Immunoregulation in Myasthenia Gravis

by

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in

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(Pathology Department)

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University of British Columbia
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Date Dec 16, 1987
Abstract:

Myasthenia Gravis (MG) is an autoimmune disorder of neuromuscular transmission. Clinically, the disease is manifested by abnormal muscle fatigue with recovery on resting. Circulating nicotinic acetylcholine receptor antibodies (nAchR Ab) are highly characteristic of myasthenia gravis. These antibodies have been shown to be directly pathogenic at the muscle endplate and are responsible for impaired neuromuscular transmission through several mechanisms.

While it is clear that the immune system does not function normally in MG, the mechanisms by which the response to nAchR is initiated and perpetuated remain unknown. Moreover, it is not clear whether immunoregulatory defects actually precede development of MG or are secondary features of the disease. The overall goal of the present investigation has been to more clearly define the nature of the immune regulatory defects existing in MG, both at the cellular level and in terms of possible relationship to disease progression.

To begin these studies it was necessary to develop an assay that could be used to measure nAchR Ab secreted by lymphocytes in culture. Thus, we modified the original nAchR Ab immunoassay described by Lindstrom (1976) for this purpose. Additionally, in order to gain access to an appropriate patient base for our study, we established a further modification with improved sensitivity for detection of serum nAchR Ab. This important diagnostic test had not been available in this country. Therefore, our assay was made available in Canada for clinical purposes.

Through the study of in vitro nAchR Ab and polyclonal IgG secretion by peripheral blood mononuclear cells (PBMNC), we were able to identify two previously unrecognized subgroups of seropositive, generalized MG patients. PBMNC from patients with long disease duration had low capacity for in vitro Ab production (Nonsecretors). Among patients of short disease duration, PBMNC produced nAchR Ab and also secreted higher than normal levels of polyclonal IgG (Secretors). The data suggested that there were nonspecific abnormalities affecting the immune response in myasthenia gravis. Moreover, regulation of B lymphocyte mediated immune function appeared to be related to disease progression.
It was hypothesized that circulating auto-antibody may contribute to deregulation of the immune response at certain stages of disease through direct interactions with leukocyte determinants. Separation/reconstitution experiments with CD4+ enriched, T-helper/inducer lymphocytes and B enriched (E- cells) lymphocytes suggested that the control of antibody production in myasthenia gravis was operative at the T-helper/inducer level. Preliminary studies with serum pretreated, CD4+ enriched, T-helper/inducer lymphocytes suggested that serum of Secretor MG patients indeed contained a factor(s) which interfered with the function of a CD4+ lymphocyte subset.

We further hypothesized that nAchR Ab would have the potential to behave as anti-lymphocyte Ab if nAchR were expressed on lymphocytes. Accordingly, direct binding studies, using the nicotinic antagonist, alpha-bungarotoxin, were carried out to look for such receptors on PBMNC. Specific, saturable binding of alpha-bungarotoxin to the rhabdomyosarcoma cell line, TE671, was confirmed and characterized. However, in parallel studies, alpha-bungarotoxin binding to PBMNC of healthy individuals or MG patients was not detected. These results suggested that nicotinic acetylcholine receptors, of the type expressed by muscle endplate, do not occur on human peripheral blood mononuclear cells.
Table of contents:

Abstract  ii.
List of Tables  vi.
List of Figures  viii.
List of abbreviations  x.

Chapter 1: Myasthenia Gravis
1.1 Introduction  2
1.2 nAchR Ab in MG  3
1.3 Regulation of the humoral immune response: general concepts  16
1.4 Regulation of the immune response in MG  26
1.7 Role of the thymus in MG  35
1.8 Rationále for studies  41

Chapter 2: Materials and Methods
2.1 Measurement of nAchR Ab  46
2.2 In vitro production of Ab  50
2.3 Direct binding studies  56

Chapter 3: Establishment of assay for nAchR Ab and occurrence of serum nAchR Ab in MG
3.1 Introduction  59
3.2 Results  61
3.3 Discussion  84
Chapter 4: In vitro production of nAchR Ab and polyclonal IgG in myasthenia gravis
4.1 Introduction 94
4.2 Results 99
4.3 Discussion 118
4.4 Summary 126

Chapter 5: Direct binding of aBT to human PBMNC and to TE671 rhabdomyosarcoma cell line
5.1 Introduction 129
5.2 Results 132
5.3 Discussion 137
5.4 Summary 138

Chapter 6: Summary and perspectives 140

References 146
List of Tables:

Table 3-1. Protein A precipitation of [125]I-aBT labelled nAchR... 62

Table 3-2. Comparison of the Precipitation of [125]I-aBT labelled nAchR by MG and Control Serum...65

Table 3-3. Validation of the SAC assay for diagnostic purposes...66

Table 3-4. Summary of results using SAC assay for determination of nAchR Ab in serum...68

Table 3-5. Comparison of human muscle preparations for detection of nAchR Ab in serum...69

Table 3-6. Comparison of human antigen with fetal calf antigen for the detection of nAchR Ab in serum...70

Table 3-7. Double antibody precipitation of immune complexes...71

Table 3-8. Estimation of nAchR in muscle extracts by maximal precipitation with MG sera...73

Table 3-9. Determination of concentration of nAchR in muscle extracts by DE-81 ion exchange technique...74

Table 3-10. Quantitative determination of nAchR Ab titer in MG serum...76

Table 3-11 Reproducibility of nAchR Ab titers measured between assays...79

Table 3-12. Precipitation of [125]I-aBT labelled nAchR in the presence of MG and control sera using a panel of human and fetal calf muscle extracts...82

Table 4-1. In vitro secretion of nAchR Ab in MG patients and controls...102

Table 4.2. Clinical characteristics, serum nAchR Ab levels and in vitro levels of nAchR Ab and IgG secreted by PBMNC in myasthenia gravis...103
Table 4.3. Correlation between disease duration and capacity of PBMNC from MG patients to secrete nAchR Ab in vitro...107

Table 4.4. Role of B and T-helper cells in IgG production among Secretors and Nonsecretors of nAchR Ab...109

Table 4.5. IgG production by thymocytes...111

Table 4.6. Effect of serum pre-treatment on con-A induced suppressor function of normal T cells...112

Table 4.7. Effect of serum pretreatment on the ability of CD4+ lymphocytes to support mitogen stimulated IgG production...114

Table 4.8. Influence of serum-pretreatment on the ability of CD4+ cells to stimulate CD8+ mediated suppression of IgG production...115

Table 5.1. Specific binding of [125]I-aBT to TE671 cells and PBMNC from myasthenia gravis patients (MG) and healthy donors (N)...134
List of Figures:

Figure 3-1. Precipitation of [125]I-aBT labelled nAchR by increasing amounts of MG serum...63

Figure 3-2. Precipitation of [125]I-aBT labelled nAchR by low titer MG serum...64

Figure 3-3. Scatchard plot for analysis of 125I-aBT binding to nAchR in muscle extracts...74

Figure 3.4. Determination of nAchR Ab titer in MG Serum...75

Figure 3.5. Quantitative determination of nAchR Ab titer in MG Serum...77

Figure 3.6. Comparison of disease severity and titer of nAchR Ab in serum of MG patients...81

Figure 3.7a. Precipitation of [125]I-aBT labelled nAchR by various MG sera...83

Figure 3.7b. Precipitation of [125]I-aBT labelled nAchR by various MG sera...84

Figure 4-1. Kinetics of nAchR Ab secretion by PBMNC of MG patients...99

Figure 4-2. Kinetics of secretion of nAchR Ab and polyclonal IgG in culture of thymocytes from MG patient...100

Figure 4.3. Correlation between levels of nAchR Ab secreted in cultures of PBMNC and in serum of MG patients...104

Figure 4.4. Levels of polyclonal IgG secreted in culture by Secretors and Nonsecretors and by controls...106
Figure 4.5. Influence of serum-pretreatment on the ability of CD4+ T-helper cells to support IgG production ...116

Figure 4.6. Influence of serum-pretreatment on the ability of CD4+ cells to stimulate CD8+ mediated suppression of IgG production ...117

Figure 5.1. Binding of [125]I-aBT to TE671 rhabdomyosarcoma cell line and human PBMNC...133

Figure 5.2. Analysis of binding of [125]I-aBT to TE671 line...136
**List of Abbreviations:**

MG
myasthenia gravis

Ab
antibody

nAchR
nicotinic acetylcholine receptor

ach
acetylcholine

aBT
alpha bungarotoxin

AchE
acetylcholinesterase

EAMG
experimental autoimmune myasthenia gravis

Thi
T helper inducer cell

Tsi
T suppressor inducer cell

INF-g
interferon gamma

IL
interleukin

Ig
immunoglobulin

IgG
immunoglobulin G

SIRS
soluble immune response suppressor

AMLR
autologous mixed lymphocyte reaction

TCR
T cell receptor

MLR
mixed lymphocyte reaction

PBMNC
peripheral blood mononuclear cell

IgSC
immunoglobulin secreting cell

HLA
human leukocyte antigen

MHC
major histocompatibility complex

CGVHD
chronic graft versus host disease

HC
Hassall's corpuscles

S1g
surface immunoglobulin

Con A
concanavalin A

PWM
pokeweed mitogen

anti-id
anti-idiotypic antibody

OND
other neurological disorder

SAC
staphylococcus aureus Cowan strain I

FCS
fetal calf serum

MIR
main immunogenic region [of nAchR]

OMD
other muscle disease

CTL
cytotoxic T lymphocyte
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>SLE</td>
<td>systemic lupus erythematosus</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>DAG</td>
<td>diacylglycerol</td>
</tr>
<tr>
<td>EBV</td>
<td>Epstein barr virus</td>
</tr>
<tr>
<td>KLH</td>
<td>keyhole limpet hemocyanin</td>
</tr>
<tr>
<td>MS</td>
<td>multiple sclerosis</td>
</tr>
<tr>
<td>mcAb</td>
<td>monoclonal antibody</td>
</tr>
<tr>
<td>TGF-B</td>
<td>transforming growth factor-beta</td>
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To
my parents
Chapter 1. Myasthenia Gravis

1.1 INTRODUCTION 2

1.2 nAchR Ab in MG 3
1.2.1 Historical perspectives 3
1.2.2 Structure of endplate nAchR 5
1.2.3 Pathogenicity of nAchR Ab at the muscle endplate 10
1.2.4 Polyclonal nature of the immune response in MG 13

1.3 REGULATION OF THE HUMORAL IMMUNE RESPONSE: GENERAL CONCEPTS 15
1.3.1.1 Positive control of humoral immunity 16
1.3.1.2 B cells 16
1.3.1.3 T-helper cells 18
1.3.2.1 Negative control of humoral immune responses 21
1.3.2.2 T-suppressor cells 22

1.4. REGULATION OF THE IMMUNE RESPONSE IN MG 26
1.4.1 Introduction 26
1.4.2 Enumeration of lymphocyte subsets in MG 27
1.4.3 Studies of immune function in MG 28
1.4.4 Studies of nAchR Ab specific immune response in MG 28
1.4.5 Non-specific defects of immune function in MG 30
1.4.5.1 Suppressor function in MG 30
1.4.5.2 Other auto Ab in MG 31
1.4.5.3 Polyclonal B cell function in MG 32

1.5. Evidence for primary immune function defects in MG 32
1.6. Evidence for secondary defects of immune response in MG 33

1.7 ROLE OF THE THYMUS IN MG 35
1.7.1 T cell development in normal thymus 35
1.7.2 Thymic hyperplasia in MG 38

1.8. RATIONALE FOR STUDIES 41
1.1 Introduction:

Myasthenia Gravis (MG) is an autoimmune disorder of neuromuscular transmission characterized by the abnormal fatigue of voluntary muscle. Clinically, MG is categorized as generalized or ocular. In the latter condition weakness is restricted to extraocular muscle, producing double vision and ptosis. MG patients often present with ocular and/or bulbar weakness and this may or may not progress to include the limb musculature (generalized MG). It is not uncommon for MG patients to spontaneously enter remission; however, the mechanisms behind this are unknown.

The overwhelming majority of MG patients with active, generalized disorder possess detectable levels of circulating antibodies (Ab) which are specific for the nicotinic acetylcholine receptor (nAchR) of muscle endplate. In fact, the presence of nAchR Ab in serum has become an essential diagnostic criterion for MG. nAchR Ab are not merely passive indicators for MG; they have been shown by numerous investigations to be directly pathogenic at the muscle endplate and to interfere with neuromuscular transmission.

Current treatments are aimed at compensating transmission defects by increasing synaptic concentrations of acetylcholine with cholinesterase inhibitors. Nonspecific immunosuppressant agents are also used. Thymectomy is frequently beneficial and may, in fact, be a more specific form of treatment than drug therapy since the gland may be acting as a renewable source of antigen (nAchR) or of specifically activated lymphocytes.

In order to gain insight into the mechanisms of autoimmunity and ultimately, to develop more specific treatments for MG, it is necessary to develop a greater understanding of the manner in which nAchR Ab production is regulated by the immune system. At present, the manner in which tolerance to the nAchR is initially lost is unknown. The most popular theories include loss of tolerance to self nAchR or nAchR-like molecules which are normally expressed in the thymus. In this scenario thymic nAchR may become altered in appearance or density preceding development of autoimmunity. Alternately, the immunizing species may be an exogenous, cross
reactive species (molecular mimicry). In either case, it is not clear whether, in MG, the immune system has behaved normally in response to a foreign antigen or if there are true defects in immune function which cause, predispose to or even arise following onset of MG.

Thus, it is of importance to analyse the function of the immune system in MG to establish the nature of defects which may exist and to design studies which will shed light on the possible mechanisms by which immune function changes over the course of the disease. The potential of disease dependent factors such as antilymphocyte antibodies or thymic hormones, to influence the function of the immune system at various clinical stages of the disease must also be examined.

1.2. nAchR Ab in MG:

1.2.1 Historical perspectives:

It had long been suspected that MG was a disease involving the immune system. Thymic pathology had been found to exist in the majority of MG patients (Castelman 1966). Moreover, the recognition that MG patients had a greater than normal incidence of putative autoimmune diseases led Simpson (1960) to hypothesize that MG was itself an autoimmune disorder.

Involvement of a circulating pathogenic factor was suspected following the observation that hemodialysis resulted in transient improvement of clinical symptoms (Stricker et al 1960). Moreover, it had been shown that serum from some MG patients could influence neuromuscular transmission to the frog sartorius muscle (Nastuk et al 1959). These investigators also showed that levels of complement in MG serum deviate significantly from normal and are correlated with disease activity (Nastuk et al 1956,1960). This finding led them to suggest that an autoimmune phenomenon was occurring (Nastuk et al 1960).

In early studies it was recognised that MG patients had circulating antibodies specific for striated muscle (Strauss et al 1960, Beutner et al 1962). Today it is known that a relatively small percentage of MG patients, mostly those with thymoma, possess antibodies to skeletal muscle antigens (Adner et al 1964, Penn et al 1986, Williams and Lennon 1986).

Simpson (1960) was the first to hypothesize that MG was due to an autoimmune response
against a muscle endplate protein. In 1966 Namba and Grob, using a complement fixation assay, reported that immunoglobulin from 48% of 63 MG sera could bind to a skeletal muscle ribonucleoprotein which had a high affinity for acetylcholine and D-tubocurarine. Binding was not observed with any of 77 serum samples from healthy controls and was observed with only 2.7% of 300 sera from patients with other diseases.

Almon et al (1974) had demonstrated that serum from some MG patients has the ability to inhibit the binding of alpha bungarotoxin (aBT), a snake toxin with extremely high affinity for nAchR (reviewed by Fambrough 1979), to detergent extracts of skeletal muscle. Histological studies by Bender et al (1975) confirmed that MG serum could displace aBT from the neuromuscular junction. To date, reports on the frequency of occurrence of these so-called blocking antibodies in serum of MG patients vary considerably (38%-98% of patients tested) (Lindstrom et al 1976c, Drachman et al 1982, Besinger et al 1983, Itoh et al 1985, Toyka et al 1986).

When nAchR Ab directed at sites other than the aBT binding site were measured, it was discovered that the majority of MG patients possess such Ab in their serum (Lindstrom et al 1976b, 1976c). This has been a consistent finding (Lefvert et al 1978, Garlepp et al, Nicholson et al 1982, Abramsky et al 1981, Vincent et al 1985). Moreover, nAchR Ab are detected only very rarely in persons who do not have clinical symptoms of MG (Lindstrom 1977, Lefvert et al 1978, Tindall et al 1981b, Garlepp et al 1982, Vincent et al 1985, Oger et al 1987). The measurement of nAchR Ab in sera of MG patients has become an extremely powerful diagnostic assay.

Diagnosis of MG is based upon clinical features (weakness of voluntary muscle with recovery of strength on resting), however, there are other disorders which have a similar presentation. The Tensilon ® test, administration of an acetylcholinesterase inhibitor (edrophonium chloride), leads to rapid, transient recovery of muscle strength in patients who have MG. This is a valuable test which is widely used. Difficulties can arise with the Tensilon test, however. This test does involve risk to cardiac patients. Furthermore, the test can sometimes be positive in other disorders, such as congenital myasthenia gravis or botulism. There is also an
element of subjectivity to this test which may render the results equivocal. Patients suspected of having MG will often be started on antiacetylcholinesterase and/or steroid medication to determine whether improvement will take place. If so, thymectomy may be carried out since this has been shown to be beneficial in a large percentage of MG patients. Thus, if the patient is incorrectly diagnosed there is the risk of performing unnecessary thymectomy and, of course, the risks associated with failure to treat the true disorder. In instances where MG exists and is not recognized there can be death due to respiratory failure. Errors in diagnosis of MG can and do occur, thus more accurate and objective testing will improve the health care that can be given to the patient.

Electromyography has been extremely useful in diagnosing MG, in which there is an impairment of neuromuscular transmission. Repetitive nerve stimulation results in sequential reduction to the amplitude of the contracting muscle action potential, which is recorded on a tracing. This technique is sensitive enough to recognize approximately 85% of MG when testing is carried out on several muscles. Single fiber electromyography (Stalberg 1978) is very sensitive for detecting mild transmission disorders. Electrical tests, however, can produce abnormal results in a variety of other muscle or nerve disorders (reviewed in Swift 1981).

The discovery that nAchR Ab directly damage the muscle endplate and are found circulating in the vast majority of MG patients has provided the basis for a powerful diagnostic test. nAchR Ab are also highly specific for MG. Routine use of this safe, simple blood test will greatly facilitate the differential diagnosis of MG.

1.2.2 Structure of endplate nAchR:

The nAchR of muscle endplate is a transmembrane glycoprotein consisting of a total of 5 subunits. There are 2 alpha subunits interspersed by a beta subunit on one side and by a gamma and a delta subunit on the other side. As illustrated in Diagram 1-A, the five subunits surround a central cation channel in an arrangement which has been likened to the staves of a barrel surrounding a central pit (reviewed in Lindstrom 1985a, Changeux and Revah 1987). The cation channel opens when acetylcholine binds to it's complementary site on the extracellular portion of
the alpha subunit. Ion flux through the "open" nAchR is critical for membrane depolarization and transmission of the neural signal.

The 4 different subunits of the nAchR have been studied in several species including human, bovine and electric eel. The primary structure of all 4 subunits has been determined through the cloning and sequencing of the genes encoding them (reviewed by Numa et al 1986). In human, primary structures of the alpha and gamma subunits have been determined (Noda et al 1983a, Shibahara et al 1985). Considerable homology exists (see Diagram 1-B) in the primary structures of the various nAchR subunits, both within and between species (Noda et al 1983b). Analysis of hydrophobicity profiles for the amino acid residues in nAchR subunits allowed recognition of 4 unusually hydrophobic regions (marked M1-4 in Diagram 1-B) (Noda et al 1982, Claudio et al 1983). The amphipathic alpha helix, MA, was detected by amphipathic analysis (Finer-Moore and Stroud 1984).

It is thought that all nAchR subunits have a similar secondary structure as deduced from their primary sequences (Noda et al 1983b). The N-terminal two thirds of the subunit molecule is the only portion containing potential glycosylation sites and thus is probably oriented extracellularly (Noda et al 1983b, Claudio et al 1983, Finer-Moore and Stroud 1984). The secondary structure of the N-terminal portion of the subunit molecule is probably composed of both alpha helices and anti-parallel beta-pleated sheets as determined from amphipathic analysis (Finer-Moore and Stroud 1984). A model of nAchR subunit structure and membrane orientation is illustrated in Diagram 1-C. In this model there are five membrane spanning domains. These include the regions denoted by M1, M2 and M3 in Diagram 1-B as well as an amphipathic alpha-helix termed M7 (not shown in Diagram 1-B) and a hydrophilic sequence termed M6 (Criado et al 1985). The unusually hydrophobic region, M4, is proposed to exist in a hydrophobic environment at the center of a globular domain of the molecule. The amphipathic helix, M7, may line the cation channel. The hydrophilic stretch, M6, was inferred to traverse the membrane because a preceding...
putative glycosylation site is assumed to occur extracellularly and several immediately succeeding sites map to the cytoplasmic side of the membrane (Criado et al 1985). The carboxy-terminal is located on the cytoplasmic side of the membrane. This placement has been confirmed by immunohistochemical and ultrastructural studies (reviewed in Lindstrom et al 1987, Young et al 1985). It is thought that the nAchR subunits are arranged in a pseudosymmetrical fashion within the membrane such that their membrane spanning regions associate to form the cation channel (Noda et al 1983b, Lindstrom 1985, Finer-Moore and Stroud 1984, Ratnam et al 1986b).

In general, this model is consistent with physical measurements of the nAchR. Electron microscopic studies indicate that the bulk of the molecule is located on the extracellular side of the membrane (Zhingshein et al 1982). X-ray diffraction data suggest the existence of intramembrane alpha-helices oriented perpendicular to the membrane surface (Ross et al 1977).

Earlier models of nAchR subunit structure differed from that shown in Diagram 1-C. A popular model had consisted of 4 membrane spanning regions which corresponded to the 4 unusually hydrophobic stretches of amino acid residues. The amino-terminal was correctly assigned to the extracellular side of the membrane. However, four turns of the subunit through the membrane had incorrectly placed the carboxy-terminal on the extracellular side of the membrane (Noda et al 1983b, Claudio et al 1983). Finer-Moore and Stroud (1984) recognized an amphipathic region (MA in Diagram 1-B) which had properties characteristic of a soluble alpha-helix. They proposed this region as a fifth membrane spanning domain whose hydrophilic sides would be in contact with the hydrophobic regions and whose hydrophilic surface would form the cation channel.

The model was refined to its present state (Diagram 1-C) by Lindstrom's group who carried out peptide mapping of the intact, membrane bound nAchR using their extensive library of nAchR monoclonal Abs (Ratnam et al 1986a and b). Using colloidal gold labelled Ab and electron microscopy, these investigators proved that the region between M3 and MA is exposed at the
cytoplasmic side of the membrane as had been proposed by others. Contrary to existing models at that time, they also found evidence that the MA and M4 regions are cytoplasmically oriented and do not traverse the membrane.

The nAchR molecule is large and complex, containing many potential immunogenic sites. It is now thought that immunogenic regions of proteins are those which are amphipathic. Hydrophobic portions of the processed antigen would thus associate with MHC molecules on the surface of an antigen presenting cell and hydrophilic portions would be accessible to the lymphocyte during an immune response. Theoretically, such regions would include portions of the nAchR which are folded into secondary structures. Recent studies of nAchR peptides with capability to stimulate lymphocytes from MG patients showed that peptides with amphipathic properties are indeed effectively presented to lymphocytes (Hohlfeld et al 1988). Recently, a strongly immunogenic domain of nAchR (main immunogenic region, MIR) has been mapped to residues 67-76 of the amino-terminal portion of the alpha subunit. The peptide corresponding to this region was predicted to have a convex shape (Tzartos et al 1988).

Diagram 1

Structure of nAchR

1-A

Model of nAchR in the membrane. The squiggly lines projecting from the extracellular surface represent carbohydrate. (Reproduced from Lindstrom 1985a)
Alignment of the amino acid sequences of mammalian, avian and fish nAChR subunit precursors. The one letter amino acid notation is used. Sets of aligned residues common to each subunit are enclosed with solid lines. Sets of identical or conservative residues at one aligned position are enclosed by dotted lines. Gaps have been inserted to achieve maximum homology. Positions in the aligned sequences including gaps are numbered beginning with the amino-terminal residue of the mature subunits and the preceding positions are indicated by negative numbers. The putative disulfide bridge shared by all subunits (S-S) and the putative transmembrane segments M1-4 and MA are indicated. (Reproduced from Numa et al 1986)
Model for the transmembrane orientation of the polypeptide chain in nAchR subunits. The numbers shown refer to the sequence of alpha subunits. Bold lines indicate a sequence whose transmembrane orientation has been explicitly demonstrated, whereas solid lines show intervening sequences whose orientations have been inferred from these observations. The authors note that residues 261-277 have now been determined to be exposed at the extracellular surface and should be indicated as a solid line instead of a dotted line. (Figure reproduced from Lindstrom et al 1987).

1.2.3 Pathogenicity of nAchR Ab at the Muscle Endplate:

Ultrastructural studies have shown that in MG the endplate region is affected by the disease process (Zacks et al 1962, Engel et al 1971). The predominant features of the healthy endplate include the highly folded post synaptic membrane, the distribution of nAchR predominantly at the crests of these folds (Bender et al 1976, Porter et al 1976) and the alignment of the crests with the axonal site of acetylcholine release (Porter et al 1976). At the healthy synapse nAchR and acetylcholine esterase (AchE), which degrades acetylcholine, are effectively compartmentalized; the depths of the sarcolemmal folds are rich in AchE and poor in nAchR.
In MG, the sarcolemmal folds are simplified (Zacks et al 1962, Engel et al 1971) which results in a widened synaptic cleft, loss of post synaptic surface area and loss of alignment. It would seem likely that, even in the absence of changes directly involving the nAchR, defects in neuromuscular transmission may arise secondary to such altered synaptic geometry (Lindstrom et al 1980). More specific studies showed that nAchR is reduced at muscle endplates in MG (Fambrough et al 1973). The degree of nAchR reduction has also been shown to correlate with clinical severity of MG (Pestronk et al 1985).

The direct involvement of the nAchR Ab in the destruction of muscle endplate became more clear following the demonstration by Patrick and Lindstrom in 1973 that rabbits which had been repeatedly immunized with nAchR developed muscle weakness which was reversible with acetylcholinesterase inhibitors. In 1975 the model was extended to rat and guinea pig and was termed experimental autoimmune myasthenia gravis (EAMG) (Lennon et al 1975). The experimental disease has been shown to be characterized by circulating Ab to the nAchR. Weakness occurs in the same locations as in human MG; the head, neck, upper limbs and respiratory muscles. Both EAMG and human MG are characterized by abnormal muscle fatigue with clinical improvement following treatment with anticholinesterases. Additionally, electrophysiological studies have revealed similar patterns of defect in both disorders.

EAMG has now been produced in a variety of animal species (Barkas et al 1979, reviewed in Lindstrom 1985b). Ultrastructural and immunohistological studies suggest that pathogenesis of endplate damage in chronic EAMG is similar to that observed in acquired, human MG (Sahashi et al 1978). Abnormal electrical findings, muscle weakness and inflammation have also been induced in some species following passive transfer of myasthenic immunoglobulin or monoclonal antibody (mcAb) specific for nAchR (Toyka et al 1975, Gomez et al 1985). Passive transfer of sublethal doses of nAchR Ab results in acute, transient EAMG which is characterized by phagocytic invasion of endplates. In fact, macrophage mediated destruction of endplates is a very important pathogenic mechanism in acute EAMG (reviewed in Ashizawa and Appel 1985),
although it is not observed in chronic EAMG or in human MG. Following recovery from passive transfer acute EAMG, a refractory period exists during which it is not possible to induce further EAMG by renewed administration of nAchR Ab (Corey et al 1985). Refractoriness may reflect prior reduction of endplate nAchR density so that immune mediated chemotaxic stimulus is insufficient to induce macrophage invasion (Corey et al 1987). These investigators speculated that similar mechanisms could conceivably account for the observed lack of cellular infiltrates at the endplate in human MG. However, it appears that passively transferred nAchR Ab contribute to development of an MG- or chronic EAMG-like endplate pathology long after the phagocytic infiltrates which characterize the acute phase have subsided (Gomez et al 1984, Gomez and Richman 1987).

Several studies have provided strong evidence that a complement mediated lytic reaction is responsible for destruction of the post synaptic membrane at myasthenic endplates. Complement fixing anti-muscle Ab were described in MG patients in 1960 by Strauss. Complement levels in serum of myasthenic patients are reduced during periods of more intense disease activity (Nastuk et al 1960). IgG and the complement components, C3 (Engel et al 1977a) and C9 (Sahashi et al 1980) have been detected at the myasthenic endplate, both on the sarcolemmal folds and on membrane debris occurring within the synaptic space. Deposition of immune complexes at the endplate is also detectable in MG patients who are seronegative for nAchR Ab (Tsujihata et al 1989). Immunoglobulin from myasthenic sera also induces complement dependent lysis of rat myotubes in vitro (Ashizawa and Appel 1985). Moreover, animals depleted of complement proteins are resistant to development of EAMG (reviewed in Ashizawa and Appel 1985).

Complement mediated lysis of the post synaptic membrane is not the sole mechanism by which anti-nAchR Ab interfere with neuromuscular transmission. nAchR Ab may increase the rate of internalization and degradation of receptors (modulation). Modulation of endplate nAchR expression by anti-nAchR Ab has been studied extensively. This mechanism is believed to involve a nAchR Ab mediated increase in the rate of internalization of nAchR that takes place without affecting the rate of re-insertion of nAchR and thus reduces the amount of postsynaptic
nAchR available for reaction with acetylcholine. Early studies showed that MG serum interfered with acetylcholine sensitivity of rat myotubes (Anwyl et al 1977) or human muscle (Bevan et al 1977) in vitro. This occurred in an irreversible manner and the activity was not dependent on the presence of complement (Bevan et al 1977). Modulation was detected in muscle cultures containing nAchR that had been labelled with [125]I-aBT and treated with myasthenic immunoglobulin by measuring the rate of appearance of [125]I-aBT breakdown products in culture media (Heineman et al 1977, Stanley et al 1978). In these studies, culture medium contained protease inhibitors and was examined for [125]I-tyrosine as a measure of [125]I-aBT degradation. It was thus determined that degradation of [125]I-aBT had occurred but must have followed receptor internalization. The rate at which [125]I-aBT was lost from mouse diaphragms was also increased following passive transfer of myasthenic immunoglobulin to mice (Stanley et al 1978). Studies using monovalent fragments of nAchR Ab showed that immunoglobulin mediated modulation of nAchR expression at the post synaptic membrane depended on bivalent Ab and involves cross-linkage of adjacent receptors (Drachman et al 1978).

There is evidence that pharmacologic blockade of acetylcholine binding by antibodies may also interfere with neuromuscular transmission in MG. Myasthenic serum has been shown to interfere directly with the binding of aBT to solubilized nAchR (Mittag et al 1984, Besinger et al 1983) as well as to in situ endplate nAchR (Bender et al 1975). AchR Ab could conceivably interfere with receptor function without directly competing for the acetylcholine binding site. Donnelly et al (1984) have described a monoclonal Ab which could bind to a noncholinergic site on nAchR and directly interfere with ion influx into AchR rich membrane vesicles.

1.2.4. Polyclonal nature of the nAchR Ab response in MG:

It is clear that nAchR Ab interfere with neuromuscular transmission by a variety of mechanisms. However, although levels of circulating nAchR Ab are correlated with changes in disease severity within a given patient (Lefvert et al 1978, Dawkins et al 1981, Besinger et al 1983, Whiting et al 1986), there is only a very loose correlation between the titer of circulating nAchR Ab and severity of disease among groups of MG patients (Lindstrom et al 1976c, Lefvert
et al 1978, Tindall et al 1981b). While such relationships are not likely to influence the
diagnostic sensitivity of the assay for nAchR Ab, they are of interest from a scientific point of
view. It is possible that the apparent lack of correlation between serum nAchR Ab titer and severity
of disease is, at least in part, a reflection of the polyclonality of the nAchR Ab response.
Differences in pathogenicity of nAchR Ab subtypes may be related to the relapsing, remitting
course of myasthenia.

