Autoimmunity to cell surface receptors : immunopathology of acetylcholine receptors in experimental autoimmune myasthenia gravis in the rat

Citation for published version (APA):

de Baets, M. H. (1984). *Autoimmunity to cell surface receptors : immunopathology of acetylcholine receptors in experimental autoimmune myasthenia gravis in the rat*. [Doctoral Thesis, Maastricht University]. Leiter-Nypels. <https://doi.org/10.26481/dis.19841026mb>

Document status and date:

Published: 01/01/1984

DOI:

[10.26481/dis.19841026mb](https://doi.org/10.26481/dis.19841026mb)

Document Version:

Publisher's PDF, also known as Version of record

Please check the document version of this publication:

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

**Immunopathology of acetylcholine receptors in experimental
autoimmune myasthenia gravis in the rat**

AUTOIMMUNITY TO CELL SURFACE RECEPTORS

Immunopathology of acetylcholine receptors in experimental
autoimmune myasthenia gravis in the rat

PROEFSCHRIFT

ter verkrijging van de graad van doctor in de geneeskunde aan de
Rijksuniversiteit Limburg te Maastricht, op gezag van de rector
magnificus, Prof. Dr. H.C. Hemker, volgens besluit van het College van
Dekanen, ter verdediging op 26 oktober 1984 om 16.00 uur

door

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CIP-DATA KONINKLIJKE BIBLIOTHEEK, DEN HAAG

De Baets, M.H.

Autoimmunity to cell surface receptors: immunopathology of acetylcholine receptors in experimental autoimmune myasthenia gravis in the rat / M.H. De Baets. — (S.l.: s.n.). — ill.

Thesis Maastricht. — With summary in Dutch.

ISBN 90-9000767-9

SISO 605.16 UDC 616.44

Subject headings: myasthenia Gravis / acetylcholine receptor / autoimmunity / cell membrane receptors.

This thesis was designed in 1978 bij Prof. P.J.C. van Breda Vriesman and originated from a collaborative study between the Departments of Immunology (University of Limburg at Maastricht, the Netherlands) and Endocrinology (University of Gent, Belgium) on autoantibody mediated receptor disease specifically Graves disease. Since the only experimental model of antibody mediated receptor disease existing at that time was experimental autoimmune myasthenia gravis it was decided to use this model in order to analyze autoantibody receptor interactions.

A great number of people contributed to this study. Dr. J. Lindstrom (Receptor Laboratory, Salk Institute, La Jolla, California) who taught me the acetylcholine receptor isolation and radioimmunoassays for determining anti-acetylcholine-receptor antibody. The *in vitro studies*, presented in Chapter III of this thesis were done in the laboratory of Prof. W.O. Weigle, Department of Immunopathology, Scripps Clinic and Research Foundation. The experiments presented in Chapter IV, V and VI were performed at the Department of Immunology, University of Limburg at Maastricht (Prof. Dr. P.J.C. van Breda Vriesman). The majority of experiments were performed by ms. L.F.M. Vlek, ms. P. Storms, and last but not least mrs. A. de Baets-Janssens. The isoelectrofocusing experiments were done in a collaborative study with A. Bionda and A.N. Theophilopoulos (Scripps Clinic and Research Foundation).

The work would not have been possible without a Fogarty fellowship, and grants from the Rijksuniversiteit Limburg and the Prinses Beatrix Foundation.

Mrs. C. Bollen and also F. Teng and R. le Doux assisted with the typing of the manuscript and Dr. G. Majoor with the translation of the summary.

Only a poet not a scientist can say "*Exegi momentum aere perennius*"
(*Horatius, Carmina III, 30, 1*)

Aan mijn ouders

Dank aan Aldegonde

ABBREVIATIONS

ab	Antibody	pI	Isoelectric point
α -BGT	α -bungarotoxin	RCM-AChR	Reduced and carboxy-methylated acetylcholine receptor
ACh	Acetylcholine	SC	Spleen cells
AChR	Acetylcholine receptor	SDS	Sodium dodecyl sulfate
BSA	Bovine serum albumin	t-AChR	Trypsinized acetylcholine receptor
cAMP	Cyclic-adenosine monophosphate	TBII	Thyrotropin binding inhibiting immunoglobulins
CFA	Complete Freund's adjuvant	TCGF	T cell growth factor
Con-A	Concanavalin A	[³ H] TdR	Tritiated thymidine desoxyribose
DNP	Dinitrophenol	TG	Thyroglobulin
DS	Dextran sulphate	TGI	Thyroid growth stimulating immunoglobulin
DTH	Delayed type hypersensitivity	TSab	Thyroid stimulating antibody
EAE	Experimental autoimmune encephalomyelitis	TSH	Thyrotropin
EAMG	Experimental autoimmune myasthenia gravis	TSI	Thyroid stimulating immunoglobulins
EAT	Experimental autoimmune thyroiditis		
ELISA	Enzyme linked immunosorbent Assay		
FACS	Fluorescence-activated cell sorter		
FCS	Fetal Calf serum		
FSH	Follicle stimulating hormone		
HBSS	Hank's balanced salt solution		
HLA	Human leucocyte antigens		
Ia	Immune response region associated		
IEF	Isoelectric focusing		
IgG	Immunoglobulin G		
IL-1	Interleukin 1		
IL-2	Interleukin 2		
i.p.	Intraperitoneal		
LNC	Lymph node cells		
LPS	Lipopolysaccharide		
MAB	Monoclonal antibody		
MEPP	Mini endplate potential		
MHC	Major histocompatibility complex		
MIR	Main immunogenic region		
MG	Myasthenia gravis		
NP-40	Nonidet-P-40		
NZB	New Zealand Black mice		
NZW	New Zealand White mice		
PAGE	Polyacrylamide gel-electrophoresis		
PBL	Peripheral blood lymphocyte		
PBS	Phosphate buffered saline		
PMSF	Phenyl methyl sulfonylfluoride		

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INTRODUCTION

Autoimmunity against a receptor defines *immunological* reactions against "self" glycoproteins which are embedded in the cell membrane. The immune reactions may be physiologic when in the course of an immune response to a given antigen, antibodies to B-cell receptors (= anti-idiotypic antibodies) are elicited (1). Autoimmunity can be pathologic when the immune reactions cause signs of disease because of receptor loss (2) or receptor stimulation (3). The clinical spectrum of these autoimmune receptor diseases is described in chapter I of this thesis.

Clinically analysis of the pathogenesis of disease, in this context autoimmune receptor disease, is usually greatly hampered by the absence of suitable animal models. This is well illustrated by two autoimmune receptor diseases: Graves' disease and myasthenia gravis. It was suggested in 1956 (4) and known since 1962 (5) that Graves' disease is an autoimmune disorder caused by circulating antibodies to TSH receptor (thyroid stimulating hormone) on thyroid cells. The absence of an animal model has insured that now, over two decades later, little is known about the TSH receptor or its interaction with autoantibodies; and scientists are still searching for a convenient assay to measure these antibodies. In contrast the hypothesis that myasthenia gravis might be caused by autoantibodies to the acetylcholine receptor was only proposed in the beginning of the seventies (6). Next Patrick and Lindstrom developed an animal model for this disease in 1973 (7) and a few years later a clinical useful assay was available for diagnosis and follow up of patients with myasthenia gravis (8).

Since we were interested in the pathogenesis and therapeutic manipulation of autoimmune receptor diseases the state of the art dictated, experimental myasthenia gravis as model system for these diseases (fig. 1). This model is described in Chapter II.

Conceptually, a therapeutic approach to autoimmune receptor diseases requires precise information about immune reactions involved and target organ characteristics. Both manipulation of the immune response or the target organ might ameliorate disease. Since Lindstrom et al. had spented great effort to characterize the acetylcholine receptor — a joint effort with Dr. J.M. Lindstrom (Receptor Biology Laboratory at the Salk Institute) and Dr. W.O. Weigle (Scripps Clinic and Research Foundation) was created in order to characterize the immune reactions involved in experimental autoimmune myasthenia gravis *in vitro*. The question asked was which humoral and cellular immune reactions were involved in experimental autoimmune myasthenia gravis. Although in any given immune reaction both T and B cells are involved there may be T or B cell preponderance in the effector arch as illustrated by the following experimental diseases. In experimental autoimmune allergic encephalitis T cells are important effector cells although antibodies to basic myelin protein are demonstrated (9). In ex-

Fig. 1 Induction of experimental autoimmune myasthenia gravis in the rat. Acetylcholine receptors are isolated from the electric organs of *Torpedo californica* by affinity chromatography on Cobra toxin coupled to agarose beads. The purified acetylcholine receptors incorporated in complete Freund's adjuvant are injected into experimental animals (rats). These animals develop experimental autoimmune myasthenia gravis, a disease that resembles myasthenia gravis in man.

perimental autoimmune myasthenia gravis antibodies are thought to be important and little is known about cellular immune mechanisms (10). At the onset of the study the rat was chosen as an experimental animal. This choice was made because inbred strains were available and the immune system is well characterized. The rats — in contrast to mice — develop clinical myasthenia after immunization with acetylcholine receptors whereas mice are rather resistant (10). In chapter III an *in vitro* lymphocyte culture system is described which allows the study of the immunogenicity of several AChR preparations and the analysis of the differing lymphocyte subsets operative in EAMG. These *in vitro* findings were next confirmed in an *in vivo* model (chapter IV).

Next, we analyzed which category of antibodies mediated the disease. Since experimental autoimmune myasthenia gravis is induced by immunizing rats with acetylcholine receptors isolated from electric eel or *Torpedo californica* the question asked was which crossreactive antibodies mediated the disease. Therefore the relationship between immunogenicity of various acetylcholine receptor preparations, crossreactive antibodies binding to rat acetylcholine receptor and myasthenia gravis was analyzed *in vivo* (chapter IV). The experiments described in chapter IV strongly supported the view of that myasthenia gravis in the experimental model was brought about by antibodies binding to a discrete part of the rat acetylcholine receptor. In other words these experiments suggested that antibody specificity might be of great importance in experimental autoimmune myasthenia gravis (EAMG). This is an important issue in terms of immunotherapy of the disease, for if the pathogenetically important antibody response could be shown to be highly specific and restricted, then therapy with anti-idiotypic antibody might influence disease (12). The studies on antibody specificity and restriction are described in chapter V. The results obtained in chapter IV and V strongly suggested that apart from crossreactive antibody titers, target organ characteristics determined the severity of disease. In chapter VI a therapeutic approach to experimental autoimmune myasthenia gravis is described which originated from efforts to increase target organ resistance to autoantibody mediated acetylcholine receptor loss from muscle.

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CHAPTER I

Autoimmune receptor diseases

1.1 INTRODUCTION

Cell surface receptors are antennas designed to receive information and stimuli carried by means of messengers residing in the extracellular fluid; these messengers include hormones, neurotransmitters, trophic factors, and immunogens. After binding of the messenger — also called ligand — to the receptor, cell function is modulated. The receptor concept therefore implies both formation of a complex between receptor and messenger (ligand), activation of the receptor, and a demonstrable biological effect on the cell function by means of activation of the receptor; in other words, the binding of a ligand to a cell membrane implies the presence of a receptor only when the binding results in a specific biological activity on the part of the cell.

The receptor concept was already introduced at the turn of the century by Langley (1) who held that striated muscle cells contained a "receptive substance" and a "chief substance"; the latter was required for the specific biological function of the striated muscle cell : contraction. In the "receptive substance"; we have a description of a true receptor, the acetylcholine receptor, "avant la lettre". Although Ehrlich - also at the turn of the century — coined the term "receptor", and Ehrlich is still alluded to today when describing immunoglobulin receptors on the surface of B lymphocytes, Ehrlich theorized only about highly specific "side chains", residing in the cell protoplasm; these unique chemical structures he held could serve as "receptors" for chemotherapeutic drugs (2). Clearly Ehrlich described no receptor in the modern sense of the word for a substance that can bind to another substance does not necessarily imply a biologically active ligand.

Several types of membrane receptors have been shown to exist. Peptide hormone receptors activate a cascade of enzymes including adenylate cyclases (1,2) and protein kinases (5). Neurological receptors are more directly involved in cellular events through ion channels allowing acute changes in intracellular ion compensation resulting in neuroactivation or muscle contraction (6). Immunological receptors are modulated by antigen or anti-antibodies (=anti idiotypic antibodies) resulting in cell proliferation and differentiation (7). These immunological receptors were the first receptors to be characterized by technology developed because of the existence of plasma cell tumors, secreting large amounts of homogenous antibodies. Recent advances in genetic engineering have made it possible to detect the subtle difference in cell bound i.e. receptor immunoglobulin and secretory immunoglobulin (8). The acetylcholine receptor was next on the list of new discoveries of cell surface receptor molecules (9). Both (purified) snake toxins and electric organ from *Electrophorus Electricus* (electric eel) were of great

help in the purification of AChR. The specific high affinity binding of cobra toxin to AChR which are present in large quantities in the eel's electric organ, allowed Karlin and several others (9) to isolate an integral membrane protein. Further research in this field was hampered by extensive proteolysis resulting in isolation of small peptides with no receptor function. In the last decade a vast array of receptors have been purified to homogeneity. First by use of ligand-affinity columns and later on by lectins and more recently also by insolubilized monoclonal anti-receptor antibodies.

The concept of autoimmunity was developed prior to most of the above mentioned technology was available. Indeed, in 1956, Roitt and Doniach demonstrated antibodies against thyroglobulin in patients with Hashimoto's thyroiditis (10). In the same year Rose and Witebsky developed an animal model for this disease (11). These authors, probably inspired by Koch's postulates, formulated new criteria for the definition of autoimmune diseases (Table I).

Table I : Criteria for establishing the autoimmune etiology of an organ-specific human disease (12)

-
- (1) Autoantibodies reactive at body temperature, or evidence of cell-mediated immunity.
 - (2) Isolation and purification of the organ-specific antigens.
 - (3) Production of autoantibodies to organ-specific antigen in experimental animals.
 - (4) Development of similar lesions in autosensitized animals.
-

In 1960, when the idea of autoimmunity inspired thinking in clinical medicine Simpson postulated that myasthenia gravis (MG) was due to an autoimmune attack upon a protein of the motor endplate (13). This concept was inspired by the fact that MG was frequently associated with disorders of the blood the reticulo-endothelial system and systemic lupus erythematosus; other arguments were abnormalities of the thymus, the presence of lymphorrhages in muscles, the fluctuating course of the disease and above all the occurrence of transient neonatal MG (13). Nastuk et al. observed that the sera of some MG patients were cytotoxic for muscle cells (14). They also found that complement levels were lower during the clinical exacerbation of MG. The idea that antibody to a membrane receptor could be a cause of disease was first put forward by Carnegie. He speculated that the encephalitogenic determinant on myelin basic protein, used to induce experimental autoimmune encephalomyelitis, was remarkably similar to the estimated requirements for a serotonin receptor (15); this hypothesis has never been substantiated however. The concept of autoimmunity to cell surface receptors has been lately extended to other diseases and referred to as immunopharmacological disease (16).

Based on these concepts animal models for the study of antireceptor diseases were developed. Patrick and Lindstrom demonstrated that antibodies against acetylcholine receptors from electric organ of an electric eel could interfere with the neuromuscular transmission in the rabbit (17). The autoimmune nature of myasthenia gravis was further demonstrated by Lennon and Lindstrom in rat and guinea pig (18). The

Table II. Autoimmune diseases associated with anti receptor antibodies

Receptor for	Disease	Reference
Acetylcholine (nicotinic)	myasthenia gravis	(17)
Thyroid stimulating hormone	Thyrototoxicosis (TSI) ^a	(20)
	Simple goitre (TGI) ^b	(21)
	Primary myxoedema (TGI-block) ^c	(22)
Insulin	Insulin resistance	(23)
	Hypoglycemia	(24)
Parathyroid hormone	Secondary hyperparathyroidism in renal failure	(25)
Gastrin	Pernicious anemia	(26)
β_2 -adrenergic agonists	Asthma and allergic rhinitis	(27)
Follicle stimulating hormone	Amenorrhoe	(28)
Transferrin	Anemia	(29,30)

^a) TSI: thyroid stimulating immunoglobulin;

^b)TGI: thyroid growth promoting immunoglobulins;

^c)TGI-block: thyroid growth inhibiting immunoglobulins.

Table III. Anti receptor antibodies exerting agonist like effect

Receptor	Cellular response	Reference
TSH	thyroid hormone secretion	(31)
Insulin	glucose uptake	(24)
Prolactin	casein synthesis	(32)
Epidermal growth factor	DNA synthesis	(33)

demonstration of antibodies against AChR's in myasthenia gravis patients proved the existence of antireceptor diseases in man (19). Since then several autoimmune receptor diseases have been identified (table II) clinically mainly through the development of assays to measure anti receptor antibodies. A number of antibodies can exert an agonist like effect (table III). These diseases are now reviewed separately.

1.2 AUTOIMMUNITY TO ACETYLCHOLINE RECEPTORS

Myasthenia gravis is a disease affecting the neuromuscular junction resulting in abnormal weakness and fatigability of some or all voluntary muscles. The weakness increases with repeated or sustained exertion and is improved by rest. These signs respond to anticholinesterase drugs which act increasing the amount of available acetylcholine. External ocular muscles are affected initially in approximately 50% and eventually in about 90% of the patients. Other voluntary muscles innervated by cranial nerves and

cervical, pectoral-girdle and hip flexor muscles are also frequently affected; proximal limb muscles are usually more severely affected than distal ones. The deep tendon reflexes are brisk or normally active. There are no objective sensory deficits. The signs of the disease fluctuate from day to day, week to week and spontaneous remission can occur for varying periods of time (34-36).

The evidence for an autoimmune etiology of MG includes clinical and epidemiological arguments. As already suggested by Simpson an association exists with other diseases of autoimmune etiology, MG has a fluctuating course, and a transient neonatal disease exists (13). MG predominates in females of 15-40 year of age (reviewed in 37) and is associated with HLA-A1-B8 (38-47) in caucasians and with HLA-B12 (48) and Gm allotypes in the japanese population (49). A favourable reaction to corticosteroids and/or immunosuppressive agents is seen (reviewed in 50). These clinical and epidemiological evidence prompted several investigators to search for immunopathological data to prove the autoimmune etiology of MG. This search resulted in the fulfillment of the four criteria of Witebsky and Rose (12). Almost all patients with myasthenia gravis have circulating antibodies against AChR as measured by a double antibody immunoprecipitation assay using ^{125}I - α -bungarotoxin-labeled human AChR (51-53,46). Anti-AChR antibodies can also be detected in the blood of babies born to myasthenic mothers (54-56) and a small number of these babies (12%) show weakness at birth (57). MG can also be transferred from man to mice by means of serum (65). There is also evidence of cell mediated immunity against AChR as measured by peripheral blood lymphocyte proliferation assays (reviewed in 58). The AChR has been purified from the electric organ of electric fish and from mammalian muscle (reviewed in 59). Injection of acetylcholine receptors in complete Freund's adjuvant (CFA) into

Table IV : Myasthenia gravis is an autoimmune disease :

A. Clinical and epidemiological evidence

1. Association with other autoimmune disease, high prevalence of autoantibodies other than AChR antibody (rheumatoid arthritis in females, hyperthyroidism)
2. Fluctuating course
3. Female predominance (3:1, 15-40 yr)
4. Genetic predisposition (HLA A1-B8; Gm allotypes)
5. Favourable reaction to corticosteroids and immunosuppressive agents

B. Immunopathological evidence (criteria of Witebsky and Rose)

1. Anti-AChR antibodies are detectable in 85-90% of patients with the disorder. There is also evidence of cell-mediated immunity against AChR.
 2. The acetylcholine receptor, one of the organ-specific antigens, has been purified.
 3. Injection of acetylcholine receptors in Freund's complete adjuvant into animals induces the production of autoantibodies
 4. The lesions present at neuromuscular endplate in animals injected with acetylcholine receptors are similar to those in patients with myasthenia gravis.
-

experimental animals induced the production of autoantibodies (reviewed in 60) and myasthenia (see chapter II). In these animals the immunopathological lesions including the anti-AChR antibody and complement deposited at the neuromuscular endplate resemble to a considerable extent the post-synaptic neuromuscular lesions observed in myasthenia gravis (61-64).

1.3 AUTOIMMUNITY TO THYROTROPIN RECEPTORS

Graves' disease is characterized by overproduction of the thyroid hormones thyroxine and triiodothyronine, resulting in a state of metabolic hyperactivity. The probable cause of these features is the interaction of thyroid-stimulating immunoglobulins (TSI) with the TSH receptor or a closely adjacent site on the membranes of the epithelial cells lining the thyroid follicles (31). Morphologically, Graves' disease is characterized by diffuse hyperplasia of the acinar tissue and usually foci of lymphocytic infiltration. Antibodies against the TSH receptors were discovered by chance when sera from patients with thyrotoxicosis due to Graves' disease were tested for thyrotropin activity in a guinea pig bioassay (66). A substance in these sera, later identified as immunoglobulin G, induced a long acting thyroid stimulation (LATS) when compared to thyrotropin (20,67). The majority of patients with Graves' disease, however, were LATS negative. A second factor, LATS-protecting factor which competes with LATS for human thyroid tissue was identified (68). LATS-Protector is a human specific thyroid stimulating antibody which does not cross-react with the bioassay in animals (68). Infusion of sera with LATS-P activity into normal human volunteers stimulated their thyroids thus proving that LATS-P was a true thyroid stimulator. (69). Since then a large array of *in vitro* assays for thyroid stimulation have been developed (70,71). Two *in vitro* assays using human thyroid membranes are clinically useful. The first measures the inhibition of binding of radiolabelled highly purified thyrotropin (TSH) to human thyroid plasma membranes by immunoglobulin from the sera of patients with Graves' disease (TBii thyrotropin binding inhibition immunoglobulins). A second assay measures the activation of the TSH receptor-adenylate cyclase complex by Graves' IgG (TSi or TSab: thyroid stimulating immunoglobulin or antibodies) on human thyroid membranes or thyroid slices (72,73).

Recently a second population of antibodies to the TSH receptor, affecting thyroid cell growth was identified (21). In the sera of patients with goitrous form of Graves' disease or with simple goitre, immunoglobulins that stimulate thyroid cell growth *in vitro* are detectable. These thyroid growth immunoglobulins (TGi) may induce goitre formation in Graves' disease and simple goitre. Patients with atrophic thyroiditis have circulating antibodies that block TSH induced thyroid cell growth *in vitro* (TGI-block) (22).

Despite the fact that these antibodies are well characterized no experimental model for Graves' disease has been developed. Immunization of rabbits with bovine thyroid membranes results in the generation of antibodies to thyroglobulin and microsomal antigens much like that in humans, the equally characteristic thyrotoxicosis (74) is not

observed in animals however. The failure to develop an experimental model does not result from target insensitivity in rodents, as the transfer of human lymphocytes from patients with Graves' disease into nude mice temporarily elevates their thyroid hormone levels (75); however restricted heterogeneity — c.q. oligoclonality of thyroid stimulating immunoglobulin — might be necessary for the development and continuation of the disease (76). Immunization of animals with TSH receptors in thyroid membranes produces a polyclonal antibody response probably combining blocking and stimulating antibodies directed against TSH receptor and other membrane constituents.

1.4 AUTOIMMUNITY TO INSULIN RECEPTORS

Some patients with diabetes display an extreme form of insulin resistance associated with acanthosis nigricans which is attributable to antibodies against the insulin receptors. These patients have presented with symptomatic diabetes, resistant to extremely high doses of insulin (up to 24,000 U/day). As with other autoimmune diseases females are more frequently affected than males. Nearly all patients have acanthosis nigricans, a skin disorder characterized by pigmented thickening of the skin over the extensor surfaces of the joints, the neck, frequently extending over the trunk and face. The skin lesions may regress simultaneously with the insulin resistance. Some periods of hypoglycemia may occur. Hypoglycemia may also occur without a previous period of insulin resistance. In patients with autoimmune disease presenting with fasting hypoglycemia and clinical evidence for an insulinoma, the diagnosis of anti receptor autoimmunity should be considered (24).

The mononuclear lymphocytes and probably all cells expressing insulin receptors in these patients have a markedly decreased ability to bind insulin. This receptor defect is due to antibodies against the insulin receptor. When tested in short term cultures these anti-receptor antibodies have an insulin-like effect. This effect predominates in patients with autoimmune hypoglycemia. However, prolonged exposure of adipocytes to anti-receptor antibodies results in desensitization of the insulin receptor, a phenomenon observed in vivo in patients with the above-described diabetic syndrome. (77). The molecular basis of these effects is unknown, but the evidence suggests that they are mainly due to allosteric changes in binding site conformation rather than to direct "competitive" effects at the binding site (78). Chronic exposure to anti-insulin receptor antibodies induced receptor loss (78). Such antibodies can be detected with competitive binding assay in which ^{125}I -Insulin is allowed to react with peripheral blood monocytes or the human cell line IM-9 in the presence of a patient's serum (23). Alternatively, an immunoprecipitation method can be used to demonstrate antibodies that react with a preparation of solubilized insulin receptors purified from placental membranes (79). An animal model for this disorder is the New Zealand obese mice (NZO), related to NZB and NZW lupus prone mice, which contain besides antibodies against single and double stranded DNA also anti-insulin receptor antibodies. These

antibodies are of low titer and IgM class. Dense glomerular deposits of IgM are detectable in the kidneys of these animals (80).

1.5 AUTOIMMUNITY TO- β -ADRENERGIC RECEPTORS

Autoantibodies to β -adrenergic receptors have been identified occasionally in sera of patients with asthma and allergic rhinitis and patients with autonomic dysfunction (27). To detect these antibodies, β -adrenergic receptors isolated from lung or placental tissues are radiolabelled with a high-affinity β -adrenergic agonist (^{125}I -hydroxybenzylpindolol) and incubated with the patient's serum. Immune complexes formed in this assay are precipitated by a second antibody. In some sera antibodies were directed at or near the ligand-binding site of the β_2 -receptor (27). The presence of these antibodies is also associated with autonomic abnormalities characterized by α -adrenergic and cholinergic hypersensitivity and β -adrenergic hyposensitivity due to imbalance between the ortho- and parasympathic system. Subjects with antibodies required higher doses of isoproterenol to elevate their pulse pressure and plasma cAMP indicating β -adrenergic hyporesponsiveness. An increased α -adrenergic and cholinergic sensitivity was present as measured by the effects of the α -adrenergic agonist and the cholinergic agent carbachol phenylephrine on pupillary dilation and constriction respectively (81). The role of anti-adrenergic antibodies in the pathogenesis of asthma is less clear. A β -adrenergic theory of atopic asthma proposes an imbalance of the autonomic control with decreased β -adrenergic sensitivity of bronchial smooth muscle, mucous glands and mucosal vessels (82). An autoantibody induced β -receptor defect may be an intrinsic feature of asthma. No experimental model exists.

1.6 AUTOIMMUNITY TO PARATHYROID HORMONE RECEPTORS

In uremic patients with secondary hyperparathyroidism a high frequency of autoantibodies can be detected which block the binding of parathormone to its receptor (25). The inhibition of parathormone binding by these antibodies is accompanied by functional alterations of the parathormone receptors detected as an impaired stimulation of cAMP formation by parathormone in kidney tubular membranes. The biological significance of these antibodies is ill defined; however, blocking of the parathormone receptor might increase the circulating levels of parathormone and its metabolites. Accumulation of these long-lived peptides might be responsible for abnormalities like hyperlipidaemia, impaired motor-nerve conduction and anemia, which have been said to be due to a uraemic toxin but may be due to high levels of non-aminoterminal containing parathormone metabolites (25).

1.7 AUTOIMMUNITY TO FOLLICLE-STIMULATING HORMONE RECEPTORS

Two patients with hypergonadotropic amenorrhea and myasthenia gravis had cir-

culating antibodies to the follicle stimulating hormone receptor (FSH) (28). The diagnosis of gonadotropin-resistant ovary syndrome was made on the basis of high serum gonadotropin levels and the presence of numerous cortical primordial follicles in an ovarian biopsy. Antibodies against the FSH receptor were demonstrable in a radioreceptor assay. In this assay the patient's immunoglobulin G competed with ^{125}I -FSH for its receptor on rat testes homogenate. In vivo these antibodies probably act by blocking FSH binding to its receptor or by down-regulating the number of receptors (28).

1.8 AUTOIMMUNITY TO TRANSFERRIN RECEPTORS

A patient with a severe acquired iron-deficiency anemia had circulating antibodies against the transferrin receptor (30). The diagnosis was made on the basis of a microcytic anemia, increased serum iron levels and normal transferrin concentration. The bone marrow had no stainable iron. Erythroid precursors could be stained by fluorescent mouse anti human IgM. Antibodies against the transferrin receptor were demonstrated by these assays. The serum contained an antibody that reduced the uptake of radiolabeled iron by erythroleukemia cells *in vitro*. The serum also inhibited the binding of a fluorescent labeled monoclonal antibody against transferrin receptor. An IgM fraction of the patient's serum immunoprecipitated the human transferrin receptor. This autoantibody was capable of down-regulating the number of transferrin receptors. A complete clinical and hematological remission occurred after treatment with azathioprine and prednisone.

1.9 AUTOIMMUNITY TO OTHER RECEPTORS

Several endocrine diseases might be induced by antireceptor antibodies. Doniach et al. have found antibodies of the surface of gastric parietal cells in sera of patients with pernicious anemia. These antibody inhibit the production of HCl by parietal cells (26). These inhibitory effects on parietal cells could be responsible for the absence of intrinsic factor secretion required for vitamin B12 resorption. In patients with pseudohypoparathyroidism in which features of hypoparathyroidism exist with normal or raised levels of parathormone a circulating factor has been identified which inhibits the biological action of parathormone in a cytochemical bioassay system. These inhibitory factors might also be antibodies which block the renal receptor for parathormone or might be responsible for the deficient receptor-cyclase coupling activity in these patients (83).

Patients with immunodeficiency, such as acquired agammaglobulinemia could also have anti-antibodies against B cell receptors. Experimentally a total ablation of B cells resulting in agammaglobulinemia can be induced by the administration of antisera to the μ chain of IgM to newborn animals (84). Depending upon the heavy chain specificity of these antibodies selective immunoglobulin deficiency could occur. Naturally oc-

curing anti-idiotypic antibodies are present in immune complexes in patients with IgA deficiency (85).

1.10 CONCEPTS ON AUTOIMMUNITY APPLIED TO ANTI-RECEPTOR DISEASES

Autoimmunity *sensu strictu* defines the termination of unresponsiveness to self antigens. This can be induced in two ways: by the loss of specific unresponsiveness (tolerance) or by the development of a primary immune response to an autoantigen. The nature of this autoantigen i.e. soluble or membrane associated antigens and its concentration in the microenvironment, may determine largely which mechanism is operational (86). Induction of B cell tolerance requires a 100-1000 fold higher concentration of an antigen compared to the concentration necessary to induce T cell tolerance. Some autoantigens e.g. albumin which circulates in large quantities in the extracellular fluid induce both T and B cell tolerance. Thyroglobulin, an autoantigen which is present in small concentrations in the extra-cellular fluid (87,88) induces no B cell tolerance and a partial T cell tolerance. Acetylcholine receptors are probably not circulating in the extracellular fluid as freely circulating glycoproteins since they are internalized after they have been incorporated in the lipid bilayer of the cell membrane (89). Therefore tolerance against these membrane receptors probably does not exist and autoimmunity against these cell membrane components is the result of a primary immune reaction against these receptors. In chapter III we will present evidence for the absence of tolerance against self AChRs.

Another important question is how a primary immune response against cell membrane receptor, specifically AChR, starts. Several hypotheses have been put forward.

Hypothesis A: The Thymus.

Wekerle et al. proposed that the primary sensitization against AChR occurs in the thymus (90). They cultured thymic epithelial cells expressing AChR in culture. These cells differentiated into myoid cells and might sensitize T cells. The thymus could participate in the pathogenesis of the disease in two genetically controlled steps. A first set of genes would control the susceptibility to signals inducing myogenesis and the expression of AChR. In murine models this hypothesis has been confirmed (90). A second set of genes probably encoded within the major histocompatibility locus would control the level of responsiveness against self-AChR in the thymus. The thymus is also the site where T cells acquire the capacity to recognize the self-MHC determinants (91). This model proposed by H. Wekerle may have clinical implications. Indeed, a particular feature of MG are the thymic abnormalities e.g. follicular hyperplasia (germinal centres) and thymoma. Germinal centres occur in 47-81% of these MG patients in whom thymectomy is performed (reviewed in 92). Germinal centres are sites of B-memory cells and may develop in sites of chronic antigenic stimulation. It is tempting to speculate that the primary sensitization against AChR occurs in the thymus since this

organ contains both the autoantigen — expressed on thymic epithelial (93) and thymic muscle cells (94) — and the antigen reactive cells. These speculations are substantiated by the isolation of two populations of lymphocytes from the MG thymus. The first population contains B cells that spontaneously secrete anti-AChR antibodies (95) and the second population contains a T helper cell subset that enhances the anti-AChR autoantibody production of peripheral blood lymphocytes of MG patients (96). These thymic abnormalities are also observed in other autoimmune diseases including thyrotoxicosis, rheumatoid arthritis and systemic lupus erythematosus (reviewed in 92). A second thymic abnormality associated with MG are epithelioid tumors of the thymus (reviewed in 92). The immunopathological mechanisms that relate these thymic tumors called thymomas to MG is not entirely clear. Thymic epithelial cells secrete a number of immunological active factors such as "facteur thymique serique" which might stimulate the immune system to produce autoantibodies to AChR. The specificity of the immune reaction to AChR might be related to the presence of the autoantigen within the thymus. Other diseases including pure red cell anaemia and hypogammaglobulinemia are associated with thymomas. Other immunological abnormalities reported in thymoma patients are the presence of antibodies to striated muscle and antinuclear antibodies (98).

Hypothesis B: MHC.

A second hypothesis applicable to autoimmune receptor disease is that of an aberrant expression of MHC class II (Ia-like) antigens on cell membrane of non-immune cells. T cells interact with antigens when presented together with Ia antigens. An interesting finding in this respect was the demonstration of HLA-DR antigens (Ia antigen) on thyroid epithelial cells of patients with Graves' disease, an other typical antireceptor autoimmune disease (99). This aberrant expression of HLA-DR antigens on epithelial cells could enable these cells to present autoantigens to non-tolerant T and B cells. The expression of Ia antigens could be induced by interferons or other local factors (100). This hypothesis links autoimmunity to viral infection, a relation that was frequently suspected but difficult to prove.

Hypothesis C: Idiotypic mimicry.

A third mechanism by which autoantibodies would be induced is by idiotype mimicry of autoantigen in the absence of antigen (fig. 1). Anti-idiotypic antibodies directed at anti-hormone antibodies can mimic hormone action by acting an internal image of the site reactive with the receptor for that hormone. Anti-hormone antibodies (ab 1) elicited spontaneously or by active immunization of experimental animals, induce anti-idiotypic antibodies (ab 2) which in turn induce anti-anti-idiotypic antibodies (ab 3) (fig. 1). The antibody combining site of ab 2 is the internal image of the hormone and may therefore mimic the hormone action on the receptor. Antibody 3 resembles antibody 1 and is able to bind to the hormone. The "internal image" hypothesis has been tested in several systems. Anti-idiotypic antibodies against insulin antibodies interact

Fig. 1 Interacting components of an idiotypic — anti-idiotypic network involving hormones (H), receptors (R) and antibodies (ab₁, ab₂, ab₃). Antibodies to the hormone (ab₁) induce anti-idiotypic antibodies which display binding sites similar to the hormone. Anti-anti-idiotypic antibodies (ab₃) direct against ab₂ will be able to bind to the hormone.

with insulin receptors and mimic insulin action. Similarly anti-idiotypic antibodies against-adrenergic ligands, thyrotropin, retinol-binding proteins, formyl peptide chemoattractant interact with the receptors for their respective ligands (reviewed in 101). This system is also relevant for autoimmune diseases. Anti-idiotypic antibodies prepared against the acetylcholine analogue Bis Q, interact with rat and Torpedo AChR (102, 103). In vivo, these anti-idiotypic antibodies block the AChR and cause myasthenia gravis. Some authors have criticized this system because of its artificial nature. Anti-hormone antibodies, however, occur spontaneously. A larger number of patients with autoimmune thyroiditis have anti-thyroid hormone antibodies (104).

