mAbs of this specificity. However, a 10 aminoacid protein epitope like the MIR can be regarded as a group of closely overlapping epitopes (5), eliciting a heterogeneous population of anti-MIR mAbs formed by several diverse heavy chains. Similar, mAbs specific for a decapeptide of Tobacco Mosaic Virus protein (56) were encoded by diverse V_H and V_L genes.
Our current library of anti-AChR mAbs comprises six mAbs directed to the α-BT binding site localized to α189-195 on the α-subunit (48). Four anti-α-BT binding site mAbs were able to inhibit α-BT binding to both binding sites on each AChR molecule whereas two mAbs inhibited only one of the α-BT binding sites confirming that the two α-BT binding sites show similar epitopes as well as epitopes unique for each of the two sites (57, 58). A partial correlation between V_H gene family usage and epitope specificity was demonstrated among anti-α-BT binding site mAbs. V_H genes from the relatively small Vgam3.8 family were found in 3 out of 6 anti-α-BT binding site mAbs. The two mAbs that inhibit only one of the two binding sites are both encoded by the Vgam3.8 family suggesting that closely related V_H chains are used.

Previously, we identified a CRI on both rat and mouse anti-AChR mAbs. Cross-reactive idiotopes were associated with the antigen binding site, i.e. paratope of anti-MIR mAb, as well as non-paratope related framework associated cross-reactive idiotopes shared by all anti-AChR mAbs (49). In order to relate expression of CRI to V_H gene family usage we determined the appearance of a CRI on anti-AChR mAbs encoded by 6 different V_H gene families. Expression of a CRI was found to be irrespective of the V_H gene family used. One explanation for expression of a CRI could be that these antibodies are formed by related primary amino acid sequences of V_H regions (23, 59); a high degree of idiotypic cross-reactivity is frequently found among antibodies encoded by V_H genes from the same V_H gene family (24, 40, 60, 61). However, expression of a CRI can also be independent of the V_H gene family used (62). V_H genes originating from different families could share small highly homologous DNA sequences encoding similar structural features of the antibody although the overall DNA sequence homology of the entire V region is less than 70 %. Expression of CRI could also be a consequence of highly homologous V_L chains. The structural basis for expression of a CRI among anti-AChR mAbs may furthermore be a consequence of shared topological elements in the three-dimensional structure which are not easily deduced from the primary amino acid structure.

The differences observed in V_H gene family utilization of C57bl/6 and Balb/c derived anti-AChR mAbs are characteristic for the used mouse strains as was previously demonstrated for LPS induced (63-65) or unstimulated splenic B-cells (65). However, the Vgam3.8 gene family was exclusively used by anti-AChR mAbs derived from EAMG sensitive C57bl/6 mice. Although these Vgam3.8 positive mAbs are directed against extracellular located epitopes (MIR, α-BT binding site), only mAb 58 cross-reacted with mAChR and may be pathogenic.
Thus, strain dependent V_H gene family usage resembles the usage of the normal repertoire and no association could be made between disease susceptibility and abnormalities in the V_H gene family usage. It was shown that susceptibility to

multiple sclerosis in both familial and sporadic MS patients is associated with a locus within the D-region proximal Ig V_H region (66), suggesting that abnormalities within the V_H region could contribute to genetic control of susceptibility to autoimmune disease.

Taken together these results indicate that the immune response against a large protein antigen like the AChR is encoded by many different V_H genes originating from different V_H gene families reflecting the polyclonal nature of anti-AChR antibodies in EAMG (67), which was also observed in MG (68). Our results are in agreement with a report of Guigou et al (69) in which the activated B cell repertoire in germinal centers of hyperplastic thymuses of MG patients closely reflected the V_H and V_κ family usage of the normal repertoire.

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Chapter 6

V_H gene family utilization of rat anti-acetylcholine receptor monoclonal antibodies

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V_H gene family utilization of rat anti-acetylcholine receptor monoclonal antibodies

ABSTRACT

The diversity of V_H genes encoding rat anti-AChR mAbs was investigated by determining the V_H gene family utilization using family specific probes for known mouse V_H gene families. Mouse V_H gene family probes could identify homologous V_H gene families in the rat. Analogous to the mouse, all tested rat hybridomas reacted with one particular mouse V_H gene family probe at the RNA level and not with the other tested V_H gene family probes. Rat V_H gene families corresponding to 11 of 12 mouse V_H gene families were found in germline DNA from four rat strains. Considerable differences in V_H gene family complexity were found between rat and mouse. A panel of 21 rat anti-AChR mAbs are encoded by 8 different V_H gene families. The V_H genes encoding anti-AChR mAbs did not follow a stochastic distribution over the different V_H gene families due to overrepresentation of the S107 and underrepresentation of the X24 V_H gene families. A partial correlation between V_H gene family utilization and mAb fine specificity was found. MAbs against the main immunogenic region, showing pathogenic potential *in vivo* or *in vitro*, were most frequently encoded by V_H genes from the Q52 family, whereas non pathogenic mAbs against intracellular epitopes were most frequently encoded by V_H genes from the PC7183 family. Anti-AChR mAbs expressing cross-reactive idiotopes were not characterized by a particular V_H gene family utilization.

INTRODUCTION

In myasthenia gravis autoantibodies against the acetylcholine receptor arise, which cause loss of AChRs and failure of neuromuscular transmission. Experimental autoimmune myasthenia gravis can be induced in rats by immunization with AChR (1), and provides an excellent model for studying the anti-AChR antibody response (2). Rat anti-AChR monoclonal antibodies have been extensively characterized and have provided much information about the antigenicity of AChR (3-6), and the pathogenic mechanisms that cause AChR loss (7-9). However, no information is available about the diversity of genes encoding rat anti-AChR mAbs. It was previously suggested that murine autoantibodies are encoded by a restricted number of V_H genes (10, 11), which led to the hypothesis that autoimmune disease may be related to a defective control of V_H gene expression (12). The diversity in V_H genes encoding rat anti-AChR mAbs was determined using mouse V_H gene family specific probes. The mouse immunoglobulin heavy chain variable region (Ig V_H) locus is well characterized. Mouse V_H genes are classified into 12 V_H gene families, containing V_H genes with more than 80 % homology whereas the homology between different families is less than 70 % (13). Therefore, we used V_H gene familiy specific probes for 12 known mouse V_H gene families to identify corresponding rat V_H gene families in the germline DNA of four different rat strains. It was verified whether members of these V_H gene families were actually expressed in rat anti-AChR hybridomas and whether these hybridomas show a random or restricted usage of the different V_H gene families. Furthermore, the relation between V_H gene family utilization and mAb fine specificity, pathogenicity and idiotype expression of anti-AChR mAbs was determined.

The complexity of the rat Ig V_H gene locus and the utilization of V_H gene families in rat anti-AChR mAbs were determined. Rat V_H gene families corresponding to 11 out of the 12 mouse V_H gene families were found in rat germline DNA. Rat anti-AChR mAbs were encoded by 8 different V_H gene families and did not show a stochastic distribution over the different V_H gene families. Members of the Q52 family were frequently found in pathogenic mAbs directed against the MIR whereas members of the PC7183 family were most frequently used by mAbs against intracellular located epitopes. No relation between expression of a CRI and utilization of a particular V_H gene family could be demonstrated.

MATERIAL AND METHODS

Genomic DNA and Southern blot analysis

High molecular weight DNA from liver of PVG, AO, BN, Louvain/A rats, and BALB/c mice was isolated as previously described (14).

Liver DNA was digested to completion with Eco RI and electrophoresed on 0.7 % agarose gels, blotted to nylon filters and hybridized to ^{32}P-labeled V_H gene probes at 65 °C for 14-16 hrs in hybridization mix, containing 0.5 M $NaHPO_4$, 1 % w/v BSA, 1 mM EDTA, 3% w/v SDS.

Filters were washed at 65 °C, once in 40 mM $NaHPO_4$, 2% w/v SDS, 1 mM EDTA and 0.5% w/v BSA and 5-8 times in 40 mM $NaHPO_4$, 1% w/v SDS, 1mM EDTA.

Sealed filters were exposed to Kodak XAR-5 films at -70 °C using an intensifying screen (Du Pont) for one to two weeks.
The complexity of the rat V_H gene families was defined as the maximal number of different bands in a Eco RI digest of liver DNA derived from PVG, AO, BN and Louvain/A rats after hybridization with a V_H gene family specific probe.

DNA probes
DNA probes representative for the mouse V_H gene families J558, PC7183, Q52, J606, 3660, S107 and X24 used in this study were previously described (15). The V_H 23-9 probe (V_H 3609) is a 500 bp Bam HI-Pst I fragment containing a V_H sequence belonging to the 3609 family. The Vgam3.8 probe contains a 550 bp Pst 1-Eco R1 fragment of the sequence of Vgam3.8. The V_H 10 probe is a 500 bp Pst I fragment from MRL-DNA4 and the V_H 11 probe is a 290 bp Dra I-Pst I fragment from clone pCP12 (16). These probes were gifts from Drs. D. Holmberg, J. Kearney, F. Alt, D. Schulze, and D. Capra.

Rat anti-AChR mAbs
Rat monoclonal antibodies were raised against human, fetal calf, electric eel and native, SDS denatured or isolated subunits of Torpedo californica AChR, as was previously described (3-5).

