Characterization of autoreactive B cells and AChR autoantibodies in myasthenia gravis

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Dedication

I dedicate this thesis to my loving parents

Abstract

Myasthenia gravis (MG) is a B cell mediated autoimmune disease that is characterized by muscle weakness and fatigue and associated with autoantibodies directed against muscle acetylcholine receptor (AChR) at the neuromuscular junction. Detection of these antibodies has an important role in the diagnosis, treatment and management of the disease. The antibodies are partly produced by the autoreactive B cells in germinal centres formed in the medulla of the myasthenia thymus, where there are also thymic muscle-like cells that express fetal AChRs. The antibodies circulate to the neuromuscular junction leading to complement mediated lysis of the membrane and internalization of AChRs. The patients are successfully treated by corticosteroids, plasmapheresis and immunotherapies that reduce the levels of circulating antibodies. The AChR antibodies are disease specific and thought to be pathogenic but the mechanisms involved in the failure of tolerance is not well understood. In addition, the treatments are non-specific. Better treatments, targeted at acetylcholine receptor specific B cells, or the plasma cells that make the antibodies, would be preferred.

The aim of the thesis was to try to characterize AChR autoantibodies from B cells in the myasthenia gravis thymus gland. The first steps were to test sera, from patients whose thymic lymphocyte populations had been archived by Prof N Willcox, in order to identify cultures that contained cells synthesizing AChR antibodies. Radioimmunoprecipitation and cell-based assays using human embryonic kidney (HEK293T) cells transfected with adult or fetal AChR with additional rapsyn-EGFP (enhanced green fluorescent protein), were used to measure the antibodies. Thymic cultures were tested from selected patients to identify AChR synthesis. The next step was to identify the AChR-antibody specific B cells; a novel approach was to use extracellular membrane vesicles prepared from the transfected cells expressing the

AChRs clustered with the intracellular protein rapsyn. Finally, the peripheral blood mononuclear cells (PBMCs) and thymic lymphocytes were characterized by fluorescence activation cell sorting (FACS), and the binding of AChR vesicles to CD19 positive B cells examined.

The radioimmunoprecipitation assay was able to detect serum AChR antibodies in MG patients' sera and to a lesser extent in thymic cell cultures. The cell-based assay also detected clustered AChR antibodies in sera of the majority but not all MG patients. Because the thymus gland contains fetal AChR rather than the adult form, fetal AChR antibodies were tested and found to be more highly represented in the patients than the antibodies to the adult form. Extracellular membrane vesicles expressed AChRs as shown by immunostaining and western blotting. FACS analysis of the AChR/rapsyn-EGFP membrane vesicle preparation showed that they could be detected by this method. To develop the methods for detecting the AChR antibody producing specific B cells, FACS analysis of lymphocyte populations was established in Raji cells and peripheral blood lymphocytes, and finally in the thymic cultures of two myasthenia patients. Analysis of CD19+ B lymphocytes from one selected thymic culture was incubated with AChR expressing vesicles which demonstrated binding of the vesicles to a population of B cells.

The work done in this thesis partially achieved the aims of developing the methods for identifying AChR-specific B cells by use of AChR-expressing membrane vesicles and has provided the basis for further work. For this confirmation of the results, fresh thymic preparations from patients would clearly be desirable rather than the archived preparations available at the time of this work.

Keywords: Myasthenia gravis; autoantibody; autoimmune; neuromuscular junction; acetylcholine receptor; cell-based assays; thymic hyperplasia; antibody synthesis; extracellular vesicles.

List of abbreviations used in the thesis

MG	Myasthenia gravis
ACh	Acetylcholine
AChR	Acetylcholine Receptors
AChE	Acetylcholine esterase
NMJ	Neuromuscular Junction
VGKC	Voltage Gated Potassium Channels
VGCC	Voltage Gated Calcium Channels
MIR	Main Immunogenic Region
MusK	Muscle-specific kinase
NMO	Neuromyelitis optica
MOG	Myelin oligodendrocyte glycoprotein
AQP4	Aquaporin- 4
PCR	Polymerase Chain Reaction
α BuTx	α-Bungarotoxin
GC	Germinal Centres
HEK293T	Human Embryonic Kidney 293T cells
EOMG	Early Onset Myasthenia gravis

CBA	Cell Based Assay
RIA/RIPA	Radioimmuno Assay/Radioimmunoprecipitation Assay
ELISA	Enzyme-linked Immunosorbent Assay)
ELISPOT	Enzyme-Linked ImmunoSpot assay
Ag	Antigen
Ab	Antibody
IBD	Inflammatory bowel disease
RA	Rheumatoid Arthritis
HLA-DR	Human Leukocyte Antigen - antigen D Related
Ig	Immunoglobulin
IgG	Immunoglobulin G
IgA	Immunoglobulin A
IgD	Immunoglobulin D
IgM	Immunoglobulin M
IgE	Immunoglobulin E
FIPA	Filter immuno-precipitation assay
SLE	Systemic lupus erthythematosus
MS	Multiple sclerosis
EMVs	Extracellular membrane vesicles
HC	Healthy control
ATG	Anti-thymocyte globulin
T1DM	Type 1 Diabetes mellitus

HSCs	Hematopoietic stem cells
NaN3	Sodium azide

Contents

Figures and Table

CHAPTER 1

Introduction

1.1 Autoimmunity

Autoimmunity is a misguided immune response to the body's own proteins with or without damage to the organ. Autoimmune diseases can have a variety of different effects on the individual. Classically, they usually have one of three characteristic pathological effects which characterize them as autoimmune diseases: damage to or destruction of tissues altered organ growth or altered organ function (Ada&Rose,1988).

The human immune system produces both T-cells and B cells that are capable of being reactive with self-antigens, but these self-reactive cells are either usually deleted in th thymus before becoming active within the immune system or suppressed within the immune system by regulatory cells. In case of failure of any of these mechanisms, self-reactive cells can become functional within the immune system. The mechanisms of preventing self-reactive T-cells from being created takes place through negative selection process in the thymus (Ada & Rose, 1988 and Yin *et al*., 2013).

Neurological autoimmunity can target virtually any cell or protein within the central or peripheral nervous system, often in a highly specific manner. As the different CNS cells or structures can be targeted, the syndromes can be diverse. The main known targets are the myelin sheath in multiple sclerosis, the astrocyte in neuromyelitis optica (NMO), the peripheral nerve myelin in immune neuropathies, and the neuromuscular junction in myasthenia gravis (MG). The diseases can result in clinical features such as blindness from optic neuritis, paralysis from spinal cord myelitis, and epilepsy or memory changes from brain inflammation. There are also a new group of conditions caused by antibodies

to receptors on neuronal cells. Understanding these disorders ultimately requires an analysis of how the target antigen molecules generate the autoimmune reaction and how the immune system produces the immune–mediated injury of the nervous system. Cytokines, autoantibodies, and other immune factors like complement factors, human leukocyte antigens (HLA) all contribute to the diseases.

The focus of this Thesis is on myasthenia gravis (MG) but a few comments will first be made about other well-characterized common autoimmune diseases.

1.1.i Systemic lupus erythematosus (lupus/ SLE) is an autoimmune inflammatory disease characterized by interferon and complement activation, autoantibodies and tissue destruction involving multiple organ systems (Rahman& Isenberg, 2008). In addition, activation of type I interferons was also prevalent in SLE and associated with distinct autoantibody profiles (Weckerle*et.al*., 2011). Common clinical symptoms in SLE include rash, nephritis, central nervous system disease, thrombocytopenia and musculoskeletal manifestations. SLE often occurs in women between 20–40 years of age and has strong genetic and environmental components. (Rahman P et.*al.,* 1999).

1.1.ii Multiple Sclerosis (MS): Although this is predominantly an inflammatory disease associated with T lymphocyte infiltrates into the brain, B cells and T cells are both major players in the pathogenesis of multiple sclerosis (MS) and cooperate at various check points. B cells isolated from CNS lesions as well as from the cerebrospinal fluid (CSF) show signs of clonal expansion and hypermutation, suggesting their local activation (Weber *et.al*., 2011). Plasmablasts and plasma cells maturing from B cells contribute to the development of oligoclonal antibodies produced within the CSF, which remain a diagnostic hallmark finding in MS. B cells, besides serving as the precursors for antibody-secreting plasma cells, are efficient antigen presenting cells for

processing of intact myelin antigen and subsequent activation and pro-inflammatory differentiation of T cells; these mechanisms are supported by the immediate clinical benefit of therapeutic B cell depletion in MS. Within the CNS, antibody deposition is associated with complement activation and demyelination. Some studies have implied a pathogenic role of antibodies directed against components of the myelin sheath. The notion of a pathogenic role for antibodies in MS was supported by the plasma exchange in patients with histologic signs of antibody deposition within the CNS but only in a minority of patients (Weber *et.al*.,2011).

1.1.iii Rheumatoid arthritis (RA): RA is a chronic autoimmune disease that mainly affects the joints. (Scott *et.al*., 2010). The immune system produces antibodies that attacks to the linings of joints in our body. A large proportion of RA patients are characterized by the presence of antibodies directed against post-translationally modified proteins, especially proteins that are citrullinated (Klareskog*et.al*., 2008). The antibodies against such proteins are called anti-citrullinated protein antibodies (ACPA) and recognize proteins only when they contain the modified, 'non-encoded', amino acid citrulline. Untreated RA gradually causes permanent damage to the joints (Burska*et.al*., 2014).

1.1.iv Chronic inflammatory demyelinating polyneuropathy (CIDP): This disease is similar to Guillian-Barre syndrome; the immune system also attacks the peripheral nerves in CIDP, but symptoms last much longer. About 30% of patients can become confined to a wheelchair if not diagnosed and treated early. Treatment for CIDP and GBS are essentially the same. Anti-thymocyte globulin, from animals that were immunized against human thymocytes, was used formerly. Intravenous immunoglobulin therapies are now the main treatments for CIDP. Immunosuppressive drugs such as

rituximab which target B cells are a more recent approach to treat CIDP from 2004 (Odaka *et.al*., 2005).

1.1.v Diabetes mellitus type 1, Diabetes mellitus (DM) is a disease of glucose metabolism characterized by chronic hyperglycemia resulting from defects in insulin secretion or insulin action (Skyler *et.al*., 2017). Type 1 (T1) DM results from an absolute deficiency in insulin caused by the loss of insulin-secreting islet cells in the pancreas, while type 2 (T2) DM is characterized by insulin resistance and relative insulin deficiency, either or both of which may be present at the time diabetes is diagnosed. According to the updated data from the international diabetes federation (IDF), the estimated global prevalence of DM reached 8.8% in 2015 and 12% of global health expenditure was due to DM in that same year (Yates *et.al*.,2016). Attempts to understand the pathogenesis of DM are still ongoing. T1DM was traditionally considered an autoimmune disease; early research demonstrated that T cells were involved in various pathogenic steps in T1DM, including the initiation of insulitis and the injury to β cells (Wong *et.al*., 1997). Moreover, recent research has also discovered the role of T cells in the development of insulin resistance (Jagannathan-Bogdan *et.al*., 2011) and various complications in T2DM. Thus, T cells may be a key component in the pathogenesis of DM as well as a potential diagnostic and therapeutic target.

1.2 Immunoglobulin molecules

Immunoglobulins, also known as antibodies, are glycoprotein molecules produced by plasma cells (white blood cells). They act as a critical part of the immune response by specifically recognizing and binding to particular antigens, principally infectious microbes, but they can also react to self to cause autoimmunity. The antibody immune response is highly complex and exceedingly specific. The various immunoglobulin

classes and subclasses (isotypes) differ in their biological features, structure, target specificity and distribution. Antibody or Ig molecules are glycoproteins composed of one or more units, each containing four polypeptide chains. Heavy and light chains are held together by a combination of non-covalent interactions and covalent interchain disulfide bonds.

Immunoglobulins occur in two main forms, soluble antibodies and membrane-bound immunoglobulins; the latter are associated noncovalently with two accessory peptides, forming the B-cell antigen receptor complex. Alternative splicing regulates the production of secreted antibodies and surface bound B-cell receptors in B cells. The receptor is a prototype of the antibody that the B cell will make. The B cell receptor (BCR) binds specific antigens. It is a heterodimer of Ig alpha and Ig beta chains that enable the cell to transduce the signal and respond to the presence of the antigen on the cell surface. The signal generated causes the growth and proliferation of the B cell which transitions to a plasma cell that secretes the antibodies.

1.3 Immunoglobulins and their functions

Antibodies are classified into classes and subclasses. The five primary classes of immunoglobulins are IgG, IgM, IgA, IgD and IgE. These are distinguished by the type of heavy chain found in the molecule. IgG molecules have heavy chains known as gamma-chains. The light chains are known as kappa and lambda.

IgG is the major immunoglobulin in blood, lymph fluid, cerebrospinal fluid and peritoneal fluid and a key player in the humoral immune response. Serum IgG in healthy humans represents approximately 15% of total protein beside albumins, enzymes, other

globulins and many more. IgG is the only class of Ig that can cross the placenta in humans.

There are four IgG subclasses described in human. The subclasses differ in the number of disulfide bonds and the length and flexibility of the hinge region. Except for their variable regions, all immunoglobulins within one class share about 90% homology, but only 60% among all the classes.

Of the five antibody classes, only significant amounts of IgG are transferred across the placenta. Whole IgG molecules or Fc fragments of IgG pass into the fetal circulation more readily than $F(ab')^2$ fragments (Brambell *et.al*., 1960). The Fc portion of IgG, but not Fab fragments, can cross the placenta of a mother and enter the fetal circulation, providing the fetus with postpartum protection (Palmeira *et.al*., 2012). It was hypothesized that IgG Fc receptors (FcγRs) on placental cells may be involved in IgG transfer across placenta. Later, it was established that this specific transport of IgG is carried out by the neonatal Fc receptor (FcRn) (Simister&Mostov.,1989). IgG molecules can react with Fcγ receptors that are present on the surface of macrophages, neutrophils and natural killer cells, and can activate the complement system. IgG is produced in a delayed response to an infection and can be retained in the body for a long time. The longevity in serum makes IgG most useful for passive immunization by transfer of this antibody. Detection of a specific IgG usually indicates a prior infection or vaccination, or sometimes an autoimmune disease. This is a very important aspect of early onset myasthenia (EOMG) gravis which is discussed further below.

In cases with prolonged or severe infections, determination of IgG levels can provide additional insight into the manifestation of disease. It is important to interpret IgG subclass concentrations in correlation to the donor's age since the immune system

matures during childhood. Due to its relative abundance and excellent specificity toward antigens, IgG is the principle antibody used in immunological research and clinical diagnostics.

IgG1 comprises 60 to 65% of the total main subclass IgG and is predominantly responsible for the thymus-mediated immune response against proteins and polypeptide antigens. IgG1 binds to the Fc-receptor of phagocytic cells and can activate the complement cascade via binding to C1 complex. IgG1 immune response can already be measured in newborns and reaches its typical concentration in infancy (Vidarsson *et.al*., 2014). IgG2, the second largest of IgG isotypes, comprises 20 to 25% of the main subclass and is the prevalent immune response against carbohydrate/polysaccharide antigens. IgG2 is most commonly associated with airway/respiratory infections in infants (Vidarsson*et.al*., 2014). IgG3 comprises around 5 to 10% of total IgG and plays a major role in the immune responses against protein or polypeptide antigens. The affinity of IgG3 can be higher than that of IgG1 (Plomp R *et.al*., 2015). IgG4 is usually less than 4% of total IgG, IgG4 does not bind to polysaccharides. Recent studies have shown that elevated serum levels of IgG4 are found in patients suffering from sclerosis, pancreatitis and interstitial pneumonia caused by infiltrating IgG4 positive plasma cells. The precise role of IgG4 was unknown (Vidarsson*et.al*., 2014) but recent studies reveal that IgG4 plays a role in autoimmune neuropathies and myasthenia gravis associated with MuSK antibodies. IgG4 are not complement activating, unlike the IgG1 and IgG3 AChR antibodies (McConville *et.al.*, 2004), and the antibodies act by direct inhibition of function of the antigen molecules (Huijbers*et.al.*, 2014; Huijbers*et.al*., 2015)

IgM is also expressed on the plasma membrane of B lymphocytes as a monomer. IgM is the first antibody built during an immune response. Pentameric structure of IgM is the largest structure among all the immunoglobulins. IgM are produced by B cells usually in the spleen. Recent studies have shown the role of IgM in CSF of multiple sclerosis (MS) patients (Beltran *et.al*., 2014).