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CHAPTER II

The experimental autoimmune myasthenia gravis animal model.

2.1 INTRODUCTION

J. Simpson, a neurologist, suggested that myasthenia gravis was caused by autoantibodies directed against proteins at postsynaptic sites (1). His idea was picked up by E. Lennox who passed Simpson's paper on to his graduate student, the biochemist, J. Lindstrom (2). Lindstrom and J. Patrick succeeded in identifying AChR biochemically but not by means of using human anti AChR antibodies although this was implicit in Simpson's hypothesis.

Progress in AChR isolation was made possible by two observations. First, the electric organs of the electric eel (*Electrophorus electricus*) and electric ray (*Torpedo Californica*) were found to be rich sources of AChR (3); second, some snake venom toxins with curare-like activity were shown to bind specifically to AChR (4).

Using these observations Patrick and Lindstrom purified AChR from solubilized electric organs on snake-toxin-agarose columns. AChRs were eluted from the columns by means of an acetylcholine antagonist. In order to test if this purified receptor contained the physiologically active AChR they decided to immunize rabbits with this AChR preparation. The immunized animals became paralyzed and died. This observation brought Simpson's theory that MG was an autoimmune disease, back to their mind (2).

2.2 THE AUTOANTIGEN: The acetylcholine receptor (AChR)

The study of organ specific autoimmune diseases necessitates a detailed study of autoantigens and their role in the induction and maintenance of the disease. The regulatory role of autoantigens in autoimmune disease had been demonstrated by I. Roitt and coworkers who showed that neonatal removal of an autoantigen such as thyroglobulin prevented the production of autoantibodies to thyroglobulin in the obese chicken strain (5).

The detailed structure of the nicotinic AChR receptors has been elucidated by combined efforts of biochemists, using recombinant DNA technology and immunologists using monoclonal antibodies. Acetylcholine receptors from the electric organ of *Torpedo Californica* are composed of four subunits associated in the molar ratio: 2- α , β , γ and δ . The apparent molecular weights of these subunits are 38,000, 49,000, 57,000 and 64,000 for α , β , γ and δ subunits respectively (6,7). All four subunits are strongly associated by hydrophobic bonds and compose a single AChR molecule with a central ion channel. This channel opens upon interaction of an acetylcholine molecule with each α -subunit.

2.2.1 Biochemistry of acetylcholine receptors

The AChR is a glycoprotein that spans the cell membrane. It can be isolated easily from the electric organs of electric fish (*Torpedo californica*, and other *Torpedo* species and *Electrophorus electricus*), mammalian and human muscle (8). The critical observation which allowed receptor purification was the discovery that α -bungarotoxin (α -BGT) present in venoms of cobras and kraits (*Naja Naja siamensis*) toxin binds with high affinity and specificity to the acetylcholine binding site or a closely adjacent site (4). Solubilized extracts of electric organs were passed over a column containing Cobra toxin linked to agarose beads. After washing away all contaminants, AChR was eluted with acetylcholine analogues such as carbamylcholine or benzoquinonium (9, 10).

The amino acid sequence of AChRs purified from electric organs of rays and eels, has been determined by partial amino-terminal amino acid sequencing (13) and by recombinant DNA technology (13, 14). There is a 35-50% amino acid sequence homology between the four subunits, indicating that the 4 subunits probably are derived by reduplication from one ancestral gene (13). This homology is only present at the amino-terminal two-thirds, whereas the carboxy terminal one-third is rather divergent (11-13). Most of this carboxy terminal part is embedded in the cell membrane or expressed at its cytoplasmic side. Each subunit is glycosylated presumably on its extracellular surface and contains small amounts of galactose, mannose, glucose and galactosamine (14, 15). These carbohydrates may play a role in folding newly synthesized peptides to form a conformation compatible with ion channel function (16).

Acetylcholine receptors have also been purified from mammalian muscle including fetal bovine AChR (17,18) and human AChR (19). Bovine and human AChR are structurally similar to those of *Torpedo* and eel electric organ. All four receptors when labeled with ^{125}I - α -BGT sediment at 9S in linear sucrose gradients and have a MW of approximately 250.000 (17). AChR from *Torpedo* also exists in dimers (20). Among the α -subunits of fetal bovine, eel and *torpedo* AChR a homology of 51% and 38% exists respectively (21).

2.2.2 Acetylcholine receptor function

The function of AChRs present at the neuromuscular junctions of striated muscle is to amplify the small current required for propagating an action potential in a myelinated nerve to the larger current necessary for the contraction of a muscle fiber. Upon binding of two acetylcholine molecules released in quanta of 10^4 molecule from the nerve terminal to each α -subunit, the ion channel opens for about 1 msec. at a conductance of about 30 pico Siemens, which corresponds to the transport of about 50.000 monovalent cations (22). Most of the acetylcholine molecules released are inactivated by acetylcholine esterase, an enzyme attached to the basement membrane located between nerve terminal (presynaptic membrane) and the muscle membrane (postsynaptic membrane) (23). Whereas activation is a rapid process, recovery from desensitization

is a slow process, requiring minutes.

When the nerve is stimulated about 60 quanta ($= 60 \times 10^4$ ACh molecules) of acetylcholine are released at the neuromuscular endplate of human intercostal muscle (24). Normally the amount of acetylcholine released and the number of AChR activated is much larger than the minimum amount necessary for a muscle fibre contraction (23). This excess of acetylcholine released provides a safety factor, which differs among species. Rabbits and rats are very susceptible to the induction of EAMG whereas mice which have a larger safety factor are more resistant to the disease.

2.2.3 Synthesis and destruction

AChRs are synthesized in the rough endoplasmic reticulum of the muscle cells, using four separate m-RNA's for each receptor subunit. The AChR is "secreted" through the Golgi apparatus and inserted in the plasmamembrane (25). Glycosylation is required for maturation of subunit structure (16, 26). Further maturation is necessary before the ligand binding site is operational.

AChRs at the neuromuscular junction are removed by endocytosis. Their half life is 5-7 days, in contrast to AChR present extrajunctionally which are removed more rapidly ($t_{1/2} = 20$ hours). Cross-linking of AChR by autoantibodies, which occurs in EAMG and MG facilitates internalization with lysosomal breakdown which takes about 90 minutes (27).

2.2.4 Antigenic anatomy of Acetylcholine receptor

Before monoclonal antibody technology was applied to acetylcholine receptors little was known about the antigenic structure of AChR from electric fish. It was found to be an extremely potent antigen since an injection of as little as $1 \mu\text{g}$ electric organ AChR incorporated in complete Freund's adjuvant (CFA) was able to induce an antibody response. Separate subunits, dissociated in sodium dodecylsulfate (SDS) are poorly immunogenic, but prolonged immunization yields antisera specific for each receptor subunit inspite of the extensive sequence homology. (Some degree of cross-reaction is detectable especially between γ and δ -subunits) (29). This suggests that most of the antigenic determinants are dependent upon the three dimensional structure of these determinants. This is also true for the main immunogenic region, a small group of determinants which were discovered with monoclonal antibodies (30). More than 50% of the antibodies in sera from patients with myasthenia gravis (31) and from animals with EAMG are directed at this region. Many other sites on AChR are immunogenic but antiAChR antibodies that cross react with AChR from other species are mainly directed at the main immunogenic region (31). This determinant consists of a continuous sequence of amino acids localized on the extracellular surface of the α -subunit but distinct from the acetylcholine binding site (32). The MIRs on α -subunits are oriented in such a way that an antibody bound to one α -subunit cannot bind to another

α -subunit on the same receptor. Thus monoclonal antibodies to the MIR can only cross link AChR molecules (38). Cross linking of AChR induces an increase in AChR turnover, a phenomenon called antigenic modulation which is probably one of the most important immunopathological mechanisms in MG and EAMG (*vide infra*). Antibodies to β , γ or δ -subunits can only cross link to receptor molecules, since only one antigenic site is present on each AChR monomer. Monoclonal antibodies to the MIR can passively transfer EAMG whereas only few antibodies with other specificities are myasthenogenic (34). Some monoclonal antibodies against the acetylcholine binding site induce EAMG when passively transferred into normal animals (35). This acetylcholine binding site is a very small antigenic site (36) and since the ligand is phylogenetically highly conserved, the binding site must be too. For these reasons it is probably not very immunogenic and therefore not very important in the pathogenesis of EAMG.

2.3 INDUCTION OF EXPERIMENTAL AUTOIMMUNE MYASTHENIA GRAVIS

Experimental autoimmune myasthenia gravis (EAMG) can be induced in mammals, birds and reptiles by the injection of purified acetylcholine receptors from electric organs incorporated in complete Freund's adjuvant (CFA) and pertussis vaccines (reviewed in 37). The animal species tested include rabbit, rat, guinea pig, goat, monkey, frogs and hens. Historically the rabbit was the first animal species used to induce EAMG (38, 39). With few exceptions rabbits developed flaccid paralysis one week after the second injection of 100 μ g Torpedo AChR given at four weeks' interval. The affected animals are unable to support their bodies, have difficulties in breathing and swallowing and usually die within 1 or 2 days from respiratory insufficiency. Anticholinesterase treatment (37 μ g/kg) produces a marked but temporary improvement (37).

Clinical symptoms and severity of disease varies widely among the species used. Monkeys developed signs characteristic of human disease including eye lid ptosis, dysphagia, facial weakness and ophthalmoplegia (40). Except for the human-like clinical signs the monkey model for EAMG is not more relevant than the rodent models for the study of the mechanisms operational in EAMG and MG and should therefore not be used for ethical reasons. We will therefore concentrate on studies using rodents including rats and mice. Rats in contrast to mice are very susceptible to clinical signs of EAMG because rats probably have a lower neuromuscular safety factor than mice. The susceptibility to EAMG in inbred and genetically defined strains of mice is linked to the immune response genes on the major histocompatibility gene (H-2) and the genes coding for the allotype (IgCh locus)* (41, 42).

* Allotypes: aside from structural heterogeneity in the heavy chains associated with a given subclass there is a further variation within a given subclass which is called allotype. This usually involves one or two aminoacids in the peptide chain. Allotypes can be used as a genetic marker.

2.3.1 Experimental autoimmune myasthenia gravis in rats

EAMG can be induced in Lewis rats by injection of AChR purified from electric organs of the electric eel (27), Torpedo, or its denaturized subunits (28), fetal calf muscle (17), and syngeneic rat AChR (43). Immunization with two injections of 15 μ g Torpedo AChR incorporated into complete or incomplete Freund's adjuvant given at a one month interval invariably induces EAMG in all Lewis rats tested. Other strains of rats including Brown Norway, Louvain and Sprague Dawley are equally susceptible to EAMG. A single report mentioned that Wistar rats were resistant to the disease (44). This seems unlikely, since susceptible Louvain rats are also of Wistar origin.

Lewis rats immunized with additional adjuvants such as *Bordetella pertussis* (10^{10} organisms of special vaccine product of Eli Lilly & Co) develop a biphasic illness (27). An acute, transient illness characterized by a temporary, sometimes fatal paralysis develops 7 to 10 days after immunization. About one month after the first (of two) immunizations with AChR a chronic disease develops. EAMG can be passively transferred from these animals into normal rats with small amounts of serum (200 μ l of a serum with a titer of 5×10^{-8} M of anti rat AChR or 1 μ g of antibody) (45).

The clinical signs of EAMG are a consequence of weakness of upper limb, head and neck, and laryngeal and respiratory muscles. The animal lays his head and chest on the floor, the back is exaggeratedly humped with the thighs partially abducted. The digits of the forelimbs are flexed and fail to extend when the rat is placed on a flat surface (fig. 1A). These symptoms can be temporarily reversed by treatment of anticholinesterase drug (Prostigmine 35 μ g./kg) (fig. 1A en 1B). Excessive growth of the teeth suggests impairment of masticatory muscles (fig. 1B). At the end stage of the disease chronic respiratory disease becomes manifest as loud expiratory wheeze and brown porphyrin staining of nasal secretion and tears. Animals with chronic respiratory disease also have a brown discoloration of the neck that is caused by wiping off their paws (27). The rats finally die from malnutrition. The endstage of disease can be delayed by trimming their teeth and placing soft food within easy reach of the animals.

2.3.2. Experimental autoimmune myasthenia gravis in mice

Mice like rats, develop EAMG upon injection with AChR from electric organ. One to two weeks after a second injection of 15 μ g AChR in CFA mice develop muscular weakness. In order to distinguish myasthenic from non-myasthenic animals behavioral and pharmacological tests are necessary. These tests include forced exercise (e.g. forced run, inverted hang, free swim) and injection of low doses d-tubocurarine which induce paralysis in subclinical myasthenia (41).

The susceptibility to EAMG in mice is genetically determined. In some strains such as C57Bl/6, SJL and AKR 50-70% of the mice develop EAMG; while in Balb/cke, SWR/J and C3H/HC only 3-15% of the mice become paralyzed. These differences in

Fig. 1A: Posture of a myasthenic rat. The animal lays his head and chest on the floor, the back is exaggeratedly humped with the thighs partially abducted.

Fig. 1B: Close-up of the head of a myasthenic rat. Note the excessive growth of the teeth due to impairment of the masticatory muscles.

susceptibility can be explained in two ways. Strain dependent differences at the neuromuscular junction might render the highly susceptible strain more sensitive to the neuromuscular blockade. However, no significant difference in sensitivity to d-tubocurarine, a competitive antagonist of acetylcholine, between high and low response strains was demonstrable. It is therefore more likely that the degree of the immune response to AChR determines the susceptibility to the disease (41).

2.4 HUMORAL IMMUNE RESPONSE TO ACETYLCHOLINE RECEPTORS

Immunization of animals with Torpedo AChR results in an antibody response against the immunogen. These antibodies can be measured by means of a double antibody immunoprecipitation assay using ^{125}I - α BT-labeled Torpedo AChR (46). Antibody titers are expressed as moles α -BGT-binding sites precipitated per liter of serum (see material and methods section). The presence of α -BGT bound to AChR does not markedly interfere with the precipitation of AChR, since antibodies that are directed against the ligand-binding site are only a small portion of the total (37). However, when unlabelled AChR is preincubated with an excess of antibody, the subsequent binding of α -BGT or acetylcholine is inhibited by 50% or more (47). Since this blockade occurs only at high antibody concentration this means that only a small portion of the total anti-AChR antibodies are directed against the ligand binding site or very close to that site (47). Another possibility is that anti-AChR antibodies change the conformation of AChR so that it can no longer bind the ligand (44).

2.4.1 Specificity of the antibodies

The humoral immune reaction against AChR from electric organs is quite species specific. A small fraction (1-5%) of these anti-Torpedo antibodies cross-react with AChRs in the rat's own muscles (46). Most of these cross-reactive antibodies are directed to the main immunogenic region (29). This population of autoantibodies is thought to be responsible for the disease. The time course of the two populations of antibodies and their interrelationship will be discussed in chapters IV and V. These antibodies cross-reacting with muscle AChR can be extracted from muscles of rats with chronic EAMG (43). Autoantibodies against muscle AChR occupy 10-90% of AChR at the neuromuscular endplate depending upon the severity of disease (see Chapter IV). When rats are immunized with syngeneic AChR, anti-AChR antibodies show extensive cross-reaction (5-80%) with AChR from both muscle (even human AChR) and electric organs (43, 48). In contrast sera from MG patients do not show a high percentage of cross-reaction with rat or electric organ AChR. It may be that some of the determinants shared by human AChR and rat AChR, like those shared with eel or Torpedo AChR, do not provoke a strong humoral response in humans (48). It is still controversial if the rats' own AChR contribute to the autoimmune response in EAMG. This question will be addressed in Chapter V.

2.4.2 Mechanisms of humoral immune attack

Electrophysiological studies in rats with chronic EAMG revealed that the amplitude of the first evoked compound muscle action potential is normal. In rats with signs of clinical weakness, a decremental electromyographic response is seen at low frequencies of stimulation. There is a posttetanic facilitation followed by exhaustion. The amplitude of the mini-endplate potential, produced by the spontaneous release of single quanta of acetylcholine at the rate of about 1 per second, is decreased (49). These electrophysiological abnormalities are provoked by several immunopathological mechanisms: complement mediated focal lysis, an increased turnover of AChR molecules and by immunopharmacological blockade of the acetylcholine binding sites.

a) Complement mediated lysis of the muscular cell membrane.

Immunoglobulin G and complement (C3 and C9) have been demonstrated by immunoelectron microscopy to be present on the terminal expansions of the junctional folds of the postsynaptic membrane in rats with chronic EAMG (50-53). The distribution of IgG and complement is similar to that of acetylcholine receptors localized by peroxidase labeled α -BGT. These findings and especially the demonstration of the final lytic component C9 on the postsynaptic folds of myasthenic muscle strongly supports an immune mediated destruction of the muscular membrane (53).

Complement can induce membrane destruction and cell death through the formation of the lytic membrane attack complex, a polymer of C9 molecules which spans the cell membrane and induces leaks through its central channel (54). The demonstration of C9 at the neuromuscular endplate is concordant with this mechanism of complement mediated membrane lysis. The activation of the complement cascade also generates anaphylatoxins (C3a and C5a) which attract polymorphonuclear leucocytes and macrophages which have receptors for those substances (55). These cells are not present in chronic EAMG (27) and MG, but are readily demonstrable in EAMG induced by passive transfer of anti-AChR from EAMG animals. Depletion of C3 with cobra venom factor prevents the clinical signs, the AChR deficiency and the infiltration with inflammatory cells after passive transfer of anti-AChR antibody (56). C4 deficient guinea pigs are also resistant to EAMG induced by passive transfer of monoclonal antibody (56). The failure to induce acute EAMG by passive transfer of anti-AChR in complement depleted rats or C4 deficient guinea pigs proves that complement plays a central role in vivo. As to the role of complement in chronic EAMG, it would be interesting to try to induce chronic EAMG by injection of AChR in CFA into C4 deficient guinea pigs, since prolonged depletion of complement by Cobra venom factor is not possible. This experiment would also clarify the role of complement in conjunction with a second immunopathological mechanism, antigenic modulation of AChR.

b) Antigenic modulation of AChR

Anti-AChR antibody increases the degradation of AChR on cultured muscle cells *in*

vitro (57, 58). This phenomenon, called antigenic modulation, results in a decrease in the number of AChRs on the muscle cell membrane and a decreased effect of available AChR to acetylcholine. Antigenic modulation can be measured *in vitro* using ^{125}I - α -BGT labeled cultured muscle cells. Addition of anti-AChR antibodies to the tissue culture induces a release of ^{125}I -tyrosine in the medium. Since ^{125}I - α -BGT irreversibly binds to AChRs, the release of ^{125}I -tyrosine from degraded α -BGT parallels AChR destruction by lysosomal enzymes. This energy dependent process is inhibited by low temperature, DNP, NaF and by inhibitors of cytoskeleton function (58). The effect of anti-AChR antisera does not require complement but depends on the divalent structure of immunoglobulin G. Univalent Fab anti-AChR fragments are ineffective, but cross-linking of AChRs on muscle cells by intact anti-AChR antibodies or Fab fragments and anti IgG induces a twofold degradation of AChR (57). Muscle cells in tissue culture display no compensatory increase of AChR synthesis, since AChR synthesis is probably already maximal. In muscle from rats with EAMG degradation of AChR occurs at approximately the same rate as when diaphragms are exposed to anti-AChR antibodies *in vitro*. It is not known whether in animals with EAMG there is a compensatory increase of AChR synthesis. It also is not known if groups of muscle fibers with increased AChR synthesis are less susceptible to the modulating effect *in vivo*.

c) Direct block of AChR function by antibody:

Conceptually, antibodies to the acetylcholine binding site should be very strong inhibitors of AChR function. Antibodies against AChR of animals with EAMG directly affect AChR function when applied to electric organ cells or muscle (59-63). Agonist induced depolarisation of eel electric organ cells is blocked by preincubation with an excess of anti-AChR antibody. Although all AChRs are bound with antibodies the acetylcholine binding site can still be labelled with ^{125}I - α -BGT and no decrease in AChR numbers can be found (64). This suggests that antibodies bound have an allosteric effect on AChR function.

The analysis of immunopharmacological blockade of AChR can best be studied by means of monoclonal antibodies which by virtue of their restricted specificity bind only to one antigenic determinant on AChR. An antigenic determinant consists of 4-6 amino-acids in a linear sequence or in three dimensional configuration. The acetylcholine binding site is, in view of the small molecular weight of acetylcholine a minute phylogenetically conserved site, probably not larger than the size of one antigenic determinant. It is therefore not surprising that most polyclonal and monoclonal antibodies bind to AChR outside this small acetylcholine binding site; antibodies that bind outside the ligand binding site can however influence AChR function as measured by altered ion channel opening time and conductance (33). The dissection of the immune response of antibodies against AChR was performed by means of monoclonal antibodies. Twenty five MABs were tested for their ability to block agonist induced $^{22}\text{Na}^+$ influx into unilamellar liposomes containing reconstituted AChR. Only two monoclonal antibodies blocked AChR function without interfering with the ligand bin-

dging site. These 2 MABs could act by interfering with the propagation of the ligand signal to the ion channel or by directly blocking the function of the ion channel. Very few monoclonal antibodies directed against the acetylcholine binding site have been isolated. These MABs compete with agonists (e.g. carbamylcholine and acetylcholine) or antagonists (α -bungarotoxin, d-tubocurarine, decamethonium) (36). Passive transfer of such a MAB induces acute EAMG within one hour after injection (34). Despite the clear pathogenicity of antibodies to this ligand-binding site, there is little evidence that they play an important pathological role in MG and EAMG. First, such antibodies are only a minor fraction of the total, which explains why only few MABs affecting AChR function have been isolated. A library of monoclonal antibodies may be considered representative for the spectrum of antibodies in polyclonal immune serum. Second, the concentration of anti-site antibodies does not correlate closely with severity of disease (65).

In summary, three mechanisms are responsible for the neuro-muscular dysfunction in EAMG, i.e. complement mediated focal muscular membrane lysis, AChR modulation, and blocking of the ligand binding site. The first mechanism is the most important because in C3 depleted rats no acute disease occurs, although 60% of the AChRs are occupied by antibody (56). In the absence of the lytic action of complement no increased AChR turnover (i.e. antigenic modulation) occurs. Thus, the intact postsynaptic membrane is not susceptible to antibody induced AChR turnover. In contrast, AChRs on cultured muscle cells are susceptible to antibody induced antigenic modulation in the absence of complement. It is likely therefore that the lytic cascade of complement factors is necessary to remove the cytoskeletal restraints of the postsynaptic membrane (56).

2.5 THE CELLULAR IMMUNE RESPONSE TO ACETYLCHOLINE RECEPTORS

T lymphocytes can be categorized into regulatory cells and effector cells. Regulatory T cells can enhance (helper T cells) or suppress (suppressor T cells) the activity of effector T cells or B cells. The function of effector T cells is either killing target cells (cytotoxic T lymphocytes) or mediating the delayed type hypersensitivity reaction. To be activated by an antigen, these T lymphocytes must recognize the antigen together with its own histocompatibility antigen.

From the preceding section one might conclude prematurely that EAMG is an antibody mediated disease. However, several T cell mediated immune reactions can be observed. Positive skin test, typical of a delayed type hypersensitivity (DTH) reaction to AChR were present in rats 3 days after immunization with eel AChR in CFA and Bordetella pertussis. This DTH reaction subsides before the animals develop chronic EAMG, suggesting that cells mediating DTH are not necessarily important effector cells in this model (66). In search for cytotoxic T cells, S. Fuchs and her colleagues found that macrophages armed with anti-AChR antibodies were cytotoxic for cultured myocytes.

The lymphocytes isolated from animals with EAMG were not cytotoxic for muscle cells (67). These findings agree with the presence of macrophages at the neuromuscular endplate of rats with acute EAMG. However, endplates of animals with chronic EAMG show no cellular infiltrate (50, 66). The detection of cytotoxic T effector cells will be difficult since muscle cells are very difficult to lyse, on account of a strong cytoskeleton. In view of this property of muscle cells it will be difficult to design assay systems for cytotoxic T lymphocytes in EAMG.

Since EAMG can be transferred by antibody alone, more attention was given to the role of regulatory T cells in EAMG. The contribution of defined lymphocyte populations to the pathogenesis of EAMG has been tested by passive transfer experiments. Transfer of EAMG to normal rats by immune lymph node cells was possible, although a very high number of cells (6×10^8) was required no antibody to rat muscle receptor was detectable (66). It was not possible to induce EAMG in adult rats which were depleted of T cells by thymectomy and X-irradiation and reconstituted with purified B cells. This suggests that T cells are involved in EAMG as helpers for the B cells in their production of antibody to AChR (66).

A new field opened upon the advent of *in vitro* techniques for the assessment of the relative contribution of T cells and their subsets and B cells in the pathogenesis of EAMG. Most of these initial studies were performed in mice which were genetically well characterized, but are relatively resistant to the induction of EAMG. In a first attempt to determine the genetic control of EAMG, Christadoss et al. tested the proliferative response by lymph node cells to Torpedo AChR. Studies with congenic and recombinant inbred strains of mice revealed that T cell responses during the induction phase of EAMG (day 7) to Torpedo AChR are controlled by an immune response gene, localised in the IA subregion of the major histocompatibility complex (69). These lymphocyte proliferative responses to AChR are dependent on cells expressing the T helper cell phenotype ($lyt\ 1^{+}2,3^{-}$) (70). Lymphocyte responses were eliminated by blocking Ia antigens on lymphocyte cell surface with anti-I-A antisera and by removal of adherent (antigen presenting) cells. A spontaneous mutation at the IA subregion (A_B chain) converted high responsiveness to AChR to a state of low responsiveness (71). These data imply the antigen presenting cell associated Ia molecule in induction of EAMG, probably in the presentation of AChR together with Ia antigen to helper T lymphocytes. These helper T lymphocytes help B lymphocytes to differentiate into anti-AChR antibody-forming cells (72). These *in vitro* results have also been tested *in vivo*. Indeed *in vivo* therapy with monoclonal anti-Ia antibody suppresses immune responses to acetylcholine receptor (73). The *in vivo* autoantibody responses to mouse muscle AChR were also roughly under immune response gene control. This H-2 control was not complete since in certain strains of mice (AKR/J, H-2^k) genes outside the H-2 complex were found to influence autoantibody responses to AChR (41).

As mentioned earlier if one wants to relate *in vitro* cellular responses to AChR to *in vivo* phenomena and severity of disease, the rat is a far better experimental animal. Work from Noguchi et al. indicated that lymphocytes from rats with chronic EAMG

proliferate *in vitro* when AChR was added to the culture (74). Hohlfeld et al. isolated AChR reactive lymphocytes from rats with chronic EAMG (75). Further culture with self-AChR resulted in the selection of autoreactive T cell clones. In these studies no attempt was made to relate these *in vitro* proliferation assays to *in vivo* phenomena. However, transfer of isolated anti-AChR specific T cells into unprimed rats did not induce EAMG unless AChR incorporated in CFA was injected along with AChR primed B cells (76). In summary, it is clear that T cells play an important role in EAMG most likely as T helper cells that cooperate in the antibody response to AChR.

2.6 PATHOLOGY OF THE NEUROMUSCULAR ENDPLATE IN EXPERIMENTAL AUTOIMMUNE MYASTHENIA GRAVIS

When the EAMG model was first developed by Patrick and Lindstrom several pathologists in the San Diego area analyzed the muscles of rabbits that died from EAMG, but they could not find clear cut lesions with the light microscope. Not until the rat EAMG model was developed by Lindstrom, Engel, an expert in neuropathology described in detail the ultrastructural lesions of the neuromuscular endplate in acute and chronic EAMG.

In *acute EAMG* induced by injection of AChR in CFA along with Bordetella pertussis or induced by passive transfer of antibodies against AChR a phase of acute weakness occurs. Light microscopy shows an inflammatory exudate composed predominantly of mononuclear cells with a lesser admixture of neutrophils and rare eosinophils. The inflammatory reaction is confined to the endplates as visualized by the cholinesterase enzyme histochemical reaction. The postsynaptic regions split away from the underlying muscle fibers and the junctional folds are destroyed by macrophages. Some muscle fibers undergo segmental necrosis and invasion by macrophages (50).

Electron microscopic examination shows degenerating postsynaptic folds that are splitting away from the underlying muscle fiber region. These folds are being degraded by macrophages. The nerve terminals remain intact but are separated from the muscle fibers by macrophages (50, 77). These lesions are typical for acute phase EAMG but are not observed in muscles from patients with MG.

The *chronic phase* of EAMG in the rat begins about one month after immunization with AChR in CFA. Light microscopic studies shows no abnormalities (50). Electron microscopic studies shows intense degeneration of the junctional folds (50, 77). This results in a widened primary synaptic cleft and a simplified postsynaptic region. Splitting of the synaptic folds from the muscle fibers and phagocytosis of the degenerating folds by macrophages are not seen. The nerve terminals, however, remain intact. Morphometric analysis of the postsynaptic regions reveals a 50-60% decrease in length of the postsynaptic membrane (50, 77). Since the AChR sites are located on tips of the synaptic folds this decrease in length implies a proportional greater decrease in the number of acetylcholine receptor sites.

2.7 RELEVANCE OF THE EAMG-MODEL FOR MYASTHENIA GRAVIS

Although Simpson's idea that myasthenia gravis was caused by autoantibodies to the acetylcholine receptor was innovating, his hypothesis was not really believed by other neurologists until J. Patrick and J. Lindstrom were able to induce anti-AChR antibodies and a myasthenic syndrome in rabbits. Experimental autoimmune myasthenia gravis was also induced in several other species, but most of the studies have been done in rats and mice. The most important clinical features of EAMG and MG is muscular weakness which increases after repeated use of certain muscle groups. Only monkeys develop signs comparable to those of human disease (40). In all animal species with EAMG, like for MG, a clear temporary improvement occurs after administration of anti-cholinesterase drugs. This treatment improves the neuromuscular blockade assessed by electromyography with repetitive stimulation. Neostigmine treatment increases the miniature endplate potential in both EAMG and MG.

Besides the electrophysiological similarities between EAMG and MG this experimental model is very useful to analyse the immunological mechanisms operational in MG. First of all, the technology developed during the isolation of Torpedo AChRs made it possible to assay antibodies against AChRs in MG (77). AChRs present in a crude extract of human muscle specifically labeled with ^{125}I - α -BGT are used as antigen. This assay has a very high sensitivity and is highly specific for MG. Using this assay Lindstrom et al. and several other investigators showed that most MG patients have antibodies whereas patients with other neurological diseases lack anti-AChR antibodies (79-22). The only exceptions are some thymoma patients, 3% of RA patients treated with d-penicillamine without MG and some asymptomatic relatives of MG patients (reviewed in ref. 83). Passive transfer of such antibodies from MG patients to mice induces acute EAMG (84). Neonatal MG, the passive transfer experiment of nature, occurs in some babies born from myasthenic mothers (85). EAMG occurs also in neonatal rats born of and nursed by EAMG mothers (86). In the sera of myasthenic pups anti-AChR antibodies can be demonstrated. In the rat most perinatal immunity is conferred via IgG passage to newborns by means of milk from the mother. Therefore neonatal EAMG occurs only in rats nursed by EAMG rats (86). In patients with MG who gave birth to a baby showing neonatal myasthenia, anti-AChR antibodies have been demonstrated in the sera from the infants but not in the mother's milk. Thus although the neonatal form of EAMG in rats appears to be similar to its human counterpart the pathogenesis differs.

The mechanisms by which anti-AChR antibodies induced myasthenia in MG and EAMG are similar. In both chronic EAMG and MG, C3 is bound to the postsynaptic membrane. The presence of the lytic component (C₉) of complement at the neuromuscular endplate provides evidence for antibody mediated, complement-mediated injury of the postsynaptic region in MG (53).

Antigenic modulation of AChR is a second mechanism by which anti-AChR antibodies cause a neuromuscular blockade in MG and EAMG. Addition of anti-AChR

antibodies to rat or human cells in culture increases the rate of AChR destruction. Cross-linking of AChRs by anti-AChR antibodies is a necessary requirement (87, 89). A third mechanism of anti-AChR induced AChR dysfunction is a direct block of AChR function by antibodies directed at the acetylcholine binding site on a closely adjacent site. The cholinergic binding site may be a major antigenic determinant in a few patients (90, 91) but in many sera the anti- α -BGT binding site antibody is only a small portion of the total anti-AChR antibody (92). Most of the antibodies in MG sera are directed at the main immunogenic region (MIR). These determinants are oriented in such a way that an antibody bound to one α -subunit cannot bind to another α -subunit on the same receptor. Antibodies to the MIR are probably very efficient in antigenic modulation and thus increase AChR degradation (32).

These mechanisms are probably responsible for the morphological and functional changes found in MG. The immunopathological lesions are similar as those in chronic EAMG and include mostly postsynaptic changes (51).

Beside humoral, cellular immunity against AChRs is present in MG and EAMG. Peripheral blood lymphocytes (PBL) of MG patients or lymph node cells of animals with EAMG proliferate in the presence of AChRs *in vitro* (93-97). These proliferating

Table I: Comparison between chronic EAMG and MG^a

	EAMG	MG
A. Clinical		
1) weakness	+	+
2) fatigability	+	+
3) eye lid ptosis	+ / -	+
B. Electrophysiological		
1) decremental electromyogram	+	+
2) effect of acetylcholinesterase inhibitors	+	+
3) reduced mepp amplitude	+	+
4) increased curare sensitivity	+	+
5) decreased acetylcholine sensitivity	+	+
C. Immunological		
1) anti-AChR antibodies in serum	+	+
2) anti-AChR antibodies at the endplate	+	+
3) complement components at the postsynaptic membrane	+	+
4) antigenic modulation by antibody	+	+
5) antibodies directed to ACh binding site	+	+
6) anti-MIR antibodies	+	+
7) neonatal transfer of disease	+	+
8) cellular immunity to AChR	+	+
9) thymic abnormalities	-	+

a) adapted from Lindstrom et al. (45)

lymphocytes probably act as T helper cells in order to cooperate with sensitized B cells to produce anti-AChR antibodies (97). This brings us to the role of the thymus in myasthenia gravis. The thymus plays a central role in the maturation process of T lymphocytes. It has been well established that thymectomy induces a remission in about 25% of MG patients (99). The thymus contains the antigen on "myoid cells" and epithelial cells. More experiments have to be done to analyze the role of the thymus in MG. Unfortunately, rats with EAMG do not develop thymic follicular hyperplasia. Here our faithful companion, the EAMG model, does not bear on the human pathology leaving unsolved questions about the aetiology of myasthenia gravis.