Analysis of cross-reactive idiotopes on rat anti-AChR mAbs
Cross-reactive idiotopes specific for anti-AChR mAbs were defined by polyclonal affinity purified anti-idiotype antibodies (anti-Id) against rat anti-AChR mAb 6 (17). Binding of anti-Id to solidphase mAbs was described in detail in chapter 4. Cross-reactive idiotopes associated with the antigen combining site were determined by a solidphase antigen binding inhibition assay. Briefly, 96 wells microtiterplates (Flow ICN, Amsterdam, The Netherlands) were coated with 50 µl anti-AChR mAb (5µg/ml) for 1 hr at 37° C. After washing 3 times with H_2O + 0.5 % v/v Tween 20, plates were incubated with PBS + 0.5 % w/v BSA and 0.5 % v/v Tween 20 for 15 minutes at roomtemperature. Subsequently, increasing amounts of affinity purified anti-Id 6 were incubated overnight. Without intermediate washing, a previously determined limiting concentration (10^{-9} mol/l) of ^{125}I-α-BT labeled Torpedo AChR (^{125}I-α-BT-tAChR) was incubated for 4 hours. After washing with PBS + 0.5 % v/v Triton X-100 + 0.02 % NaN_3, bound radioactivity was counted with a gamma counter. The percentage inhibition of AChR binding was calculated as follows:

[(Average of duplicate wells with ^{125}I-α-BT-tAChR alone)-(Average of duplicate wells in which ^{125}I-α-BT-tAChR was tested in the presence of anti-Id 6)/Average of duplicate wells with ^{125}I-α-BT-tAChR alone]x100

RNA slot-blot hybridization
Total cytoplasmic RNA of 10^8 viable hybridoma cells was prepared according to Maniatis et al (18). RNA samples were diluted in 20 x SSC to a range of 1, 0.5, 0.25 µg and immobilized on B85 nitrocellulose filters (Scheicher and Schuell, 's-Hertogenbosch, The Netherlands) using a Minifold II slot-blot manifold. Nitrocellulose filters were air dried and baked at 80 °C for 2 hours. Filters were prehybridized for

4 hours at 42 °C then hybridized with ^{32}P-labeled DNA probes (approximately 5x10^6 cpm/ml) for 16 hours at 42 °C and subsequently washed in 3 X SSC, 0.1 % w/v SDS and twice in 1 X SSC, 0.1 % w/v SDS at 42 °C.

Statistical analysis
The V_H gene family utilization of rat anti-AChR mAbs was compared to a stochastic distribution by the χ^2 test (19).

RESULTS

V_H gene family expression in rat anti-AChR mAbs

In order to verify whether rat V_H gene families analogous to known mouse V_H gene families are expressed in rat hybridomas, we determined the V_H gene family ultilization of a panel of 21 rat anti-AChR hybridomas using probes specific for mouse V_H gene families. All tested rat anti-AChR hybridomas showed a clear positive hybridization signal to a single mouse V_H gene family probe. All nine tested mouse V_H gene families except for the X24 V_H gene family, were represented in these rat anti-AChR mAbs. Hybridization signals representative for each V_H gene family are shown in figure 1.

The complexity of the rat V_H gene locus

After demonstrating that mouse V_H gene family probes are capable of discriminating between different rat V_H gene families, the Ig V_H gene locus in the rat was characterized by Southern blot analysis of genomic DNA of PVG, AO, BN and Lou/a rats using probes representing 11 of the 12 known mouse V_H gene families. All of the tested mouse V_H gene family probes hybridized to nonoverlapping patterns of Eco RI restriction fragments of rat liver DNA (fig. 2). The number of rat Eco RI fragments hybridizing with each mouse V_H gene family probe is an estimate for the minimum size or complexity of the corresponding rat V_H gene family. A total of 132 Eco RI fragments hybridizing with the 11 mouse V_H gene family probes was comparable to the 127 fragments found in the mouse. However, considerable differences in the complexity of individual rat V_H gene families corresponding to the 11 mouse families were found (table I). The rat V_H gene families corresponding to the mouse families PC7183, J606, S107, X24, V_H10 and V_H11 showed more fragments than in the mouse. Especially the 2 member mouse families X24, V_H10 and V_H11 are much larger in the rat showing 18, 11 and 11 fragments respectively. The Q52, 3609, Vgam3.8 and 3660 families are equal of size in both rat and mouse. The J558 is the largest mouse V_H gene family containing at least 60 members (47 %). The corresponding rat V_H gene family only showed 16 fragments (12 %). The largest rat V_H gene family is analogous to the PC7183 family and showed 19 fragments making up 14 % of the V_H genes in the rat. The one member mouse V_H gene family V_H12 was not yet tested on rat DNA.

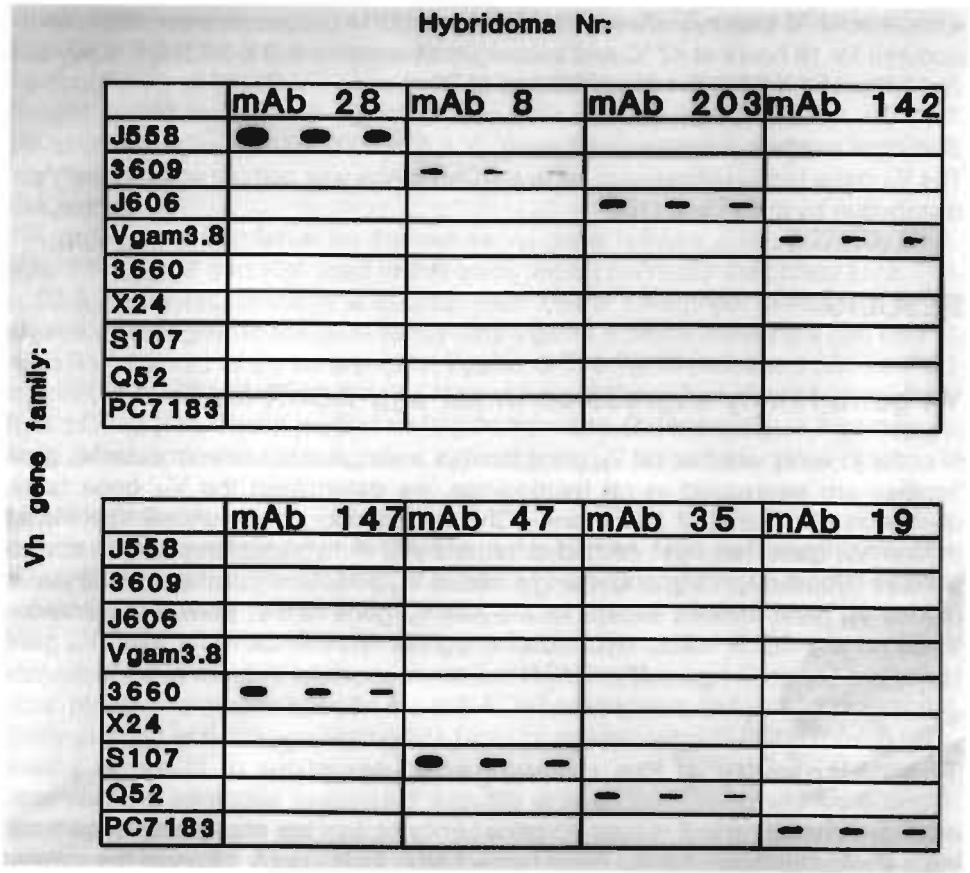

Figure 1: Slot-blot hybridizations of rat anti-AChR mAbs
Representative examples of slot-blot hybridization of rat anti-AChR hybridoma RNA to mouse V_H gene family specific probes. Aliquots of 1, 0.5 and 0.25 µg hybridoma RNA were immobilized to nitrocellulose and hybridized to probes specific for 9 V_H gene families.

The V_H gene family usage of rat anti-AChR mAbs is non-stochastic

The V_H gene family utilization found among rat anti-AChR mAbs was compared to the stochastic distribution over the different V_H gene families based on the family complexity in the rat (fig. 3). The V_H gene family utilization of rat anti-AChR mAbs showed several deviations from a stochastic distribution. None of these mAbs used a V_H gene from the X24 family, whereas the small S107 family was markedly overrepresented. The V_H gene family utilization was significantly different ($p<0.05$) from a stochastic distribution mainly due to overrepresentation of the S107 family.

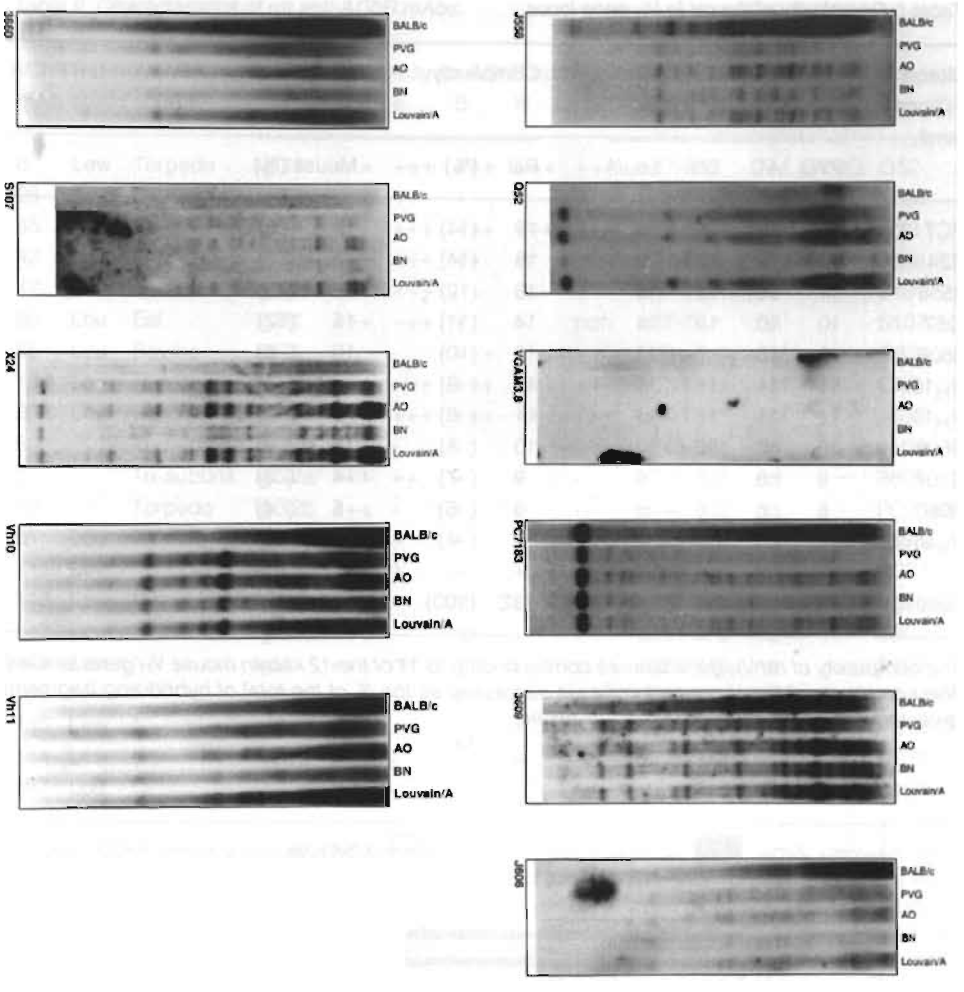

Figure 2: Rat V_H gene families corresponding to mouse V_H gene families detected in germline DNA by Southern-blot analysis.
Liver DNA from PVG, AO, BN, Lou/A rats and Balb/c mice was digested with Eco RI, separated on a 0.7 % agarose gell, blotted and hybridized to probes representative for 11 known mouse V_H gene families. Each mouse V_H gene family probe displays a different set of fragments. The number of restriction fragments is an estimate for the minimun size of the rat V_H gene family.