IgE is primarily involved in defence against parasitic invasion but is also responsible for allergic reactions. IgE protects against various parasitic infections (Capron *et.al*.,1977) but it is known to mediate type I hypersensitivity since it binds the high affinity receptor for IgE on mast cells and basophils. (Blank & Rivera, 2004).

IgD is the major antigen receptor isotype on the surface of most peripheral B cells, where it is coexpressed with IgM (Van Boxel*et.al*., 1972).

The role of secretory IgA is to prevent passage of foreign substances into the circulatory system. IgA has been relatively associated with various autoimmune diseases such as MG, SLE, RA and type 1 diabetes but, although its prevalence differs between ethnic groups, there are no known disease associations with IgA antibodies (Wang *et.al*., 2011).

1.4 B cells in autoimmunity

B cell development and function is tightly regulated, including the induction of B cell memory and antibody secreting plasmablasts and plasma cells, one of the major roles of B cell lineage. B cells subsets and markers were based on the developmental stages of a B cell and its exposure to antigens. As B cells mature and differentiate, they give rise to functionally distinct subsets. Differential expression of cell surface and intracellular

markers, as well as their distinct immunoglobulin and cytokine secretion profiles, provide understanding of diverse nature and function of the different B cell subsets. B cells originate from hematopoietic stem cells (HSCs) located in the bone marrow, migrate to secondary lymphoid tissues for their development into follicular B cells. When the B cell receptor complex of a mature B cell, consisting of membrane bound Ig, interacts with an antigen, the cell becomes activated and differentiates into an ab secreting plasma cell. Autoreactive B cells can potentially be generated at several stages during B cell development and there is some evidence that a promoter FcYRIIB mediates its function during the antigen dependent stages of maturation (Dörner, Jacobi& Lipsky., 2009).

B cells evolve under tightly regulated mechanisms along with other immunocompetent cells and historically, B cells were not considered to play a major regulatory function in the development of autoimmunity or autoimmune disease. However, the identification of autoantibodies and their pathogenicity is now widely accepted.

1.5 Role of the thymus

The normal thymus is an epithelial organ that can be divided into cortex, medulla and corticomedullary zone. The cortex contains immature lymphocytes alongside epithelial cells and macrophages. The medulla is less cellular containing more mature T lymphocytes, epithelial cells, dendritic cells and rare myoid cells. There are some B cells but germinal centres are not frequent. It plays a critical role in self-tolerance with a balance between the generation of T lymphocytes and deletion of autoreactive T cells. Some MHC molecules have strong associations with autoimmune disease. These MHC molecules are polymorphic, which influences the amino acids involved in binding peptide. One possible explanation for the disease association is that self-peptides do not

bind as well to these MHC molecules, which then compromise the efficiency of negative selection of self-reactive T cells in the thymus.

1.6 Immunological tolerance

T cells which react to self-antigens are usually tolerant to normal, physiological levels of self-antigen. However, in some people, self-destructive autoimmune disease develops because of the breakdown in tolerance mechanisms. This appears to be due to multiple factors, with contributions from gene defects, which confer autoimmune susceptibility, and environmental factors. Infectious agents or tissue damage from some other cause may activate the immune system, alter expression of self-antigen, or lead to expression of self-antigens which the immune system normally does not see.

Normally immune responses against our own tissue are not made, a concept known as self-tolerance. Determining how the immune system distinguishes between self and foreign antigens to make the decision between tolerance and immunity has been a subject of detailed investigation during the past 50 years. A crucial observation, that suggested that tolerance to self-antigens occurred because the immune system was exposed to these antigens during early development, was made by R.D. Owen in 1945 and the theory modified by J. Lederberg, who suggested correctly that it was the stage in the development or ontogeny of the lymphocyte that was critical, rather than the stage in development of the animal (see Basten, 1989). Central tolerance refers to mechanisms of tolerance acting by clonal deletion during lymphocyte development in the thymus or bone marrow whereas peripheral tolerance refers to mechanisms acting on mature lymphocytes after they have left the primary lymphoid organs. Peripheral tolerance acts by deletion and inactivation (anergy). Not all genes are expressed in the thymus so developing T cells cannot be exposed to all self-antigens.

B cell tolerance is most profound to soluble antigens expressed in the serum or on the surface of cells. Normally, B cells specific for these self-antigens are deleted in the bone marrow or shortly afterwards. By contrast, there are high affinity autoantibodies to antigen normally isolated within cells, suggesting that B cells specific for intracellular self-proteins may escape clonal deletion. However, some antibodies recognize extracellular cell-surface self-antigens. For instance, B cells secreting the IgM haemolytic antibody are also present in normal individuals, but the level of expression is so low that there is no direct cell destruction. When the level of IgM antibody is increased by infection, autoimmune haemolysis occurs.

Mechanisms of peripheral B cell self-tolerance are particularly necessary because after stimulation with antigen B cells expand and undergo somatic mutation, generating a population of B cells with new antigen specificities. Some of these cells may be specific for self-antigens.

Although the principal mechanisms of tolerance to self-antigens are clonal deletion and anergy, there is also a back-up mechanism called suppression. This is a form of dominant tolerance because suppressor cells specific for a given antigen are able to inactivate other lymphocytes specific for the same antigen.

There is now a large amount of evidence to support the Clonal Selection Theory for both T and B cells, but deletion of lymphocytes specific for self-antigens is not the only mechanism of self-tolerance. Both T and B cells specific for self-antigens can be identified in healthy people, so there must be mechanisms that prevent such cells from becoming activated and damaging self-tissue, thereby causing autoimmune diseases.

The existence of autoimmune diseases reveal that lymphocytes reactive to certain self-antigens do exist in the body as mentioned above. In some circumstances, the self-antigen may have been previously hidden from the immune system so that lymphocytes have not had an opportunity to become tolerant. Therefore, immunocompetent B cells which recognize the self-antigen and which require T cell help in order to be activated are able to respond. Finally, these potentially autoreactive cells may be activated by a potent inflammatory stimulus.

Pathogenic autoantibodies are more commonly associated with non-organ-specific systemic autoimmune diseases. Transfer of autoantibodies to experimental animals may confirm their pathogenic role, although this approach is limited by inter species differences in target antigens. Work on myasthenia gravis in particular, however, has demonstrated the validity of this approach.

There are five principle mechanisms by which autoantibodies can produce autoimmune disease:

1. Complement dependent lysis of the target cell.

2. Opsonisation. This is the mechanism of most forms of haemolytic anaemia as the density of Ig is insufficient to allow cross-linking and activation of C1q.

3. Formation of immune complexes in the circulation.

4. Blockade of receptors for physiological ligands or loss of surface expression by internalization. e.g. Myasthenia gravis (acetylcholine receptor antibodies), pernicious anaemia (intrinsic factor antibodies).

5. Stimulation of cell surface receptors. e.g. Graves' disease.

1.7 Antibody mediated autoimmune neurological diseases

1.7.1 Neuromyelitis optica (NMO)

NMO is an idiopathic, demyelinating disease of the central nervous system that affects the optic nerve and spinal cord and causes optic neuritis (ON) and longitudinally extensive transverse myelitis. NMO has a worldwide distribution, poor prognosis, and has long been thought of as a variant of multiple sclerosis; however, clinical, laboratory, immunological, and pathological characteristics that distinguish it from multiple sclerosis are now recognized. The presence of a highly specific serum autoantibody marker (NMO-IgG) differentiates neuromyelitis optica from multiple sclerosis and has helped to define a neuromyelitis optica spectrum of disorders. NMO-IgG reacts with the water channel aquaporin 4. (Lennon *et.al* 2004; Bradl *et.al*., 2009). Autoantibodies to aquaporin 4 derived from peripheral B cells cause the activation of complement, inflammatory demyelination, and necrosis that is seen in neuromyelitis optica. The knowledge gained from further assessment of the exact role of NMO-IgG in the pathogenesis of neuromyelitis optica will provide a foundation for rational therapeutic trials for this rapidly disabling disease (Lennon *et.al*., 2005).

1.7.2. Myasthenia gravis

MG is an autoimmune syndrome caused by the failure of neuromuscular transmission, which results from the binding of autoantibodies to proteins involved in signalling at the neuromuscular Junction. The antibodies to the acetylcholine receptors (AChR) cause complement mediated destruction of the post-synaptic folds of the neuromuscular junction (NMJ) and internalization of the AChRs. This results in reduced muscle-nerve synaptic transmission and fatigable muscle weakness. It affects the voluntary muscles

including muscles of the neck, eyelids, limb and diaphragm. Fatigable weakness, involving these susceptible muscle groups, is the most characteristic feature of MG; fatigue becomes more evident on exertion and improves with rest. The course of MG is variable. Many patients experience intermittent worsening of symptoms triggered by infections, emotional stress, surgeries, or medications, particularly during the first year of the disease (see Vincent, 2002; Vincent *et.al*., 2008).

The prevalence of MG is 1–2/10,000 persons and is increasing probably due to increased longevity and improved diagnostic and treatment strategies that prolong life. The incidence varies between 1.7 and 21 per million inhabitants. The incidence had increased in older individuals (Carr*et.al*., 2010).

In autoimmune myasthenia gravis, the majority of cases are caused by antibodies targeting the acetylcholine receptor as measured by radioimmunoprecipitation assays. The role of the antibodies that cause myasthenia gravis was clearly established in the 1970s. Immunisation against purified AChR produced evidence of myasthenic weakness in rabbits and mice (Patrick & Lindstrom, 1973), and injection of monoclonal antibodies to AChR produced similar effects in laboratory animals (Lennon & Lambert, 1980). In addition, injection of patient IgG antibodies into mice also produced signs of MG (Toyka*et.al*., 1977). Finally, plasma exchange, that removes circulating antibodies, lead to a substantial but transient improvement in muscle function lasting up to 2 months (Newson-Davis *et.al*., 1978).

Approximately 15% of patients have a previously unexplained form of the disease. One cause was found to be antibodies to the protein MuSK in some of these individuals (Hoch *et.al*., 2001). Later more specific tests were developed named 'cell-based' antibody tests, which have helped to improve the sensitivity of both AChR and MuSK

antibody tests, and to provide tests for more antigens, leading to improved diagnosis of MG and related disorders. These will be discussed below.

1.7.3 Diagnostic and clinical classification of MG

Autoantibodies against AChR are typically present in 85% sera of MG patient (Lindstrom *et.al*., 1976) and antibodies to MuSK in only 40-70% of AChR-antibody negative patients (McConville *et.al.*, 2004). There is convincing evidence that MuSK antibodies are pathogenic. They bind to the extracellular domain of MuSK and inhibit agrin-induced AChR clustering in cultured muscle myotubes (Hoch *et.al*., 2001). There are still, however, a number of 'seronegative' MG (SNMG) patients. About 60% have low-affinity antibodies against AChR undetectable by the radio-immunoprecipitation assays but which can be demonstrated on cell based assays where the AChR is clustered (Leite*et.al*, 2008).

1.8.1 The neuromuscular junction in MG

The NMJ has three basic components, the presynaptic motor nerve terminal where acetylcholine is synthesised, stored, and released; the synaptic space where acetylcholine esterase is localized; and the postsynaptic muscle membrane where AChRs are densely packed (see Fig 1.1). Neuromuscular transmission begins with the entry of a nerve action potential into the nerve terminal. Exocytosis of synaptic vesicles containing acetylcholine require calcium, which enters the depolarised nerve terminal via voltage-gated Ca^{2+} channels. Acetylcholine diffuses across the synaptic cleft and interacts with the AChRs on the postsynaptic muscle membrane, leading to opening of the AChR ion channel and depolarisation. The action of acetylcholine on the AChRs is terminated by acetylcholinesterase. In MG, loss of functional AChRs results in the decrease of the threshold required for generation of the muscle nerve fibre action

Potential which, during repetitive nerve depolarisations, results in neuromuscular transmission failure. The NMJ with different antigenic targets are described diagrammatically in Fig 1.2 (see also Vincent, 2002).

1.8.2 Structural characterization of AChRs

AChR remains the major antigenic target in MG followed by MuSK, LRP4 and agrin. AChR is a pentameric membrane protein. Two isoforms of AChR, fetal and adult differ in the composition of five subunits: each contains two α1, one δ and one β1 subunits but the fetal receptor contains a γ that is replaced by a ε subunit in adult receptor. The subunits are organized around a central cation channel (Fig 1.3). The two binding sites between α and ε or γ and α and δ need to be occupied by the ACh for full activation to occur (Vincent, 2002).

1.8.3 Autoantibodies in MG

The antibodies in most MG cases are against the α1 subunit of the AChR (Kalamida *et.al*., 2007), and bind to the main immunogenic region (MIR) that is located on the extracellular domain of the two α subunits (Lindstrom *et.al*., 2000) (Fig 1.3). The binding sites for the MG antibodies were described by competition with monoclonal antibodies raised against the human AChR (see Jacobson *et.al*., 1999; Fig 1.4). The MG antibodies are mainly the complement fixing IgG1 or IgG3, which recognize the native conformation of the AChR. The conventional assay to detect AChR antibodies in the sera is a radioimmunoprecipitation assay which is based on the binding of ^{125}I-α-Bungarotoxin (BuTx) to an equal mixture of fetal and adult AChR extracted from human muscle cell lines. The non-radioactive cell-based assay (CBA) uses human embryonic kidney cells (HEK cells) co-transfected with the AChR subunits and rapsyn that clusters AChR at the NMJ (Leite *et.al*., 2008).