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CHAPTER III:

Immunogenicity of Acetylcholine receptors: *in vitro* studies

3.1. INTRODUCTION

The immunogenicity of glycoproteins such as acetylcholine receptors can be tested *in vivo* and *in vitro*. *In vivo* immunogenicity depends on the assay systems, — e.g. specific antibody detection — used and is operationally defined since induction of specific unresponsiveness may give false negative results (1). Consequently *in vitro* assays which measure specific stimulation and proliferation of T cells and/or antibody production are preferred; these *in vitro* assays allow in addition to measurements of antibody a precise analysis of antigen presentation, and of the responding T cell subsets (2). *In vitro* assays for immunogenicity can be performed using primed or unprimed lymphocytes. The first to develop *in vitro* "immunization" of unprimed mouse lymphocytes to sheep erythrocytes were Mishell and Dutton using spleen cell suspensions (containing antigen presenting cells, T lymphocytes and B lymphocytes) and greatly improved tissue culture conditions (3). Using these culture conditions, however, primary "immunizations" to soluble antigens are difficult to achieve and consequently primed lymphocytes from (immunized animals) are mostly used. The first to describe a lymphocyte proliferation assay using sensitized lymphocytes and soluble antigens was Pearmain (4) using lymphocytes from tuberculin positive individuals and PPD. Corradin (5) scaled the culture system down to microtiter plates in order to analyse the proliferative response of lymph node cells of primed mice to protein antigens. Since this technique is economical and allows the handling of many samples we decided to adapt it in order to study the immunogenicity of AChR receptor, using primed lymphocytes. In order to answer our questions the assay system had to be specific and sensitive and had to allow analysis of responding T cell population and of specificities of the antibodies produced *in vitro*. The questions asked in terms of AChR immunogenicity in the secondary immune response *in vitro* were:

- 1) whether or not the way in which the receptor was presented to the lymph node cells influenced immunogenicity. This question was asked because *in vivo* the acetylcholine receptor is inserted into the bilayer of the cell membrane whereas the isolated AChR is solubilized in detergents which may influence immunogenicity.
- 2) whether or not antigen presenting cells were required to signal primed T and B cells to respond to AChR. This question was asked in order to differentiate antigen processed T cell mediated B cell responses from direct B cell activation.
- 3) whether or not the T and B cell responses were directed to the main immunogenic region (MIR) of the AChR since *in vivo* at any rate the antibody response is mainly directed to the MIR region (36, 37).

3.2. DESIGN OF THE EXPERIMENTS: The assay system

We developed an antigen-induced lymphocyte proliferation system with a double read-out (6). First we measured the antigen-induced proliferative capacity of primed lymphocytes *in vitro*, taken from animals with chronic EAMG, by [³H]-thymidine desoxyribose incorporation. The level of incorporation of this nucleoside is a measure for the rate of DNA synthesis. Secondly, we measured the amount of auto-antibody produced against AChR in each culture, by means of a sensitive radioimmunoassay using ¹²⁵I- α -BGT-AChR as antigen (see material and methods section, p.155).

The culture conditions for both read-out systems were very similar and therefore always performed in parallel for each experiment.

3.2.1 Lymphocyte proliferation culture system

Popliteal, inguinal and paraaortic lymph nodes and spleens were removed under aseptic conditions from Lewis rats with chronic EAMG i.e. 42 days after the primary injection with 15 μ g AChR in CFA and 7 days after the booster injection with the same amount of antigen. Single cell suspensions were prepared by forcing the lymph nodes or spleens through a stainless screen (60 mesh opening). The cell suspension was subsequently filtered through a Nitex screen (100 mesh opening) followed by three washes in Hanks' balanced salt solution (HBSS). The cells were resuspended at a concentration of 1×10^6 cells/ml in RPMI 1640 (Flow laboratories) supplemented with 5% Lewis rat serum, 100 U penicillin, 100 μ g streptomycin, and 5×10^{-5} M 2-mercapto-ethanol.

Triplicate cultures of 2×10^5 cells/0.1 ml were incubated in flat-bottomed microtiter plates (3040 microtest II, Falcon Plastics) for 5 days at 37°C in 5% CO₂ in a humidified atmosphere. One hundred microliters of AChR previously dialyzed against 250 ml PBS and diluted in RPMI 1640 was added to each well at the start of the culture to give a concentration ranging from 0.005 μ g/culture (2×10^{-10} M) to 0.5 μ g/culture (2×10^{-8} M). The cultures were pulsed with 1 μ Ci tritiated thymidine [³H] TdR, 5 Ci/mM, Amersham, England), 24 hr before the cells were harvested with an automated cell harvester. The filters (Titertek harvest filters no. 78-105-05) were dried, placed in 3 ml of scintillation fluid (OCS, Amersham, U.K.) and counted in a scintillation counter. The results were expressed as mean counts per minute (cpm) minus background of triplicate cultures $\pm$ standard error. In some instances the background values (although subtracted) are also given. Peripheral blood lymphocytes were purified from heparinized (40 IU/ml, preservative free) blood obtained by cardiac puncture. Whole blood was diluted 1:2 with HBSS and lymphocytes were purified on Ficoll-Hypaque (Pharmacia) and a sucrose gradient according to the procedure of Perper et al. (7) (for details see appendix, p.153). These cells were cultured at a density of 1×10^6 cells/ml under identical conditions as were used for lymph node and spleen cells.

3.2.2 Antibody synthesis *in vitro*

Immune lymphocytes (1×10^6 cells/ml) were cultured in triplicate in the presence of increasing amounts Torpedo AChR in 200 μ l RPMI 1640 (supplemented as described in the proliferation assay) for 5 days in a humidified atmosphere of 5% CO₂. The cells were then centrifuged in microtiter plates at 200 g at roomtemperature and washed once with 200 μ l RPMI 1640 to remove AChR that might interfere with the radioimmunoassay. After resuspending the cells in 200 μ l supplemented medium, the cultures were incubated for an additional 24 hours. Aliquots (50 μ l) of the tissue culture fluid were assayed for antibody to AChR by radioimmunoassay (see material and methods section p.155).

3.3. AChR INDUCED LYMPHOCYTE PROLIFERATION

3.3.1 The proliferative response of lymphoid cells from various anatomic sites

Since immunogens may in a dose dependent fashion specifically suppress or elicit immune responses (7) a dose response curve was made; we looked in addition to the anatomic origin i.e. lymph node, spleen and peripheral blood, of the lymphoid cells used, since this may influence the proliferative response observed (5).

Results and comments.

The addition of AChR *in vitro* to AChR primed rat lymphocytes induces proliferation. The [³H]-thymidine incorporation increases linearly with the log of the AChR concentration added *in vitro* starting at 2,5 ng/ml to an AChR concentration of 0.25 μ g/ml (2×10^{-9} M). A plateau is reached at a concentration of 2.5 μ g/ml (2×10^{-8} M) (fig. 1). A culture period with AChR of 4 to 5 days is found to be optimal with a sharp decline thereafter (fig. 2). This is not the result of cell death but probably of exhaustion of the media since the viability of the cells after 5 days in culture is still greater than 85%. In view of these findings, the cells were cultured for 5 days throughout the study. Because of the limited amounts of affinity-purified rat muscle AChR available, extended dose or kinetic studies were not carried out. Instead the conditions optimal for Torpedo AChR were used (2,5 μ g muscle AChR/ml (2×10^{-8} M) and a culture period of 5 days). Peripheral blood lymphocytes proliferated in the presence of Torpedo AChR, but the extent of this response was only 10% of that of lymph node cells (fig. 3). Spleen cells cultured under identical conditions did not proliferate significantly.

AChR- induced proliferation is extensive with lymph node cells, minimal with peripheral blood lymphocytes, and absent with spleen cells. Several factors can account for these differences. First, the route of immunization preferentially stimulates the popliteal, inguinal and paraaortic lymph nodes. Because very low concentrations of the immunogen are used, most of the AChR preparation may be retained by macrophages

Fig. 1 : AChR concentration dependency of the proliferative response *in vitro*. Forty-two days after immunization with 15 μg of Torpedo AChR lymph node cells were cultured in the presence of the indicated concentrations of Torpedo AChR ($0.02 - 2 \times 10^{-8}\text{M}$) for 5 days.

Fig. 2 : Kinetics of the proliferative response to Torpedo AChR. Rats were immunized with 15 μg AChR and sacrificed 45 days later. AChR was added to the cultures at a concentration of 2.5 $\mu\text{g/ml}$ ($2 \times 10^{-8}\text{M}$).

Fig. 3. Comparison of the proliferative response to Torpedo AChR of peripheral blood lymphocytes (PBL), lymph node cells (LNC), and spleen cells (SC) in rats with EAMG. In each case, 2×10^5 cells were used per well.

in lymph nodes. This compartmentalization has also been shown in other systems (5). Secondly, T suppressor cells might be responsible for the inhibition of T proliferation in spleen cells, in that suppressor cells preferentially home in this organ (8). Rat spleen cells also contain suppressor macrophages. Indeed when these cells are removed by filtration on G-10 Sephadex columns, spleen cells do not suppress the secondary *in vitro* response to AChR (data not shown). In experimental allergic encephalitis, Richert et al. also found no proliferation, measured by [³H]-thymidine uptake, after incubation of spleen cells with basic protein. However, antigen-reactive cells were present in the spleens of these animals, shown by the observation that small numbers of cells (2.5×10^6) stimulated *in vitro* were able to proliferate *in vivo* after transfer to naive recipients (10). Peripheral blood lymphocytes of rats with EAMG, however, do show proliferation. This population presumably represents circulating lymphocytes that leave the draining sites. Thus, the responsiveness of peripheral blood lymphocytes in EAMG and possible also in MG does not reflect the responsiveness in regional lymphoid tissue.

3.3.2 The immunogenicity of soluble AChR, native membranes and liposome incorporated AChR

Since AChRs are *in vivo* incorporated into the lipid bilayer of the postsynaptic membrane at the neuromuscular junction it is essential — in terms of assessing immunogenicity — to compare solubilized AChR with AChR incorporated into mem-

Fig. 4 : Proliferative responses of lymph node cells *in vitro* after exposure to AChR. Lymph node cells (2×10^5 cells/well) were cultured for 5 days in the presence of the indicated concentration of soluble AChR, AChR incorporated into regular liposomes, AChR incorporated into liposomes supplemented with 20% w/w cholesterol, or native electric organ membranes.

branes. Membrane incorporated AChR was used in two forms: native membranes rich in AChR from the electric organ of *Torpedo californica* and purified, soluble AChR incorporated into model membranes (liposomes). These membrane incorporated AChRs differ in size and density. AChR rich native membranes (0.4μ (11)) are four times larger than reconstituted liposomes ($0.06 - 0.14 \mu$ (12)) and AChRs are about 5 fold more densely packed in native membranes compared to liposomes containing AChR (11,12). That these liposomes contain physiologically active AChR was demonstrated by the finding of agonist induced sodium uptake that can be blocked by neurotoxins, curare and local anaesthetics (13).

Since liposomes can be visualized ultrastructurally we studied in addition to the proliferative T cell response, the ultrastructure of macrophages in order to analyze ultrastructurally if macrophage processing of AChR containing liposomes had occurred and if this could be related to immunogenicity.

Results and comments.

AChR incorporated into lipid vesicles (i.e. liposomes) elicit a 25 fold larger response than soluble AChR when added to cultures of immune lymphocytes at concentrations of less than 3 ng/culture (fig. 4). A good proliferative response against soluble AChR is observed at doses between 3-30 ng/cultures. AChRs in liposomes, however, still provide at least a 7 fold enhancement of lymphocyte proliferation in this range of concentrations. When added at concentrations larger than 30 ng/culture (approximately 2-4 μ g of lipid /culture is added at this point) these reconstituted vesicles appear toxic to the cells. Presence of cholesterol (20% w/w) in these proteoliposomes appears to shift this toxic effect to higher doses (around 300 ng/culture) (fig. 4A) Native AChR-rich membranes from Torpedo electric organ appear to be no more immunogenically potent than soluble AChR. (fig. 4B). When liposomes prepared in the absence of AChRs are added to lymphocyte cultures, no proliferative responses above background values can be detected.

After exposure of lymphocyte cultures to AChRs in reconstituted liposomes, macrophages can be observed which have accumulated large quantities of cytoplasmic vacuoles, ranging in size between 1 and 7 μ in diameter (fig. 5). These structures apparently result from phagocytosis of the liposomes and subsequent liposomal fusions with the cytoplasm of the cell. These macrophages are surrounded by numerous lymphocytes with which they appear to interact extensively through cellular processes and pseudopods enfolding the lymphocytes (details of these interactions can be seen in the insets to fig. 5). The lymphocytes themselves appear to be devoid of the cytoplasmic vacuoles observed in the interior of the macrophages. The phagocytosed vacuolar structures occasionally appear to be most prominent near the sites of macrophage lymphocyte interactions. Liposomes devoided of AChRs are also accumulated by macrophages, but these macrophages are not surrounded by lymphocytes.

AChR reconstituted into vesicles containing soybean lipid plus cholesterol (20% w/w) appear to confer to the macrophages a lightrefractive appearance after phagocytosis, allowing their identification by phase contrast microscopy (fig. 6A). The macrophages can then be visualized as single highly refractive cells within large colonies of proliferating lymphocytes. This is not observed when soluble AChR is presented as antigen to the culture (fig. 6B) or when cholesterol is not present as a component of the AChR containing liposomes (data not shown).

This experiment shows first that in terms of immunogenicity solubilized AChR is equal to AChR in plasma membranes of Torpedo californica (= native membranes); thus solubilized AChR was used for further studies (vide infra) on immunogenicity. The experiment shows in addition that AChR incorporated in liposomes are highly immunogenic and circumstantial morphological evidence suggests that antigen presenting cells (macrophages) are involved in triggering the T cell proliferation in this model. This issue will be analyzed in separate experiments with defined cell populations (see section 3.5).

Fig. 5 : Electron microscopic observation of the interaction between macrophages and lymphocytes in cultures exposed to AChR containing liposomes. Lymphocytes (2×10^5 cells/well) were cultured for 5 days in the presence of AChR incorporated in cholesterol (20% w/w) supplemented liposomes.

(A) A macrophage surrounded by several lymphocytes. The cytoplasm of the macrophage is filled with electron translucent lipid droplets (L), many of which appear in the process of fusion. Although a phagosomal membrane is not evident in this stage, these vacuoles are likely to represent modified forms of phagocytosed liposomes. X 2,400, the bar indicates 8.0μ

(B) Details of the interactions between macrophage and a lymphocyte. The inset displays a low magnification view of the field. The arrow in the inset indicates the interaction site, displayed in the figure. X 10,800, the bar indicates 1.5μ Inset : X 900, the bar indicates 10μ

(C-F) Details of the interactions between a macrophage and surrounding lymphocytes. Arrows 1, 2 and 3 in C indicate areas enlarged in D, E and F, respectively. The contacts between the macrophage and the surrounding lymphocytes are made via macrophage filopodia as shown in B, both macrophage and lymphocyte filopodia as demonstrated in D and F, and through a large area of apposed membrane as shown in D.

C : x 1,200, the bar indicates 15.0 μ

E : x 1,200, the bar indicates 1.5 μ

D : x 1,200, the bar indicates 1.5 μ

F : x 1,200, the bar indicates 2.0 μ

Fig. 6 : Lymphocyte proliferation in cultures exposed to AChR as observed by phase-contrast microscopy. Immune lymph node cells were cultured for 5 days in the presence of AChR incorporated into liposomes supplemented proteoliposomes. X25.

3.3.3 Effect of the main immunogenic region (MIR), the sites outside the MIR, and the subunits of the Torpedo AChR on the *in vitro* proliferative response

In this experiment the hypothesis was tested that the main immunogenic region (MIR) of the AChR — to which 50-70% of the autoantibodies elicited *in vivo* bind — was most likely responsible for the proliferative responses described in the experiments above. Therefore the main immunogenic region of the AChR molecule was selectively destroyed by reduction and carboxymethylation (RCM-AChR) or alternatively the antigenic sites outside the MIR were removed by trypsinisation.

The rationale of this approach is as follows:

Torpedo AChR, a complex pentameric molecule is irreversibly denatured under reducing conditions. Recombinations of the opened disulphide bridges was prevented by iodoacetamide. (for details see material and methods section, p.151). The resulting reduced and carboxymethylated AChR (RCM-AChR) is still assembled as a pentamer when analysed under nondenaturing conditions (see chapter IV). Functionally however RCM-AChR is altered in that it does not bind α -bungarotoxin or other cholinergic ligands (14, 15). Immunologically RCM-AChR is also different from native-AChR (see Chapter IV). Mapping experiments using a panel of monoclonal antibodies revealed that RCM-AChR lacks the main immunogenic determinant (MIR), an immunodominant epitope on native AChR against which most of the circulating autoantibodies of patients with MG or animals with EAMG, are directed. It is therefore not surprising that RCM-AChR does not induce EAMG although it is a good immunogen that in-

Fig. 7A : Dose response curve of the proliferative response *in vitro* against RCM-AChR (○) compared to native AChR (●).

Thirty five days after immunization with 15 μg native Torpedo AChR lymph node cells were cultured in the presence of the increasing amounts of RCM-AChR or native AChR.

Fig. 7B : Dose response curve of the proliferative response *in vitro* to trypsinized AChR (squares) compared to native AChR (circles). Thirty-five days after immunization with 15 μg native Torpedo AChR lymph node cells were cultured in the presence of the indicated amounts of trypsinized or native AChR.

duces high titers of antibodies which crossreact with native AChR (14-16).

Mild tryptic digestion of AChR on the other hand, does not change the functional activity of AChR (17) but alters its immunological anatomy (16). Indeed after trypsinization most antigenic determinants are removed with the exception of the MIR, when analysed with a panel of monoclonal antibodies. These monoclonal antibody mapping experiments also revealed that trypsinization removes the antigenic determinants in opposite to reduction and carboxymethylation (RCM-AChR; see Chapter IV). Trypsinized AChR still induces myasthenia while RCM-AChR does not (15, 16). Apparently trypsinization similarly to papain cleaves some protein bonds in AChR without profoundly altering its three dimensional structure (17). Whereas reduction and carboxymethylation induces conformational changes.

Results and comments.

When RCM-AChR is added to lymphocytes primed *in vivo* with native AChR no dif-

Fig. 8 : Concentration dependency of the proliferative response to denatured subunits of Torpedo AChR. Note the decreased response with respect to native AChR. Lymph node cells ($1 \times 10^6/\text{ml}$) were cultured for 5 days in the presence of increasing amounts of native AChR ($4\text{--}400 \times 10^{-14}$ mol/culture) or denatured subunits (α , Δ ; β , $\circ$; γ , $\blacktriangle$; δ , $\blacksquare$). Rats were sacrificed 45 days after the primary immunization with $15 \mu\text{g}$ of Torpedo AChR in CFA.

ferences between native and denatured AChR on the proliferative response of the primed lymphocytes was observed (fig. 7A). We examined in addition the effect of the four purified receptor subunits on the proliferative response and found them to be equal on a molar basis each subunit giving a proliferative response about 50% of that induced by native AChR (fig. 8). Also the slopes of the dose response curve of the various subunits was similar.

Trypsinized AChR also induced a proliferative response of lymphocytes primed with native AChR. The proliferative dose response curve elicited by native and trypsinized AChR were identical (fig. 7B).

Surprisingly the absence of the main immunogenic region (RCM-AChR) or deletion of antigenic epitopes outside the MIR did not influence the immunogenicity when compared to native AChR. This suggests that in our model the AChR is broken down at the level of the antigen presenting cells (e.g. macrophages) prior to presentation to the T cells for the loss of the three dimensional structure of the AChR and the MIR does not influence the proliferative response of primed T cells. This view is supported by the fact that isolated subunits also elicit a proliferative cell responses (fig. 8). These

Fig. 9 : AChR concentration dependency of anti-AChR antibody production *in vitro*. Lymph node cells were removed from rats 45 days after immunization with $15\text{ }\mu\text{g}$ of Torpedo AChR and cultured for 6 days. The two symbols indicate very similar response of cells from two different rats.

fig. 10 : *In vitro* the proliferative and antibody responses are directly proportional. Lymph node cells of 15 rats immunized with $15\text{ }\mu\text{g}$ Torpedo AChR with EAMG were cultured under optimal conditions for T cell proliferation ($2.5\text{ }\mu\text{g/ml}$) and for antibody production (25 ng/ml).

results were not to be expected on the basis of the antibody response elicited *in vivo* with native AChR which show the main autoantibody response to be directed to the MIR.

3.4. AChR-INDUCED SPECIFIC ANTIBODY PRODUCTION *IN VITRO*

3.4.1 The effect of chemical alteration of AChR on the *in vitro* antibody production

In the previous experiment no restriction of the T cell proliferation response (restriction in terms of MIR) was seen although such a restriction appears present when studying autoantibody (to native AChR) *in vivo*. In order to elucidate this discrepancy the antibody response of lymph node cells (from rats primed with native AChR) to native and chemically modified AChR was examined *in vitro*. The question asked was whether or not the presence of the MIR was required to elicit an antibody response by primed lymph node cells by AChR.

Fig. 11 : Antibody production by lymph node cells *in vitro* after exposure to AChR. Lymph node cells were removed from rats 5 weeks after they received a single immunization with 15 μ g soluble AChR. The cells were cultured in the presence of the indicated concentrations of soluble AChR (left bars), AChR incorporated into regular liposomes (middle bars), or AChR incorporated into liposomes supplemented with 20% (w/w) cholesterol (right bars). The coefficient of variation between individual cultures was within 10%.

Results and comments.

A dose response curve showed that addition of native AChR to primed lymph node cells elicited antibody production; maximal antibody production was observed when 25 ng/ml (2×10^{-10} M) of Torpedo AChR was added. Higher dosages of receptor were inhibitory (fig. 9); The inhibition might have been nonspecific due to low levels of detergent; however the observation that much higher antigen concentrations did not inhibit thymidine incorporation (fig. 1) suggests that the inhibition may have been specific. The amount of antibody produced *in vitro* was directly proportional to the proliferative response of the lymph node cells (fig. 10). Reduced and carboxymethylated AChR as well as trypsinized acetylcholine receptor elicited an amount of antibody comparable to native AChR (table I). Finally AChR incorporated in artificial membranes (liposomes) were more immunogenic in terms of antibody production compared to native AChR (fig. 11). Liposomes without AChR had no effect. Inclusion of cholesterol (20 % w/w) as a component of the AChR containing liposomes lead to a substantial additional enhancement of antibody production.

It is clear from this experiment that the antibody response elicited by adding chemically modified Torpedo californica AChR to cultures of *in vivo* primed lymphocytes yields

similar results compared to the proliferation assay. The fact that proliferative response is directly proportional to the amount of antibody produced suggest the proliferating cells to be of T-helper cells. These results then suggest that the main immunogenic region or an intact three dimensional structure of AChR are not required for — presumably T cell mediated — B cell responses. Definitive evidence for this assumption requires of course study of antibody specificity. In the culture system used the small amounts of antibody produced were not sufficient to answer this question.

Table I : Anti-AChR antibody response induced *in vitro* by native AChR, trypsinized AChR and RCM-AChR.

	anti-Torpedo AChR antibody ^a		(nM) induced by RCM-AChR
	native AChR	trypsinized AChR	
animal 1	18.8 ± 3	15.8 ± 2	N.D.
animal 2	8.1 ±	N.D.	13.9 ±

^a) the amount of antibody (nM) to native AChR produced *in vitro* by 2×10^5 cells was measured by radioimmunoassay (mean ± s.e.).

3.4.2 Immunogenicity and pathogenicity of AChR

In the previous experiments the immunogenicity of AChR from Torpedo Californica was assayed by means of (secondary) *in vitro* assays using chemical modifications of the AChR to determine the most immunogenic sites. These experiments were not conclusive since the fine specificities of the antibody and proliferative responses could not be determined in the system used. Clinically however immunogenicity of the receptor should be related to pathogenicity. Thus only the immune T and B cells that (after immunization with Torpedo AChR) crossreact with mammalian (e.g. rat) AChR determine the immunogenicity clinically.

In order to examine this question the following experiment was designed:

AChR used for immunization		Source of AChR used in assay system
Species	Dose	
Torpedo californica	2 x 15 µg	Torpedo californica fetal calf muscle rat muscle
Fetal calf	2 x 15 µg	fetal calf muscle rat muscle Torpedo californica
Rat	1 µg	rat muscle fetal calf muscle Torpedo californica

Rats were immunized with equal amounts of mammalian (fetal calf muscle) or Torpedo AChR and the cross-reactive T and B cells were assayed as shown above. Since only a limited amount of rat muscle AChR was available only about 6% of the dose of Torpedo AChR was used as immunogen.

Results and comments.

Immunization with a single dose of Torpedo AChR (15 μ g) elicits a strong antibody response ($5.0 \pm 0.75 \times 10^{-6}$ M, $n = 11$) as measured by radioimmunoassay (see material and methods section, p.155). Cross-reactive antibody titers to fetal calf and syngeneic rat AChR average, 1.2% and 1.0% of the anti-Torpedo AChR titer respectively, when assayed at 1×10^{-9} M AChR. Cross-reaction in the proliferative response appears considerably greater (25%) (fig. 12). Similar proliferative and serologic cross-reactivity were observed with affinity purified rat AChR, but because of the limited availability of rat AChR, extensive studies were not possible. (table II).

Immunization of rats with fetal calf AChR (15 μ g AChR in complete Freund's adjuvant injected twice, 1 month apart) induces severe EAMG. The titers of serum antibody to rat muscle AChR and to Torpedo AChR were $7.9 \pm 1.1 \times 10^{-7}$ M and $1.1 \pm 0.2 \times 10^{-7}$ M respectively. These anti-Torpedo AChR antibody titers are significantly lower compared to animals immunized with Torpedo AChR, but these sera cross-react to a greater degree with fetal calf and rat AChR than the sera from rats immunized with Torpedo AChR. To ascertain the cross-reaction at the T cell level, lymph node cells and peripheral blood lymphocytes were allowed to proliferate in the presence of fetal calf AChR (fig. 13). The proliferative response of lymph node cells to Torpedo was only 2.7% of that to fetal calf AChR compared to a 13% cross-reaction at the antibody level. Similar results were obtained in rats immunized with syngeneic AChR showing a cross-reaction to Torpedo californica of 32% in antibody specificity and 5% of the proliferative response.

Results show the crossreactive lymphocyte proliferation and antibody responses to rat AChR to be determined by the species from which the immunogen derives. Thus Torpedo AChR elicits hardly any crossreactive antibody (1%) but a substantial crossreactive T cel proliferative response (25%). Fetal calf AChR and rat AChR on the

Table II : Cross-reactivity of the proliferative response to AChR in Torpedo AChR^a induced EAMG

Torpedo AChR ^b	Fetal Calf AChR ^b cpm/culture (m $\pm$ S.E.)	Rat AChR ^b
229,898 $\pm$ 21,843	23,842 $\pm$ 4,412	22,560 $\pm$ 3,767

^a) All animals were injected once with 15 μ g Torpedo AChR and sacrificed 40 days later.

^b) The response was measured on day 5 of culture in the presence of 2.5 μ g/ml (2×10^{-8} M) of AChR from the indicated species.

Fig. 12 : Cross-reaction of the proliferative response and serum anti-AChR of rats immunized with Torpedo AChR and stimulated with either Torpedo or fetal calf muscle AChR. Rats immunized and boosted with $15 \mu\text{g}$ of Torpedo AChR were sacrificed after 39 days for lymph node cells. Both the proliferative response and the serum titers were assayed with $2.5 \mu\text{g}/\text{ml}$ of Torpedo or fetal calf muscle AChR ($2 \times 10^{-8} \text{ M}$). In this figure antibody is assayed at the same high concentration of AChR ($2 \times 10^{-8} \text{ M}$) used in the proliferative assay, and the anti-Torpedo AChR titers divided by 1.7 to correct for the presence of dimers in Torpedo AChR, but not muscle AChR. Still the extent of antibody cross-reaction is smaller than the extent of proliferative cross-reaction (25%).

Fig. 13 : Cross-reaction of the proliferative response and serum anti-AChR antibody of rats immunized and boosted with $15 \mu\text{g}$ fetal calf muscle AChR and stimulated with either Torpedo or fetal calf muscle AChR. The proliferative response and the serum antibody concentrations were assayed with $2.5 \mu\text{g}/\text{ml}$ ($2 \times 10^{-8} \text{ M}$) Torpedo or fetal calf muscle AChR. Lymph node cells taken 41 days after the initial immunization were cultured for 5 days.

other hand elicited a substantial crossreactive antibody response to Torpedo AChR (13%) and rat AChR (32%) respectively, but not at the level of the T cells (2.7% and 5% respectively). Thus at the level of the T cells no tolerance to mammalian AChR exists since a good T cell proliferative reaction to muscle AChR occurs regardless the source of AChR used for immunization. In addition these experiments show that in rats, immunized with muscle AChR, the restriction of the crossreaction occurs at the T cell level, whereas Torpedo AChR induces a restriction at the B cell level (table III).

Table III: Schematic representation of cross-reactivity patterns of T and B cells in EAMG induced by Torpedo or rat muscle AChR.

Immunogen	Cross reaction of			
	T-cells		B-cells	
	Torpedo AChR	Mammalian AChR	Torpedo AChR	Mammalian AChR
Torpedo AChR	+	+	+	-
Rat AChR	-	+	+	+

These differences in T and B cell responsiveness to self antigens are well known from other models of autoimmune disease e.g. autoimmune thyroiditis (1). We hold that the difference between autoimmune thyroiditis and EAMG resides in the fact that in contrast to thyroglobulin the AChR does not circulate and consequently does not maintain unresponsiveness of T cells as shown by the fact that immunization with rat AChR elicits a clearcut T cell proliferative response.

In conclusion then, Torpedo californica AChR is a superior immunogen compared to mammalian AChR in terms of crossreactive T cell proliferation; mammalian AChR however, is the superior immunogen clinically for, although the absolute amount of antibody elicited is less compared to Torpedo AChR, the percentage crossreactivity is much greater and these antibodies are responsible for disease clinically.

3.5. ON THE CELLULAR MECHANISMS OF THE SECONDARY *IN VITRO* RESPONSE TO AChR

3.5.1 The cellular requirements for the secondary *in vitro* proliferative and antibody response to AChR

Our previous experiments yielded indirect evidence that antigen presenting cells were required for proliferation and that the proliferating cells were most likely T-helper cells. Therefore the effect of AChR on the proliferative response and antibody production of primed lymph node cells was measured after removal of accessory cells (e.g. macrophages) from the lymph node cell suspensions.

Results and comments.

Removal of T cells from native AChR primed lymph node cell suspensions with a T cell specific anti rat brain serum in the presence of complement resulted in a dramatic reduction of both AChR induced proliferation (fig. 14) and antibody production (Table IV) indicating that both responses were T cell dependent.

Fig. 14 : T cell depletion inhibits the proliferative response. Five rats were immunized with 15 µg AChR and sacrificed 40 to 45 days later. Lymph node cells were cultured either without (cross-hatched bars) or after treatment (stippled bars) with a rabbit anti-rat brain serum and complement.

Table IV : T cells are required for AChR induced antibody production *in vitro*.

Lymph node Cell ^a Treatment	Torpedo AChR (2.5 µg/ml)	Anti-Torpedo AChR ^b Ab (x 10 ⁻⁹ M)
None	—	0.36
None	+	69
Anti-rat brain serum + C'	—	0.31
Anti-rat brain serum + C'	+	2.8

^a) The total lymph node cell population was treated with anti-rat brain serum and C' or left untreated before stimulation with AChR.

^b) The response was measured on day 6 of culture after removal of AChR by washing 24 hr before harvesting.

Table V : W3/25-Positive cells are required for proliferation and antibody production *in vitro*.

Lymph Node Cell ^a Preparation	[³ H]TdR Uptake ^b (cpm/culture)	Anti-Torpedo AChR ^c Antibody (x 10 ⁻⁹ M)
Unseparated	230,455	34
W3/25 depleted	8,421	0.6
W3/25 positive	142,940	2.1

^a) Immune lymph node cells were incubated with anti-W3/25 monoclonal antibody and a fluoresceinated goat anti-mouse IgG rendered mouse IgG-specific by absorption on insolubilized rat IgG. Subsequently, cell separation was performed with a fluorescence-activated cell sorter. This is one representative experiment out of 3.

^b) The response was measured on day 5 of culture.

^c) Anti-Torpedo AChR antibody concentration in culture was measured on day 6 of culture after removal of the antigen by washing 24 hr before harvesting.

Direct evidence that the proliferating cells are of the T-helper variety was obtained by removal of T-helper cells with fluorescence activated cell sorting techniques as follows. Lymph node cells from rats primed with native AChR were incubated with a monoclonal mouse antibody (W 3/25) which recognizes rat T-helper cells and macrophages (18); next these cells were incubated with fluoresceine labeled F(ab)₂ fragments of goat IgG anti mouse IgG. This suspension was passed through a fluorescence activated cell sorter as follows:

Both sorted populations were brought to a cell concentration of 10^6 cells/ml and cultures of 2×10^5 cells/microwell were set up with native AChR added. Depletion of W 3/25 positive cells completely abolished the proliferative and antibody response to native AChR (table V). The W 3/25 positive lymphocytes on the other hand were capable of proliferation in the presence of AChR but not capable of antibody synthesis, because few antibody producing cells were retained in this fraction. The proliferative capacity of the W 3/25 positive population was reduced compared to the control cultures because this fraction contained no dendritic cells since these cells do not express the W 3/25 phenotype (Dr. L. Nagelkerken, personal communication).

In order to demonstrate that both proliferative and antibody responses required the presence of macrophages (antigen presenting cells), primed lymph node cells were depleted of macrophages by filtration through G-10 Sephadex column (19); next cultures were set up with native AChR added.

Results (table VI) show that macrophages are required for both proliferative and antibody responses since macrophage depletion results in a reduction in T cell proliferation and an even more pronounced fall in antibody production.

The results show clearly that both proliferative and antibody responses require accessory cells (macrophages) and T lymphocytes. The cell sorting experiments show that

Fig. 15 : Schematic representation of the immune response to AChR in a lymph node of rats with EAMG. T and B cells recognize AChR and proliferate and differentiate into T blasts and plasmacells respectively (insert). Symbols : Ia : immune response gene associated antigen; IL-1: interleukin 1; IL-2: interleukin 2.

Table VI : Macrophages are required for AChR-induced proliferative and antibody responses.

LNC Treatment ^a	Torpedo AChR 2.5 µg/ml	[³ H]TdR Uptake (cpm/culture)	Anti-AChR ^b Antibody (x 10 ⁻⁹ M)
None	—	2,778	0.30
None	+	149,460	14.00
G-10 filtered	—	253	0.01
G-10 filtered	+	59,311	1.00

^a) Lymph node cells (LNC) were depleted from macrophages by passage over a G-10 column.

^b) Anti-Torpedo AChR antibody concentration in culture was measured on day 6 of culture after removal of the antigen by washing 24 hr before harvesting.

addition of native AChR to W 3/25 positive cells — macrophages and T-helper cells — elicits a proliferative response. These results taken together indicate that the proliferative response of primed lymph node cells to native AChR is mediated by accessory cells (macrophages) and T-helper cells.

3.5.2 The role of lymphokines, specifically interleukin-2

It is well known that T cells produce polypeptides ("interleukins") which act upon other lymphocytes and thus induce proliferation and differentiation (see figure 15). A large number of lymphokines have been described and partially characterized (20) specifically interleukin 2 (or T cell growth factor (TCGF)) which has been produced by means of recombinant DNA technology (21, 22) and is produced in the rat exclusively by T-helper cells (J. Rozing, personal communication). Interleukin 2 is produced by T-helper cells and acts on T cells (inducing proliferation and differentiation) provided they have a receptor for interleukin 2 (23). The receptor for Interleukin 2 on T cells is induced by antigen presenting cells (e.g. macrophages) which also secrete lymphokines called Interleukin 1 (24).

Since the proliferation and antibody response observed in our *in vitro* system required the presence of macrophages and T cell the question asked was whether Interleukin 2 production was demonstrable in our system thus confirming the presence of T-helper cells.

Results and comments.