There is experimental evidence that different nAchR Ab have different degrees of
pathogenicity. The varying ability of monoclonal anti-nAchR Ab to induce EAMG upon passive
transfer is related to antibody avidity and immunoglobulin class and subclass in some cases
(Gomez et al 1985). Differing pathogenicity of nAchR Ab may, in part, be related to the varying
abilities of different Ig isotypes to fix complement (Ishizaka et al 1967) as complement fixation is
essential for one of the main pathogenic mechanisms in MG. Some monoclonal nAchR Ab also
enhance the immune response to nAchR in vitro (Zhang et al 1987). The enhancing effect is
related to the target epitope on the receptor as well as to the isotype of the mcAb. Of 29 mcAb,
only those recognizing epitopes on the alpha subunit and of isotype IgG2b had enhancing
properties. These effects may have been mediated through opsonization of the receptor. In general
terms, it is possible that the relative lack of correlation between nAchR Ab titer and disease
severity in MG is due, in part, to differences at the level of pathogenicity of nAchR Ab.

A better correlation exists between disease severity and the titer of Ab directed against
extracellular determinants of nAchR (Oda et al 1986). Main immunogenic region (MIR) directed
nAchR Ab from MG serum are directly pathogenic to muscle. Monovalent Fab fragments of MIR
mcAbs are able to shield muscle nAchR from modulation induced by MG sera (Tzartos et al
1985). In fact, the efficiency with which MIR Ab fragments protect against modulation suggests
that, in MG, nAchR Ab directed towards the MIR may be the predominant species of auto-Ab
responsible for this pathogenic mechanism. However, as the authors pointed out, the study was
carried out using mouse muscle cultures and so detection may have been limited to cross reactive
species of Ab. Other pathogenic mcAbs, with specificities outside of the MIR, have been
described. These Ab interfere with the functioning of the nAchR (Wan et al 1985, Blatt et al 1986).

If nAchR Ab subtypes have varying pathogenicity then one might expect to observe changes in their proportions in parallel with clinical changes. This is a question which has not been widely addressed and at the present time it is not clear whether this is the case (Tzartos et al 1982, Besinger et al 1983, Lang et al 1985, Heidenreich et al 1988). Studies of nAchR Ab may also eventually bring clues as to the initiation of the autoimmune response. For example, if immunity initially develops against a foreign, cross reactive antigen, as has been speculated by proponents of the molecular mimicry hypothesis of autoimmunity, then it is possible that nAchR idiotypes might be observed to change as the immune response developed against genuine self antigen. Studies of nAchR idiotypes in EAMG have shown that, following immunization with xenogenic nAchR, nAchR Ab develop which exclusively recognize host and not immunizing species of nAchR (Bearman et al 1980).

Production of anti-idiotypic Ab (anti-id) for nAchR may also be an important clinical variable in MG. Anti-idiotypic (anti-id) nAchR Ab have been detected in serum of MG patients (Dwyer et al 1983, Lefvert et al 1984) and were shown to be produced in vitro by PBMNC (Lefvert et al 1986). An inverse relationship exists between disease severity and prevalence of anti-id nAchR (Lefvert 1987b) indicating that anti-idiotypic Ab may have a protective influence in MG. Experimental autoimmune MG induced by immunization with nAchR is regulated by anti-idiotypic nAchR Ab (Fuchs et al 1981, Agius and Richman 1986). Rats treated with anti-idiotypic nAchR Ab prior to immunization had normal levels of endplate nAchR and lower titers of serum nAchR Ab compared with nontreated rats (Agius and Richman 1986). Furthermore, EAMG induced by passive transfer of a monoclonal nAchR Ab is preventable by prior administration of the corresponding anti-idiotypic Ab and reversal of weakness occurs if anti-ids are administered following development of EAMG (Souroujan et al 1986). Anti-id nAchR Ab may play a role in MG by suppressing production of idiotype or by combining with idiotype to enhance the clearance rate. Combination of anti-id with idiotype was reported to have the potential to interfere with
quantitation of nAchR Ab levels (Dwyer et al 1983).

1.3. Regulation of the immune response: General concepts

Generation of a T-dependent humoral immune response involves the cooperation of B and T lymphocytes. B cells become activated following association with T cells. They then proliferate and differentiate into plasma cells which secrete immunoglobulins. T cell and T cell products regulate the development of B cells into Ab producing cells as well as the isotype of Ig which is produced. T cells are grouped into two broad classes, T helper/inducer and T cytotoxic/suppressor cells. The T-helper/inducer subset expresses the CD4 surface molecule and the T-cytotoxic/suppressor subset expresses the CD8 molecule (Jandinski et al 1976, Reinherz et al 1979a). Monoclonal Ab binding is the conventional means by which these subsets are identified, isolated and studied.

1.3.1.1. Positive control of humoral immunity:

The MHC class II gene products play a critical role in regulating the humoral immune response. Class II molecules are expressed on B cells as well as on other antigen presenting cells, such as cells of the dendritic/macrophage lineage and endothelial cells. Expression of functional class II molecules can also be induced, under certain conditions, on non immune cells such as thyrocytes (Londei et al 1984) or astrocytes (Fierz et al 1985), a phenomenon which may be involved in autoimmune processes (Todd et al 1986).

The class II molecule consists of two glycoprotein chains, alpha and beta, which are noncovalently associated. The molecule spans the cell membrane expressing a large extracellular domain and a cytoplasmic tail of approximately 12-15 amino acids in length. The alpha and beta chains of class II molecules are polymorphic which results from polymorphism at the level of the genes which encode them (reviewed in Germain and Malissen 1986).

Early studies showed that MHC compatibility was required for effective interaction between B and T helper cells (Anderson et al 1980). Class II molecules are critically important
for the interaction of B cells with T helper cells, which occurs, in the presence of antigen, through recognition of complexed antigen-class II by the T cell receptor (Kappler et al 1981, Bevan et al 1981).

1.3.1.2 B cells:

Under appropriate circumstances a B cell will proliferate and differentiate into a clone of plasma cells which secrete antibody of a single specificity. The phenomenon of linked recognition (Mitchison 1971a and b) considered together with the more recently demonstrated antigen presenting capacity of B cells (Kakiuchi et al 1986, Janeway et al 1987, Pierce et al 1988) and, indeed, physiologic significance (Ron et al 1987a, 1987b, Janeway et al 1987) of antigen presentation by B cells has led to the hypothesis that antigen presentation by B cells to T helper cells may be requisite in the development of T-dependent humoral responses (Jones 1987, Abbas 1988).

Events occurring at the level of antigen presentation are important in determining responsiveness to antigens. An antigen presenting cell internalizes antigen and, following processing, expresses portions of the antigen on its surface in noncovalent association with the class II molecule (Ishikura et al 1988). Evidence from studies of synthetic peptides suggest that immunodominant regions of an antigen are stretches of amino acids which form amphipathic helices so that a hydrophobic domain may associate with the class II molecule and a hydrophilic portion may be accessible to the T cell receptor (Berzofsky 1987).

In vivo, B cell activation begins when antigen combines with membrane associated immunoglobulin (mIg) of resting B cells. B cell activation has been studied extensively in vitro using anti-Ig to mimic the effects of antigen (reviewed in Cambier et al 1987). Under experimental conditions, supernatants from cultures of activated T cells can induce polyclonal proliferation among resting B cells (DeFranco et al 1984, LeClercq et al 1986) but seem to induce appreciable B cell differentiation only in the presence of antigen (Marrack and Kappler 1980) implying that resting B cells must first become activated, through triggering of mIg and/or through
triggering through class II during antigen presentation to T helper cells, in order to respond to differentiation factors.

Steps in the activation pathway triggered through mIg begin with phospholipase hydrolysis of membrane phosphoinosides and release into the cytoplasm of inositol trisphosphate and diacylglycerol (DAG). Inositol trisphosphate mediates the release of Ca$^{2+}$ from intracellular and extracellular compartments. Ca$^{2+}$ and DAG are involved in the activation of protein kinase C (PKC) leading to a chain of phosphorylation events and proliferation of B cells (For a review of this subject see Cambier et al 1987, Klaus et al 1987). Crosslinking of surface immunoglobulin also results in an increased expression of the HLA class II molecules which are necessary for antigen presentation.

Events pursuant to cognate T-B interaction include activation of the T cell, through its T cell receptor, and resultant secretion of lymphokines that have activational influences on B cells. A variety of T cell lymphokines are secreted including some which promote proliferation and/or differentiation of B cells into Ig secreting cells. It is not clear whether direct contact between B and T helper cell is an absolute requirement for Ig production in vivo or whether it serves to orient and activate the T helper cells to secrete the appropriate lymphokines in a highly localized manner. However, under experimental conditions, direct stimulation through class II has been shown to be involved in signal transduction in B cells. An inhibition of Ia expression and proliferation among mitogen stimulated murine B cells has been observed in the presence of Ia binding ligands (reviewed in Cambier 1987). Moreover, it has recently been shown that stimulation of B cells with anti-Ia results in translocation of protein kinase C to the nucleus of the cell (Chen et al 1987), an effect that is mediated by cAMP (Cambier et al 1987a). Thus, triggering through Ia may be interpreted as a differentiation signal by the B cell (Cambier et al 1987b).

1.3.1.3. T-helper cells:

Class II restricted antigen recognition by T helper cells is critical in mounting a humoral
immune response. The T cell receptor (TCR) expressed by the majority of T cells is a disulfide linked heterodimer (for a review of structure see Kronenberg et al 1986, Weiss et al 1986). The alpha and beta chains each contain constant and variable domains that are encoded by separate genes analogous to the situation for immunoglobulin. There are domains which recognize antigen and domains which recognize MHC within the T cell receptor molecule. Upon MHC restricted recognition of antigen, a signal is transduced through the T cell receptor resulting in activation. The CD3 differentiation antigen is closely associated with the antigen receptor and is involved in mediating the signal. Chemical properties of TCR may be important in autoimmunity. For example, the genes encoding the alpha and beta TCR subunits are very closely similar in rat and murine T cell clones recognizing different encephalitogenic epitopes of myelin basic protein in the context of different MHC molecules (Burns et al 1989).

T cells become activated and secrete lymphokines following stimulation through their CD3/antigen receptor complex. In vivo, this would occur through interaction of TCR with the antigen- MHC complex. Under experimental conditions, activation has been studied using anti-TCR Ab. Interleukin 1 (IL1) may also be required for T cell activation. This lymphokine has been shown to be produced by antigen presenting cells, including B cells and by T cells themselves following interaction with antigen presenting B cells (Tartakovsky et al 1988). The biochemical pathway leading to increased expression of lymphokine genes in activated T cells has been partially worked out and involves the hydrolysis of membrane phospholipids, production of diacylglycerol and inositol triphosphate, mobilization of Ca\(^{2+}\) and activation of protein kinase C (PKC) (for recent review see Weiss et al 1987).

Recent evidence suggests that T cell secretion of lymphokines during T-B association is polar in nature which may represent a control mechanism for delivering high concentrations of lymphokines in a very localized manner. Ultrastructural studies have indicated that, during histocompatible T-B association in the presence of specific antigen, submembranal cytoskeletal proteins, microtubule organizing centers and Golgi apparatus become reoriented to face the area
of contact (Kupfer et al 1986a and b). Further, the TCR and the adhesion molecule, LFA-1, and CD4 differentiation markers also move through the membrane and orient at the face of contact (Kupfer et al 1987). Presumably, clustering of these molecules plays a role in adherence; however, the exact nature of the interaction has not been fully worked out although CD4 is now thought to associate with non polymorphic determinants of class II (reviewed by Swain and Dutton 1987). CD4 probably also mediates signal transduction (Wassmer et al 1985) by triggering a membrane tyrosine kinase (Veillette et al 1989).

A multitude of T cell lymphokines which influence B cell function have been described. Many of these lymphokines can act independently or may synergize with other lymphokines. The study of these factors has been further complicated by the observation that lymphokines may have different effects based on the activational state of the responding cell and may also have effects on cell types other than B cells. The interleukins, notably IL2, IL4 and IL5, have been widely studied for their capacity to influence the T dependent humoral immune response. IL4 induces an increase in class II expression on resting B cells and induces proliferation among activated B cells (reviewed in Swain et al 1988). IL5 (BCGF-II) is a factor which causes proliferation of, and differentiation of, activated B cells into Ab secreting cells (Swain et al 1985, Alderson et al 1987). IL2 has been shown to promote proliferation and differentiation among activated B cells and also to synergize with gamma interferon in promoting differentiation (reviewed in Swain et al 1988a). However, other studies have suggested that these effects of IL2 and IFN-gamma in impure populations of B cells may be mediated through other cell types (Julius et al 1987). Antigen specific T helper factors have also been described although they have not been fully characterized (Christados et al 1986, reviewed in Asherson et al 1986).

Based on functional studies and differentiation marker phenotype, a subset of CD4+ T-helper/inducer cells were identified which mediate T helper function in the humoral immune response (Morimoto et al 1985). These cells have been called T-helper-inducers (Thi) and are characterized by the lack of the CD45R antigen (associated with T-suppressor inducers or naive cells, see below) (Morimoto et al 1985a, Clement et al 1988) and by the expression of the surface
antigen, CDw29, reactive with the mcAb, 4B4 (Morimoto et al 1985a) and CD45, reactive with UCHL 1 (Smith et al 1986). Human CD4+, CDw29+,CD45R- cells have also been called memory cells because this population contained cells capable of proliferating in response to soluble tetanus toxoid (Tedder et al 1985). The CD4+, CDw29+, CD45R- cell, which has helper function for humoral immune responses, has been shown to derive sequentially from the CD4+, CDw29-, CD45R+ cell type upon activation (Smith et al 1986, Clement et al 1988, Byrne et al 1988, Akbar et al 1988). CD4+, CD45R-, CDw29+ cells also secrete IL2 and express IL2 receptors upon mitogenic stimulation using anti-CD3 mcAb whereas CD4+, CD45R+,CDw29- cells do not (Byrne et al 1988).

1.3.2.1 Negative control of humoral immune responses:

Regulation of plasma cell Ig production occurs by a variety of mechanisms. Antigen is more readily cleared by macrophages when bound to specific Ab (opsonization). Antigen bound Ab is also no longer accessible for binding to mIg on B cells. These effects limit the continued humoral response to a given antigen. Immunoglobulin molecules may also give specific negative feedback to the cells which produce them. B cells express Fc-gamma receptors (FcR) which bind to the Fc portion of IgG and it is thought that when IgG bound in this fashion is cross-linked either by specific antigen or by anti-idiotypic Ab a partial "abortive" activation of B cells occurs (reviewed in Klaus et al 1987). Anti-idiotypic Ab (Jerne 1974), may play a role in controlling the humoral immune response to a particular antigen either through neutralization of the idiotype once it has been secreted by the plasma cell and/or through interactions at the level of mIg on the B cell. Indeed, anti-idiotypic Ab have been shown to have protective influences and even to reverse symptomatology associated with idiotypic responses to auto-antigens in vivo (Fuchs et al 1981, Agius et al 1986, Souroujan et al 1986).

Components of the complement cascade, or their fragments, are also directly involved in regulating the immune response. Studies with EBV and mcAb have linked the C3d,g-C3d receptor to an activation pathway in B cells (Nemerow et al 1985, Mold et al 1986). However, the exact role of C3d and C3d,g in activation of B cells is not clear. C3d,g does not activate normal,
human B cells (Nemerow et al 1985) and C3d inhibits mitogen (Tsokos et al 1984) and antigen (Schenkein et al 1979) induced lymphocyte activation. Other investigators reported that C3d and C3d,g could induce growth in an EBV positive, pre-B cell line (Raji cells) (Hatzfeld et al 1988).

1.3.2.2. T-suppressor cells:

Convention holds that there exists a population(s) of CD8+ lymphocytes which are responsible for mediating suppression of humoral immune responses through the action of soluble factors that they elaborate. Multitudes of functional studies have shown that addition of CD8+ T cells to in vitro assay systems inhibits the immune response (Brideau et al 1980, Gatenby et al 1982, Brieva et al 1983, Antel et al 1984, Morimoto et al 1985b, 1986a, Miedema et al 1985, Elmasry et al 1987). Similarly, in vivo experiments have also demonstrated the effectiveness with which the CD8+ T cells can diminish the immune response (Kanellopoulos-Langevin et al 1984, Mann and Neilson 1986). Moreover, CD8+, antigen-specific suppressor T cell lines and clones have been developed (Modlin et al 1986, Pachner et al 1987, Clementi et al 1987, Takeuchi et al 1988). These cells have been maintained in vitro and express the surface marker phenotype and have the functional properties of suppressor cells. At present, the mechanisms by which suppressor cells exert their effects have not been fully worked out. Cloned T suppressor cells should prove to be very valuable tools in evaluating the immunoregulatory role of the suppressor cell and as a source of soluble factors which can be characterized.

Suppressor cells have been shown to produce soluble factors which can mediate suppression. Both antigen-specific and nonspecific soluble factors have been described (reviewed in Germain and Benacerraf 1980, Asherson et al 1986).

Antigen specific factors are believed to function in the development of suppressor effector cells. Studies in a variety of mouse models designed to assess B and T cell function have suggested that antigen dependent suppression is mediated by a family of related cell types which secrete soluble factors in a cascade leading ultimately to the generation of non-specific suppressor factors by an accessory cell (reviewed in Dorf and Benacerraf 1984 and in Asherson et al 1986,
Green et al 1987, O'Hara et al 1988, Chue et al 1989). Several variations have been described and the details are not well worked out. In general, however, three intermediate T cells have been proposed to interact sequentially in a complicated pathway which may also involve a variety of antigen presenting cells (APC) and possibly, in some systems, anti-idiotypic B cells. Initially, the inducer or TS1 cell, which is CD4+, responds in a class II restricted manner to antigen presentation by an Ia+, I-J+ APC. The TS1 secretes a non-Ab factor (TSF1) which has idiotypic determinants for the immunizing antigen and expresses I-J. It is thought that the soluble T suppressor factors studied in most cascade systems are two chain, possibly disulfide bonded, molecules which express either idiotypic or anti-idiotypic antigen binding sites and express both I-J determinants as well as complementary regions which bind I-J. TSF1 is presented by an I-J+ APC to a second population of suppressor cells, the TS2 or trans-suppressor, in an I-J restricted manner. There may also be an Igh VH restriction associated with the expression of TS2 activity in some systems (Yamauchi et al 1982, Dorf and Benacerraf 1984). The CD8+ TS2 express receptors and secrete suppressor molecules with anti-idiotypic determinants for antigen. Soluble factors secreted by TS2 are then presented, in an I-J restricted fashion, to a CD8+, I-J+ effector-suppressor cell, TS3, by Ia-, I-J+ APC. This results in production of an antigen specific, soluble factor which then binds to antigen expressed on an I-J+ APC, the "nonspecific acceptor cell", thereby mediating the production of nonspecific suppressor factors. Thus, regardless of the antigen specific nature of the TSF's within the cascade, the final suppressor factors in the pathway are nonspecific in nature.

The mode of action of T-suppressor-inducer cells has not been completely worked out. Fairchild et al (1986) reported that the activity of an L3T4+ T cell necessary for the induction of suppression could be replaced by IL1. Others have reported that CD4+ T cells could be replaced by interferon-gamma in PWM stimulated suppressor assays (Elmasry et al 1987). O'Hara et al have disputed this; in their system IL1, 3, 4 or interferon-gamma alone could not replace soluble factors elaborated by CD4+ suppressor inducer cells. These investigators have studied the murine system where a mcAb to a mouse equivalent of CD45R has not yet been developed. The CD4+
population which they have described, however, has the characteristics of the CD45R+ T-suppressor-inducer described by Morimoto and others in the human (see below).

The human CD4+ CD45R+ T-suppressor-inducer (Tsi) cell is required to induce CD8+ mediated suppression in antigen specific (Morimoto et al 1986a) as well as pokeweed mitogen driven Ig secretion assay systems (Morimoto et al 1985b, Takeuchi et al 1986). Tsi also develop during the autologous mixed lymphocyte reaction (AMLR) by 6 days (Takeuchi et al 1987). Tsi can be differentiated phenotypically from CD4+ T cells which behave as T helper inducers by expression of the CD45R and relative lack of the CDw29 (see section 1.3.1.3) surface molecules to which a variety of monoclonal Ab have now been produced (Tedder et al 1985, Morimoto et al 1985b, Smith et al 1986). Several groups have shown that the CD45R+ Tsi develops directly into the CD45R-, T-helper-inducer cell type following activation (Smith et al 1986, Byrne et al 1988, Akbar et al 1988, Clement et al 1988).

The Tsi has also been implicated as a weak link in the regulation of immune responses toward auto-antigens. Several enumerative or functional studies have suggested that this cell type is reduced in autoimmune disorders (Morimoto et al 1987a and 1987b, Emery et al 1987, Sobel et al 1988, O'Gorman and Oger 1989b).

The direction of response to antigen may be determined by the APC. Macrophages are necessary for suppression of the immune response in some systems (Whitham et al 1989). Further, it has been reported that the state of differentiation of macrophage clones determines whether immunity or suppression results from their interaction with lymphocytes (Ishikura et al 1988). Monocyte/macrophage requirements for induction of CD8+ mediated suppression in a PWM stimulated system was also examined by Elmasry et al (1987) who found that supernatants containing prostaglandins could replace these cellular requirements. Others have found that irradiation of APC results in their failure to induce immunity in T-helper/inducer clones but instead allows development of T-suppressor inducer function (Green et al 1987). The development of suppressor function was mediated by a soluble antigen-binding factor which was specific for each T cell clone among several of different antigen specificity that were tested. This latter study also
brings the possibility that a single CD4+ cell may have the potential to induce either immunity or suppression upon appropriate stimulation, although, of course, it would remain to be determined whether the individual cells of the clone had attained variable maturational states during the maintenance period.

Recently, the existence of T-suppressor cells as distinct cellular entities has been challenged on philosophical grounds. This skepticism is based largely on the inability to identify the I-J gene region as discussed by Moller (1988). As further pointed out by Moller (1988), progress in characterization of the soluble suppressor factors has indeed been slow and, to date, surface molecules which can differentiate between CD8+ cells that function as cytotoxic T cells or suppressor cells have not been identified. Further, T-suppressor cell hybridomas were reported to lack genes or meaningful rearrangements of genes for T cell receptors (Kronenberg et al 1985, Hedrick et al 1985), although reports to the contrary have also appeared in the literature (Modlin et al 1987, Takeuchi et al 1988).

Suppression of humoral immune responses may be carried out by cytotoxic lymphocytes in vivo. The existence of a population of antigen specific CD8+, class II restricted cytotoxic T cells was reported recently (Shinohira et al 1988). This cell line was developed from lymph nodes of BALB/c mice immunized with keyhole limpet hemocyanin (KLH) and was able to lyse a trinitrophenol (TNP)-specific B cell line in the presence of TNP-KLH but not KLH. It was hypothesized that these cells may be antigen specific, class II restricted suppressor cells which act through cytolysis of the antigen presenting B cell. Interestingly, Clayberger et al (1987) have described a CD4+ T cell clone which had developed during an AMLR, and which could induce help for B cell mediated immune function or could lyse Ia+, activated B cells or other APC at high ratios of clone to target. Others have described antigen specific CD4+, class II restricted T cell clones with cytotoxic or uncharacterized suppressive function (Bottomly et al 1983, Ozaki et al 1987, Lakacher et al 1985). Class II restricted CD4+ cytotoxic activity also develops among CD4+ T-helper clones with prolonged culture (Fleisher et al 1984, Pawelec et al 1986). As postulated by Lanzavecchia (1989), it is indeed possible that helper or suppressor influences may
be mediated by the same cell at different stages of differentiation.

Some nonspecific in vitro assay systems for measurement of suppression by unseparated T cell populations have been shown to be related simply to kinetics of IL2 usage by the cells behaving as suppressors. This has been shown for the con A stimulated suppressor cell assay system (Palacios et al 1981). Con A activated T cells with suppressive potential in vitro also exert helper function in vivo (Moller et al 1985). Others, however, have partially characterized soluble immune response suppressor (SIRS) from murine and human systems. This is a lymphokine generated by T cells stimulated with IFN-gamma (Schnaper et al 1984) or con A (Devans et al 1988). APC (macrophage) are required to process and thus activate SIRS (Schnaper et al 1984). SIRS was shown to exert its effects through interference with the capacity of T and B cells to positively respond to lymphokines (Aune et al 1984).

Clearly, much more work is needed to clarify the exact cellular and molecular mechanisms by which CD4+ and CD8+ T cell subsets mediate suppression of immune responses in vitro and in vivo.

1.4. Regulation of the immune response in myasthenia gravis:

1.4.1 Introduction:

A major question concerning the pathogenesis of MG is whether or not failure of normal immunoregulation is responsible for initiation and/or maintenance of the immune response against nAchR. Studies of the immune system in MG have shown that abnormalities do exist but they have not been well characterized.

In MG, B cell production of nAchR Ab is regulated by T cells. Thus overproduction of auto Ab could be linked to intrinsic changes at the level of the B cell or could involve regulatory T cell function. It has been suggested that "forbidden clones" of nAchR Ab producing B cells may arise. It has also been suggested that the thymus produces clones of nAchR specific T helper cells (see section 1.7.2. Thymic hyperplasia in MG). In SLE, it has been postulated that a mitogenic
agent may enhance the auto Ab production which is observed in that disorder. Although MG is conventionally thought of as an autoimmune disorder involving a specific auto Ab, the nAchR Ab, there is some evidence that a heightened polyclonal response does in fact exist.

T regulatory function in MG has been assessed by a variety of in vitro techniques. In general, information to be gained by such studies has been limited by the gross level of inquiry. In particular, studies of unseparated populations of T cells have not adequately assessed the various contributions of regulatory T cell subsets. Furthermore, aside from results of nAchR Ab measurements, results of immune function assessments in MG usually show considerable variability and overlap with control groups. Results of this nature suggest that MG is a heterogeneous disorder, as is also suggested by epidemiologic studies and studies of HLA patterns. Additionally, disease dependent variables, such as anti-lymphocyte Ab, may influence immune functioning at different clinical stages of disease. These observations emphasize the need to study well-defined patient groups in MG.

1.4.2 Enumeration of Lymphocyte Subsets in MG:

Studies involving enumeration of lymphocyte subsets have yielded variable results and, in general, are of limited usefulness since changes in lymphocyte functioning are not necessarily reflected in changes in conventional cell surface markers. Abnormal findings from such studies will be reviewed briefly here and a more extensive discussion of functional evaluations of immune response in MG will follow.

Decreased percentages of peripheral CD8+ T cells, encompassing the T-suppressor and cytotoxic lymphocytes, have been reported in MG (Berrih et al 1981, Skolnik et al 1982). However, there have been reports to the contrary (Miller et al 1982). The T-gamma suppressor subset has also been found to be elevated in several studies (Piantelli et al 1979, Tindall et al 1981a). The CD4+, T-helper/inducer lymphocyte population may be increased (Berrih et al 1983) or decreased (Chiu et al 1985). The controversy generated from these studies probably reflects
differences in clinical status of the patients studied. Interestingly, a recent study showed that the percentage of the CD4+ T-helper inducer subset, defined by the mcAb,4B4, was increased in MG (Grob et al 1989). There are also reports of increased CD4+CD8+ T cells of unknown function (Berrih et al 1981). Levels of this subset returned to normal following successful therapeutic thymectomy. Increased levels of circulating thymosin alpha 1+ T-cells have also been reported (Dalakas et al 1983). Thymosin alpha 1 is a hormone secreted by the thymus and has been shown to be overproduced by the myasthenic thymus (Dalakas et al 1981). Glucocorticoid receptor expression among lymphocytes was also reported to be elevated in MG (Brentani et al 1985).

1.4.3 Studies of immune function in MG:

Studies of immune function in MG have proven to be more informative than enumerative studies. Two major points have emerged from such studies. There does, in fact, seem to be an acquired or inherited non-specific hyperactivity of B cell function as demonstrated by studies of Ab production and Ab producing cells. Additionally, the evidence suggests that there is an impairment of T-suppressor function in some MG patients.

1.4.3.1 Studies of nAchR Ab specific immune response in myasthenia gravis:

mechanisms.

Studies of specific Ab production in myasthenia gravis have indicated that nAchR is a T-dependent antigen (Shinomiya et al 1981 and 1984). nAchR Ab production was shown to depend on the presence of CD4+, T-helper cells in vitro (Shinomiya et al 1984). Work in this area has been advanced through the development of T cell lines from MG patients which are specific for nAchR. Studies of the functioning of T cell lines in nAchR specific systems have indicated that normal mechanisms of immune response to T-dependent antigens are operative. Several T-helper lines have been characterized which operate in a typical class II MHC restricted manner (Hohlfeld et al 1986a, Hohlfeld et al 1987). They also had the surface markers characteristic of helper T cells (i.e. CD2+, CD4+) (Hohlfeld et al 1987). Polyclonality of nAchR specific T cell lines developed from MG patients has been demonstrated by capacity for recognition of a variety of epitopes on the nAchR molecule (Hohlfeld et al 1988). Christadoss et al (1986) have also partially characterized an antigen specific T helper factor from lymphocytes of mice with experimental myasthenia gravis.

Consistent with the recently established role of B cells in antigen presentation (reviewed in Jones 1987, Abbas et al 1988), immortalized B cells from myasthenia gravis patients were shown to have the capacity to present antigen to nAchR specific T cell lines (Hohlfeld et al 1986b). Some nAchR specific B cell hybridomas also can preferentially present nAchR to T cells (Zhang et al 1987).

Recently, T-suppressor cell lines have been developed from mice with EAMG. Functional studies showed that these cells produced an antigen-specific soluble factor which could suppress the immune response to nAchR in vivo or in vitro (Pachner et al 1987, Clementi et al 1987). Line cells possessed the surface marker phenotype characteristic of T-suppressor lymphocytes (Clementi et al 1987). nAchR specific suppressor factors have also been partially characterized by others studying lymphocytes from MG patients or EAMG animals (Shinomiya et al 1984, Bogen et al 1984, McIntosh et al 1986).

Shinomiya et al have shown that T cells from MG patients, previously stimulated with
purified nAchR, did not suppress nAchR Ab production in nAchR stimulated heterologous cultures of thymocytes or PBMNC. Interestingly, normal T cells were very suppressive under these circumstances (Shinomiya et al 1981 and 1984). Lisak et al (1986a) have studied the effect of removal of CD8+ cells on the mitogen stimulated production of nAchR Ab by PBMNC in MG. Levels of nAchR Ab were increased, indicating that non-specific T-suppressor function was indeed present, yet these studies did not indicate whether the level of response was normal. Removal of T-suppressor cells from cultures of normal PBMNC did not result in nAchR Ab production (Lisak et al 1984).

1.4.3.2 Non specific defects of immune functions in MG:

i) Suppressor function:

There is evidence suggesting that a T-suppressor defect exists in MG and is not specific for the production of nAchR Ab. T suppressor function was reduced in some MG patients in studies of con A induced suppression of DNA synthesis by mitogen stimulated lymphocytes (Mischak et al 1976, Zilco et al 1979, Mischak et al 1981, Salzner et al 1981); however, this observation was not confirmed by Koethe (1981). Irradiation of T cells, which inactivates suppressor function, did not induce the same degree of enhancement of IgG production among MG as compared with normal. These results were also interpreted as indicating impaired suppressor function (Kelley et al 1981) although the contribution by T-helper cells was not adequately controlled. Shore et al (1981) showed reduced suppressor function among MG patients in an ovalbumin specific plaque forming assay. A study by Birnbaum et al (1976) showed reduced mixed lymphocyte reaction (MLR) among young, non thymectomized MG patients but since it was normal in age matched thymectomized patients, it was taken as indirect evidence that T suppressor function was more active in some MG patients. The finding of reduced MLR in non thymectomized MG was not confirmed by Wijermans et al (1980).

The 6-day autologous mixed lymphocyte response (AMLR), which is a measure of CD4+, T-cell mediated suppressor-inducer function (Takeuchi et al 1987), was shown to be impaired in
MG (Richards et al 1986, 1987). An earlier study, however, by Greenberg et al (1984), indicated an enhanced 6-day AMLR in MG.

ii) Other auto Ab in MG:

Auto antibody production in MG is not restricted to nAchR Ab, although certainly anti-nAchR Ab are of primary importance in terms of their pathogenic capacity and the frequency of their occurrence. A variety of serum auto antibodies have been detected including anti-thyroglobulin (Adner et al 1964), anti-nuclear (Adner et al 1964, Kornguth et al 1970), rheumatoid factor (Adner et al 1964) and anti-acetylcholinesterase (Keese y et al 1986). Zimmerman et al (1987) studied serum immunoglobulin binding to a variety of muscle proteins. While there was evidence for auto-antibodies in normal serum, as has been described by others (Dighiero et al 1986), it was clear that MG sera had different binding patterns. Circulating antibodies to striated muscle in MG have been described by many investigators but these usually occur infrequently (Strauss et al 1960, Beutner et al 1962, Adner et al 1964) unless thymoma is also present (Penn et al 1986, Williams et al 1986). Yamamoto et al (1986) reported that antibodies to filamin, an actin binding protein which occurs at the neuromuscular junction, were elevated over control levels in 93% of MG patients but also in 100% of polymyositis patients.