V_H gene family utilization in relation to mAb specificity

The panel of rat anti-AChR mAbs used for determination of V_H gene family utilization comprises 9 mAbs directed against the MIR on the extracellular surface of AChR and 12 against well defined epitopes on the intracellular surface of AChR (table II). MAbs with fine specificity for the MIR located at residue 67-76 of the α-subunit were encoded by at least 4 V_H gene families (J558: 2/9, Q52: 4/9, S107: 2/9, J606: 1/9).

Table I: Complexity of the rat Ig V_H gene locus.

Mouse V_H gene family	Number of hybridizing Eco RI bands				Complexity			
	PVG	AO	BN	Lou/A	Rat	(%)	Mouse	(%)
PC7183	16	19	8	18	19	(14)	12	(9)
X24	18	18	13	18	18	(14)	2	(2)
J558	14	16	12	16	16	(12)	60	(47)
Q52	10	10	13	14	14	(11)	15	(12)
J606	13	13	7	13	13	(10)	10	(8)
V_H10	11	11	11	11	11	(8)	2	(2)
V_H11	11	11	11	11	11	(8)	2	(2)
3609	10	10	10	10	10	(8)	10	(8)
S107	9	8	7	9	9	(7)	4	(3)
3660	6	6	6	6	6	(5)	5	(4)
Vgam3.8	1	5	5	5	5	(4)	5	(4)
Total					132	(100)	127	(100)

The complexity of rat V_H gene families corresponding to 11 of the 12 known mouse V_H gene families. The complexity of the V_H gene families is expressed as the % of the total of hybridizing fragments detected with all V_H gene family probes tested.

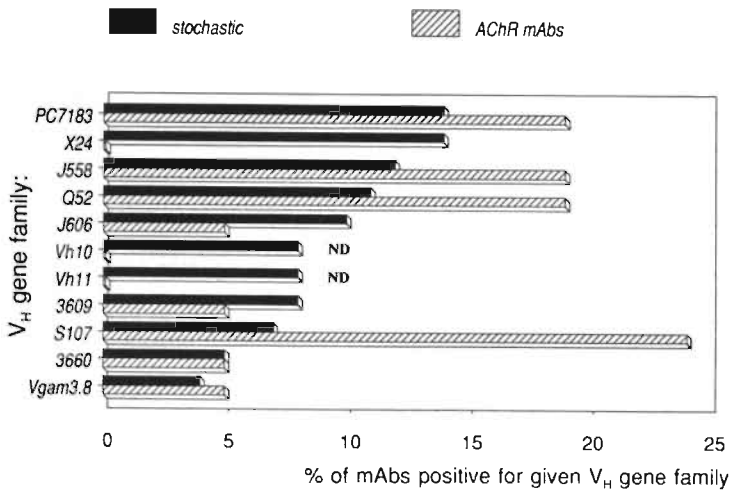

Figure 3: The V_H gene familiy utilization of rat anti-AChR mAbs.
The distribution of rat anti-AChR mAbs over the nine tested V_H gene families. The V_H gene family utilization was expressed as the percentage of mAbs positive for a given V_H gene family, and was compared to a stochastic distribution based on the complexity of these families in the rat. ND: not done.

Table II: Characteristics of rat anti-AChR mAbs.

AChR mAb	Rat strain	AChR[a] source	Isotype	Specificity for AChR from[b] T	E	B	R	H	Fine specificity[c]	Patho-genicity[d]	V_H gene family[e]
6	Lew	Torpedo	IgG1	+++	+++	+++	+++	+++	α67-76	AM, EAMG	Q52
28	Lew	Eel	IgG2a	++	+++	+	+		α67-76	nd	J558
35	Lew	Eel	IgG1	+++	+++	+++	+++	+++	α37-85	AM, EAMG	Q52
42	Lou	Eel	IgG2a	+++	+++	++	+	-	α67-76	EAMG	J558
47	Lou	Eel	IgG2a	+++	+++	-	-	-	α67-76	nd	S107
50	Lou	Eel	IgG1	+++	+++	+	+	nd	α67-76	nd	S107
65	Lou	Bovine	IgG1	+	-	+++	-	+	MIR	AM	Q52
198	Lou	Human	IgG2a	+++	+++	+++	+++	+++	α67-76	AM	Q52
203	Lou	Human	IgG2a	+++	+++	+++	++	+++	α67-76	AM	J606
5		Torpedo	IgG2b	+++	-	-	-	-	α349-357	nd	J558
8		Tα-subunit	IgG2a	+++	++	+	+	-	α366-372	nd	3609
19		Torpedo	IgG2b	+++	-	-	-	-	α348-362	nd	PC7183
61	Lou	Eel	IgG2a	+	+	+	+		α371-378	nd	J558
111	Lew	*	IgG1	+++	+	+	+	±	α368-406	nd	PC7183
142	Lew	*	IgG2a	+++	-	nd	-	-	α353-359	nd	Vgam3.8
147	Lew	*	IgG2a	+++	-	nd	-	-	α364-370	nd	3660
149	Lew	*	IgM	+++	nd	-	-	±	α339-346	nd	S107
153	Lew	*	IgG2a	++	+	++	++	+	α371-378	nd	PC7183
155	Lew	*	IgG2a	++	+	+	+	+	α371-378	-	PC7183
164	Lew	*	IgG2a	+	-	-	+	+	α371-379	nd	S107
187	Lou	*	IgG2a	+++	-	nd	-	nd	α339-346	nd	S107

*Isotype, AChR cross-reactivity, fine specificity, pathogenicity and V_H gene family utilization of rat anti-AChR mAbs.[a] The source of AChR to which mAb is elicited; Torpedo: Torpedo californica AChR, Eel: electric eel AChR, Bovine: fetal bovine AChR, Human: denervated muscle AChR, Tα-subunit: the α-subunit of Torpedo AChR, *: SDS denatured AChR + isolated AChR subunits.[b] Cross-reaction with AChR from different species; T: Torpedo AChR, E: eel AChR, B: bovine AChR, R: rat AChR, H: human AChR.[c] Fine specificity determined by binding of mAbs to synthetic peptides of the α and β subunits of the AChR; numbers denote aminoacid residues.[d] The pathogenicity of anti-AChR mAbs determined by their ability to increase AChR turnover by antigenic modulation in vitro using muscle cell cultures, or induction of EAMG in vivo by passive transfer of anti-AChR mAb. AM: antigenic modulation in vitro, EAMG: mAb inducing EAMG in vivo, nd: not done.[e] The V_H gene family used by mAb determined with mouse V_H gene family probes.*

MAbs against closely adjacent or overlapping epitopes at the intracellular surface of AChR were encoded by 6 different V_H gene families (J558: 2/12, S107: 3/12, PC7183: 4/12, Vgam3.8: 1/12, 3660: 1/12, 3609: 1/12). The panel of anti-AChR mAbs comprises mAbs that can induce experimental myasthenia gravis *in vivo* by passive transfer (mAb 6, 35 and 42) or increase AChR turnover *in vitro* by antigenic modulation (mAb 6, 35, 65, 198, 203). MAbs against intracellular epitopes are not pathogenic *in vivo*. Pathogenic mAbs were encoded by V_H genes from the Q52 (4/6), J558 (1/6) and J606 (1/6) families.