Lymph node cells of animals with EAMG were restimulated *in vitro* with increasing amounts of AChR under conditions as described for the T cell proliferative assay. Aliquots of the supernatants (50 μ l) of these cultures were assayed for Interleukin-2 production by means of a cytotoxic T cell line (CTLL cell line) that is absolutely dependent on IL-2 for its growth (see material and methods section, p.158). The amount of IL-2 present in the culture supernatants is directly proportional to the height of the proliferative response of the CTLL cell line as measured by [3 H]-TdR incorporation. The amount of IL-2 produced by immune lymphocyte upon addition of AChR peaks at day 3 (fig. 16). When the proliferation of immune T cells reaches its maximum, IL-2 concentration decreases below detection capacity. These results suggest that IL-2 is produced and subsequently consumed by AChR induced T cell proliferation.

To demonstrate that IL-2 produced *in vitro* is necessary for AChR induced proliferation we added exogenous IL-2, isolated from culture of concanavalin A stimulated normal rat splenocytes (kindly donated by Dr. L. Nagelkerken, Dept. Immunology, University of Limburg, Maastricht) and IL-2 obtained by recombinant DNA technology (kindly donated by Dr. R. de Vos and Prof. W. Fiers, University of Gent, Belgium). The addition of increasing amounts of rat IL-2 purified from concanavalin-A stimulated lymphocyte supernatants, stimulates the proliferation of immune lymph node cells from animals with EAMG in the absence of AChR (fig. 17a). In ad-

Fig. 16 : Kinetics of interleukin 2 production (solid line) compared to T cell proliferation (broken line). Lymph node cells (2×10^6 cells/well) were cultured for indicated time in the presence of $2.5 \mu\text{g}$ AChR/ml. Interleukin-2 production was measured by using the CTL-L cell line.

dition to proliferation these supernatants can also stimulate AChR specific antibody production *in vitro* (data not shown). Similar results were obtained with IL-2 produced by recombinant DNA technology (fig. 17b)

It is clear from these experiments that in our culture system the amounts of endogenous produced IL-2 is rate limiting in terms of T helper cell proliferation since exogenous added Interleukin 2 is capable of an aspecific amplification of both proliferation and antibody production indicating the presence of an abundance of IL-2 receptors.

3.6. DISCUSSION

Lymph node cells from rats with EAMG proliferate and differentiate into anti-AChR antibody producing cells upon stimulation with Torpedo and mammalian AChR *in vitro*. It is demonstrated that the cells which proliferate *in vitro* in response to AChR are T helper cells. The evidence that they are T helper cells is that:

1. they are killed by antisera to T cells plus complement.
2. the cells produce interleukin-2 (T cell growth factor) *in vitro*
3. antibody production *in vitro* depends on their presence
4. antibody production *in vitro* is proportional to their proliferative capacities.
5. they express the W3/25 antigen previously characterized in a classical adoptive transfer system as a T helper cell membrane marker.

Fig. 17 : Effect of Interleukin 2 on the T cell proliferative response of immune lymph node cells from rats with EAMG. Lymphocytes (2×10^5) were cultured in the presence of Interleukin 2 purified from supernatants of Con A stimulated rat spleen cells (panel A; 1250 U/ml) or obtained by recombinant DNA technology (panel B; 780,000 U/ml). T cell proliferation was measured on day 3 (solid line) and day 5 (broken line) of culture in the absence of AChR.

Previous studies (25-27) provided only indirect evidence for the role of T helper cells in EAMG, in that no attempt was made to show the effect of depletion of T helper cells on antibody production to AChR in this antibody mediated disease. The proliferating T helper cell is very sensitive to AChR *in vitro*; only minute amounts of AChR (0.5 ng/culture, i.e., 4×10^{-15} mol/culture) are required to produce proliferation *in vitro* compared to 70×10^{-12} mol ($\pm 8 \mu\text{g}$) present in the muscles of a normal rat. However, *in vitro* 12000 AChR monomers are available per lymphocyte and no doubt several orders of magnitude more AChR per antigen reactive cell, because non-specific recruitment of T cells can occur via lymphocytotropic factors (28). In the systems in which other T cell-dependent antigens were used, relatively large concentrations of antigen (10-500 $\mu\text{g/ml}$) were required (5, 29-35).

Chemically modified AChR i.e. reduced and carboxymethylated (RCM-AChR) or trypsinized AChR did not differ in their immunogenicity *in vitro* when compared to native AChR. These results were surprising in view of the fact that RCM-AChR lacks the main immunogenic region (MIR) towards which 50-70% of the autoantibodies are directed (36, 37). The similarity between native and chemically modified AChRs in terms of specific T cell activation is based on the notion that enzymatic processes take place in antigen presenting cells (macrophages) prior to presentation of the antigen to antigen specific T cells (38-41) such enzymatic processes lead to the loss of the three dimensional conformation of AChR and also of the MIR. Small protein fragments generated by trypsinization, could be utilized by macrophages and presented to specific T cells. In contrast to the high identity at the T cell level of these chemically modified AChR preparations a much lower cross-reaction is observed at the antibody level. As seen in fig. 8, the cross-reactivity between the isolated AChR subunits and native AChR at the T cell level can be as high as 60% unlike the cross-reaction at the serum antibody level ($<1\%$) (33). Similar cross-reactivity patterns are seen when native and denatured cytochrome, lysozyme, insuline, myoglobin, bovine serum albumin, ovalbumin, staphylococcal nuclease, are used for *in vitro* activation of primed T cells (38-48). From these reports and our study it can be concluded that T and B cells differ from one another in the way they recognize native and denatured forms of antigen. An intact threedimensional structure of AChR is a prerequisite for B cell activation and the production of specific antibodies.

Lymph node cells from rats with chronic EAMG mature into anti-AChR antibody producing cells upon stimulation with native or chemically modified AChR *in vitro*. The elicitation of anti-AChR antibody *in vitro* requires smaller amounts of the antigen with inhibition at higher antigen concentrations, possibly as a result of antigen blockade or suppressor generation in cultures. These observations have been confirmed by Bogen et al. in murine EAMG (49). These investigators could generate suppressor T cells in cultures using high doses of AChR. When we stimulate immune lymph node cells *in vitro* with optimal (i.e. low) doses of AChR, we preferentially induce the differentiation of T helper cells since the amount of antibody produced *in vitro* is directly proportional to the height of the proliferative response. Indeed the removal of cells expressing

the helper phenotype W3/25 abolishes the *in vitro* antibody production.

As seen with other T cell dependent antigens, the AChR-stimulated proliferation and antibody production of primed lymph node cells *in vitro* as the same cellular requirements as reported for antigens not involved in autoimmune diseases (see fig. 15). The requirement of macrophages was demonstrated in two experiments. First, the proliferative and *in vitro* antibody responses are dependent upon their presence. Second, AChR incorporated in lipid membranes (liposomes) is accumulated by the macrophages (50). The latter probably accounts for the higher antigenic potency of AChR in liposomes as compared to soluble AChR. The requirement of macrophages probably reflects the requirement for antigen presentation to T cells in the context of major histocompatibility complex determinants (51) and more specifically, class II (Ia molecules) as shown for EAMG in mice (51). Indeed antibodies to Ia gene products of the immune response gene complex in the mouse attenuate both humoral and cellular responses to acetylcholine receptor both *in vitro* and *in vivo* and appears to suppress clinical manifestations of murine EAMG (52, 53).

Measurement of cross-reactivity of serum antibodies by radioimmunoassay and T cell proliferation *in vitro* showed marked differences of cross reactivity at T and B cells depending upon the AChR type used for immunization. When rats are immunized with Torpedo AChR the cross-reactivity with fetal calf AChR at the antibody level is only 1%, whereas cross-reactivity of the proliferative response is 25%. In contrast, when rats are immunized with fetal calf AChR, which is immunologically very similar to rat AChR, the reverse cross-reactivity pattern is observed. In the latter situation considerable cross-reactivity (13%) is observed with anti Torpedo AChR and anti fetal calf AChR antibody, whereas less cross-reactivity is seen with T cell proliferation (3%). Although the absolute levels of anti Torpedo AChR antibody and anti Torpedo AChR T cell proliferation are low, these observations could best be explained by a restricted response to mammalian AChR in the rat, in which antibody is produced to a limited number of epitopes on the mammalian AChR in comparison to a more unrestricted response to the more foreign Torpedo AChR. Most of the cross-reactive epitopes between Torpedo AChR and mammalian AChR as characterized by means of monoclonal antibodies are localized on a region of that subunit (36, 37, 54). The restrictions in the response to mammalian AChR are in agreement with other reports on the restrictions in the response to syngeneic thyroglobulin (55). Antibodies to thyroglobulin present in sera of patients with autoimmune thyroiditis, are directed to probably not more than four (55) or six (57) antigenic determinants. In patients with MG and rats with EAMG, the majority of autoantibodies are similarly restricted to a small region on the subunits (36, 37).

The analysis of the immune status of T and B cells against self antigens may lead to a better understanding of initial events leading to autoimmune diseases. Whether a given self antigen is vulnerable to an autoimmune attack depends on the specific immune status of T and B cells to that antigen; this, in turn, is determined by the concentration of self-antigen in the extra cellular fluid. This suggestion and the observation

Table VI: Relationship between levels of self-proteins in the serum, immune status of T and B cells to autoantigens and autoimmune disease.

experimental ^a autoimmune disease	autoantigen ^b (concentration)	Immune status of ^c		Effector cells
		T cell	B cell	
thyroiditis (EAT)	thyroglobulin (low)	TOL	IC	T/B
encephalomyelitis (EAE)	myelin basic protein undetectable	IC	IC	T
myasthenia (EAMG)	AChR undetectable	IC	IC	B

^a) EAT: experimental autoimmune thyroiditis; EAE: experimental encephalomyelitis; EAMG: experimental autoimmune myasthenia gravis.

^b) The degree of tolerance in both T and B cell compartments is dependent on the concentration of the self-antigen in the micro environment. For the examples given, the autoantigen concentration is low, or the autoantigen is not detectable.

^c) TOL: tolerant; IC immunocompetent.

that B cells require much higher concentrations of self antigen than T cells for the maintenance of unresponsiveness are supported by data obtained using an experimental model of acquired tolerance against thyroglobulin. (1, 58). The mechanisms proposed for breaking tolerance in model autoimmune diseases are outlined schematically as follows (Table VI)

3.7. Summary and conclusions

In order to analyze the immune reactions involved in experimental autoimmune myasthenia gravis (EAMG) an *in vitro* system was developed in which sensitized lymphocyte cells from rats with chronic EAMG were cultured in the presence of differing acetylcholine receptor preparations. The proliferative cellular responses and the amount of antibody secreted *in vitro* were quantitated. The results obtained using this *in vitro* lymphocyte culture system can be summarized as follows.

1. The physicochemical form in which AChR was presented influenced its immunogenicity *in vitro*. AChR incorporated in liposomes — a form reminiscent of the postsynaptic membrane — elicited far greater proliferative and antibody responses compared to solubilized AChR.
2. The cellular requirements for an optimal proliferative and antibody response were defined. The culture system required AChR, sensitized T helper cells, antigen presenting cells (macrophages). The T helper nature of the proliferating cells was suggested by the finding that they produced Interleukin 2 and by the fact that antibody producing cells required their presence and that

the amount of antibody *in vitro* was proportional to the cellular proliferative responses. Conclusive evidence was derived by determining the phenotype of the proliferating cells which was that of T helper cells (W3/25).

3. Finally, the fine specificities of the proliferating T helper cells were determined. Most of the serum antibodies in EAMG appear to be directed against a small group of determinants on the α -subunit of AChR, called the main immunogenic region (MIR). The T cell responses however, were not directed to the MIR since a AChR preparation lacking the MIR (RCM-AChR) was equally effective as native AChR in eliciting T cell proliferation. A second set of specificities were studied by comparing the immunogenicity of Torpedo, fetal calf and rat muscle AChR *in vitro* and *in vivo*. It was found that no tolerance at the T cell level AChR exist. The cross reactive T cell proliferation and antibody response to rat AChR is determined by the species from which the immunogen derives.

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CHAPTER IV

Immunogenicity and Pathogenicity of Acetylcholine Receptors *in vivo* studies

4.1 INTRODUCTION

We have shown previously (Chapter III) that T helper and B cells sensitized *in vivo* by immunization with acetylcholine receptor from *Torpedo californica* can be induced to proliferation (T helper cells) and antibody secretion (B cells) *in vitro* provided AChR and accessory cells are present. Interestingly fish acetylcholine receptor devoided of the main immunogenic region (RCM-AChR), elicited *in vitro* responses equal to unmodified (native) fish acetylcholine receptor (AChR). Since destruction of the main immunogenic region by reduction and carboxymethylation (RCM-AChR) from the acetylcholine receptor abolishes its capacity to elicit myasthenia gravis *in vivo* in rabbits (1) it is clear that these *in vitro* results cannot be related to pathogenicity *in vivo* unless antibody specificities are determined. In fact prior immunization of rabbits with reduced and carboxymethylated *Torpedo californica* acetylcholine receptor (RCM-AChR) has been reported to protect against the induction of experimental autoimmune myasthenia gravis (EAMG) with native AChR (1).

It is clear from the observation alluded to above that immunogenicity (ability of an AChR preparation to elicit a primary immune response) and pathogenicity (ability to elicit EAMG) require both precise information about the antigenic binding sites of the target organ as well as the quality and quantity of the immune reactions involved, and possibly the ability of the target organ to withstand the immunological assault.

Consequently the relationship between immunogenicity and pathogenicity of a given AChR preparation should be studied in two ways. In the *in vivo* approach animals are immunized with various AChR preparations and the antibody response in the serum and at the neuromuscular endplate is quantitated and related to signs of myasthenia; the assumption being that the myasthenia is brought about by antibody (2). Alternatively animals are first immunized, next the immune reactions elicited are dissected *in vitro* by physicochemical separation of the immune cells in order to be transferred passively *in vivo* to naive recipients for measuring the capacity to elicit EAMG. Although the last approach is superior in terms of yielding concise answers the logistics are prohibitive. This is so because it not only implies *in vitro* separation of various (*in vivo*) sensitized cell populations (T suppressor, T helper, cytotoxic T and delayed type hypersensitivity cells) and characterization of their "fine" specificities, but above all it requires that cell populations of defined phenotype and antigenic specificity are generated in sufficient numbers to be used in passive transfer experiments. A similar reasoning holds for antibodies which would first have to be separated into diverse classes

and subclasses and next have to be characterized in terms of antigenic specificities in order to give clear answers in passive transfer studies.

It is clear from the considerations outlined above that an abundant supply of antibodies and immune cells of known phenotype and specificity are required in order to study pathogenicity in a passive transfer model. In practice this requires the presence of libraries of monoclonal antibodies (3) and T cell lines or clones (4). Recently however, the use of cloned T cells for studying pathogenesis of disease has been questioned and short term cultures of *in vitro* expanded T cells have been shown to be superior in this regard (5).

We therefore decided to approach the problem of immunogenicity and pathogenicity in three ways. First in the experimental model we characterized native and biochemically altered immunogens and next determined in a quantitative way the antibody response elicited and its relation to the severity of the myasthenia. Next monoclonal antibodies were used in passive transfer studies in order to characterize the pathogenetically important binding sites on the neuromuscular endplate; finally, a passive transfer system using immune cells expanded in short term cultures was developed in order to verify the *in vitro* observation that T helper cells are important for the development of EAMG and in order to rule out a contribution of effector T cells in mediating AChR loss.

4.2. DESIGN OF THE EXPERIMENTS

Experimental autoimmune myasthenia gravis (EAMG) was elicited in Lewis rats by means of active immunization and passive transfer of antibody or immune lymphocytes.

Active immunization with AChR

Acetylcholine receptors purified from electric organs of *Torpedo californica* muscle, fetal calf AChR and rat muscle AChR incorporated in complete Freund's adjuvant were injected into Lewis rats. At given time intervals following immunization, sera were obtained from these animals and assayed for antibody against the immunogen and against autologous AChR. In a separate set of experiments the *in vivo* immunogenicity of chemically modified AChR (reduced and carboxymethylated — RCM-AChR — and trypsinized AChR) was analyzed.

Passive transfer with monoclonal antibody

Monoclonal antibodies derived from splenocytes of rats with EAMG were passively transferred into normal rats in order to elicit acute EAMG. In these experiments the role of antibody with established specificities in respect to the main immunogenic region (MIR) on AChRs was examined in terms of their capacity to induce EAMG.

Passive transfer with immune lymphocytes

Immune lymphocytes obtained from animals with chronic EAMG were cultured *in vitro* in the presence of AChR in order to expand AChR specific T cells following the protocol described in Chapter III (pg. 00). These cultured cells were next injected into normal Lewis rats without antigen or adjuvant and antibody titers against AChR which are a measure of T helper activity on B cells were assayed at regular time intervals.

In all rats treated according to these three protocols the severity of myasthenia was measured clinically and biochemically i.e. by quantitating the amount of AChR in the rats' muscles and the amount of antibody complexed to the AChR at the neuromuscular endplate.

4.3 INDUCTION OF EXPERIMENTAL AUTOIMMUNE MYASTHENIA GRAVIS (EAMG) BY ACTIVE IMMUNIZATION WITH NATIVE ACETYLCHOLINE RECEPTOR

4.3.1. Immunogenicity of AChR and kinetics of the serum antibody response.

Because rat AChR and the antibodies directed to it are the relevant auto-antigen and autoantibodies we compared the immunogenicity of rat, fetal calf and Torpedo AChR by immunizing female Lewis rats, 8 to 12 weeks of age, with 1 μ g of Torpedo fetal calf muscle, rat muscle AChR, given twice with a one month interval. Antibodies to the 3 immunogens were measured 5 days after the booster injection. It is clear from the results in table I that all these types of AChR elicit an immune response. All sera reacted better with the immunogen than with AChR from another species. Torpedo AChR is a better immunogen than mammalian AChR. The lower immunogenicity of rat AChR per mol AChR in this experiment was probably due to fragmentation of this immunogen when analyzed by analytical polyacrylamide gel electrophoreses (data not shown). Antisera raised against rat muscle AChR cross-react very well with AChR from electric organs. Thus, an important fraction of antibodies to mammalian AChR are directed against phylogenetically conservative determinants of AChR.

These findings prompted us to use Torpedo AChR for our further studies, since the electric organ is a rich source of AChR, which can be prepared in its native, non-proteolyzed form. In order to characterize the chronic EAMG model, the kinetics of the antibody response elicited with Torpedo AChR was determined.

Antibody titers against Torpedo AChR and rat AChR were measured in 4 rats at several time points after immunization with 15 μ g Torpedo AChR incorporated in complete Freund's adjuvant (CFA). The primary immune response reached a maximal antibody concentration about 20 days after immunization (fig. 1A). The titer against rat AChR ranged between 0.5 to 1% of that to Torpedo AChR with an immunizing dose of 15 μ g Torpedo AChR. The kinetics of the immune response show that the titer of

Fig. 1: Kinetics of the immune response against Torpedo and rat AChR. These rats were immunized once with 15 μg Torpedo AChR. Anti Torpedo AChR (panel A) and anti rat antibody titers (panel B) were measured at given time intervals after immunization. Note the difference in scale between anti-Torpedo and anti-rat antibody titers.

Table I: Cross reactive antibody and acetylcholine receptor immunogenicity

Acetylcholine receptor	no. rats	Antibody titer in nM/l		
		Torpedo	fetal calf	rat
Torpedo	4	54.6 ± 8.2	N.D.	.30 ± .08
Fetal calf	5	16.9 ± 9.0	28.1 ± 1.0	5.3 ± 3.0
Rat	4	.45 ± .11	.68 ± .02	.84 ± .13

Female LEW rats, 8 to 12 weeks of age, were immunized with 1 μ g of AChR given on days 1 and 30; animals were bled on day 35 and antibody to AChR was quantitated (mean $\pm$ S.D.). Anti-Torpedo AChR antibody titers were divided by 1.7 to correct for the presence of dimers.

antibodies against rat AChR is still increasing at day 35 whereas anti Torpedo AChR antibodies reach a plateau at day 20 after immunization (fig. 1B).

The differences in kinetics between serum antibodies against Torpedo AChR and self AChR suggest that cross-reactive antibodies are produced later in time. It cannot be excluded that the delay is due to AChR at the neuromuscular endplate acting as an *in vivo* immunoabsorbent.

4.3.2 Binding of antibody cross-reactive with muscle AChR to the endplate

The differences in kinetics of antibodies to Torpedo and muscle AChR suggested that cross-reactive antibody bound to the neuromuscular endplate might be related to the pathogenesis of the myasthenia. Therefore the next experiment was designed to relate the presence of (cross-reactive) antibody bound to the neuromuscular endplate with signs of myasthenia gravis.

Results and comments.

A group of nine animals was immunized twice with 15 μ g AChR incorporated in CFA with an interval of 30 days. Five days after the booster injection only one animal (no. 1) showed discrete signs of myasthenia clinically. All animals were sacrificed 5 days after the booster injection. As seen in table II the amount of AChR extracted from the myasthenic rats' muscles is reduced by 60% compared to the AChR concentration in rats injected with CFA alone. About one half of the AChRs in the myasthenic rats' muscles are complexed with antibody.

It is clear from this study that (cross-reactive) antibody is demonstrable at the neuromuscular junction complexed to the receptor; this antigen-antibody interaction at the neuromuscular endplate is associated with a marked decrease in acetylcholine receptor concentration with or without clinical signs of myasthenia. The receptor loss is brought about by antibody mediated increased receptor turnover (6, 7) and antibody

Table II Rats with chronic EAMG* have reduced amounts of extractable AChR in their muscles.

Animal number	AChR concentration per animal (pmol) ^a	% AChR loss ^b	Antibody-AChR ^c complexes (pmol per rat)	Serum anti rat AChR titer (n Mol/l)
1	20	72	13	25
2	25	65	10	43
3	32	56	22	13
4	38	49	21	79
5	27	64	10	110
6	22	69	15	102
7	37	50	16	104
8	38	49	22	36
9	24	67	22	119
$\bar{m} \pm S.E.$	29 ± 2.4	60 ± 3.1	17 ± 1.7	70 ± 14

*) All animals were immunized twice with 15 μ g AChR incorporated in CFA at day 1 and day 30. The rats were sacrificed 5 days after the last booster injection. Only one rat (no. 1) showed signs of myasthenia clinically.

^a) The AChR concentration of age matched control rats injected with CFA alone is 71 ± 3 pmol (n = 4).

^b) Amount of AChR loss versus control animals.

^c) Amount of anti AChR antibody complexed with AChR.

mediated complement dependent receptor destruction (8, 9, 10). It is clear from this experiment that the biochemical quantitation of receptor loss rather than the clinical symptoms is a better parameter for establishing the presence or absence of EAMG, for clinically myasthenia is only apparent when over 70% of the receptors are lost, whereas biochemically all animals suffered from substantial losses of AChR. Results obtained in this experiment are in agreement with experiments of Lindstrom et al.(11, 12) using eel acetylcholine receptor. This was to be expected since the antigenic structure of AChR from electric eel and *Torpedo californica* is very similar (13).

4.3.3 Dose dependency of the immune response against AChR and myasthenia

In the previous experiment an immunizing dose of 15 μ g *Torpedo californica* AChR given twice, 30 days apart, caused invariably a loss of AChR at the neuromuscular junction when measured 5 days after the booster injection. We hold this receptor loss to be brought about by antibody cross reactive with rat AChR. The finding at the time of sacrifice of appreciable amounts of AChR at the neuromuscular junction not covered with antibody is not in contradiction with this view; for the presence of high titer crossreactive antibody in the serum cannot be taken to indicate that conditions of relative antibody excess have to be present at the site of the endplate. This is so because

transcapillary passage of IgG molecules, their transit through the negative charged interstitial colloidal matrix (14), the capacity of the neuromuscular endplate to interiorize antigen/antibody complexes and generate new receptors are all (unknown) variables which determine at the site of interstitial fluid bathing the neuromuscular junction conditions of relative antibody excess. It is clear from direct measurements that the protein concentration of the interstitial fluid of the limb musculature is well below that of serum and about 1 to 2 gram per liter (15) with — compared to serum — an increase in the albumin/globulin ration (16).

For this reason we decided to test the hypothesis that EAMG is mediated by crossreactive antibody in an indirect way by means of a dose response curve. Assuming that the "local factors" alluded to above are rather fixed variables, a direct relationship between the concentration of crossreactive antibody in the serum and a loss of AChR at the neuromuscular junction would support this hypothesis that myasthenia is brought about by crossreactive antibody.

Results and comments.

Fifteen rats, divided in 3 groups of 5 animals were immunized once with respectively 5, 10 and 20 μg Torpedo AChR incorporated into CFA. Serum titers of anti Torpedo AChR antibody were determined by radioimmunoassay on days 30, 37 and 41 after the initial immunization. The serum antibody titers against Torpedo and rat AChR increased with the amounts of AChR used for immunization (fig. 2) and reached a plateau between 30 and 41 days after immunization. In some animals the antibody titer was already declining at day 41. The animals were sacrificed 41 days after the initial immunization except for one animal (no.14) which died at 39 days from severe myasthenia. All animals had reduced amounts of AChR in their endplates indicating that all rats had albeit varying degrees of myasthenia (fig. 3). The loss of AChR depended on the dose of immunogen used; animals immunized with 10 μg of Torpedo AChR showed 1.5x more loss of AChR at the neuromuscular junction compared to those rats injected with 5 μg . ($P < 0.05$). Animals immunized with 20 μg showed a receptor loss exceeding that of 10 μg with one animal dying from severe myasthenia indicating a receptor loss well over 70% (n.s.). That the receptor loss was mediated by crossreactive antibody and dependent on the total amount of circulating crossreactive antibody present is suggested by finding a direct relationship between anti-AChR antibody bound at the neuromuscular junction and total circulating antibody (fig. 4.). These results prove a direct relationship between cross-reactive antibody in the intravascular compartment and local binding capacity at the site of the endplate over a wide range of serum antibody concentrations. These results are compatible with the view that cross-reactive antibody mediated the myasthenia.

Fig. 2: Kinetics of the immune response against Torpedo AChR in animals with chronic EAMG. Three groups of five animals were immunized with 5 (A,B), 10 (C,D) and 20 μ g (E,F) of Torpedo AChR respectively. Antibody titers against Torpedo AChR (A, C, E) and rat AChR (B, D, F) were assayed at the indicated time points after immunization.

Fig. 3: Comparison between the immunizing dose of AChR and the severity of myasthenia. Three groups of five rats immunized with 5, 10 and 20 μg AChR respectively were sacrificed 42 days after immunization with Torpedo AChR. Loss of AChR from muscle was used as an index of severity of EAMG. The asterisk indicates an animal that died from EAMG, it was assumed that AChR loss is greater than 70%.

Fig. 4: Comparison between the concentration of antibody to AChR in serum and the amount of antibody bound to muscle AChRs *in vivo*. The same rats were studied as in figure 3.

4.4. THE ROLE OF THE MAIN IMMUNOGENIC REGION IN EAMG

In order to relate cross-reactive antibody specificity to pathogenicity the Torpedo californica AChR was first altered biochemically in such a way that deletion of antigenic sites within the main immunogenic region or outside was accomplished. Next, these altered AChR were assayed *in vivo* for immunogenicity and pathogenicity.

4.4.1 The antigenic anatomy and physicochemical characteristics of biochemically altered AChR

Native Torpedo AChR was reduced and carboxymethylated (RCM-AChR) as described in material and methods section (p.151). Torpedo AChR was also subjected to mild proteolysis with trypsin (t-AChR) in such a way (see material and methods) that the subunit structure as well as the molecular morphology (17) does not change. In order to show RCM-AChR was still intact after reduction and carboxymethylation native and RCM-AChR were labeled with I^{125} (see material and methods) and analyzed for molecular weight by exclusion chromatography using high performance liquid chromatography on a TSK 4000 column with the appropriate precolumn (Beckman). As seen in fig. 5A/B I^{125} -native AChR consists mainly of AChR dimers (D) with some monomers (M); the dimer peak is preceded by some aggregates (between 15 and 20 minutes). I^{125} RCM-AChR shows essentially the same profile but contains somewhat more aggregates and degradation products (fig. 5B). Thus, reduction and carboxymethylation does not dissociate AChR into its subunits. In order to analyse the antigenic differences between native AChR and RCM-AChR we used a panel of 29 monoclonal antibodies (see material and methods section, p.159). These monoclonal antibodies were derived from rats immunized with native, SDS denatured individual subunits of AChR. The specificities of some of these monoclonal antibodies were already determined by Tzartos et al. in terms of their reactivity with the MIR, isotype and crossreaction with mammalian AChR and is specified in the material and methods section.

Results and comments.

The mapping experiments were performed by radioimmunoassay (see material and methods section) at an AChR concentration of 1 nM and antibody concentrations of 3 nM in 1 ml 0.5% Triton X-100-PBS- $NaNa_3$. The results are expressed as:

$$\frac{\text{net cpm } ^{125}\text{I-}\alpha\text{-bungarotoxin-RCM-AChR precipitated}}{\text{net cpm } ^{125}\text{I-}\alpha\text{-bungarotoxin native AChR precipitated.}} \times 100$$

Parallel experiments were performed using AChR subjected to partial proteolysis using trypsin. The results with t-AChR were likewise expressed as:

$$\frac{\text{net cpm } ^{125}\text{I-}\alpha\text{-bungarotoxin-t-AChR precipitated}}{\text{net cpm } ^{125}\text{I-}\alpha\text{-bungarotoxin-native AChR precipitated.}} \times 100$$

As seen in fig. 6 (right panel) 9 out of 29 monoclonal antibodies bound well to RCM-AChR, three bound moderately and 17 did not bind to RCM-AChR. The polyclonal antibody, which was obtained by pooling the serum of a large number of rats with EAMG, precipitated only 40% of the ^{125}I -RCM-AChR. Thus in the polyclonal

Fig. 5: HPLC profile of native AChR (panel A) and reduced and carboxymethylated AChR (RCM-AChR, panel B). A 20 μ l sample was injected and eluted with 0.2% cholate-PBS buffer pH 7.4 with a flow 0,4 ml/min. Absorption was set at 0.01 AUFS.

Symbols: D: AChR dimers, M: AChR monomers.

response against native AChR only 40% of the antibodies were directed against RCM-AChR.

Surprisingly the results obtained with trypsinized-AChR (t-AChR) are the opposite of those with RCM-AChR with an exception for the determinants recognized by monoclonal antibody 61 that is absent on both t-AChR and RCM-AChR. Fifteen monoclonals bind well, one moderate and 13 do not bind t-AChR (fig. 6, left panel). Seventy five percent of the polyclonal antibodies reacted with t-AChR.

Fig. 6: Analysis of antigenic structure of t-AChR (left panel) and RCM-AChR (right panel) by means of a panel of monoclonal antibodies. The relative amount of monoclonal or polyclonal (EAMG serum) antibody bound to either t-AChR and RCM-AChR is expressed as a percentage compared to the binding of that antibody to native AChR.

The combined results obtained with RCM-AChR and t-AChR are interesting in several aspects. First, reduction and carboxymethylation — a procedure that destroys conformational dependent determinants — has an opposite effect compared to trypsinization, a procedure that probably removes linear polypeptides in between conformational dependent determinants. Second, RCM-AChR lacks the MIR whereas t-AChR probably contains the MIR only. Finally, the results obtained with polyclonal antibodies suggest that 75% of the polyclonal antibody response is directed against the MIR which is in agreement with monoclonal antibody blocking experiments reported by Tzartos et al. (18, 19).

4.4.2 Immunogenicity and pathogenicity of RCM-AChR

In order to assess the importance of the main immunogenic region (MIR) on AChR in the rat model for EAMG, female LEW rats were immunized with Torpedo RCM-AChR in CFA and the crossreactive antibody in the serum, as well as receptor density at the neuromuscular junction was measured.

Results and comments.

Female LEW rats (no. 1 through 5) were immunized with 15 μ g Torpedo RCM-AChR in complete Freund's adjuvant on day 1 and 30; positive controls were female rats immunized with native Torpedo AChR (rats no. 6 through 8) and negative controls were rats (rats no. 9 through 11) immunized with CFA only (see table III). Animals were sacrificed at 42 days after the primary immunization.

Rats immunized with RCM-AChR showed serum antibody concentrations against Torpedo and rat AChR of $2.7 \pm 4 \mu$ M and 353 ± 69 pM respectively. The antibody concentration of antibodies crossreactive with rat AChR of rats immunized with native Torpedo AChR was 760 ± 41 pM. Thus, deletion of the MIR reduced the crossreactive antibody elicited by (denatured) Torpedo AChR about 50%. None of the rats immunized with RCM-AChR showed acetylcholine receptor loss (table IV), although compared to animals immunized with native Torpedo AChR greater quantities of antibodies were complexed to the receptor at the neuromuscular junction. ($p < 0.02$)

Surprisingly rats immunized with native as well as denatured (RCM-AChR) Torpedo AChR show crossreactive antibody bound to the neuromuscular junction; this is associated with receptor loss (and thus EAMG) in rats immunized with native AChR only. These results can be interpreted in several ways. First, the crossreactive antibodies elicited by RCM-AChR bind to sites outside the MIR on the extracellular side of AChR, since RCM-AChR lack this antigenic determinant. Antibodies as well as MABs with this specificity are less likely to cause receptor aggregation (20). The results of Bartfeld and Fuchs (24) who found a protective effect in rabbits on EAMG by prior immunization with RCM-AChR are compatible with this view. The second explanation is that the crossreactive antibodies elicited by RCM-AChR bind to intracellular determinants not accessible *in vivo* (22). In this view crossreactive antibodies raised against

Table III RCM-AChR does not induce EAMG

Rat no.	AChR type		AChR conc/ pmol/animal	% AChR loss	Anti-AChR complexed pmol/animal	% ab complexed to AChR
	1st injection	Boost				
1	RCM	RCM	63	0	21	33
2	RCM	RCM	74	0	24	37
3	RCM	RCM	73	0	30	41
4	RCM	RCM	75	0	36	48
5	RCM	RCM	80	0	43	53
6	native	native	38	36	8,7	23
7	native	native	31	47	14	45
8	native	native	24	59	13	54
9	CFA	CFA	69	0	0	0
10	CFA	CFA	55	0	0	0
11	CFA	CFA	53	0	0	0

Female LEW rats were immunized at days 1 and 30 and sacrificed 42 days after the 1st injection. Both native and RCM AChR were given in complete CFA 15 μ g per immunization.

denatured receptor and residing in the interstitial fluid binds to the cytoplasmic side of AChR during the extraction procedure: the binding of anti-RCM-AChR to muscle AChR is therefore an *in vitro* artefact. Finally, but less likely, the receptor loss in EAMG is mediated by immune cells and not antibody. In order to refute or confirm the hypotheses outline above an additional series of experiments was performed.

4.4.3 RCM-AChR does not protect against EAMG

Ten female LEW rats were immunized with 15 μ g of RCM-AChR in complete Freund's adjuvant; after 30 days 5 rats received a booster injection of 15 μ g of RCM-AChR (group I) and the other 5 rats received a booster of 15 μ g of native AChR (group II). Animals were bled every week from the tail vein and the antibody titer to native Torpedo AChR was measured and found to be identical in both groups of rats (fig. 7). At the time of sacrifice the animals in group I had no receptor loss in contrast to the animals in group II (table IV), who showed a receptor loss of about 25%. Both groups had comparable amounts of crossreactive antibody bound at the neuromuscular junction.

These results show that prior immunization with RCM-AChR does not protect against EAMG induced with native Torpedo AChR. Also immunization with RCM-AChR does not in our hands protect against an ongoing EAMG as claimed by others using rabbits (18) ($66 \pm 7,5\%$ AChR loss in animals with ongoing EAMG injected with 15 μ g RCM-AChR in CFA versus $67 \pm 5,8\%$ AChR loss for control animals injected with 15 μ g native AChR in CFA.)

Fig. 7: Time course of anti-Torpedo AChR antibody titers in rats immunized with their RCM-AChR (open circles) or with RCM-AChR and native AChR (closed circles). The booster injection was given on day 28.