Production of auto-antibodies to muscle antigens other than nAchR may arise as a result of nAchR Ab and complement mediated damage to nAchR rich membranes. There is evidence that membrane debris containing nAchR is shed into the synapse following postsynaptic complement mediated lysis (Engel et al 1977a, Sahashi et al 1980). Circulating immune complexes have also been recognized in MG (Barkas et al 1980). Consistent with this scenario, Penn et al (1986) reported that anti-muscle Ab do not occur in MG who are seronegative for nAchR Ab. It is also possible that Ab to self antigen other than nAchR could result from somatic mutation during the immune response to nAchR. With ongoing immune response Ab diversity and potential for cross reactivity are usually increased (Steward et al 1983, Manser et al 1985).
iii) Polyclonal B cell function:

Studies of polyclonal B cell mediated immune function indicate that abnormalities of immune function in myasthenia gravis are not limited to production of specific Ab. Evidence that polyclonal immunoglobulin (Ig) production might be enhanced in vivo comes from studies of spontaneous production in culture (Kelley et al 1981, Limberg et al 1985) and from studies involving enumeration of circulating Ig secreting cells (Levinson et al 1981) although such differences have not been consistently detected (Harfast et al 1981, Lisak et al 1983). Lisak et al (1976) have reported increased levels of IgG in serum of some patients with autoimmune disease including MG. PWM stimulated IgG secretion among MG did not differ from or was increased over control (Harfast et al 1981, Lisak et al 1983).

As will be described in Chapter 3, studies from our laboratory have demonstrated for the first time that two subgroups of MG patients exist among whom there is a correlation between disease duration and capacity of PBMNC to secrete antibodies in vitro (polyclonal IgG or nAchR Ab).

1.5. Evidence for primary defects of immune response in MG:

There is evidence that hereditary factors may influence development of myasthenia gravis. There appears to be at least two groups of MG patients. The HLA antigens A1, B8 (Fritze et al 1974, Pirskanen et al 1976, Compston et al 1980) and DRW3 occur more frequently among early onset patients, most of whom are females (Fritze et al 1974, Compston et al 1980). HLA A3, B7 (Fritze et al 1974, Compston et al 1980) and DRW2 (Compston et al 1980) are characteristic of males with late onset MG. There is also an association of HLA B8 and DR3 with other autoimmune diseases; in a study of patients with Graves disease Stenszky et al (1986) suggested that the presence of HLA B8, as a marker of immunological defect, forces the Graves disease patient into a more severe illness. In their study HLA B8 was present in 80% of severe Graves patients, but in only 9% of the patients with a less severe disease which was actually less than in the healthy population (18%). Naeim et al (1978) reported that anti-AchR Ab titers were higher.
among MG patients without thymoma expressing the HLA B8 and DR3 alleles.

Significantly, there are suggestions that immune function may be associated with HLA type. Healthy individuals who carry the B8 and DR3 alleles have higher numbers of circulating immunoglobulin producing cells (IgSC) and decreased Con A induced suppressor function in a PWM stimulated plaque forming assay for detection of IgSC (Ambinder et al 1982). It was thought that the immune function of DR3+ individuals might predispose them to development of a variety of immunological disorders which had been observed to be more prevalent among individuals of this HLA type. Fc-receptor mediated clearance of immune complexes and Fc-receptor bearing T cells were reduced in individuals with HLA B8 and HLA DR3 (Lawley et al 1981). McComb et al (1982) also reported that mitogen stimulated proliferation was reduced among healthy individuals of the HLA B8 phenotype.

There is also evidence that HLA type may be associated with defects of immune function in MG. Zilco et al (1979) have studied con A induced suppressor function in MG. Some patients showed reduced T-suppressor function compared with controls; reduced suppression was also correlated with the occurrence of the HLA B8 and DR3 alleles. An association between reduced T suppressor function and HLA B8 has also been reported among patients with Graves disease (Balazs et al 1979).

1.6. Evidence for secondary defects of immune response in MG:

It is also possible that dysregulated immunity in MG may be secondary to the disease itself. There are several possibilities as follows.

Some of the observed abnormalities in immune function in MG are consistent with changes that would occur with ongoing stimulation of the immune response. It was proposed that the apparent decrease in Tsi activity in some immune-mediated diseases, including MG, may reflect ongoing immunologic response with conversion of naive (CD45R+, CDw29-) T-cells to memory T-cells (CD45R-,CDw29+) and the attendant change in functional status from T-suppressor-inducer to T-helper-inducer or cytotoxic cells (reviewed in Sanders et al 1988).
Autoimmune disease may be associated with reduced T-helper function as well as reduced T-suppressor function. In SLE, reduced T-helper function is accompanied by reduced IL2 production (reviewed in Zubler et al 1986). This defect is reversible following an in vitro culture period, presumably to allow time for recovery of lymphocytes which had been chronically stimulated in vivo (exhaustion). In MG, IL2 receptor (TAC) expression and proliferative response of lymphocytes to IL2 in vitro is higher than normal (Berrih-aknin et al 1988). In that study, however, TAC expression did not exceed normal levels prior to culture, indirectly suggesting that IL2 production in vivo may be insufficient to induce appropriate levels of TAC expression. IL2 production by EBV infected PBMNC was lower than normal in patients with autoimmune disease, including 3 patients with MG (Lotz et al 1986). In murine EAMG, IL2 production by splenic T cells is also lower than normal (Christadoss et al 1983).

When EAMG was studied over time, a sustained enhancement of macrophage-mediated suppression was seen (Christadoss et al 1983). This may be a natural regulatory response during chronic immune reaction and could conceivably influence humoral immunity in long standing disease. Chronic graft versus host disease (CGVHD) is a condition which often leads to the development of a variety of autoimmunity disorders including MG (reviewed in Bolger et al 1986). In CGVHD there is evidence for increased T-suppressor function (Tsoi et al 1979, reviewed in Witherspoon et al 1984).

During the course of an autoimmune disease it is possible that anti-lymphocyte Ab could arise. Anti-lymphocyte Ab have been described in autoimmune disorders such as juvenile rheumatoid arthritis (Morimoto et al 1981) and SLE (Morimoto et al 1984). Anti-lymphocyte Ab might alter the immune response either by directly killing lymphocytes together with complement or by leading to an enhanced clearance of lymphocytes (Seaman et al 1988). Further, anti-lymphocyte Ab might interfere with normal mechanisms of lymphocyte activation or even modulate immune function directly. Auto-Ab have been described which actually stimulated their target receptor (Kohn et al 1986).

Anti-lymphocyte Ab have been described in MG. Complement fixing antibodies which
bind to normal lymphocytes are found in the sera of some MG patients (Zilco et al 1979). Fuchs et al (1980), using indirect immunofluorescence, demonstrated that MG serum, anti-torpedo nAchR anti-sera and affinity purified nAchR Ab bind to thymic lymphocytes. Others have also demonstrated cytotoxic action of MG serum on lymphocytes (Lisak et al 1979). In fact, percentages of CD4+ cells among human T-lymphocytes are reduced following treatment with MG serum or with an nAchR mcAb (Chiu et al 1985).

Hormonal influences on lymphocyte function could be important disease variables in MG. It is well known that the thymic hormones are very important in influencing the function of mature lymphocytes as well as developing lymphocytes (Trivers et al 1979). Thymic hormones, including thymosin alpha 1, are overproduced in MG (Twomey et al 1979, Dalakas et al 1981). Thymosin alpha 1 enhances humoral immunity against specific antigens in primed individuals (Lowell et al 1980, Ershler et al 1984). Thus, it is possible that thymic hormones may play a role in dysregulation of the immune response in MG. Passive transfer of EAMG serum induces pathology in the thymus of mice (Dalkara et al 1983). Thus, it is possible that damage to the thymic microenvironment in MG might be mediated by nAchR Ab and one might speculate that anti-nAchR Ab, through interaction with target cells in the thymus, might influence production of immunoregulatory hormones. Thymic nAchR has been shown to bind MG serum directly (Van der Geld et al 1964, Lindstrom et al 1976b). Another possibility is that thymic hormones may interfere directly with neuromuscular transmission. Indeed, thymopoietin binds nAchR with high affinity (Venkatasubramanian et al 1986) and interferes with with neuromuscular transmission at physiologically relevant doses (Goldstein et al 1975).

1.7. Role of the thymus in myasthenia gravis:

Pathology of the thymus has been widely recognized in MG and thymectomy is often a very successful treatment. Before turning to a discussion of the thymus in MG a brief overview of the role of the thymus in normal T cell development will be given.

1.7.1. T cell development in normal thymus:
Undifferentiated stem cells destined to become T cells enter the thymus from the bone marrow. The vast majority of these cells will die in the thymus before being allowed to enter the recirculating pool of lymphocytes. The thymus is morphologically divided into the cortex and medullary regions. Within the thymic cortex T cells develop in an orderly progression, acquiring increasingly more mature surface phenotype and functional properties. The TCR develops first followed by the orderly appearance and disappearance of surface differentiation antigens including, ultimately, the appearance of the CD4+ and CD8+ antigens (for recent review see von Boehmer 1988).

The thymic cortex itself is protected from the circulation by a blood/thymus barrier (Kissler 1979). Epithelial cells throughout the cortex express MHC class I and class II and this is thought to be very important for the two main functions of the thymus, development of tolerance and development of self MHC restricted recognition of antigen (Haynes 1984).

Upon reaching maturity, the T cells will leave the cortex and enter the medulla or the recirculating pool (Weissman 1973). Emigration probably occurs through the venules of the corticomedullary junction through which lymphocytes recirculate from the periphery to the medulla of the thymus.

The bone marrow derived dendritic cells are thought to be important for induction of self tolerance in the thymus. Dendritic cells occur exclusively in the regions of the corticomedullary junction and the medulla (Kaiserling et al 1974). They have been shown to be accessible to circulating antigens (Kyewski et al 1984) and to be capable of antigen presentation (Kyewski et al 1986). The thymic medulla is also accessible to lymphocytes from the circulation and the population of T cells which occur there are largely of mature phenotype.

The development of MHC restriction is controlled by thymic stromal cells (reviewed in Lo et al 1986). Experiments in the murine model have shown that if homozygous parental strains a and b are crossed and the F1 marrow is transplanted to one of the parental types (irradiated to destroy lymphoid cells) then the immune response to foreign antigen only occurs when antigen is presented in context with MHC of the recipient parental type but not the MHC of the other parental
Tolerance to self MHC also takes place in the thymus. Studies of mixed lymphocyte reaction (MLR) in parent-F1 chimeras have suggested that marrow derived macrophage/dendritic cells act as a "filter" at the corticomedullary junction for elimination of self reactive thymocytes (Lo et al 1986). Veto cells, proposed as cells of the T lineage with the capability of suppressing potentially auto reactive T cells, may also be involved in induction of intra-thymic tolerance. Veto cells are thought to operate by cytolysis of cells which bind them following unidirectional recognition of veto cell surface antigens (Fink et al 1988b).

During fetal life, exposure to non-MHC antigen results in a life long state of tolerance and under certain circumstances tolerance can also be induced in the adult (reviewed in Nossal 1983). The mechanisms by which this occurs are not worked out but it appears that clonal anergy/deletion and/or active T cell mediated suppression may be involved (Nossal 1983, Fink 1988a). Introduction of foreign antigen in the perinatal period results in a reduction of antigen specific cytotoxic T clones and this occurs most rapidly in the thymus compared with other lymphoid organs. This suggests that functional silencing takes place in the thymus followed by seeding of peripheral lymphoid organs by the selected lymphocyte populations (Nossal and Pike 1981).

Several recent studies provide evidence that MHC may be constitutively expressed as a complex with self-peptides that may, in some cases, be cell-specific (Marrack and Kappler 1988, Bjorkman et al 1987). Accordingly, a "peptide" model (Marrack and Kappler 1987) of self tolerance has been proposed. This is a variation of the more conventional affinity model in which all thymocytes that recognize self MHC would first be positively selected but subsequently undergo a negative selection if the thymocyte TCR binding to MHC occurred with moderate to high affinity. In the peptide model, thymocytes first undergo positive selection in the cortex via interaction with epithelial MHC expressed as a complex with cortex epithelial-specific peptides. In a second step, thymocytes meet with marrow derived macrophage/dendritic cells which express
MHC in association with ubiquitous self-peptides. In this latter case, the thymocyte recognizing self antigen in the context of self MHC will be negatively selected through, as yet, unknown mechanisms. In support of the peptide theory, a mcAb has been recognized which does not bind to thymic cortex class II but is specific for class II expressed in medulla and on peripheral B cells (Murphy et al 1989). It appears more likely that the negative selection of self reactive thymocytes is due to a unique response of the immature T cell rather than to unique antigen presenting properties of medullary dendritic cells. Studies of splenic dendritic cells, which productively present antigen to mature T cells, cause reduced cytotoxic activity among thymocytes (Matzinger and Guerder 1989).

1.7.2. Thymic hyperplasia in MG:

A hyperplastic thymus is frequently found in MG patients (Sloan et al 1943, Castelman 1966, Habu et al 1971, Thomas et al 1982, Palestro et al 1983). Hyperplasia is restricted to medullary changes; the cortex is often condensed, apparently due to compression from the expanded medulla. The most prominent features of the hyperplastic thymus is the abundant germinal centers in the medulla (Sloan et al 1943, Castelman 1966, Habu et al 1971, Thomas et al 1982, Palestro et al 1983) which are of normal appearance (reviewed in: Levine et al 1978, Henry 1981). Germinal centers occur much less frequently in thymus of healthy individuals (Sloan 1943, Habu et al 1971) and only rarely in persons over 40 years of age or in persons who have been hospitalized (Middleton 1967). Their extensive occurrence in thymus of MG patients, regardless of age (Habu et al 1971), suggests that there is an abnormal degree of thymic lymphocyte activation in this disease.

Several in vitro studies have suggested that a high degree of activation towards nAchR exists in the thymus of MG patients. Unstimulated cultures of thymic lymphocytes from MG patients often secreted higher levels of nAchR than did PBMNC from the same individual (Newsom-Davis et al 1984, Lisak et al 1987). Fujii et al (1986) studied spontaneous secretion of nAchR Ab by PBMNC, thymocytes and several other lymphoid organs of MG patients. The ratio of nAchR Ab to polyclonal IgG was markedly higher among cultures of thymic lymphocytes.
Cultured thymic lymphocytes are also able to secrete nAchR Ab without help from CD4+ cells (Shinomiya et al 1981, Newsom-Davis et al 1984). The degree to which spontaneous nAchR Ab production is increased in B cell enriched cultures is much greater in cultures prepared from thymus than from lymph node (Fujii et al 1985a). Levels of nAchR Ab secreted in vitro by thymic lymphocytes also correlate with the degree of hyperplasia of the thymus (Scadding et al 1981, Safar et al 1987).

The arrangement of germinal centers in relation to other thymic structures suggests that an active immune response is occurring against in situ thymic nAchR. Significantly, medullary germinal centers occur in close proximity to myoid cells, which are normal, muscle-like cells of unknown function that bear nAchR (Henry 1981, Palestro et al 1983). This arrangement suggests that myoid cells may be involved in the autoimmune response in MG.

Germinal centers also exist in close association with Hassall's corpuscles (HC) (Levine 1978, Henry 1981, Palestro et al 1983, Pizzighella et al 1983, Chilosi et al 1986, Wekerle 1986). There are reports of increased numbers of HC in MG thymus (Palestro et al 1983). The role of HC in the normal thymus is unknown but they produce immunoregulatory hormones (Dalakas et al 1981) and their appearance ultrastructurally is consistent with an active secretory function (Vetters et al 1973). HC aggregate near the superficial pole of medullary germinal centers (Henry 1981, Scadding et al 1981, Palestro et al 1983). This is the region of highest antigen concentration in normal lymphoid tissue (Millikin 1966). Experimental work suggests that HC can accumulate antigen in-vivo (Blau 1967). Moreover, HC express what are considered to be nAchR-like molecules that bind aBT and cross react with some nAchR mcAb (Kirchner et al 1988).

A variety of other pathological changes occur within thymus in MG. These include hyperplasia of epithelial structures (Henry 1981, Dalakas et al 1981), increased reticular network (Henry 1981, Palestro et al 1983, Savino et al 1984) and increased numbers of interdigitating reticular cells, the main antigen presenting cell of the thymus (Thomas et al 1982, Gilhus et al 1986).

Because the morphological and immunological evidence strongly suggest the presence of
an active immune response to nAchR in thymus of MG, many have hypothesized that loss of tolerance to nAchR or to a cross reactive molecule occurs within this gland (Henry 1981, Wekerle 1981, Palestro et al 1983). This is an important question and merits some consideration. There is no clear evidence at the present time to establish whether thymic changes may be primary or secondary with respect to disease development. In fact, some pathologic changes in thymus are observed following passive administration of EAMG sera (Dalkara et al 1983). nAchR can be detected on myoid cells (Kao and Drachman 1977, Wekerle et al 1978, Kirchner et al 1988) and HC (Kirchner et al 1988) and serum nAchR Ab do bind to thymic tissue (Van der Geld 1964, Lindstrom et al 1976b). Importantly, thymic hyperplasia occurs in EAMG where the immunizing species is known to be a xenogenic, cross reactive nAchR (Ueno et al 1982). Thus, certain aspects of thymic pathology may be caused by autoantibodies, dysregulated local hormone production or other factors which become relevant after disease has been established.

Studies of nAchR idiotypes in EAMG showed that, with time, idiotypes are produced that only recognize host nAchR and do not recognize the immunizing species of nAchR (Bearman et al 1980). This demonstration is important because it provides evidence that, regardless of the exact nature of immunizing antigen, the host immune system can generate a response against self nAchR. In MG, it is possible that the immune response may begin against a cross reactive molecule but, following Ab mediated tissue damage at muscle endplate and/or thymic medulla, self nAchR becomes available to drive the immune response. Interestingly, recent studies have shown that anti-nAchR Ab from sera of MG cross react with certain bacterial antigens of the normal gut flora (Stefansson et al 1987). Dwyer et al (1986) reported that anti-id to alpha 1,3 dextran (a bacterial surface determinant) cross reacted with nAchR, a finding which led them to suggest, as have others (Cleveland et al 1983, Souan et al 1985), that MG may result from an auto-anti-idiotype response.

Although less likely, it is conceivable that an aberrant intra-thymic immune response may be generated against a foreign, nAchR-cross reacting antigen in MG. Antigen can accumulate in thymus under experimental conditions (Blau 1967, Kyewski et al 1984) and activated, antigen
specific lymphocytes may enter the thymus (Naparstek et al. 1983) where they then become quiescent (Ben-Nun et al. 1982). In fact, thymic lymphocytes from MG patients secrete tetanus toxoid Ab if patients are boosted prior to thymectomy (Lisak et al. 1986b).

Removal of thymus in treatment of myasthenia gravis is often very beneficial although it has been suggested that the contribution to total nAchR Ab by thymus is insufficient to explain the beneficial effects of thymectomy and, as discussed by Berrih et al. (1981), levels of nAchR Ab do not fall consistently or rapidly after thymectomy. It is therefore possible that the thymus may provide a renewable source of antigen and/or be a source of recirculating, specifically activated lymphocytes. In view of the overproduction of thymic hormones which influence lymphocyte function in MG (Twomey et al. 1979, Dalakas et al. 1981) it is also possible that thymectomy may be beneficial in this regard.

1.8. Rationale for studies:

In MG nAchR Ab are directly pathogenic at the muscle endplate. Mechanisms contributing to the initiation of, and perpetuation of, the autoimmune response to nAchR are unknown. It is clear that defects in immune function do exist in MG, however, studies designed to evaluate immune function in MG have been conducted at a gross level. The use of heterogeneous lymphocyte populations and incompletely understood functional assays has made data interpretation difficult. Thus, the exact nature of immune response defects at the cellular and molecular level remain unknown. Moreover, it is not clear whether immune function defects in MG exist as primary and/or secondary aspects of disease. This is a very important question to address because secondary alterations in immune function may be underlying factors in influencing the course of myasthenia gravis.

Our working hypothesis has been that immune function defects do indeed occur in MG and that these defects occur secondary to the establishment and/or continuation of disease. Further, we have hypothesized that anti-lymphocyte Ab arise and may constitute at least one
mechanism by which immune function varies over the course of MG. The overall aim of our studies has thus been to clarify the nature of immune regulatory defects existing in MG, both at the cellular level and in terms of their possible relationship with disease progression. We have also carried out studies to examine the possibility that lymphocytes, by analogy with their known expression of muscarinic AchR, may express nAchR on their surfaces which would thus be potential targets for nAchR Ab.

At the time these studies were undertaken it was not clear whether nAchR Ab production was regulated in concert with the polyclonal humoral response. The only group at that time to have studied the relationship between polyclonal and specific immune response did not observe a correlation (Lisak et al 1984). Therefore, to study the immune response in MG in a meaningful way, it was first necessary to establish an assay with which to quantitate levels of the pathogenic Ab, nAchR Ab. In phase I of our research, we developed a modification of Lindstrom's nAchR Ab immunoassay for nAchR Ab. Because this assay had been unavailable in Canada and was not in routine use for clinical evaluation of MG we further modified the assay for maximum sensitivity and made it available for diagnostic purposes. Through the use of our clinical assay we were also able to select a group of seropositive, generalized MG patients to include in our study of in vitro nAchR Ab secretion.

In phase II of our research we studied the in vitro production of nAchR Ab and polyclonal IgG in cultures of PBMNC from seropositive, generalized MG patients and controls. We identified, for the first time, two subgroups of seropositive, generalized MG patients (Secretors and Nonsecretors) in whom B cell mediated immune function varied with duration of disease. These findings were consistent with our hypothesis that changes in immune function occurred secondary to MG. We have also shown for the first time that polyclonal immunoglobulin production is enhanced in early MG suggesting either that MG occurred preferentially among persons with high capacity for immunoglobulin production or that capacity for immunoglobulin production increased significantly following the onset of MG.

Mixing studies were carried out to determine whether defects in immune function were
operative at the level of the B cell or the T cell. B cells from MG patients whose PBMNC produced low levels of Ab (nAchR Ab and polyclonal IgG) in vitro were able to produce substantial amounts of IgG when stimulated by T-helper cells (CD4+) from individuals whose PBMNC produced high levels of Ab producers in vitro, suggesting that changes to immune function were controlled at the level of the T regulatory cell.

In view of these findings, and in keeping with our hypothesis that immune function in MG may be altered by anti-lymphocyte Ab, we carried out preliminary experiments to test the effect of MG sera on the functioning of normal T-lymphocyte subpopulations. We examined sera from the 2 categories of MG patients which we had identified (Secretors and Nonsecretors). Lymphocyte functioning was assessed in well defined PWM-stimulated, IgG secretion assays so that serum effects could be characterized at the level of T-regulatory cell subpopulations. Our data suggested that regulatory function of CD4+, T suppressor-inducer lymphocytes was inhibited by a factor present in sera of Secretors (patients with short disease duration and high in vitro Ab production by PBMNC). These studies were interesting, however, as we experienced difficulties obtaining large amounts of sera and cells required to do separation/recombination experiments of this nature, we concentrated on exploring our hypothesis that lymphocytes may be targets for auto-Ab in MG through expression of nAchR.

Thus, in phase III of our research, we conducted direct binding experiments using the classical nicotinic antagonist, alpha bungarotoxin, to probe for the nAchR on human PBMNC from healthy individuals and MG patients. There had been a single report of aBT binding to lymphocytes from MG patients (Morell 1976) which we had intended to confirm and extend through analysis of subset distribution and examination of potential relationships between expression of nAchR and clinical variables. In view of several functional studies which have been reported in the literature indirectly showing nAchR on normal human lymphocytes, we also expected to find some degree of aBT binding to normal lymphocytes. As discussed in Chapter 5, our study did not, in fact, confirm the earlier report of aBT binding to MG lymphocytes nor was aBT binding to normal lymphocytes observed. We did, however, in parallel studies, confirm and
characterize aBT binding to the rhabdomyosarcoma cell line, TE671, which has been viewed with considerable interest recently for its potential as a living model system in which to study nAchR and nAchR Ab.
## Chapter 2. Materials and Methods

### 2.1 Measurement of nAchR Ab 46

#### 2.1.1 Preparation of antigen for assay of nAchR Ab 46

#### 2.1.2 Acetylcholine receptor antibody detection in Protein A precipitation assay 46

#### 2.1.3 Optimization of double antibody precipitation of immune complexes for quantitative measurement of nAchR Ab 47

#### 2.1.4 Quantitative Determination of nAchR Ab titer 47

#### 2.1.5 Determination of nAchR concentration in muscle extracts 48

   i) binding of [125]I-aBT labelled nAchR to DE-81 ion exchange paper 48

   ii) maximal precipitation of [125]I-aBT labelled nAchR with high titer MG serum 49

#### 2.1.6 Quantitative measurement of nAchR Ab in supernatant fluids of PBMNC cultures 49

### 2.2 In vitro Ab production 50

#### 2.2.1 Patients and controls for study of in vitro Ab secretion 50

#### 2.2.2 Culture of peripheral blood mononuclear cells 50

#### 2.2.3 Isolation and culture of thymic lymphocytes 50

#### 2.2.4 Enrichment of lymphocyte subsets for functional studies 51

   i) T and B cell enrichment 51

   ii) T helper/inducer cells 51

   iii) T-suppressor cells 52

#### 2.2.5 Separation / Recombination experiments with Secretors and Non-secretors 52

#### 2.2.6 Serum pretreatment of lymphocytes for IgG secretion assays 53

#### 2.2.7 Culture of lymphocytes for IgG secretion assays 54

   i) T-helper assay 54

   ii) T-suppressor assay 54

#### 2.2.8 Measurement of IgG in supernatant fluids of PBMNC cultures 54

#### 2.2.9 Con A suppressor function assay 55

### 2.3 Direct binding studies 56

#### 2.3.1 Preparation of peripheral blood mononuclear cells for direct binding studies with aBT 56

#### 2.3.2 TE671 cell line: maintenance and preparation for binding assay 56

#### 2.3.3 Direct binding experiments 56
2.1 Measurement of nAchR Ab

2.1.1 Preparation of antigen for assay of nAchR Ab:

Acetylcholine receptor (nAchR) was solubilized from human or fetal calf muscle for use as antigen in the assay. Samples of skeletal muscle were obtained from amputated limbs or autopsy muscle material. Muscle was stored at -70 °C until used. Frozen muscles were thawed at 4 °C, cut into small pieces and homogenized in a blender for 2 to 3 min. with cold buffer A (0.1 M Na Phosphate buffer pH 7.0, containing 1 μg/ml pepstatin A (Sigma), 0.01 M sodium azide, 2 X 10^{-4} M phenylmethylsulfonylfluoride and 10^{-4} M Benzethonium chloride) in a 2.5:1 (v/v). The homogenate was then centrifuged at 27 X 10^3 g for 30 min at 4 °C. The pellets were recovered and rehomogenized in the same buffer with 2% Triton X-100 (Buffer A- Triton X) to solubilize the nAchR from the cell membrane. The mixture was stirred overnight at 4 °C. Centrifugation was carried out the next day at 39 X 10^3 g for one hour at 4°C. The supernatant was filtered through glass wool. Extracts were stored in aliquots at -70 °C.

2.1.2 Acetylcholine receptor antibody detection in Protein A precipitation assay:

A modification of the procedure of Tindall et al (1981c) was used to assay for precipitating nAchR Ab. Muscle extracts were incubated 30 min. at room temperature in the presence of an excess of [125]I-alpha bungarotoxin (aBT) (1.5 X 10^{-8} M, on average 50 - 100 times the concentration of aBT binding sites in the muscle homogenate). Serum was added to triplicate 12 X 75mm test tubes containing 100μl of [125]I-aBT (NEN or Amersham) labelled muscle extract. After incubation overnight at 4 °C, a 7% (weight/volume) solution of crude, lyophilized Cowan I strain Staphylococcus aureus (SAC) (Sigma P9151) was added. SAC had been pretreated by heating for 30 min (95 °C) in the presence of 3% SDS and 10% beta-mercaptoethanol. This treatment resulted in a more stable preparation which allowed higher and more reproducible precipitation. After 20 min at room temperature, tubes were washed three times using Buffer A-Triton X.
washes centrifugation was carried out for 5 min at 2000 g. Precipitates were counted in a Beckman Gamma 5500. In preliminary experiments, optimal amounts of test serum and of SAC were determined to be 10 μl and 100 μl respectively. For each assay five sera were randomly selected from a group of 50 samples obtained from healthy individuals. Sample values between 2 and 3 standard deviations over the mean of controls were considered "borderline". Sample values 3 SD over controls were considered positive. Borderline sera were systematically re-assayed using another preparation of human antigen.

2.1.3 Optimization of double antibody precipitation of immune complexes for quantitative measurement of nAchR Ab:
A panel of goat and rabbit anti-human-IgG antibody products were screened for their ability to precipitate [125]I-aBT labelled nAchR immune complexes formed with nAchR Ab in MG sera. The following products were tested: Cappel rabbit-anti-human immunoglobulin, Kent goat-anti-human IgG, Jackson ImmunoResearch rabbit-anti-human IgG, Research Product goat-anti-human IgG and New England Immunology Associates rabbit-anti human IgG. 100 μl of various dilutions of anti-human IgG (second antibody) were incubated in triplicate with a mixture of 100 μl of [125]I-aBT labelled human muscle extracts which had been allowed to react, overnight at 4°C, with 2μl of a known positive MG serum. Incubation with second antibody was allowed to proceed overnight at 4°C and the following day immune complexes were precipitated by centrifugation and were washed three times before counting. Second Ab were compared for their efficiency in precipitating immune complexes.

2.1.4 Determination of nAchR Ab titer quantitatively:
A modification of the precipitation assay of Lindstrom et al (1977) was used to measure nAchR Ab. Human muscle extract was incubated with excess [125]I-aBT (Amersham) at room temperature for 30 min. 20 μl of an appropriate dilution of serum to be tested was added with
excess carrier serum in triplicate 12 X 75 mm plastic tubes overnight at 4 °C. Serum was diluted as necessary to remain within a predetermined region of antigen excess (< 30% of maximum precipitable cpm as determined from concentration of nAchR (see below) in muscle extract and specific activity of aBT on day of testing). The next day, normal human serum, in excess over test serum (2 μl), was added as carrier to standardize the amount of IgG in the mixture. IgG was precipitated by addition of optimal concentrations of goat-anti-human IgG second Ab. Background was determined in the absence of serum. Serum titers were reported in moles of aBT binding sites precipitated per liter of serum. When nAchR Ab were measured in supernatants of lymphocyte cultures, triplicate 500 μl samples of unconcentrated supernatant were incubated with 100 μl aliquots of labelled muscle homogenate. 2 μl normal human serum was used as a carrier and immune complexes were precipitated with goat-anti human IgG as in serum assay. After three washes in PBS, precipitates were counted in a Beckman Gamma 5500. Background cpm was determined on RPMI-1640 or on 1-day lymphocyte culture supernatant. Samples were considered positive if they were increased over background (95% confidence limit). nAchR Ab titers for lymphocyte cultures were reported as fmol [125]I-aBT binding sites precipitated per milliliter supernatant. Known positive MG serum, diluted to the range of Ab found in supernatants, was included as control in each assay. Titers of nAchR Ab were calculated according to the formula below and corrected for decay of [125]I:

\[
\text{net dpm} + 4.54 \times 10^{7} \, \text{dpm/μci (constant)} + \text{specific activity aBT (μci/μg)} + 8 \times 10^{9} \\
\text{μg/mol aBT (molecular weight)} + \text{volume of serum tested (μl)} \times 10^{6} \, \text{μl/liter}
\]

2.1.5. Determination of nAchR concentration in muscle extracts:

i) binding of [125]I-aBT labelled nAchR to DE-81 ion exchange paper:
100 μl of muscle extract was incubated with serial dilutions of [125]I-aBT (Amersham) in the
presence or absence of an excess of unlabelled aBT (1 X 10^{-4}M) (Sigma) in a final incubation volume of 150 μl. Following incubation at room temperature for 60 min., the mixture was filtered over DE-81 ion exchange filters (Whatman). At neutral pH, DE-81 ion exchange paper has ionic affinity for the nAchR protein but allows the aBT to pass through its pores. Filters were rinsed with 12 ml of Buffer A-Triton X and counted in a Beckman Gamma 5500. Nonspecific binding was determined in the presence of excess, unlabelled aBT and subtracted from the total binding. Values were corrected for the decay of [125]I.

ii) Maximal precipitation of [125]I-aBT labelled nAchR with high titer MG serum:
A panel of high titer MG sera (10μl) were incubated overnight with 100 μl of [125]I-aBT labelled muscle extract. Immune complexes were precipitated using an excess of Staph A as described above. Pellets were washed and counted. Cpm were converted to moles of [125]I-aBT and values were averaged for all MG sera tested in order to obtain an approximation of the concentration of nAchR in a given muscle extract. This technique was used routinely.