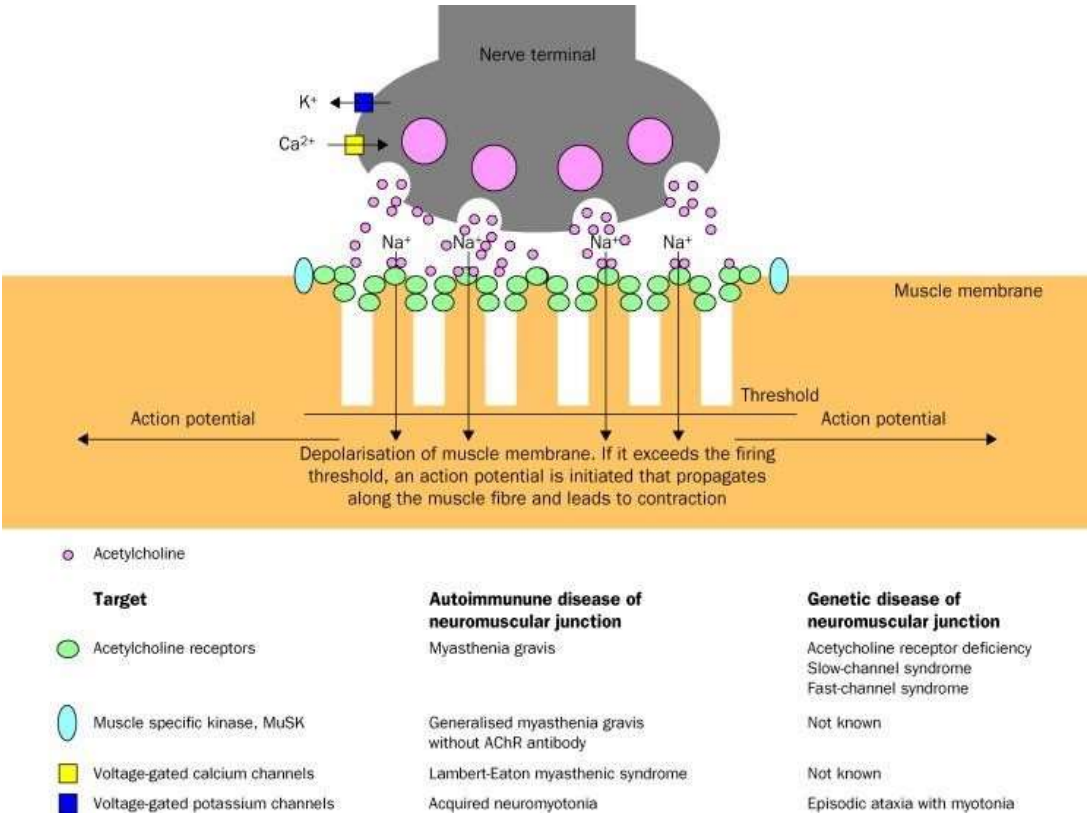


Figure 1.1. Structure of NMJ, the ion channels involved in neuromuscular transmission. Acetylcholine containing vesicles (pink) are released from the motor nerve terminals. Acetylcholine receptors present on the postsynaptic side of muscle membrane interact with acetylcholine leading to depolarisation of muscle membrane. MuSK present at the AChR, maintains AChR on the post synaptic side of NMJ. (Vincent *et.al.*,2001)

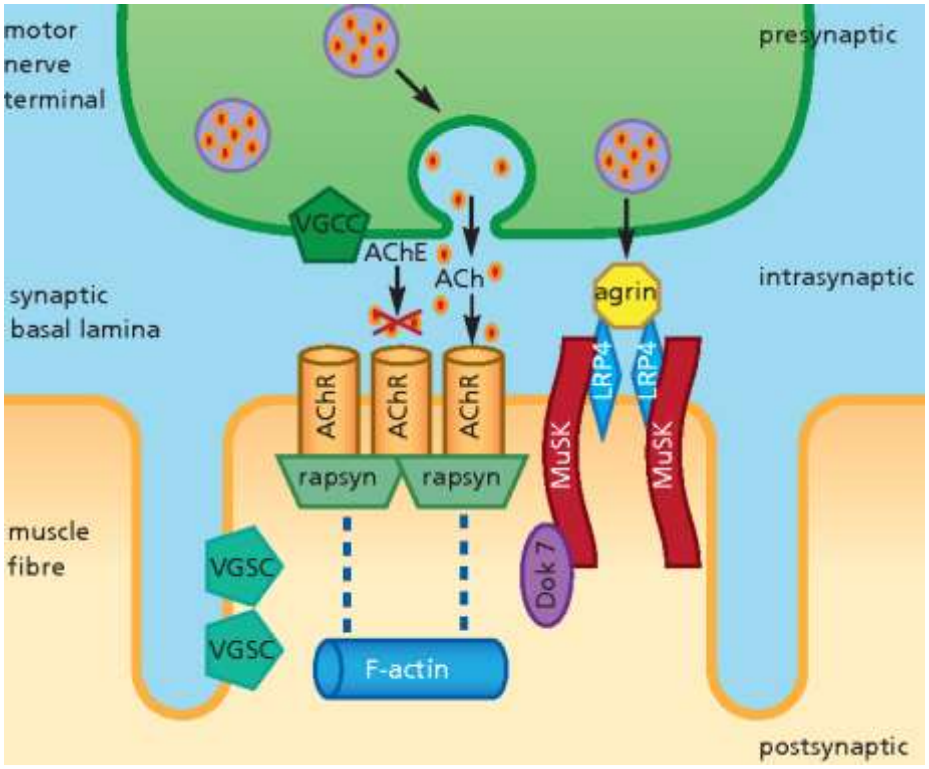


Figure 1.2. Signaling pathways at the neuromuscular junction. AChR - acetylcholine receptor; rapsyn – the AChR clustering molecule; AChE - acetylcholine esterase; LRP4 - Low density lipoprotein 4; Dok7 - downstream of tyrosine kinase 7. (Taken from Barber C., 2017) Diagnosis and management of myasthenia gravis.

A

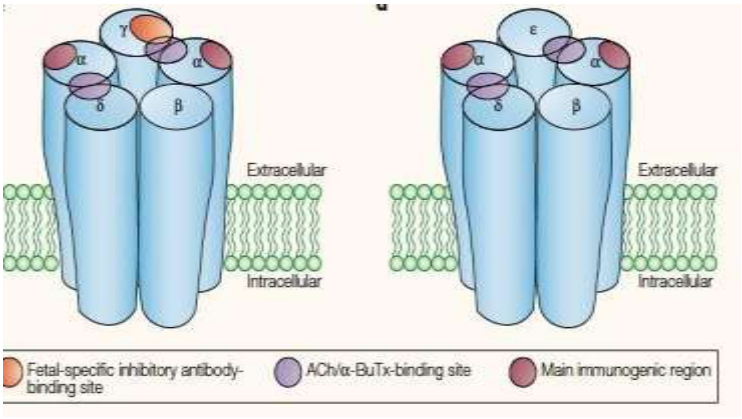


Figure 1.3. Structure of the AChR. Each subunit of AChR consists of a large extracellular domain and four transmembrane domains (above) that occur in an adult and foetal isoform. Most of the antibodies in MG bind to a main immunogenic region on the two α AChR subunits. In addition, some MG patients' antibodies bind to fetal-AChR and can inhibit its function. (taken from Vincent, 2002).

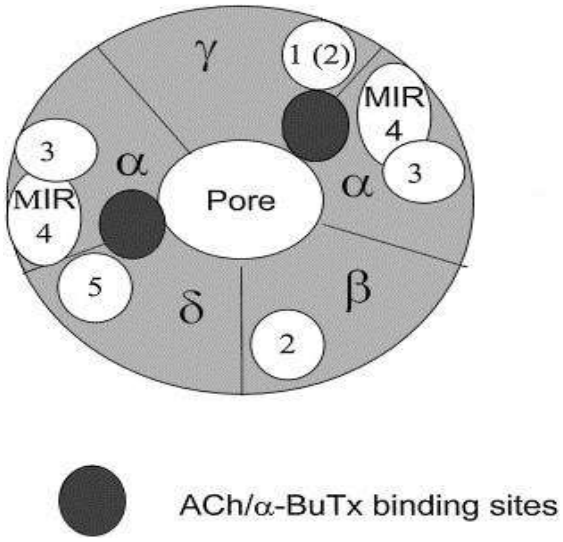


Figure 1.4. A diagrammatic representation of the five binding sites defined by monoclonal antibodies on the extracellular surface. The MIR and α-BuTx binding sites are located on the extracellular surface. None of the mAbs inhibit α-BuTx binding (taken from Jacobson *et.al*., 1999).

1.8.4 Different subgroups of MG

Early-onset myasthenia gravis is defined as presenting before age 40 years and is more common in women. The majority are positive for AChR antibodies, and the thymus gland is enlarged and contains germinal centres. These patients also have antibodies to other muscle antigens, and some have other organ-specific autoantibodies (Oosterhuis, 1989). About 60% of early-onset patients are HLA-B8 and DR3 positive (Compston*et.al.,* 1980; Garlepp *et.al*., 1982).

Late-onset myasthenia gravis is defined as its first presentation in people older than 40 years. The thymus gland is not enlarged, but there is an HLA association with B7 and DR2 (Compston *et.al*., 1980). Thymoma-associated myasthenia gravis can present at any age but the peak onset is during the fourth to sixth decades. There are no clear HLA associations. The patients usually have antibodies to other muscle antigens such as titin and ryanodine receptor (Aarli., 1999).

Ocular weakness, presenting as fluctuating ptosis and/or diplopia, is the most common initial presentation of MG, occurring in approximately 85% of patients but around 80% will then develop generalized symptoms. Ocular myasthenia gravis is restricted to the eye muscles. The titres of antibodies to AChR are lowest in this subgroup, and undetectable in 40–60% of patients. However, electromyography and in-vitro studies on muscle biopsy samples indicate that the disease is probably present sub-clinically in other muscles (Sommer *et.al*., 1993).

About 10–15% of patients have thymoma, while 30% of thymomas are associated with MG. Thymoma is equally common in men and women and is almost always positive for AChR antibodies. Thymoma is often associated with antibodies to titin or especially ryanodine receptor, both intracellular muscle proteins, and these patients may have more

severe disease course often characterized by progressive oropharyngeal weakness. Although the tumour must be removed, the symptoms usually persist after thymectomy unless immunotherapy treatments are given (Skeie, Romi., 2008).

Neonatal MG occurs to about 10% of the babies born to the women having MG regardless of its presentation at the time of pregnancy. It is caused by placental transfer of maternal IgG AChR antibodies. The mothers tend to have raised titres of antibodies specific for the fetal isoform of the AChR (Riemersmae *et.al*., 1996) and the AChR antibodies are transferred to the fetus via the FcRn (as mentioned above).

1.8.5. Muscle-specific kinase MG

MUSK is a transmembrane protein involved in a signaling pathway that maintains the normal functional integrity of the NMJ (see Fig 1.2; Valenzuela *et.al.*, 1995). MUSK antibodies are mainly IgG4 and are not complement activating, unlike the IgG1 and IgG3 AChR antibodies (Koneczny *et.al*., 2013). MUSK antibodies adversely affect the maintenance of AChR clustering at the muscle endplate, leading to reduced numbers of functional AChR (Shiraishi *et.al*., 2005). There is a radioimmunoprecipitation assay for MuSK and a highly sensitive and specific cell-based assay using MuSK-transfected HEK cells. Some of the sera of MuSK-MG reacted with clustered AChRs as well, showing that low-affinity IgG and IgM antibodies to AChR may be present in a few MuSK-MG patients (Leite *et.al*., 2008). IgG4 antibodies were previously known in autoimmune disease and thought to occur as a benign phenomenon in conjunction with resolution of allergic reactions. However, they are recognized in other diseases, such as forms of pemphigus (Huijbers*et.al*., 2015).

1.8.6 Other antibodies

LRP4 is as essential as MusK in the development and function of the adult NMJ, where it performs both anterograde and retrograde signaling roles (Fig 1.2; Koneczny *et.al*., 2014). These roles highlighted LRP4 as a putative antigen of interest, and LRP4 antibodies were reported in a small proportion of SNMG Japanese patients (Higuchi *et.al*., 2011) and more often in European patients (Pevzner *et.al*., 2012). The antibodies were of the complement-activating IgG1 type (Higuchi *et.al*., 2011) and impeded agrin-induced clustering of AChRs in one study. Agrin and Collagen Q, antibodies to agrin have been identified in a small number of 'triple negative' MG sera (samples negative for AChR, MuSK and LRP4 antibodies) at proportions from 15 to 50%, but these antibodies, usually at low titres, were often found also in patients with AChR or MuSK antibodies (Cossins *et.al*., 2012; Zhang *et.al*., 2014). ColQ localises the acetylcholine esterase within the synapse and is thought to interact also with MuSK. ColQ antibodies were reported in 3–4 % of all MG patient sera tested but only 5.5 % of the AChR/MuSK/LRP4 negative samples (Vincent *et.al*., 2018).

1.9.1 Medical treatment of MG

Treatment often relies on institutional preferences and the experience of the prescribing clinician as well as course of the disease. Below are listed various treatments for MG which are still non-specific till date. Acetylcholinesterase inhibitors are usually first line therapy, providing symptomatic relief in ocular and generalized myasthenia. Side effects of acetylcholinesterase inhibitors are both muscarinic (abdominal cramps, salivation and lacrimation) and nicotinic (muscle cramps). Short term immunosuppression and long-term immunosuppression are dependent on prednisolone (Sathasivam*et.al*., 2008; Schneider-Gold *et.al*., 2005) and azathioprine (Palace *et.al*.,1998) respectively. Long-term immunomodulation involves thymectomy in non-thymoma patients which has been shown to be effective (Wolfe *et.al.*, 2016). There

are a number of other drugs and they are described below. Therapeutic monoclonal antibodies are considered later.

Patients with MuSK positive MG tend not to respond so well to acetylcholinesterase inhibitors and are more sensitive to their side effects (Kawakami *et.al*., 2011). The basis of this cholinergic hypersensitivity in MuSK positive MG is thought to relate to the interference of MuSK binding to Col-Q which leads to a reduction of acetylcholinesterase at the neuromuscular junction (Kawakami *et.al*., 2011).

Corticosteroids:

The majority of patients with generalised MG will require immunosuppression and patients with MuSK antibody positive MG may require early and aggressive immunosuppression. Corticosteroids are generally the first line immunosuppressive treatment. It is thought that early use of steroids in ocular MG may prevent generalisation (Monsul*et.al*., 2004). Azathioprine, methotrexate and mycophenolate are among the most frequently used oral immunosuppressant agents.

Azathioprine:

Azathioprine is one of the most widely used second line immunosuppressant agents. It has been shown in two randomized controlled trials to have a steroid sparing effect. The maximal efficacy of azathioprine treatment may only be achieved after one year of treatment (Palace *et.al*., 1998).

Methotrexate:

Methotrexate has also been recommended in MG by the European Federation of Neurological Sciences (EFNS) as a second line agent. It has been found to have a

similar steroid sparing effect to azathioprine in a randomized controlled trial (Heckmann *et.al*., 2011).

Cyclosporine:

Cyclosporine has been found to be effective. In MG in a small randomised trial and it has a fast onset of action compared to other steroids in MG (Tindall *et.al*., 1987). However, it was used only in particularly steroid-resistant cases.

Cyclophosphamide

Cyclophosphamide is an alkylating agent that is rarely used due to its adverse effect profile and is generally reserved for truly refractory cases of MG. It has been shown to be of benefit, showing marked improvement in myasthenic weakness in a small group of myasthenic patients (Drachman*et.al*., 2003).

Plasma Exchange and IVIG

Plasma Exchange (PLEX) and intravenous immunoglobulin (IVIG) are treatments with a rapid but transient effect and are used in certain situations such as myasthenic crisis and rapidly worsening myasthenia (Newsom-Davis *et.al*., 1978).

IVIG has been demonstrated to be effective for the treatment of worsening myasthenic Weakness (Zinman Ng, Brill., 2007), its mechanism of action is complex and involves inhibition of cytokine competition with autoantibodies, inhibition of complement deposition, interference with the binding of Fc receptor, and interference with antigen recognition by sensitized T cells.

1.9.2 Thymectomy in the management of MG

Removal of the thymus gland, thymectomy, is a frequently used treatment for MG. There is a rationale for the use of thymectomy in non-thymomatous AChR antibody positive MG as the B cell follicles and the germinal centres of the thymic tissue are the likely site of antibody production. Since these early reports, thymectomy has become an accepted treatment for early onset MG. Non-randomized studies suggested that thymectomy was associated with an improvement in myasthenic symptoms and an increased likelihood of remission (Gronseth&Barohn., 2002). A recent randomized trial has shown that thymectomy combined with prednisolone, compared to prednisolone alone, does result in an improvement of myasthenic weakness, and allows a lower dose of prednisolone and enhanced quality of life by reducing symptoms and adverse events (Wolfe *et.al*,.2016).