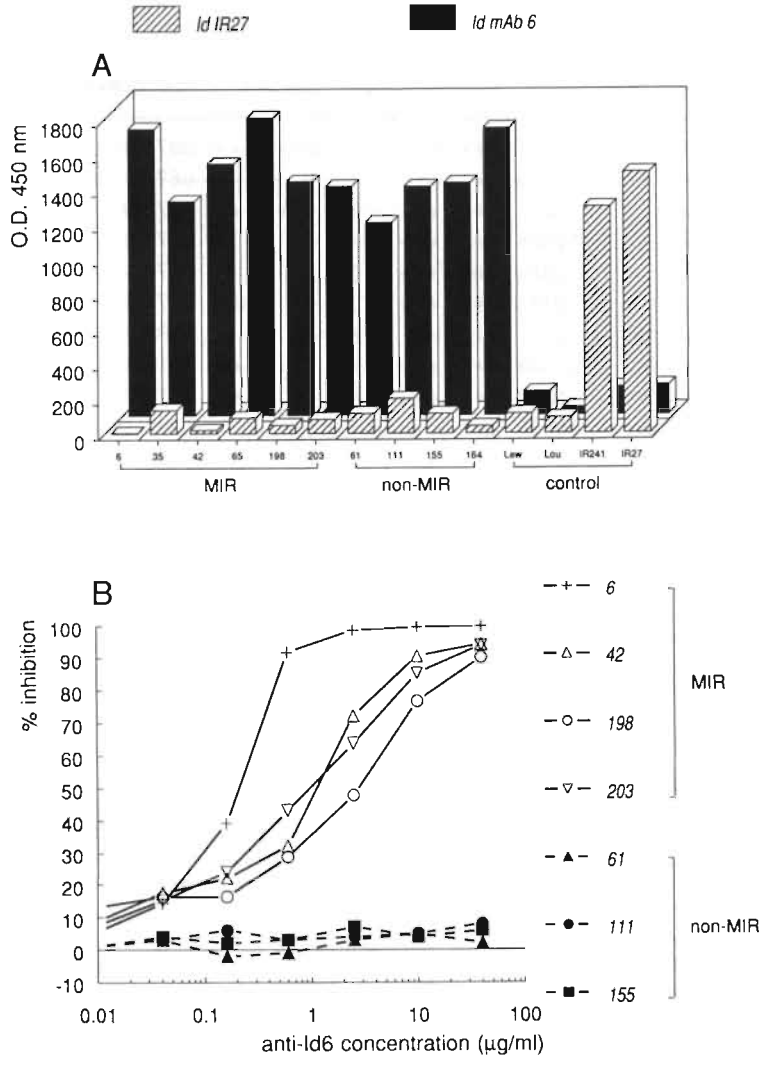

Figure 4: Rat anti-AChR mAbs express cross-reactive idiotopes.
(A) *Expression of a CRI was determined by binding of affinity purified polyclonal anti-idiotype antibodies to rat anti-AChR mAbs bound to microtiter plates. Anti-Id 6 is directed against the CRI expressed by mAb 6 and binds to all anti-AChR mAbs but non of the controls. Control anti-Id IR27 only recognized CRI on rat myeloma protein IR27 and IR241. Lew: polyclonal lewis IgG, Lou: polyclonal louvain IgG.*
(B) *CRI determinants associated with the antigen binding site were detected by a solidphase antigen binding inhibition assay. Anti-AChR mAbs bound to microtiter plates were incubated with increasing amounts of anti-Id 6 prior to incubation with ^{125}I-α-BT-AChR. The binding to anti-MIR mAbs can be inhibited by anti-Id 6. The binding of ^{125}I-α-BT-AChR to anti-AChR mAbs against intracellular located epitopes of AChR was not inhibited by anti-Id 6.*

V_H gene family utilization in relation to idiotype expression

Expression of a cross-reactive idiotype (CRI) by rat anti-AChR mAbs was determined for a panel of mAbs directed to the MIR or intracellular epitopes. All tested anti-AChR mAbs but non of the controls expressed a CRI as was demonstrated by binding of polyclonal anti-idiotype (anti-Id) antibodies raised against mAb 6 (fig 4A). None of the anti-AChR mAbs bound to a control anti-Id raised against rat myeloma protein IR27. Furthermore, it was demonstrated that only anti-MIR mAbs share idiotypic determinants associated with the antigen combining site. The binding of Torpedo AChR to anti-MIR mAbs was inhibited almost completely by anti-Id 6. The binding of AChR to mAbs recognizing intracellular epitopes distinct from the MIR was not inhibited by anti-Id 6 (fig 4B). The mAbs tested for expression of a CRI were encoded by the Q52, J558, J606, S107, and PC7183 V_H gene families. No relation between expression of a CRI and utilization of a particular V_H gene family could be demonstrated.

DISCUSSION

In this study the diversity of V_H genes encoding rat anti-AChR mAbs was determined. The V_H gene family utilization of anti-AChR mAbs directed against the MIR or intracellular epitopes on AChR was analyzed using mouse V_H gene family probes. V_H gene family utilization by a panel of 21 rat anti-AChR mAbs revealed that these mAbs are encoded by V_H genes from 8 different families. Thus, V_H genes expressed by rat B cell hybridomas can be identified by mouse V_H gene family probes. Messenger RNA transcripts of the Q52, PC7183 and J606 families were previously demonstrated in different rat mAbs against tubular basement membrane and the neuropeptide substance P (20, 21). Furthermore, members of the S107 V_H gene family were demonstrated in rat anti-phosphorylcholine antibodies (22). These results indicate that V_H gene families analogous to mouse V_H gene families exist in the rat.

In order to investigate the V_H gene family utilization of rat anti-AChR mAbs it was necessary to characterize the number and complexity of V_H gene families in the rat. The Ig V_H gene locus has already been exstensively characterized in the mouse (13). Using the mouse V_H gene family specific probes it was shown that rat V_H gene families homologous to 11 out of 12 known mouse V_H gene families exist in the rat, indicating that much resemblance between the rat and mouse Ig V_H gene loci exists. Although some families are similar in complexity in mouse and rat, some rat families are expanded whereas others are contracted compared to the complexity of the mouse V_H gene families. Homologous but unequal recombination between genes of the same family probably leads to expansion of a V_H gene family whereas unequal recombination between genes of different families leads to contraction of the recombining V_H gene families (23). The relative position of the rat V_H gene families on chromosome 6 in the rat is still unkown.

The V_H gene family utilization of rat anti-AChR mAbs was compared to the complexity of the rat V_H gene families and did not follow a stochastic distribution over the different families, indicating that a non-random selection of V_H genes has taken place. Especially the S107 family was overrepresented whereas V_H genes from the X24 were not expressed by anti-AChR mAbs in this panel. Utilization of V_H genes from the S107 family was however not related to a particular mAb fine specificity.
Anti-AChR mAbs with pathogenic potential *in vivo* or *in vitro* were most frequently encoded by V_H genes from the Q52 family, whereas non pathogenic mAbs against intracellular epitopes were most frequently encoded by V_H genes from the PC7183 family.
Murine autoantibodies of various specificities were found to be encoded by a restricted number of V_H genes in particular from the PC7183 and Q52 families (10). Furthermore, hybridomas selected by PC7183 V_H gene expression produce autoreactive antibodies at a high frequency (11). Therefore, it was concluded that autoimmune disease may be related to a defective control of V_H gene expression (12). The kind of V_H gene family restriction found among rat anti-AChR mAbs is more likely to be related to structural requirements for the generation of particular binding sites than an indication for a possible defect in V_H gene usage. Genetic restriction of V_H genes was found to be common among antibodies against defined haptens but has also been observed in antibody responses to epitopes on complex antigens (24, 25). However, mAbs against the MIR are only partially restricted to the Q52 family and were also encoded by at least 3 other V_H gene families. This may be a result of the heterogeneity among anti-MIR mAbs which were demonstrated to interact with different combinations of residues within the sequence α67-76 (26, 27).

The presence of framework associated cross-reactive idiotopes was demonstrated on mAbs of different fine specificity whereas paratope related idiotopes were only demonstrated on MIR specific mAbs. Expression of a CRI was not related to a particular V_H gene family utilization. Expression of a CRI by all tested anti-AChR mAbs indicate some form of shared determinants and one might expect a restricted V_H gene usage (28, 29, 30). However, apparently sharing of idiotopes can be found among mAbs of different specificities and encoded by different V_H gene segments (10, 31, 32). A definitive conclusion whether expression of CRI is derived from conformity in the tertiar structure or similarities in primary structure can only be made by assessment of the DNA sequence of the V_H and V_L genes encoding these mAbs.

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Summary and general discussion

The immune system is able to recognize and eliminate virtually all foreign organisms invading the body. In contrast, a state of unresponsiveness towards self is maintained by the immune system. In some instances the unresponsiveness towards self constituents is abrogated resulting in an autoimmune response destroying the body's own structures. Myasthenia gravis (MG) is an autoimmune disease in which autoantibodies develop against a receptor protein involved in the signal transmission from the nerve to the muscle. The neurotransmitter acetylcholine is released from the nerve ending upon nerve stimulation and binds to the acetylcholine receptor (AChR) at the muscle membrane, which subsequently activates the muscle to contract. In MG the number of available functional AChR molecules is diminished by autoantibody mediated degradation of these receptors. Signal transmission is therefore impaired, resulting in muscle weakness and paralysis. MG is caused by autoantibodies as was demonstrated by transfer of antibodies from patients to experimental animals or placental transfer from mother to child. The events that initiate this autoimmune response in MG are still unkown. Much of the information about myasthenia gravis is obtained from an experimental model for this disease in which animals are immunized with purified AChR. In this thesis the experimental autoimmune model was studied to gain information about the nature of the immune response directed against the AChR on the one hand, and on the other hand the role of the target organ in determining the severity of the disease. Therefore two main subjects were addressed in this thesis. I The contribution of immunological and non-immunological factors involved in age related resistance to experimental autoimmune myasthenia gravis (EAMG). II The specificity and genetic diversity of anti-AChR antibodies, isolated from mice strains which have high or low susceptibility for EAMG, were investigated.

The first chapter contains a review of current facts about the AChR, MG and EAMG. The autoantigen in MG is the AChR which is localized at the motor nerve endplate in high density. Much of the information about this transmembrane receptor protein has been revealed by gene sequence analysis and functional studies. The AChR is composed of five subunits arranged around a central ion channel. The relevant antigenic determinants recognized by the immune system have been characterized for anti-AChR antibodies and AChR specific T lymphocytes. Several antigenic determinants recognized by antibodies (B lymphocyte epitopes) have been characterized by binding studies of anti-AChR monoclonal antibodies (mAb) to overlapping synthetic peptides of AChR subunits. The majority of anti-AChR antibodies from MG patients or experimental animals with EAMG are directed to a fragment of 10 aminoacids on the α-subunit called the main immunogenic region (MIR). AChR specific T lymphocytes recognize multiple short peptide sequences from the ex-

tracellular, transmembrane and intracellular domains of the AChR. Determinants recognized by T lymphocytes are mostly different from those recognized by antibody.
A description of MG is given and possible aetiological factors including molecular mimicry, drug induced transient loss of tolerance, and the role of the thymus are discussed. Detailed information is given about the EAMG model, since all experiments in this thesis are performed in this model. A chronic form of EAMG can be induced in several animal species by immunization with AChR isolated from electric organs of the electric ray Torpedo californica. EAMG can also be induced by some (poly)peptide fragments of the α-subunit of AChR or passive transfer of anti-AChR antibodies. EAMG mirrors MG in several aspect except for the thymic abnormalities associated with MG. The mechanisms of the autoimmune attack against the AChR and the pathology observed at the motor nerve endplates is identical for MG and EAMG. Three major immunopathological mechanisms of AChR degradation by autoantibodies result in a loss of functional AChRs in MG and EAMG. I Antibodies bound to the AChR induce antigenic modulation of AChR molecules through cross-linking by antibody, resulting in an increased internalization and degradation. II Complement mediated destruction of muscle membrane at the motor nerve endplate. III Interference with AChR function by blocking acetylcholine binding or the ion channel function of the AChR.