2.1.6 Quantitative measurement of nAchR Ab in supernatant fluids of PBMNC cultures:
To quantitate nAchR Ab in supernatants of lymphocyte cultures, 100 μl [125]I-aBT labelled human nAchR solution was incubated with 500μl of unconcentrated culture supernatant overnight at 4°C. Immune complexes were precipitated in the presence of goat-anti-human IgG adjusted to equivalence with normal human carrier serum. nAchR Ab titers were expressed as femtomoles of [125]I-aBT binding sites precipitated per milliliter of culture supernatant. Known positive MG serum, diluted to the range of Ab found in supernatants was included in each assay. Background cpm were determined in the presence of RPMI or supernatant from one day cultures. Limit of detection in this assay was 8.5 X 10^{-17} mol [125]I-aBT binding sites. In terms of concentration of
nAchR Ab in culture supernatants, this was .0002 nmol/liter.

2.2 In vitro production of Ab:

2.2.1. Patients and controls in study of in vitro Ab secretion:
Thirty six MG patients were selected on the basis of having nAchR Ab in their serum. All patients had generalized MG and 3 were in clinical remission at the time of study. The control group consisted of 20 healthy volunteers and two patients with other neurological disorders.

2.2.2. Culture of peripheral blood mononuclear cells:
PBMNC were isolated from heparinized blood by centrifugation over Ficoll/Hypaque (Pharmacia). This technique has been reported to yield 95% mononuclear cells and less than 5% granulocytes or erythrocytes. We have assumed that degree of enrichment of mononuclear cells by centrifugation over Ficoll/Hypaque does not differ between MG and normal and that percentage monocyte/macrophage do not differ between MG and control (Kelley et al 1981). Random testing of mononuclear cell viability, by staining with trypan blue, following centrifugation over Ficoll/Hypaque indicated that viability was >98%. PBMNC were washed in Hanks balanced salt solution, and resuspended in RPMI-1640 supplemented with L-glutamine (2 X 10^{-5} M), gentamycin (0.1 µg/ml), 5% sodium bicarbonate and 10% heat inactivated fetal calf serum. Triplicate 1.0 ml cultures containing 10^6 cells were set up in 12 X 75 mm plastic tubes for 10 days at 37° C in an atmosphere of 5% CO_2. Some cultures were stimulated with pokeweed mitogen, a lyophilized extract of *Phytolacca americana* root (PWM, Gibco) at a final dilution of 1/300. Supernate from triplicate cultures were pooled and stored at -70 °C prior to assay for IgG and nAchR Ab.

2.2.3. Isolation and culture of thymic lymphocytes:
Thymic lymphocytes were obtained from MG patients at the time of thymectomy. Tissue was
minced under sterile conditions in Hanks balanced salt solution. Debris was separated by
decantation and thymocytes were collected from the supernatant by centrifugation. Thymocytes
were cultured in RPMI-1640 supplemented with L-glutamine (2 X 10^{-5} M), gentamycin (0.1
μg/ml), 5% sodium bicarbonate and 10% heat inactivated fetal calf serum. Thymocytes were
resuspended to 10^6/ml and cultured in a volume of 1.0, 10.0 or 25.0 ml for 10 days at 37° C in an
atmosphere of 5% CO2 in the presence or absence of PWM (1/300, Gibco). Supernatants from
triplicate cultures were pooled and stored at -70 °C prior to assay for IgG and nAchR Ab.

2.2.4. Enrichment of lymphocyte subsets for functional studies:

i) T and B cells: Mononuclear cells were depleted of monocytes by plastic adherence at 37 °C for
45 min. T and B cell populations were then enriched by a standard E-rosette technique (Saxon et al
1976). Separation was based on the ability of T cells to form rosettes with 2-
aminoethylisothiouronium bromide (AET) treated sheep red blood cells (E+ cells).

E- and E+ populations were studied for surface markers (cells were stained with various
mcAb and enumerated using a fluorescence activated cell sorter). E- cells contained (averaged over
several experiments) 85% surface Ig positive (SIg) cells. E+ cells were 86% positive for CD3+.
As a criterion of purity of E+ and E- cells, we have routinely assessed their function in IgG
secretion assays. E- (B enriched) or E+ (T enriched) lymphocytes did not secrete IgG when
cultured alone. Rosetting for E+ and E- populations does not yield 100% pure populations of T
and B cells. The demonstration that T or B enriched cells did not produce IgG when cultured in the
absence of the other was the most important control for our functional studies on IgG secretion. It
was not feasible to routinely perform enumeration of lymphocyte differentiation markers due to
consumption of lymphocytes needed for the assays, time constraints and cost.

Hereafter, for simplicity, E+ and E- cell populations will be referred to as T cells and B
cells respectively.
ii) T-helper/inducer cells: CD4+ lymphocytes were enriched from T-cells by negative panning to remove CD8+ lymphocytes. Briefly, serum pretreated T-lymphocytes were washed extensively and then labelled with the CD8 directed, murine mcAb, OKT8 (Ortho), by incubation for 20 min at 4 °C. Labelled cells were then incubated for 1.5 hr at 4 °C on goat-anti-mouse IgG coated petri dishes to allow adherence of CD8+ T-cells. The floating cells were harvested by gentle swirling. CD4+ enriched T-cells were shown to stimulate IgG production in a dose dependent manner. CD8+ deprived lymphocytes contained < 2% CD8+ cells as determined by staining with OKT8 or Leu 2a.

iii) T-suppressor cells: CD8+ lymphocytes were enriched from T-cells by negative panning to remove CD4+ cells following labelling of T cells with the CD4 directed, murine mcAb, OKT4 (Ortho). CD8+ enriched T-cells were shown to induce a dose dependent inhibition of IgG production in cultures of E- and CD4+ enriched T-cells. T cells from which CD8+ cells were obtained had been stored frozen at -70 °C for less than 2 weeks and were recovered with 70% viability by trypan blue exclusion.

2.2.5. Separation / Recombination experiments with Secretors and Nonsecretors:

Mononuclear cells were recovered following centrifugation over Ficoll/Hypaque. Cells were washed in Hanks balanced salt solution and were incubated in plastic flasks in medium at 37°C for 45 min. to allow monocytes to adhere to the plastic. Nonadherent cells (lymphocyte enriched) were rosetted with AET-treated sheep red blood cells (see section 2.2.4 i) to enrich B and T cell population. T enriched lymphocytes were enriched for T helper/inducer cells as described above. Mixing experiments were carried out using B enriched and CD4+ enriched (CD8+ depleted) lymphocytes from MG patients or controls. In experiment #1, 1.5 X 10^5 B cells and 1.5 X 10^5 CD4+ lymphocytes were cultured in a final volume of 1.0 ml in 12 X 75 mm test tubes. In
experiment #2, 5 X 10^4 B cells were added with 3 X 10^5 CD4+ and cultured in a final volume of 200 µl round bottom microwell culture plates in triplicate. All cultures were maintained for 10 days in an atmosphere of 5% CO2 at 37°C in RPMI-1640 supplemented with L-glutamine (2 X 10^{-5} M), gentamycin (0.1 µg/ml), 5% sodium bicarbonate and 10% fetal calf serum and were stimulated with PWM at 1/300. Supernates were harvested by centrifugation and assayed for IgG.

2.2.6. Serum pretreatment of lymphocytes for IgG secretion assays:

Normal human T- cells were incubated in RPMI-1640 supplemented with L-glutamine (2 X 10^{-5} M), gentamycin (0.1 µg/ml) and 5% heat inactivated fetal calf serum (FCS) for 3 days at 37°C. The incubation media also contained fetal calf serum or sera pooled from at least 4 individuals in each of the following categories (final concentration in the culture media was 10%):

- Sera pooled from MG patients who were in vitro secretors of nAchR Ab
- Sera pooled from MG patients who were in vitro nonsecretors of nAchR Ab
- other non-autoimmune, neurological disease controls (OND)

Sera from MG patients who were either Secretors or Nonsecretors of nAchR Ab in vitro were selected on the basis that the disease variables for the patient were representative of the respective categories. The average disease duration among patients in the secretor category from whom sera were taken was 64 mos. and the average IgG secretion in 10 day PWM stimulated PBMNC cultures was 3037 ng/ml. Average disease duration among patients in the nonsecretor category was 153 mos. and the IgG secretion was 155 ng/ml.

Separate sets of sera were used in each experiment. Sera had been pretreated by heat inactivation at 56 °C for 30 min to inactivate complement and ultracentrifuged at 100,000 g for 30 min to remove aggregated IgG which might influence suppressor function of T cells. All patient and control sera were screened by examination of patient records to exclude any individual having autoimmune disorders (other than MG) since abnormalities of immune function and anti-lymphocyte Ab have been reported in some autoimmune diseases.
During incubation of T cells with sera, autologous B cells, simultaneously obtained from PBMNC at the time of rosetting, were stored frozen in 10% DMSO (dimethylsulfoxide) solution at -70°C until used in the assay. Viability was 90% after freezing as determined by trypan blue exclusion.

2.2.7. Culture of Lymphocytes for IgG secretion assays:

i) T-helper assay:

5.4 X 10^4 B cells were cultured with graded numbers of CD4+ enriched T-cells (T-helper/inducer) in a total volume of 310 μl in round bottom microwells for 10 days in RPMI-1640 supplemented with 10% heat inactivated FCS, L-glutamine (2 X 10^-5 M) and gentamycin (0.1 μg/ml) in an atmosphere of 5% CO2, 37 °C. Cultures were stimulated with PWM at a final dilution of 1/300. Supernatants were assayed for IgG secreted.

ii) T-suppressor assay:

5.4 X 10^4 B cells and 5.4 X 10^4 T-helper cells were cultured as above. Graded numbers of CD8+ enriched lymphocytes (T-suppressors) were added in triplicate to the microwells. All cultures contained PWM at 1/300. Cultures were maintained for 10 days in an atmosphere of 5% CO2 at 37 °C. The supernatants were assayed for IgG.

2.2.8 Measurement of IgG in supernates of PBMNC cultures:

ELISA microculture plates were coated, overnight at 4°C, with 220μg goat-anti-human IgG (Cappell, 1:1 mix of Fc specific and heavy and light chain specific) in 0.015M Na2CO3, 0.035 M NaHCO3, 0.003M NaN3, pH 9.6. Following washing, wells were coated with PBS containing 0.1% bovine serum albumin by incubation for 1 hour at room temperature. Following washing, appropriately diluted (RPMI) test samples were added to wells in triplicate and incubated for 1
hour at room temperature. After washing, alkaline - phosphatase labelled goat-anti-human IgG (2.5 units, Tago #2490) was added to each well and incubated for 1 hour at room temperature in the dark. Forty four micrograms of p-nitrophenyl phosphate was then added to each well (Sigma) for a minimum of 30 min or until yellow color develops. Absorbance was read on a Dynatech MR600. Standard curves were included in each assay and consisted of serially diluted (1248 ng/ml-1.2 ng/ml) human IgG (Calbiochem).

2.2.9. Con A Suppressor Assay:
Peripheral blood mononuclear cells were isolated from healthy individuals by centrifugation over Ficoll/Hypaque. Cells were cultured in paired flasks for 3 days at 2 X 10^6/ml in RPMI-1640 supplemented with 5% fetal calf serum (FCS), L-glutamine (2 X 10^{-5} M) and gentamycin (0.1 μg/ml). Culture media also contained 10% heat inactivated serum from MG, control or FCS. For each pair of flasks, one flask contained concanavalin A (con A) (6 μg/ml) and the other one was unstimulated. Following 3 days in culture the cells were treated with 0.08 mg/ml mitomycin C (to inhibit DNA synthesis). Effectiveness of mitomycin C treatment was verified by a ³H-thymidine uptake test. Cells were washed extensively and re-cultured in triplicate microwells (5 X 10^4) with 1 X 10^5 freshly obtained heterologous responder cells in a final volume 250 μl containing 3μg/ml Con A and 10% FCS. After 2 days the wells were pulsed with 1 μci ³H-thymidine for 6 hours and then harvested onto filter paper which was then allowed to air dry. Five ml scintillation cocktail was added (Econofluor) and the next day samples were counted on a Beckman scintillation counter (LS9000).
2.3. Direct binding studies:

2.3.1 Preparation of peripheral blood mononuclear cells for direct binding studies with aBT:
PBMNC were isolated from heparinized blood by centrifugation over Ficoll/Hypaque. Before
assay, cells were washed three times and resuspended in phosphate buffered saline (PBS).

Six of the seven MG patients had active, generalized disease; one was in clinical remission. All
patients had nAchR Ab in their serum. Five patients were receiving pyridostigmine bromide and
corticosteroids. Two patients were not receiving any medication at the time of study.

To assess the effects of culture on the expression of aBT binding sites, PBMNC from 3
MG patients and one control were cultured, in an atmosphere of 5% CO2, at 10^6/ml in RPMI-1640
supplemented with L-glutamine (2 x 10^{-5} M), gentamycin (0.1µg/ml) and 10% heat inactivated
fetal calf serum for 1, 3 or 6 days. One MG patient and one control were also tested following a 3
day incubation in the presence of 6 µg/ml Concanavalin A (Con A).

2.3.2. TE671 cell line: Maintenance and preparation for binding assay
The human rhabdomyosarcoma cell line, TE 671, was purchased from American Type Culture
Collection and maintained in Dulbecco's modified Eagle's medium with 4.5 g/liter glucose, 10%
fetal calf serum (Gibco) and 0.1µg/ml gentamycin in an atmosphere of 5% CO2 at 37°C. Prior to
binding studies, cells were treated with 1% trypsin, washed in media and resuspended at 10^7/ml in
PBS.

2.3.3 Direct Binding Experiments:
One hundred microliters of cell suspension was added to various concentrations (0.5 - 64 nM) of
[^125I]aBT (100µl) in the presence or absence of an excess (1.1 x 10^{-5} M) of unlabeled aBT
(Sigma). Final reaction volume was 220 µl. Incubation was allowed to proceed for 50 min. at
room temperature. Cells were filtered over cellulose acetate filters (Millipore AAWP024) that had been pretreated by soaking at 4°C in PBS, 0.5% bovine serum albumin. Filters were washed four times with 3 ml cold PBS containing 0.5% BSA and counted in a Beckman gamma 5500.
Chapter 3. Establishment of assay for nAchR Ab and occurrence of serum nAchR Ab in MG

Data reported in Ann New York Acad Sci 475, 413-414 1986
and Can J Neurol Sci 14, 297-302 1987

3.1 INTRODUCTION 59

3.2 RESULTS 61
3.2.1 Protein A precipitation assay for serum nAchR Ab: 61
A) Optimization of Protein A precipitation assay for detection of nAchR Ab in serum 61
   i) Protein A precipitation of [125]I-aBT labelled nAchR 61
   ii) Precipitation of [125]I-aBT labelled nAchR by increasing amounts of MG serum 62
      iii) Capacity for testing large serum volumes increases sensitivity of Protein assay for nAchR Ab 64
   iv) Comparison of the precipitation of [125]I-aBT labelled nAchR by MG and control sera 65
B) Validation of the qualitative assay for diagnostic purposes 66
C) Summary of clinical results using qualitative assay for detection of nAchR Ab in serum 67
3.2.2 Comparison of different antigens for detection of nAchR Ab: 68
   i) Comparison of human muscle preparations for detection of nAchR Ab in serum 69
   ii) Comparison of human antigen with fetal calf antigen for the detection of nAchR Ab in serum 70
3.2.3 Double Ab precipitation assay for quantitative determination of nAchR Ab: 71
   A) Optimization of double Ab precipitation assay 71
      i) Double Ab precipitation of immune complexes 71
      ii) Quantitation of nAchR in muscle extract 72
3.2.4 Quantitative determination of nAchR Ab titer 75
3.2.5 Reproducibility of nAchR Ab titers measured between assays 78
3.2.6 Comparison of disease severity and titer of nAchR Ab in serum of MG patients 80
3.2.7 Polyclonal nature of nAchR Ab in MG serum 81

3.3 DISCUSSION 84
3.3.1 Measurement of nAchR Ab in serum 84
3.3.2 Protein A precipitation assay for detection of nAchR Ab in serum 85
3.3.3 Frequency of occurrence of circulating nAchR Ab in MG of different disease categories 86
3.3.4 Comparison of alternate antigen sources for nAchR Ab assay 88
3.3.5 Quantitative assay for nAchR Ab 89
3.3.6 Heterogeneity of serum nAchR Ab 90
3.3.7 Relationship between nAchR Ab titer and severity of disease 91

3.4 SUMMARY 92
3.1 Introduction:

To explore the regulation of nAchR Ab production in vitro it was necessary to have access to a set of patients diagnosed as having MG. At the time this study was begun, the vast majority of MG patients in the Vancouver area had been diagnosed on purely clinical and electrical criteria. It is known that nAchR Ab can be detected in serum from the overwhelming majority of MG patients with generalized, active disease (Lefvert et al 1978, Tindall et al 1981b, Vincent et al 1985). The detection of serum nAchR Ab is an extremely sensitive and specific diagnostic tool. This led us to establish an nAchR Ab immunoassay using a modification of the procedure originally described by Lindstrom (1976c).

The determination of serum nAchR Ab is based on the in vitro binding of nAchR Ab to nAchR solubilized from human muscle and precipitation of those immune complexes by a double antibody technique (Lindstrom et al 1976c). We have made improvements to the assay in order to circumvent the following problems.

For diagnostic work, the double antibody technique has the disadvantage of being slow. Its sensitivity may also be limited because of the necessity to test dilute serum in the presence of normal carrier serum. The double antibody technique has been used by some investigators to assay several μl of test serum without carrier serum (Lindstrom et al 1976c, McAdams et al 1980, Nicholson et al 1982, Vincent and Newsom-Davis 1985). Such a practice, however, assumes that variations in test serum immunoglobulin concentration will not result in variable efficiency of immune complex precipitation. As concentrations of immunoglobulin in patient serum vary more than in normal (Lisak et al 1976), such techniques may result in false negatives for low titer sera. We have used Protein A to precipitate the immune complexes in our assay system, which is rapid and allows testing of larger volumes of serum.

To minimize the potential occurrence of false negative results for sera of very low nAchR Ab titer it is desirable to assay a relatively high concentration of patient serum with the largest
amount of nAchR (human muscle extract) that is practical to use. This will maximize the amount of immune complexes that are available for detection. Our use of Protein A to precipitate immune complexes has allowed the testing of large volumes of serum. Thus we have increased the formation of immune complexes by increasing the concentration of nAchR Ab in the reaction mixture. Up to now, others have carried out nAchR Ab testing on only small volumes of serum under dilute conditions.

To quantitate levels of nAchR Ab in serum, measurements must be made in the so-called "linear region" of the precipitation curve where solublized nAchR is in large excess over serum nAchR Ab. To work on a practical scale this necessitates that routine testing be carried out on very dilute sera. This will, of course, minimize the sensitivity of the assay for low titer sera. Others have practiced scaling up assay reagents, presumably to partially compensate for large serum dilutions through increasing the amount of precipitable nAchR (Lindstrom et al 1976c, Tindall et al 1981c, McAdams et al 1980, Nicholson et al 1982). Such a practice is certainly not cost or labor efficient. Moreover, it is well known that serum nAchR Ab titer does not exhibit a meaningful relationship with disease activity (see Chapter 1). Recognizing that there is little value to quantitation of nAchR Ab for diagnosis of MG, we have placed the emphasis on maximization of sensitivity and on running the assay in an economically feasible manner.

In the present study, our first goal was to optimize the nAchR Ab assay for maximal sensitivity and to make it available on a wide scale. We have used Protein A to precipitate immune complexes in our assay. This technique has the advantage that large volumes of serum can be reliably tested thus maximizing the sensitivity of the assay. This technique is also rapid, requiring half the time of the double antibody technique. Our assay is now available throughout Canada for diagnostic purposes. A quantitative, double Ab nAchR Ab assay has also been established which has permitted us to measure nAchR Ab secreted in vitro (see Chapter 4).

Human limb muscle homogenate is presently the reagent of choice for detection of nAchR Ab. However, there are technical difficulties associated with its use in that it cannot be reliably obtained, is difficult to standardize, and is biohazardous. For experimental purposes, investigators
have often made determinations of nAchR Ab using nAchR derived from electric organs of *Electrophorus electricus* or *Torpedo Californica* which are rich and readily accessible sources. Unfortunately, due to limited cross reactivity, this reagent is not useful for diagnostic work in humans.

In an attempt to find a suitable replacement for human muscle tissue, fetal calf muscle derived antigen was studied for its potential to bind human nAchR Ab. The correlation between assay results using human and fetal calf muscle was good. However, because we found 29% false negatives using fetal calf derived antigen, we concluded that its substitution for human muscle derived antigen in the diagnostic assay was not feasible.

We have also had an interest in the degree of polyclonality of circulating nAchR Ab in MG. Theoretically, variability in pathogenic capacity of nAchR Ab idiotypes could be a factor in controlling disease expression. We have taken advantage of our nAchR Ab assay to study binding patterns of nAchR Ab in serum of MG patients against cross reactive antigen preparations and have thus confirmed the polyclonal nature of the auto Ab response.

### 3.2 Results:

#### 3.2.1. Protein A precipitation assay for serum nAchR Ab:

To study the immunoregulation of nAchR Ab production in MG it was necessary to develop an assay for measurement of nAchR Ab. We developed a Protein A precipitation assay which was very sensitive for detection of nAchR Ab in serum. Although this assay was not quantitative, it was used to screen for seropositive MG patients who were then included in subsequent studies of in vitro nAchR Ab production. Further, since the detection of nAchR Ab in serum is an extremely important diagnostic test for MG which was not available in Canada, we optimized the Protein A precipitation assay for maximum sensitivity and made it available for diagnostic use.

Ultimately, it was found that, while the Protein A precipitation assay was most sensitive for detection of nAchR Ab in low titer sera, a double Ab precipitation technique was most efficient for quantitative work. Thus, a double Ab precipitation assay was developed and routinely
employed for measuring levels of nAchR Ab in serum and in media of PBMNC cultures.

A) Optimization of Protein A precipitation assay for detection of nAchR Ab in serum:

i) Protein A precipitation of [125]I-aBT labelled nAchR:

We used Protein A to precipitate immune complexes in our assay. It was necessary to be certain that the Protein A was precipitating all of the available immune complexes so we studied the effects of adding increasing amounts of Staphylococcus aureus (SAC) to the reaction mixture. Increasing amounts of SAC were added to a mixture containing [125]I-aBT labelled nAchR and a constant amount of known positive MG serum (Table 3.1). For each amount of SAC the background was determined in the presence of normal human serum. Background increased as the size of the SAC pellet increased, presumably due to nonspecific trapping of [125]I-aBT.

Table 3-1. Protein A precipitation of [125]I-aBT labelled nAchR:

<table>
<thead>
<tr>
<th>Volume of SAC Added (µl)</th>
<th>CPM Precipitated by NHS (a)</th>
<th>CPM Precipitated by one MG Serum (b)</th>
<th>Net Counts (b-a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>470</td>
<td>2860</td>
<td>2390</td>
</tr>
<tr>
<td>25</td>
<td>820</td>
<td>5080</td>
<td>4260</td>
</tr>
<tr>
<td>50</td>
<td>1200</td>
<td>5470</td>
<td>4270</td>
</tr>
<tr>
<td>100</td>
<td>1840</td>
<td>5900</td>
<td>4060</td>
</tr>
<tr>
<td>200</td>
<td>2310</td>
<td>6530</td>
<td>4220</td>
</tr>
<tr>
<td>300</td>
<td>2640</td>
<td>7060</td>
<td>4420</td>
</tr>
</tbody>
</table>

Precipitation of immune complexes by Protein A. Human [125]I-aBT labelled nAchR was incubated with 10 µl of a high titer MG serum overnight at 4 °C. Following addition of SAC the mixture was incubated for 20 min. at room temperature, pellets were washed three times and counted. Numbers represent cpm.

From the net counts column it can be seen that precipitation was maximal at 25µl of 7% SAC solution. 100µl was chosen for routine use in the assay in order to allow a safety margin for optimal precipitation of immune complexes.
Precipitation of [125]I-aBT labelled nAchR by increasing amounts of MG serum:

To determine whether binding of MG serum to aBT labelled muscle extracts was saturable, increasing amounts of a known positive MG serum were added to [125]I-aBT labelled extracts. Immune complexes were precipitated with SAC.

Figure 3-1. Precipitation of [125]I-aBT labelled nAchR by increasing amounts of MG serum:

Precipitation of [125]I-aBT labelled nAchR from a detergent solubilized extract of human muscle tissue (mean cpm±SD). Increasing amounts of MG serum were added to extract and incubated overnight at 4 °C. Immune complexes were precipitated with 100μl 7% SAC solution, washed and counted.

Figure 3.1 shows that, indeed, the SAC precipitation of [125]I-aBT labelled nAchR increased with increasing amounts of MG serum until a plateau was reached. At the plateau the nAchR Ab had combined with all the available nAchR in the extract.
iii) Capacity for testing large serum volumes increases sensitivity of SAC assay for nAchR Ab:

In order to maximize the sensitivity of the SAC precipitation assay, several low titer MG sera were studied at varying serum concentrations for binding to [125]I-aBT labelled nAchR. Figure 3.2 shows that, when 1 μl was used, all 6 sera tested fell in the range of the negative controls; however, when 10μl were used 3 were positive and 1 was borderline.

Figure 3-2. Precipitation of [125]I-aBT labelled nAchR by low titer MG serum:

Cpm precipitated in the presence of increasing amounts of human sera having low reactivity. The dotted lines represent the range of the controls run in the same experiment. A high titer MG serum is included as a positive control (interrupted line //).

The SAC assay system has the advantage of being able to handle large volumes of serum and thus the sensitivity of the assay for detection of circulating nAchR Ab was maximized. The limit of
detection in this assay was approximately $3 - 9 \times 10^{-16}$ moles of aBT binding sites (average of $5 \times 10^{-16}$), depending on the muscle extract used. The detection limit for concentration of nAchR Ab which could be detected in serum was $0.03 - 0.09$ (average of 0.05) nanomoles of aBT binding sites per liter of serum.

**iv) Comparison of the precipitation of [125]I-aBT labelled nAChR by MG and control sera:**

We studied the precipitation of [125]I-aBT labelled nAchR by sera from MG patients and controls as illustrated in Table 3.2.

**Table 3.2. Comparison of the Precipitation of [125]I-aBT labelled nAchR by MG and Control Serum:**

<table>
<thead>
<tr>
<th>cpm precipitated</th>
<th>control</th>
<th>MG</th>
</tr>
</thead>
<tbody>
<tr>
<td>2577</td>
<td>4789</td>
<td></td>
</tr>
<tr>
<td>2370</td>
<td>2737</td>
<td></td>
</tr>
<tr>
<td>2191</td>
<td>4932</td>
<td></td>
</tr>
<tr>
<td>2270</td>
<td>4515</td>
<td></td>
</tr>
<tr>
<td>2429</td>
<td>4339</td>
<td></td>
</tr>
<tr>
<td>2351</td>
<td>4921</td>
<td></td>
</tr>
<tr>
<td>2174</td>
<td>4613</td>
<td></td>
</tr>
<tr>
<td>2017</td>
<td>3095</td>
<td></td>
</tr>
<tr>
<td>1966</td>
<td>4225</td>
<td></td>
</tr>
<tr>
<td>2113</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1940</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

mean±SD: 2218±194

*mean+3SD: 2800

*Test sample is positive if over mean of controls plus 3 SD.

Precipitation of [125]I-aBT labelled nAchR from a detergent solubilized extract of human muscle tissue. 10µl of serum from either an MG patient or a normal control were added to 100µl of muscle extract containing an excess of [125]I-aBT. Incubation proceeded overnight at 4 °C and the following day immune complexes were precipitated with an excess of SAC.
The MG sera precipitated significantly more immune complexes than control sera. For routine purposes, sera to be analyzed for nAchR Ab were always tested in parallel with a panel of control sera. Test sera which were over the mean of controls plus 3SD were considered to be positive (99% confidence level) and between 2-3 SD over control were considered to be borderline.

B) Validation of the SAC assay for diagnostic purposes:
Before the SAC precipitation assay could be used for clinical purposes it was necessary to validate its potential for detection of nAchR Ab in patients with MG. We carried out a retrospective study of circulating nAchR Ab among MG patients. Diagnosis had been made by university affiliated neurologists who were asked, without knowledge of the nAchR Ab results, to classify their MG patients into ocular, generalized active or remission. Diagnoses of these patients had been based on clinical findings and results of Tensilon ® testing and/or electromyography (emg). The controls were recruited among healthy hospital personnel. Results are shown in Table 3.3.

<table>
<thead>
<tr>
<th></th>
<th>+</th>
<th>±</th>
<th>-</th>
</tr>
</thead>
<tbody>
<tr>
<td>Active generalized MG (43)</td>
<td>40</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>MG generalized remission (21)</td>
<td>5</td>
<td>5</td>
<td>11</td>
</tr>
<tr>
<td>Ocular MG (37)</td>
<td>19</td>
<td>3</td>
<td>15</td>
</tr>
<tr>
<td>Healthy controls (55)</td>
<td>0</td>
<td>0</td>
<td>55</td>
</tr>
</tbody>
</table>

101 MG patients were tested for the presence of nAchR Ab in their serum using the qualitative assay (human nAchR). These patients had been previously diagnosed on clinical grounds by neurologists in the Vancouver area. Diagnostic criteria included the demonstration of weakness on exercise affecting ocular, bulbar and limb musculature and a positive tensilon test (improved strength following injection of acetylcholinesterase inhibitor).
Among 43 patients having active, generalized MG, 40 (93%) were positive, 2 (4.7%) were borderline and 1 (2.3%) was negative using the SAC assay. Of 37 patients with ocular MG, 19 (51.8%) were positive, 3 (8.1%) were borderline and 15 (40.5%) were negative. Five of 21 (24%) of MG in clinical remission were positive, 5 (24%) were borderline and 11 (52%) were negative. Circulating nAchR Ab were not detected in sera from any of 55 healthy controls.

These results confirmed the potential diagnostic usefulness of this test.

C) Summary of clinical results using SAC assay for detection of nAchR Ab in serum:

Following validation of our Protein A precipitation assay we made it available for clinical purposes. We went on to study nAchR Ab in sera of 51 patients with possible MG who had not been diagnosed at the time of testing. Of these, 13 were positive, 5 were borderline and 33 were negative. A diagnosis of MG was arrived at for 12 of the 13 seropositive patients. These diagnoses were arrived at with knowledge of the nAchR Ab testing. Diagnosis was based on clinical findings, tensilon testing and/or emg and results of nAchR Ab testing. Diagnoses were confirmed by clinical follow up and response to Mestinon® (pyridostigmine bromide), an acetylcholinesterase inhibitor. The seropositive patient who did not receive a diagnosis of MG was diagnosed with botulism on clinical and epidemiological grounds, however was seronegative for Botulinum Toxin. All 33 patients whose sera were negative for nAchR Ab were subsequently determined to have other neuromuscular or psychiatric disorders. The 5 sera which were borderline in Protein A assay for nAchR Ab were systematically reassayed. Borderline sera which were negative on repeated testing were considered to be negative. Those which re-tested borderline or positive were considered to be positive. Three of the borderline sera were determined to be negative and were subsequently diagnosed as having other neuromuscular disorders. Two remained borderline on repeated testing and were thus considered to be positive. Final diagnosis on these patients included one MG patient in clinical remission and one rheumatoid arthritis patient who was being treated with penicillamine.
We have summarized the results generated with the Protein A precipitation assay. Table 3.4 includes results of the retrospective study together with results generated through clinical use of the assay.