1.10.1. Therapeutic targeting of B cells

Better treatments for autoantibody-mediated diseases require further work on the autoreactive B cells and antibodies that are involved in a variety of neurological diseases. Their causative roles have been formally established in some disorders and are likely involved in the pathogenesis of others. During the immune response against an antigen, B cells bearing antigen-specific receptors proliferate and differentiate into antibody-secreting plasma cells within the germinal centres. This requires the presence of follicular dendritic cells (FDCs) and activated CD4 T-helper 1(Th-1) cells, CD40/CD40 ligand interaction, and a cocktail of cytokines to create the microenvironment necessary for a germinal centre reaction. A few B cells bearing an appropriate antigen receptor are stimulated to undergo clonal proliferation in the dark zone of the germinal center and differentiate into centroblasts, centrocytes, memory B cells, and plasma cells. Antibody-secreting plasma cells migrate out of the follicle into the surrounding tissue (Stott *et.al*., 1998).

Several markers are expressed on B cells during their proliferation, differentiation and development, and help characterize them into subsets. CD 20, CD19, CD27 and CD138 which are found on the surface of B cells and plasmablasts, are currently in research to develop B cell targeted immunotherapy to treat MG and other related autoimmune neurological disorders (Fig 1.5).

1.10.2 Response of B cells to therapeutic abs

Targeting surface antigens expressed on tumor cells and B cells, monoclonal antibodies have demonstrated to be effective as treatments in cancer and autoimmune diseases respectively. Recent successful antibody-based strategies have focused on enhancing anti-tumor immune responses by targeting immune cells, irrespective of tumor antigens or self antigens.

Rituximab is a chimeric monocloncal antibody directed against CD20, a molecule expressed on B cells. It has an established role in the treatment of multiple sclerosis (MS). A number of retrospective studies have demonstrated its efficacy in MG (see Collongues*et.al*., 2012). It reduces circulating B-cell counts, and on the basis of its potential for targeting autoreactive B-cell clones, has a therapeutic role in antibody-mediated autoimmune diseases. It has been a useful treatment in IgG4-related diseases which eliminates a population of B or plasma cells responsible for the production of IgG4 antibodies (Huijbers*et.al*., 2015).

Ofatumumab, is a fully humanized monoclonal anti CD20 antibody which inhibits early B cell development. Ofatumumab induces efficient depletion of peripheral B lymphocytes in rheumatoid arthritis on retreatment after rituximab (Quattrocchi*et.al*., 2016). It has been approved for treating chronic lymphocytic leukemia.

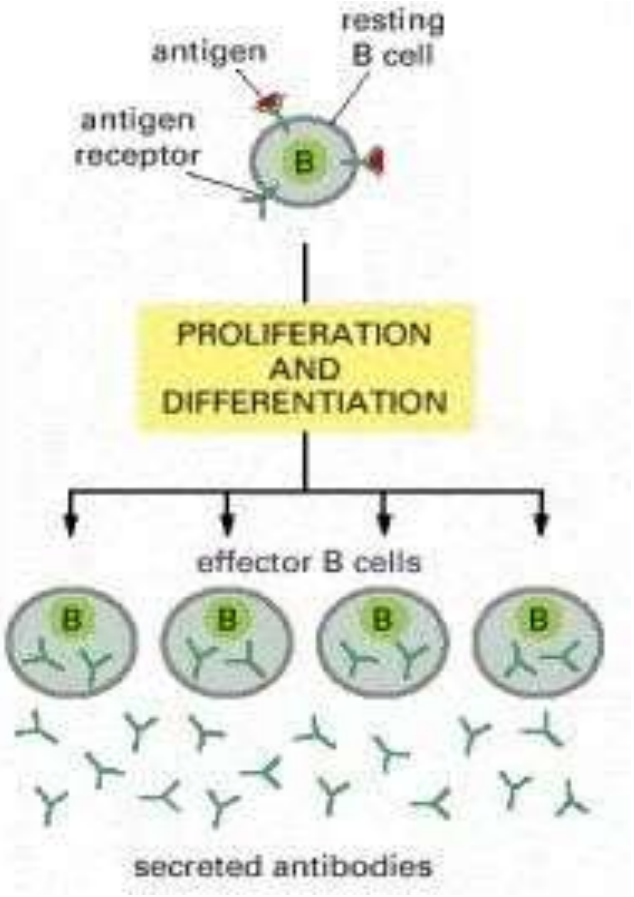


Figure 1.5 Naïve or memory B cells are activated by an antigen binding to surface IgG. The B cells proliferate and differentiate into effector B cells. The effector cells produce and secrete antibodies with a unique antigen-binding site, which is the same as that of their original membrane-bound IgG that served as the antigen receptors. (from Alberts B, Johnson A, *et. al*., 2002).

Its effect on B cells from MG patients' thymus is currently under study. There are several emerging therapies for MG, including tacrolimus, antigen-specific apheresis and other treatments that await evidence of their efficacy and role in the treatment of MG. In addition, complement inhibitory therapy has been shown to be effective in experimental MG (Zhou *et.al*., 2007) and might prove promising in myasthenic crisis and particularly in ocular MG, because of the low expression of complement regulators in extraocular muscle (Kaminski *et.al*., 2004). Eculizumab is a humanized monoclonal antibody that cleaves the complement component C5 and hence blocks the formation of the terminal complement complex. (Howard *et.al.*,2013).

Recent findings suggest that B cells have critical positive and negative roles in autoimmune disease (Matsushita *et.al.,* 2008). Therapeutic strategies that specifically target AChR antibody-producing memory B cells would be a major advance, but for this it is necessary to identify and characterize the AChR-antibody specific B cells.

The myasthenia gravis thymus includes germinal centres and immune cells that are involved in the pathogenesis of the disease, providing a potentially valuable source of B cell and plasmablasts that can be studied in vitro.

1.11 Membrane vesicles used as an antigen

Membrane vesicles (MVs) are released from different cells and are an emerging interest as biomarkers in a whole range of disorders. Classification, isolation, detection and the biological functions of membrane vesicles is still under investigation in these different fields (reviewed by Gyorgy *et.al*., 2011). The intracellular space of multicellular organisms contains solutions of metabolites, ions, proteins and polysaccharides. MEVs include exosomes, activation- or apoptosis-induced microvesicles, microparticles and apoptotic bodies (Fig 1.6).

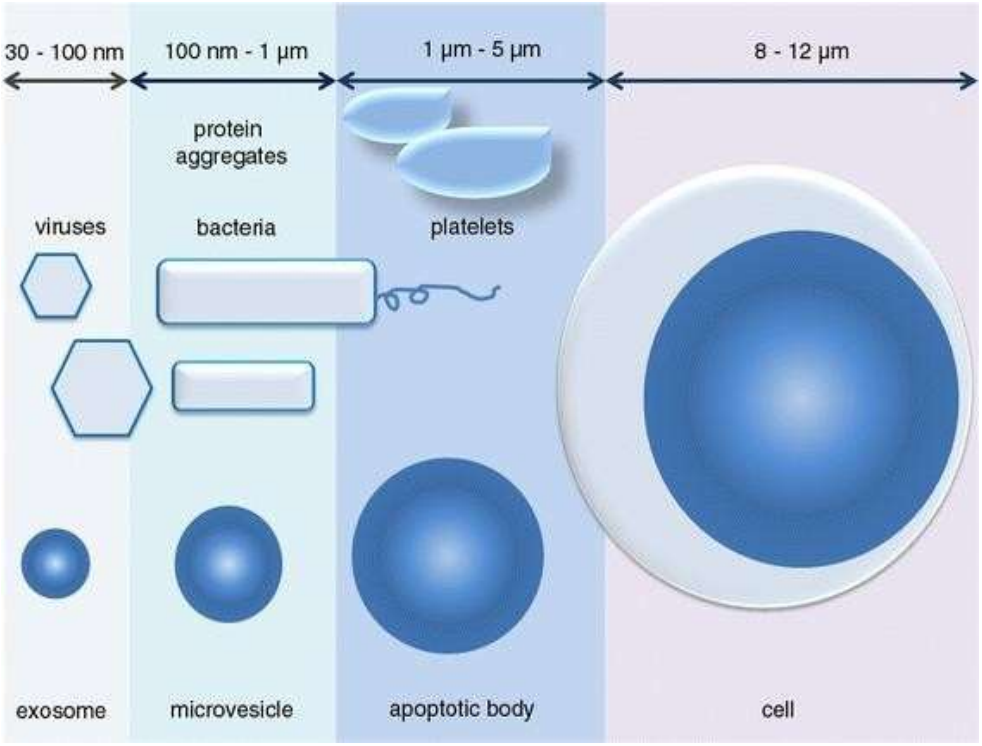


Figure 1.6. Size ranges of typical membrane vesicles in comparison with viruses, bacteria and cells. Exosomes share size with viruses, microvesicles share with bacteria or protein aggregates and then largest is a cell. (from Gyorgy *et.al.*, 2011)

The MVs to be used in this thesis lie between 100nm to 1µm and derive mainly from the cell surface membrane of transfected cells.

Vesiculation may be classified as a type of trogocytosis. The transfer of membrane components between donor and acceptor cells was coined “trogocytosis” (from Greek“trogo”, meaning “gnaw” or “bite”). Some authors suggest that trogocytosis challenges classical theories of cell autonomy (Rechavi*et.al.*, 2009), as cells may receive membrane and cytoplasmic fragments from other cells. Two forms of membrane transfer (trogocytosis) have been described: via nanotubes or via membrane vesicles (Davis, 2007). This project focuses on membrane vesicles formed from the membrane of mammalian cell lines. The biogenesis of membrane vesicles essentially distinguishes exosomes from MVs and apoptotic bodies.

Aim of the thesis:

There were three aims of this study,

1. To characterize AChR abs in MG sera and in their thymic suspensions for the selection of suitable patients for studying the synthesis of the antibodies.

2. To produce AChR expressing membrane vesicles to restimulate antigen specifc B cells in the thymic suspensions.

3. To identify B cells (memory B cell subset) which are specific for AChR antibodies using AChR membrane vesicles, as a source of antigen presentation and for identification of AChR-specific.

CHAPTER 2

Materials and Methods

2.1 Introduction

Although much is now known about autoimmune diseases, more specific therapies are needed. This study aimed to characterise the B cells in myasthenia gravis in order to provide a more directed and cell-targeted approach to treat MG.

To detect antibodies and their specificity, several tests are available including western blotting and, more recently, line blots, both of which use linear, denatured targets, and are often used to detect antibodies to intracellular targets. Newer assays have been developed that maintain the native structure of membrane proteins to permit detection of antibodies that recognize extracellular targets; specifically cell based assays (CBAs), radioimmunoprecipitation assays (RIPA), and fluorescence activated cell sorting (FACS).

2.2 Patients providing serum and thymic samples

Patients selected for this study were mainly seropositive Early Onset Myasthena gravis (EOMG), selected to cover a wide range of clinical parameters including age at the onset of disease/thymectomy, prior MG durations and AChR antibody titers. The origin, demographic and clinical details of patients were available from Prof Willcox but only the demographics are shown in Chapter 3. All samples were obtained with informed consent under approved ethical guidelines.

2.3 Samples for analyses

Serum samples were stored at --20^{o}C, and thymic cells and thymic tissues from MG patients, thymectomized between 1970 and 2007, had been stored in liquid nitrogen

(LN_2) by Prof Willcox and colleagues. None of the thymectomized patients had received immunosuppressive drugs or steroids before thymectomy. Blood samples of the patients were collected at regular intervals over the years between 1970 and 2007, before and after thymectomy, in order to measure the antibody levels in the sera. Some EOMG samples were plasmas obtained from anti-coagulated blood samples, and there was always some fibrin clot evident on thawing. These samples were centrifuged, and the supernatants transferred into clean tubes avoiding as much of the floating fibrin as possible. They were then carefully aliquoted into smaller tubes or vials, labelled, stored at -20^{o}C and each vial used once only to avoid deterioration on further freezing and thawing.

2.4 Preparation and transformation of E.coli for plasmid preparations

This project started with CBAs of antibodies binding to AQP4 and AChR receptors expressed on human embryonic kidney 293T cells. AQP4 is a single subunit protein which has a single transmembrane protein and expresses well on the plasma membrane. Binding of antibodies can be detected easily and read with an immunofluorescence microscope.

To provide cDNA for transfection, plasmids needed to be produced by highly competent *E. coli* cells, (JM109), a K strain that is *rec*A and *end*A to minimize recombination and improve the quality of plasmid DNA. These competent cells were available as standard format 200µl aliquots provided by Promega.

Five hundred mL of Luria Broth (LB) was prepared by adding tryptone (10mg/mL), yeast extract (0.5mg/mL), NaCl (0.5mg/mL) and agar (0.75mg/mL) to RNase free water. Broth was autoclaved and antibiotics, ampicillin (100µg/mL) and kanamycin (50µg/mL), were added. Broth was then poured into two different kinds of antibiotic

resistant petri dishes filling 3/4th of the dish, under the bacterial culture hood. Petri plates were separately marked with a red/black stripe indicating Amp^R and a blue/black stripe indicating $Kan^{R.}$ All the plates were left in the hood until dried completely.

2.4.1. Isolation and preparation of plasmid DNA from *E.Coli*

The different cDNAs for the AChR subunits and the clustering molecule rapsyn-EGFP were originally designed and produced by Prof. David Beeson *et.al* in the laboratory and were to be used for transient transfection of HEK cells. First, pcDNA 3.1 hygro (Invitrogen) and pEGFP-N1 were purchased from Clontech Laboratories, Inc. pGEM®-T Vectors provided by Prof David Beeson were used as they contain numerous restriction sites within the multiple cloning region (Fig 2.1). The pGEM®-T Vector multiple cloning region is positioned by recognition sites for the restriction enzymes EcoRI, BstZI and NotI, providing three single-enzyme digestions. These vectors have high copy numbers and contain SP6 RNA polymerase promoters positioning a multiple cloning region within the α-peptide coding region of the enzyme β-galactosidase.

cDNAs for AChR subunits α, β, γ or ε, δ and rapsyn-EGFP were expressed by transforming pGEM-T easy vector containing pDNA into High Efficiency Competent Cells on the sterile agar plates. pDNA was diluted with RNase free water in the ratio of 1:10, at an optimal working concentration of pDNA between 80-100 ng/µl. For each preparation, a single colony was used to inoculate 600 ml of Luria Broth (LB) and grown overnight in a shaking incubator at 37°C. cDNAs were purified using the Promega kit for Maxi preps (provided by Cat. L2001, Promega). DNA yield was finally measured using ND100 Nanodrop software, in nanograms. The optimum working concentrations of plasmid DNAs were 0.8-1.2 µg/mL.

2.5 Cell Culture techniques

General conditions and reagents

HEK 293T cells (human embryonic kidney), TE671 (human rhabdomyosarcoma with muscle like properties) and CN21 were grown in DMEM (Dulbecco's modified Eagle's essential medium, Sigma Aldrich, UK) supplemented with 10% fetal calf serum (FCS, HYclone, USA), with 100 units/mL each of antibiotics (A+A; penicillin and streptomycin from Invitrogen, UK). All the cells were cultured at 37^{o}C in a humid atmosphere with 5%CO_2.