Table IV RCM-AChR does not protect against EAMG

Group	Immunogen (AChR)		% AChR loss (m ± S.E.)	% AChR complexed with ab (m ± S.E.)
	1st	2nd		
Group I	RCM	RCM	0 ± 4	42 ± 3.6
Group II	RCM	native	25 ± 5	55 ± 6.4

Two groups of rats were immunized with 15 µg of RCM-AChR in CFA. A second injection with either RCM-AChR (group I) or native AChR (group II) was given 25 days after the initial immunization. Animals were sacrificed on day 42.

4.4.4 Cross-reactive antibody elicited by RCM-AChR binds to *in vivo* non-accessible antigenic sites on rat AChR

In order to relate the lack of pathogenicity of RCM-AChR to antibody specificity we analyzed if anti-RCM antibody bound to extracellular parts of AChR incorporated in native membranes or to buried determinants.

Table V Specificities of antisera^a against RCM and native AChR

serum	immunogen	titer (nM)	% of antibody activity that	
			binds to membranes ^b	remains in supernatant ^c
1	RCM-AChR	430	23	87
2	RCM-AChR	320	39	61
3	RCM-AChR	320	29	71
4	RCM-AChR	470	34	66
5	native AChR	99	88	12
6	native AChR	370	70	30
7	native AChR	83	79	21
8	native AChR	380	78	22

^a) Antisera against RCM or native AChR diluted 1/20 were incubated with an approximately 100-fold excess of native Torpedo membranes (vesicles)

^b) The antibody activity absorbed by Torpedo membranes

^c) The antibody activity not absorbed by Torpedo membranes

Serum (20 μ l) from rats immunized with RCM-AChR (15 μ g) or native AChR was diluted (1/20) in PBS-BSA-NaN₃ (10 mM Na phosphate, 5 mg/ml Bovine serum albumin, 10 mM azide) and incubated with an excess of native Torpedo membranes (30 μ mol) in a total volume of 100 μ l overnight at 4°C. The antibody activity in the supernatant was determined by radioimmunoassay. The amount of antibody remaining in the supernatant was compared to that of a serum sample to which no membranes had been added.

It is clear from table V that most of the anti RCM antibody activity (69%) does not bind to native membranes and is therefore directed to determinants buried in the membranes or to cytoplasmic surface of AChR. In contrast, the antibody activity against native AChR was absorbed by native membranes and thus directed to extracellular parts of AChR.

These results suggest that RCM-AChR is not pathogenic because its most immunogenic parts are located on the cytoplasmic surface of native AChR. Consequently, the majority of antibodies against RCM do not bind to AChR *in vivo* and can only bind to solubilized AChR which is freed from its surrounding membrane. The majority of anti-native AChR antibodies bind to the MIR located on the extracellular part of AChR (23). Similar results were obtained by Froehner with polyclonal antisera and monoclonal antibodies against sodium dodecyl sulfate denatured receptor or its subunits (22). Only 10-20% of the determinants recognized by these antisera are accessible to antibodies when the receptor is membrane bound (22). Most of the monoclonal antibodies to denatured AChR bound to AChR only when membranes were made permeable with saponin (24, 25).

4.4.5 Passive transfer of EAMG by monoclonal antibody

The previous experiments show that crossreactive antibody elicited by RCM-AChR binds to the cytoplasmic side of the AChR which is *in vivo* inaccessible. Thus immunization with RCM-AChR does not cause receptor loss and the crossreactive antibody bound to the rat AChR must be regarded as *in vitro* phenomenon or — in terms of pathogenesis of disease — as an "artefact". In order to substantiate this view monoclonal antibodies directed against RCM-AChR (no. 157, 158, 164) and against the MIR (no. 35) were infused into LEW rats; 48 hours later they were sacrificed and the receptor concentration was determined. Only rats infused with monoclonal antibody directed against the MIR (table VI, rats 10, 11, 12 group D) had AChR loss from muscle. No significant amounts of ab — above background IgG — were found complexed to the receptor regardless of the monoclonal antibody used. This is probably due to the fact that relatively small quantities of antibody (40 μ mol) were infused, compared to total AChR receptor present in rat muscle (about 70 μ mol)

These results are consistent with the findings of Froehner and Sargent that antibodies to denatured AChR, and as we have shown RCM-AChR, bind to cytoplasmic domains of AChR (21, 22). These results explain why RCM-AChR does not induce EAMG.

Table VI Passive transfer of EAMG with monoclonal antibody against AChR

Group	MAB	specificity	amount inj. (pmoles anti rat AChR ab)	animal no.	(AChR) fmol/g muscle
A	157	RCM-AChR	100	1	0.46
				2	0.48
				3	0.45
B	158	RCM-AChR	150	4	0.41
				5	0.46
				6	0.49
C	164	RCM-AChR	100	7	0.42
				8	0.55
				9	0.47
D	35	native AChR (MIR)	40	10	0.28
				11	0.25
				12	0.30
E	saline	—	—	13	0.53
				14	0.54
				15	0.49

Monoclonal antibodies were infused into female LEW rats. Rats were sacrificed 48 hours later and AChR was quantitated.

4.5. PASSIVE TRANSFER OF EAMG WITH IMMUNE LYMPHOCYTES

Although EAMG can be passively transferred by polyclonal (26) as well as monoclonal antibody (18, 27, 28) a role of immune lymphocytes in terms of effector cells (e.g. cytotoxic T or delayed type hypersensitivity cells) in this model of chronic EAMG has not been ruled out. Attempts by Lennon et al. to transfer rat EAMG passively with 6×10^6 cells were only partially successful and no antibody to muscle AChR could be detected (20) suggesting that antibody was required for producing EAMG. These findings of Lennon et al. in EAMG can be put in juxtaposition with those of Panitch who transferred experimental autoimmune encephalomyelitis to LEW rats with small numbers (2×10^6) of *in vitro* cultured immune lymphocytes provided these cells were cultured in presence of myelin basic protein or in the presence of a T lymphocyte mitogen: concanavalin A.(30) Thus in the autoimmune encephalomyelitis model, which is known to be mediated by immune T lymphocytes (31), passive transfer with immune lymphocytes is possible and cloned T cell lines, which are also effective in this regard (32) are only needed for determining fine specificities.

Using the approach of Panitch et al. we reexamined the issue of cellular immunity in EAMG by means of passive transfer with *in vitro* cultured immune lymphocytes using AChR, or T cell mitogens (concanavalin A) or B cell mitogens (lipopolysaccharide (LPS) and dextran sulfate (DS) to expand the *in vitro* cultured immune lymphocytes. Immune cells were isolated from the lymph nodes of rats with chronic EAMG induced by immunizing LEW rats with 15 μ g of Torpedo and cultured under conditions as described for the *in vitro* proliferation assays (Chapter III, pg.54) as follows. Immune lymph node cells (20×10^6) were cultured in 10 ml medium (5% FCS/RPMI/1640) supplemented with penicillin, streptomycin, and 5×10^{-5} M mercapto-ethanol. Torpedo AChR was added at a concentration of 0.5 μ g/ml, a concentration previously found to be optimal for *in vitro* stimulation (Chapter III, p. 54). Alternatively concanavalin A (2 μ g/ml), or LPS/dextran sulfate (50 and 25 μ g/ml respectively) were added instead of AChR. Cells were next cultured for 3 days (ConA or LPS/dextran) or 5 days (AChR) at 37°C in 5% CO₂ and 95% air, harvested, washed in medium to remove mitogens or AChR, and next transferred passively into 10 to 12 week old rats. The recovery of cells thus cultured varied from 50 to 60%. The sera of the recipient rats were assayed for anti AChR antibody.

Results and comments.

Immune lymph node cells were expanded *in vitro* with Torpedo AChR, or T cell mitogen (concanavalin A) or B cell mitogen (LPS/dextran sulfate) and next varying numbers of expanded cells were passively transferred to groups of two to three female LEW rats. The antibody response of the recipients was monitored over a 50 day period and rat muscle AChR was quantitated at 52 days after passive transfer. Clinical myasthenia was not observed in this passive transfer system regardless of the way the immune cells were expanded. Only in the presence of AChR (table VII) in the *in vitro*

Table VII Passive transfer of EAMG with *in vitro* expanded immune lymph node cells

no. cells	no. anim.	<i>in vitro</i> expansion			antibody titer (nM/l) at 7 days	% AChR loss
		AChR	ConA	LPS/DS		
10x10 ⁶	2			+	20	ND
20x10 ⁶	2			+	54	ND
40x10 ⁶	2			+	61	ND
10x10 ⁶	3		+		<0.5	0
1x10 ⁶	3	+			41	ND
10x10 ⁶	2	+			400	1,22
20x10 ⁶	2	+			560	28,31
40x10 ⁶	2	+			560	38,28

Immune lymph node cells were taken from rats immunized with 15 μ g of Torpedo californica AChR and cultured *in vitro* for 5 days with Torpedo AChR (0.5 μ g/ml) or T cell mitogen (Concanavalin A) or B cell mitogens (LPS/DS) for 3 days prior to passive transfer. In the recipients antibody to Torpedo AChR was assayed after 7 days. None of the recipients developed myasthenia gravis clinically over a period of 52 days. Controls included passive transfer of 60x10⁶ immune lymph node cells not expanded and passive transfer of 40x10⁶ immune lymph node cells expanded with AChR but sonicated prior to transfer. Neither of these procedures yielded measurable quantities of antibody on the recipients.

cultures significant quantities of anti-AChR antibodies were produced provided sufficient numbers of cells (10x10⁶ or more) were transferred. These antibody titers declined linearly after passive transfer (fig. 8). At 52 days significant receptor losses were observed in the animals that had produced antibody. The controls excluded that the results observed were due to passive transfer of AChR or antibody.

These results show that EAMG can be passively transferred provided the immune lymph node cells are expanded *in vitro* with antigen (AChR). It is also clear that in absence of the immunogen the passively transferred response "burns out" in the (virgin) recipients: thus in the model of chronic EAMG the self AChR is not capable of expanding immune T helper (and thus primed B) cells. The results do not suggest that — like in experimental allergic encephalomyelitis — delayed type hypersensitivity cells or cytotoxic effector T cells play a role.

4.6. DISCUSSION

The series of experiments described in this chapter provides new evidence in favour of antibody in the pathogenesis of chronic EAMG. First, a relationship was shown between the severity of the receptor loss and the (intravascular) concentration of antibody directed to the rat AChR. This relationship is a "rough" one since the intravascular

Fig. 8: Time course of anti-Torpedo AChR antibodies in rats receiving *in vitro* expanded immune lymphocytes from animals with EAMG. Normal Lewis rats were injected i.p. with 10×10^6 (●), 20×10^6 (○), 40×10^6 (▲), or 60×10^6 (□) immune lymphocytes cultured for 5 days in the presence of AChR. Anti-Torpedo AChR antibody concentration was measured at the indicated time points.

immunoglobulin concentration differs from the interstitial one (15), but more importantly because we have shown that native Torpedo AChR elicits antibody which binds both to the extracellular and cytoplasmic sites of the AChR (section 4.4.5.). Only the antibodies binding to the extracellular sites, however, can be considered to be of pathogenetic significance for two reasons.

Immunization with Torpedo AChR stripped of the main immunogenic region (MIR) by reduction and carboxymethylation (RCM-AChR) elicited antibodies which bound to rat AChR *in vitro* but failed to cause receptor loss *in vivo*, because all antibody bound to the cytoplasmic *in vivo* non accessible site of the AChR. This was confirmed by passive transfer studies with monoclonal antibodies directed against native and RCM-AChR, which showed receptor loss only with monoclonal antibody directed to the extracellularly located MIR but not with monoclonal antibody directed against RCM-AChR (section 4.4.5.). These results agree in part with the findings of Bartfeld and Fuchs (21) on the failure to elicit EAMG in rabbits injected with RCM-AChR. They do not substantiate the other observations of Bartfeld and Fuchs et al. (21), who showed a protective effect in only two rabbits of immunization with RCM-AChR on the development of EAMG (21). Our results do agree with work of others who hold antibodies to the MIR to be of particular pathogenetic importance in EAMG (18, 23). In our view the failure of RCM-AChR to protect against EAMG can best be explained by a carrier effect. This view holds native AChR to contain a "carrier protein" and a "MIR" hapten. Since RCM-AChR contains only the carrier, preimmunization cannot be expected to prevent an immune response to the hapten in the absence of induction of tolerance (to the carrier); on the contrary carrier priming enhances the immune response to a hapten (33).

The second set of arguments in favour of antibody mediating chronic EAMG derives from passive transfer studies with immune lymph node cells. Using techniques identical to others in studying experimental allergic encephalitis (30) we failed to transfer EAMG with concanavalin A expanded immune lymph node cells. Provided immune lymph node cells were expanded *in vitro* with AChR, however, disease could be transferred with immune lymph node cells. Since antibody was produced in the recipients, these passive transfer studies favour T helper cells — together with primed B cells — mediating the effects. This is in agreement with our *in vitro* work (chapter III). This evidence is preliminary, however, since the expanded immune lymph node cells were not dissected into various T cell subsets and B cells prior to passive transfer.

Wekerle et al. using cell lines derived from lymphocytes of rats with EAMG, have shown that their lines express the T helper cell phenotypes. These cells were effective in inducing EAMG when injected along with primed B cells and AChR in CFA (34). In our lymphocyte transfer we did not need to inject AChR in CFA to induce disease. This can best be explained by the fact that our *in vitro* activated lymphocytes crossreact with the rat AChR, whereas the cell lines of Wekerle et al. only react with Torpedo AChR (personal communication). Interestingly the antibody titers produced by *in vitro* expanded, passively transferred, immune lymph node cells in virgin recipients decreas-

ed gradually after transfer. These data suggest that the autologous AChR does not expand these primed cells *in vivo*. This observation agrees with our hypothesis (Chapter III, pag.78) that probably no tolerance exists to membrane antigens that do not circulate in the extracellular fluid. This implies that the passively transferred immune lymphocytes are not expanded by circulating antigen. Apparently mammalian (c.q. rat) AChR is recognized as "foreign" which results in a vigorous immune response with a high percentage (section 4.2.1.) of antibodies crossreacting with Torpedo AChR suggesting that the immune response is directed to a group of restricted highly conservative determinants to which no tolerance exists; the region has been defined as the main immunogenic region on Torpedo and mammalian AChR (18).

The experiments in this chapter support the three stage model of pathogenesis of myasthenia gravis of Wekerle (34). This author proposed that the primary event in the development of MG is the appearance of myogenic cells expressing AChR on their cell membrane in the thymus. In a second step, intrathymically developing T cells are sensitized against AChR after appropriate presentation by antigen presenting cells. Then these cells migrate to peripheral lymphoid organs where they cooperate with AChR specific B cells to induce autoantibody synthesis (some of these autoantibodies can also be synthesized within the thymus). The immunopathological lesions observed at the neuromuscular membrane are brought about by these autoantibodies against AChR.

4.7 SUMMARY AND CONCLUSIONS

In this chapter the immunogenicity of differing AChR preparations were related to their *in vivo* pathogenicity. For these purpose three protocols were used, each allowing the analysis of differing pathogenetic mechanisms.

1. EAMG induced by active immunization with AChR in CFA.

We first established the kinetics of the experimental *in vivo* model. The immunizing dose of native Torpedo AChR was proportional to the amount of circulating antibodies against both Torpedo and self AChR (rat), and the amount of antibody bound at the site of the neuromuscular junction. Removal of the MIR (RCM-AChR) did slightly reduced the immunogenicity but abolished pathogenicity. In animals immunized with RCM-AChR, lacking the MIR, most of the serum antibodies were shown to be directed to cytoplasmic, *in vivo* non accessible sites of muscle AChR. In contrast to suggestions in the literature preimmunization of rats with RCM-AChR failed to protect against EAMG induced by immunization with native AChR nor was an ongoing EAMG cured by immunizations with RCM-AChR.

2. Induction of EAMG by passive transfer with monoclonal antibodies.

The data obtained by active immunizations with RCM-AChR were extended using monoclonal antibodies against native and RCM-AChR. Only antibodies directed to the

extra-cellularly located MIR (present on native AChR but absent on RCM-AChR) were capable of causing AChR loss from muscle.

3. Induction of EAMG by passive transfer of immune lymphocyte.

In order to demonstrate that this crossreactive immune response was mediated by antibody only passive transfer studies were performed using *in vitro* expanded immune cells isolated from lymph nodes of animals with EAMG. *In vitro* expansion of immune T cells with T cell mitogen (in a manner identical to that used for passive transfer of effector T cells in experimental allergic encephalitis) failed to induce an antibody response to AChR in the recipient. Passive transfer was only successful when the immune cells were expanded *in vitro* in the presence of AChR; the receptor loss observed was mediated by actively secreted and crossreactive antibody. The autoantibody levels in the serum of the recipients of passively transferred cells decreased rapidly suggesting that the autologous AChR is not capable of expanding these primed T (helper) cells possibly because the membrane antigens residing on the receptor do not circulate in the extra cellular fluid.

The studies described in this chapter therefore support the view that chronic EAMG is an antibody mediated disease requiring the continuous presence of immunogen and primed helper T cells.

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CHAPTER V

Anti-AChR antibody - Acetylcholine receptor interactions: Analysis of mechanisms that influence the severity of myasthenia

5.1 INTRODUCTION

Myasthenia gravis (MG) and its experimental animal model are autoimmune diseases caused by antibodies to AChR in muscle that impair neuromuscular transmission resulting in muscular weakness and fatigue (1). In both MG and EAMG the primary factors impairing neuromuscular transmission is antibody-induced loss of AChR. AChR loss occurs by both antigenic modulation (2-4) and complement mediated focal lysis of the neuromuscular endplate (5-7). Conceptually the anti-AChR antibody AChR interactions in chronic EAMG are influenced by antibody specificity and concentration, and target cell characteristics including the density of antigenic epitopes on the receptor as well as the capacity of the cell to regenerate AChR.

It was shown in chapter IV that the endplate in chronic EAMG contains AChR not complexed with antibody, in spite of conditions of marked antibody excess to rat AChR suggesting that antibody specificities and target organ characteristics must be important for the development of myasthenia. The problem of antibody specificity in EAMG has been approached by blocking assays using libraries of monoclonal antibodies (8, 9), by passive transfer of monoclonal antibodies (8, 10, 11) or by means of immunizing with altered AChR (chapter IV) (12). All of these indirect approaches suggest that in acute and chronic EAMG as well as myasthenia gravis antibodies directed to a small area on the extracellular surface of the α -subunit, the so called main immunogenic region (MIR) are important in the pathogenesis of disease. These approaches have not ruled out however that low concentrations of antibodies directed to other antigenic epitopes on the extracellular surface of AChR outside of the MIR might not contribute to disease; e.g. the question whether or not antibodies directed to the acetylcholine binding site -which is located outside of the MIR on the α -subunit- exist remains a topic of debate (1, 13). For this reason we have approached the problem of antibody specificity in two ways.

First in a direct approach we assayed sera from animals with chronic EAMG for the presence or absence of antibodies directed to the acetylcholine binding site on the receptor since antibodies with such a specificity would be of crucial importance in terms of pathogenesis of disease.

Secondly, we approached the problem of antibody specificity indirectly by looking at antibody diversity in the sera of rats with chronic EAMG by first separating immunoglobulins on the basis of charge and next by determining antigen binding capacity of these separated immunoglobulins. By virtue of this manoeuvre low concentrations

of antibodies secreted by a small number of B cell clones possibly of importance for the pathogenesis of EAMG, might be detected theoretically (15). For clarity the literature dealing with the approaches alluded to above is cited separately under the appropriate heading in the result section.

5.2 ROLE OF ANTIBODY TO THE ACETYLCHOLINE BINDING SITE IN CHRONIC EAMG

EAMG can be induced by immunization with native AChR or with any of the four subunits composing AChR, and in animals immunized with intact AChR antibodies are produced to all subunits (16). However, studies employing monoclonal antibodies (MABs) have shown that, although rats with EAMG make antibodies to many parts of the AChR molecule, most are directed at a small part of the extracellular surface of the α -subunit, referred to as the "main immunogenic region" (8, 9). The acetylcholine binding sites are also located on the α -subunit, but are not part of the main immunogenic region (8, 9, 10). The use of MABs with known specificities as competitive inhibitors of the binding of autoantibodies from MG patients to human AChR showed that these patients have a similar pattern of anti AChR specificities as rats with EAMG, and that most antibodies from MG patients are also directed to the main immunogenic region (8,9). Antibodies of this specificity are thought to be pathogenetically important because MABs can passively transfer the complement-dependent, acute form of EAMG (8, 18, 19) as well as crosslink AChR and induce antigenic modulation of AChR (18, 20). It seems likely that these antibodies cause AChR loss by fixing complement or by crosslinking AChRs to induce AChR modulation, antibodies that directly impair AChR function competitively or noncompetitively are rarely identified (18). Recent reports however suggest that inhibition of AChR activity may be important in situations where the number of AChR is already reduced (12). Differing results have been reported in testing the ability of anti AChR antisera to inhibit α -bungarotoxin or acetylcholine binding to AChR (1, 13, 14). All these studies used immunoprecipitation techniques. In this type of assay two phenomena are analysed simultaneously: binding of antibodies to AChR and immunoprecipitation of AChR-antibody complexes. Several factors that interfere with the immunoprecipitation reaction could give false positive results for the existence of anti-acetylcholine binding site antibody. In order to answer this question we developed a solid phase assay where AChR with or without blocked acetylcholine binding site, is attached to plastic (microtiter plates) and subsequently incubated with anti-AChR antibodies in serial dilutions from animals with chronic EAMG. The binding of these antibodies was revealed by means of an enzyme labeled second antibody (see material and methods section, p.157).

Results and comments.

When 1×10^{-12} mol (in bicarbonate buffer 0.01 M, pH 9.6) AChR was incubated in microtiter wells, 44×10^{-15} mol (4.4% of the amount AChR added) effectively bound

Fig. 1 Effect of acetylcholine on ^{125}I - α -BGT binding to AChR in a solid phase radioimmunoassay (for explanation see text).

to the plastic as assessed by ^{125}I α -bungarotoxin binding sites available for binding. We next determined the amount of acetylcholine in the fluid phase necessary to block α -bungarotoxin binding. As seen in fig. 1 concentrations of $3 \cdot 10^{-4}$ M acetylcholine almost completely blocked the binding for ^{125}I α -bungarotoxin; the binding of ^{125}I α -BGT could not completely be blocked by concentrations of acetylcholine as high as 20 mM. The failure to completely block α -BGT binding to AChR can be explained by the high association constant of α -BGT (10^{14} /mol). Antibodies however, have association constants which are several orders of magnitude lower compared to α -BGT. We therefore incubated AChR coated microtiter plates with acetylcholine (5 mM), together with the acetylcholine-esterase inhibitor neostigmine bromide (Sigma Co, St. Louis), at $5 \cdot 10^{-3}$ M in order to prevent acetylcholine breakdown, and different sera taken from rats with chronic EAMG. The binding of the anti-AChR antisera was only marginally decreased in the presence of acetylcholine (fig 2). From these experiments it can be concluded that circulating antibodies against the acetylcholine binding site are present in sera from animals with EAMG, but that the titer of these antibodies is extremely low. It is conceivable that these antibodies against the acetylcholine binding site are capable to bind to AChR in the presence of high concentrations of antibodies against the MIR, since both the MIR and the binding site are present on the extracellular part of the α -subunit. It is therefore possible that antibodies against the acetylcholine binding site play a minor role in determining disease severity. This view

Fig. 2 Anti-acetylcholine binding site antibodies. Three sera from rats with EAMG were assayed for anti-AChR antibody by ELISA in the presence (dashed line) or absence of acetylcholine and neostigmine bromide.

receives some support from the literature where *in vivo* injection of monoclonal antibodies to the acetylcholine binding site produced a clinical picture differing from chronic EAMG in that these monoclonal antibodies produced an acute paralysis in chicken hatchlings (21).

5.3 THE ROLE OF ANTIBODY DIVERSITY IN EAMG

Isoelectric focusing (IEF) is a method frequently used to analyse the diversity of antibodies in terms of V-region variability involved in an immune response (15), but so far not applied to the study of EAMG. In isoelectric focusing, an electric current is applied across a continuous pH gradient, causing proteins such as immunoglobulins to run and concentrate as narrow bands at their isoelectric point (pI), the pH at which their net charge is zero. The resolution power of IEF is so high that even a single myeloma protein can be separated into several bands, representing sets of molecules altered post-translationally (deamination, glycosylation, e.a.)(22). Isoelectric focusing is therefore useful for distinguishing immunoglobulin species with different variable regions and different pI's. The problem with the technique is that when a serum contains too many molecular species, the isoelectric spectrum or spectrotyping (i.e. series of bands appearing after a Coomassie blue staining for proteins) is too complex. These problems can be partially overcome by using an ^{125}I antigen overlay technique for revealing the spectrotyping (22).

We have used IEF to analyze the diversity of anti AChR antibodies to Torpedo and muscle AChR in the sera and bound to the endplate of rats during the course of EAMG. This is important to determine because it is possible that antibody specificities which appeared pathologically unimportant because of their low concentrations in serum might very well bind to AChR in muscle and in fact be quite important in impairing neuromuscular transmission.

In view of these considerations an isoelectric focusing technique was adapted to analyze anti-AChR antibodies. In order to validate this technique we had to demonstrate first that antibodies complexed to antigen could be separated by isoelectric focusing, and next we had to determine the sensitivity and specificity of the method to detect antibodies to Torpedo AChR. Finally the isoelectric spectrum of antibodies to Torpedo and rat AChR in serum as well as at the site of the neuromuscular junction was analyzed.

5.3.1 Separation of complexed antibodies from antigens by isoelectric focusing

To analyze spectrotypes of antibodies that may have been complexed *in vivo* with their respective antigens it was necessary to develop procedures for separation of antibody from antigen prior to analysis. To determine whether a reversed electrofocusing technique could accomplish this, (see material and methods section), we examined the patterns obtained with anti-BSA antibody alone or anti-BSA complexed *in vitro* with BSA. Crystalline BSA (Calbiochem-Behring) repurified on Sephadex G150 column was used for the formation of model BSA-anti BSA complexes *in vitro*. Affinity purified IgG fraction of rabbit anti-BSA antibody was incubated with BSA at equivalence or in 5-fold antigen excess. The reaction mixture, containing trace amounts of ^{131}I labeled BSA and ^{125}I labeled rabbit anti-BSA IgG was incubated for 2 hours at room temperature and then overnight at 4°C . After centrifugation at 1000 g for 10 minutes the precipitate was washed repeatedly and resuspended with IEF sample buffer.

Results and comments.

In this experiment antigen (BSA) and antibody were trace labeled with ^{131}I and ^{125}I respectively (fig. 3, lane 1 - 3), before assessment and electrofocused separately or as antigen antibody complexes. Unlabeled antibody complexed with unlabeled antigen was focused too and next reacted with radiolabeled antigen (fig. 3, lane 4). The resulting banding pattern was identical whether or not the antibodies had initially been complexed with antigens. Thus, the IEF pattern of autoantibodies should not be altered by any complexes between these antibodies and autoantigens which might be present. Using a reverse isoelectric focusing technique, the samples migrate through a zone of acid pH ($\text{pH} = \pm 3$) and immune complexes are separated in their respective constituents. Subsequent incubation with radiolabeled antigen reveals the full spectrotrope of antibodies.

Fig. 3 Separation of constituent antigen and antibodies from immune complexes by reversed IEF. Lane 1, ^{131}I -BSA; lane 2, ^{125}I anti-BSA; lane 3, ^{131}I -BSA- ^{125}I anti-BSA complexes; lane 4, unlabeled BSA-anti BSA complexes, overlayed with ^{125}I -BSA.

5.3.2. Sensitivity and specificity of analytical IEF of antibodies to Torpedo AChR

The sensitivity and specificity of this IEF technique was determined by using monoclonal antibodies to AChR. The preparation and characterization of rat monoclonal antibodies used in this study is described in the material and method section (see addendum). Classes and subclasses of MABs to AChR were determined by radial double immunodiffusion using monospecific antisera to rat immunoglobulins kindly provided by Prof. H. Bazin (University of Louvain, Brussels, Belgium) (table II).

Table II. Characterization of MABs used for IEF

MAB number	Torpedo AChR	specificity	animal strain	Isotype
1	Torpedo AChR	α (MIR)	Lewis	IgG2b
3	Torpedo AChR	α	Lewis	IgG2b
5	Torpedo AChR	α	Lewis	IgG2b
6	Torpedo AChR	α (MIR)	Lewis	IgG1
17	Eel & Torpedo AChR	α, β	Lewis	IgG2b
22	Eel AChR	α	Lewis	IgG2b
35	Eel AChR	α (MIR)	Sprague Dawley	IgG1
42	Eel AChR	α (MIR)	Louvain	IgG2a

Results and comments.

Polyclonal antisera from rats immunized with Torpedo AChR, and monoclonal antibodies to Torpedo AChR were first electrofocused in the gel; next the gel was incubated with radiolabeled AChR, (^{125}I - α -bungarotoxin-AChR), in order to image the binding sites by autoradiography.

Even a single MAB produced a pattern of at least 6 bands (fig. 4). Thus single IEF bands cannot be thought of as a sole product of a single anti-AChR clone. Note also that the pattern of bands given by MAB 6 does not resemble the pattern of bands given by a serum pool. Yet MAB 6 is directed at the main immunogenic region and can inhibit the binding of 70% of the antibodies into Torpedo AChR (8). This indicates that there is no obvious correlation between the isoelectric points at which a particular antibody binds and its binding specificity. As little as 170 ng (1.1×10^{-12} mol) of a monoclonal antibody to AChR (MAB 6) could result in a full expression of antibody spectrotype as seen following overlay with labeled antigen (fig. 4, lane 3-6). Similar experiments with affinity purified anti-BSA showed that as little as 200 ng could result in full spectrotype expression (not shown).

The specificity of this IEF technique is also shown in fig. 4. First, normal rat serum did not bind radiolabeled AChR (fig. 4, lane 7) and, second, immune serum preadsorbed with AChR-rich membranes failed to bind radiolabeled AChR (fig. 4, lane 2) in contrast to unabsorbed serum (fig. 4, lane 1). Absorption of the immune sera with an unrelated antigen (BSA) had no effect on the binding of AChR (not shown). MABs against AChR were used to define the sensitivity and the discriminating ability of this technique (fig. 5). We found that all of the MABs tested had a restricted appearance at neutral pIs with a limited number of bands. The presence of more than one band by our MABs should not be considered as a disproof of their monoclonality. Others (22) have reported similar findings with MABs analyzed by IEF and attributed this phenomenon to carbohydrate heterogeneity, deamination and minimal amino acid sequence variability. This microheterogeneity of MABs makes these reagents less useful for the dissection of the clonal serum antibody response by IEF. Nevertheless, as expected the MABs pI ranges were included within the pIs of the polyclonal antibodies

in sera of rats with EAMG. Therefore, a panel of MABs can be taken to represent the spectrum of the serum antibody response to AChR.

5.3.3 Isoelectric spectra of antibodies to Torpedo and muscle AChR

The previous experiments showed reversed isoelectric focusing with ^{125}I labeled antigen overlay to be a specific and sensitive tool for the analysis of the anti AChR antibody spectrum. In order to determine whether early and late spectrotypes of antibodies directed to muscle and Torpedo AChR differed from each other, early and late sera were obtained from rats immunized with $15\text{ }\mu\text{g}$ of Torpedo AChR in CFA. The question of cross reactivity — antibodies binding to muscle AChR — was raised because restriction of the spectrotypes of self antibodies would suggest the involvement of a limited number of B cell clones. We determined spectrotypes in both Brown Norway (BN) and Lewis rats since preliminary results (Lindstrom, personal communication) suggested that like in experimental allergic encephalitis BN rats are rather resistant to EAMG.

Results and comments.

The isoelectric spectrum of sera derived from 4 Lewis rats with EAMG is composed of a multitude of bands observed in the range pI 6.5 to 7.5 (fig. 6). When sera from Lewis and Brown Norway rats immunized with AChR were compared, Lewis rats showed bands in the pI range 5.1 to 7.3, whereas the Brown Norway rats showed bands largely confined to the range pI 6 to 7.5 (fig. 7); although the range of pI between LEW and BN rats differed, the number of bands was similar. These results do not suggest that BN rats are poor responders in terms of anti AChR compared to Lewis. This was confirmed by immunizing BN and LEW rats with $15\text{ }\mu\text{g}$ of Torpedo AChR given twice 30 days apart. Both LEW and BN had comparable loss of AChR at the neuromuscular junction when measured 5 days after the booster injection ($67 \pm 7\%$ AChR loss).

Fig. 4 Sensitivity and specificity of analytical electrofocusing of anti-AChR antibodies. Lane 1, polyclonal unabsorbed rat anti AChR antibodies; lane 2, polyclonal serum shown in lane 1 absorbed prior to focusing with AChR-rich membranes. Lanes 3 to 6, containing decreasing amounts of monoclonal (MAB 6) antibody to AChR (lane 3, 550×10^{-12} moles, lanes 4, 11×10^{-12} moles, lane 5, 5.5×10^{-12} moles and lane 6, 1.1×10^{-12} moles). Lane 7, normal rat serum. All gels following focusing of sera or monoclonal antibodies were reacted with ^{125}I - α -BGT-AChR and then imaging was obtained by autoradiography.

Fig. 5 Isoelectric patterns of MABs to Torpedo AChR (no. 17, 42, 22, 6, 35, 5, 3 and 1). Lane 1: Five μl normal rat serum. lane 2-5: five μl concentrated tissue culture supernatants. Both were focused and overlayed with ^{125}I - α -BGT labeled Torpedo AChR.

Fig. 6 IEF patterns from our individual Lewis rats with EAMG induced by immunization with 15 μ g Torpedo AChR. Animals were bled at several time intervals after injection as indicated. Five μ l of serum from each rat was focused and reacted with 125 I- α -BGT labeled Torpedo AChR.

Fig. 7 IEF patterns of immune sera from Lewis (lanes 1-4) and from Brown Norway rats (lanes 5-8) obtained 42 days postimmunization ($15 \mu\text{g}$ Torpedo AChR in CFA). The number of bands is similar but the range of pI's differs.

As seen in fig. 8 between 30 and 45 days after immunization of rats with Torpedo AChR rather identical reactivities are observed against Torpedo AChR; when muscle AChR is used the bands appear later and are in addition fewer in number. Since we have shown in kinetic experiments (Chapter IV, pg. 86, fig. 1) using Torpedo AChR as immunogen that antibodies cross-reactive with rat AChR appear late and are present in lower titers compared to antibodies to Torpedo AChR, the differences observed in spectrotypes to Torpedo and rat AChR appear to be due to differences in concentration rather than restriction in the antibody spectrum. This interpretation is supported by an experiment in which first a serum obtained at 34 days from a rat with chronic EAMG was submitted to preparative isoelectric focusing (for details see material and methods section, p.161) next individual fractions were eluted and assayed for binding to Torpedo and rat AChR by radioimmunoassay. Antibody activities to both Torpedo and rat AChR coincide.

Our analysis of experimental sera revealed that the antibody response to Torpedo and muscle AChR was of polyclonal origin with a banding pattern characteristic for a given strain. These results indicate that genetic background not only influences immune responsiveness to AChR but it also affects the pI of the antibodies produced.

Fig. 8 Electrofocusing of sera obtained from two rats at the indicated time points following immunization with Torpedo AChR (15 μ g in CFA). Focused sera (5 μ l) were reacted with 125 I- α -BGT-labeled AChR from either Torpedo or muscle. Note the delayed appearance of anti-muscle AChR bands compared to spectrotypes against Torpedo AChR used for immunization.