### Table 3.4. Summary of results using SAC assay for determination of nAchR Ab in serum:

<table>
<thead>
<tr>
<th>Acetylcholine Receptor Antibodies:</th>
<th>Positive</th>
<th>Negative</th>
<th>% positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Generalized M.G. Active (55)</td>
<td>54</td>
<td>1</td>
<td>98.2</td>
</tr>
<tr>
<td>Generalized M.G. remission (21)</td>
<td>8</td>
<td>13</td>
<td>38</td>
</tr>
<tr>
<td>Ocular M.G. (37)</td>
<td>21</td>
<td>16</td>
<td>56.7</td>
</tr>
<tr>
<td>Other neuro-musc. disorders (38)</td>
<td>2*</td>
<td>36</td>
<td>5.2</td>
</tr>
<tr>
<td>Healthy controls (55)</td>
<td>0</td>
<td>55</td>
<td>0</td>
</tr>
</tbody>
</table>

* includes 1 case of botulism and 1 case of penicillamine treated rheumatoid arthritis.

nAchR Ab were not found in any of 55 healthy individuals and in only 2/38 (5.2%) of patients with other neuro-muscular disorders. We did find nAchR Ab in 54/55 (98.2%) of generalized active MG, in 8/21 (38%) of MG in remission and in 21/37 (56.7%) of ocular MG.

#### 3.2.2. Comparison of different antigens for detection of nAchR Ab:
We have compared the capacity of different muscle extracts to bind to serum nAchR Ab. We have thus defined a set of criteria by which to select muscle extracts that will be acceptable for use in the clinical assay. We have also evaluated fetal calf derived nAchR for its potential use in detection of nAchR Ab in human serum.
i) **Comparison of human muscle preparations for detection of nAchR Ab in serum:**

The reactivity of sera from MG patients and controls with different human nAchR preparations was studied. We tested four different muscle extracts for reactivity with a panel of 4 positive MG sera, one negative MG serum and 5 healthy controls (Table 3.5). The immune complexes formed between [125]I-aBT labelled nAchR and nAchR Ab were precipitated by SAC.

<table>
<thead>
<tr>
<th>Muscle Preparation:</th>
<th>20</th>
<th>1</th>
<th>12</th>
<th>16</th>
</tr>
</thead>
<tbody>
<tr>
<td>MG positive: 1</td>
<td>7175</td>
<td>3568</td>
<td>1846</td>
<td>1837</td>
</tr>
<tr>
<td>2</td>
<td>9792</td>
<td>2800</td>
<td>1739</td>
<td>1802</td>
</tr>
<tr>
<td>3</td>
<td>8503</td>
<td>3519</td>
<td>1518</td>
<td>1766</td>
</tr>
<tr>
<td>4</td>
<td>2599</td>
<td>3377</td>
<td>1604</td>
<td>2046</td>
</tr>
<tr>
<td>Mean± SD:</td>
<td>7017±3133</td>
<td>3316±353</td>
<td>1676±145</td>
<td>1862±126</td>
</tr>
</tbody>
</table>

| MG negative: 5      | 1807 | ND | 1374 | 1712 |
| Control:            |     |    |     |     |
| 1                   | 1803 | 1645 | 1449 | 1811 |
| 2                   | 1774 | 1495 | 1396 | 1771 |
| 3                   | 1745 | 1689 | 1392 | 1797 |
| 4                   | 1706 | 1669 | 1392 | 1797 |
| 5                   | 2071 | 1534 | 1443 | 1777 |
| Mean ± SD:          | 1819±144 | 1605±86 | 1419±26 | 1793±19 |

Reactivity of 5 MG sera and 5 Control sera with different human nAchR preparations (cpm precipitated)

Cpm precipitated by the positive MG sera differed considerably between the different muscle preparations. Maximum precipitable counts represents the maximum available nAchR in a given preparation. Cpm precipitated in the presence of control sera, also differed between preparations.

For diagnostic work, preparations were routinely screened using MG serum known to contain high and low levels of nAchR Ab and sera from a group of healthy controls. A muscle preparation was considered to be acceptable for clinical use if, on average, a panel of known positive MG sera
precipitated twice the cpm of the control sera and if maximum precipitable cpm was at least 3000 cpm/100 μl (eg., preparations #20 and #1 in Table 3.5).

ii) Comparison of human antigen with fetal calf antigen for the detection of nAchR Ab in serum:
Considerable variability was observed in the ability of human muscle derived antigen to be precipitated by serum nAchR Ab. For this reason and also because of technical difficulties associated with obtaining human limb muscle we decided to study fetal calf muscle, another mammalian source of antigen, in our assay system (Table 3.6).

A series of MG and control sera were assayed on muscle homogenates prepared both from human and from fetal calf (4 mos. gestation).

Table 3-6. Comparison of human antigen with fetal calf antigen for the detection of nAchR Ab in serum:

<table>
<thead>
<tr>
<th>Fetal Calf Antigen</th>
<th>Human Antigen</th>
<th>No. of Sera</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>+</td>
<td>15</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>45</td>
</tr>
<tr>
<td>-</td>
<td>+</td>
<td>6</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
<td>0</td>
</tr>
</tbody>
</table>

4 MG gen. active
1 ? Botulism
1 Ocular MG

Of 21 sera positive with human antigen, 6 were negative when studied with fetal calf antigen. Of 45 sera which were negative on human antigen none were found to be positive with fetal calf antigen.

Thus, while a reasonably good correlation was found between the ability of human and fetal calf muscle to detect nAchR Ab in serum we conclude that the sensitivity of the assay was reduced by the use of fetal calf muscle.
3.2.3. **Double Ab precipitation assay for quantitative determination of nAchR Ab:**

The relatively high nonspecific precipitation in the Protein A assay interfered with the quantitative determination of nAchR Ab since quantitation must be carried out on very dilute sera in order to retain a linear relationship between precipitation of immune complexes and concentration of antibody in serum tested. Thus, it was necessary to develop a further modification of the nAchR Ab assay with a more sensitive limit of detection so that nAchR Ab could be measured quantitatively and so that very dilute concentrations of nAchR Ab secreted into PBMNC cultures would be detectable.

**A) Optimization of double Ab precipitation assay:**

**i) Double Antibody Precipitation of immune complexes:**

We originally compared the ability of different anti-human Ig sera (second Ab) to precipitate a constant amount of [125]I-aBT labelled immune complexes. It can be seen (Table 3.7) that the various commercial anti-human IgG products differed widely in their ability to precipitate the IgG in human serum.

**Table 3.7. Double antibody precipitation of immune complexes:**

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1/2.5</td>
<td>513</td>
<td>238</td>
<td>168</td>
<td>378</td>
<td>117</td>
</tr>
<tr>
<td>1/5</td>
<td>487</td>
<td>246</td>
<td>175</td>
<td>475</td>
<td>139</td>
</tr>
<tr>
<td>1/10</td>
<td>429</td>
<td>273</td>
<td>254</td>
<td>478</td>
<td>152</td>
</tr>
<tr>
<td>1/50</td>
<td>131</td>
<td>382</td>
<td>379</td>
<td>192</td>
<td>127</td>
</tr>
<tr>
<td>1/250</td>
<td>166</td>
<td>437</td>
<td>113</td>
<td>124</td>
<td>116</td>
</tr>
<tr>
<td>1/500</td>
<td>120</td>
<td>125</td>
<td>124</td>
<td>112</td>
<td>101</td>
</tr>
<tr>
<td>1/2500</td>
<td>134</td>
<td>141</td>
<td>154</td>
<td>129</td>
<td>108</td>
</tr>
</tbody>
</table>

100 μl [125]I-aBT labelled nAchR were incubated with 10 μl 1/50 MG serum overnight at 4°C. The following day 100μl goat or rabbit-anti-human immunoglobulin antisera was added. Incubation proceeded overnight at 4°C. Immune complexes were collected by centrifugation and washed three times before counting. Numbers represent cpm.
Research Product goat-anti-human IgG was selected for routine use since it was the most cost efficient. It was determined that a ratio of 2.0 for final volume of second antibody (1/250 dilution in figure 3-7) to volume of serum was optimum for precipitation of immune complexes. This ratio was subsequently verified using a pool of normal human serum (2.0 µl) as carrier to establish equivalence with the second antibody in the presence of 0.2 µl MG serum. Carrier serum was used routinely in order to minimize possible variations that might have resulted from abnormalities in serum IgG concentration in MG. In later assays, a goat serum anti-human IgG prepared by our laboratory was found to react equally as well as Research Product goat anti-human IgG. Precipitation was maximal at a ratio of 2.3 for volume of second antibody to normal serum.

ii) Quantitation of nAchR in muscle extract:
For routine quantitation of nAchR Ab it was necessary to have an estimate of the nAchR concentration within the muscle extract that was being used. Table 3.8 illustrates the technique which was routinely used to estimate the concentration of nAchR in a muscle extract. Known high titer MG sera were used to precipitate all the available [125]I-aBT labelled nAchR in a muscle extract. The concentration of the nAchR in the muscle preparations was reported as moles [125]I-aBT binding sites/liter.
Table 3-8. Estimation of nAchR in muscle extract by maximal precipitation with MG sera:

<table>
<thead>
<tr>
<th>Experiment</th>
<th>concentration of nAchR in muscle extract (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.067</td>
</tr>
<tr>
<td>2</td>
<td>0.086</td>
</tr>
<tr>
<td>3</td>
<td>0.060</td>
</tr>
<tr>
<td>mean±SD:</td>
<td>0.071±0.013</td>
</tr>
</tbody>
</table>

Maximal precipitation of [125]I-aBT labelled nAchR from human muscle extracts in the presence of 10µl of known high titer MG serum.

Table 3.8 shows that the mean±SD of the concentration of nAchR in the muscle extract was 0.07±0.01n M. For routine work, the concentration of nAchR in the muscle extract was always approximated from the average of the maximal precipitation in at least three separate assays.

In order to confirm the validity of this technique for estimating the nAchR concentration in muscle preparations we used an alternate technique, the DE-81 ion exchange technique. Concentration of nAchR in the muscle extract was determined by binding increasing amounts of [125]I-aBT to muscle extracts and filtering immune complexes over DE-81 ion exchange paper. Nonspecific binding was determined in the presence of an excess of unlabelled aBT. The muscle homogenate studied (Table 3.9 and Figure 3.3) was the same one described in Table 3.8.
Table 3.9. Determination of concentration of nAchR in muscle extract by DE-81 ion exchange technique:

<table>
<thead>
<tr>
<th>concentration [125]I-aBT (nM)</th>
<th>net dpm</th>
</tr>
</thead>
<tbody>
<tr>
<td>32</td>
<td>3541*</td>
</tr>
<tr>
<td>8</td>
<td>2187</td>
</tr>
<tr>
<td>4</td>
<td>1380</td>
</tr>
<tr>
<td>2</td>
<td>789</td>
</tr>
</tbody>
</table>

*net dpm determined by subtracting dpm in the presence of excess, unlabelled aBT from total dpm

Figure 3.3. Scatchard plot for analysis of [125]I-aBT binding to nAchR in muscle extract:

The concentration of nAchR in this muscle extract was determined to be 0.10 nM (B max, figure 3-3) which was comparable with the value determined using MG serum precipitation (0.07nM).
3.2.4. Quantitative determination of nAchR Ab titer:

We then turned to assaying sera using the double Ab technique. The method for quantitative determination of nAchR Ab titer is illustrated in Figures 3.4A and B and 3.5. In figure 3.4A, increasing amounts of MG serum were incubated with [125]I-aBT labelled nAchR. Immune complexes were precipitated with goat-anti-human IgG in the presence of a constant amount of normal carrier serum. In Figure 3.4B serum had been diluted to remain in the "linear region" where antigen was in excess over serum nAchR Ab. In this region the nAchR Ab titer can be determined quantitatively and expressed as moles of [125]I-aBT binding sites precipitated per liter of serum.

Figure 3.4. Determination of nAchR Ab titer in MG serum:

A. Counts precipitated in the presence of increasing amounts of MG serum. Immune complexes were precipitated using goat-anti-human IgG. Each point represents the mean ± SD of triplicate determinations in the same assay.
Table 3.10. Quantitative determination of nAchR Ab titer in MG serum (corresponds to figure 3.4B):

<table>
<thead>
<tr>
<th>Dilution of MG serum</th>
<th>CPM±SD</th>
<th>Net CPM*</th>
<th>Titer (nM) **</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/100</td>
<td>439±11</td>
<td>370</td>
<td>12.9</td>
</tr>
<tr>
<td>1/200</td>
<td>279±6</td>
<td>210</td>
<td>14.7</td>
</tr>
<tr>
<td>1/400</td>
<td>182±11</td>
<td>113</td>
<td>15.8</td>
</tr>
<tr>
<td>1/800</td>
<td>129±10</td>
<td>60</td>
<td>16.8</td>
</tr>
<tr>
<td>1/1600</td>
<td>105±6</td>
<td>36</td>
<td>20.2</td>
</tr>
<tr>
<td>no serum</td>
<td>69±11</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table corresponds to figure 3.4B
*net cpm determined by subtracting nonspecific binding (in the absence of serum) from total cpm
**titer calculated according the formula: net dpm / 4.54 X 10^7 dpm/μCi + specific activity aBT (μci/μg) + 8 X 10^9 μg/mol aBT + volume of serum tested(μl) X 10^6 μl/liter of serum

nAchR Ab titer determined from slope in Figure 3.4B was 17.6 X 10^{-9} moles of [125]I-aBT binding sites/liter MG serum.

Another example of the quantitation of nAchR Ab in MG sera is seen in Figure 3.5. nAchR Ab was quantitated in MG sera of high or low titer. The normal serum control did not precipitate more labelled nAchR than was precipitated in the absence of serum.
Figure 3.5. Quantitative determination of nAchR Ab titer in MG serum:

Cpm ± SD precipitated in the presence of increasing amounts of various MG sera or a normal control serum.

Titers of nAchR Ab in MG serum were determined from the slopes of the lines in figure 3.5.

For MG-1 the titer was 72.0 nmol [125]I-aBT binding sites per liter of serum and for MG-2 the titer was 9.8 nmol [125]I-aBT binding sites per liter of serum.

Routine measurements of nAchR Ab were carried out below 30% maximum precipitable cpm to remain in the linear region as described in Material and Methods. If initial values were outside the linear region, sera were further diluted and re-assayed.
The nonspecific precipitation of [125]I-aBT was much lower using the double Ab technique (Table 3.10) than it was in the Protein A assay. Presumably this was due to the much smaller pellet size with less trapping of [125]I-aBT. The limit of detection in this assay was approximately \(8.5 \times 10^{-17}\) mole aBT binding sites on average, depending on the muscle preparation used. This is the amount which can be detected over background (determined in the absence of serum) at the 95% confidence level. The maximum amount of serum which could be assayed in this system without interfering with the zone of equivalence established between carrier serum and second antibody, was arbitrarily considered to be 0.4 µl. Thus, the limit of detection in terms of concentration of serum nAchR Ab was 0.08 - 0.34 nM (average of 0.24 nM). It is noteworthy that, due to restrictions on the amount of sera which could be assayed in the double Ab system, the Protein A precipitation assay was considerably more sensitive for detection of nAchR Ab in serum of MG patients (limit of detection was an average of 0.05 nM).

3.2.5. Reproducibility of nAchR Ab titers measured between assays:
In order to assess the reproducibility of nAchR Ab quantitation between assays we made determinations of serum nAchR Ab for individual test samples, over a range of titers, in multiple assays. nAchR Ab titers for several sera are reported in Table 3.11. For a given serum, all measurements of nAchR Ab titers were carried out using the same muscle homogenate so that results are directly comparable.
Table 3.11. Reproducibility of nAChR Ab titers measured between assays:

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Muscle Extract</th>
<th>Serum</th>
<th>nAChR Ab Titer (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>222</td>
<td>MG1</td>
<td>62.5</td>
</tr>
<tr>
<td>2</td>
<td>222</td>
<td>MG1</td>
<td>102.4</td>
</tr>
<tr>
<td>3</td>
<td>222</td>
<td>MG1</td>
<td>68.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>mean±SD: 77.8±21.5</td>
</tr>
<tr>
<td>4</td>
<td>7B</td>
<td>MG2</td>
<td>8.6</td>
</tr>
<tr>
<td>5</td>
<td>7B</td>
<td>MG2</td>
<td>10.9</td>
</tr>
<tr>
<td>6</td>
<td>7B</td>
<td>MG2</td>
<td>8.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>mean±SD: 9.3±1.4</td>
</tr>
<tr>
<td>7</td>
<td>22</td>
<td>MG3</td>
<td>688.1</td>
</tr>
<tr>
<td>8</td>
<td>22</td>
<td>MG3</td>
<td>778.8</td>
</tr>
<tr>
<td>9</td>
<td>22</td>
<td>MG3</td>
<td>797.5</td>
</tr>
<tr>
<td>10</td>
<td>22</td>
<td>MG3</td>
<td>611.9</td>
</tr>
<tr>
<td>11</td>
<td>22</td>
<td>MG3</td>
<td>989.8</td>
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<td>MG3</td>
<td>1294.1</td>
</tr>
<tr>
<td>13</td>
<td>22</td>
<td>MG3</td>
<td>944.9</td>
</tr>
<tr>
<td>14</td>
<td>22</td>
<td>MG3</td>
<td>mean±SD: 872.2±228.5</td>
</tr>
<tr>
<td>7</td>
<td>22</td>
<td>MG4</td>
<td>77.5</td>
</tr>
<tr>
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<td>22</td>
<td>MG4</td>
<td>83.6</td>
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<td>56.6</td>
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<td>86.9</td>
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<td>106.3</td>
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<td>13</td>
<td>22</td>
<td>MG4</td>
<td>67.8</td>
</tr>
<tr>
<td>14</td>
<td>22</td>
<td>MG4</td>
<td>mean±SD: 80.3±14.4</td>
</tr>
<tr>
<td>7</td>
<td>22</td>
<td>MG5</td>
<td>8.3</td>
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<td>MG5</td>
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<td>MG5</td>
<td>5.3</td>
</tr>
<tr>
<td>14</td>
<td>22</td>
<td>MG5</td>
<td>mean±SD: 6.8±1.4</td>
</tr>
</tbody>
</table>

MG sera were assayed quantitatively for nAChR Ab. Multiple determinations were made on each serum in individual assays using the same human muscle extract. Average variation for all sera was: 21.3%

The average nAChR Ab titer was 77.8±21.5 for MG1, 9.3±1.4 for MG2, 872.2±228.5 for MG3,
80.3±14.4 for MG4 and 6.8±1.4 for MG5. Thus, the average variation for measurement of nAchR Ab titer between assays was 21.3%.

3.2.6. Comparison of disease severity and titer of nAchR Ab in serum of MG patients:

Others have shown that quantitative measurement of levels of serum nAchR Ab in MG patients is not a useful predictor of disease severity. We have studied a group of MG patients to determine whether a relationship existed between serum nAchR Ab levels and category of severity. Serum nAchR Ab was measured quantitatively in 27 MG patients in different disease categories who had previously been determined to be seropositive using the Protein A precipitation assay. Patients were classified for disease severity according to the criteria of Osserman; this information was obtained from the patients' neurologist. All measurements of nAchR Ab were made using the same human muscle as antigen so that results would be comparable (Figure 3.6).
Titers of MG sera ranged from .85 - 578.0 nM aBT binding sites. It can be seen from figure 3.6 that there was considerable overlap of nAchR Ab titer between the groups.

**3.2.7. Polyclonal nature of nAchR Ab in MG serum:**

It is important to appreciate whether the autoimmune response in MG is polyclonal because this may have important implications for the pathogenesis of MG as well as for the laboratory determination of nAchR Ab levels. A group of seropositive MG sera were studied for patterns of cross reactivity exhibited in binding to [125]I-aBT labelled nAchR from different muscle extract (Table 3.12).
Table 3.12. Precipitation of [125]I-aBT labelled nAchR in the presence of MG and control sera using a panel of human and fetal calf muscle extracts:

**Human Muscle Preparations**

<table>
<thead>
<tr>
<th>Sera</th>
<th>Prep A</th>
<th>Prep B</th>
<th>Prep C</th>
<th>Prep D</th>
<th>Prep E</th>
<th>Fetal Calf A</th>
</tr>
</thead>
<tbody>
<tr>
<td>MG1</td>
<td>1398±45*</td>
<td>1478±6</td>
<td>4469±90</td>
<td>2814±77</td>
<td>2554±70</td>
<td>12750±14</td>
</tr>
<tr>
<td>MG2</td>
<td>1176±63</td>
<td>1314±51</td>
<td>3810±35</td>
<td>2118±46</td>
<td>2167±25</td>
<td>1610±15</td>
</tr>
<tr>
<td>MG3</td>
<td>1301±31</td>
<td>1519±11</td>
<td>4587±107</td>
<td>2527±87</td>
<td>2547±40</td>
<td>8495±265</td>
</tr>
<tr>
<td>MG4</td>
<td>1254±24</td>
<td>1499±53</td>
<td>4262±20</td>
<td>2329±55</td>
<td>2318±120</td>
<td>1784±61</td>
</tr>
<tr>
<td>MG5</td>
<td>1255±17</td>
<td>1401±83</td>
<td>2994±84</td>
<td>1784±31</td>
<td>1899±77</td>
<td>2771±87</td>
</tr>
<tr>
<td>N1</td>
<td>935±53</td>
<td>1132±23</td>
<td>1536±30</td>
<td>1827±79</td>
<td>1157±26</td>
<td>1791±11</td>
</tr>
<tr>
<td>N2</td>
<td>996±75</td>
<td>1136±12</td>
<td>1555±55</td>
<td>1784±37</td>
<td>1228±19</td>
<td>1753±98</td>
</tr>
<tr>
<td>N3</td>
<td>967±41</td>
<td>1171±48</td>
<td>1515±56</td>
<td>1722±55</td>
<td>1223±52</td>
<td>1804±27</td>
</tr>
<tr>
<td>N4</td>
<td>991±2</td>
<td>1538±380</td>
<td>1715±125</td>
<td>1717±119</td>
<td>1203±11</td>
<td>1855±111</td>
</tr>
<tr>
<td>Buffer</td>
<td>962±14</td>
<td>1246±66</td>
<td>1654±75</td>
<td>1085±114</td>
<td>1192±65</td>
<td>1700±57</td>
</tr>
</tbody>
</table>

* numbers represent cpm precipitated in the presence of 10μl of serum. Serum was incubated overnight at 4°C with [125]I-aBT labelled muscle extract. Immune complexes were precipitated with excess SAC, washed and counted.

The heterogeneous composition of nAchR Ab in MG serum is evident from Table 3.12 (as well as Table 3.5) where it was seen that patterns of precipitation of [125]I-aBT labelled nAchR varied among individuals. For example, comparing MG1 and MG2 in Table 3.12, we see that reactivity patterns differed. MG1 serum showed the highest precipitation of immune complexes with the Fetal Calf- A muscle preparation, followed by preparations C>D>E>B>A. MG2 reacted best with preparation C, followed by E>D>Fetal Calf- A>B>A. This indicated that serum nAchR Ab idiootypes occurred in variable proportions among MG patients tested.

The polyclonal nature of nAchR Ab in serum of MG patients can also be seen from
Figures 3.7a and b. Saturating amounts of MG sera were used to bind [125]I-aBT labelled nAchR in muscle extracts and immune complexes were precipitated by SAC.

Figure 3.7a. Precipitation of [125]I-aBT labelled nAchR by various MG sera:

![](image)

Net cpm precipitated in the presence of increasing amounts of MG serum for several MG patients. MG sera were incubated with 100μl [125]I-aBT labelled human muscle extract overnight at 4°C. Immune complexes were precipitated with an excess of SAC.
Maximal precipitation of [125I]-aBT labelled nAchR in a single muscle extract was lower with some MG sera than with others indicating heterogeneity of serum nAchR Ab. For example, in Figure 3.7a three of the four MG tested appeared to plateau at approximately the same level (at least 1700 cpm). One of the MG sera (represented by triangles in the figure), however, plateaued at a much lower level (approximately 800 cpm). Thus, there must be some degree of heterogeneity to the nAchR within the muscle preparation and further, some MG sera lack nAchR Ab which are capable of recognizing one of the variants (or fragments) of nAchR in the preparation.

3.4 Discussion:

3.4.1 Measurement of nAchR Ab in serum:

To achieve our aim of studying immunoregulation in MG it was first necessary to develop an assay with which to measure nAchR Ab. We have established two modifications of the nAchR Ab originally described by Lindstrom et al (1977). Our Protein A precipitation assay was more sensitive than the double Ab assay for detection of low levels of nAchR Ab in serum. This assay
was validated, in a retrospective study of MG sera, for its potential use as a diagnostic test for MG and was subsequently made available for this purpose. This assay was used to screen for seropositive MG patients to be included in further studies of in vitro nAchR Ab production. A double antibody precipitation assay was used to quantitate levels of nAchR Ab in serum and in lymphocyte culture supernates.

3.4.2. Protein A precipitation assay for detection of nAchR Ab in serum:

A sensitive, diagnostic assay for MG, modified from the procedures of Lindstrom et al (1977) and Tindall (1981c), has been developed in our laboratory. We have taken advantage of the ability of the Protein A reagent to bind to the Fc portion of immunoglobulin in order to precipitate immune complexes in our assay. IgG molecules are the predominant class of nAchR Ab in MG (Lefvert et al 1977). The protein A of the Staphylococcus aureus Cowan I strain cell wall binds to the Fc portion of human IgG subclasses 1, 2 and 4 as well as to IgM but does not bind IgG 3 (Brunda et al 1977, Langone et al 1978, Duhamel et al 1979). Tindall et al (1981c), studying a panel of sera from known seropositive MG patients, found a 100% correlation between the ability of Protein A and goat-anti-human IgG to precipitate immune complexes. It was evident from their study, as well as from the present study, in which 98.2% of patients with generalized, active MG were positive for nAchR Ab, that a patient having nAchR Ab which are exclusively IgG3 exists only rarely if at all.

We have chosen to test 10μl of serum in the Protein A assay system because this improved the sensitivity of the assay for diagnostic purposes. nAchR Ab in sera of very low titer (down to 0.03 nmol per liter) were detectable with the Protein A precipitation assay. We have modified the nAchR Ab assay so that efficient testing of very concentrated sera could be carried out. It therefore was not possible to determine levels of nAchR Ab without an impractical scaling up of reagents. Others carrying out clinical studies have geared their nAchR Ab assays to report titers quantitatively and in so doing have carried out testing on much more dilute sera (Lindstrom et al 1976c, Lefvert et al 1978, Tindall et al 1981b,c). At one time, such studies were useful because it
was necessary to analyze the relationship between nAchR Ab titer and disease severity. These studies clearly showed that a reliable relationship does not exist, thus quantitation of serum nAchR is not useful when diagnosing MG.

To validate our assay, a retrospective study was carried out on sera from 101 MG patients. These patients had been diagnosed, prior to nAchR Ab testing, as having MG by neurologists in the Vancouver area. The diagnoses of these patients had been based on clinical findings and, in some cases, results of tensilon testing and/or electromyography. 93% of 43 patients with active, generalized MG were positive in the Protein A precipitation assay. Two patients were borderline and one was negative. The two patients who were borderline were systematically reassayed with no change. These patients had disease of short duration (< 9 mos) and were reassayed 3 months later. At that time, one patient had become positive and the other remained borderline. The patient whose serum was negative remained negative on repeated testing. The diagnosis of MG had been based on the clinical findings which included muscle weakness affecting bulbar and limb muscles as well as ptosis. Tensilon test was positive. Thymectomy was performed; however, no pathologic features were observed. 51% of 37 patients with ocular MG were also positive in our assay as were 24% of patients in clinical remission. We did not find nAchR Ab in any of 55 sera from healthy controls. The results of our retrospective study were similar to those reported by others (Lefvert et al 1978, Tindall et al 1981b, Vincent et al 1985b) and have recently been published (Oger et al 1986).

3.4.3. Frequency of occurrence of circulating nAchR Ab in MG of different disease categories:

Following validation of our Protein A precipitation assay we made it available for diagnostic purposes. We then studied a group of patients in whom MG was suspected. 13/14 of these patients who were positive for nAchR Ab received a final diagnosis of MG. Diagnoses in these patients was arrived at by considering nAchR Ab results along with clinical findings, tensilon testing and/or emg. Diagnosis of MG was confirmed by response to appropriate treatments and clinical follow up.
When we summarized the data from our retrospective study with data generated through use of the assay for clinical purposes, we found nAchR Ab in 54/55 (98.2%) of generalized active MG, in 8/21 (38%) of MG in remission and in 21/37 (56.7%) of ocular MG. nAchR Ab were not found in any of 55 healthy individuals and in only 2/38 (5.2%) of patients with other neuro-muscular disorders. These two patients included one with botulism and one who had rheumatoid arthritis treated with penicillamine. Treatment with penicillamine is known to induce MG in a small percentage of individuals who take this medication.

Although there is only a loose correlation between serum titer of nAchR Ab and severity of MG, a correlation does exist between percentage positivity and increasing category of severity (Lefvert et al 1978, Tindall et al 1981b, Vincent et al 1985b). Generalized, active MG is the most highly positive group. In the present study we have found 98.2% positive in this category. This is somewhat higher than in similar studies carried out by others (Lefvert et al 1978, Tindall et al 1981b, Vincent et al 1985b, Garlepp et al 1981, Nicholson et al 1982) and may reflect the improvements we have made to the technique. In our study, among patients with ocular MG, the percent positivity for serum nAchR Ab was 56.7%. This is also similar to other reports although positivity in the ocular category is increased when antigen is prepared from ocular muscle instead of leg muscle (Oda et al 1987). In our study 38% of patients who had generalized disease in remission were positive for circulating nAchR Ab. Again, this is similar to the reports of others. The fact that circulating nAchR Ab can still be detected during clinical remission may reflect the differing pathogenic capacities of nAchR Ab subtypes. Alternately, it may be an indication that there are other, as yet undefined, factors which contribute to the defect in neuromuscular transmission and/or have a protective influence on synaptic function. This latter possibility is supported by the studies of Howard et al (1987) who showed that circulating nAchR Ab from MG patients in remission can actually modulate nAchR in human fetal muscle cultures. Additionally, passive transfer to mice of serum containing nAchR Ab from patients in remission causes a reduction in nAchR content of muscle (Pirskanen et al 1987).

We have not found nAchR Ab in any of 55 sera from healthy individuals and in only 2 of

Circulating nAchR Ab have been reported to exist in the absence of clinical features of MG under certain circumstances. For instance, they do rarely occur in healthy relatives of MG patients (Pirskanen et al 1981, Lefvert et al 1984, Lefvert et al 1985). Lefvert et al (1987a) also reported that 40% of 51 asymptomatic patients who had undergone autologous or allogeneic bone marrow grafting had measurable nAchR Ab in their serum. This procedure is known to result in development of autoimmune conditions, including MG, in some patients. Transient neonatal MG is a well recognized entity and is accompanied by serum nAchR Ab when it exists. Circulating nAchR Ab, however, are often transiently found in healthy infants born to myasthenic mothers (Morel et al 1988). Finally, serum nAchR Ab also occur in animals immunized with nAchR even in the absence of clinical signs of EAMG (Fuchs et al 1976, Ueno et al 1982, Scadding et al 1986).

3.4.4. Comparison of alternate antigen sources for nAchR Ab assay:

We have studied the cross reactivity of several different human muscle preparations with a panel of MG sera. Indeed, there was considerable variability between preparations. This may have been due to differing densities of endplate nAchR or to post mortem degradation of tissue prior to processing. Variability could also have been due to muscle pathology among donors (some muscle was obtained from patients with peripheral nerve disease). Patients with peripheral nerve disease develop extrajunctional nAchR and it has been reported that higher titers for circulating nAchR Ab were measured when muscle preparations rich in extrajunctional receptors are used as antigen (Weinberg et al 1979). Since MG sera are polyclonal for nAchR Ab, individual sera may have differing capacities to bind to the nAchR in a given muscle preparation.