Examples of the HEK 293T cells are shown in Fig 2.2. The cells were split at around 80% confluency. Culture medium was aspirated and the cells were detached by trypsinization (0.5% trypsin, 0.5mM EDTA, Invitrogen) at RT. Trypsinised cells were washed at 1500 rpm for 5 mins. Cell pellets were resuspended in culture medium (DMEM + FCS + antibiotics as above) and diluted 1:9 into the flasks for propagation.

2.6 Thawing of cells

Cryo-vials containing frozen cells including thymic cells and peripheral blood mononuclear cells (PBMCs) stored in liquid nitrogen were transported in dry ice for further use. Cells were thawed at 37 °C in a water bath in the cell culture hood. Culture medium (RPMI) was added dropwise into the vial up to 0.5 mL, and the content transferred into larger tubes containing 80-100µL of DNase for 300x 10^6 cells, followed by slow addition of more culture medium to the tubes until the volume reached 10 mL, taking around 3 minutes. Cells were further washed and pelleted at 1200xg for 7

minutes, then re-suspended in complete medium (RPMI + 5%FCS + A+A) of 10 ml and cultured in 24 well plates and 96 well plates provided by Corning Inc.

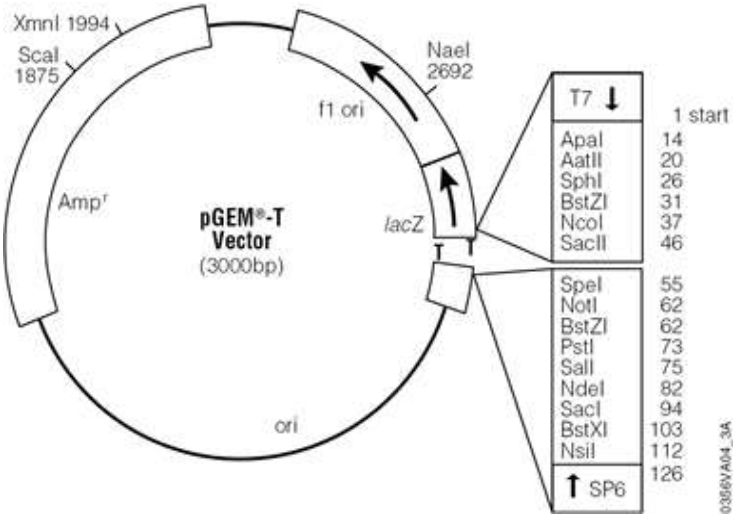


Figure 2.1. The pGEM®-T Vector System II. This contains JM109 Competent Cells in addition to all of the pGEM®-T Vector System I

2.7 Enzymatic/mechanical disintegration of thymic cells

Fresh thymus tissues were dispersed mechanically by chopping and passing through a 1mm mesh. During the subsequent washing, clumps were pelleted by centrifugation at low speed (800 rpm) and the single cell suspension was washed once and the cells used as Thy conv (conventional thymic cells) and frozen in LN_2 for future use. From some Thy conv samples, low and medium density fractions were separated by ficoll gradient, with recovery of 15% and 40% of the total of 300 $\times 10^6$ cells.

2.8 Splitting HEK293T, CN21 and TE671 Cell lines

HEK cells were grown in the incubator at 37ºC in 5% CO_2 usually in (175 cm^2) culture flasks. When 80-90% confluent, the medium was aspirated under sterile conditions in the category II hood. 1 ml of 10 X trypsin in PBS was added to the cells carefully so that cells were not damaged. The flask was rotated gently, so that the trypsin solution covered all the cells, and after 1 minute the trypsin was discarded. The process was repeated with another 3 ml of 10 x trypsin. The cells were left for 30-90 seconds in the culture hood. Then, gentle rotation was applied to the flask until the cells could be seen beginning to detach from the plastic surface. The flask was tapped gently to dislodge the cells. 8 ml of fresh DMEM with 10% FBS/1xPSA or antibiotic/antimycotic was added. The medium containing cells was then transferred to 50 ml falcon tubes. Cells were centrifuged at 1100 x g for 4 minutes at RT. The supernatant was aspirated and the pellet was resuspended in 9 ml of medium in the same falcon tube. 1 mL containing around 0.45-0.50x10^5 cells of resuspended cells from the falcon tube were added to 39 ml DMEM/10% FBS/PSA in a new 175cm^2 flask. This method was optimized by counting the HEK293T cells using the Neubauers counting chamber.

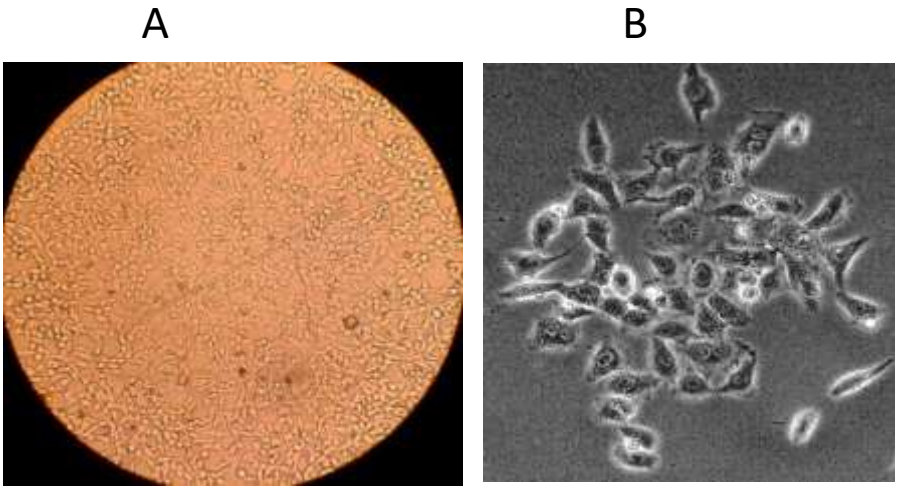


Figure 2.2. Cultured HEK 293 T cells. As seen in a 175cm^2 flask of cells grown in DMEM with FCS. B. HEK 283T cells taken 48 hours after first passage at the magnification of 40X under inverted microscope. Cells were usually harvested at 80% confluency for CBAs.

To obtain HEK 293T cells at high confluency for the following day, 3 ml/1.5 x 10^5 cells were added to a new flask with 25 mL of fresh culture medium in 175cm^2 flask. Cells were transferred carefully to the incubator set at 5%CO_2,37^oC.

2.9. Preparation of cells for Immunofluorescence assay

Transfection of cells for the expression of AChR and rapsyn:

For transfections, HEK 293T cells at 80% confluency were washed at 1500 rpm and the pellet was re-suspended in 5 or 10 mL of culture media. These were then seeded at a concentration of 0.45 x 10^6 cells per well in 6 well plates pre-coated with poly-L-Lysine and with 4 plastic coverslips in each well and grown for 24- 30 hours under 5% CO2 at 35^oC.

The plasmid DNAs were designed for transfections as described above. pDNAs for human adult and fetalAChR and rapsyn-EGFP were transfected at 3µg total DNA per well (dividing between α, β, δ, γ or ε, with rapsyn-EGFP) in 2.5 mL of DMEM with 1.5 µL of transfecting reagent polyethylenimine (PEI). After 14-16 hours, the medium with PEI was discarded by carefully aspirating and replaced with fresh culture medium and the cells kept in the incubator for another 21-24 hours before use.

Similarly, for MuSK and AQP4, HEK 293T cells were transfected as above with 3µg of each plasmid DNA per well using polyethylene imine (PEI). After 14-16 hours the medium was replaced with fresh culture medium and left for 21-24 hours in the incubator. After 24 hours, cells were ready for testing/immunofluorescence assay.

Antibody testing using the AChR, AQP4, or MUSK transfected cells:

The techniques used for measuring antibodies by CBA are shown graphically in Fig 2.3. Specific antibodies were identified in the sera of MG patients by immunofluorescence

cell based assay (CBA). Patients' sera were diluted 1:20 and incubated with the cells for 1 hr at RT before washing to remove any unbound antibody. The cells were then fixed with 4% formaldehyde for 5 mins and washed gently three times with DMEM only. After the third wash, fluorescent-labelled goat anti-human IgG (Alexa-Fluor 568 (red) or 488 (green)) was incubated with the cells for 45 mins at RT or at 4^0C. After final wash with PBS, any cells with specific antibodies were identified visually by fluorescence microscopy using DAPI to identify the HEK cells. Sera of 10 patients, identified with high antibody levels were diluted in a serial order of 1:100, 1:200, 1:400, 1:800, 1:1600, 1:3200 to check their antibody levels at highest dilution.

Healthy control sera were from healthy laboratory individuals and were tested in parallel. After optimization all the sera were repeated in similar conditions to ensure accurate comparisons.

2.10 Thymic cell cultures

Thymic cells were cryopreserved in 5% DMSO with 50% v/v FCS and stored in aliquots in liquid Nitrogen (LN2, at -150^{o}C to -190^{o}C; usually set at -160^{o}C), information provided by Prof. Willcox. The thymic cells were transferred to dry ice before thawing as described in 2.6. The live thymic cells obtained after counting on Neubaurer counting chamber were pooled and plated at 6 million cells per well in 24 well plates or 3 million cells per well in 200 µL in 96 well plates. Over the consecutive four weeks, a quarter (50 liters) of each supernatant was collected and replaced with the same quantity of fresh medium (RPMI+ 10% FCS + A+A + L-Glutamine) and the three supernatants for each condition pooled. During the first week, the supernatant was collected three times at two-day intervals to compare the antibody levels in the first week versus the consecutive three weeks.

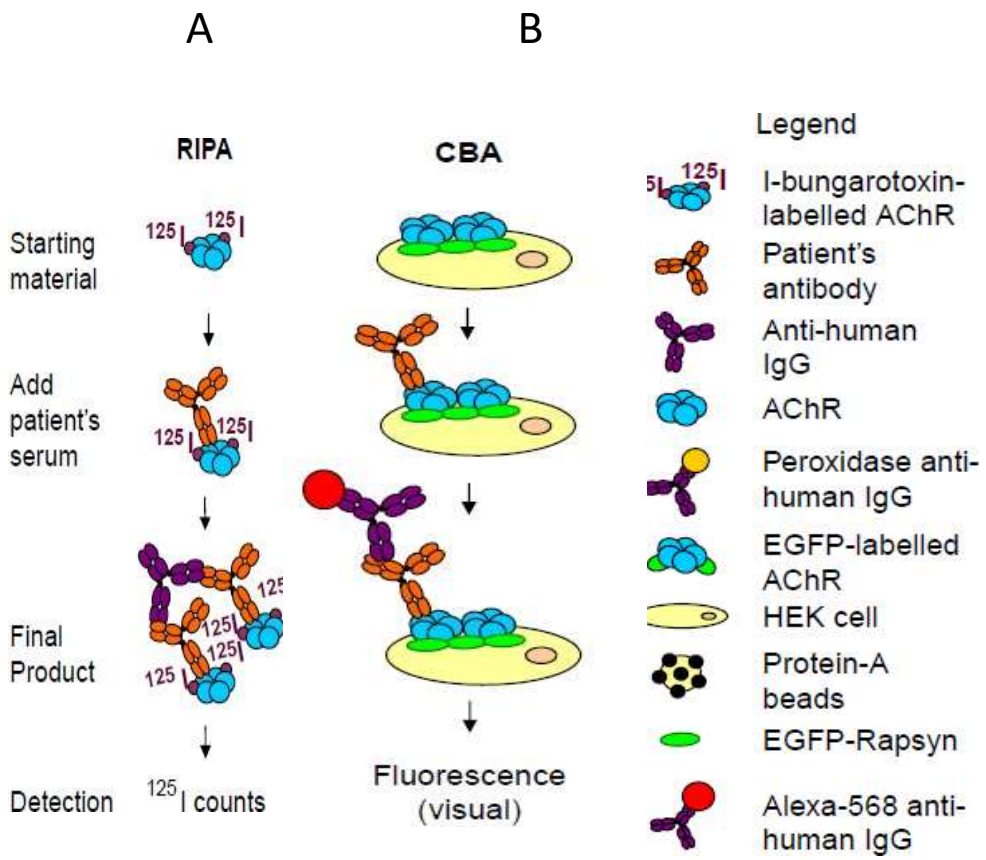


Figure 2.3. Different techniques used to detect AChR antibodies in patients' sera. A. RIPA shows patient IgG binds to ^{125}I labelled AChRs and is precipitated by excess anti-human IgG. B. CBA shows AChRs expressed on HEK cells binds patient IgG antibody, which is then detected by Alexa fluor 568 anti-human IgG. C. Symbols used to show ab and antigens in this figure. Taken from Leite, Waters & Vincent, 2010.

Pokeweed Mitogen at 1/10 and 1/100 dilution were used to stimulate the cells and cycloheximide at 1/100 was used to inhibit the production of antibodies (cycloheximide is a potent inhibitor of protein synthesis whereas pokeweed mitogen stimulates proliferation and activation of B lymphocytes). AChR antibody in the supernatants was measured using ^{125}I- labelled α-bungarotoxin AChR.

A total of 10 patients' thymic cells were cultured and their supernatants tested by CBA. Supernatants collected from thymic cell cultures were incubated in 150 µLs with the transfected HEK 293Tcells and binding of any antibodies identified visually under fluorescence microscope as above. None of the cultures were positive by CBA and only three patient cultures, with some RIPA positivity (see below), were used for the subsequent experiments. Supernatant from thymic cell cultures of the 10 patients were selected on the basis of high antibody levels in sera CBA and RIPA.

2.11 Radioimmunoprecipitation assay for AChR antibodies in sera or culture supernatants.

The RIPA assay is illustrated in Fig 2.3. ^{125}I-α-bungarotoxin AChR (RSR Ltd Cardiff, UK) was incubated with 5 µl of test sera and 50 µl of culture supernatants, in separate tubes, under cold temperature overnight. After 16 hours, normal human serum (to provide carrier IgG) was added, if required, and anti-human IgG (from RSR Ltd.) and left for 2 hours at RT. Any resulting complex of labelled receptor and AChR antibody were immunoprecipitated by pelleting in the bench centrifuge at 13000 x g for 5 mins, washed with PTx and counted on gamma counting chamber using ^{125}I-α AChR protocol. Selection of the serum samples for serial dilutions was based on the high titres of antibody levels on CBA and RIPA.

2.12 Preparation, production and staining of membrane vesicles

Extracellular membrane vesicles were obtained because they have been shown to express high levels of certain antigens such as AQP4, and could be used as a source of the proteins for further studies. Transfection of HEK293T cell line with the plasmids containing AQP4 or AChR subunit genes were performed as above to provide cells expressing the AQP4 or AChRs. After 4 days of incubation at 5% CO2 at 37.5°C**,** the supernatants were spun and suspended in 50 µL of PBS with100 mM sucrose + 0.02% of Sodium Azide (NaN_3) + 1:100 protease Inhibitor cocktail (PI). To check for the presence of AChRs, the cells or vesicles were incubated with high titre serum and anti-human Alexa fluor 568 IgG (H+L) to detect antigen binding of patient's antibody to the vesicles. The concentrated stock was preserved at -20 °**C** for future use. In some experiments, the vesicles were used fresh, without NaN_3 or inhibitors added.

Cell lysates with and without the expression of AChRs were prepared using HEk293T cells incubated for 1-3 days after transfection. The cells were detached from the surface of the flask by using RIPA buffer 1x solution which contains 150mM NaCl, 1.0% IGEPAL, 0.5% sodium deoxychelate, CA 630, 0.1%SDS, 50mM Tris and pH is 8.0. The suspensions were centrifuged at low speed at 800 to 1000xg and the nuclear debris discarded.