Chapter 2 describes the observation that aged rats are resistant to the induction of both chronic and acute passive transfer EAMG. The mechanisms underlying this age related resistance were investigated in detail using the passive transfer model, in which young and aged rats were injected with a mAb directed against the MIR of the AChR. Passive transfer of this anti-AChR mAb induced severe weight loss and electromyographic abnormalities in young but not in aged rats. In addition, aged rats in contrast to young rats did not show loss of AChRs. Possible mechanisms responsible for resistance to EAMG were evaluated. Uptake of intraperitoneal injected mAb into the circulation was similar in both age groups. However, 48 hours after injection the concentration of mAb declined more rapidly in young compared to aged rats indicating a higher consumption of mAb in young rats. This could not be explained by a difference in antibody clearance from the circulation since both age groups showed a comparable clearance of immunoglobulin G. The significantly larger decline in mAb concentration in the circulation of young rats suggested that the AChR degradation by antigenic modulation is higher in young rats, consuming more antibody. Complement component C3 was demonstrated at endplates in muscle biopsies of both age groups indicating that complement mediated lysis of the postsynaptic membrane occurs in both age groups. However, infiltrating macrophages were only found at the endplates and in necrotic muscle fibers of young animals.
In summary these results suggest that the age related resistance against induction of EAMG is attributable to resistance of the AChR at the postsynaptic membrane (target organ of the autoimmune response) against autoantibody mediated degradation. One of the mechanisms underlying this observed resistance may be the absence of AChR degradation due to cross-linking of adjacent AChR molecules by antibody. At the normal density of AChR molecules at the postsynaptic membrane,

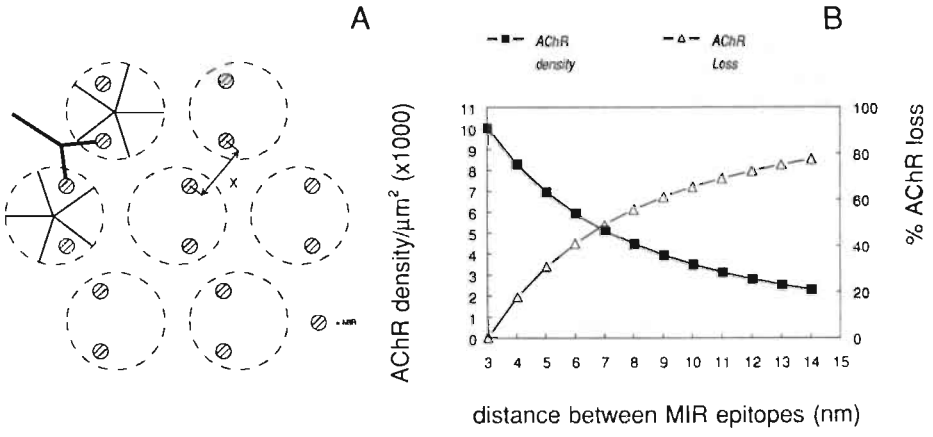


Figure 1: A: Cross-linking of AChR molecules by antibody.
The presumed arrangement of AChR molecules at the endplate at a density of 10000 molecules/µm². At this AChR density, the distance between adjacent MIR epitopes is ± 3 nm and anti-MIR antibody is able to cross-link AChR molecules. X: the distance between adjacent MIR epitopes.
B: The distance between adjacent MIR epitopes is related to the AChR density.
The distance between adjacent MIR epitopes is calculated in relation to AChR density. An AChR density of 10000 molecules/µm² is considered physiological. A decrease in AChR density is also expressed as percentage AChR loss (right abscissa). At decreasing AChR densities, the distance between MIR epitopes increases until ± 12 nm at which the antibody is no longer able to simultaneously bind two MIR epitopes and cross-linking is no longer possible.

eptitopes (like the MIR) on the α-subunits of adjacent AChR molecules are in close proximity (±3 nm), which enable anti-AChR antibodies to cross-link these AChR molecules and accelerate their internalization and breakdown (fig. 1a). When the AChR density decreases the distance between adjacent epitopes increases (fig. 1b). At distances larger than 12 nm the antibodies are no longer able to cross-link AChRs and AChR degradation by antigenic modulation is averted. The maximum distance of 12 nm between adjacent epitopes is attained at a decrease of the AChR density of about 70 % (fig. 1b). It was demonstrated in rats that the AChR density at the endplate diminishes up to 80 % with increasing age (1). Interestingly, the maximum AChR loss observed in rats with chronic EAMG seldom exceeds 70 % (2,3). Moreover, rats that become resistent to a second episode of passive transfer EAMG show more than 60 % AChR loss, and regain susceptible to EAMG when the AChR density increases again (4). Therefore, the AChR density is apparently important for antigenic modulation to take place and the AChR density at the aged endplate is possibly below a critical level to accomplish AChR degradation by antigenic modulation. In future studies the AChR degradation rate *in vivo* will be measured in young and aged rats during passive transfer EAMG, to evalute this hypothesis.
Another possible mechanism may be an increased rigidity of the postsynaptic membrane in aged rats due to a different lipid composition. A rigid membrane could impede internalization of the AChR-antibody complex. The influence of the lipid composition on the rate of AChR degradation shall be investigated by manipulation

of the membrane lipid contents by treatment with phosphatidylcholine/sphingomyelin containing liposomes which were demonstrated to reverse the lipid composition of rat myocytes from "aged" to "young" (5).
Finally, a possible defect of macrophage function in aged animals (6), that could influence the susceptibility for EAMG can not be ruled out. It remains to be determined what te actual contribution of these cells is to the pathophysiology of the disease.

Chapter 3 introduces the second part of this thesis and gives an overview of the immunoglobulin structure and the generation of antibody diversity. Antibodies are capable of specifically recognizing virtually any antigenic determinant. The immunoglobulin molecule is composed of a heavy- and light chain. Each heavy- and light chain is composed of a constant, and a variable region (V_H and V_L) which forms a large part of the antigen binding site. The genetic elements encoding the variable regions are grouped in families of related V genes. The complexity (size) and the organization of the V_H gene families are discussed. It was previously postulated that murine autoantibodies are frequently encoded by V_H genes from a restricted number of families. A review is given about current literature concerning the utilization of V_H genes in autoantibodies from normal or autoimmune prone animals. The immunoglobulin molecule itself is a protein, bearing antigenic determinants. The antigenic determinants associated with the variable region of an antibody molecule together form the idiotype, that can be recognized by other antibodies (anti-idotypes). Antibodies of different specificity can share a cross-reactive or common idiotype that suggests that these antibodies are encoded by related variable region genes. The structural basis for cross-reactive idiotypes is discussed.

Chapter 4 describes the production and characterization of mAbs obtained from two mouse strains with high (C57bl/6) and low (Balb/c) susceptibility for the induction of EAMG. MAbs directed against several extra- and intracellular located epitopes including mAbs directed against the MIR, and the α-bungarotoxin/acetylcholine binding site (α-BT), were isolated from both mouse strains. The relative frequencies of hybridomas cross-reactive with autologous mouse AChR, or directed against the MIR and the α-BT binding site were higher in susceptible C57bl/6 than in low susceptible Balb/c mice, suggesting a possible difference in antibody repertoire. This observed difference in the anti-AChR antibody repertoire was evaluated in the polyclonal anti-AChR response upon induction of EAMG by immunization with AChR. The mean antibody titer against mouse AChR was similar in both mouse strains. In addition, the concentrations of antibodies directed against the MIR and α-BT binding site in the sera of both mouse strains were found to be similar after secondary immunization with AChR. Moreover, the concentrations of anti-MIR and anti-α-BT antibodies in C57bl/6 mice with or without clinical signs, were found to be comparable. These results indicate that strain specific differences in disease susceptibility in murine EAMG are not related to differences in the overall concentration of antibodies against the MIR or α-BT/cholinergic binding site. Fine specificity, affinity and the combined effect of several antibody specificities binding to the AChR at the same time determine the net effect on the AChR function and

degradation rate. This probably explains the poor correlation of the total anti-AChR antibody titer with the clinical state; pathogenic antibodies may form a variable subset of the total anti-AChR antibody pool. Furthermore, several physiological factors like the AChR reserve and the acetylcholine quantal content, which together determine the safety margin of neuromuscular transmission, influence the outcome of the disease.

In Chapter 5 the genetic diversity of heavy chain variable region genes encoding anti-AChR mAbs was determined. The V_H genes are grouped in families of homologous genes. To investigate the diversity of V_H genes encoding anti-AChR mAbs in the EAMG model, we determined the V_H gene family usage of 65 anti-AChR mAbs and 20 anti-KLH mAbs isolated from susceptible C57bl/6 and low susceptible Balb/c mice, by RNA slot-blot analysis using probes specific for nine V_H gene families. Anti-AChR mAbs were found to be encoded by V_H genes from at least 6 different families. The V_H gene family usage of anti-AChR mAbs approached a stochastic distribution over the different V_H gene families in which the frequency of utilization of a V_H gene from a particular family is proportional to the size of that family. Significant deviations from this distribution were found for the J606 and Vgam3.8 families. V_H genes from the J606 family were not found in any of the tested *anti-AChR mAbs*, while the Vgam3.8 family was markedly overrepresented in mAbs directed to the α-BT binding site. In contrast, no preferential V_H gene familiy was utilized to encode mAbs directed to the MIR.
Anti-AChR mAbs only specific for Torpedo AChR and mAbs recognizing both Torpedo and mouse AChR showed no significant difference in V_H gene family utilization. In addition, there was no significant difference in V_H gene family utilization between anti-AChR mAbs obtained from high and low susceptible mice. Therefore, differences in disease susceptibility could not be attributed to a deviant V_H gene family usage.
Anti-AChR antibodies sharing a cross-reactive idiotype (CRI) were found to be encoded by V_H genes from at least 6 different families suggesting no direct relation between expression of a CRI and the utilization of a restricted number of different V_H genes. Taken together, these results indicate that anti-AChR antibodies are encoded by multiple different V_H genes and no bias in V_H gene family utilization as reported for other autoantibodies, could be demonstrated for anti-AChR mAbs.