In an attempt to replace human muscle with a more accessible and reliable source of antigen for measurement of nAchR Ab, we compared muscle extracts prepared from fetal calf muscle or human muscle. In general, there was a good correlation between results obtained using human or
fetal calf antigen. However, we did find 6 seropositive patients who were negative with fetal calf antigen: 4 were generalized, active MG, 1 was ocular MG and 1 was thought to have botulism. We concluded that, while the correlation was good, this significant reduction in sensitivity precluded the use of fetal calf antigen on a routine basis. Fetal calf antigen was also studied by Gotti et al (1984) who reported similar results, although their false negative rate (6%) with fetal calf antigen was slightly less than ours (Kaufman and Oger 1986).

More recently, the use of the rhabdomyosarcoma cell line, TE671, as a standard source of nAchR has been proposed (Lindstrom et al 1987). An excellent correlation between nAchR Ab titers measured against human leg muscle and TE671 derived nAchR was observed by these investigators.

3.4.5 Quantitative assay for nAchR Ab:

We have used a double Ab assay to measure levels of nAchR Ab quantitatively. A very efficient precipitation of immune complexes was accomplished using goat-anti-human IgG and the background in the double Ab assay was approximately 5-10 times less, on average, compared with the Protein A assay. Thus, the limit of detection was improved from 0.5 fmol to 0.09 fmol aBT binding sites on the average. On average, sera with titers as low as 0.24 nMol per liter serum could be measured quantitatively. nAchR Ab titers observed among our group of MG patients were in the nanomolar range (.85 - 578.0 nM). This is similar to reports by others (Lindstrom et al 1976c, Tindall et al 1981b,c, Vincent et al 1985b, Garlepp et al 1982).

MG sera were tested in the presence of an excess of standard, normal human carrier serum to minimize error that might have arisen due to variability in concentration of IgG in MG sera (Lisak et al 1976). Thus, since the double Ab assay could only handle relatively small amounts of MG serum it was much less suitable for use as a diagnostic assay compared with the Protein A precipitation assay.

The quantitative assay was important for our study of in vitro nAchR Ab secretion (see Chapter 4). Because of the extremely low concentration of IgG in culture supernatants it was
possible to measure nAchR Ab in a large volume of culture supernatant (500 µl). Thus, the concentration of nAchR Ab that could be measured in culture supernatants was, on average, 0.0002 nmol/liter.

3.4.6 Heterogeneity of serum nAchR Ab:

Our present study has confirmed that the humoral response to the nAchR is indeed polyclonal. At saturation, some MG sera did not precipitate as much [125]I-aBT labelled nAchR as did others which indicated dissimilarity between serum nAchR Ab among MG patients. In addition, variable patterns of cross reactivity were observed among MG serum when the precipitation of nAchR in different muscle preparations was studied.

Patterns of cross reactivity of nAchR Ab with antigen prepared from different species have also been studied by others (Lindstrom et al 1978, Garlepp et al 1981). Individual MG sera exhibit unique binding patterns suggesting that nAchR Ab exist as heterogeneous populations. Antidiotypic nAchR Ab inhibition of nAchR Ab binding to human receptor also varies considerably among patients (Lang et al 1985, Whiting et al 1986). More recently, a variety of human monoclonal Ab (mcAb) with differing affinities for the receptor has been developed (Blair et al 1986, Lefvert 1987b).

A region of the nAchR has been identified which apparently is very immunogenic (Tzartos et al 1980, 1981, 1983). This is a multideterminant, extracellular region of the nAchR on the alpha subunit, not encompassing the acetylcholine binding site. It appears to have been highly conserved through evolution (Tzartos et al 1981, Tzartos et al 1983). In most MG patients, the majority of nAchR Ab measurable by conventional immunoassay have specificity for this region, which is termed the main immunogenic region (MIR) (Tzartos et al 1982). Among rats immunized with human nAchR, the Ab to MIR are also the predominant species of nAchR Ab (Tzartos et al 1982). Interestingly, however, MIR Ab titer was not correlated with disease severity in 86 MG patients studied by this group (Tzartos et al 1982).
3.4.7 Relationship between nAchR Ab titer and severity of disease:

We have compared nAchR Ab titer with disease severity in a small group of MG patients. As anticipated, titers fell over a wide range and overlapped between groups. Other investigators have carried out studies of this nature on much larger groups of patients. Mean titers are often observed to correlate with increasing category of severity; however, the overlap is characteristically very pronounced which probably reflects the polyclonal nature of the immune response against nAchR Ab.

Polyclonality can influence the apparent relationship between nAchR Ab titer and disease duration in several ways. It is possible that nAchR Ab which, in vivo, are directed against physiologically relevant epitopes on the receptor may not be detected by conventional immunoassay which uses a denatured (detergent solubilized) nAchR as antigen. Oda et al (1986) reported that a better correlation between nAchR Ab titer and severity of MG existed when only those nAchR Ab directed against extracellular determinants of the receptor were measured using cultured rat myotubes as antigen in the assay. Interestingly, nAchR Ab specific for intracellular determinants of the receptor were also recognised in MG patients. Production of antibodies recognizing intracellular determinants in vivo may occur following the shedding of receptor rich membrane debris into the synapse (Sahashi et al 1980). nAchR Ab specific for epitopes that are not available in an intact endplate are not likely to be involved in any of the major pathogenic mechanisms. While the occurrence of such antibodies might reflect a more aggressive immune response, it could also be argued that the titer of a pathologically irrelevant antibody would not be expected to correlate with disease activity.

It has also been suggested that nAchR among humans may be polymorphic (Garlepp et al 1981). If this was the case, then it would be expected that some nAchR Ab which were pathogenic to one individual may not be pathogenic to, or even recognize, nAchR from another. Such factors could influence apparent titer of nAchR Ab measured in conventional immunoassay.

Immunoassays for nAchR Ab employ iodinated aBT, a nicotinic antagonist, to trace the nAchR. Such a practice precludes measurement of nAchR Ab which might be directed at or near
the aBT binding site. Such nAchR Ab that block aBT binding have been reported to be pathogenic in rat muscle cultures (Drachman et al. 1982). However, they are considered to represent only a small proportion of total nAchR Ab present in most patients (Morel et al. 1988).

As discussed in detail in Chapter 1, there exists a considerable body of evidence that different nAchR Ab have different pathogenic capacity and this may, of course, also influence the apparent relationship between nAchR Ab titer and severity of MG.

3.5 Summary:

Before proceeding with studies on the regulation of nAchR Ab production in MG, it was necessary to identify a group of seropositive MG patients on whom further study could be carried out. Thus, we developed a rapid, sensitive modification of Lindstrom's immunoassay for nAchR Ab and validated it for diagnostic purposes. As nAchR Ab testing was not routinely available at the time, we made this assay available in Canada for diagnostic purposes. nAchR Ab were detected in serum of 98.2% of patients with generalized, active MG. The presence of nAchR Ab was highly specific for MG; we did not detect nAchR Ab in sera of any healthy controls and only in 2/38 patients having other neuromuscular disorder (one was treated with penicillamine, known to induce MG in some persons).

A quantitative version of the nAchR Ab assay has also been established in our laboratory. We have used this assay to study nAchR Ab titers in serum and have confirmed that a clear relationship between titer and severity of MG does not exist. The quantitative assay was particularly geared towards measurement of very low levels of nAchR Ab that would be secreted in studies of lymphocyte cultures described in Chapter 4.
Chapter 4. In vitro production of nAchR Ab and polyclonal IgG in Myasthenia Gravis

Data reported in Neurology 38, 818-821 1988

4.1 INTRODUCTION 94
4.1.1 Use of pokeweed mitogen to study immunoregulatory systems 94
4.1.2 Immunoregulation in MG 97

4.2 RESULTS 99
4.2.1 In vitro production of nAchR Ab in MG 99
4.2.1.1 Rate of nAchR Ab secretion by lymphocytes from MG patients 99

4.2.2 Study of Ab production by PBMNC of MG patients and controls 101
4.2.2.1 nAchR Ab secreted in vitro by PBMNC 101
4.2.2.2 Correlation between levels of nAchR Ab secreted in vitro by PBMNC and levels of nAchR Ab in serum of MG patients 104
4.2.2.3 Polyclonal IgG secretion among Secretors and Nonsecretors and in controls 105
4.2.2.4 Disease variables among Secretors and Nonsecretors 107
4.2.2.5 Relationship between disease duration and capacity for polyclonal IgG secretion in vitro 108
4.2.2.6 Relationship between disease duration and levels of nAchR Ab in sera of MG patients 108
4.2.2.7 Role of B and T-helper cells in IgG production by Secretors and Nonsecretors 108

4.2.3 Ab production by thymocytes 110

4.2.4 Assessment of the effects of MG sera on the function of normal lymphocytes 112
4.2.4.1 Effect of pretreatment of normal PBMNC with MG serum in the Con A suppressor assay 112
4.2.4.2 Effect of pretreatment of normal CD4+ lymphocytes with MG serum in IgG secretion assay 113
   i) Helper assay 113
   ii) Suppressor-inducer assay 114

4.3 DISCUSSION 118
4.3.1 In vitro secretion of Ab by PBMNC in myasthenia gravis 118
4.3.2 T-lymphocyte regulation of B cell mediated immune function in MG 121
4.3.3 In vitro secretion of nAchR Ab by thymocytes of MG patients 122
4.3.4 Influence of MG serum on immune function 123

4.4 SUMMARY 126
4.1 Introduction:

4.1.1 Use of Pokeweed mitogen to study immunoregulatory systems:

Pokeweed mitogen (PWM) is a plant lectin from Phytolacca americana root. It is a polyclonal mitogen for T and B lymphocytes and has been used extensively as an in vitro model system in which to study Ig production. The PWM induced activation of B cells is dependent on T cells (Reinherz et al 1979b, Thomas et al 1980, Miedema et al 1985, Suzuki et al 1986) and monocyte/macrophages (reviewed in Waldmann and Broder 1982). In this regard, the in vitro model of Ig production resembles the physiologic response to T-dependent antigens. The mechanisms of action of PWM have been partially worked out and are discussed below.

PWM induced B cell activation is dependent upon the CD4+ subset of T-lymphocytes (Reinherz et al 1979b, Miedema et al 1985, Suzuki et al 1986). CD4+ T-helper/inducer lymphocytes proliferate (Puck and Rich 1984) and secrete lymphokines, including IL2, in response to PWM (Miedema et al 1985, Nakagawa et al 1987). That IL2 is critical for lymphocyte activation in PWM stimulated systems is suggested by studies showing that addition of mcAb directed against IL2 receptors to PWM stimulated cultures drastically inhibits Ig production (Miedema et al 1985, Ceuppens and Stevens 1986). Exogenous IL2 reverses the inhibition of PWM-induced B cell activation caused by early addition of cyclosporin A to mononuclear cell cultures. In addition to its role in stimulating T cells to produce lymphokines, IL2 also acts directly to activate B cells (Ceuppens and Stevens 1986, Nakagawa et al 1985, Zubler et al 1984, Nakagawa et al 1987). Addition of IL2 to slg+ cells (B cells), enriched from mononuclear cell cultures pre-activated with PWM, resulted in their differentiation to Ig secreting cells (Nakagawa et al 1987). However, early activation of B cells is dependent on the presence of T cells and PWM in culture. IL2 can not effectively replace T cells or PWM, suggesting that the role of PWM in in vitro B cell activation involves more than the simple induction of CD4+ cells to secrete IL2 (Nakagawa et al 1987).
Although it is well known that PWM induced B cell activation is genetically unrestricted (reviewed in Waldmann et al 1982), the physical presence of T cells in PWM stimulated cultures appears to be a requirement for the early activation of B cells (Suzuki et al 1986 Nakagawa et al 1987). Supernatants of PWM stimulated mononuclear cells, which contain IL2 and other lymphokines, can not replace the requirement for T cells in inducing differentiation of B cells into Ig secreting cells. In ultrastructural studies, T cell blasts physically associated with macrophages after 24 hours of culture in the presence of PWM-latex conjugates (Suzuki et al 1986). In fact, these investigators proposed that cognate interaction between antigen presenting cells and CD4+ cells is a requirement for B cell activation in PWM stimulated cultures. In their study, requirement for T cells to be present was limited to the first 6 hours of culture. B cells subsequently proliferated and differentiated into Ig secreting cells (IgSC) over the next 5 days in the presence of activated T cell supernatants. B cell responses were specifically inhibited by addition of mAb to Ia or CD4+ during the preliminary culture of B cells and monocytes with T cells (Suzuki et al 1986). An alternate explanation for these results could be that CD4+ cells secrete a very short lived substance early in the culture period which is required for B cell activation. Blockade of Ia or CD4 with mAb might impede binding of PWM to these sites.

Other lymphokines influence the immune response to PWM. Interferon (IFN) beta, but not IFN alpha, acts directly on B cells to enhance PWM stimulated Ig secretion (Wasserman et al 1985). IFN gamma does not have a positive influence on PWM stimulated Ig production in mononuclear cells cultures (Nakagawa et al 1987). IFN gamma probably enhances suppressor function in this system and has been shown to enhance 7 day PWM induced blastogenesis (Aoki et al 1985), known to involve mostly CD8+ lymphocytes (Puck and Rich 1984).

There have been few investigations into the exact nature of the B cell which responds to PWM. IgM, IgG or IgA are secreted in roughly equal amounts in PWM stimulated cultures. It has been estimated that only 2% of all circulating B cells are reactive to PWM (Stevens et al 1981). This cell type is surface IgD-negative and may be positive for surface IgG, IgA or IgM (Kuritani and Cooper 1982). There does not appear to be any Ig isotype class switch in PWM
stimulated plasma cells (Stevens et al 1981, Levitt and Dagg 1981). Thus, it has been hypothesized that the PWM-reactive B cells may be memory cells generated through prior in vivo immune response (Stevens et al 1981). PWM-reactive B cells are also larger and of lower density than other circulating B cells (Kuritani and Cooper 1982, Dagg and Levitt 1981). Because it is known that activated B cells increase in size, Kuritani and Cooper (1982) suggested that PWM-reactive B cells have been pre-activated in vivo.

CD8+ cells also become activated in response to PWM (Miedema et al 1985, Fox et al 1986, Morimoto et al 1985b, Takeuchi et al 1986). CD4+ cells are required for proliferation of CD8+ cells in PWM stimulated cultures (Puck and Rich 1984). CD4+ lymphocytes have been shown to secrete a growth factor for CD8+ cells (Fox et al 1986). This protein factor induces IL2 receptors on CD8+ lymphocytes and, in the presence of IL2, the CD8+ cells proliferate extensively (Fox et al 1986). CD8+ cells from PWM stimulated mononuclear cell cultures behave as suppressor cells for CD4+ cell proliferation (Puck and Rich 1984) and for B cell Ig secretion (Miedema et al 1985, Thomas et al 1980, Morimoto et al 1985b, Takeuchi et al 1986). Suppression by PWM stimulated CD8+ cells may, to some extent, be related to their ability to deplete available IL2 in culture (Puck and Rich 1984).

The PWM induced Ig secretion model has been used to analyze the relationship between disease activity and immune response in a variety of immunological disorders. Multiple sclerosis (MS), for example, has been well studied in this manner. MS is a demyelinating, CNS disorder in which there is an overactive humoral immune response in vivo. In relapsing remitting MS, the tendency for clinical relapse has been found to be correlated with heightened Ig secretion response to PWM (Oger 1989). In MS, as in normals, a high Ig secretion response to PWM is related to a low functional capacity of CD8+, T-suppressor cells (Antel et al 1984, O'Gorman and Oger 1988).
4.1.2. Immunoregulation in MG:

nAchR Ab have a directly pathogenic influence on neuromuscular transmission. Therefore, it is of primary importance to understand the mechanisms by which auto antibody production is controlled. It remains to be determined whether the immune system functions abnormally prior to development of MG and/or whether immunoregulatory function becomes dysregulated following establishment of disease.

The overall aim of the present study was to define more clearly the nature of immune function defects at the cellular level and to examine the possible relationship between immune functional status and clinical stage.

At the time these studies were begun it was not clear whether nAchR Ab production by lymphocytes was differentially regulated relative to overall humoral immunity. A short study by Lisak et al (1984) had suggested that this may be the case; levels of nAchR Ab secreted in vitro did not correlate with levels of polyclonal immunoglobulin. Other studies had demonstrated that abnormalities of humoral immune function in MG were not limited to the production of nAchR Ab (Kelly et al 1981, Limberg et al 1985, Levinson et al 1981, Lisak et al 1986b) suggesting that immune dysfunction in MG may, in fact, be polyclonal to some degree. Thus, in the present study, we compared the antigen specific humoral response with the polyclonal response in MG. It was toward this end that we had developed a very sensitive, quantitative assay with which to measure nAchR Ab secreted into culture by PBMNC.

In MG, assessment of the contribution of immunoregulatory defects to the disease process has been complicated by the heterogeneity of the disease. Such heterogeneity is evident from epidemiological studies. Also, many studies of immune function reveal heterogeneity among MG patients (Richman et al 1976, Zilco et al 1979, Mischak and Dau et al 1981, Rauch et al 1985). Results of immune function tests are frequently abnormal, yet there is often considerable overlap
with control groups.

We have chosen to study a clinically well defined patient group. All of the patients we studied had generalized MG and, to avoid ambiguity in diagnosis, were seropositive for nAchR Ab. We analyzed the immune response data in relation to disease variables and discovered that two subgroups of seropositive MG patients were identifiable based on the capacity of their PBMNC to secrete Ab (antigen specific and polyclonal) in vitro. Comparison of clinical variables between these patients suggested that B cell mediated immune function had become altered as MG progressed over time.

A number of studies by others suggest that an impairment of T-suppressor function exists in some MG patients. The situation is far from clear, however. Interpretation of the studies designed to assess T-suppressor function has been limited by the gross level of inquiry. In particular, the molecular and cellular bases for suppression in the assay systems which have been used by others are not well understood. Since unseparated lymphocytes are used in these systems, it is inappropriate to assume that an apparent reduction in suppressor function is due to impaired suppression rather than to enhanced "helper" function. Moreover, recent work by Morimoto and others (see Chapter 1) has brought an understanding that CD4+ helper/inducer lymphocytes are actually composed of cells which "help" to generate a humoral immune response and cells which help to induce suppressor function by CD8+ lymphocytes.

We designed studies to explore the regulation of Ab production, at the level of the lymphocyte subset, in the two groups of MG patients which we had defined. We have also hypothesized that anti-lymphocyte Ab may be involved in dysregulation of the immune response at certain stages of MG. Indeed, preliminary experiments, in the well defined PWM stimulated IgG secretion assay, suggested that serum from MG patients impaired the function of normal CD4+ T-lymphocytes.
4.2. Results:

4.2.1. In vitro production of nAChR Ab in MG:
Because nAChR Ab are directly pathogenic at the muscle endplate in MG it is important to study the regulation of their production in vitro. We have studied in vitro nAChR Ab and polyclonal IgG production by peripheral blood mononuclear cells from MG patients and controls.

4.2.1.1. Rate of nAChR Ab secretion by lymphocytes from MG patients:
Figure 4.1 represents a typical experiment in which the production of nAChR Ab by PBMNC was studied over time in culture. PBMNC (10^6/ml) were cultured in triplicate in the presence or absence of PWM. Culture supernates were assayed for nAChR Ab in the double Ab assay.

Figure 4.1. Kinetics of nAChR Ab secretion by PBMNC of MG patients:

nAChR Ab (fmol aBT binding sites per ml) secreted in vitro by PBMNC from MG patient.
Rate of production of nAchR Ab was increased in the presence of PWM. Optimal time in culture for detection of nAchR Ab was chosen to be 10 days.

Figure 4.2 illustrates the kinetics of in vitro nAchR Ab and IgG production by thymic lymphocytes from an MG patient.

Figure 4-2. Kinetics of secretion of nAchR Ab and polyclonal IgG in culture of thymocytes from MG patient:

nAchR Ab Titer (fmol/ml) vs. Days in culture

nAchR Ab (fmol aBT binding sites/ml) secreted by thymic lymphocytes from an MG patient (patient HL). Thymocytes were cultured at 10^6/ml in the presence or absence of PWM.
Comparison of nAchR Ab and IgG secretion reveals that PWM markedly increased the production of IgG in thymocyte cultures but the effect on production of nAchR Ab was much smaller. The ratio of nAchR Ab to IgG in PWM stimulated cultures at 14 days was $7.4 \times 10^{-4}$ fmol aBT/ng IgG. However, in unstimulated cultures the ratio was $1.9 \times 10^{-2}$ fmol aBT/ng IgG. We interpret this as evidence that nAchR Ab specific B cells from thymus were already activated in vivo.

4.2.2. Study of Ab production by PBMNC from MG and control:
We have studied the in vitro production of nAchR Ab and polyclonal IgG among MG patients in order to gain insight into possible regulatory mechanisms of the production of auto-Ab in MG.

4.2.2.1. nAchR Ab secreted in vitro by PBMNC:
Thirty six patients with seropositive (titers ranged from 0.5 - 578.0 nM), generalized MG were studied for the ability of their PBMNC to secrete nAchR Ab in 10 day PWM stimulated
cultures (Table 4.1 and 4.2).

Table 4.1. In vitro secretion of nAchR Ab in MG patients and controls:

<table>
<thead>
<tr>
<th></th>
<th>MG Patients</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>AchR Ab: secretor</td>
<td>21</td>
<td>0</td>
</tr>
<tr>
<td>AchR Ab: nonsecretor</td>
<td>15</td>
<td>20</td>
</tr>
</tbody>
</table>

Secretion of nAchR Ab by cultures of PBMNC among MG patients and controls. Cultures were maintained in an atmosphere of 5% CO2 at 37 °C for 10 days in the presence of 1/300 PWM. Culture supernatants were assayed for nAchR Ab by quantitative immunoassay.
Table 4.2. Clinical characteristics, serum nAchR Ab levels, and in vitro levels of nAchR Ab and IgG secreted by PBMNC in MG:

<table>
<thead>
<tr>
<th>Patient</th>
<th>sex</th>
<th>age(yr)</th>
<th>Disease duration (mos)</th>
<th>IgG secretion (ng/ml)</th>
<th>nAchR Ab secretion (fmol/ml)</th>
<th>serum nAchR Ab (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
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<td>3</td>
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<td>3</td>
<td>224</td>
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</tr>
<tr>
<td>22</td>
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<td>75</td>
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<tr>
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<td>1255</td>
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<td>115</td>
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<td>M</td>
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<td>139</td>
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</tr>
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<td>M</td>
<td>62</td>
<td>360</td>
<td>ND</td>
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<td>F</td>
<td>62</td>
<td>624</td>
<td>720</td>
<td>344</td>
<td>13.1</td>
</tr>
</tbody>
</table>

*0 indicates that level of nAchR was undetectable in culture
ND = no data
M- = cultures not stimulated with mitogen
PBMNC from 21 patients (58.3%) exhibited in vitro secretion of nAchR Ab (0.2 - 27.0 fmol/ml/10 days) in the presence of PWM (Secretors). PBMNC from 15 MG patients did not secrete detectable levels of nAchR Ab in culture (Nonsecretors). Fourteen of 33 patients tested secreted detectable levels of nAchR Ab in non-stimulated cultures (0.2 - 23.7 fmol/ml/10 days). None of the 20 controls tested were positive for secretion of nAchR Ab in vitro; nor did sera of control individuals contain nAchR Ab. There was no correlation between levels of nAchR Ab secreted in the presence and absence of PWM among MG patients (r= 0.45).

4.2.2.2. Correlation between levels of nAchR Ab secreted in vitro by PBMNC and levels of nAchR in serum of MG patients:

We have compared the levels of nAchR Ab secreted into PBMNC culture media with the levels found in the serum of the same patient (Figure 4.3).

Figure 4.3. Correlation between levels of nAchR Ab secreted in cultures of PBMNC and in serum of MG patients:
Among MG patients who were positive for in vitro secretion of nAchR Ab, levels of nAchR Ab secreted in culture by PBMNC (0.2 - 27.1 fmol/ml/ 10 days) were positively correlated with the levels found in serum (0.5 - 578.0) from the same patient (r= 0.82, p< 0.0001, n=16). This observation confirmed the validity of our measurements of nAchR Ab in culture media.

We have demonstrated that nAchR Ab are produced by thymocytes and by PBMNC. It is probable that nAchR Ab are also produced in spleen and lymph node. The fact that nAchR Ab levels in serum are correlated with capacity for nAchR Ab secretion by PBMNC would suggest that the activity of the recirculating lymphocyte population reflects regulatory events occurring at the level of the lymphoid organ in MG.

4.2.2.3. Polyclonal IgG secretion among Secretors and Nonsecretors and controls:

To determine whether production of nAchR Ab by PBMNC has escaped regulatory control, we compared levels of polyclonal IgG and nAchR Ab secreted into the same culture supernatants (Figure 4.4).
Figure 4.4. Levels of polyclonal IgG secreted in culture by Secretors and Nonsecretors and by controls:

Levels of IgG (mean ± sem ng/ml) secreted in-vitro by PBMNC of MG patients and controls. MG patients were classified according to the ability of their PBMNC to secrete detectable levels of nAchR Ab (Secretor or Nonsecretor). Cultures were maintained in an atmosphere of 5% CO2 at 37 °C for 10 days in the presence of 1/300 PWM to stimulate IgG secretion. IgG was determined by ELISA.

There was no difference between PWM stimulated IgG production among MG (2455.0 ± 544 ng/ml) and control PBMNC (1799.0 ± 392.1 ng/ml). However, as shown in Figure 4.4, levels of PWM induced IgG secretion differed between MG patients who were in vitro secretors and those who were nonsecretors of nAchR Ab. The mean IgG among MG patients who were positive for in vitro secretion of nAchR Ab (Secretors) was 3840.3 ± 793.9 ng/ml (mean±sem). Mean IgG secreted by PBMNC from Nonsecretors was 516.5 ± 195.2 ng/ml and was 1799.0 ± 392.1 ng/ml in the controls. IgG secretion was higher in the Secretor group than in the Nonsecretor group (p< 0.0001 ). Secretors and Nonsecretors also differed from the control group (p< 0.04 and p< 0.001 respectively) (data analysed by one way ANOVA ).
The actual levels of IgG and nAchR Ab within individual culture supernatants were not correlated (r = 0.08).

4.2.2.4. Disease Variables among Secretors and Nonsecretors:

Among myasthenic patients, in vitro secretors and nonsecretors of nAchR Ab did not differ with respect to age, age of onset, sex or treatment, including thymectomy. However, we found that 16 of 21 Secretors had a short disease duration (≤ 5 years) while only 6 of 15 Nonsecretors had a short disease duration (p< 0.05, Chi square) (Table 4.3).

Table 4.3. Correlation between disease duration and capacity of PBMNC from MG patients to secrete nAchR Ab in vitro:

<table>
<thead>
<tr>
<th>Duration</th>
<th>≤5 years</th>
<th>&gt;5 years</th>
</tr>
</thead>
<tbody>
<tr>
<td>AchR Ab:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Secretor</td>
<td>16*</td>
<td>5</td>
</tr>
<tr>
<td>AchR Ab:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nonsecretor</td>
<td>6</td>
<td>9</td>
</tr>
</tbody>
</table>

p< 0.05, * represents number of individuals belonging to each class

The correlation between disease duration and capacity for Ab production by PBMNC suggests that immune function may have become altered with progression of disease over time.
4.2.2.5. Relationship between disease duration and capacity for polyclonal IgG secretion in vitro:

Disease duration was also correlated with capacity for in vitro secretion of polyclonal IgG by PBMNC. Among MG patients who were high responders (>1000 ng/ml) for in vitro PWM stimulated IgG production, the mean disease duration was 52.5 ± 10.9 months and among MG patients who were low responders the mean disease duration was 144.9 ± 42.3 months (students t-test, p< 0.05).

4.2.2.6. Relationship between disease duration and levels of nAchR Ab in sera of MG patients:

Disease duration was correlated with levels of nAchR Ab in patients' sera (p< 0.01). The mean disease duration (months) among MG patients who had high (>25 nM) levels of nAchR Ab in their sera was 26.7 ± 5.8 but was 114.1 ± 25.3 among patients with low serum levels of nAchR Ab.

4.2.2.7. Role of B and T-helper cells in IgG production by Secretors and Nonsecretors of nAchR Ab:

For Ab production to occur in the PWM driven system which we have studied, cooperation at the level of the B cell, T-helper cell and monocyte/macrophage is necessary. Thus, in our system, regulation of Ab production may have been operative at any one or all of these levels. We studied lymphocyte subsets from three MG patients in order to determine whether B cell mediated Ab production was under control by T regulatory cells.

Preliminary results were generated from two experiments in which lymphocyte separation and recombination was carried out between Secretors (MG patients whose PBMNC secreted nAchR Ab), Nonsecretors (MG patients whose PBMNC did not secrete nAchR Ab) and healthy controls (Table 4.4).
Table 4.4. Role of B and T-helper cells in IgG production among Secretors and Nonsecretors of nAchR Ab:

<table>
<thead>
<tr>
<th>Experiment #1:</th>
<th>B cells (E-)</th>
<th>T-helper (CD4+)</th>
<th>IgG (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>nonsec-1</td>
<td>nonsec-1</td>
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<td></td>
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<td>sec</td>
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<td></td>
</tr>
<tr>
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<td>sec</td>
<td>3240</td>
<td></td>
</tr>
<tr>
<td>sec</td>
<td>nonsec-1</td>
<td>50</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Experiment #2:</th>
<th>B cells (E-)</th>
<th>T-helper (CD4+)</th>
<th>IgG (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>nonsec-2</td>
<td>nonsec-2</td>
<td>750</td>
<td></td>
</tr>
<tr>
<td>control</td>
<td>control</td>
<td>3682</td>
<td></td>
</tr>
<tr>
<td>nonsec-2</td>
<td>control</td>
<td>1330</td>
<td></td>
</tr>
<tr>
<td>control</td>
<td>nonsec-2</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>

Various combinations of B cells (5 X 10^4) and CD4+ enriched T-cells (5 X 10^4) from MG patients who were Secretors (sec) or Nonsecretors (nonsec) or control individuals were cultured for 10 days in the presence of PWM. Supernatants were assayed for IgG.

As expected, the combination of B cells (adherent cell depleted, E- population) and T-helper cells (CD4+ enriched cells) from Nonsecretors produced little IgG in both experiments (70 ng/ml and 750 ng/ml). In experiment #1, the combination of Secretor B cells with autologous T-helper cells produced high levels of IgG in culture (1845 ng/ml). Mixed cultures containing B cells from a
Nonsecretor and T-helper cells from the Secretor also produced high levels of IgG (3240 ng/ml). However, the combination of B cells from the Secretor with T-helper cells from the Nonsecretor produced very low levels of IgG (50 ng/ml). Similarly, in experiment #2 the combination of healthy, high responder B cells with autologous T-helper cells produced high levels of IgG (3682 ng/ml). The combination of Nonsecretor B cells (a different patient from experiment #1) with healthy, high responder T-helper cells produced high levels of IgG (1330 ng/ml). However, cultures of healthy B cells with Nonsecretor T-helper cells produced only low levels of IgG (100 ng/ml).

These data suggest that in MG, the mechanism of T cell dependency of IgG secretion by B cells is intact.

4.2.3. Antibody production by thymocytes:
The thymus has been implicated in the pathogenesis of MG. In order to study Ab production by thymocytes we obtained thymic tissue from patients who underwent thymectomy. Thymocytes from 6 MG patients and one patient with other muscle disease (OMD) were cultured for 10 days in the presence or absence of PWM (Table 4.5). Three of the 4 thymocyte cultures which were tested for secretion of nAchR Ab were positive. Thymocyte cultures were also tested for secretion of polyclonal IgG.
Table 4.5. IgG production by thymocytes:

<table>
<thead>
<tr>
<th>Type of MG (PBMNC Secretor (S) or Nonsecretor (NS) of nAchR Ab in-vitro):</th>
<th>Thymocytes:</th>
<th>IgG (ng/ml/10 day):</th>
<th>nAchR Ab (fmol/ml/10 day):</th>
</tr>
</thead>
<tbody>
<tr>
<td>PWM</td>
<td>unstimulated</td>
<td>PWM</td>
<td>unstimulated</td>
</tr>
<tr>
<td>S</td>
<td>2010</td>
<td>189</td>
<td>1.2</td>
</tr>
<tr>
<td>S</td>
<td>2615</td>
<td>19</td>
<td>12.2=</td>
</tr>
<tr>
<td>S</td>
<td>7299</td>
<td>ND</td>
<td>7.3</td>
</tr>
<tr>
<td>S</td>
<td>2281</td>
<td>149</td>
<td>ND</td>
</tr>
<tr>
<td>ND</td>
<td>3658</td>
<td>441</td>
<td>ND</td>
</tr>
<tr>
<td>NS</td>
<td>137</td>
<td>22</td>
<td>0**</td>
</tr>
<tr>
<td>control (OMD*):</td>
<td>3977</td>
<td>45</td>
<td>0</td>
</tr>
</tbody>
</table>

* other muscle disease, ** undetectable, = nAchR Ab measured in 10.0 ml culture
ND= not done

Secretion of IgG and nAchR Ab in 10 day cultures of human thymocytes. Thymocytes were cultured at 10⁶/ml for 10 days in the presence or absence of PWM. Identification of MG patients as Secretors or Nonsecretors of nAchR Ab by PBMNC were made in a separate experiment.