Moreover, the combined results indicate that the pIs of anti-AChR antibodies are not the determining factor in their ability to bind the target organs and induce disease. During the course of immunization, a discrete expansion of the number of antibodies is seen and it takes approximately 45 days to obtain the full spectrotypes. Others (23) have shown that the peak response to a given antigen levels at 45 days which corresponds with our data. From our sequential studies, the recruitment of clones against Torpedo AChR seemed to precede the response to self AChR. This asynchrony may be more apparent than real, since our studies by preparative IEF (fig. 9) of serum antibodies show that immune activities to both Torpedo and rat muscle AChR coincide. This last finding is consistent with the idea that clones reactive with rat AChR only are not detectable and that cross-reaction between AChR from various species occurs at several determinants on the AChR molecule (1).

5.3.4 Analysis of tissue deposited antibodies

In order to determine whether certain antibody specificities are preferentially bound to muscle AChR, we examined the IEF profile of antibodies eluted from the muscles of Lewis rats with EAMG. This is important to determine since it is possible that antibody specificities are not present in serum because they have been bound to AChRs in mus-

Fig. 9 Preparative IEF of serum from a rat with EAMG. Serum was focused under the pH gradient as indicated. Thereafter, the gel was sliced into 19 bands and each band was eluted with buffer. The dialyzed eluate was subsequently assayed against Torpedo AChR and rat muscle AChR. This is a representative graph obtained out of five individual sera analyzed.

cle; these antibodies may in fact be quite important in terms of pathogenesis of disease.

The carcasses (muscle and bones) of individual Lewis rats with EAMG were homogenized in a Waring blender in two volumes 10 mM phosphate buffer pH 7.0, 100 mM NaCl and 10 mM NaN₃ (PBS-NaN₃) supplemented with protease inhibitors (see material and methods) for one minute at high speed. The homogenate was resuspended in a Waring blender in medium at low speed for 15 seconds and centrifuged at 10,000 g for 30 min. In order to remove all "trapped serum" this procedure was repeated six times. Antibody bound AChR complexes were extracted by gently shaking at 4°C in 2 volumes of 2% Triton X-100-PBS-NaN₃ in medium supplemented with protease inhibitors for 2 hrs. Insoluble material was removed by centrifugation first at low speed (10,000 g, 30 min), then high speed centrifugation (100,000 g, 30 min). The supernatant was incubated with 0.5 ml goat anti rabbit IgG coupled to Sepharose CL4B (10 mg IgG/ml of gel) overnight at 4°C. The immunoabsorbent was washed with 0.5% Triton X-100 in PBS. Finally, bound anti-AChR antibody as well as AChR were eluted with 0.1 M glycine-HCl buffer pH 2.8 and neutralized immediately with 3M Tris-HCl, pH 10.

To monitor any possible contamination of the extract with anti-AChR antibody in "trapped serum" we injected a rat six hours prior to sacrifice with ¹²⁵I labeled rat IgG (2.6 μg , 6×10^6 cpm). We found no radioactivity in the final Sepharose-eluted material prepared as described above, thus precluding contamination of the tissue eluate with serum antibodies.

Fig. 10 Comparison of IEF profiles of serum antibodies versus antibodies eluted from a diseased animal (EAMG). After focusing both serum (lane 2) and tissue eluate (lane 1) following focusing were reacted with ^{125}I - α -BGT Torpedo AChR.

Results and comments.

As depicted in fig. 10 the profiles obtained with antibody eluates from the muscles of rats with EAMG did not differ from the profile of serum antibodies of the same animal. Thus, the cross-reacting antibodies eluted from AChRs at the neuromuscular endplate displayed a similar banding pattern to the circulating antibodies to rat AChR and were also cross-reacting with Torpedo AChR. These observations are important for several reasons: first, they show that the spectrotpe of serum antibodies is identical to that of antibodies reacting with the target organ. Second, all clones reacting with self are activated by the xenogeneic antigen. Finally, no particular class or subclass is responsible for the induction of EAMG. Although the majority of the antibody clones appeared to be represented at the neuromuscular junction of myasthenic animals, this does not necessarily imply that the antibody response to Torpedo and muscle AChR are quantitatively similar. In fact the serum antibody titer to muscle AChR is only a fraction ($\pm 0.5\%$) of the titer to Torpedo AChR used as immunogen (1). However, the neuromuscular endplate acts as an *in vivo* immuno absorbent for cross-reactive anti-AChR antibodies.

5.4 DISCUSSION

The muscular weakness characteristic of myasthenia gravis and its experimental animal model is caused by autoantibodies to acetylcholine receptors in skeletal muscle (1). The primary effect of these antibodies is to reduce the amount of receptor at the neuromuscular endplate (1, 4). We investigated the properties and specificities of these

antibodies that determine the severity of disease, c.q. the amount of AChR loss from muscle. The characteristics of antibodies are discussed in terms of titer, specificity, isotypes and spectrotypes. The autoimmune attack at the neuromuscular endplate also induces reactive changes in the muscle cells which are also important in the disease process.

There is a correlation between total anti-AChR *antibody concentration* in serum and severity of muscular weakness. Animals immunized with 5 μ g AChR have lower anti-AChR antibody titers and less severe disease than rats immunized with 10 μ g AChR (see chapter IV). With higher doses of AChR (15 μ g) the anti-AChR antibody titers further increase, but no further AChR loss from muscle is seen.

Apart from antibody concentration antibody *specificity* may influence the severity of disease. Indeed, antibodies against the main immunogenic region on the α -subunits are pathogenic since antibodies with this specificity are able to cross-link receptors at the muscle membrane and reduce the number AChR at the neuromuscular endplate, whereas antibodies against other subunits do not cross-link receptors (18, 20). Antibodies against the MIR are the most frequent specificity (8, 9) in both MG and EAMG. However, since no variation in antibody specificity occurs during the disease process (9) it is unlikely that variation in the titer anti-MIR antibodies influences disease severity. It is therefore necessary to analyse the role of antibodies with other specificities. In the previous chapter (IV) we have demonstrated that antibodies against reduced carboxymethylated AChR (RCM-AChR) are not pathogenic because they bind mainly to the cytoplasmic surface of AChRs. Since antibodies do not enter cells, anti-RCM-AChR antibodies are not pathogenic. As shown in the previous chapter boosting animals with RCM-AChR following a primary immunization with native AChR does not significantly alter the disease process compared to two immunizations with native AChR. Such alterations in antibody specificities in the presence of an excess of anti-MIR antibodies does not affect the antibody attack at the endplates. A third specificity of antibodies that may influence disease are antibodies that inhibit receptor function. This category of antibodies can be divided into a population of antibodies directed at the acetylcholine binding sites and antibodies that influence directly or allosterically the ion channel opening (18). We were not able to demonstrate high titer antibodies against the acetylcholine binding site in the serum of our rats with chronic EAMG using a solid phase assay system. In the past several groups demonstrated anti-acetylcholine binding site antibodies using immunoprecipitation assays, sucrose gradients (13, 24-29) or a DEAE disc assay. In the immunoprecipitation assays, receptors may become aggregated by antibodies, resulting in an impaired access of radiolabeled α -BGT or acetylcholine. In the disc assay α -BGT-AChR complexes could be prevented from binding to the discs by serum proteins. Although for the disc assay Claudio et al. carefully titrated the assay system when no such interference was seen. The inhibition of toxin binding is only seen at high serum concentrations. Finally Barkas et al. unequivocally demonstrated that inhibition of toxin binding can be mediated by antibodies which are not directed against the toxin-binding sites (30).

In our solid phase assay system (ELISA) no substances could be found that interfere with the binding of α -BGT to the solid phase AChR. The binding of α -BGT however, can be effectively inhibited by acetylcholine (fig. 1) or its analogues (results not shown) which demonstrated that the acetylcholine binding site was oriented in such a way that α -BGT or antibodies to the acetylcholine binding site, if present, could bind.

Antibodies that effect AChR function by interfering with the ionchannel function are also not widely represented in the polyclonal response against AChR. Only 2 of 25 MABs tested by Lindstrom et al. interfere with receptor function perhaps by occluding the lumen of the channel (18). Since a panel of monoclonal antibodies may be taken to be representative for the serum polyclonal response it is unlikely that antibodies that interfere with AChR function are important in EAMG. In MG antibodies that possibly directly affect receptor function by virtue of their binding to the α -BGT binding site have been demonstrated using immunoprecipitation assays (31-34). As mentioned above, this result should be interpreted with caution of possible methodological errors. Moreover, no good correlation was found between the titer of antibodies against the acetylcholine binding site and the disease severity (34), with a larger overlap between the different stages of disease.

Since the most frequent specificity of antibodies is directed towards the MIR which renders these antibodies important in terms of pathogenesis and because these antibodies are circulating in large excess over the amount of AChR in muscle, parameters other than titer or antibody specificity determine disease severity. We therefore analyzed *the diversity of the antibody response* in terms of variable region variability by isoelectric focusing. In all EAMG sera tested, the immune response against Torpedo AChR is polyclonal, this means that antibodies against similar antigenic determinants of Torpedo AChR are produced by different B cell clones. The full spectrotpe was expressed early after immunization and remained constant during the immunization period (similar results have been obtained by L. Nye (35) in rats immunized with thyroglobulin). The results show that the same B cell clones produced anti-Torpedo AChR antibodies in the period preceeding the disease and during the chronic phase of the disease (starting at about day 30 after immunization). These results suggest that the spectrotpe of anti-Torpedo AChR antibodies does not affect the expression of the disease.

The antibodies reactive with rat muscle AChR are a cross-reacting fraction of those to the Torpedo AChR used as immunogens. The population of antibodies bound to receptors in muscle is the same as the population of antibodies to Torpedo AChR in serum and cross-reactive with muscle AChR. This refutes the existence of a particular antibody clone with a greater affinity or specificity for the muscle AChR than others and, therefore, of higher pathogenic potential. This conclusion is consistent with the observation that there was no correlation between the pattern of anti-AChR specificities (as analyzed with MABs) in MG patients and the severity of their muscular weakness (9). These conclusions are important because the correlation between serum anti-AChR concentration and weakness in MG is poor (36-40), and one possible explanation could

be that only a small fraction of the anti-AChR antibodies is pathogenetically significant. Our experiments provide additional evidence that this is not the case. Thus, it would appear that diverse clinical responses to the same serum concentration of antibodies is influenced by other factors such as acetylcholine synthesis and release or AChR synthesis or destruction rates.

5.5 SUMMARY AND CONCLUSIONS

In this chapter anti-AChR antibodies that mediate AChR loss from muscle in rats with chronic EAMG were examined in terms of specificity and spectrotype (isoelectric point). This analysis yielded two conclusions :

1. Antibodies to the acetylcholine binding site on AChR were hardly detectable. These data are compatible with the observations that the main category of antibodies that induce EAMG are directed against the MIR.
2. Analysis of antibody diversity revealed that the serum anti-AChR antibodies are polyclonal. Sequential studies on sera from rats with chronic EAMG showed the full spectrotype to be expressed early after immunization and not to change with the onset of disease suggesting that the spectrotype of anti Torpedo AChR antibodies does not influence disease. This was confirmed by finding the spectrotype of anti rat muscle AChR antibodies eluted from the neuromuscular junction to be identical with those of the serum.

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CHAPTER VI

Effect of AChR-synthesis in the severity of EAMG

6.1 INTRODUCTION

Conceptually myasthenia gravis is determined by the immune response to the acetylcholine receptor (AChR) and the capacity of the target organ to withstand the immunological assault. Immunological factors such as antibody titer and antibody specificity are important determinants in causing AChR loss. Conversely the rate of acetylcholine synthesis should also influence myasthenia. The present therapeutic strategies in myasthenia gravis are designed to suppress antibody or inflammatory reactions by aspecific means, e.g. plasmapheresis and immunosuppressive drugs; alternatively the amount of acetylcholine present at the motor endplate is enhanced pharmacologically (1).

Although the issue of specific immunotherapy with antiidiotypic antibodies has been raised in EAMG these results are by and large inconclusive in terms of ameliorating the disease although crossreactive idiotypes have been demonstrated (2). We therefore raised the question whether procedures known to stimulate protein synthesis in general and AChR in particular influenced EAMG. For this reason the effect of unilateral limb denervation and administration of anabolic steroids in the AChR loss in EAMG was examined. The ratio for choosing these procedures was as follows. Although in normal muscles all AChR resides at the neuromuscular junction, denervation of muscle tissue results not only in an increased turnover of AChR at the neuromuscular junction but also in a spread of AChR to extrajunctional sites; the entire muscle plasmamembrane may in fact be covered with AChR. The postsynaptic area membrane remains however an area packed with AChR and thus rapid replacement of AChR occurs at the former junctional area (3).

Apart from denervation the effect of anabolic steroids (nandrolone) on EAMG was examined for two reasons. First anabolic steroids have a clearcut effect on muscle mass and androgens (such as danazol) have been shown to enhance the synthesis of deficient proteins in patients with hereditary angioneurotic edema, C1q esterase inhibitor (4), α 1-antitrypsin deficiency (5) or deficient coagulation factors (6). Thus we assumed it might also influence AChR synthesis in EAMG. In addition however epidemiological evidence in humans and experimental evidence of spontaneous animal models of autoimmune diseases show sex hormones to play a role in a variety of autoimmune diseases including myasthenia gravis.

Myasthenia gravis has a female preponderance (3:1, females to males)(7). Other autoimmune diseases in which immune abnormalities are predominantly present in the female are systemic lupus erythematosus (SLE) (10:1) (8), rheumatoid arthritis (3:1)

(9), scleroderma (3:1) (10), Sjogren's syndrom (9:1) (11), chronic active hepatitis (non infectious origin) (9:1) (12), primary biliary cirrhosis (9:1) (13), chronic idiopathic thrombocytopenic purpura (4:1) (14) and another receptor autoimmune disease: Graves' disease (6:1) (15). In addition, the age of the patient plays a role in the manifestation of the disease. The ratio of 10:1 for female to male SLE applies only to females in the childbearing years (8). When prepubertal and post-menopausal females are included this ratio falls to 3:1 (16). These epidemiological data suggest that both in female and male oestrogens or progesterons may have a deleterious effect on these autoimmune diseases. Patients with SLE for example have an increased hydroxylation of estradiol at C-16 towards the more feminizing metabolites 16 α -hydroxyestrone and estriol. These metabolites might contribute to the severity of SLE (17, 18). Androgens in contrast to female sex hormones have been shown to have protective effects against the manifestation of autoimmune diseases in animal models. Nandrolone (19-nortestosterone) and 5- α -dihydrotestosterone given to female NZB/NZW mice (murine model for systemic lupus erythematosus) improved survival, reduced anti-DNA autoantibody levels and glomerulonephritis (19, 20). The administration of the oestrogen antagonist nafoxidine retarded the development of murine SLE (21). Based on these beneficial effects of androgens on autoimmune disease and their anabolic capacities we analyzed a possible effect of nandrolone on EAMG.

6.2 EFFECT OF UNILATERAL DENERVATION (N. ISCHIADICUS) ON THE SEVERITY OF EAMG INDUCED BY PASSIVE TRANSFER OF MONOCLONAL ANTIBODY

In order to analyse the effect of enhanced AChR synthesis on acute EAMG we denervated the left hindleg of 8 female Lewis rats by cutting the left nervus ischiadicus. All animals were operated under ether anesthesia. The skin was incised on the midline over the lower back. The incised skin was moved to the left of the musculus gluteus maximus which was incised. Under that muscle, the n. ischiadicus, was removed over a length of 5 mm. The muscle was not sutured; the skin was closed with three metal clips. On day 8 after denervation four animals (group A) were injected intraperitoneally with 40 pmol of anti AChR monoclonal antibody directed against the MIR (MAB no. 35; see table material and methods section, p.); the 4 other rats (group B) were injected with an irrelevant monoclonal antibody (100 μ g of a monoclonal anti-human thyroglobulin antibody). Forty-eight hours later all rats were sacrificed; the skin was removed and the body was divided above the place of insertion of the hindleg muscles. Both legs were then separated from the backbone and frozen on dry ice. AChR and anti AChR antibody concentration was measured in each leg, according to the methods described in the material and methods section (p.161).

Table I. Passive transfer of EAMG by monoclonal antibody into rats with unilaterally denervated (left) legs.

group	MAB ^a	leg ^b	concentration ^c of AChR (pmol/leg)	concentration ^c of anti-AChR ab (pmol/leg)	% AChR complexed with ab
A(n=4)	anti-TG	left	24.5 ± 1.0	0	0
		right	6.2 ± 0.4	0	0
B(n=4)	anti-AChR	left	24.2 ± 1.4	0.62 ± 0.08	2.6
		right	4.7 ± 0.5	0.31 ± 0.13	6.5

^a) the rats were infused i.p. with monoclonal anti-thyroglobulin antibody (anti-TG) or monoclonal, anti-acetylcholine receptor antibody (anti-AChR).

^b) the left leg was denervated by cutting the nervus ischiadicus.

^c) concentrations of AChR and anti-AChR antibody were measured by radioimmunoassay, results were expressed in p mol/leg (mean ± S.E.)

Results and comments.

Both groups of rats had a higher concentration of AChR in the denervated leg when compared to the normal leg indicating that the denervation was complete.

Rats infused with monoclonal anti AChR antibody (group B) had less AChRs in their normally innervated legs than animals injected with an irrelevant monoclonal antibody (group A) (n.s.). The denervated legs contained the same amount of AChR in rats infused with anti-AChR MAB and anti thyroglobulin MAB. The ratio of the AChR content of the denervated leg versus the normal leg, however, was significantly increased in the animals injected with an anti-AChR MAB (5.2 ± 0.4 , mean ± SEM) when compared to control animals (3.9 ± 0.1) ($p < 0.05$). The amount of anti-AChR antibody deposited in the denervated leg was twice that found in the normal leg. The number of AChR covered with antibody was 2.5 times higher (6.5%) in the normally innervated leg than in the denervated leg (2.6%)(Table I). These experiments suggest that denervation prevents AChR loss because of an increased AChR turnover in the denervated leg. Possibly this protective effect was due to the low AChR occupancy with antibody in the denervated leg. It was therefore necessary to perform the same experiments in large antibody excess. These high antibody levels can be easily achieved in chronic EAMG.

6.3. EFFECT OF UNILATERAL DENERVATION (N. ISCHIACHIDUS) ON THE SEVERITY OF EAMG INDUCED BY ACTIVE IMMUNIZATION WITH TORPEDO AChR

In order to further analyze the protective effect of denervation on antibody mediated AChR loss observed in the experiment described above, rats were subjected to unilateral limb denervation prior to the induction of chronic EAMG. Six female Lewis rats were

Table II: Effect of unilateral limb denervation on the AChR loss in chronic EAMG ^a

	%AChR loss ^a from muscle	AChR concen- tration ^b (pmol/leg)	Concentration of anti-AChR ab ^b (pmol/leg)	% AChR com- plexed with (pmol/leg)
normal leg	53.0 ± 4.8	2.5 ± 0.25	0.67 ± 0.16	27.0 ± 6
denervated leg	13.5 ± 4.8	12.3 ± 1.12	2.10 ± 0.50	17.1 ± 3.8

^a) AChR loss (mean ± S.E.M.) from muscle (leg) in animals immunized with AChR in CFA is given in % of AChR concentration of control animals immunized with CFA only.

^b) Actually measured in the experimental animals.

denervated unilaterally by cutting the left ischiadic nerve. Four animals were immunized that same day with 15 µg AChR in CFA and two rats were injected with CFA alone. All animals were sacrificed 35 days after immunization. AChR and AChR-antibody concentration was measured in each leg.

Results and comments.

All animals immunized with AChR in CFA had high anti-Torpedo AChR antibody titers ($5.2 \pm 0.3 \mu\text{M}$) in the serum whereas control animals did not have circulating anti-AChR antibodies. All animals immunized with AChR showed significant AChR loss in their normally innervated legs whereas the denervated legs showed minimal AChR loss ($p < 0.005$) (table II). The ratio of the AChR content of the denervated leg versus the normal leg was 4.9 ± 0.17 (mean ± S.E.).

The amount of antibody deposited in the denervated leg is 3 times higher compared to the innervated leg ($p < 0.01$). The AChR occupancy with antibody however, is somewhat lower in the denervated leg since it contains five times more AChR than the normally innervated leg. This lower AChR occupancy agrees with the fact that in spite of the large circulating antibody excess the AChR incorporation in the muscle membrane occurs faster in the denervated leg.

This experiment and the previous one (see table I) demonstrate that a non-immunological factor such as the rate of AChR resynthesis is a limiting factor in chronic EAMG. This factor may account for differences in susceptibility observed between different animal strains from the same species. It is also likely that during an autoimmune attack against the neuromuscular endplate, AChR synthesis is increased until a new equilibrium between synthesis and antibody mediated degradation is achieved.

6.4 EFFECT OF ANABOLIC STEROIDS NANDROLONE ON EAMG

Female Lewis rats were immunized with 15 µg of Torpedo AChR incorporated in CFA. One group of animals (group A, $n = 5$) was treated with nandrolone (40 mg/kg, incor-

Table III: Effect of nandrolone on the immune response to AChR in Lewis rats

Group	n	Serum antibody titer against	
		Torpedo (nM)	rat (nM)
A (nandrolone)	5	898 $\pm$ 130	3,76 $\pm$ 2.0
B (arachis oil)	4	1670 $\pm$ 523	10,90 $\pm$ 4.1

All animals (group A and B) were immunized with 15 μ g of Torpedo AChR on day 0. Group A was treated on day 0, 14 and 28 with 40mg/kg of nandrolone in arachis oil; group B was given arachis oil only on days 0, 14 and 28. Serum antibody titers were measured on day 28 after immunization.

porated in Arachis oil) on days 0, 14 and 28 after immunization with AChR. A second group (group B, n=4) was treated with Arachis oil only according to the same treatment schedule. Control animals (not immunized with AChR) received nandrolone or arachis oil only. All animals were bled 28 days after immunization with AChR and sacrificed 4 days later. Anti-AChR antibody titers were determined in the serum by radioimmunoassay; AChR and anti-AChR antibody complexes were measured in muscle tissue (see material and methods section, p.161).

Results and comments.

One month after immunization with AChR the nandrolone treated rats (group A) had significantly lower serum anti-Torpedo AChR and anti-rat AChR antibody titers than animals in the control group (group B) ($p < 0.01$). The serum anti-rat AChR titers were threefold higher in the animals treated with arachis oil only (table III). None of the five nandrolone treated rats developed clinical signs of EAMG; 3 out of 4 rats injected with Arachis oil, however, showed severe EAMG one month after immunization with Torpedo AChR. At that time all animals were sacrificed. A significant difference in weight of the animal carcasses was found between the groups A and B ($134,6 \pm 3,5$ gr versus $97,4 \pm 7,7$ gr. respectively) ($p < 0.01$). The increase in weight after nandrolone treatment was not due to fluid retention but due to a visible increase in the muscular mass.

Next the amount of AChR and anti-AChR antibody complexes was measured in rat muscles. As shown in table IV nandrolone treated animals showed less AChR loss compared to the animals treated with arachis oil only (n.s.). Nandrolone had no effect on the AChR concentration in rats not immunized with Torpedo AChR in CFA as shown by a concentration of 45.6 ± 2.2 pmol/leg (n = 4) in rats given nandrolone versus 48 ± 3.0 pmol/leg in rats given arachis oil only (n = 4). Striking were the differences in AChR anti-AChR complexes. Nandrolone treated animals of group A had only 44% of their AChR's covered with antibody whereas in the arachis oil treated (group B) 82,2% of the receptors was complexed with antibody (table IV).

Table IV: Effect of nandrolone on EAMG

group	n	clinical EAMG	% AChR loss ^a	% AChR complexed with antibody
A (nandrolone)	5	0/5	43 ± 5	44.2 ± 12
B (arachis oil)	4	3/4	53 ± 3	82.2 ± 8.6

^a) AChR loss from leg muscle in animals of group A and B is given in % of AChR concentration of normal rats with nandrolone (controls for group A) or arachis oil (controls for group B) only.

These data suggest that nandrolone is effective in the treatment of EAMG by virtue of an immunosuppressive effect on anti-AChR antibody synthesis. It is also possible that nandrolone stimulates the AChR synthesis after the immune attack on the endplate, which could account for the low AChR occupancy with antibody.

6.5 DISCUSSION

Animals in which the AChR-synthesis in muscle was increased by denervation were protected against AChR loss in the denervated muscles after injection with monoclonal anti AChR antibody or AChR in CFA.

These experiments clearly demonstrate that non-immunological factors such as AChR synthesis can be rate limiting, in the presence of a limiting amount of (monoclonal) antibody and also in larger (polyclonal) antibody excess after immunization with AChR. Since muscle is capable of large increases in rate of receptor synthesis during embryonal development and after denervation it is likely that after an immunological attack a reactive increase of AChR synthesis occurs at the neuromuscular junction.

This proces of antibody mediated attack and AChR synthesis reaches an equilibrium. The set-point of this equilibrium can probably be changed by immunological factors such as an increase in antibody titer or by factors influencing AChR synthesis. These factors have been identified in the central and peripheral nervous system. A small polypeptide with a molecular weight of 1700, extracted from embryonic chick brain increased both AChR and acetylcholinesterase 3-5 fold (22). Other factors have been partially characterized but the mechanism of action of the various factors remains to be worked out (23, 24). Aspecific factors such as the external calcium concentration (25) and cyclic nucleotides are also regulators of AChR levels.

The finding that non-immunological factors affecting AChR-synthesis have a protective effect against AChR loss from muscle might have important therapeutic implications for myasthenia gravis. The present therapeutic strategies all aim at a decrease in anti-AChR antibody concentration and an increase of the amount of acetylcholine at the neuromuscular endplate; these therapeutic manoeuvres could be supplemented by adding drugs that stimulate AChR synthesis. Candidates are phosphodiesterase in-

hibitors such as theophyllin or coffee that raise AChR synthesis in culture by increasing intracellular cAMP levels (25). Anabolic steroids such as nandrolone might also be effective by increasing the general protein synthesis.

We therefore examined the effect of nandrolone on the induction of EAMG. Indeed, nandrolone had a clear clinical effect on EAMG. The mechanisms responsible for this protective effect may be dual. First, nandrolone had an immunosuppressive effect on mainly the anti-self AChR antibody titers. These data are in agreement with the immunosuppressive effects of nandrolone on anti-DNA titers and disease in murine systemic lupus erythematosus (20). Secondly, nandrolone has a clear effect on muscle which appeared from an increase in muscular mass. The anabolic effect possibly also protects the animals against clinical signs of EAMG. Nandrolone treated rats however had AChR loss from their muscle, which was however less than in control animals. More striking was the effect on the AChR occupancy with antibody. In spite of anti-AChR antibody excess in their serum the nandrolone treated animals showed a much lower number of AChR complexed with antibody. This can best be explained by an increase of AChR synthesis induced by nandrolone. These results open new perspectives for the treatment of MG and possible for anti-receptor disease in general. Before a clinical trial is started it will be necessary to analyze whether nandrolone has also a protective effect on ongoing EAMG. Studies with nandrolone in murine SLE suggest that this approach might yield success. (20).

6.6 SUMMARY AND CONCLUSIONS

In this chapter the effect of target organ manipulation on EAMG was studied. AChR synthesis was increased in two ways:

1. Unilateral limb denervation by removal of the ischiadic nerve resulted in a significant increase in AChR synthesis. This manoeuvre protected the denervated limb against antibody mediated AChR loss induced by passive transfer of monoclonal anti AChR antibody or by active immunization with AChR.
2. The administration of anabolic steroids protected rats against the induction of clinically apparent EAMG. In the control rats the clinical myasthenia was associated with a 50% muscle AChR loss; of the remaining muscle AChR 80% was complexed with antibody. Compared to the controls the rats treated with anabolic steroids showed significantly less cross reactive antibody in the serum. Striking were the differences in immune complex formation at the neuromuscular junction : the rats treated with anabolic steroids showed somewhat less muscle AChR loss (43%) compared to controls (53%) but of the remaining muscle AChR only 44% was complexed to antibody. These results suggest anabolic steroids to be beneficial in rats with chronic EAMG because of decreasing antibody concentration; in addition however a beneficial effect on the increase in AChR synthesis induced by antibody mediated AChR cross linking appears likely.

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SUMMARY

This thesis originated from a clinical study on autoantibody mediated receptor disease specifically Graves' disease. Since the only experimental model of antibody mediated receptor disease existing at that time — 1978 — was experimental autoimmune myasthenia gravis it was decided to use this model in order to analyze autoantibody receptor interactions.

The questions asked were:

1. Is this a true antibody mediated disease or do immune cells contribute to the lesions?
2. If only antibody causes the disease is there a causal relationship between antibody concentration, antibody specificity and symptoms of disease?
3. Can the auto-antibody receptor interaction be manipulated in a therapeutic usefull way?

Chapter I

The introductory chapter reviews the literature of clinical autoimmune anti-receptor diseases. Although the mechnisms operational in these diseases may be similar, the clinical spectrum varies from endocrine diseases and neurological disorders to asthma and allergic rhinitis. Conceptually antibodies against cell membrane receptors may bring about disease in three different ways. First, antibodies may bind to the receptor and block ligand induced receptor stimulation. Although myasthenia gravis was originally believed to belong to this category it is now known that blockade of the acetylcholine binding site is not the main cause of disease. Second, antibodies may mimic hormone or neuro-transmitter action and thus stimulate the receptor in the absence of the natural ligand eg. in Graves' disease and some cases of hypoglycaemia; experimental evidence suggest that these stimulating antibodies may be auto-anti-idiotypic antibodies. A third mechanism, operative in myasthenia gravis and autoimmune insuline resistance, down regulates receptor molecules yielding reduced numbers of receptors left for stimulation. The spectrum of autoimmune receptor diseases will probably be extended in the near future. Candidate diseases might be hypogammaglobulinemia, and pseudo hypoparathyroidism. For all of the anti-receptor autoimmune diseases only one well characterized animal model exists : experimental autoimmune myasthenia gravis.

Chapter II

This chapter is a literature study which summarizes the "state of the art" of the experimental autoimmune myasthenia gravis (EAMG) model. Disease is induced by im-

munizing animals with acetylcholine receptors isolated from the electric organ of fish: *Torpedo californica* an electric ray residing off the Californian coast or *Electrophorus electricus* an electric eel found in the Amazon river. Clinically this experimental model is relevant in terms of pathogenesis of disease (myasthenia gravis) but not in terms of etiology.

Detailed analysis of the acetylcholine receptor by biochemical and immunological means has elucidated the structure of the receptor as well as its antigenic structure. A small part of the acetylcholine receptor residing on the α -subunit, the so called main immunogenic region (MIR), is of crucial importance for the development of disease in the experimental animal model and in man.

The electrophysiologic abnormalities observed in the rat model of EAMG — specifically the acute model — are induced by three different immunopathological mechanisms; complement mediated focal lysis, of the neuromuscular membrane increased turnover of acetylcholine receptor (AChR) at the neuromuscular endplate, and possibly by blockade of the acetylcholine binding site. Next to antibodies cellular immune reactions must be important in EAMG although their role is undefined. No clear evidence exists for a role of effector T cells and the role of T helper cells is assumed but not proven in the model of chronic EAMG. The humoral autoimmune reactions observed in EAMG have also been demonstrated in man. Thus EAMG is a useful model in terms of pathogenesis of myasthenia gravis in man.

Chapter III

In order to analyze the immune reactions involved in EAMG the first approach was to determine the phenotype and specificity of the cellular immune reactions of rats with chronic EAMG *in vitro*. For this purpose an *in vitro* culture system was developed in which sensitized lymphoid cell suspensions from rats with chronic EAMG were cultured in the presence of acetylcholine receptor (AChR) preparations. Next the proliferative cellular responses and anti-AChR antibody secreted was quantitated. The culture system finally adopted required antigen (AChR), sensitized (T and B) lymphocytes, and antigen presenting cells. The cells that showed a proliferative response to AChR were T cells since they could be lysed by anti T cell serum in the presence of complement. Their T helper cell nature was suggested by finding that they produced interleukin-2, and by the fact that antibody producing cells required their presence and that the amount of antibody produced *in vitro* was proportional to the cellular proliferative response. Conclusive evidence derived from an experiment which showed by means of phenotypic separation of T cells with a fluorescence activated cell sorter that the proliferating cells were T helper cells. The T helper cell proliferation had an absolute requirement for accessory cells and the proliferative response was influenced by the physicochemical nature of the AChR added to the culture as illustrated by the finding that AChR incorporated in lipid membranes (a form reminiscent of the postsynaptic membrane) elicited far greater proliferative T helper cell responses compared to

solubilized AChR.

Since most of the serum antibodies in EAMG appear to be directed towards the main immunogenic region (MIR) of the AChR the specificity of the T helper cell proliferative response was studied in terms of pathogenetically important specificities : those residing on the MIR and cross reacting with rat AChR. For this purpose Torpedo AChR was chemically altered by reduction and carboxymethylation (RCM-AChR) or trypsinization (t-AChR). These procedures yielded two differing receptor preparations, RCM-AChR with a selectively destroyed MIR and t-AChR lacking antigenic determinants outside the MIR. When these chemically altered AChR were added to cultures of immune lymph node cells taken from rats with chronic EAMG, a high proliferative T helper cell response and antibody synthesis was observed regardless of the absence or presence of the MIR. These surprising results suggest that in the *in vitro* culture system the accessory cells first break down the AChR with a concomitant partial loss of primary (t-AChR) or tertiary structure (MIR) prior to presentation to the T cells. That here the accessory cells present the tertiary structure to the T cells.

The cross-reactive specificities were studied by inducing EAMG with Torpedo californica, fetal calf muscle and rat muscle AChR; the sensitized lymph node cells were next cultured *in vitro* with the three different AChR preparations and the proliferative response as well as the antibody production to each individual AChR was determined. Rats immunized with Torpedo AChR showed a high degree of cross-reactive T helper cell proliferation to mammalian muscle AChR whereas rats immunized with muscle AChR react with muscle AChR (but do not cross-react with Torpedo AChR at the T cell level). The antibody response showed the opposite effects. These findings can only be interpreted to indicate that at the T cell level no tolerance to mammalian AChR exists. They show in addition that cross reactive T cell proliferation and antibody responses to rat AChR are determined by the species from which the immunogen derives.

Chapter IV

In this chapter the immunogenicity of differing AChR preparations was related to their *in vivo* pathogenicity. For these purposes an *in vivo* model of chronic EAMG was used allowing precise quantitation of the immunopathological lesions at the neuromuscular junction rather than scoring the signs of myasthenia.

First an *in vivo* model was developed using Torpedo californica AChR as immunogen and a dose response curve was made relating the amount of Torpedo californica AChR used for immunization to antibody cross reactive with rat AChR in the serum and bound at the site of the neuromuscular junction. Next the immunizing dose of Torpedo AChR was related to AChR loss and clinical signs of myasthenia. Immunizing doses between 5 μ g and 15 μ g caused a dose dependent increase in crossreactive antibody in the serum, immune complex formation at the neuromuscular enplate and AChR loss. AChR loss from the neuromuscular junction was a far better measure of EAMG compared to clinical signs of myasthenia which was only apparent with AChR losses in ex-

cess of 70%. In crossreactive antibody excess however — achieved by immunizing rats with dosages of Torpedo californica AChR exceeding 15 μ g — AChR unoccupied by antibody remained at all demonstrable at the neuromuscular junction which suggested that target organ resistance to antibody mediated receptor loss had occurred. This observation was the basis of the experiments described in Chapter VI.