In addition to mouse anti-AChR mAbs, the V_H gene family utilization was determined of a panel of rat anti-AChR mAbs with well defined fine specificity and pathogenicity. In Chapter 6 the V_H gene family utilization of rat anti-AChR mAbs was determined using probes for known V_H gene families in the mouse. Slot-blot analysis of 21 rat anti-AChR mAbs revealed that these mAbs are encoded by V_H genes from at least 8 different families. It was verified whether V_H gene families analogous to the mouse V_H gene families exist in the rat. Southern blot hybridization of DNA of different rat strains with probes for 11 mouse V_H gene families confirmed the existance of analogous V_H gene families in the rat. The complexity of these rat V_H gene families was found to be different from analogous families in the mouse; the J558 family was smaller whereas the PC7183, X24, Q52, V_H10, and V_H11 families were larger than in the mouse.

The V_H genes encoding rat anti-AChR mAbs did not follow a stochastic distribution over the different V_H gene families, due to overrepresentation of members of the S107 family and underrepresentation of the X24 V_H gene family. The panel of rat anti-AChR mAbs consists of anti-MIR mAbs and mAbs against overlapping epitopes on the intracellular surface of the AChR. A partial correlation between V_H gene family utilization and mAb fine specificity was found. MAbs against the MIR showing pathogenic potential *in vivo* or *in vitro* were most frequently encoded by V_H genes from the Q52 family, whereas non pathogenic mAbs against intracellular epitopes were most frequently encoded by V_H genes from the PC7183 family. The frequent utilization of V_H genes from a particular V_H gene family suggests the use of related V_H gene sequences to encode these antibodies of related fine specificity.
Rat anti-AChR mAbs were shown to express a CRI consisting of framework associated cross-reactive idiotopes shared by all tested anti-AChR mAbs and paratope associated cross-reactive idiotopes shared only by anti-MIR mAbs. Both anti-AChR mAbs expressing framework- as well as paratope associated cross-reactive idiotopes were encoded by V_H genes from several different V_H gene families.
Taken together, the V_H gene family utilization of rat anti-AChR mAbs showed that probably many different V_H genes can be used to encode anti-AChR antibodies. This is an unexpected finding in the light of the fact that a large fraction of the anti-AChR antibodies in MG and EAMG are directed against the MIR, which comprises a small group of epitopes. Furthermore, anti-AChR antibodies express a CRI that suggests some form of structural resemblance between these antibodies. Expression of a CRI idiotype by anti-AChR mAbs may be related to the V_L chain; sequence analysis of the V_L chain of 4 anti-MIR mAbs revealed an amino acid homology of more than 90 % (Dr. S.J. Tzartos, personal communication). Furthermore, anti-AChR mAbs could be encoded by highly homologous diversity or joining gene segments. In future studies the sequence of anti-AChR mAbs will be determined to further reveal structural similarities between anti-AChR antibodies.
Structural resemblance between immune receptors like antibody and the T cell receptor which are specific for AChR reactive B or T lymphocytes may provide a tool for manipulating the autoimmune response against the AChR (7, 8).
In addition, sequence analysis of anti-AChR antibodies may provide information about the origin of these autoantibodies; are these autoantibody sequences part of the germline encoded repertoire ? (9, 10). Furthermore, comparison of the anti-AChR antibody sequences with known sequences of antibodies against exogenous antigens, may reveal information about the possibility that anti-AChR antibodies evolve from such non-pathogenic antibodies by limited somatic mutation (11).

Samenvatting en algemene discussie

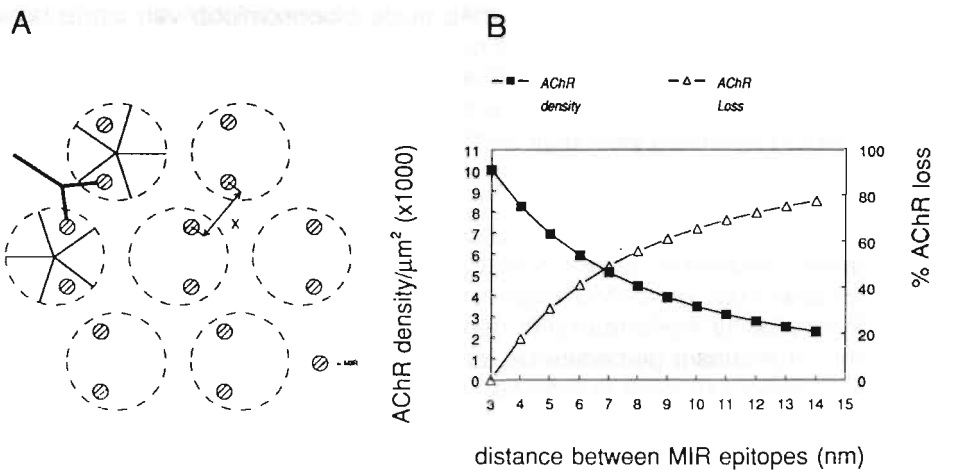
Het immuunsysteem is in staat vrijwel elk lichaamsvreemd organisme, dat het lichaam binnendringt, te herkennen en te elimineren. In tegenstelling tot deze reactiviteit met lichaamsvreemde structuren, reageert het immuunsysteem niet met eigen structuren. In sommige gevallen wordt deze tolerantie voor 'eigen' opgeheven en worden lichaamseigen structuren afgebroken. Myasthenia gravis (MG) is een auto-immuun ziekte, waarbij zich auto-antilichamen ontwikkelen gericht tegen een receptor-eiwit, dat betrokken is bij de overdracht van zenuwsignalen van de zenuw naar de spier. Bij stimulatie van de zenuw wordt de neurotransmitter acetylcholine aan het zenuwuiteinde vrijgemaakt en bindt aan de acetylcholine receptor (AChR), gelegen in de spiermembraan welke vervolgens de spier aanzet tot samentrekking. Bij MG wordt het aantal beschikbare functionele AChR moleculen verminderd door auto-antilichaam gemedieerde afbraak van deze receptoren. Hierdoor treedt een verslechtering op van de signaal overdracht, wat resulteert in spierzwakte en verlamming. Dat MG veroorzaakt wordt door auto-antilichamen is aangetoond door passieve overdracht van antilichamen afkomstig van MG patiënten naar proefdieren, of door overdracht van moeder op kind via de placenta. De oorzaak voor het onstaan van deze auto-immuun respons bij MG is nog niet bekend. Veel van de informatie over MG is verkregen met behulp van een proefdiermodel voor deze ziekte, waarbij proefdieren worden geïmmuniseerd met gezuiverde AChR. Enerzijds wordt in dit proefschrift het proefdier model bestudeerd, om meer informatie te verkrijgen over de aard van de auto-immuun respons gericht tegen de AChR en anderzijds om informatie te verkrijgen over de rol die het doelorgaan speelt bij de ernst van de ziekte.
Dit proefschrift is daarom in twee hoofdonderwerpen onder te verdelen. I de bijdrage van immunologische en niet immunologische factoren betrokken bij leeftijds afhankelijke resistentie tegen experimentele auto-immuun maysthenia gravis (EAMG). II De specificiteit en de genetische diversiteit van anti-AChR antilichamen, verkregen uit muizen met hoge en lage gevoeligheid voor EAMG.

Het eerste hoofdstuk omvat een overzicht van recente gegevens met betrekking tot de AChR, MG en EAMG.
Het auto-antigeen bij MG is de AChR, die in hoge dichtheid gelokaliseerd is ter hoogte van de motor-eindplaat. Veel van de informatie over dit transmembraan receptor-eiwit is verkregen door analyse van de AChR coderende genen en door functionele studies. De AChR is samengesteld uit vijf subunits gegroepeerd rond een centraal ion kanaal. De belangrijkste antigene determinanten die herkend worden door het immuunsysteem, zijn gekarakteriseerd voor zowel anti-AChR antilichamen als AChR specifieke T lymfocyten. Verscheidene antigene determinanten die herkend worden door antilichamen (B lymfocyt epitopen), zijn bepaald door

binding van anti-AChR monoklonale antilichamen (mAb) aan overlappende synthetische peptiden van AChR subunits. Het grootste deel van de anti-AChR antilichamen van MG patiënten of proefdieren met EAMG, zijn gericht tegen een fragment van 10 aminozuren gelegen op de α-subunit, genaamd 'main immunogenic region' (MIR). AChR specifieke T lymfocyten herkennen verscheidene korte peptiden afkomstig van extracellulaire, transmembraan en intracellulaire gedeelten van de AChR. Determinanten die herkend worden door T lymfocyten, zijn vaak verschillend van die welke herkend worden door antilichamen.
Er wordt een beschrijving gegeven van MG en mogelijke factoren, die een rol spelen bij het onstaan van de ziekte waaronder moleculaire mimicry, tijdelijke geneesmiddel geinduceerd verlies van tolerantie en de rol van de thymus. Het EAMG model wordt meer gedetailleerd beschreven, daar het experimentele werk van dit proefschrift in het EAMG model is uitgevoerd. Een chronische vorm van EAMG kan in verschillende diersoorten opgewekt worden d.m.v. immunisatie met AChR, afkomstig van de electrische organen van de sidderrog Torpedo californica. EAMG kan eveneens worden opgewekt door immunisatie met sommige (poly)peptiden van de α-subunit van de AChR, of d.m.v. passieve overdracht van anti-AChR antilichamen. EAMG en MG vertonen veel overeenkomst, behalve wat betreft de thymus afwijkingen die geassocieerd zijn met humane MG. De mechanismen van de auto-immuun aanval gericht tegen de AChR en de pathologie van de motorische zenuw-eindplaat zijn identiek in MG en EAMG. De afbraak van functionele AChR door auto-antilichamen bij MG en EAMG wordt hoofdzakelijk veroorzaakt door drie immunopathologische mechanismen. I Antilichamen gebonden aan de AChR kunnen resulteren in antigene modulatie van AChR moleculen d.m.v. het koppelen van twee AChR moleculen door een antilichaam ("cross-linken"), waarna dit complex versneld wordt geïnternaliseerd en afgebroken. II Complement gemedieerde afbraak van de spiercelmembraan ter hoogte van de motorische zenuw-eindplaat. III Inhibitie van de receptor functie d.m.v. het blokkeren van de acetylcholine binding of de ion kanaal functie.