6/7 thymocyte cultures secreted high levels of polyclonal IgG. Cultured thymocytes from one MG patient secreted only very low levels of IgG. Interestingly, this patient had also been determined to be Nonsecretor in earlier studies with PBMNC. In PBMNC studies, four of the other 5 MG patients were Secretors. PBMNC from one MG patient had not been studied. All thymocyte cultures secreted much higher levels of IgG in response to PWM compared with unstimulated cultures. Thymocytes cultured from the Nonsecretor secreted low levels of IgG and also did not produce detectable levels of nAchR Ab. Thymocytes cultured from the OMD patient did not produce nAchR Ab.
4.2.4. Assessment of the effects of MG sera on the functioning of normal lymphocytes:

4.2.4.1. Effect of pretreatment of normal PBMNC with MG serum in the Con A suppressor assay:

Following our observation that immune function in MG appeared to change with time over the course of disease we considered the possibility that auto-anti-lymphocete Ab may arise in MG and may be responsible for some or all of the abnormalities in immune function which have been reported. We thus designed several experiments to examine the effects of MG sera on the functioning of normal lymphocytes. The con A induced suppressor function assay was used in an early study for this purpose. Suppression was calculated by comparing proliferation in cultures containing untreated responder cells (R) and mitomycin C treated suppressor (S) cells (incubated 3 days with con A to induce suppressor function) with proliferation in cultures of responder cells plus mitomycin C treated control (C) cells (incubated 3 days in the absence of con A).

Table 4.6. Effect of serum pre-treatment on con-A induced suppressor function of normal T cells:

<table>
<thead>
<tr>
<th>SERUM</th>
<th>FCS</th>
<th>N1</th>
<th>N2</th>
<th>N3</th>
<th>N4</th>
<th>MG1</th>
<th>MG2</th>
<th>MG3</th>
<th>MG4</th>
<th>MG5</th>
</tr>
</thead>
<tbody>
<tr>
<td>S+R:</td>
<td>40299</td>
<td>31775</td>
<td>36832</td>
<td>42388</td>
<td>50990</td>
<td>19448</td>
<td>72873</td>
<td>40623</td>
<td>33323</td>
<td>76190</td>
</tr>
<tr>
<td>C+R:</td>
<td>74568</td>
<td>56473</td>
<td>73614</td>
<td>49880</td>
<td>41192</td>
<td>29810</td>
<td>49776</td>
<td>26063</td>
<td>65687</td>
<td>45051</td>
</tr>
<tr>
<td>%SUP:</td>
<td>46</td>
<td>43</td>
<td>50</td>
<td>15</td>
<td>-23</td>
<td>34</td>
<td>-46</td>
<td>-56</td>
<td>49</td>
<td>-69</td>
</tr>
</tbody>
</table>

N: 21.3+16.6 (mean±sem)

MG: -17.6+24.5 (mean±sem)

Lymphocytes from healthy individuals were pretreated for 3 days with or without con A in the presence of sera from MG patients, normals or fetal calf serum. Cells were mitomycin C treated, washed and re-cultured with fresh heterologous responder cells from a healthy individual in a mitogen stimulated proliferation assay (³H-thymidine uptake) to assess the role of serum during the induction phase of suppressor function. S+R refers to the combination of responder (R) cells with con A pretreated PBMNC (S, suppressor cells). C + R refers to the combination of responder cells with PBMNC that had not been previously exposed to con A (C, control cells). Numbers represent cpm. FCS=fetal calf serum, N=normal serum, MG=myasthenia gravis serum.
Pretreatment of normal PBMNC with serum from some MG patients appeared to impair the Con A stimulated induction of suppressor function (Table 4.6). The percent suppression among cultures which contained cells that had been pretreated with normal serum was 21.3 ± 16.6% (mean ± SEM). Suppressor function in some cultures which contained cells that had been pretreated with MG sera appeared to be impaired. Pretreatment with 3 of the 5 MG sera tested resulted in values of suppression that were lower than the normal controls (95% confidence limit).

4.2.3.2. Effect of pretreatment of normal CD4+ lymphocytes with MG serum in IgG secretion assay:

Analysis of the con A induced suppression assay was rendered complex because of the use of unseparated PBMNC. To determine whether the observed effects of MG serum in impairing suppressor function were mediated through the CD4+ or the CD8+ subpopulation of T lymphocytes, T cells were pretreated with MG serum prior to measuring the function of enriched populations of CD4+ or CD8+ lymphocytes in 2 assay systems: one designed to measure help and the other to study induction of suppression of PWM stimulated IgG production.

i) Helper assay:

The results of two helper assays are shown in table 4.7. In these experiments serum-pretreated, CD4+ enriched lymphocytes were added to autologous B cells and IgG was measured in supernatant fluids of 10 day PWM stimulated cultures. Increasing the numbers of CD4+ cells in cultures resulted in increased production of IgG. This is consistent with theoretical expectations and thus serves as an internal control for assessing the functional purity of lymphocyte subpopulations. Control cultures were also set up to monitor the efficiency of lymphocyte subset enrichment procedures. Cultures containing only E- cells (B enriched) did not secrete IgG. Cultures containing only CD4+ enriched (CD8+ depleted) lymphocytes also did not secrete significant amount of IgG (119 ng/ml).
Table 4.7. Effect of serum pretreatment on the ability of CD4+ lymphocytes to support mitogen stimulated IgG production:

<table>
<thead>
<tr>
<th>Serum pretreatment of CD4+ cells</th>
<th>IgG secretion (ng/ml):</th>
</tr>
</thead>
<tbody>
<tr>
<td>per well</td>
<td>Secretor</td>
</tr>
<tr>
<td>Experiment #1:</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>55±0.1</td>
</tr>
<tr>
<td>5940</td>
<td>323±14</td>
</tr>
<tr>
<td>13500</td>
<td>446±4</td>
</tr>
<tr>
<td>27000</td>
<td>982±71</td>
</tr>
<tr>
<td>40500</td>
<td>471±35</td>
</tr>
<tr>
<td>Experiment #2:</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>142±1</td>
</tr>
<tr>
<td>5940</td>
<td>932±70</td>
</tr>
<tr>
<td>13500</td>
<td>1088±101</td>
</tr>
<tr>
<td>27000</td>
<td>808±124</td>
</tr>
<tr>
<td>40500</td>
<td>1924±113</td>
</tr>
<tr>
<td>54000</td>
<td>2418±65</td>
</tr>
<tr>
<td>81000</td>
<td>1155±29</td>
</tr>
<tr>
<td>94500</td>
<td>1081±55</td>
</tr>
</tbody>
</table>

Levels of IgG secreted by 54000 B (E-) cells in the presence of autologous, serum pretreated CD4+ T cells. Sera used for pretreatment of CD4+ lymphocytes was obtained from MG patients who were either Secretors or Nonsecretors, patients with other neurological disease (OND) or fetal calf serum (FCS). Different sera were used in each of the two experiments. Cultures were maintained for 10 days in the presence of PWM. In experiment #2 serum pretreated CD4+ enriched T cells were incubated alone as controls for IgG production. Levels of IgG secreted in these cultures were 119, 119 and 118 for Secretor, Nonsecretor and OND respectively. Each point was determined on supernatant pooled from triplicate microwell cultures and assayed in triplicate by ELISA (mean ± SD).

There was no difference in the ability of CD4+ lymphocytes to support IgG production irrespective of pretreatment with MG sera, sera from other neurological disease controls or fetal calf serum.

ii) Suppressor-inducer assay:

The effect of pretreatment of CD4+ enriched T lymphocytes with serum from MG patients was studied in suppressor assays which were run in parallel with the helper assays (Experiments #1 and #2). It is known that a subpopulation of CD4+ lymphocytes is required for induction of CD8+
mediated suppression in PWM stimulated systems. Thus, we have cultured B cells and CD4+ cells which had been treated with various sera in the presence of increasing numbers of CD8+ enriched lymphocytes in order to study the function of this CD4+ cell type (Table 4.8).

The addition of CD8+ enriched T-cells to cultures containing B and CD4+ enriched T-cells resulted in a dose dependent inhibition of IgG production in experiment #2. In Experiment #1 this dose dependency was somewhat less clear. This was probably due to the fact that the level of IgG production in this experiment was not very high and so had been potently suppressed by addition of even very small amounts of CD8+ enriched T-cells.

Table 4.8. Influence of serum-pretreatment on the ability of CD4+ cells to stimulate CD8+ mediated suppression of IgG production:

<table>
<thead>
<tr>
<th>CD8+ cells per well:</th>
<th>Secretor</th>
<th>Serum pretreatment of CD4+ cells:</th>
<th>OND</th>
<th>FCS</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Experiment #1:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>810</td>
<td>574±33</td>
<td>ND</td>
<td>ND</td>
<td>305±16</td>
</tr>
<tr>
<td>1620</td>
<td>439±18</td>
<td>ND</td>
<td>ND</td>
<td>325±9</td>
</tr>
<tr>
<td>3240</td>
<td>662±55</td>
<td>ND</td>
<td>ND</td>
<td>370±12</td>
</tr>
<tr>
<td>6750</td>
<td>687±45</td>
<td>ND</td>
<td>ND</td>
<td>205±11</td>
</tr>
<tr>
<td>13500</td>
<td>417±20</td>
<td>ND</td>
<td>ND</td>
<td>402±17</td>
</tr>
<tr>
<td>27000</td>
<td>375±82</td>
<td>ND</td>
<td>ND</td>
<td>403±10</td>
</tr>
<tr>
<td><strong>Experiment #2:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>2115±337</td>
<td>2020±211</td>
<td>1799±362</td>
<td>ND</td>
</tr>
<tr>
<td>189</td>
<td>954±97</td>
<td>856±99</td>
<td>854±44</td>
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</tr>
<tr>
<td>378</td>
<td>1397±26</td>
<td>1184±96</td>
<td>1243±135</td>
<td>ND</td>
</tr>
<tr>
<td>810</td>
<td>1598±287</td>
<td>705±70</td>
<td>1019±131</td>
<td>ND</td>
</tr>
<tr>
<td>1620</td>
<td>986±122</td>
<td>774±36</td>
<td>755±107</td>
<td>ND</td>
</tr>
<tr>
<td>3240</td>
<td>1007±43</td>
<td>781±4</td>
<td>543±17</td>
<td>ND</td>
</tr>
<tr>
<td>6750</td>
<td>446±21</td>
<td>326±10</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>13500</td>
<td>381±15</td>
<td>285±6</td>
<td>278±23</td>
<td>ND</td>
</tr>
<tr>
<td>27000</td>
<td>274±20</td>
<td>292±7</td>
<td>171±14</td>
<td>ND</td>
</tr>
</tbody>
</table>

Levels of IgG (mean±SD) secreted by a constant number of autologous B (54,000) and serum pretreated CD4+ T lymphocytes (54,000) in the presence of increasing numbers of untreated CD8+ T lymphocytes. Sera used for pretreatment of CD4+ lymphocytes was obtained from MG patients who were either Secretors or Nonsecretors, patients with other neurological disease (OND) or fetal calf serum (FCS). Cultures were maintained for 10 days in the presence of PWM. Each point was determined on supernatant pooled from triplicate microwell cultures and assayed in triplicate by ELISA (mean ± SD).
Cultures containing CD4+ enriched T-cells which had been pretreated with serum from Secretors showed less inhibition of IgG secretion in the presence of CD8+ enriched T-cells in both experiments.

Figures 4.5 and 4.6 display the normalized (% maximum), pooled data of experiments #1 and #2.

Figure 4.5. Influence of serum-pretreatment on the ability of CD4+ T-helper cells to support IgG production:

Production of IgG in cultures of normal B cells and autologous CD4+ T-cells that had been pretreated with heat inactivated sera from MG patients who were Secretors or with other sera (FCS,OND, Nonsecretors). Data represents the mean ± sem from two separate experiments (Experiment #1 and #2). Cultures contained B cells (E-cells) and graded numbers of serum pretreated CD4+ cells. Cultures were maintained for 10 days in the presence of PWM and supernatants measured for IgG by ELISA. The x-axis represents amount of CD4+ cells on a ranked scale.
Figure 4.6. Influence of serum-pretreatment on the ability of CD4+ cells to stimulate CD8+-mediated suppression of IgG production:

Suppression of PWM induced IgG secretion by CD8+ lymphocytes in the presence of CD4+ lymphocytes which had been pretreated with sera from MG patients who were Secretors or other sera (FCS, OND, Nonsecretor). Data represents mean ± sem from 2 separate experiments (Experiment #1 and #2). Graded numbers of heterologous CD8+ T lymphocytes were added to cultures which contained a constant ratio of B and autologous CD4+ lymphocytes. Cultures were maintained for 10 days in the presence of PWM and supernatants were measured for IgG by ELISA. The x-axis represents amount of CD8+ cells on a ranked scale.

Overall, we observed that treatment of T-cells with Secretor sera did not influence the capacity of CD4+ cells to support IgG production by B cells. However, these same CD4+ cells exhibited an impaired ability to support CD8+-mediated suppression of IgG production. These results suggest that functioning of the CD4+ T-suppressor-inducer cell may be impaired by a heat-resistant serum factor from Secretors.
4.3. Discussion:

4.3.1. In vitro secretion of Ab by PBMNC in myasthenia gravis:

We have studied in vitro production of nAchR Ab and IgG by PBMNC from MG and controls in order to gain insight into possible mechanisms by which auto Ab production is regulated. Our Ab secretion data support the view that non-specific defects of immunity do indeed occur in MG and may be related to disease progression (Kaufman and Oger 1987, 1988).

The levels of nAchR Ab secreted in culture by PBMNC that were detected in the present study (picomolar range/10^6 cells/10 day culture) were very similar to levels reported in small studies by others (Vincent et al 1978, McLachlan et al 1981) and have subsequently been confirmed (Fujii et al 1986, Lisak et al 1986a, Safar et al 1987). We have been able to measure nAchR Ab secreted in vitro without first concentrating culture media as others have had to do (Lisak et al 1983, McLachlan et al 1981).

We have found that secretion of nAchR Ab in vitro occurred in 21/36 PWM stimulated myasthenic PBMNC cultures tested (58.3%). None of the 20 control cultures that were tested secreted nAchR Ab. The frequency with which nAchR Ab was detected in myasthenic PBMNC cultures was higher than that reported earlier by Vincent et al (1978a) for a small group of patients and somewhat less than that recently reported for larger groups by Lisak et al (1987) (26+/34) and Safar et al (1987) (29+/37).

Among patients who were positive for in vitro secretion of nAchR Ab, it was observed that a positive correlation existed between the levels of nAchR Ab secreted in culture and the levels of nAchR Ab found in serum from the same patient. This had also been reported by Lisak's group (1983, 1984) and, more recently, by others (Fujii et al 1986, Safar et al 1987).

In our study, polyclonal IgG was measured in parallel with determinations of nAchR Ab secreted into culture media. Although there was no difference in the levels of IgG secreted by controls and by MG patients as a group, it was discovered that PBMNC cultures which were positive for nAchR Ab secretion (Secretors) also secreted high levels of polyclonal IgG.
secretion in this group was higher than in the control group. Levels of IgG secretion in cultures from Nonsecretors of nAchR Ab were very low compared with either Secretors or with the control group, suggesting that nAchR Ab production may be regulated in a nonspecific manner.

Others have studied mitogen stimulated polyclonal IgG secretion by PBMNC in MG, however the findings have not been consistent. In vitro production of IgG in PWM stimulated cultures either does not differ from (Kelley et al 1981, Lisak et al 1983, Limburg et al 1985) or is increased over (Harfast et al 1981) control PBMNC. In studies of Ig forming cells there was no difference between MG and control (Levinson et al 1981).

In the present study, the secretion by PBMNC of detectable levels of nAchR Ab was correlated with high levels of IgG production. The actual levels of IgG and nAchR Ab within the individual cultures were not correlated, however. Lisak et al (1984), studying a smaller group of patients, had earlier reported that levels of IgG and nAchR Ab secreted in PBMNC cultures were not correlated. This has more recently been confirmed on 11 patients by Lefvert et al (1986). It is not surprising that levels of IgG and nAchR Ab secreted by PBMNC would appear not to be correlated when one considers the following. The range of IgG responses among the healthy population is known to be quite broad (Rosenkoetter et al 1984, O'Gorman et al 1988). Ideally, one would need to measure the change in IgG secretion before onset and at different time points after onset of MG to determine whether a correlation existed between changes in level of IgG secretion and levels of nAchR Ab secreted in culture. Another point to consider is that there are certain limitations regarding the quantitation of nAchR Ab. The observed lack of correlation between levels of IgG and levels of nAchR Ab secreted in vitro by PBMNC maybe due to artifacts inherent in the determination of nAchR Ab by the immunoprecipitation technique.

We have studied the clinical variables of MG patients to determine whether any correlations existed with the capacity of the PBMNC to secrete nAchR Ab and IgG in culture. No differences were found with respect to age, sex, age of onset or treatment, including treatment with steroids as well as treatment by thymectomy. Notably, however, there was a correlation between disease duration and secretion of Ab in vitro. Among MG patients, those with short disease
duration secreted higher levels of Ab (nAchR Ab and IgG) in vitro. Moreover, disease duration was correlated with the levels of nAchR Ab present in patients' sera; patients with MG of short duration had higher levels of nAchR Ab in their serum compared with patients having a long history of MG. Vincent et al (1985a) have also reported that serum titers of nAchR Ab were significantly higher in MG patients with short disease duration.

Subsequent studies by others comparing PBMNC secretion of nAchR Ab with clinical variables have turned up correlations with disease severity (Lefvert et al 1987c) and with patient age (higher secretion of nAchR Ab by patients under 40 yrs) (Safar et al 1987). Studying cultures of thymic lymphocytes from MG patients, Scadding et al (1981) reported that disease duration of greater than one year was correlated with a higher degree of in vitro secretion of nAchR Ab.

Data from the present study suggested not only the occurrence of non-specific defects in B cell mediated immune function in MG, but that such defects might be related to disease progression over time. We have seen that MG patients with a short disease duration who secrete nAchR Ab in-vitro have unusually high levels of IgG production in response to PWM. This raises the question of whether or not MG may occur preferentially among individuals who are high producers of immunoglobulin. There is some suggestion from the literature that a genetic predisposition to development of MG does indeed exist. Certain HLA types occur more frequently among MG patients than among the general population. These HLA types are also associated with abnormalities of immune function (see Chapter 1). An alternate speculation concerning the abnormally high IgG production in this group of MG patients is that there may have been an early increase in B cell mediated immune function following onset of disease. Of the six patients who had short disease duration and low levels of polyclonal IgG production 3 had a disease duration of less than 12 months and one was subsequently shown to be a consistently high responder for IgG and thus probably represented a spuriously low point. This trend suggested that occurrence of MG was not related to phenotype for IgG secretion, but rather that immune function became dysregulated shortly after onset of MG. A similar trend has been observed by Newsom-Davis's group; it was found that, among several MG patients in whom serum nAchR Ab were not
detectable, the disease duration was only a few months (Vincent et al 1985a). We have made similar observations concerning serum levels of nAchR Ab on a small number of patients. Lindstrom et al (1976b), studying a larger group of patients, found that serum nAchR Ab levels were lower in patients with a very short duration, increased in patients with duration between 1 and 5 years and lower again in patients with a duration of more than 5 years. Those results did not reach statistical significance however.

The relationship of disease duration to immune function within the group of MG patients strongly suggested that B cell mediated immune function had become altered during the course of the disease. Therefore, it appears that some immune function changes were secondary to the development of MG and were related to aspects of disease progression. We have speculated that immune function may become altered secondary to circulating auto antibodies which recognize lymphocytic determinants. It is also conceivable that chronic overuse of the immune system may influence immune function over time. Theoretically, in a mitogen stimulated culture system, changes in regulation of B cell mediated immune function could be operative through diminished or enhanced activity at the level of the B cell itself, the CD4+ T-helper/inducer subset and/or the CD8+ T-suppressor cells. Monocyte/macrophage immune function could also be involved. Others have proposed that PWM stimulated IgG production may be controlled at multiple levels (Antel et al 1983). As described below, preliminary experiments addressing this question in MG were carried out in our laboratory.

3.4.2 T lymphocyte regulation of B cell mediated immune function in MG:

Preliminary data were generated through separation/recombination experiments in a PWM stimulated IgG secretion assay. Results suggested that regulation of Ab production was operative at the level of the CD4+ T-helper cell among MG patients. This would seem to be consistent with the situation regarding regulation of in vitro IgG production in the normal population. As mentioned, there are two populations among normals, high and low responders,
based on ability of PBMNC to secrete IgG in vitro. Our group has shown that, among CD4+ T cells, the ratio of T-helper inducer to T-suppressor inducer (CD4+ CDw29+ / CD4+ CD45R+) was correlated with levels of IgG production among normal, high and low responders (O'Gorman and Oger 1988). However, among normal, high and low responders, IgG production has also been shown to be regulated by CD8+ mediated T-suppressor function (Rosenkoetter et al 1984).

Future studies into the regulation of Ab production in MG should be aimed at assessing both T-helper and T-suppressor function. Studies of the type described in this chapter, which are carried out using enriched subpopulations of lymphocytes to assess functional status, have a distinct advantage over the majority of studies which have been carried out in this area in that the ambiguity of measuring net effects from unseparated populations of T cells has been minimized. The very recently available mcAbs for CD4+ T-suppressor inducer and T-helper inducer cells should allow for even more detailed analysis in the future.

3.4.3 In vitro secretion of nAchR Ab by myasthenic thymocytes:

We have generated data on the secretion of IgG and nAchR Ab by thymic lymphocytes. Thymocytes from 6 MG patients and one patient with other neuromuscular disorder secreted detectable levels of IgG in vitro. PWM stimulated levels of IgG were always much higher than levels in unstimulated cultures. This pattern of secretion of IgG by normal or myasthenic thymocytes has also been reported by others (Willcox et al 1983, Fujii et al 1986). In fact, lymphocytes from human thymus have been shown to be very susceptible to mitogen activation, more so than blood lymphocytes even though the percentage of B cells in thymus is much lower (Lisak et al 1987).

We have studied a small sample of MG patients and found the nAchR Ab secretion response to be consistent between cultures of thymocytes and cultures of PBMNC. Correlations between ability of PBMNC and thymocytes to secrete nAchR Ab have been reported by others (Lisak et al 1987). Also, in our study, 4/5 MG patients whose thymocytes secreted high levels of
IgG in PWM stimulated cultures (2010-7299 ng/ml) were Secretors (of nAchR Ab) when their PBMNC were studied (one was not studied therefore Secretor status was unknown). PWM stimulated thymocyte cultures from 1/6 MG patients studied produced very low levels of IgG. This patient was also identified as a Nonsecretor (of nAchR Ab) in studies of PBMNC. These data suggested that, in MG, thymic B cells may be regulated in a manner similar to that of peripheral B cells.

We have only had the opportunity to compare PWM stimulated and unstimulated nAchR Ab production by thymocytes for one MG patient. We observed substantial production of nAchR Ab even in the absence of PWM. Others have reported that PWM stimulation of thymocyte cultures results in inhibition of nAchR Ab production (Newsom-Davis et al 1981). A variable nAchR Ab response to PWM was observed by others (Lisak et al 1987, Fujii et al 1981).

3.4.4 Influence of MG serum on immune function:

Several assay systems were designed to test our hypothesis that serum factors in MG interfere with normal immunoregulatory function. Initially, the con A proliferation assay was adapted for this purpose. A panel of sera from healthy controls and MG patients was tested for possible influence on con-A induced suppressor function. The results suggested that, indeed, some MG sera had an inhibitory influence on suppressor function in this assay. While these results were interesting, the design of the con-A suppressor assay is limiting in terms of information that can be gained. It was not possible to discern whether serum was acting at the induction or the effector level of suppression. Therefore, we designed cell subset separation/recombination experiments which could provide more detailed information about this process. At the same time we expanded the study to examine the possibility that serum factors might differ with the clinical stage of MG.

In a second series of experiments, we generated preliminary evidence that pretreatment of healthy T lymphocytes with sera from Secretors (Kaufman and Oger 1989b) impaired the ability of CD4+ enriched T cells to support suppression of IgG secretion by autologous B cells in the
presence of heterologous CD8+ enriched T cells. The most likely target, among CD4+ enriched lymphocytes, for the pathogenic serum factor would be the recently described T-suppressor-inducer lymphocyte.

Functional studies have identified the CD4+, T suppressor-inducer cell (Tsi) as being required for induction of suppressor function in the con A stimulated suppressor assay (Morimoto et al 1986b), PWM induced Ig production assay (Morimoto et al 1985b, Takeuchi et al 1986) and in antigen specific stimulation assays (Morimoto et al 1986a). Phenotypically, this cell type has been identified as bearing the CD4+ surface antigen as well as the CD45R antigen.

Recent studies have shown that impaired suppressor inducer function and/or reduced percentage of peripheral CD4+ CD45R+ lymphocytes occur in autoimmune disorders such as SLE and juvenile rheumatoid arthritis (Morimoto et al 1987b, Emery et al 1987). Morimoto et al (1987b) recognized that the degree of decreased expression and function of the CD4+ CD45+ T-suppressor-inducer population is correlated with the severity of SLE. Percentages of circulating CD4+ CD45R+ lymphocytes are also reduced in some multiple sclerosis patients (Morimoto 1987a, O'Gorman and Oger 1989b) and CD45R antigen is reduced in MS lesions (Sobel et al 1988). So far, reports of decreased CD45R+ cells have not appeared for MG. However, the very recent report of an increase in cells bearing the T-helper-inducer (Thi) phenotype (CD4+ CDw29+) suggests that more extensive study of subgroups of MG patients might reveal such a defect; other studies have shown that activation of the CD45R+ subset causes transformation into the Thi or memory cell type (Byrne et al 1988, Clement et al 1988).

At the present time we cannot exclude the possibility that the serum factor has influenced the function of a CD4+ subset other than the Tsi. Studies have shown that CD4+ lymphocytes, while not essential (Woodcock et al 1986, Sprent et al 1988), are able to regulate the development of CD8+ mediated cytotoxicity (Sprent et al 1988, Roopenian et al 1988). In the system we have studied CD8+ cells were heterologous to CD4+ cells and B cells, thus, it is possible that a portion of the observed suppression of IgG production may have been due to cytotoxicity by CD8+ lymphocytes. Since treatment of CD4+ lymphocytes with MG serum influenced the effectiveness
of CD8+ mediated suppression, it is possible that the target CD4+ cell was interacting with pre-cytotoxic lymphocytes. However, given that mitogen stimulation of cells early in culture has been shown to induce suppression for CTL development (Peavy et al 1974) it would seem that this latter possibility is less likely. Others have also shown that nonspecific, soluble T-suppressor factors, which were induced in culture by mitogen or by interferon, acted to dampen development of CTL (Devan et al 1988).

An interesting extension of our work for future studies would be to identify the CD4+ subtype which is the target for serum factors in MG. Indeed, the existence of multiple anti-lymphocyte Ab, specific for different target cell populations, was reported in SLE (Morimoto et al 1984). Recently, monoclonal Ab to the CD45+ Tsi subset have become commercially available so this subset could be enriched and studied directly.

Evidence from the literature suggests that auto-Ab may interfere with lymphocyte function in MG. Studies by Shore et al (1979) have shown that the IgG fraction of MG serum directly interfered with suppressor function among healthy lymphocytes, although that study had not been designed to determine whether effects were operative at the induction or effector level. This same study also showed that an IgG factor could influence the ability of T cells to form rosettes with sheep red blood cells, an activity that was blocked by D-tubocurarine, thus implicating a role for nAchR as the target receptor. Sagar et al (1980) showed that an unidentified factor in serum of some MG patients impaired mitogen induced proliferation of normal lymphocytes. Autoantibody mediated reduction of Tsi function has also been recognised in other autoimmune disorders. Anti-lymphocyte antibodies from patients with active SLE bind to T cell subsets which are involved in the generation of T-suppressor activity (Sakane et al 1978, Morimoto et al 1979, Morimoto et al 1983). This has also been recognised in juvenile rheumatoid arthritis (Morimoto et al 1981).

In the present study the identity of the MG serum factor which impaired CD4+ cell mediated lymphocyte function has not been established. We have hypothesized that this factor is an antibody, however, an expansion of this work to look at the effects of the immunoglobulin fraction on CD4+ function will make for interesting future study. Since all sera were heat treated to
inactivate complement this leaves the possibility that we have been observing the effects of Ab which influence function by interacting with surface receptors as opposed to mediating lysis of cells. Such Ab could have very pathogenic effects in vivo and would not depend on availability of complement for their action.

Our demonstration of the existence of this factor in sera of patients with relatively recent onset and high in vitro capacity for Ab production suggests that the high production of Ab which we have observed among patients in this category may be a secondary manifestation of disease rather than a pre-existing defect.

At the present time we cannot exclude the possibility that this factor is non-Ab and it may, in fact, turn out to be hormonal in nature. It would also be very interesting to systematically study MG patients in different categories of disease to determine the stage at which this factor is clinically important. This could lead to novel forms of therapy.

Summary:

We have studied in vitro Ab (nAchR Ab and polyclonal IgG) production by PBMNC in a group of 36 seropositive MG patients with generalized disease for whom complete clinical data were available. These patients had been screened prior to study (Protein A precipitation assay, see Chapter 3) in order to select only those patients who were seropositive for nAchR Ab. Fifty eight percent of seropositive MG patients secreted detectable levels of nAchR Ab. We have compared polyclonal and antigen specific Ab production in the MG patients to clarify whether nAchR Ab is regulated separately. Capacity for production of nAchR Ab and IgG were correlated suggesting that similar regulatory mechanisms existed.

These studies have demonstrated for the first time that two subgroups of MG patients can be recognized based on capacity of PBMNC to secrete Ab in vitro and on disease duration. No other relation of Ab secretion in vitro to clinical variables (including treatment) was observed. Our data suggest that B cell mediated immune function had become altered as MG progressed over time.
Moreover, preliminary experiments, using separation/recombination techniques, showed that B cells from MG patients who were low Ab producers were fully capable of significant Ab production when cultured in the presence of CD4+ enriched T-lymphocytes from individuals whose PBMNC were high Ab producers. These data implicated the CD4+ T-cell in regulating the changes to humoral immunity which occurred over time during the course of MG.

Data from this study of in vitro production of nAchR Ab and IgG suggested strongly that changes in B cell mediated immune function occurred in a manner secondary to the prior establishment and persistence of MG. We therefore hypothesized that auto-anti-lymphocyte Ab may arise and be involved with alterations to immune function in MG. In initial investigations, the effects of MG serum on the functioning of normal lymphocytes was examined. Following our observation that some MG sera could interfere with suppressor function in the con A- induced suppressor assay, we developed a more specific assay system to determine which cell types were involved in mediating these effects. Preliminary experiments in the PWM-stimulated IgG secretion system suggested that the CD4+ T-cell was the target for a heat resistant serum factor in Secretor MG patients. The CD4+ T-cell target may be the T-suppressor-inducer lymphocyte however, further investigations will be necessary to clarify this point.
Chapter 5. Direct binding of aBT to human PBMNC and to TE671 Rhabdomyosarcoma cell line

Data reported in J Neuroimmunol 23, 83-87 1989

5.1 INTRODUCTION 129
5.1.1 Lymphocytes as potential targets for auto-antibodies in MG 129
5.1.2 nAchR expression by lymphocytes 129
5.1.3 TE671 Rhabdomyosarcoma cell line: Living model system for study of nAchR and nAchR Ab 131

5.2 RESULTS 132
5.2.1 PBMNC as potential targets for auto Ab in MG 132
5.2.1.1. Binding of [125]I-aBT to PBMNC and TE671 132
5.2.1.2. Study of [125]I-aBT binding to PBMNC under different conditions 135
5.2.1.3. Scatchard analysis of binding of [125]I-aBT to TE671 cell line 135

5.3 DISCUSSION 137

5.4 SUMMARY 138
5.1 Introduction:

5.1.1 Lymphocytes as potential targets for auto-antibodies in myasthenia gravis:

Our previous study of in vitro Ab production by PBMNC (Chapter 4), in which we demonstrated that patients with long disease duration produced abnormally low levels of Ab, suggested that changes in immune function may occur secondary to the establishment and continuation of MG. We have hypothesized that auto-anti-lymphocyte Ab may arise and may be involved in alterations to immune function in MG. Anti-lymphocyte Ab have been described for other autoimmune diseases (Sakane et al 1978, Morimoto et al 1979, 1981, 1983). Preliminary studies from our laboratory suggested that MG serum may impair CD4+ mediated T regulatory functioning of normal lymphocytes (Chapter 4).