The specificity of the cross-reactive antibodies causing the AChR loss *in vivo* was examined next by immunizing rats with reduced and carboxymethylated Torpedo californica AChR (RCM-AChR). This procedure selectively removes the MIR of AChR as mapped by means of a panel of thirty monoclonal antibodies. Destruction of the MIR did not reduce the immunogenicity of the AChR *in vivo* since high titers of anti native AChR antibodies were elicited but abolished pathogenicity as shown by the fact that none of these rats developed myasthenia or AChR loss. In these rats the antibodies crossreactive with rat AChR were shown to be directed to the cytoplasmic (*in vivo* non accessible) sites of the muscle AChR and thus to be irrelevant in terms of pathogenesis of disease. These observations were extended in passive transfer studies using monoclonal antibodies against native and RCM Torpedo AChR. Only antibodies directed to the extracellularly located MIR (present on native but absent from RCM-AChR) were capable of causing AChR loss. In contrast to suggestions in the literature preimmunization of rats with RCM-AChR failed to protect against native Torpedo AChR induced EAMG nor was an ongoing EAMG cured by immunizations with RCM-AChR. These observations are compatible with the view that in terms of immunogenicity RCM-AChR is the equivalent of the carrier and that the MIR functions as hapten. The cross-reactive immune response to the hapten determines antibody specificity and thus pathogenicity. In order to demonstrate that this cross-reactive immune response was mediated by antibody only passive transfer studies were performed using *in vitro* expanded immune cells isolated from lymph nodes of rats with EAMG. *In vitro* expansion of immune T cells with a T cell mitogen (in a manner identical to that used for passive transfer of effector T cells in experimental allergic encephalitis) failed to induce AChR loss in the recipient. Passive transfer was only successful when the immune cells were expanded *in vitro* in the presence of AChR; the receptor loss observed was mediated by actively secreted and cross-reactive antibody. The autoantibody levels in the serum of the recipients of passively transferred cells decreased rapidly suggesting that the autologous AChR is not capable of expanding these primed T (helper) cells possibly because the membrane antigens residing on the receptor do not circulate in the extra cellular fluid. The studies described in this chapter therefore support the view that chronic EAMG is an antibody mediated disease requiring the continual presence of immunogen and primed helper T cells.

Chapter V

In chapter V the anti-AChR antibodies that mediate the AChR loss from muscle in rats with chronic EAMG were examined in terms of specificity and spectrotype (isoelectric

point) in order to rule out that restricted specificities (e.g. the products of limited number of B cell clones not readily apparent in serum) contributed to the disease. This possibility had to be investigated because in conditions of considerable excess of antibody to the MIR in the serum of rats immunized with large doses of Torpedo AChR unoccupied AChR (capable of binding antibody) was at all times demonstrable at the neuromuscular junction of rats with chronic EAMG. Apart from target organ resistance (e.g. increased synthesis of AChR) this observation could also be due to a restriction of antibodies bound to the muscle AChR, when compared to the antibodies present in the serum. Differences in charge of anti-AChR antibody molecules could allow the more positively charged antibody to bind to the interstitial matrix consisting of mucopolysaccharides carrying a negative charge at pH 7.4. Alternatively the antibody response itself might be restricted and thus low concentrations of antibody of a certain specificity might be concentrated at the neuromuscular junction and thus escape detection when measuring the antibody specificities in the serum. We first determined whether antibodies directed to the acetylcholine binding site contributed — in addition to those directed to the MIR — to the disease. Using a solid phase assay system almost no antibody to the acetylcholine binding site was demonstrable in EAMG suggesting that results of others using precipitation assays might be artifacts. Differences in charge (isoelectric point) of anti-AChR antibody molecules from the serum and in immune complexes from the neuromuscular junction were analyzed by reversed isoelectric focusing followed by radiolabeled AChR overlay. This technique allows analysis of the antibody response in terms of variable chain diversity and is capable of detecting low concentrations of a limited number of B cell clone products. The procedure dissociates antigen-antibody complexes and thus allows spectrotypic analysis of antibody residing in these complexes. Sequential studies on sera from rats with chronic EAMG showed the full spectrotypic to be expressed early after immunization and not to change with the onset of disease suggesting that the spectrotypic of anti Torpedo AChR antibodies does not influence disease. This was confirmed by finding the spectrotypic of anti-rat muscle AChR antibodies eluted from the neuromuscular junction to be identical with those of the serum. These results show the antibody response in chronic EAMG to be polyclonal; also in myasthenia is brought about by a polyclonal antibody response which is directed towards the MIR. The observation that unoccupied AChR receptor is demonstrable in condition of apparent crossreactive antibody excess suggests that target organ characteristics in addition to cross reactive antibody determines AChR loss.

Chapter VI

In chapter VI the effect of target organ manipulation on EAMG was studied; the results obtained were the basis for designing a new strategy for the treatment of myasthenia.

Since the muscle is capable of large increases in AChR synthesis during embryonal

development and after denervation the hypothesis was advanced that secondary to an immunological attack resulting in a cross-linking of AChR at the neuromuscular junction a reactive increase in AChR occurs. If this were so not only suppression of the antibody response by aspecific or specific means but also enhancement of the AChR synthesis would be expected to have beneficial effects in EAMG.

In order to test this hypothesis rats were given a unilateral limb denervation by removal of the ischiadic nerve and 8 days later monoclonal antibody directed towards the MIR was infused. The denervated limb contained 4 times as much AChR compared to the control leg and more importantly showed no AChR loss in contrast to the control leg. This experiment suggested that antibody mediated AChR loss in the denervated leg was compensated for by enhanced AChR synthesis. Next the effect of unilateral limb denervation performed at the time of induction of chronic EAMG, was studied. All rats had high titers of anti-AChR antibody but the amount of AChR loss from the control leg was 4 times that of the denervated leg; the percentage of AChR complexed with antibody in the denervated leg (17%) however was far less than the control leg (27%). This lower AChR occupancy with antibody in the denervated leg agrees with the view that in this leg a greater reactive increase in AChR synthesis had occurred compared to the normal leg.

The effect of anabolic steroids (nandrolone) on chronic EAMG was examined next for two reasons. First anabolic steroids have a clearcut positive effect on muscle mass and androgens enhance the synthesis of deficient proteins in some hereditary human diseases. Thus we hypothesized that anabolic steroids might also influence the reactive increase in AChR synthesis after antibody mediated AChR cross linking in analogy to the experiments with denervation. In addition, epidemiological evidence in man and experimental evidence from spontaneous animal models show sex hormones to play a role in autoimmune diseases inclusive myasthenia gravis.

Female Lewis rats were immunized with Torpedo AChR and a dose of anabolic steroids were given every two weeks; animals were sacrificed 30 days after the first immunization with AChR. At this time 3 out of 4 control rats showed severe myasthenia clinically whereas none of the treated rats were myasthenic. In the control rats the clinical myasthenia was associated with a 53% muscle AChR receptor loss; of the remaining 80% AChR was complexed with antibody. Compared to the controls the rats treated with anabolic steroids showed significantly less cross reactive antibody in the serum. Striking were the differences in immune complex formation at the neuromuscular junction: the rats treated with anabolic steroids showed somewhat less muscle AChR loss (43%) compared to controls (53%) but of the remaining AChR only 44% was complexed to antibody. These results show anabolic steroids to be beneficial in rats with chronic EAMG because of decreasing antibody concentration; in addition however a beneficial effect on the increase in AChR synthesis induced by antibody mediated AChR cross linking appears likely.

SAMENVATTING

Dit proefschrift is tot stand gekomen naar aanleiding van klinische vraagstellingen op het gebied van onderzoek naar ziekten welke veroorzaakt worden door auto-antilichamen gericht tegen receptoren, in het bijzonder zoals bij de ziekte van Graves. Omdat in 1978 alleen experimentele autoimmuun myasthenia gravis bekend was als model voor een dergelijke ziekte (welke dus veroorzaakt wordt door antilichamen gericht tegen receptoren) werd besloten dit model te gebruiken om interacties tussen auto-antilichamen en receptoren te analyseren. Daartoe werden de volgende vraagstellingen geformuleerd:

1. Wordt deze ziekte uitsluitend door antilichamen veroorzaakt of dragen immunologisch actieve cellen ook bij tot de weefselbeschadiging?
2. Indien auto-antilichamen de enige veroorzakers van de ziekte zijn, bestaat er dan een causaal verband tussen de hoeveelheid geproduceerde antilichamen, hun specificiteit en de ernst van de symptomen van de ziekte?
3. Kan op een therapeutisch relevante wijze geïnvenierd worden in de interactie tussen autoantilichaam en receptor?

Hoofdstuk I

Dit inleidende hoofdstuk geeft een overzicht over de literatuur van de kliniek van auto-immuunziekten tegen receptoren. Hoewel de mechanismen die bij deze ziekten betrokken zijn veel gemeen kunnen hebben, beslaan deze ziekten een breed klinische spectrum dat loopt van endocriene ziekten en neurologische afwijkingen tot asthma en allergische rhinitis. Er zijn drie manieren waarop antilichamen tegen receptoren in de celmembraan ziekte kunnen veroorzaken. Ten eerste: antilichamen kunnen zich aan de receptor binden en (daardoor) stimulatie van de receptor door het ligand verhinderen. Hoewel men oorspronkelijk meende dat myasthenia gravis tot deze categorie behoorde wordt nu gesuggereerd dat blokkade van de bindingsplaats voor acetylcholine niet de belangrijkste oorzaak van de ziekte is. Ten tweede: antilichamen kunnen de werking van een hormoon of neurotransmitter imiteren en zodoende de receptor stimuleren in afwezigheid van het natuurlijke ligand! Dit is bijvoorbeeld het geval bij de ziekte van Graves en in sommige gevallen van hypoglycaemie; resultaten uit experimenten geven aan dat deze "stimulerende" antilichamen in feite anti-idiotypische auto-antilichamen zouden kunnen zijn. Het derde mechanisme dat werkzaam is in myasthenia gravis en in een door auto-immuniteit veroorzaakte vorm van insuline-resistentie heeft een negatief effect op de aanmaak van receptor moleculen zodat minder receptoren beschikbaar zijn voor stimulatie. Waarschijnlijk zal het scala van door auto-immuniteit veroorzaakte ziekten waarbij receptoren betrokken zijn in de nabije toekomst nog worden uitgebreid. Als kandidaten voor deze uitbreiding kunnen hypogammaglobulinaemie en

pseudo-hypoparathyreoidie worden genoemd. Voor al deze zogenaamde "anti-receptor auto-immunziekten" bestaat maar een goed uitgewerkt proefdiermodel: experimentele auto-immuun myasthenia gravis (EAMG).

Hoofdstuk II

Dit hoofdstuk is gebaseerd op literatuur-onderzoek; het vat de meest recente bevindingen in het EAMG-model samen. De ziekte wordt opgewekt door proefdieren te immuniseren met acetylcholine-receptoren welke geïsoleerd worden uit het elektrische orgaan van een sidder-rog (*Torpedo californica*) welke voor de Californische kust leeft, of van een sidder-aal (*Electrophorus electricus*) welke in de Amazonerivier voorkomt.

Dit proefdiermodel is klinisch relevant voor zover het de pathogenese van myasthenia gravis betreft, maar niet voor het bestuderen van de etiologie. Nauwgezet biochemisch en immunologisch onderzoek van de acetylcholine receptor heeft zowel de biochemische als de antigene structuur van de receptor aan het licht gebracht. Op een beperkt gedeelte van de receptor (de α -subunit) bevindt zich zowel de bindingsplaats voor acetylcholine als de plaats waar antilichamen worden gebonden. Dit laatste gebied, het zogenaamde dominante immunogene gebied ("main immunogenic region": MIR) is van cruciaal belang voor de ontwikkeling van de ziekte, zowel in het proefdiermodel als in de mens.

De electro-fysiologische afwijkingen welke met name in het acute rattemodel voor EAMG worden gevonden, worden door drie verschillende immunopathologisch mechanismen veroorzaakt, te weten door complement teweeg gebrachte focale lysis van de receptor, versnelde afbraak en aanmaak van acetylcholine receptoren (AChR) in de zenuw-eindplaatjes op de spier en mogelijk door blokkade van de bindingsplaats voor acetylcholine. Naast antilichamen kunnen ook cellulaire immuunreacties van belang zijn in EAMG hoewel hun rol nog niet precies bekend is. Er is geen onweerlegbaar bewijs voor een rol van effector T cellen terwijl van helper T cellen alleen maar wordt aangenomen dat ze een rol spelen in het chronische EAMG model. De humorale auto-immuun reacties welke in EAMG zijn gevonden zijn ook bij de mens aangetoond. Vandaar dat EAMG een bruikbaar model is voor de pathogenese van myasthenia gravis in de mens.

Hoofdstuk III

Voor het analyseren van de immuunreactie die deel uitmaakt van het chronische EAMG model is in eerste instantie het fenotype en de specificiteit van de cellulaire immuunreacties *in vitro* bepaald. Hiertoe werd een *in vitro* kweekstelsel ontwikkeld waarin suspensies van (gesensitizeerde) lymfoïde cellen, verkregen uit ratten met chronische EAMG, gekweekt werden in aanwezigheid van AChR. Vervolgens werd de proliferatieve respons van de cellen gemeten en de geproduceerde hoeveelheid anti-AChR antilichamen gekwantiteerd. Het uiteindelijke door ons gebruikte kweekstelsel bleek

afhankelijk te zijn van de simultane aanwezigheid van antigeen (AChR), gesensitizeerde (T en B) lymfocyten en antigeen-presenterende cellen. De cellen die in respons op AChR prolifereerden, bleken T cellen te zijn; het was mogelijk ze te lyseren met behulp van een anti-T cel serum en complement. Dat het hier om helper T cellen ging, werd aannemelijk door de bevinding dat deze cellen interleukine-2 produceerden en door het feit, dat de antilichaamproducerende cellen van hun aanwezigheid afhankelijk waren. Bovendien correleerde de hoeveelheid geproduceerde antilichamen positief met de proliferatieve respons van de T cellen. Het doorslaggevende bewijs werd geleverd in een experiment waarin met behulp van een "Fluorescence-Activated Cell sorter" T cellen op basis van hun fenotype werden gescheiden, waarna bleek dat de helper T cellen de prolifererende cellen waren. Proliferatie van de helper T cellen was volstrekt afhankelijk van de aanwezigheid van antigeen-presenterende cellen; bovendien werd de proliferatieve respons beïnvloed door de fysico-chemische vorm waarin de AChR aan de celkweek werd toegevoegd. Dit laatste punt werd geïllustreerd door de bevinding, dat de AChR ingebouwd in lipide-membranen (een vorm die doet denken aan de post-synaptische membraan) een sterkere proliferatieve T cel respons opwekte dan AChR in opgeloste vorm. Aangezien de meeste antilichamen in EAMG tegen de MIR van de AChR gericht zijn, werd de specificiteit van de proliferatieve helper T cel respons met name onderzocht op pathogenetisch belangrijke specificiteiten: die tegen MIR en die welke kruisreactiviteit vertonen tegen de ratten-AChR. Hiertoe werd Torpedo AChR chemisch veranderd door middel van reductie en carboxylatie (RCM-AChR) of door middel van trypsinisatie (t-AChR). Deze procedures leverden twee verschillende receptor-preparaten op: RCM-AChR met een selectief vernietigd MIR en t-AChR zonder antigen-determinanten buiten de MIR. Als deze chemisch gemodificeerde AChR werden toegevoegd aan kweken van lymfekliercellen uit ratten met chronische EAMG (opgewekt met natief Torpedo AChR) werd een flinke proliferatieve respons van helper T cellen alsmede antilichaamproductie waargenomen, zulks onafhankelijk van de aan- of afwezigheid van de MIR. Dit verrassende resultaat doet vermoeden dat de antigeen-presenterende cellen in het *in vitro* kweekstelsel eerst de AChR afbreken, waarbij dan de primaire (zoals bij t-AChR) of tertiaire structuur (zoals van MIR) gedeeltelijk verloren gaat, om vervolgens het afgebroken antigeen aan te bieden aan de T cellen. De kruis-reagerende specificiteiten werden bestudeerd door EAMG op te wekken met AChR uit Torpedo californica, uit spieren van runderfoetussen of uit rattespieren. Vervolgens werden de daardoor gesensitizeerde cellen uit de lymfeklieren *in vitro* gekweekt met de drie verschillende AChR-preparaten en werden de proliferatieve respons en antilichaamproducties tegen elk der AChR preparaten bepaald. Ratten welke met Torpedo AChR waren geïmmuniseerd lieten een hoge mate van helper T cel kruis-reactiviteit zien in hun proliferatieve respons tegen AChR uit spieren van zoogdieren, terwijl ratten geïmmuniseerd met spier-AChR wel tegen spier-AChR reageerden maar geen kruis-reactiviteit op het niveau van de T cel tegen Torpedo AChR vertoonden. De antilichaamrespons liet echter het tegenovergestelde zien. Deze bevindingen leidden tot de enig mogelijke interpretatie dat er op het niveau van de T cel geen tolerantie tegen

AChR uit zoogdierweefsel bestaat. Bovendien blijkt hieruit dat de kruis-reactiviteit van T cel proliferatie en van antilichamen tegen de AChR van de rat bepaald wordt door de diersoort waaruit het immunogeen wordt geïsoleerd.

Hoofdstuk IV

Allereerst werd door ons in dit deel van het onderzoek de immunogeniciteit van verschillende AChR-preparaten onderzocht in relatie tot hun pathogeniciteit *in vivo*. Hier toe werd een *in vivo* model voor chronische EAMG gebruikt waarin, eerder dan af te gaan op het registreren van de symptomen van klinisch manifeste myasthenie de immunopathologische beschadigingen van de neuro-musculaire eindplaat exact gekwantiteerd kunnen worden.

Gebruik makend van Torpedo AChR als immunogeen werd allereerst het *in vivo* model ontwikkeld. Voor dit model werd in een dosis-respons curve de relatie tussen de voor de immunisatie gebruikte hoeveelheid Torpedo AChR en de opgewekte (met rat AChR kruis-reagerende) vrije antilichamen in het serum, plus die welke gebonden waren in de neuromusculaire eindplaat, vastgelegd. Zodoende kon vervolgens de dosis Torpedo AChR gebruikt voor de immunisatie gerelateerd worden aan het verlies aan AChR en de klinische verschijnselen van myasthenie. Doses variërend van 5 tot 15 μg Torpedo AChR veroorzaakten zowel een dosis-afhankelijke toename van kruis-reactieve antilichamen in het serum, de vorming van immuuncomplexen in de neuro-musculaire eindplaat als een verlies van AChR. Verlies van AChR in de neuro-musculaire eindplaat bleek een betere maat voor EAMG te zijn dan registratie van de klinische verschijnselen van myasthenie welke pas manifest werden wanneer meer dan 70% van de AChR verloren waren gegaan. In aanwezigheid van een overmaat kruis-reagerende antilichamen (bereikt door ratten te immuniseren met hoeveelheden Torpedo AChR boven de 15 μg) bleven echter in de neuromusculaire eindplaat nog steeds receptoren aantoonbaar die niet bezet waren door anti-AChR antilichamen; dit zou er op kunnen wijzen dat zich in de eindplaat resistentie tegen het door antilichamen veroorzaakte verlies van receptoren had ontwikkeld. Deze waarneming ligt ten grondslag aan de proeven welke in Hoofdstuk VI beschreven zijn.

Vervolgens werd de specificiteit van de kruis-reagerende antilichamen, die het verlies van AChR *in vivo* veroorzaken, nagegaan door ratten te immuniseren met (chemisch gemodificeerde) RCM-AChR. Zoals door gebruik van een aantal monoclonale antilichamen tegen AChR kon worden aangetoond is op deze RCM-AChR geen MIR meer aanwezig terwijl de antigene determinanten buiten de MIR niet aangetast worden. Vernietiging van de MIR verminderde de *in vivo* immunogeniciteit van de AChR niet aangezien er hoge titers anti-AChR antilichamen mee konden worden opgewekt; wel bleek dit immunogeen niet pathogeen meer te zijn want geen van deze ratten ontwikkelde myasthenie of verloor AChR. De met ratte-AChR kruis-reagerende antilichamen bleken in deze ratten tegen intracellulair gelegen plaatsen van de spier-AChR gericht te zijn. Deze plaatsen zijn *in vivo* onbereikbaar en de ertegen gerichte antilichamen zijn derhal-

ve irrelevant voor het ontstaan van de ziekte. Deze waarnemingen werden nog verder uitgebreid in experimenten waarin monoclonale antilichamen tegen natief AChR en RCM-AChR uit Torpedo "passief" werden toegediend aan ratten. Uitsluitend antilichamen gericht tegen de extracellulair gelegen MIR — aanwezig op de natieve, maar niet op RCM-AChR — veroorzaken verlies van AChR. In tegenstelling tot aanwijzingen in de literatuur bleek immunizatie van ratten met RCM-AChR geen bescherming te bieden tegen inductie van EAMG met natieve Torpedo AChR; ook een bestaande EAMG werd niet genezen door immunizatie met RCM-AChR. Deze waarnemingen zijn verenigbaar met de hypothese dat RCM-AChR qua immunogeniciteit als de zgn. "carrier" kan worden gezien en dat de MIR als haptene fungeert. De kruis-reactiviteit van de immuunrespons tegen het haptene bepaalt dus de specificiteit van de antilichamen en daarmee dus de pathogeniciteit. Om te bewijzen dat deze kruis-reactieve immuunrespons uitsluitend door antilichamen tot stand wordt gebracht, werden studies verricht waarin gesensitizeerde cellen uit de lymfeklieren van dieren met EAMG na vermenigvuldiging *in vitro* passief werden doorgegeven aan onbehandelde ratten. Indien de gesensitizeerde T cellen *in vitro* met behulp van een T cel mitogeen werden vermenigvuldigd (zoals ook elders werd toegepast voor de passieve overdracht van experimentele allergische encephalitis met behulp van effector T cellen) werd geen antilichaamrespons tegen AChR in de ontvanger gevonden. Dergelijke passieve overdracht had alleen succes indien de gesensitizeerde cellen *in vitro* werden gekweekt in de aanwezigheid van AChR; het gevonden verlies aan AChR in deze ontvangers werd veroorzaakt door de actief geproduceerde kruis-reactieve antilichamen. De spiegels van auto-antilichamen in het serum van de ontvangers van passief doorgegeven cellen nam snel af, wat erop wijst dat autologe AChR niet in staat was deze gesensitizeerde (helper) T cellen *in vivo* te laten delen. Dit kan het gevolg zijn van het feit dat receptor-geassocieerde membraanantigenen niet vrij in de extra-cellulaire vloeistof voorkomen. De in dit hoofdstuk beschreven onderzoeken ondersteunen dus de idee dat chronische EAMG een ziekte is die veroorzaakt wordt door antilichamen. De aanmaak hiervan is afhankelijk van de ononderbroken aanwezigheid van immunogeen en specifiek geactiveerde helper T cellen.

Hoofdstuk V

In het onderzoek beschreven in Hoofdstuk V werden de anti-AChR antilichamen die tot verlies van spier-AChR in ratten met chronische EAMG aanleiding geven nader onderzocht op specificiteit en op iso-electrisch punt. Dit onderzoek werd verricht om uit te sluiten dat juist antilichamen met een beperkt aantal specificiteiten (bijvoorbeeld omdat zij zijn voortgekomen uit een beperkt aantal B cel clonen en dus niet zijn aan te tonen in het serum) bijdragen tot het ontstaan van de ziekte. Deze mogelijkheid diende te worden onderzocht omdat van ratten, die geïmmuniseerd waren met grote doses Torpedo AChR ondanks een aanzienlijke overmaat antilichamen in het serum tegen de MIR toch steeds vrije AChR in de neuro-musculaire eindplaat konden worden aange-

toond (AChR die nog steeds in staat waren antilichamen te binden). Afgezien van opbouw van resistentie (bijvoorbeeld door versnelde synthese van AChR) zou deze observatie ook het gevolg kunnen zijn van een beperkte mogelijkheid van antilichamen om zich aan de spier-AChR te binden.

Ladingsverschillen tussen anti-AChR antilichaam moleculen zou de meer positief geladen antilichamen in staat stellen de interstitiele matrix te passeren welke bestaat uit mucopolysacchariden welke bij pH 7.4 negatief geladen zijn. Een andere mogelijkheid is, dat de antilichaamrespons van (zeer) beperkte specificiteit zou kunnen zijn en dat een (absoluut gezien) geringe hoeveelheid antilichamen met een bepaalde specificiteit geconcentreerd wordt in de neuro-musculaire eindplaat, zodat ze niet kunnen worden aangetoond in het serum. Eerst werd door ons nagegaan of antilichamen gericht tegen de bindingsplaats voor acetylcholine, alsook die welke tegen de MIR gericht zijn, bijdragen tot het ontstaan van ziekte. Met gebruikmaking van een "solid phase" test-systeem waren in EAMG echter vrijwel geen antilichamen tegen de acetylcholine-bindingsplaatsen aantoonbaar, wat doet veronderstellen dat de resultaten van anderen die gebruik maakten van precipitatie-testsystemen op artefacten zouden kunnen berusten. Ladingsverschillen (ofwel verschillen in iso-electrisch punt) tussen anti-AChR antilichamen uit het serum en uit immuuncomplexen geïsoleerd van de neuromusculaire eindplaat werden nagegaan door omgekeerde iso-electrische focussering gevolgd door incubatie met radioactief-gemerkt AChR. Deze techniek maakt het mogelijk de diversiteit van de antilichaamrespons te analyseren; lage concentraties van produkten van een beperkt aantal B cel clonen kunnen er mee worden opgespoord. Bij het toepassen van deze procedure vallen antigeen-antilichaam-complexen uit elkaar zodat het mogelijk wordt het spectrotipe (isoelectrische punten) van de antilichamen uit deze complexen te bepalen. Met tussenpozen herhaalde bepalingen op serummonsters van ratten met chronische EAMG lieten in de tijd steeds een constante expressie van alle spectrotypen zien. Dit was reeds het geval vroeg na de immunisatie en het veranderde niet bij het manifest worden van de ziekte wat erop wijst dat het spectrotipe van de anti-Torpedo AChR antilichamen geen invloed heeft op het verloop van de ziekte. Dit werd eens te meer bevestigd door de constatering dat het spectrotipe van antilichamen gericht tegen rattespier-AChR geëluëerd van immuuncomplexen uit de neuro-musculaire eindplaat identiek was aan die van het serum. Deze resultaten tonen aan dat de antilichaamrespons in chronische EAMG polyclonaal is; ook in de mens wordt myasthenie veroorzaakt door een polyclonale antilichaamrespons die tegen de MIR gericht is. De bevinding dat onder omstandigheden waarbij kennelijk een overmaat kruis-reactieve antilichamen aanwezig is toch onbezette AChR aantoonbaar zijn wijst erop dat behalve eigenschappen van de kruis-reactieve antilichamen ook die van de eindplaat van belang zijn.

Hoofdstuk VI

In Hoofdstuk VI wordt het effect van manipulatie van het doelwit van de immuun-

respons in EAMG beschreven; de in deze studie verkregen resultaten hebben als uitgangspunt gediend voor het ontwerpen van een nieuwe strategie voor de behandeling van myasthenia.

Aangezien de spier zowel gedurende de embryonale ontwikkeling als na denervatie in staat is tot een grote toename in de aanmaak van AChR werd de hypothese gelanceerd dat ook na een aanval door immunologische effectormechanismen (resultierend in het ontstaan van onderlinge koppeling tussen AChR in de neuro-musculaire eindplaat) "cross-linking" als reactief proces zal toenemen. Mocht dat zo zijn dan zou dus niet alleen specifieke of a-specifieke onderdrukking van de antilichaamrespons een gunstig effect op het verloop van EAMG hebben, maar ook behandelingen welke de synthese van AChR versnellen.

Deze hypothese werd getoetst door bij ratten een achterpoot te denerveren door de nervus ischiaticus te verwijderen; 8 dagen later werden deze ratten ingespoten met monoclonale antilichamen tegen de MIR. In de gedenerveerde poot werd 4 maal zo veel AChR gevonden vergeleken met de niet-gedenerveerde controle poot. Nog belangrijker was de bevinding dat, in tegenstelling tot in de controle poot, in de gedenerveerde poot geen verlies aan AChR was opgetreden. De resultaten van dit experiment suggereerden dat het door de antilichamen veroorzaakte verlies van AChR in de gedenerveerde poot werd goedge maakt door de reactieve toename van de AChR synthese. Vervolgens werd het effect van denervatie uitgevoerd op het moment van de inductie van chronische EAMG, op het verlies aan spier AChR gemeten. Alle ratten ontwikkelden hoge anti-AChR antilichaam titers maar in de controle poten werd procentueel 4 maal zoveel verlies aan AChR gemeten als in de gedenerveerde poten. In de gedenerveerde poten was het percentage antilichaam dat gebonden was aan de AChR echter lager (27%). Deze lagere bezettingsgraad van AChR door antilichamen in de gedenerveerde poten steunt de visie dat in deze poten een sterkere reactieve toename van de AChR synthese had plaatsgevonden dan in de controle poten. Vervolgens werd om twee redenen het effect van anabole steroïden (nandrolone) op chronische EAMG nagegaan. Ten eerste, omdat anabole steroïden een duidelijk positief effect op de spiermassa hebben en omdat androgenen de synthese van ontbrekende eiwitten bevorderen in bepaalde erfelijke ziekten bij de mens. We veronderstelden dat anabole steroïden, naar analogie van denervatie-experimenten, ook de reactieve toename van de AChR synthese na de door antilichamen tot stand gebrachte cross-linking van individuele AChR zouden kunnen beïnvloeden. Bovendien is er epidemiologisch bewijsmateriaal betreffende de mens, als experimenteel bewijs uit spontane proefdier modellen, dat geslachtshormonen een rol spelen in auto-immuunziekten, met inbegrip van myasthenia gravis. Vrouwelijke Lewis ratten werden geïmmuniseerd met Torpedo AChR en kregen om de twee weken een dosis anabole steroïden. Deze dieren werden 30 dagen na de eerste immunisatie met AChR afgemaakt; 3 van de 4 ratten vertoonde toen klinisch gesproken ernstige myasthenie. In de controle ratten werd met de klinische myasthenie 50% verlies van de spier-AChR gezien; 80% van de resterende AChR was echter bezet door antilichamen. In vergelijking met de controles werden in het serum van de met anabole steroïden behandelde

ratten statistisch significant minder kruis-reagerende antilichamen gevonden. De verschillen in de mate van immuuncomplexvorming in de neuro-musculaire eindplaat waren echter het meest in het oog springend: hoewel de met anabole steroïden behandelde ratten iets minder spier-AChR hadden verloren dan de controles (43% vs. 53%) was in deze dieren van de overgebleven AChR slechts 44 % bezet door antilichamen. Deze resultaten tonen aan dat anabole steroïden in ratten een gunstig effect hebben op chronische EAMG omdat ze de concentratie van de antilichamen verlagen; daarnaast lijkt het waarschijnlijk dat er tevens een gunstig effect is op de toename van de AChR synthese in reactie op de door antilichamen tot stand gebrachte verbinding van individuele AChR.

ADDENDUM: Material and methods

1. ANIMALS

Inbred female Lewis and Brown-Norway rats were obtained from Charles River laboratories (Wilmington, MA) or from our own breeding colony (Dept. of Experimental Animal Services of the University of Limburg, Maastricht, The Netherlands). The rats were bred under special pathogen free circumstances. All animals were supplied with normal tap water and rat chow ad libitum. Rats purchased from both suppliers did not differ in their *in vivo* or *in vitro* reactivity to AChR. Torpedo californica electric ray and electro-phorus electricus (electric cel) was obtained from Pacific Biomarine (Venice, California U.S.). The main electric organ was removed and frozen on dry ice and kept at -70°C .

2. ACETYLCHOLINE RECEPTOR PREPARATION

2.1 Soluble AChR

AChR solubilized with Triton X-100 from the main electric organ of Torpedo californica or Electrophorus electricus, fetal bovine muscle or denervated rat muscles was purified on an affinity column of Naja Naja Siamensis (Miami Serpentarium, Florida, USA) coupled to sepharose CL4B, followed by specific elution with an acetylcholine analogue benzoquinonium (gift of Stirling Winthrop). In order to avoid proteolysis, EDTA, iodoacetamide and phenylmethanesulfonylfluoride (PMSF sigma) were used as protease inhibitors. The entire procedure was performed at 4°C in a cold room and completed within 24 hr.

A typical procedure as carried out in Dr. J.M. Lindstrom's laboratory is as follows (1): Typically 400 gr frozen Torpedo californica electric organ was broken into chunks and mixed with 1.5 volumes of 10 mM Na phosphate pH 7.5, 10 mM NaN_3 , 5mM EDTA, and agitated occasionally over about 15 min while the tissue thawed.

Iodoacetamide (to 5mM) and PMSF (to 1mM) were added and the mixture was homogenized in a 1-gallon Warring blender for 8 x 15 sec at high speed. The initial homogenate was then centrifuged at low speed, 10 min., at 5000 rpm in a Beckman JA-14 rotor. The supernatant was strained through a wire mesh to remove large fragments. The pellet was rehomogenized 1 x 30 seconds in an equal volume of the initial buffer, re-centrifuged, and the strained supernatant pooled with the first. The low speed pellet was discarded and the supernatant was centrifuged in a Beckman type JA-20 rotor at 19000 rpm for 60 min. This crude membrane pellet was resuspended with a Virtis homogenizer in 10 mM Na phosphate buffer pH 7.5, 10 mM NaN_3 , 1 mM EDTA. Sodium cholate (10% in dH_2O) was added to the suspension to a final concentration of 2%. After

30 min. shaking at 4°C, the mixture was centrifuged for 30 min at 30.000 rpm in a Beckman no. 30 rotor. The supernatant was added to toxin-sepharose (0,5 mg of Naja Naja Siamensis toxin II/ml of sepharose CL4B) at about 5 nmol ($\pm 0,6$ mg) of AChR/ml of toxin-sepharose and shaken gently for 1 h before being poured into a column. The column was washed with 300 column-volumes of 0.2% cholate buffer (0.2% Na Cholate, 100 mM NaCl, 4 mM Na Phosphate, pH 7.5). AChR was eluted with 2 mM Benzoquinonium chloride in 0.2% cholate buffer overnight into a small DEAE cellulose (40 ml) column in a pumped closed system at 4°C. The DEAE column was washed with one column of 0.2% cholate buffer, then eluted with 0.5 M NaCl 0.2% cholate buffer. Torpedo AChR had a specific activity of about 10 nmol α -bungarotoxin binding sites/mg protein.

2.2 Subunits

The four *subunits* composing Torpedo AChR were purified by preparative electrophoresis in sodiumdodecylsulfate (SDS) (2). The AChR preparation was concentrated in an Amicon XM 100 apparatus in 0.2% cholate buffer plus 0.5 M NaCl to 5 ml. After addition of 5 ml dH₂O it was reconcentrated to 5 ml. The sample was (30-40 mg) made up to 14 ml with sample buffer (2.3% SDS, 5% mercaptoethanol, 10% glycerol and 62,5 mM TRIS-HCl pH 6.8) and applied to a preparative gel of 10% acrylamide containing 0.1% SDS (0,5x34x27 cm) with a stacking gel of 4,75% acrylamide (0,5x5x27 cm). The gel was run at 125 mA at 4°C for 16 hrs. The gel was stained with 0,25% Coomassie Brilliant Blue in 50% methanol and 0,5% acetic acid for 2 minutes and destained minimally with 50% methanol and 0,5% acetic acid. The gel was then sliced and the individual slices comprising the subunits were homogenized in 100 ml dH₂O in a Warring blender (155). Then 200 ml dH₂O is added and finally 1,5 ml 10% SDS. This gel mixture was shaken overnight at 4°C and subsequently for 2 hrs at room temperature and filtered. The filtrate was lyophilized, resuspended in 5ml dH₂O and lyophilized again to total dryness. The Coomassie blue dye was removed by methanol extraction with freezing. The sample was then brought up in 1% SDS in 100 mM NaCl, 10 mM Na phosphate pH 7.5 10 mM NaN₃ and dialyzed against 10 mM Na phosphate pH 7.5 containing 100 mM NaCl 0.05% SDS and 10 mM NaN₃.