Hoofdstuk 2 beschrijft de observatie dat oude ratten resistent zijn tegen het opwekken van zowel chronische als acute EAMG d.m.v. passieve overdracht van anti-AChR antilichaam (passieve transfer EAMG). De mechanismen die aan deze leeftijds afhankelijke resistentie ten grondslag liggen werden in detail onderzocht, gebruik makend van het passieve transfer model, waarbij jonge en oude ratten werden geïnjecteerd met een mAb gericht tegen de MIR van de AChR. Passieve transfer van dit anti-AChR mAb induceerde ernstig gewichtsverlies en electromyographische afwijkingen bij jong volwassen ratten. Oude ratten vertoonden daarentegen geen klinische verschijnselen. Bovendien vertoonden oude ratten in tegenstelling tot jonge ratten geen verlies aan AChRs. Mogelijke mechanismen die ten grondslag liggen aan deze resistentie tegen EAMG werden nagegaan. De opname van intraperitoneaal geinjecteerd antilichaam in de bloedsomloop was vergelijkbaar in beide leeftijdsgroepen. Echter 48 uur na injectie, nam de concentratie van het mAb sneller af bij jonge ratten vergeleken met oude ratten, wat wees op een hoger verbruik van het mAb bij jonge ratten. Dit kon niet verklaard worden door een verschil in antilichaamklaring uit de bloedsomloop, daar beide leeftijdsgroepen een overeenkomstige klaring van immunoglobuline G vertoonden. De significant grotere

afname van de concentratie van het mAb in de bloedsomloop van jonge ratten suggereerde dat de afbraak van AChR d.m.v. antigene modulatie hoger is in jonge ratten, waardoor meer mAb werd verbruikt.
De C3 component van complement werd aangetoond ter hoogte van de eindplaat in spierbiopten afkomstig van beide leeftijdsgroepen, wat liet zien dat complement gemedieerde lysis van de postsynaptische membraan in beide leeftijdsgroepen optrad. Infiltrerende macrophagen werden echter enkel aangetoond bij eindplaten en in necrotische spiervezels van jonge dieren.
Samengevat suggereren deze resultaten dat de leeftijds afhankelijke resistentie tegen het opwekken van EAMG berust op resistentie van de AChR ter hoogte van de postsynaptische membraan (het doel orgaan van de auto-immuun respons), tegen auto-antilichaam gemedieerde afbraak. Een van de mechanismen die ten grondslag liggen aan deze resistentie zou kunnen zijn, het ontbreken van AChR degradatie door het koppelen van naburige AChR moleculen door antilichamen. Bij een normale AChR dichtheid ter hoogte van de eindplaat, zullen epitopen (zoals de MIR) gelegen op de α-subunits van naburige AChR moleculen dicht bij elkaar liggen (±3 nm), zodat anti-AChR antilichamen AChR moleculen kunnen koppelen en zodoende de internalisatie en afbraak kunnen versnellen (fig 1a). Als de AChR dichtheid afneemt zal de afstand tussen naburige epitopen toenemen (fig 1b). Wanneer de afstand groter wordt dan 12 nm zullen anti-AChR antilichamen niet langer in staat zijn om AChRs te kunnen koppelen waardoor antigene modulatie wordt voorkomen. Deze maximum afstand tussen naburige epitopen wordt bereikt wanneer de AChR dichtheid afneemt tot ±70 % (fig 1b). Aangetoond werd dat de AChR dichtheid ter hoogte van de eindplaat afneemt met ±80 % bij ratten met toenemende leeftijd (1). Opmerkelijk is bovendien dat het maximale verlies aan AChR waargenomen bij ratten met chronische EAMG zelden groter is dan 70 % (2, 3). Bovendien vertoonden ratten, die resistent waren tegen een tweede inductie van passieve transfer EAMG, meer dan 60 % AChR verlies en werden hernieuwd gevoelig voor passieve transfer EAMG nadat de AChR dichtheid weer toenam (4). De AChR dichtheid is daarom blijkbaar belangrijk voor het optreden van antigene modulatie en mogelijkerwijs is de AChR dichtheid ter hoogte van de "oude" eindplaat beneden een kritische dichtheid, waarbij AChR afbraak door antigene modulatie niet meer mogelijk is. Deze hypothese zal worden nagegaan in een toekomstige studie waarbij de AChR afbraak *in vivo* zal worden gemeten bij jonge en oude ratten waarbij passieve transfer EAMG is opgewekt.
Een ander mogelijk mechanisme zou een verhoogde rigiditeit van de postsynaptische membraan bij oude ratten kunnen zijn, als gevolg van een veranderde lipide samenstelling. Een rigide membraan zou de internalisatie van AChR-antilichaamcomplexen kunnen bemoeilijken. De invloed van de lipide samenstelling op de afbraaksnelheid van AChR zal worden onderzocht d.m.v. manipulatie van het membraan lipide, door behandeling met fosfaditylcholine/sphingomyeline bevattende liposomen waarvan, is aangetoond dat deze de lipide samenstelling van rat myocyten kunnen omzetten van 'oud' naar 'jong' (5). Tenslotte kan de invloed van een mogelijk defect van de macrophagen bij oude dieren (6) op de gevoeligheid voor EAMG niet worden uitgesloten. De werkelijke bijdrage van deze cellen aan de pathofysiologie van de ziekte zal in toekomstig onderzoek worden bepaald.



Figuur 1: A: Het koppelen van AChR moleculen door antilichaam.
De veronderstelde rangschikking van AChR moleculen ter hoogte van de eindplaat bij een dichtheid van 10000 moleculen/µm². Bij deze AChR dichtheid is de afstand tussen naburige MIR epitopen ± 3 nm en zijn anti-MIR antilichamen in staat AChR moleculen te koppelen. X: de afstand tussen naburige MIR epitopen.
B: De afstand tussen naburige MIR epitopen is gerelateerd aan de AChR dichtheid
De afstand tussen naburige MIR epitopen werd berekend in relatie tot de AChR dichtheid. Een AChR dichtheid van 10000 moleculen/µm² werd beschouwd als fysiologisch. Een afname van de AChR dichtheid werd tevens uitgedrukt als percentage AChR verlies (rechter Y-as). Bij een afnemende AChR dichtheid neemt de afstand tussen MIR epitopen toe tot ± 12 nm waarbij antilichaam niet langer in staat is om twee MIR epitopen simultaan te binden waardoor het koppelen van AChR moleculen niet meer mogelijk is.

Hoofdstuk 3 vormt de introductie van het tweede deel van dit proefschrift en geeft een overzicht van de opbouw van het immunoglobuline molecuul en het genereren van antilichaam diversiteit. Antilichamen zijn in staat vrijwel elke denkbeeldige antigene determinant te herkennen. Het immunoglobuline molecuul is samengesteld uit een zware en een lichte keten. Een zware en lichte keten bestaan elk uit een constante en een variabele regio (V_H en V_L) welke het grootste gedeelte van de antigeen bindingsplaats vormt. De genetische elementen die voor de variabele regio coderen zijn gegroepeerd in families van verwante V genen. De complexiteit (grootte) en de organisatie van deze V_H gen families worden besproken. In voorgaande studies werd gepostuleerd, dat muize auto-antilichamen frequent worden gecodeerd door V_H genen van een gerestricteerd aantal families. Er wordt een overzicht gegeven van recente literatuur betreffende het gebruik van V_H genen in auto-antilichamen afkomstig van normale muizen en muizen met een auto-immuun predispositie.
Het immunoglobuline molecuul is opzichzelf een eiwit dat antigene determinanten draagt. De antigene determinanten geassocieerd met het variabele gedeelte van het antilichaam molecuul, vormen samen het idiotype dat herkend kan worden door andere antilichamen (anti-idiotype antilichamen). Antilichamen met verschillende specificiteit kunnen een kruisreactief of publiek idiotype delen wat suggereert dat

deze antilichamen zijn gecodeerd door verwante variable regio genen. De structurele basis voor het voorkomen van een kruisreactief idiotype wordt besproken.