The purpose of the present study was to test our hypothesis that human PBMNC express nAchR. For the study of MG, this question is of critical importance. If nAchR are expressed by PBMNC they would be potential targets for nAchR Ab in MG. One can speculate that alteration of lymphocyte function by auto-anti-lymphocyte Ab may be part of a feed-back loop in which immune function in MG is further dysregulated.

The demonstration that nAchR is expressed by PBMNC would be consistent with rapidly accumulating evidence for extensive interactions and similarities between the nervous system and the immune system. There is increasing evidence that many substances which have functional effects on the nervous system also act on cells of the immune system. Muscarinic acetylcholine receptors are expressed on leukocytes (Gordon et al 1978, Lopker et al 1980, Strom et al 1981, Zalcman et al 1981, Maslinski et al 1983, Bidart et al 1983) as are receptors for noradrenaline (Williams et al 1976), dopamine (Ovadia et al 1987) and peptide neurotransmitters (reviewed in Morley et al 1987).

5.1.2 nAchR expression by lymphocytes:

Several functional studies have provided evidence that a nicotinic acetylcholine receptor (nAchR) is present on leukocytes (Richman and Arnason 1979, Richman et al 1981, Whaley et al
direct binding studies, however, have been equivocal (Morrell 1976, Davies et al 1982, Atweh et al
1984). Although saturable, specific binding of nicotine to human PBMNC has been observed
(Davies et al 1982) it was considered to be noncholinergic because carbachol and several classical
nicotinic antagonists did not effectively inhibit nicotine binding. The receptor they were studying
may have had pharmacological properties more similar to neuronal nAchR which does not bind
(1984) reported evidence for an unusual AchR on human leukocytes which specifically bound the
muscarinic ligand, quinuclidinyl benzilate, but which also was blocked by the nicotinic cholinergic
ligand, D-tubocurarine.

The nicotinic antagonist, alpha bungarotoxin, has been reported to bind to PBMNC from
myasthenia gravis patients, but not healthy individuals (Morrell 1976, 1981), suggesting that
nAchR expression on leukocytes may be enhanced in this disease. Since MG is an auto immune
disease which is characterized by circulating nAchR Ab it is conceivable that these Ab might also
bind to nAchR on immunocytes and thus disturb immune function. Indeed, affinity purified nAchR
Ab have been shown to bind to thymocytes (Fuchs et al 1980) and impaired suppressor function
has been induced in normal lymphocytes following pretreatment with MG sera (Shore et al 1979).

We have carried out direct binding studies using the nicotinic antagonist, aBT, to probe for
a muscle endplate-like nAchR on human PBMNC. The aim of our study was to confirm and
extend the studies of Morrell who showed that aBT binding occurred on mononuclear cells from
MG patients but not from normal controls. We intended to characterize the subset distribution of
nAchR and to examine the possibility that clinical variables such as disease duration or capacity for
Ab production in vitro may have been correlated with expression of nAchR in MG. We also
considered it unlikely that de novo expression of nAchR would occur in MG and felt that a more
sensitive assay might allow detection on normal PMMNC as well. We did not detect aBT binding
to PBMNC from either healthy controls or from MG patients, although we were able to measure
binding of aBT to the TE671 cell line in parallel studies.
5.1.3. TE671 Rhabdomyosarcoma cell line: Model system for studying nAchR and nAchR Ab:

Recently, the TE671 rhabdomyosarcoma cell line has generated much interest among MG researchers. This cell line was reported to have been developed from the cerebellar medulloblastoma of a 6 year old girl (McAllister et al 1977), however, more recent studies have shown this line to be a rhabdomyosarcoma. This cell line has been shown to express a nAchR which is pharmacologically and immunologically very similar to endplate nAchR. Syapin et al (1982) demonstrated that aBT binds to TE671 in a saturable, specific manner, although the affinity constant (1.4 nM) was somewhat lower than that for endplate nAchR, which is approximately 0.20 nM (Lindstrom et al 1976). As hypothesized in a review article by Lindstrom et al (1987), the lower binding constant may have been due to variations at the level of receptor glycosylation.

Lindstrom's group showed that TE671 nAchR is similar in size to muscle nAchR and that monoclonal Ab which recognize human muscle nAchR, including mcAb to the main immunogenic region, bind to TE671. Furthermore, TE671 nAchR is functional in electrophysiological studies using acetylcholine and various acetylcholine agonists and antagonists (Syapin et al 1982, Lukas et al 1986, Lindstrom et al 1987).

The TE671 cell line has great potential as a convenient source of nAchR for study and as a human model system in which to study regulation of nAchR expression, and the pathogenic influences of Ab and/or other pathogenic factors in MG. In a very recent study, the TE671 line has been used successfully as an in vitro model system in which to study the pathogenic effects of nAchR Ab on membrane bound nAchR (Lang et al 1988). Others have characterized the kinetics of aBT binding to TE671 cells (Walker et al 1988). aBT binding to TE671 cells is strikingly higher early in the growth phase, although these investigators did not determine the binding constants.

It has been proposed by Lindstrom, who originally developed the nAchR Ab immunoassay in 1975, that TE671 cell nAchR may be a useful antigen for the determination of serum nAchR Ab in the diagnosis of MG (Lindstrom et al 1987). This group has directly
compared the nAchR Ab titers measured on TE671 nAchR with those measured on human muscle nAchR and found the correlation to be strong. The TE671 antigen would have the advantage of being readily accessible and could be standardized between different laboratories. Studies are currently underway in our laboratory using intact TE671 cells to measure specific nAchR Ab which recognize extracellular domains of the receptor. As previously discussed, such auto Ab may be more closely related to severity of disease and it will be very interesting to compare their distribution between patients in clinical remission and those having active disorder.

We selected the TE671 cell line for its property of binding aBT as a positive control in our study of aBT binding to human PBMNC. We have also characterized the binding constants for the high affinity binding of aBT to TE671 nAchR and have confirmed earlier reports (Syapin et al 1982, Lukas 1986) that the affinity constant is somewhat lower than that of muscle endplate nAchR.

5.2. Results:

5.2.1. PBMNC as potential targets for auto Ab in MG:
We have hypothesized that lymphocytes may be the target of auto Ab in MG. Since MG is characterized by nAchR Ab and since, in recent years, lymphocytes have been shown to express receptors for many molecules which are neurotransmitters, we decided to study PBMNC for binding of the nicotinic antagonist, aBT.

5.2.1.1. Binding of [125]I-aBT to PBMNC and TE671:
Freshly obtained PBMNC (1 -10 x 10^6) from 3 healthy controls and 4 patients with generalized MG were studied for binding of [125]I-aBT. PBMNC were incubated in the presence of varying concentrations of [125]I-aBT. Specific binding was not observed in any case. When aBT binding to PBMNC was studied in parallel with binding to the medulloblastoma cell line, TE671, saturable, specific binding to the cell line, but not to the PBMNC, was observed (Figure 5.1 and
Table 5.1).

**Figure 5.1. Binding of [125I]-aBT to TE671 rhabdomyosarcoma cell line and human PBMNC:**

![Graph showing binding of [125I]-aBT to TE671 and PBMNC](image)

Binding of [125I]-aBT to $10^6$ intact human PBMNC or $10^6$ TE671 cells. Binding was carried out at room temperature for 50 min in PBS, pH 7.2. Cells were filtered onto cellulose acetate filters (Millipore AAWP024). Background binding in the presence of excess ($1.1 \times 10^{-5}$ M), unlabelled aBT was subtracted. Each point represents the mean ± SEM from three separate experiments (2 healthy donors and one MG patient).
Table 5.1. Specific Binding of [125]I-aBT to TE671 cells and PBMNC from myasthenia gravis patients (MG) and healthy donors (N):

<table>
<thead>
<tr>
<th>Sample</th>
<th>Cells/tube (X10^6)</th>
<th>Concentration of aBT (nM)</th>
<th>Net binding (dpm/10^6 cells/nM aBT)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Experiment #1:</strong> MG-1</td>
<td>2.5</td>
<td>4</td>
<td>-32</td>
</tr>
<tr>
<td>N-1</td>
<td>2.5</td>
<td>4</td>
<td>14.8</td>
</tr>
<tr>
<td>TE671</td>
<td>0.3</td>
<td>4</td>
<td>*510.4</td>
</tr>
<tr>
<td><strong>Experiment #2:</strong> N-2</td>
<td>10</td>
<td>8</td>
<td>-2.6</td>
</tr>
<tr>
<td>N-2</td>
<td>3</td>
<td>8</td>
<td>20.8</td>
</tr>
<tr>
<td>N-1</td>
<td>1</td>
<td>8</td>
<td>23.2</td>
</tr>
<tr>
<td>TE671</td>
<td>1</td>
<td>8</td>
<td>*740.9</td>
</tr>
<tr>
<td><strong>Experiment #3:</strong> MG-2</td>
<td>2</td>
<td>10</td>
<td>-13.4</td>
</tr>
<tr>
<td>MG-3</td>
<td>2</td>
<td>10</td>
<td>12.5</td>
</tr>
<tr>
<td>TE671</td>
<td>1</td>
<td>10</td>
<td>*556</td>
</tr>
<tr>
<td><strong>Experiment #4:</strong> MG-4</td>
<td>1</td>
<td>32</td>
<td>-50.6</td>
</tr>
<tr>
<td>TE671</td>
<td>1</td>
<td>32</td>
<td>*111.9</td>
</tr>
<tr>
<td>MG-LP°</td>
<td>15.5</td>
<td>64</td>
<td>-3.5</td>
</tr>
<tr>
<td>TE671</td>
<td>1</td>
<td>64</td>
<td>*176.7</td>
</tr>
<tr>
<td><strong>Experiment #5:</strong> N-3</td>
<td>1</td>
<td>16</td>
<td>9</td>
</tr>
<tr>
<td>TE671</td>
<td>1</td>
<td>16</td>
<td>*257.3</td>
</tr>
</tbody>
</table>

Net binding of [125]I-aBT (dpm/10^6 cells/nM aBT) to PBMNC from MG patients and healthy donors (N) measured in parallel with binding to the TE671 rhabdomyosarcoma cell line.

◊ Cells from MG-LP were obtained following leukopheresis and stored frozen at -70°C, viability was 80% as determined by trypan blue exclusion.

*statistically significant (t-test applied to dpm in the presence or absence of excess unlabelled aBT).

For binding of aBT to the TE671 cells, specifically bound counts represented 90% of the total counts bound at low [125]I-aBT concentration and 40% at high concentration. The limit of detection in this assay was approximately 40 x 10^6 aBT binding sites.
5.2.1.2. Study of [125]I-aBT binding to PBMNC under differing conditions:

In order to address the possibility that binding of [125]I-aBT to PBMNC in our system did not reach the limit of detection (40 x 10^6 aBT binding sites i.e. an average of 40 per cell) larger numbers of cells were studied. PBMNC from 3 individuals were studied from 10^6 - 10^7 cells/tube. Further, we examined the possibility that expression of detectable levels of [125]I-aBT binding sites among PBMNC was dependent on the state of activation of the PBMNC. PBMNC were maintained in culture for 1, 3 or 6 days at 37°C in an atmosphere of 5%CO2 prior to assessment of [125]I-aBT binding. PBMNC from 1 MG patient and 1 control were stimulated for 3 days in the presence of Con A prior to binding assay.

Specific binding of [125]I-aBT was not observed following culture for varying periods. Additionally, stimulation with Con A did not result in detectable binding of aBT by PBMNC from MG or control. Binding was also not detected at any cell number tested.

5.2.1.3. Scatchard analysis of binding of [125]I-aBT to TE671 cell line:

We have made use of the TE671 cell line as a positive control for our assay of [125]I-aBT binding to living cells. In addition to its use as a positive control in this assay, characterization of the TE671 cell nAchR is also important since this cell line has potential as a source of standardizable antigen in clinical nAchR Ab assays. We therefore analysed the binding of aBT to TE671 cells according to the method of Scatchard (Figure 5.2).
Figure 5.2. Analysis of binding of [125]I-aBT to TE671 line:

An average $K_d$ of 3.68 nM and a $B_{max}$ of $1.62 \times 10^{-20}$ mole/cell (approximately 10,000 sites per cell) were determined for binding of aBT to the TE671 cell.
5.3. Discussion:

Recently, it has become clear that substances which have biological activity in the nervous system can also act directly on cells of the immune system. This has been shown to be the case for acetylcholine; muscarinic AchR on leukocytes have been shown by direct binding studies in a number of laboratories (Gordon et al 1978, Lopker et al 1980, Zalcman et al 1981, Strom et al 1981, Bidart et al 1983, Maslinski et al 1983) although there have been reports to the contrary (Maloteaux et al 1982). Muscarinic AchR have also been shown to be involved in a variety of cell functions including early rosette formation (Galant et al 1976) and cell mediated cytotoxicity (Strom et al 1974).

Nicotinic AchR of the neuro-muscular junction have been studied extensively using the antagonist, aBT (reviewed in Fambrough 1979), although the relationship between neuronal aBT binding sites and functional nAchR is not clear (Marks et al 1982, Clarke et al 1985). In the present study we have found no specific binding of aBT to PBMNC in any of 3 healthy controls or 7 MG patients although we confirmed specific, high affinity binding of aBT to TE671. The binding constants were similar to those reported by other investigators (Syapin et al 1982, Lukas 1986).

Morrell did not observe specific binding of aBT to normal human PBMNC (Morell 1976) but he found saturable, specific binding to PBMNC from patients with MG. It is not clear why our results for MG patients should differ from those of Morrell. Our patients do not seem to differ appreciably from his. He reported that aBT binding to PBMNC occurred in both steroid treated and untreated MG patients with active disease (Morrell 1981). In the present study 6 of the 7 patients had active, generalized disease and five of our patients were on treatment with steroids. With reference to our earlier study of nAchR Ab secretion in vitro (Chapter 4); our MG patient group consisted of 3 Secretors and 4 Nonsecretors.

It is also unlikely that variation in technique would explain these discrepancies. Using the TE671 cell line we have shown that our technique was sensitive enough to recognize the presence
of 40x106 aBT binding sites (i.e. an average of 40 per cell). We cannot, however, exclude the possibility that nAchR are expressed by only an extremely small subpopulation of PBMNC and cannot be detected by a conventional binding assay before subset purification. A final possibility is that our patients had a population of high affinity nAchR Ab which could block binding of aBT. However, incubation in medium at 37°C, a maneuver that would release bound antibody, did not result in expression of aBT binding sites.

Functional studies have been carried out to look for nAchR on leukocytes. Whaley et al (1981) have shown that production of the complement component, C2, by cultured human monocytes was enhanced in the presence of acetylcholine or carbachol; this effect was dose dependant and was blocked by low doses of D-tubocurarine and aBT. E-rosette formation (Mizuno et al 1982) and leukocyte proliferation (Richman and Arnason 1979) were shown to be influenced by carbachol. These effects were blocked by D-tubocurarine and, in the latter study, by aBT. Leukocyte proliferation was also influenced by succinylcholine (Pizzighella et al 1982) and nicotine (Menard and Rola-Pleszczynski 1987). Moreover, immunoglobulin from MG patients interfered with the development of E-rosettes by normal lymphocytes and this activity was blocked by D-tubocurarine suggesting that the IgG may have been directed toward nAchR which were present on lymphocytes (Shore 1979).

These studies raise the possibility that expression of nAchR by leukocytes could be related to the state of activation of the cell. However, our results showed no evidence that aBT binding sites could be revealed on leukocytes by culturing in vitro, either alone or in the presence of ConA, a standard activator of lymphocytes. It would be interesting to examine lymphocytes from different lymphoid organs to determine if aBT binding site expression would be transiently expressed at certain locations.

5.4. Summary:

In our previous studies we generated evidence that some defects in immune function may
be secondary to the development of MG. In particular, we have hypothesized that anti-lymphocyte Ab may arise in MG and may alter immune function. We carried out direct binding studies to determine if nAchR, theoretically a target for circulating nAchR Ab, are expressed on human PBMNC. Using the classical, endplate nicotinic antagonist, aBT, we confirmed binding of aBT to the TE671 rhabdomyosarcoma cell line. This binding was saturable and of high affinity, although the affinity constant was lower than would be expected for endplate nAchR. Human PBMNC did not express detectable levels of [125]I-aBT binding sites.

This data does not support the hypothesis that nAchR, of the type expressed at the muscle endplate, are expressed by normal human PBMNC either spontaneously or after nonspecific activation. We were also unable to confirm the presence of nAchR on PBMNC in patients with MG.
Chapter 6
Summary and Perspectives

MG is an autoimmune disorder of neuromuscular transmission. Clinically, the disease is manifested by abnormal muscle fatigue with recovery on resting. Circulating nAchR Ab are highly characteristic of MG. These Ab have been shown to be directly pathogenic at the muscle endplate and are responsible for impaired neuromuscular transmission through several mechanisms.

In order to gain insight into the mechanisms of autoimmunity and to develop more specific treatments for MG, it is necessary to develop a greater understanding of the manner in which nAchR Ab production is regulated by the immune system. At present, the identity of the antigen to which tolerance is initially lost is unknown. The most popular theories include loss of tolerance to self nAchR or nAchR-like molecules which are normally expressed in the thymus. In this scenario, thymic nAchR may become altered in appearance or density preceding development of autoimmunity. Alternately, the immunizing species may be an exogenous, cross reactive species (molecular mimicry). In either case, it is not clear whether, in MG, the immune system has behaved normally in response to foreign antigen and/ or there are true defects in immune function which cause, predispose to or even arise following onset of MG.

A substantial body of evidence exists which suggests that the immune system does not function normally in MG, although the exact nature of the defects at the molecular and cellular level have not been determined. Moreover, there is evidence that some immune system defects may predispose certain individuals to development of MG. It cannot be excluded, however, that some defects in immune response may arise as secondary features of the disease. This is a very important question to examine because secondary variations in immune function may be related to the heterogeneous course of MG. Possibilities include the development of anti-lymphocyte antibodies, attack on the thymus by nAchR Ab with disruption of normal hormonal output or chronic "burn out" of the immune system. We have favored the anti-lymphocyte antibody
hypothesis as this would be consistent with the manner in which secondary immune system defects arise in other autoimmune conditions.

At the time these studies were undertaken it was not clear whether nAchR Ab production was regulated in concert with the polyclonal humoral response. Therefore, in order to study the immune response in MG in a meaningful way, it was first necessary to establish an assay with which to quantitate levels of the pathogenic Ab, nAchR Ab. In addition, it was necessary to have access to a set of patients diagnosed as having MG by immunologic criteria (i.e. nAchR Ab determinations). Thus, in the initial phase of our research, we established a modification of the nAchR Ab immunoassay originally described by Lindstrom (1976c).

For diagnostic work, we developed a rapid, sensitive immunoassay based on the Protein A precipitation of immune complexes formed by a large volume of serum in the presence of radiolabelled nAchR solubilized from human muscle. This assay differs from other nAchR Ab assays in that a larger serum volume is tested for nAchR Ab to maximize the sensitivity of detection in sera of low nAchR Ab titer. The use of Protein A has also reduced the assay time by half compared with the standard double Ab precipitation technique. Moreover, we have recognized that quantitative determination of serum nAchR Ab, which may render the assay less sensitive or necessitate impractical squandering of reagents, has no value in diagnosis of MG. Thus, we have carried out the assay on a qualitative basis which has facilitated improvement of sensitivity through testing of larger serum volumes and also allowed us to run an economically efficient assay on a wide scale basis. Through our effort, the nAchR Ab assay is now available throughout Canada for clinical purposes.

We tested serum from 55 patients who had generalized, active MG and detected nAchR Ab in 54 (98.2%). nAchR Ab were also detected in 21/37 (56.7%) patients with ocular MG and in 8/21 (38.0%) of patients with MG in clinical remission. nAchR Ab were not detected in any of 55 healthy controls and were detected in only 2/38 (5.2%) patients who had other neuro-muscular diseases. One of these patients probably had penicillamine-induced MG, a recognized disorder that occasionally manifests in response to treatment with that medication.
We also developed a quantitative version of the nAchR Ab assay which involved a double antibody precipitation of immune complexes. This assay was modified so that it could be used to measure, without preliminary concentration (thus avoiding the risk of denaturation), the very low concentration of nAchR Ab secreted into culture media by PBMNC from MG patients. Having identified a group of seropositive, generalized MG patients on whom to carry out further study and having developed a quantitative assay with which to measure nAchR Ab we moved to the second phase of our research which involved the study of in vitro nAchR Ab production in MG.

We have studied in vitro Ab (nAchR Ab and polyclonal IgG) production by PBMNC in a group of 36 seropositive MG patients with generalized disease for whom complete clinical data were available. Fifty eight per cent of MG patients secreted detectable levels of nAchR Ab. We have compared polyclonal and antigen specific Ab production in the MG patients to clarify whether nAchR Ab is regulated separately. Capacity for production of nAchR Ab and IgG were correlated suggesting that similar regulatory mechanisms exist.

These studies have demonstrated for the first time that two subgroups of MG patients can be recognized in whom there is a correlation between disease duration and capacity of PBMNC to secrete Ab in vitro. No other relationship between Ab secretion in vitro and clinical variables (including treatment) was observed. PBMNC from patients who had short disease duration secreted high levels of Ab in vitro compared with controls or with MG patients who had long disease duration. In MG of long disease duration, in vitro PBMNC production of Ab was very low. These findings were confirmed by serum Ab levels in the same patients. The data from the present study suggest that B cell mediated immune function becomes altered as MG progresses over time.

Lymphocyte subset separation / recombination experiments showed that B cells from MG patients who were low Ab producers were fully capable of significant Ab production when cultured in the presence of CD4+ enriched T - lymphocytes from individuals whose PMBNC were high Ab producers. These data implicated the CD4+ T-cell in regulating the changes to humoral immunity which occurred over time during the course of MG.
We hypothesized that auto-anti-lymphocyte Ab may arise and alter immune function in MG. Following our initial observation that some MG sera could interfere with suppressor function in the con A-induced suppressor assay, we developed a more specific assay system to determine which cell types were involved in mediating these effects. The study was also designed to compare sera from the two categories of MG patients which we defined, Secretors and Nonsecretors, for the presence of an inhibitory factor.

Preliminary experiments in the PWM-stimulated IgG secretion system suggested that a CD4+ T-cell was the target for a heat-resistant serum factor in Secretor MG patients. The CD4+ T-cell target may be the T-suppressor-inducer lymphocyte (Tsi). It is possible to envisage a scenario in which an inhibitory serum factor, such as an anti-lymphocyte Ab, may bind to and inactivate the Tsi cell. Tsi are critical for stimulating the CD8+ T-suppressor cell to suppress humoral immune responses and thus loss of and/or functional impairment of the Tsi cell would lead to an impairment of CD8+ mediated T-suppressor function. One may speculate that the following series of events would take place in some MG patients. Following development of MG a circulating, inhibitory factor arises as a secondary feature of the disease. This factor is responsible for directly damaging the Tsi cell which leads to impaired suppressor function in patients possessing the factor and to the development of a more aggressive immune response. This may lead to increased autoimmunity and early progression of disease. Such a situation would help to explain demonstrations by other investigators, using unseparated lymphocyte assays, that suppressor function may be impaired in some MG patients. Moreover, this hypothesis would be consistent with our present demonstration that there is an abnormally high polyclonal immune response in the Secretor category of MG patients, from whom the serum factor was obtained. In fact, very recent studies from our laboratory have shown that the percentage of peripheral blood Tsi cells is markedly reduced in MG compared with normal (unpublished observations). It would be of interest, through the study of larger patient groups, to ascertain whether the serum factor is indeed exclusive to Secretor MG patients. Studies correlating the factor with clinical variables, such as disease duration, might lead to important insights into the pathogenesis and course of MG.
The nature of the serum factor from MG patients which affected the function of normal lymphocytes was not determined in the present study. Identification could be approached through the testing of fractionated serum in the IgG secretion assay. If the serum factor which affects lymphocyte function in MG turns out to be Ig in nature, it would remain to determine the target epitope. The mechanisms of action of an anti-lymphocyte Ab could include the following. If antibodies are of the isotype to fix complement then this could result in cell lysis. The present studies actually provide evidence that non-complement mediated mechanisms may (also) be involved in impairing suppressor function because the sera were heat inactivated at 56°C, a treatment known to destroy complement activity. Other possible mechanisms of action include that the Ab could bind to a receptor and behave as an antagonist of the physiologic ligand. It is also possible that an anti-lymphocyte Ab, binding to a receptor on lymphocytes, could behave as an agonist; such auto-Ab have been described in other diseases. Alternately, the serum factor may turn out to be non-Ab in nature. Other possibilities include an immunoregulatory, thymic hormone. Thymic hormones are actually overproduced in MG. Dysregulated hormone output by thymus may be secondary to nAchR Ab mediated influences on thymic epithelia which produce the hormones. This hypothesis would be consistent with the demonstrations that nAchR are expressed by thymic epithelia.

Further investigations will still be necessary to verify the nature of CD4+ cell(s) which are influenced by the serum factor. The recent availability of monoclonal Abs specific for the Tsi subset will allow testing of this subset in a PWM stimulated IgG secretion assay following direct treatment with the MG serum factor.

We have also identified a subcategory of MG patients who exhibit an abnormally low humoral immune response in vitro and who are of long disease duration. These data suggest that immune function has changed from early to late disease by disease dependent mechanisms. One might speculate as to the type of mechanisms that may be involved in such changes. It is conceivable that auto-anti-lymphocyte Ab eventually arise which recognize a T cell possessing helper function. Perhaps the development of such an auto Ab is measurably time dependent.
because the nature of the target, lymphocytic epitope is sufficiently different from early auto-
immunogens that a larger amount of somatic mutation will be necessary before such an antigen can
be brought into the immune repertoire. If these patients have a diminished humoral immune
response, why are they not in clinical remission? One must consider that the immune response is
continually refining itself through somatic mutation. This will allow the natural selection of higher
affinity antibodies. Perhaps when disease continues uninterrupted for many years the immune
response refines itself to the extent that only a few particularly pathogenic nAchR Ab are
produced. Thus the overall nAchR Ab titer could be low, but particularly potent. This argument
does not take into account the low polyclonal IgG response that is also manifest in these
individuals. The humoral immune response appears to be nonspecifically reduced yet we have
shown that B cells from these individuals are capable of substantial Ab production in the presence
of appropriate T-help and therefore it is suspect that T regulatory mechanisms have become altered.
Our preliminary results of mixing studies between Nonsecretors and Secretors or controls would
also suggest that the monocyte/macrophage cell type is not implicated in the depressed immunity of
Nonsecretors since the monocytes contained in those cultures were autologous to the B cells,
having segregated with them during the E-rosette step. It is possible that T helper function has
become diminished following chronic stimulation i.e. "burn out" of the immune response.
Literally, this would translate into a deficiency of cellular production of lymphokines. For
example, some of the interleukins would be likely candidates in this type of phenomenon. It would
be possible to test this by adding the appropriate lymphokines into the deficient cultures to try and
restore the immune response. Finally, an alternate hypothesis is that the T-suppressor function has
become enhanced in the patients who are Nonsecretors. The two mixing experiments which were
carried out do suggest that the phenomenon is related to the CD4+ lymphocyte subset, however, it
would be interesting to expand this study to include larger numbers of patients and to assess
separately the functioning of their helper and suppressor lymphocytes. It cannot be ruled out at the
present time that the helper function of the CD4+ lymphocytes may in fact be diminished in vitro
secondary to an enhanced in vivo suppressor function which has down regulated their capacity to
provide help in vivo or in vitro. The mechanism behind enhanced suppressor function could also involve anti-lymphocyte Ab which perhaps might have an agonistic influence at a suppressor cell receptor thus stimulating those cells to suppress more actively. Alternately, suppressor function may become enhanced secondary to chronic stimulation of the immune system. It is likely that continued immune stimulation through CD4+ helper/inducer mediated pathways would also initiate stimulation of suppressor responses as a normal means of controlling an immune response. Perhaps such regulatory pathways have become abnormally amplified under situations of chronic stimulation. Transforming growth factor-beta (TGF-B) is a cytokine which is secreted by activated macrophages and T -cells. Under certain in vitro conditions this cytokine inhibits the expression of IL2 receptor by T cells and inhibits B cell proliferation and Ig production. TGF-B may influence lymphocyte function by interfering with the production of other cytokines. TGF-B inhibits production of IL1, tumor necrosis factor and IFN-gamma by PBMNC (Espevik et al 1987, Chantry et al 1989). The mode of action of TGF-B seems to involve post-transcriptional events (Chantry et al 1989). At present, it has not been established whether TGF-B acts directly to down regulate cytokine production by activated lymphocytes or whether it may act through stimulation of suppressor cells which then interact with T helper and/or B cells.

By extension of the previous studies, we have hypothesized that human lymphocytes may express an endplate-like nAchR which would be a potential target for nAchR Ab in MG. nAchR Ab could thus behave as anti-lymphocyte Ab in MG. As the lymphocyte subset separation/recombination studies were technically difficult a more direct approach, involving binding studies with cholinergic ligands, was used to examine this hypothesis.

Alpha-bungarotoxin (aBT), the classical nicotinic antagonist, was used to probe for the nAchR on human PBMNC from healthy individuals and from MG patients. We were able to confirm earlier reports of saturable, high affinity binding of aBT to the TE671 rhabdomyosarcoma cell line. However, we did not detect specific aBT binding to human PBMNC. In total, we studied PBMNC from 7 MG patients and from 3 healthy individuals; all were negative in our assay. Thus, our data did not support the hypothesis that nAchR, of the type expressed at muscle endplate, are
expressed by human PBMMC. The possibility that lymphocytes may express an immunologically cross reactive nAchR which differs pharmacologically from endplate nAchR cannot be ruled out. Indeed, nAchR in brain and in thymus differ biochemically and pharmacologically from endplate nAchR. This is a question which could be addressed through the techniques of molecular biology using endplate or brain nAchR cDNA as probes, if one had access to such probes. Binding studies could also be carried out using nicotinic antagonists specific for neuronal nAchR (aBT does not bind neuronal nAchR well). Additionally, it would be worth examining the lymphocytic expression of nAchR during different activational states which should also include the examination of lymphocytes from different lymphoid organs. In particular, it may be prove worthwhile to examine thymic lymphocytes for the expression of nAchR. It is widely believed that locally produced thymic hormones influence development of thymocytes and one of these, thymopoietin, has recently been shown to bind nAchR with very high affinity.

Overall, our studies have lent support to the hypothesis that alterations to immune function may occur as secondary features of MG. We have initiated preliminary investigations into the possible mechanisms by which secondary immunoregulatory defects may arise. Mixing studies showed that in vitro Ab production is controlled at the level of the T cell. Studies using MG serum to pretreat normal CD4+ lymphocytes suggested that a serum factor in Secretor patients (short disease duration and high in vitro PBMNC mediated Ab production) may inhibit T-suppressor-inducer lymphocyte functioning, a situation that could lead to enhanced immune response and may explain the elevated IgG secretion response to PWM that we also observed in this category of patient. We have hypothesized that the serum factor may be an anti-lymphocyte Ab. Indeed, we addressed the possibility that nAchR Ab may have the potential to behave as anti-lymphocyte Ab if lymphocytes expressed nAchR. Direct binding studies to search for endplate-like nAchR on human PBMNC suggested that such receptors were not present. We have not, of course, ruled out the possibility that a lymphocytic nAchR which does not strictly resemble muscle endplate nAchR exists on human lymphocytes.
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174

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