2.13. Isolation of Peripheral Blood Mononuclear Cells

Cells were separated from whole blood samples in heparinised tube obtained from informed consent healthy donors by density centrifugation over Lymphoprep which is a readymade endotoxin tested solution especially made to isolate PBMCs. The protocol was provided by company Axis Shield.

After washing with FACS buffer 0.5-1.0 x 10^5 cells/mL in a test tube were incubated for 15 minutes with 20 µl of human Fc receptor binding inhibitor on ice, followed by buffer wash and subsequently stained with primary antibodies (fluorochrome labelled anti-CD3, CD19) at optimized dilutions. The samples were washed and followed by incubation with the secondary antibodies as listed in Table 2.2. After final washes at 3500 rpm for 5 mins, the cells were resuspended in FACS buffer, called as staining buffer and the cells were fixed, kept at 4^0C in the dark, and analysed the next day or immediately after resuspension in 100 µL of FACS staining buffer. The results were analyzed using Summit software.

The thawed thymic cells were stained on day 0 and analyzed using the same method as for PBMC.

2.14 Western Blotting

AChR specific membrane vesicles harvested after 4-5 days of transfection were tested by western blotting to check for AChR in the membrane or vesicle preparations. Lysates obtained from preserved AChR vesicles and freshly prepared cell lysates from AChR transfected HEK293T cells were obtained in volumes of 20uL and 50uL respectively. 1:10 dilution of reducing agent and 1:4 dilution of loading dye were added for each gel electrophoresis well and proteins were denatured at 90ºC for 5 mins. 10µL of samples were run in each well in 10 x sodium dodecyl sulphate buffer (ME-SDS) at 150 V for one and a half hour. The proteins were transferred to PVDF membrane in the presence of transfer buffer containing 1x methanol at 50 V for an hour and then blocked in 10% of non-fat milk with 0.1% of tween 20 in PBS for an hour at room temperature.

Nitrocellulose membrane was incubated overnight with AChR β subunit cytoplasmic anti-mouse monoclonal antibody in (1: 1000 dilution), while shaking. After 12 hours of

incubation the membrane was washed thrice with 0.1% tween 20 in PBS and incubated with HRP conjugated secondary antibody (goat anti-mouse at 1:2000 dilution) for one hour at room temperature, while shaking, and washed three times. After the final wash, Enhanced Chemiluminescence (ECL) was used and the film was developed in the dark room.

2.15 Flow cytometry

For immunostaining of thymic cells and sorting of antigen specific B cells and plasmablasts by FACS, Raji cells, a cancer B cell line, was first used in order to set up the B cell detection analysis on the FACS analyser. Later the Raji cells were used as a positive control with HEK293T in one of the experiments.

B and T lymphocytes from PBMC's of healthy donors were analyzed on the FACS by labelling them with specific CD19, CD20 and CD3 fluorescently labelled antibodies. Anti-Fc block (20 µl/test) was first used to block the Fc receptors present on B cells and left for 20 minutes in cold. The same method was used for identification of B and T lymphocytes in thymic cultures.

To try to identify AChR specific B cells, AChR transfected HEK 293T cells were used to provide AChR-expressing vesicles. The HEK cells were first transfected with EGFP (green) and IRES (red) and analyzed on the Cyan Analyser to check the expression of the DNA in the cell line. The lymphocytes were then pre-incubated with AChR-EGFP transfected HEK293T cells for 45 minutes or with AChR specific vesicles for 45 minutes in different tubes.

After a wash with staining buffer (human serum in PBS at 1:100 dilution) the cells were incubated for 30 minutes on ice with fluorochrome labelled cell markers (Becton and

Dickinson and Miltenyi biotech). Cells were washed and analysed on CyAn™ ADP Analyzer and AChR specific memory B cells were sorted with BD FACSARIA III. In some experiments the cells were fixed and resuspended in staining buffer and analysed after 24 hours.

CHAPTER 3

Characterization of acetylcholine receptor antibodies in MG

Introduction

Antibodies to AChR in MG (sometimes called AChR-MG) have been recognized over many years and are important for the diagnosis of MG. The most commonly used diagnostic technique to detect AChR antibodies in patient's sera is the radioimmunoassay (RIPA) (Vincent *et.al*., 1985). It is now based on a mixture of fetal and adult ^{125}I-α–BuTx labelled AChR extracted in detergent from two human muscle cell lines, one expressing fetal AChR (TE671) and the other expressing mostly adult AChR (CN21). The assay does not distinguish between antibodies binding one or other of the two isoforms. Traditionally, RIA and ELISA are used to test for antibody-mediated diseases at the NMJ especially MG, but these techniques may limit the sensitivity of antibody detection as some antibodies bind poorly to the detergent-solublised AChR, and bind more effectively to the intact native receptors, expressed on the cell surface as they are at the NMJ. These limitations led to the development of assays specific for pathogenic extracellular epitopes, which are termed cell-based assays (CBAs). The expression of proteins on intact live cells avoids the detection of antibodies to intracellular epitopes, which are unlikely to be pathogenic. CBAs as diagnostic tests have now been developed for many different diseases at the NMJ and in the central nervous system (CNS), e.g. MG, neuromyelitis optica with AQP4 antibodies and limbic encephalitis with LGI1 antibodies (see Vincent *et.al.*,2011for a review).

Past studies suggest that autoantibodies against AChRs were present in 85% sera of MG patients (Lindstrom *et.al.*,1976; Merriggoli& Sanders, 2009). The 15% of patients with generalized MG whodo not have AChR antibodies are often referred to as seronegative

MG, among these a variable % are associated with MuSK antibodies, (McConville *et.al.*,2004).

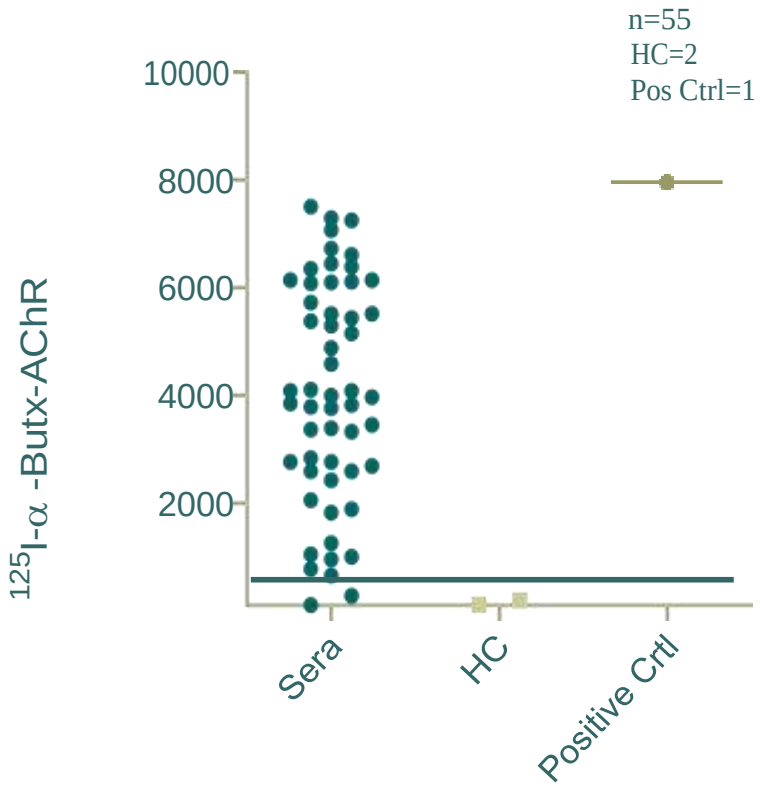
It was previously hypothesized that clustering of the AChR might improve the detection of AChR antibodies. Cell based assays (CBA) were established to test the antibody levels against clustered AChRs and these can also be used for identifying IgG subclasses (Leite*et.al.*,2008). These assays can also be modified to look for either adult or fetal AChRs in patients' sera (Vincent *et.al.*, 2012 Vincent *et.al.*, 2018). Here the binding of antibodies to adult and fetal AChRs were studied in MG sera and supernatants from thymic lymphocyte cultures.

3.1 Patient samples

RIPA and CBA were both used for autoantibody detection. There were 210 sera available for testing but on the advice of Prof Willcox, only 55 were used in RIPA. These included 30 from whom he had thymic cultures. 20 samples from healthy laboratory individuals were used as healthy controls. For the CBAs, 146 sera samples were tested including 15 AChR positive sera from the laboratory routine diagnostics. In addition, 30 NMO-IgG sera (some negative, some very strongly positive) were used to set up the CBA. Table 3.1 shows the examples of the patients studied with their antibody test results. The patients were of all ages including a few children, but mainly EOMG as older patients are not usually thymectomised. From the clinical details available from Prof N Willcox, 80% of the early onset patients had thymic hyperplasia and 20% had thymoma.

3.2 AChR identification on ^{125}I-α BuTx through RIPA

Fig 3.1 shows the results of the RIPA assy for AChR antibodies in 55 MG sera and by two healthy control sera, and one known AChR-positive control. The two healthy



Sera = MG patients (n=55)
HC= Healthy controls (n=2)
Positive control (n=1)

Figure 3.1 Radioimmunoprecipitation assay for AChR antibodies. Immunoprecipitation of ^{125}I-α-BuTx labelled AChR with sera from 55 MG patients and 2 healthy controls. A serum from routine clinical assay was used as positive control. The cut-off was based on the two healthy controls, and similar to that used in routine clinical assays. All but two of the 55 MG patients were positive by this RIPA.

Table 3.1 Patients samples used and AChR antibody results

Patient	Activity of AChR(cpm) 12000 Serum 5 µL	Activity of AChR(cpm) 12000 Serum 1 µL	AChR antibody CBA score
Patient 1	1665	1392	4
Patient 2	1579	1170	3.5
Patient 3	7977	7399	4
Patient 4	904	801	0
Patient 5	2981	2019	3.5
Patient 6	1676	679	3
Patient 7	4944	3277	4
Patient 8	4588	4345	4
Patient 9	1907	1108	0
Patient 10	9199		3.5
Patient 11	9936		3.5
Patient 12	7317		3.5
Patient 13	5078		3.5
Patient 14	3473		3
Patient 15	10365		4
Patient 16	1004		0

control sera immunoprecipitated less than 200 cpm which was subtracted from all results for plotting. There was a wide range of antibody levels in the MG patients with two within the range of the healthy controls (apparently negative).

3.3 Cell-based assays for AQP4, fetal and adult AChR antibodies

To establish the CBA technique, the well-expressed antigen, AQP4, was used. cDNA for AQP4 was transfected into HEK 293T cells and 30 NMO sera were tested for antibody binding. The cells showed very clear binding of antibodies in many of the NMO sera (for examples with a range of scores, see Fig 3.2). With the strongest AQP4 antibody serum, the fluorescence was so strong that some appeared to be intracellular but the linear membrane staining could also be clearly seen in Fig 3.2. Interestingly, this serum also bound to small vesicles between the cells; these are likely to be the membrane extracellular vesicles to be investigated in Chapter 4.

For the AChR assays the CBAs used cells co-transfected with fetal or adult AChR subunits and rapsyn that clusters AChR at NMJ. This assay detected AChR antibodies in a few patients, earlier regarded as seronegative with conventional RIPA (Leite et al., 2008). HEK cells were grown on glass coverslips in 6-well plates and then transfected with the appropriate adult and fetal AChR subunit DNAs and rapsyn-EGFP as described in Methods.

The following day medium was changed, and binding of antibodies tested one day later. The live cells were exposed to the patient samples, before fixing and detection of bound IgG using anti-human IgG. All the 161 samples were tested by CBA and 10 HCs starting with 1:20 dilution of the sera. Fig 3.3A shows the high density of HEK293T cells identified by DAPI staining of the nuclei suggesting good cell survival. Fig 3.3B shows the green fluorescence given by rapsyn-EGFP. In Fig 3.3C, shows the red

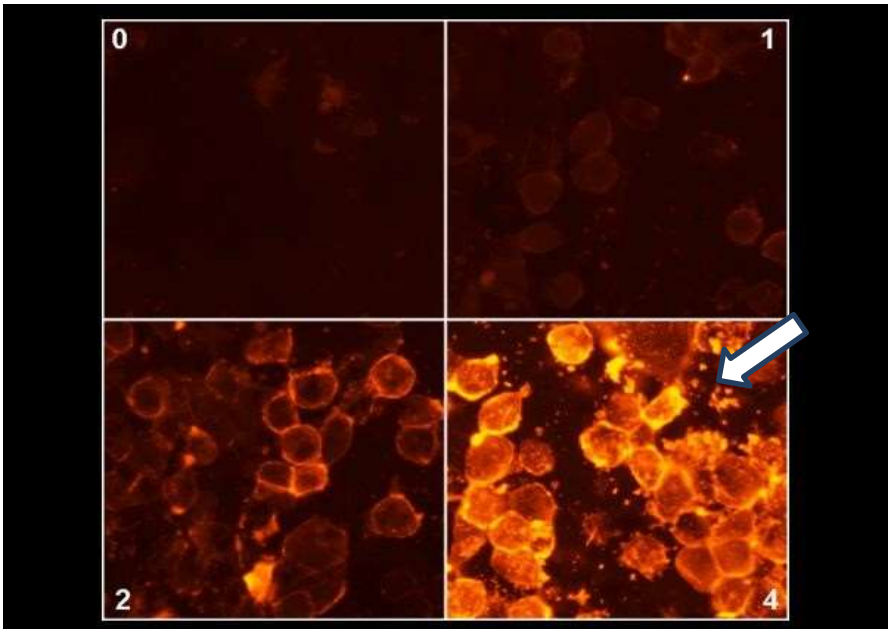


Figure 3.2. Binding of AQP4 antibodies from NMO patients' sera detected using Alexa fluor anti-human IgG (red). Results show binding of four different sera, scoring from 0 (negative) to 4 (very strong). Binding is clearly on the cell membrane of the highly transfected cells. The strongest binding sample (score 4) includes also detects small particles around the cells (arrow), most likely to be membrane vesicles that are produced by AQP4-transfected cells.