2.3 Fetal bovine and denervated rat AChR

Fetal bovine and denervated rat AChR were isolated similarly as electric organ AChR (3). Special care was taken to avoid proteolysis since muscle tissue is rich in proteases. Muscle tissue was homogenized with 3-6 volumes of 10 mM Na Phosphate buffer pH 7.5, containing 10 mM NaN₃, 10 mM iodoacetamide, 10 mM imidazole, 1 mM phenylmethanesulfonylfluoride (PMSF), 1/1000 aprotinin (Trasylo[®], Bayer) in a Warring blender for 1 min. at high speed and centrifuged at 10.000 rpm in a Beckman JA-10 rotor. The pellet was resuspended in the same buffer and centrifuged again. The pellet

was resuspended with a Warring blender for 15 sec at low speed in 2% Triton X-100 phosphate buffer and the extraction was continued by gently shaking. The homogenate was centrifuged for 30 min. at 10,000 rpm in a JA-10 rotor. The supernatant was centrifuged at 30,000 rpm for 30 min. in a Beckman no. 30 rotor. The pellet was discarded and the supernatant applied to a Toxin-sepharose column run and eluted similarly as for electric organ AChR. The muscle AChR appeared equally pure on a 10% polyacrylamide SDS gel using a discontinuous buffer system according to Laemmli (4).

2.4 Chemical alteration of Torpedo AChR

Reduced and carboxymethylated AChR (RCM-AChR) (5)

Six ml affinity purified Torpedo AChR (0.8 mg/ml) was dialyzed against 6 M guanidine HCl in 0.2 M Tris buffer pH 7.0. Next the AChR preparation was reduced in 0.1 M 2- β -mercaptoethanol for 60 minutes at 37°C under nitrogen. Then iodoacetamide was added to a final concentration of 0.15 M maintaining the pH at 8.2 for 15 minutes with 2M Tris buffer. The sample was then dialysed twice against 100 volume 0.2% cholate, 10 mM Tris-HCl pH 7.5, 100 mM NaCl, 1 mM EDTA, 1 mM PMSF and 10 mM NaN₃. The recovery, as measured by a Lowry protein assay was 100%. The RCM-AChR did not bind ¹²⁵I- α -BGT when assayed under conditions as for AChR determinations.

Trypsinized Torpedo AChR

Torpedo AChR was proteolyzed with trypsin under controlled conditions. To one ml Torpedo AChR (1mg/ml) in 0.2% cholate-PBS, 20 μ l 0.01 mg/ml trypsin and 1 mM HCl was added and incubated at room temperature for 2 hr. The reaction was stopped by addition of 2 nmoles of PMSF (8 μ l of 1 mM PMSF in isopropanol). This trypsinized AChR retained the capacity to bind ¹²⁵I- α -BGT.

2.5 Purification of AChR-rich vesicles

AChR-rich vesicles were prepared by Dr. R. Anholt in the Receptor biology laboratory (director Dr. J.M. Lindstrom) at the Salk Institute (San Diego, USA) according to a published procedure (1). The crude membrane pellet as described above was resuspended in 3 volumes of 10 mM Na phosphate, pH 7.5, 1 mM EDTA, 10 mM NaN₃ in a Virtis homogenizer 4 x 30 sec. at high speed on ice. Then 4 M NaCl was added to a final concentration of 0.4 M and 60% sucrose to a final concentration of 32% (w/w) and the mixture was homogenized again for 2 x 30 sec. This suspension was layered at 11.5 ml/tube over 14.5 ml of 36% (w/w) sucrose in 0.4 M NaCl, 10mM Na phosphate, pH 7.5, 1 mM EDTA, 10 mM NaN₃. After centrifugation for 50 min at 50,000 rpm in a Beckman Ti-60 rotor, the floating fat pad and pellet were discarded. The band of AChR-rich membranes was collected, diluted to 30% sucrose, and then centrifuged for 45 min at 50,000 rpm in the Ti-60 rotor. For further purification of AChR in intact

membranes, this pellet was resuspended in a minimal volume of flux buffer (145 mM sucrose, 10 mM Na phosphate buffer, pH 7.5, 5 mM NaN₃) using a Polytron homogenizer for 3 x 30 sec. at high speed on ice. Then aliquots of approximately 12 ml were layered on 54 ml of linear 31 to 39% sucrose gradients (in 10 mM Na phosphate buffer, pH 7.5, 1 mM EDTA, 10 mM NaN₃). These gradients were centrifuged overnight at 25,000 rpm in a Beckman SW 27 rotor. The upper layer and pellet were discarded and the midband diluted 1:1 with flux buffer and then centrifuged at 50,000 rpm for 50 min in the Ti-60 rotor. The resulting pellet was resuspended in a minimal volume of flux buffer using an 18-gauge needle and syringe, and then homogenized in a motor-driven homogenizer with a Teflon pestle. This was the purified vesicle fraction.

2.6 Reconstitution of AChR in liposomes

AChR containing and control liposomes (containing no AChR) were also prepared by R. Anholt according to a published procedure (1). Reconstruction buffer (2% Na Cholate, Interchem, Montlucon, France), 25 mg/ml of soybean L- α -phosphatidylcholine (Sigma, commercial grade), 100 mM NaCl, 10 mM phosphate buffer, pH 7.5, 10 mM NaN₃) was prepared by three cycles of stirring the ingredients together at room temperature, freezing, and thawing. The solution was then centrifuged 30 min at 30,000 rpm in a Beckman no. 30 rotor to remove any unsolubilized lipid. This solution was then aliquoted and stored frozen. Reconstitution buffer was also made by dilution of a 150 mg/ml stock of lipid in water sonicated under argon to clarity in a bath-type sonicator at 4°C. The method by which cholate/lipid mixtures were made had no effect on the activity of AChR in the reconstituted membranes. Vesicles were resuspended in reconstitution buffer at concentrations of 0.1 to 6 mg of protein/ml. After blending on a Vortex mixer and stirring to provide homogeneous suspension, this mixture was dialyzed overnight against at least 100 volumes of 100 mM NaCl, 10 mM Na phosphate, pH 7.5, 10 mM NaN₃ followed by an additional overnight dialysis against flux buffer.

3. IMMUNIZATIONS

Rats were injected with Torpedo AChR (5 to 20 μ g) or fetal calf AChR (1 or 15 μ g) in 0.1 ml phosphate-buffered saline emulsified in an equal volume of complete Freund's adjuvant containing nine parts Marcol-52 to one part of Arlacel A with 1 mg mycobacterium tuberculosis H 37 RA (Difco Laboratories, Detroit, MI). The emulsion was injected subcutaneously in the footpads, the base of the tail, and several other sites on the lower back. In some experiments the animals were boosted 4 weeks after initial sensitization with an equal amount of the same emulsion. The animals were sacrificed 5 to 6 weeks after the primary immunization.

4. LYMPHOCYTE CULTURE SYSTEM

4.1 preparation of crude lymphocyte suspensions

Popliteal, inguinal and para-aortic lymph nodes and spleens were removed under aseptic conditions. Lymph nodes were freed of fat. Simple cell suspensions were prepared by forcing the lymph nodes or spleens through a stainless screen. The suspension was subsequently filtered through a nitex screen (110 μm opening) followed by three washes in Hanks' balanced salt solution (HBSS). The viability of the cells assessed by Trypan blue exclusion was between 60-70%. Peripheral blood lymphocytes were purified from heparinized (40 IU/ml) blood obtained by cardiac puncture. Whole blood was diluted 1:2 with HBSS. Lymphocytes were purified on Ficoll-Hypaque (Pharmacia) and freed from platelets on a discontinuous sucrose gradient (12 and 16% sucrose) according to the procedure of Perper et al. (6) with a yield of 1 to 1.5×10^6 cells/ml whole blood. Viability assayed by trypan blue exclusion was 95%.

4.2 Depletion of T lymphocytes

Lymph node cells were treated with anti-rat brain serum (anti-RB-serum) and complement to deplete T cells before culture. The method of Golub (7) as modified by Ortiz-Ortiz et al. (8) was used to prepare rabbit antiserum to brain-associated thymus antigen of rats (anti-RB serum). The antiserum was sequentially adsorbed on 1/3 volume Lewis rat erythrocytes, 1/3 packed volume of washed homogenized syngeneic kidney tissue, and 1/9 packed volume of rat Y3-Ag 1.2.3 myeloma cells. The specificity of the anti-RB antiserum was evaluated by using a complement-dependent cytotoxicity test on thymus, bone marrow and lymph node cells labeled with ^{51}Cr as described by Golub (7). By this technique the antiserum was proved to be T cell-specific. Lymph node cells ($5 \times 10^7/\text{ml}$) were incubated with an equal volume of anti-RB serum diluted 1/10 in HBSS for 30 min at 4°C . The cells were then washed in HBSS and incubated with 1 ml of a mixture of low toxicity rabbit complement and guinea pig complement (1:2. v/v) for 30 min at 37°C . This treatment abolished the proliferative response to concanavalin A by greater 90%.

4.3 Depletion of macrophages

Lymph node cells were filtered through Sephadex G-10 columns (Pharmacia) by the method of Ly and Mishell (9). Lymph node cells (5×10^7) in 2 ml 5% FCS-RPMI 1640 were applied to a column of Sephadex G-10 (4.5 x 0.5 cm) and allowed to penetrate. The column was eluted with the same media and the first 3 ml of effluent were discarded. Approximately 40% of the cells were recovered in the next 4 ml. The number of macrophages, measured by nonspecific esterase staining (10), was reduced from 3% to less than 0.1% after passage over the column.

4.4 Depletion of T helper cells by fluorescence activated cell sorting (FACS)

Antibodies.

A monoclonal mouse anti-rat T cell antibody (W3/25) described by Williams et al. (11) (Sera Lab Ltd. Sussex, England) was used to identify and isolate rat T lymphocytes by indirect immunofluorescence. Fluorescence-labeled F(ab)₂ fragments of goat anti-mouse IgG (Cappel Laboratories) were passed over a rat IgG Sepharose 4B column to render it specific for mouse IgG. This preparation stained less than 1% of rat lymphocytes directly by fluorescence.

Cell staining.

Lymphocytes obtained from animals with chronic EAMG were stained by incubating 30×10^6 cells purified by Ficoll-Hypaque (Pharmacia) with 200 μ g of W3/25 in a volume of 1 ml of RPMI 1640, supplemented with 5% rat serum, and were incubated for 30 min at 4°C. Cells were washed two times and resuspended in RPMI 1640 containing 5% rat serum and 0.01% NaN₃, incubated with 120 μ g fluorescein-labeled goat F(ab)₂ anti-mouse IgG for 30 min and washed two times in RPMI with 5% rat serum.

Analysis and cell sorting.

Fluorescence intensity and light scatter were determined in a fluorescence-activated cell sorter (FACS IV, Becton Dickinson, FACS Systems, Mountain View, CA). The preamplifier and laserbeam were adjusted the way that the fluorescence peak was at channel 180 and the light scattering peak was at channel 85 when 4.25 μ m fluorescent beads were tested (Duke Scientific, Palo Alto, CA). Lymphocytes were sorted in a FACS into W3/25⁺ and W3/25⁻ populations. Cells in channels 1 to 25 were deflected left and those in channels 50 to 225 were deflected right. Of the W3/25⁺ population, 98% were positively stained after examination of the sorted cells, whereas the W3/25⁻ deflected population was contaminated with less than 5% W3/25⁺ cells.

4.5 Lymphocyte proliferation (12)

The cells obtained by the procedures described above (4.1-4.4) were resuspended at a concentration of 1×10^6 cells/ml in RPMI 1640 (Flow Laboratories) supplemented with 5% Lewis rat serum, 100 U penicillin, 100 μ g streptomycin, and 5×10^{-5} M 2-mercaptoethanol. Triplicate cultures of 2×10^5 cells/0.1 ml were incubated in flat-bottomed microtiter plates (3040 microtest II, Falcon Plastics, Oxnard, PA) for 5 days at 37°C in 5% CO₂ in a humidified atmosphere. One hundred microliters of AChR previously dialyzed against 250 ml PBS and diluted in RPMI 1640 is added to each well at the start of the culture to give a concentration ranging from 0.005 μ g/culture (2×10^{-10} M) to 0.5 μ g/culture (2×10^{-8} M). The cultures were pulsed with 1 μ Ci tritiated thymidine ([³H] TdR, 5 Ci/mM, Amersham, England), 24 hr before the cells were harvested with an automated cell harvester. The filters (Titertek Harvest filters no. 78-105-05) were

dried, placed in 3 ml of scintillation fluid (OCS, Amersham, England) and counted in a scintillation counter. The results are expressed as mean counts per minute (cpm) minus background of triplicate cultures $\pm$ standard error. In some instance the background values (although subtracted) are also given.

4.6 Antibody synthesis *in vitro*

Immune lymphocytes (1×10^6 cells/ml) obtained by the procedures described above (4.1-4.4) are cultured in triplicate wells in the presence of increasing amounts of Torpedo AChR in 100 μ l RPMI 1640 supplemented as described in the proliferation assay for 5 days in a humidified atmosphere of 5% CO₂. The cells are then centrifuged in the microtiter plates at 200 g at room temperature and washed once with 200 μ l RPMI 1640 to remove AChR that might interfere with the radioimmunoassay. After resuspending the cells in 200 μ l supplemented medium, the cultures are incubated for an additional 24 hours. Aliquots (50 μ l) of the tissue culture fluid were assayed for antibody AChR by radioimmunoassay (vide infra).

5. RADIOIMMUNOASSAY OF ANTI-ACHR ANTIBODY

Titers of serum antibodies to Torpedo, rat or fetal bovine muscle were measured by indirect immunoprecipitation using ¹²⁵I- α bungarotoxin-labeled AChR as antigen and goat anti-rat immunoglobulin as second antibody (13).

5.1 Preparation of antigen

Crude extracts of AChR of electric organ, fetal bovine muscle or denervated rat muscle were prepared as described for the crude membrane preparation of electric organ. Minced half thawed muscle was homogenized in a Warring blender at 4°C in 2 volumes 10 mM Na phosphate buffer, 1mM iodoacetamide and 1 mM PMSF, pH 7.4, 100 mM NaCl, 10 mM NaN₃, for 1 minute at high speed then centrifuged in a Beckman JA 10 or JA-14 rotor at 10,000 rpm or 14,000 rpm respectively. The resulting pellet was extracted with 2 volumes of 2% Triton X-100 in 10 mM Na phosphate pH 7.4, 100 mM NaCl, 10 mM NaN₃, 1 mM iodoacetamide and 1 mM PMSF by shaking for 2 hrs. The supernatant after centrifugation for 60 min at 100,000 g was used as antigen in the radioimmunoassay.

5.2 Radiolabeling of α -bungarotoxin

Alfa-bungarotoxin (α -BGT) from Bungarus multicinctus was purified according to Lee et al. (14) or purchased purified from Miami Serpentarium (Miami, Florida, USA). Radiolabeling of α -BGT was performed as described by J. Lindstrom et al. (13). To 5 mCi carrier-free ¹²⁵I (New England Nuclear or Amersham) was added 10 μ l 0.1

M Na phosphate buffer pH 7.0 followed by a few drops 1M HCl to neutralize the NaOH in which ^{125}I was shipped. α -Bungarotoxin ($\pm 0,5$ mg or $2,4 \times 10^{-8}$ moles) and NaI (28 nmoles) were added in 50 μl of 0.1 M Na phosphate pH 7.0. Chloramine T was added (10 μl containing 28 nmoles) and the solution was mixed on a magnetic stirrer at 4°C for 10 minutes. The sample along with 100 μl of buffer was applied to a disposable G25 column (0,5x10 cm) preequilibrated with 2 ml BSA (1mg/ml) and was washed with 5 column volumes of 0.1 M Na phosphate buffer pH 7.0. The column was eluted with the same buffer. ^{125}I - α -bungarotoxin elutes in the void volume along with 90% of radioactivity. The ^{125}I - α -BGT was adjusted to a concentration of $2 \times 10^{-8}\text{M}$, BSA was added to a concentration of 1 mg/ml and the samples (200 μl) stored frozen at -70°C . Sometimes smaller badges of α -BGT (+ 50 μg) were labeled by omitting NaI and reducing chloramine-T to 2,4 nmoles and ^{125}I to 1mCi. The specific activity of ^{125}I - α -BGT varied from $2\text{-}3 \times 10^{17}$ cpm/mole.

5.3 Measurement of AChR concentration

Concentration of AChR was estimated by measuring ^{125}I - α -BGT binding (13,15). Aliquots of receptor (10-100 μl) were incubated 4 hr with $2 \cdot 10^{-9}\text{M}$ ^{125}I - α -BGT in 0,5% Triton X-100-PBS- NaN_3 or with 10^{-3}M acetylcholine and 10^{-3}M neostigminebromide to inhibit the binding of ^{125}I - α -BGT to the acetylcholine binding site of AChR. The ^{125}I - α -BGT-AChR complex was incubated overnight with 5 μl of pooled serum of rats with EAMG at 4°C . Then 100-150 μl of goat anti-rat gammaglobulin serum (50% ammoniumsulfate precipitate) was added to precipitate immune complexes. After 4 hr at 4°C the samples were centrifuged in a Eppendorf microfuge (± 10.000 g) for 2 min, the pellet was washed with 1 ml 0,5% Triton X-100-PBS- NaN_3 and counted in a gamma counter. ^{125}I - α -BGT bound in the presence of acetylcholine/neostigmine bromide (10^{-3} M) was subtracted from the total before computing the concentration of AChR following the equation:

$$\frac{\text{cpm/l}}{\text{specific activity of } ^{125}\text{I}-\alpha\text{-BGT (cpm/mol)}} = \text{cpm} \times \frac{10^6}{\text{sample volume}} \times \frac{1}{\text{specific activity of } ^{125}\text{I}-\alpha\text{-BGT standard (cpm/mole)}}$$

AChR concentration is expressed in moles toxin binding sites/l.

5.4 Measurements of anti-AChR antibody

Anti-AChR antibody concentration was measured by immunoprecipitation using ^{125}I - α -BGT-AChR as antigen (13) AChR ($1 \times 10^{-9}\text{M}$) was labeled with ^{125}I - α -BGT ($2 \times 10^{-9}\text{M}$) for a minimum of 4 hr. ^{125}I - α -BGT-AChR was incubated in a volume of 1 ml 0,5%

Triton X-100-PBS- NaN_3 buffer with 5 μl diluted serum (1/100 or 1/500) overnight at 4°C. The resulting complexes were precipitated along with carrier immunoglobulin (5 μl normal rat serum) by addition of 100-150 μl goat anti rat immunoglobulin (50% ammoniumsulfate precipitate). After 4 hr incubation at 4°C, the samples were centrifuged for 2 min in an Eppendorf centrifuge and the resulting pellet was washed once with 1 ml 0,5% Triton-X-100-PBS- NaN_3 . Radioactivity in the resulting pellet was counted in a gamma counter. The value for ^{125}I - α -BGT-AChR pelleted in the presence of normal rat serum (5 μl) was subtracted from the assay value. The antibody titer was calculated from the moles ^{125}I - α -BGT precipitated using the following equation:

$$= \text{cpm} \times \frac{10^6}{\text{sample volume}} \times \frac{\text{dilution of serum sample}}{\text{specific activity of } ^{125}\text{I}-\alpha\text{-BGT standard (cpm/mole)}}$$

The titer is expressed in moles of toxin/liter serum.

Example:

assay values: 20500, 21000, 20750 cpm

background: 1280, 1265, 1300 cpm

sample volume: 5 μl

dilution: 1/100

specific activity of the ^{125}I - α -BGT standard: 3.25×10^{17} cpm/ml (corrected for radioactive decay)

equation for calculating titer:

$$= 19468 \times \frac{10^6}{5} \times \frac{100}{3,25 \times 10^{17}} = 1.198 \times 10^{-6} \text{ moles toxin binding sites/l serum.}$$

When anti-Torpedo AChR antibody titers were compared to anti-fetal calf AChR antibody titers, the anti-torpedo AChR titers were divided by 1,7 to correct for the presence of dimers (70%) present in Torpedo AChR preparations.

6. DETERMINATION OF ANTI-ACHR ANTIBODY SPECIFICITY BY ENZYME IMMUNOASSAY

Anti-AChR antibody binding to solid phase AChR was measured using glucosoxidase-goat anti rat Ig conjugates as described by R. Johnson (16). Microtiter plates (Dynatech Immulon type I, flat bottom) were incubated with 1×10^{-12} mol affinity purified (125 ng) Torpedo AChR in 50 μl of 0.01 M Na bicarbonate buffer pH 9.6 and dried under vacuum overnight at room temperature. Under these conditions 5.5 ng AChR (44 pmoles) or $4.4\% \pm 0.5$ ($m \pm \text{SD}$, $n = 16$) of the AChR added bound to the well. The wells were then incubated with 200 μl BSA-PBS-Tween-buffer (5 mg/ml bovine serum albumin,

0.5% Tween 20, 10 mM phosphate pH 7.5, 100mM NaCl and 10 mM NaN₃) for 15 minutes at room temperature to saturate unbound sites on the plastic. Wells were then washed three times with PBS-Tween-buffer. The acetylcholine binding site was then saturated by incubation with an excess of acetylcholine (10^{-3} M) and neostigmine bromide (10^{-3} M) in a volume of 50 μ l for 2 hr. Then 50 μ l of serial dilutions of EAMG serum in BSA-PBS-Tween buffer were added without washing the wells. After 2 hr incubation on a rocker platform, wells were washed 3 times with 200 μ l PBS-Tween-buffer prior to addition of 50 μ l goat anti-rat IgG conjugates to glucosylidase (a gift of R. Johnson, Reagent Applic. Inc., San Diego, USA) and incubated 1 hr at room temperature. After three washes with PBS-Tween-buffer, 200 μ l enzyme substrate was added. This substrate consisted of 24 volumes of 10 mM Na phosphate buffer, 100 mM NaCl, 3 volumes of 18% glucose, one volume of 0.02% horseradish peroxidase (type VI, sigma) and 0.2 volumes of 2% ABTS (2,2'-Azino-di-3-ethyl-benzthiazoline-6-sulfonate). After 60 minutes the O.D._{414nm} was read using a titertek multiscan (Flow laboratories). Background values for AChR coated wells not exposed to antibody were subtracted.

7. INTERLEUKIN-2

7.1 Assay of Interleukin-2 (IL-2)

IL-2 activity was assayed on aliquots of tissue culture supernatants using an IL-2 dependent cytotoxic T cell line (CTLL-2 line)(17). This cell line was maintained in bulk cultures at 1×10^5 cells/ml in RPMI-1640 medium supplemented with 5% fetal calf serum, 1% glutamine, 100 U penicillin, 100 μ g streptomycin and 5×10^{-5} M 2- β -mercaptoethanol in a humidified atmosphere of 5% CO₂. Before the assay, the cells were washed 3 times with PBS and pipetted in microtiter wells at 5×10^3 cells/well in RPMI 1640 supplemented as described above. Aliquots of the tissue culture supernatants (10-100 μ l) to be assayed for IL-2 activity, were added and the cells were cultured for 24 hrs in 5% CO₂. The next day the cells were pulsed with 1 μ Ci ³H-TdR six hours before harvest with an automated cell harvester. The filters were dried, placed in 3 ml of scintillation fluid (OCS, Amersham or Atomlight, NEN) and counted in a liquid scintillation counter. The results are expressed as mean counts per minute minus the background of triplicate cultures $\pm$ standard error.

7.2 Purification of interleukin-2

Interleukin-2 was partially purified from tissue culture supernatants of Con A stimulated spleen cells by gel permeation chromatography on a ACA-44 column. Spleen cells (5×10^6 /ml) from Lewis rats were cultured for 48 hrs in serum-free RPMI- 1640 medium supplemented with Con A (2 μ g/ml) and 0.01% polyethyleneglycol (MW 4000). The culture supernatants were concentrated under nitrogen on a YM 5 Amicon concentrating apparatus. The concentrated supernatants were applied on a ACA 44 column equi-

librated with PBS and eluted with the same buffer. IL-2 containing fractions (MW 12,000-18,000) were pooled. The IL-2 preparation used for this study contained 1250 U/ml (one unit being the amount of supernatant that gives a half maximal proliferation of 5×10^3 CTLL cells).

8. MONOCLONAL ANTIBODIES

The monoclonal antibodies used in this study prepared by Dr. S.J. Tzartos (Institute Pasteur Hellenic, Athens, Greece) in the Receptor Biology laboratory at the Salk Institute (Director Dr. J.M. Lindstrom) (18,19). Lewis, Sprague Dawley and Louvain rats received several subcutaneous injections of purified, native, or SDS denatured AChR in CFA. Animals were killed 3 to 5 days after the last booster injection and their spleen cells fused with either the mouse myeloma cell line S 194 (non secreter line) or with the rat LOU myeloma Y3 cell line (kappa chain secretor line). Spleen cells (1×10^8) were fused with S 194 cells (1.5×10^7) or with Y3 cells (5×10^7) using 50% polyethylene glycol 1500. Hybrids derived from S 194 myeloma form tight colonies in agar; therefore they were isolated using a "direct cloning method" (19). Briefly after fusion and washing, the cells were suspended in 15 ml warm 0.25% Agar in HAT medium. The resulting cell suspension in agar was quickly dispensed into 48 (2 ml) wells (0.3 ml/well). The next day 1.5 ml of HAT medium was added to each well. Three to four half-replacements of the HAT medium followed during the next 2 weeks. Then the supernatants were assayed for anti-AChR antibody. Colonies in antibody-containing wells were transferred into microtiter wells (0.2 ml) using a pasteur pipette. After 2 days the supernatants were assayed for anti-AChR antibody. This direct cloning technique avoids that the faster growing non producing hybrids overgrow the slow growing antibody producing hybrids. Recloning was performed by several dilutions in agar. Hybrids derived from Y3 cells all were cloned by the classical limiting dilutions technique (20) because they do not form condensed colonies in agar. Immunoglobulin fractions of the culture media from the cloned hybrids formed in large volumes were isolated and concentrated via 40-45% ammoniumsulfate precipitation and dialyzed against 10 mM Na phosphate buffer pH 7.5, 100 mM NaCl, 10 mM NaN_3 . Determination of Ig class and subclass was done by immunodiffusion using anti-rat Ig subclass antisera (a gift from H. Bazin, University of Louvain, Belgium) and known controls. The characteristics of the rat monoclonal antibodies used in this study are depicted in table I.

Table 1: Characteristics of monoclonal antibodies against AChR

MAB nr.	Immunogen (AChR)	Isotype	Cross reaction anti mammalian	subsequent specificity
1	Torpedo	IgG 2b	—	α (MIR)
2	Torpedo	IgG 2a	—	α (MIR)
3	Torpedo	IgG 2b	—	α
4	Torpedo	IgG 2b	—	α (MIR)
5	Torpedo	IgG 2b	—	α
6	Torpedo	IgG 1	+	α (MIR)
7	Torpedo	IgG 1	—	α
8	α -subunit	IgG 2a	—	α
11	α -subunit	IgM	—	β
12	Torpedo/Eel	IgG 2b	+	α (MIR)
14	Torpedo/Eel	IgA 2a	+	α (MIR)
16	Torpedo/Eel	IgG 1	+	α (MIR)
17	Torpedo/Eel	IgG 2b	+	$\alpha \beta \gamma$
19	Torpedo/Eel	IgG 2b	0	$\alpha \beta \gamma$
22	Eel	IgG 2b	0	α (MIR)
28	Eel	IgG 2a	+	α (MIR)
35	Eel	IgG 1	+	α (MIR)
37	Eel	IgG 2a	+	α (MIR)
61	Denatured Eel	IgG 2a	+	$\alpha \gamma$
65	Fetal Calf	IgG 1	+	$\alpha \beta$ (MIR)
71	Fetal Calf	IgG 1	+	α (MIR)
152	Subunit (Torp.) + Denatured AChR	nd	+	α
153	Subunit (Torp.) + Denatured AChR	nd	+	α
155	Subunit (Torp.) + Denatured AChR	nd	+	α
157	Subunit (Torp.) + Denatured AChR	nd	+	α
158	Subunit (Torp.) + Denatured AChR	IgM	+	α
164	Subunit (Torp.) + Denatured AChR	nd	+	α
198	Human	IgG 2a	+	α
203	Human	IgG 2a	+	nd

9. ISOELECTRIC FOCUSING

9.1 Analytical isoelectric focusing

Isoelectric focusing was performed according to the method of Briles and Davie (21) with some modifications (22). The vertical slab electrophoresis of 3.6% polyacrylamide gel containing 3% (pH 3-11) Ampholines (LKB Products, Promma, Sweden), 8M urea and 2% Nonidet P-40 (NP-40) was carried out at constant current of 5 mA for the first 120 minutes. After the prefocusing period the samples (diluted to a final volume of 150 μ l in buffer containing 8M urea, 2% NP-40 and 3% ampholyte) were applied to the anodic end and electrophoresis was carried out at a constant current (5 mA) for 3 hours and subsequently at constant power (1W) for 12 hours at 12°C. The focused proteins were precipitated by sequential exposure of two changes of 21% sodium sulfate (4 hours). After two changes of PBS (2 hours) to remove the gel by incubation in 0.4% dimethylsuberidimate hydrochloride (Sigma Chemical Co., St. Louis MC) in PBS for 2 hrs. The gels were then washed for 14 hours with 4 changes of PBS before incubation with the 125 I-labeled proteins, consisting of either 50×10^{-12} moles AChR conjugated with 100×10^{-12} moles 125 I- α -BGT in 50 ml 0.5% Triton-X-100-PBS- NaN_3 or with 10 μ Ci 125 I-labeled BSA in PBS (specific activity 1 μ Ci/ μ g). After incubation at 4°C for 14 hours, the gels were rinsed with PBS until radioactivity was absent in the washing solution and then dried at room temperature. Autoradiography of the gels used Kodak XRP-1 film (for 1-7 days).

9.2 Preparative Isoelectric Focusing

The gels were prepared and elctrophoresed under identical conditions as for analytical focusing, except that the spacer was omitted during the casting of the gels. After focusing and pH measurement the gels were sliced into 0.5 cm bands. Each band was eluted in 2 ml of PBS pH 7.5 at 4°C for 48 hours and the fractions dialyzed against PBS and concentrated by negative pressure dialysis. Antibodies to AChR from Torpedo electric organ and rat muscle were measured in each fraction by radioimmunoassay.

10. QUANTITATION OF ACHR AND ANTI-AChR ANTIBODY COMPLEXES IN MUSCLE

The concentration of AChR in Triton-solubilized rat muscle was determined according to Lindstrom et al (23). Each rat was processed separatly after removing intestines, skin, paws and tail. Each carcas was frozen, then minced and homogenized in 250 ml 10mM Na phosphate pH 7.5, 100 mM NaCl, 10 mM NaN_3 , 10 mM iodoacetamide and 1 mM PMSF in a Warring blender for 1 minute at high speed. The homogenate was centrifuged at 10,000 rpm in a Beckman JA-10 rotor or at 14,000 rpm in a JA-14 rotor for 30 min. The resulting pellet was resuspended in 150 ml 0.5% Triton X-100-PBS-

NaN₃ buffer with the same additions as used for the first homogenization. After extraction (2 hrs on a Dubnoff shaker at low speed to avoid excessive foaming) the samples were centrifuges for 30 min in a JA-10 or JA-14 rotor at respectively 10,000 rpm or 14,000 rpm. The supernatant containing the solubilized AChR was filtered through glass wool to remove the lipid. An aliquot (5 ml) was specifically labeled with 5 μ l ¹²⁵I- α -BGT (2x10⁻⁶M) units or with 10⁻³M acetylcholine and 10⁻³M neostigmine bromide overnight at 4°C. Labeled AChR was then precipitated by addition of excess rat anti-AChR serum followed by goat anti-rat Ig to measure AChR concentration or with goat anti-rat antibody only to measure AChR complexed with antibody.

10.1 Measurement of AChR in muscle

Half ml aliquots of labeled extracts (both with or without an excess acetylcholine and neostigmine bromide) were mixed with 5 μ l of pooled high titer rat anti-AChR serum obtained from rats with chronic EAMG. After overnight incubation, 100 μ l goat anti rat-immunoglobulin (50% ammoniumsulfate precipitate) was added and incubated for a minimum of 4 hr at 4°C. The tubes were centrifuged for 2 minutes in an Eppendorf microfuge and the resulting pellet was washed once with 1 ml 0.5% Triton X-100-PBS-NaN₃ buffer and pelleted again. The pellet was counted in a gamma-counter and the values with acetylcholine/neostigmine bromide were subtracted from the test samples. The concentration of AChR per rat was expressed as moles of ¹²⁵I- α -BGT binding sites/rat calculated using the following equation

$$\text{net cpm} \times \frac{10^6}{500} \times \frac{\text{volume of extract (liter)}}{\text{s.a. of } ^{125}\text{I-}\alpha\text{-BGT (cpm/mol)}}$$

10.2 Measurements of AChR and anti-AChR antibody complexes

One ml aliquots of labeled extracts (both with or without an excess of acetylcholine and neostigmine bromide) were mixed with 5 μ l normal rat serum. Then the samples were processed identical as those for the measurement of AChR. The concentration of anti-AChR antibody complexed with AChR expressed as moles ¹²⁵I- α -BGT binding sites per rat using the following equation:

$$\text{net cpm} \times \frac{10^6}{1000} \times \frac{\text{volume of extract (liter)}}{\text{s.a. of } ^{125}\text{I-}\alpha\text{-BGT (cmp/mol)}}$$

The percentage of AChR complexed with antibody was calculated for the following equation

$$\frac{\text{amount of anti-AChR antibody bound to AChR per rat}}{\text{amount of AChR per rat}} \times 100$$

11. Clinical Observations

Animals were observed for signs of muscular weakness which was graded according to the following score: 0, no definite weakness: +, weak grip or cry with fatigability: ++, hunched posture with head down, movements uncoordinated, and forelimb digits flexed: and ++++, severe generalized weakness, no cry or grip, tremulous, and moribund. (23).

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CURRICULUM VITAE

M.H. De Baets werd geboren op 2 april 1950 te Gent (België). Vanaf 1961 bezocht hij de humaniora afdeling van het St. Lievens College te Gent, alwaar hij in 1967 het eind-diploma Latijn-Wetenschappen (gymnasium beta) behaalde. Vervolgens studeerde hij geneeskunde aan de Rijksuniversiteit te Gent waar hij in 1974 afstudeerde. Hij ving op 1 oktober 1974 zijn opleiding tot internist aan de Rijksuniversiteit te Gent. De eerste 2 jaar van deze opleiding was hij werkzaam op de afdeling inwendige ziekten van de H. Hartkliniek te Eeklo (België) met als opleider Dr. R. de Keyser die hem naast de inwendige geneeskunde ook de interesse van de immunologie bijbracht. Vanaf 1979 was hij aangesteld als assistent in de kliniek inwendige ziekten van het Akademisch Ziekenhuis te Gent. Van 1979 tot 1981 werd hij uitgezonden naar de afdeling Immunologie van de "Scripps Clinic and Research Foundation" met als begeleider Dr. W.O. Weigle (La Jolla, Verenigde Staten van Amerika) en het laboratorium voor receptor biologie van het "Salk Institute for the Biological Sciences", met als begeleider Dr. J.M. Lindstrom (San Diego, Verenigde Staten van Amerika). Sinds november 1981 is hij als wetenschappelijk medewerker in dienst van de Rijksuniversiteit Limburg, capaciteitsgroep Immunologie, waar dit proefschrift tot stand kwam onder leiding van Prof. Dr. P.J.C. van Breda Vriesman.