Hoofdstuk 4 beschrijft de produktie en karakterisering van monoclonale antilichamen verkregen van twee muizenstammen, welke een hoge (C57bl/6) en lage (Balb/c) gevoeligheid vertonen voor de inductie van EAMG. MAbs gericht tegen verscheidene extracellulair of intracellulair gelegen epitopen, waaronder mAbs gericht tegen de MIR en de α-bungarotoxine/acetylcholine bindingsplaats (α-BT) werden geïsoleerd uit beide muizenstammen. De relatieve frequenties van hybridomas kruisreactief met autologe muis AChR of gericht tegen de MIR of de α-BT bindingsplaats waren hoger in gevoelige C57bl/6 dan in minder gevoelige Balb/c muizen. Dit suggereert een mogelijk verschil in het beschikbare antilichaam repertoire. Het geobserveerde verschil in anti-AChR antilichaam repertoire werd eveneens nagegaan in de polyclonale anti-AChR response die ontstaat na immunisatie met AChR. De gemiddelde titer van antilichamen gericht tegen muis AChR was vergelijkbaar in beide muizenstammen. Daarbij was de concentratie van antilichamen gericht tegen de MIR en de α-BT bindingsplaats in sera van beide muizenstammen vergelijkbaar na de tweede immunisatie met AChR. Bovendien waren de concentraties van anti-MIR en anti-α-BT bindingsplaats antilichamen bij C57bl/6 muizen, met en zonder klinische verschijnselen vergelijkbaar. Deze resultaten laten zien dat stam afhankelijke verschillen in gevoeligheid voor de ziekte in het muize EAMG model, niet gerelateerd zijn aan verschillen in de totale concentratie van antilichamen tegen de MIR en α-BT bindingsplaats. De fijne specificiteit, affiniteit en het effect van antilichamen met verschillende specificiteit die gezamenlijk binden aan de AChR, bepalen het netto effect op de AChR functie en de AChR afbraaksnelheid. Mogelijkerwijs verklaart dit het feit dat de totale anti-AChR antilichaam titer nauwelijks correleert met de mate van klinische verschijnselen; pathogene antilichamen omvatten waarschijnlijk een variabel gedeelte van de totale anti-AChR antilichaampopulatie. Bovendien zijn er verschillende fysiologische factoren zoals de AChR reserve en het aantal acetylcholine moleculen per quantum, welke samen de 'veiligheids marge' van de neuromusculaire signaaloverdracht bepalen. Deze factoren hebben eveneens invloed op het ontstaan van de ziekte.

In hoofdstuk 5 werd de genetische diversiteit bepaald van genen, die coderen voor het variabele gedeelte van de zware keten van anti-AChR mAbs. Om de diversiteit aan V_H genen te inventariseren werd het V_H gen familie gebruik van 65 anti-AChR en 20 anti-KLH mAbs, geïsoleerd uit EAMG gevoelige C57bl/6 en EAMG ongevoelige Balb/c muizen, bepaald d.m.v. RNA slot-blot analyse, waarbij gebruik werd gemaakt van probes specifiek voor negen V_H gen families. Anti-AChR mAbs werden gecodeerd door V_H genen, afkomstig van minstens 6 verschillende families. Het V_H gen familie gebruik benaderde een stochastische verdeling over de verschillende V_H gen families, waarbij de frequentie van gebruik van V_H genen afkomstig uit een bepaalde familie proportioneel is met de grootte van die familie. Significante afwijkingen van deze verdeling werden gevonden voor de J606 en Vgam3.8 families. V_H genen afkomstig uit de J606 familie werden in geen van de geteste anti-AChR mAbs aangetroffen, terwijl de Vgam3.8 familie duidelijk was oververtegenwoordigd in mAbs gericht tegen de α-BT bindingsplaats. In tegenstelling tot

deze bevinding werd geen bepaalde V_H gen familie preferentieel gebruikt om mAbs gericht tegen de MIR te coderen. Anti-AChR mAbs specifiek voor enkel Torpedo AChR en mAbs die zowel Torpedo als muis AChR herkennen, vertoonden geen significant verschil in V_H gen familie gebruik. Bovendien was er geen significant verschil in V_H gen familie gebruik aantoonbaar tussen anti-AChR mAbs afkomstig van muizen met hoge of lage gevoeligheid voor EAMG. Verschillen in gevoeligheid voor de ziekte zijn daarom niet toe te wijzen aan een afwijking in het V_H gen familie gebruik.
Anti-AChR mAbs die een kruisreactief idiotype delen, werden gecodeerd door V_H genen afkomstig uit minstens 6 verschillende families, wat suggereert dat er geen directe relatie bestaat tussen de expressie van dit kruisreactief idiotype en het gebruik van een beperkt aantal verschillende V_H genen. Samenvattend laten deze gegevens zien, dat anti-AChR antilichamen worden gecodeerd door meerdere verschillende V_H genen en dat geen verschuiving in het V_H gen familie gebruik kan worden geobserveerd, zoals dat werd beschreven voor andere auto-antilichamen.

Naast de bepaling van het V_H gen familie gebruik van muizen anti-AChR mAbs werd eveneens het V_H gen familie gebruik bepaald van een panel van rat anti-AChR mAbs, waarvan de fijne specificiteit en pathogeniciteit in detail zijn gedefinieerd.
In hoofdstuk 6 werd het V_H gen familie gebruik van rat anti-AChR mAbs onderzocht met behulp van probes voor bekende muizen V_H gen families. Slot-blot analyse van 21 rat anti-AChR mAbs liet zien, dat deze mAbs werden gecodeerd door V_H genen afkomstig uit minstens 8 verschillende families. Er werd nagegaan of V_H gen families analoog aan de muize V_H gen families bestaan in de rat. Southern blot hybridisatie van DNA afkomstig van verschillende rattenstammen met probes voor 11 muize V_H gen families, bevestigde het bestaan van analoge V_H gen families in de rat. De complexiteit van deze rat V_H gen families was verschillend van die van analoge families in de muis; de J558 familie was kleiner terwijl de PC7183, X24, Q52, V_H10 en V_H11 families groter waren dan bij de muis.
De V_H genen die coderen voor rat anti-AChR mAbs, volgden niet een stochastische verdeling over de verschillende V_H gen families, als gevolg van oververtegenwoordiging van leden van S107 familie en ondervertegenwoordiging van de X24 familie. Het panel van rat anti-AChR mAbs bestond uit anti-MIR mAbs en mAbs gericht tegen overlappende epitopen gelegen op het intracellulaire oppervlak van de AChR. Een gedeeltelijke correlatie van het V_H gen familie gebruik met de fijne specificiteit van sommige mAbs werd gevonden. MAbs gericht tegen de MIR met pathogeen potentieel *in vivo* of *in vitro*, werden frequent gecodeerd door V_H genen van de Q52 familie, terwijl niet pathogene mAbs gericht tegen intracellulair gelegen epitopen frequent werden gecodeerd door V_H genen van de PC7183 familie. Het frequente gebruik van V_H genen uit een bepaalde V_H gen familie suggereert het gebruik van verwante V_H gen sequenties voor het coderen van antilichamen met overeenkomstige fijne specificiteit.
Rat anti-AChR mAbs brengen een kruisreactief idiotype tot expressie, dat bestaat uit kruisreactieve idiotopen, geassocieerd met de basisstructuur (framework) van de variabele regio, welke voorkomen op alle geteste anti-AChR mAbs en kruisreactieve idiotopen, geassocieerd met de antigeen bindingsplaats (paratoop), die enkel voorkomen op anti-MIR mAbs. Anti-AChR mAbs die zowel framework geassocieer-

de als paratope geassocieerde kruisreactieve idiotopen tot expressie brengen, werden gecodeerd door V_H genen afkomstig van verschillende V_H gen families. Samenvattend laat het V_H gen familie gebruik van rat anti-AChR mAbs zien, dat waarschijnlijk vele verschillende V_H genen gebruikt kunnen worden voor het coderen van anti-AChR antilichamen. Dit is een onverwachte bevinding in het licht van het feit, dat een groot gedeelte van de anti-AChR antilichamen gericht zijn tegen de MIR welke uit een kleine groep van epitopen bestaat. Bovendien brengen anti-AChR mAbs een kruisreactief idiotype tot expressie, dat een of andere vorm van structurele overeenkomst tussen deze antilichamen suggereert. De expressie van een kruisreactief idiotype door anti-AChR mAbs zou gerelateerd kunnen zijn aan de V_L keten; sequentie analyse van V_L ketens van 4 rat anti-MIR mAbs liet een onderlinge aminozuur homologie zien van meer dan 90 % (Dr S.J. Tzartos, persoonlijke mededeling). Anti-AChR mAbs zouden eveneens kunnen worden gecodeerd door 'diversity' en/of 'joining' gen segmenten met grote overeenkomst. In toekomstige studies zal de aminozuur sequentie van anti-AChR mAbs bepaald worden om eventuele structurele overeenkomsten tussen anti-AChR antilichamen aan te tonen. Structurele overeenkomsten tussen immuunreceptoren zoals antilichaam en de T cell receptor, welke specifiek zijn voor AChR reactieve B en T lymfocyten, kunnen wellicht een aangrijpingspunt zijn voor specifieke manipulatie van de auto-immuun respons tegen de AChR (7, 8).
Sequentie analyse zou bovendien informatie kunnen geven over de oorsprong van deze auto-antilichamen; maken deze auto-antilichaam sequenties deel uit van het repertoire zoals dat in de 'germline' genen wordt gecodeerd ? (9, 10). Verder zou het vergelijken van anti-AChR antilichaam sequenties met reeds bekende sequenties van antilichamen gericht tegen exogene antigenen, informatie kunnen geven over het ontstaan van anti-AChR antilichamen uit niet-pathogene antilichamen d.m.v. beperkte somatische mutaties (10).

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Curriculum vitae

Yvo Graus werd geboren op 1 maart 1963 in Heerlen. Hij behaalde in 1981 en 1983 respectievelijk het diploma HAVO en Atheneum β aan het Sintermeerten college te Heerlen. Vervolgens studeerde hij medische biologie aan de Universiteit van Amsterdam. In 1988 behaalde hij het doctoraal examen met als afstudeer opdrachten moleculaire biologie bij het Nederlands kanker instituut in Amsterdam, onder leiding van Dr. R. Nusse en immunologie bij de vakgroep immunologie van de rijksuniversiteit Limburg in Maastricht, onder leiding van Dr. M.H De Baets. Van maart 1988 tot maart 1992 was hij werkzaam bij de vakgroep immunologie van de Rijksuniversiteit Limburg. Onder leiding van Dr. M.H. De Baets en Prof. Dr. P.J.C. van Breda Vriesman werd gedurende deze periode het onderzoek verricht beschreven in dit proefschrift. Vanaf maart 1992 is hij als post-doctoraal onderzoeker werkzaam bij de vakgroep immunologie van de Rijksuniversiteit Limburg.

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