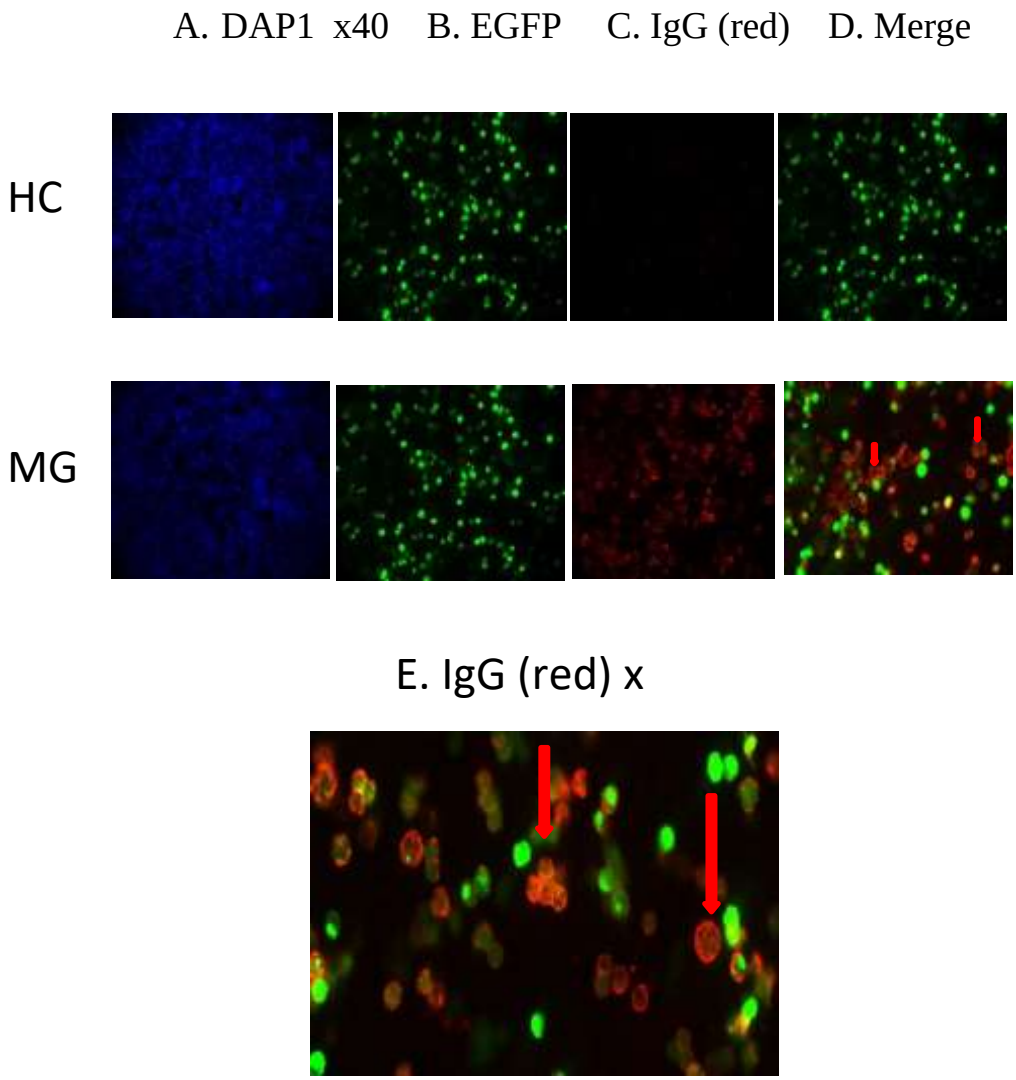


Figure 3.3. Binding of antibodies to the AChRs on the extracellular domains of the membrane. Clustered AChR expression on the cell surface of HEK 293T cells. A. DAPI binding shows the live cells. B. Rapsyn EGFP (green) cells which demonstrates good transfection rates. C. Anti-human IgG Alexa fluor 568 (red) binds to IgG on the clusters of AChRs. D, E. Merged images of IgG binding to the AChR clusters on the membrane of the cells at 40X and 100X magnification. The HC serum (top) did not bind to the cells, whereas the MG serum bound strongly, scoring 4.

fluorescence of anti-human IgG demonstrating that some of the cells have astrong IgG antibody bound to the surface. Fig 3.3D and E show two examples of stronger IgG antibody (red) with merged green EGFP fluorescence. The very strong green cells often did not have detectable IgG bound, but many of the less strong EGFP-expressing cells showed multiple clusters of IgG on the cell surface. Green clusters seen at higher magnifications represent the rapsyn-EGFP that clusters the AChRs.

The binding of the human IgG was scored on a range from 0 – 4 as shown in Fig 3.2 for AQP4 antibodies. The results of the AChR antibodies tested for binding to both fetal and adult AChRs (expressed independently but in parallel for testing) are shown in Fig 3.4A and C. Many of the MG sera showed very strong binding of antibodies (score 4). Healthy control sera showed no detectable binding and were scored 0 or 0.5, but a few of the MG sera were also negative on these CBAs.

The CBAs could therefore be used to compare the antibody binding to fetal and adult AChR in MG patients. Although many sera bound strongly to both AChR forms the scores were not the same (Fig 3.4A), and overall, there were more high scores on the fetal isoform ($p<0.001$ student's t test). Fig 3.4C, D shows the results from thirteen sera of thymoma MG patients. Although the average scores were a little lower than the non-thymoma patient scores all were positive with slightly higher scores for fetal AChR ($p<0.035$); this is illustrated by the frequency distributions that show that the peak thymoma scores are lower than the non-thymoma scores (Fig 3.4 B, D).

3.4 Accurate AChR antibody levels in sera measured by CBA

To identify accurate titres of the antibodies on the CBAs, the positive sera were tested at different dilutions from 1:20 to 1:3200. Fig 3.5 shows representative results of 6 sera.

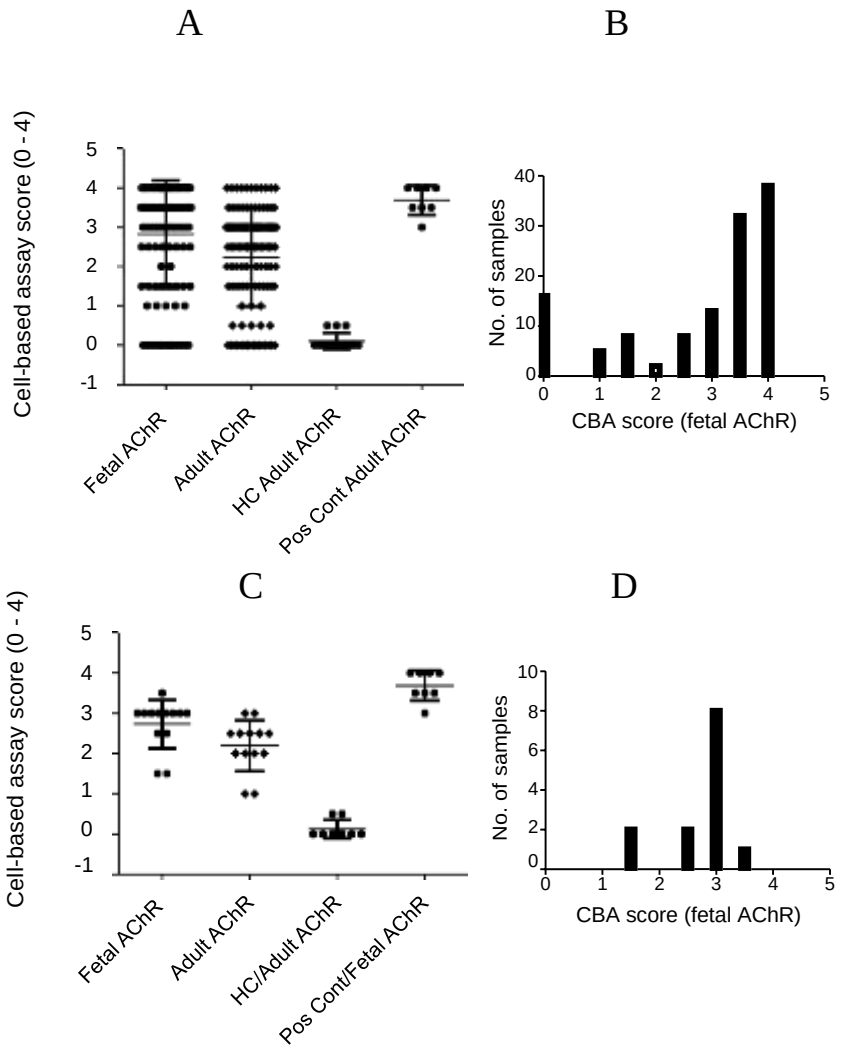


Figure 3.4**.** CBAs for fetal and adult AChR antibodies in non-thymoma (A) and thymoma (C) patients measured by CBAs. Scores lie between 0-4, indicating low to high antibody level in MG sera. Score above 1 were considered positive and below 1 as negatives. Many of the sera had high antibody levels against both clustered fetal and adult AChRs, but a number of non-thymoma sera were negative or very low positive for both forms. The frequency distributions of the fetalAChR antibody scores are shown in B and D. All thymoma samples were positive but with slightly lower scores.

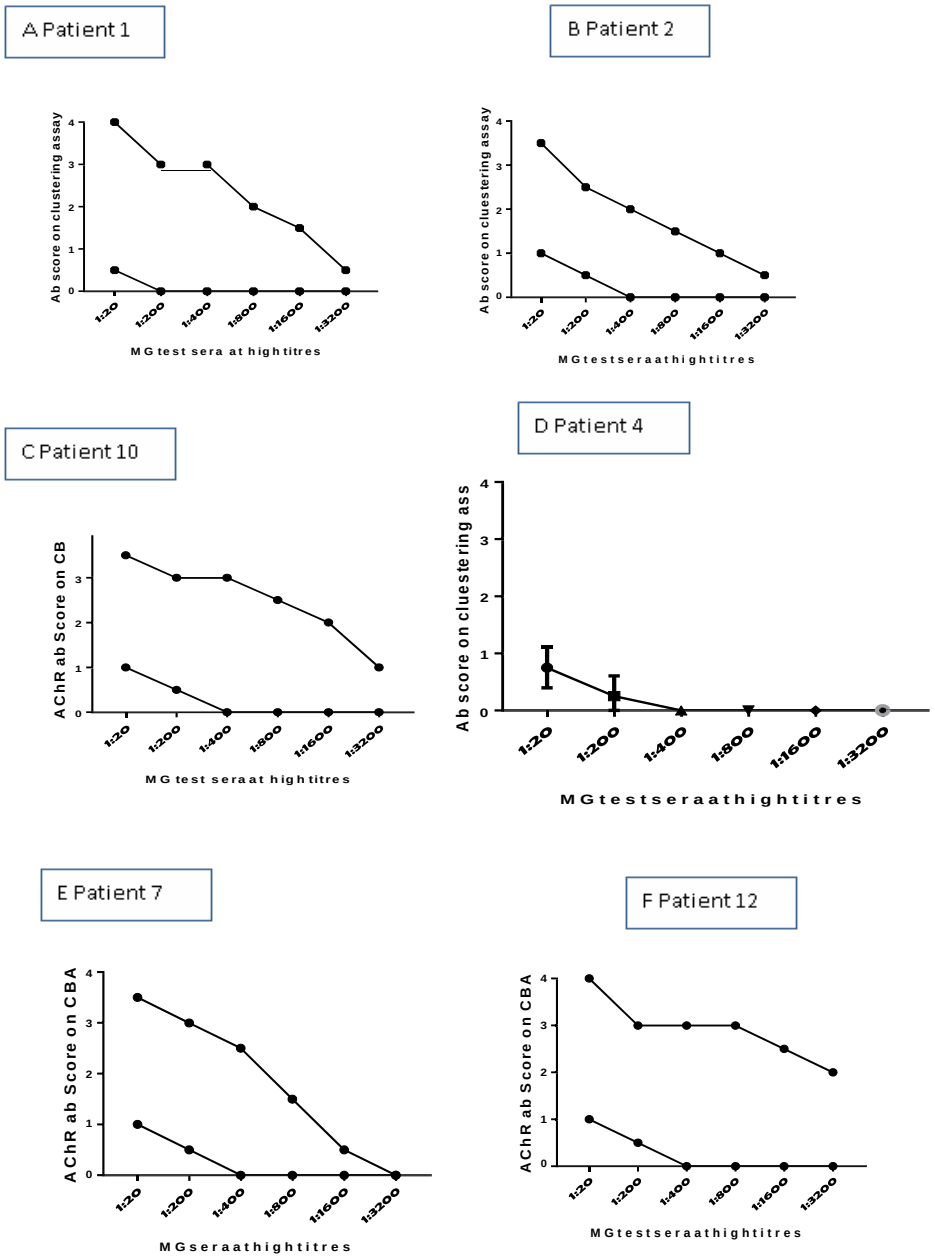


Figure 3.5. Antibody titrations in selected sera. Antibody titres can be measured by the dilution at which the serum becomes negative (defined as score of 1). This end-point dilutions ranged between <1:20 (patient 4, (D) to >1:3200 (patient 12 (F)). The control serum run in the same experiments usually scored close to positive, at 0.5 – 1 at 1:20.

At 1:3200 dilution four of the sera shown still bound detectably to the cells, whereas two sera scored 0 at that dilution. On each graph the HC serum is plotted; this was unusual in showing a score of 1 to 0.5 at 1:20 (most HC do not bind detectably). Patients 1, 2, 10, 7 and 12 showed high antibody levels with endplate dilutions (scoring 1) from 1:800 to >1:3200 (Fig 3.5). However, patient 4 did not have detectable antibody above HC

3.5 AChR antibodies in thymic cultures

Serum and cryopreserved thymic cells were provided by Prof N WIllcox. Fig 3.6 shows the serum AChR antibodies tested by RIA. Patients 3, 10, 12, and 15 had the highest RIPA results, and of these 10 and 12 had been strongly positive by CBA (Fig 3.5). Because of the number of archived samples available, however, the first cultures to be thawed were from Patient 2, 3 and 7. These were then cultured for 28 days pooling triplicate samples at weekly intervals. Cultures were either unstimulated, or treted with pokeweek mitogen (PWM) at two concentrations or cycloheximide (CHX) which was used to inhibit antibody synthesis and to provide a negative control. The supernatants collected after 7 days showed patients cells produced very little if any antibody detected by RIA. The results are shown in fig 3.7 and only patient 3's cells showed some production of AChR antibodies, with 400 cpm immunoprecipitated compared with only 50 cpm in CHX. Ten further thymic lymphocytes were thawed and cultured for four weeks in 200 µL well plates and 2000 µL well plates in triplicate but the results were not better.

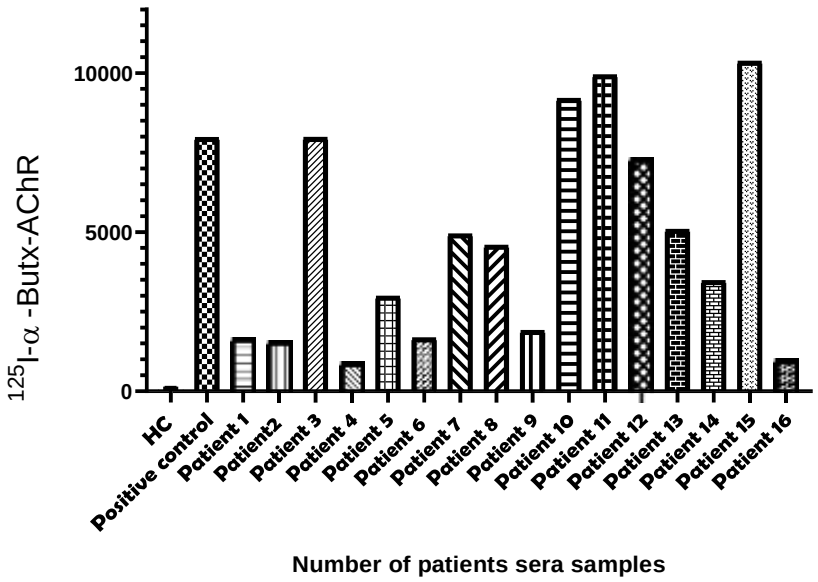


Fig 3.6. The high positive samples on the CBA measured by RIPA. RIPA results from 16 samples were re-tested to compare with the CBA results. The highest RIPA results were found in samples 3,10,11,12,15. Samples 7, 8 and 13 had intermediate binding. Samples 7, 10 and 12 had been highest in the CBA (see Fig 3.5).

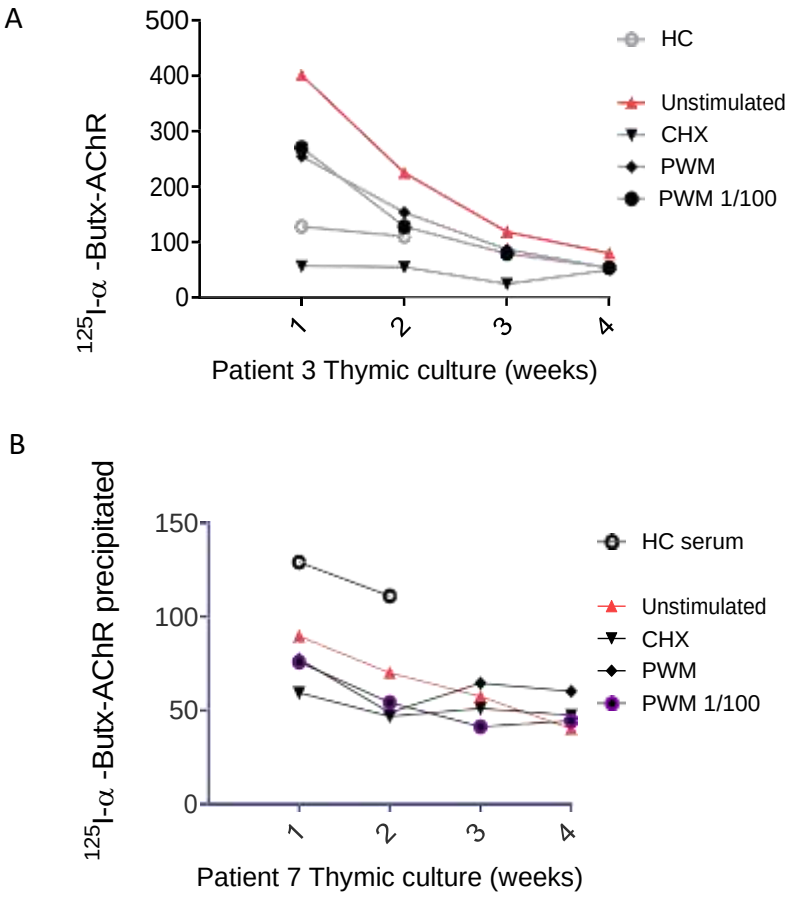


Figure 3.7 Detection of AChR antibody by RIPA in culture supernatants of two patients. A. Patient 3 cultures produced some antibodies during the first week (around 350 cpm above the CHX (cyloheximide) contros, with decreasing amounts subsequently. B. Patient 7 produced very little, not clearly increased over the CHX of around 60 cpm. In addition, Patient 2 cultures (data not shown) produced no detectable antibodies during the four weeks of about 50 cpm consistent with the very low serum levels shown in Fig 3.6.

Discussion

The ultimate aim of this chapter was to establish the thymic cultures in which to study the presence of B cells that were specific for AChR antibodies. Two different antibody assays were used to test the sera from patients whose sera and thymic cultures had been archived. Although the serum antibodies were high in around half of the patients studied in detail, the archived thymic cultures produced were small amounts of antibody and only two were considered suitable for further study. Nevertheless, the establishment of CBA for clustered AChR set the scene for the work that follows where the transfected cells were used to derive membrane vesicles expressing AChR for studying antigen-specific B cells.

Cell-based antibody assays have been developed previously (Leite*et al.*, 2008). Antibodies binding to clustered AChRs on CBAs have improved the diagnosis of a proportion of previously AChR-Ab–negative patients because they detect antibodies in patients who are not necessarily positive in the RIPA where the AChR is in solution. This was not a specific problem with the sera tested here as almost all sera tested were positive by RIPA, but it was possible that the CBA would detect antibodies that bound to rapsyn-clustered AChRs that could be different to those found in the RIPA.

The MG antibodies were overall a little higher for binding to fetal than adult AChR. Testing for fetal AChRs specifically has been useful as these antibodies can be strongly associated with arthrogryposis multiplex congenital in babies born to mothers with (or sometimes without) MG (Riemersma et.al., 1997). Using the mouse model of maternal-to-fetal transfer of these antibodies established earlier, a potential drug was shown to reduce transfer of the AChR antibodies from mother to developing fetus; if further developed, this drug might provide a useful treatment for subsequent pregnancies in similar cases (Coutinho, Jacobson and Vincent, unpublished findings). These observations, as well as some earlier studies in AChR antibody–negative MG, emphasize possible functional and antigenic differences between fetalAChRs during development and adult AChRs in mature muscle, where they are strongly clustered at the NMJ.

Placental transfer of maternal IgG antibodies to the fetus is an important mechanism that provides protection to the infant while his/her humoral response is inefficient. IgG is the only antibody class that significantly crosses the human placenta. This crossing is mediated by FcRn expressed on syncytiotrophoblast cells. There is evidence that IgG transfer depends on the following: (i) maternal levels of total IgG and specific antibodies, (ii) gestational age, (iii) placental integrity, (iv) IgG subclass, and (v) nature of antigen, being more intense for thymus-dependent ones. These features represent the basis for maternal immunization strategies aimed at protecting newborns against neonatal and infantile infectious diseases. In some situations, such as mothers with primary immunodeficiencies, exogenous IgG acquired by intravenous immunoglobulin therapy crosses the placenta in similar patterns to endogenous immunoglobulins and may also protect the offspring from infections in early life. Conversely, harmful

autoantibodies may cross the placenta and cause transitory autoimmune disease in the neonate (Palmeira *et.al.,* 2012). As it was clear that adult form of AChR is replaced by fetal AChRs at the time of birth. henceforth this study also confirms the presence of fetal AChRs in adults with MG.

Numerous immunohistochemistry studies have shown the presence of B cells and germinal centres or lymphocytic infiltrates in the thymus tissue and plasma cells of MG patients. It was shown that the thymic lymphocytes could produce AChR antibodies in Vitro (Vincent *et.al*., 1978; Scadding *et.al*., 1981). It was therefore very unfortunate that there was little AChR antibody present in thymic cultures from the archived samples examined here. This may be because of the very long years of liquid nitrogen storage and may be due to death of thymic cells as a result of moving them from liquid nitrogen at -135^{o}C to -80^{o}C freezers.

Identifying the antigen-specific B cells in the germinal centres and in the circulation is not easy if the antigen is a membrane protein which is normally expressed only in muscle, as it was shown in the fig 3.2. To do this for myasthenia B cells requires a source of AChR antigen that could be used to label the B cells and to separate them from other subset of B cells with other antigen specificity. Chapter 4 describes a novel approach to providing a source of antigen.

CHAPTER 4

Production of AChR membrane vesicles as a source of AChR for binding to lymphocytes

Introduction

Identification of the antigen-specific B cells is a necessary step in order to target treatments against the antigen-specific cells. Identifying the antigen-specific B cells in the germinal centres and in the circulation is not easy if the antigen is a membrane protein, as discussed in chapter 3. To do this for myasthenia gravis, a source of AChR antigen is required that can be used to label the B cells and to separate them from B cells with other antigen specificities. A potential source of AChR is extracellular membrane vesicles (EMVs) from AChR transfected HEK293T cells.

Production of membrane vesicles as aEMVs from AQP4 transfected cells have been successfully used to detect antibodies to AQP4 and other neurological antigens (Vincent, Waters unpublished results) and can offer a potential new method for detecting patients' antibodies. It was hypothesised that membrane vesicles from AChR- transfected HEK293T cells could contain enough AChR on their extracellular surface to identify B cells.

EMVs are micro- or nano-sized particles released from the endosomal or plasma membranes of cells. Vesicles may deliver proteins, lipids and other signaling molecules to other cells. The structure, biochemical/biophysical properties, analytical techniques, and physiological/pathological roles of membrane vesicles have been extensively characterized (Tang Q *et al.*,2017). Surface plasma extracellular vesicles are released by a variety of cells into the environment and membrane vesicles are shred

off from the plasma membrane of the cells expressing transiently transfected proteins (Vincent, Waters unpublished). The first step was to develop the techniques for preparing extracellular membrane vesicles (EMVs) from HEK293T cells expressing AQP4, which express the antigen very strongly, and then to apply the approach to EMVs from AChR-transfected cells.

4.1 Expression of AQP4 on HEK293T cells and membrane vesicles

To obtain AQP4-expressing EMVs, HEK293T cells were transiently transfected with DNA for AQP4, which well expressed (see also Fig 3.2). The cells were incubated with the AQP4-antibody serum (1:20) followed by secondary anti-human IgG (red). Fig 4.1A-C shows the DAPI stained nuclei, and immunofluorescence staining shows localisation of AQP4 patient IgG (red) on HEK 293T cells. The merge shows variable binding to the cells, some very strong.

The next step was to harvest membrane vesicles from the AQP4-transfected HEK293T cells, taken at later times to increase expression and EMV production, following the protocol described in the Methods (Chapter 2).

4.2 Membranes vesicles expressing AQP4

AQP4 membrane vesicles were harvested from the supernatants of AQP4 transfected HEK293T cells collected 2, 3 4 and 5 days after transfection. After a low speed spin to pellet the nuclei and large membrane particles in the supernatant, the AQP4 vesicles were pelleted at 30,000 rpm in the ultracentrifuge. The vesicle pellet was resuspended in 50 μl of PBS and 0.5 or 1 μl spotted on to PLL coated glass slides and then allowed to dry (20 mins) before testing for binding of antibody. A 175 cm^2 flask of fully grown HEK293T

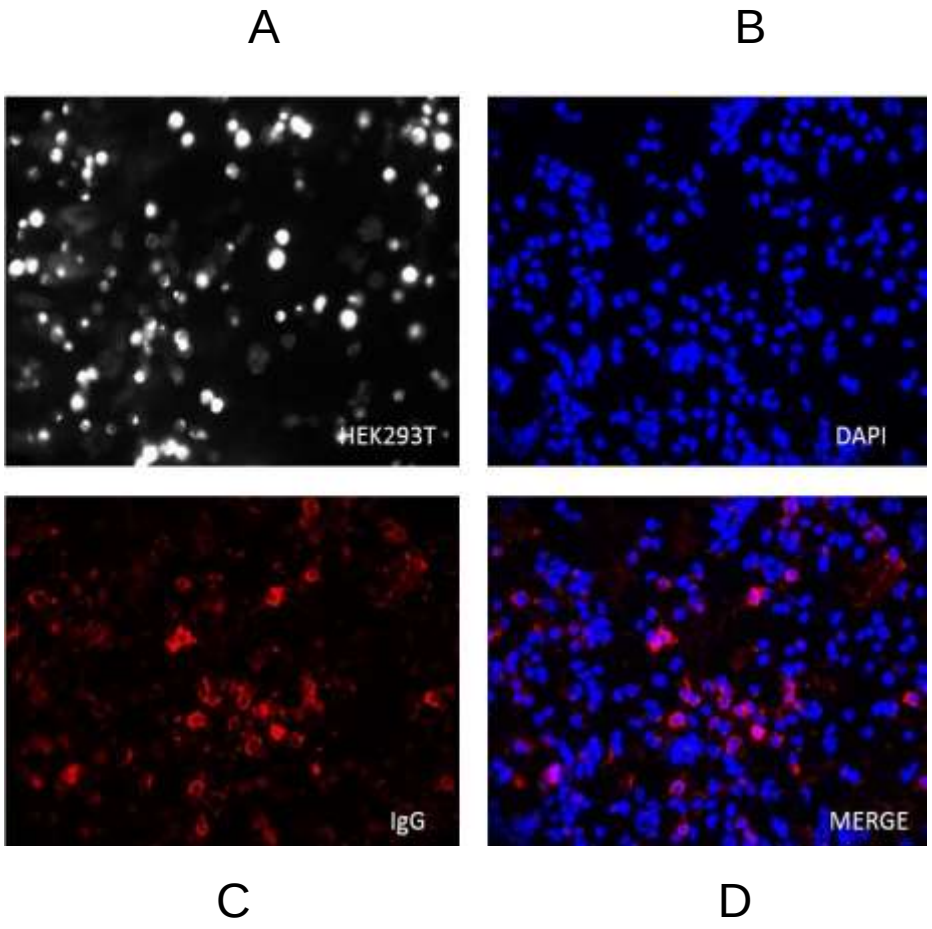


Figure 4.1 Immunofluorescence staining of HEK 293T cells expressing AQP4 from patient with NMO. A. HEK293T cells visualised by dark field microscopy. A. Nuclei of the treated cells were stained with DAPI. C. Patient IgG binding to the cells as shown by red Alexa fluor anti-human IgG. D. IgG (red) binds only to a proportion of the DAPI labelled cells, probably limited by the transfection rate in this experiment.

cells produced a good yield of AQP4 vesicles within 4-5 days after transfection. Figure 4.2 shows examples of membrane vesicles from cells transfected with AQP4 which were exposed to AQP4 antibody positive patient serum and the IgG bound identified with Alexa Fluor 488 (green) anti-human IgG. The vesicles typically spread to the periphery of the spot and there was a distinct band of green fluorescence around at the edge as seen in Fig 4.2.

To confirm that the binding was specific for AQP4 antibody positive sera, Figure 4.3 shows results of one healthy control serum and one AQP4 antibody positive serum binding to the vesicles.

4.3 Production of AChR membrane vesicles

The same approach was used to prepare AChR membrane vesicles. HEK293T cells were transfected with the adult AChR subunits together with EGFP-rapsyn. Figure 4.4a shows the very strong binding of AChR antibodies to the clustered AChRs on the surface of the HEK cells. The vesicles prepared from these cells are shown in Figure 4.4b. There is nuclear material (DAPI stained) within the EMV spot co-localising with EGFP-rapsyn. The patient's AChR antibody did not show strong binding although there was a faint suggestion of binding on the merge (Figure 4.4bD).

To try to improve the AChR membrane vesicles, in some preparations AQP4 was co-transfected with AChRs to see if it would increase the yield of membrane vesicles. Results of experiments, conducted on fresh vesicles and run with the same HC and MG serum in parallel, are shown in Fig 4.5. Control vesicles showed only faint IgG (red) binding at the periphery, as did healthy control serum suggesting some non-specific binding of anti-human IgG to the vesicle preparation rim (Figure 4.5A,B). AQP4 antibodies bound well to AQP4 vesicles (Figure 4.5C). AChR antibodies bound

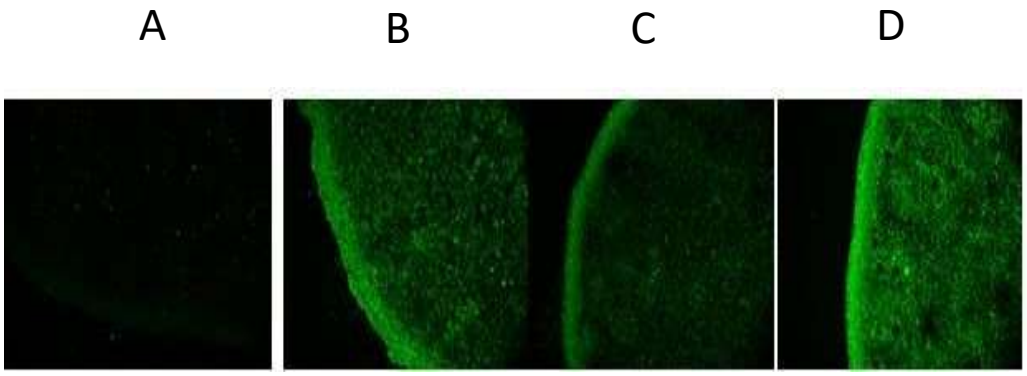


Figure 4.2 Vesicles prepared from AQP4-transfected HEK293T cells bind AQP4 antibodies. 0.5 µl of AQP4 vesicles were spotted onto glass slides. The microvesicles tend to spread to the periphery of the droplet. After drying the slides, AQP4 antibody positive serum was added at 1:20 and binding detected with 488 goat anti human (green) antibody. A shows vesicles from untransfected cells. B, C and D show AQP4-Ab binding to the rim of three vesicle spots.

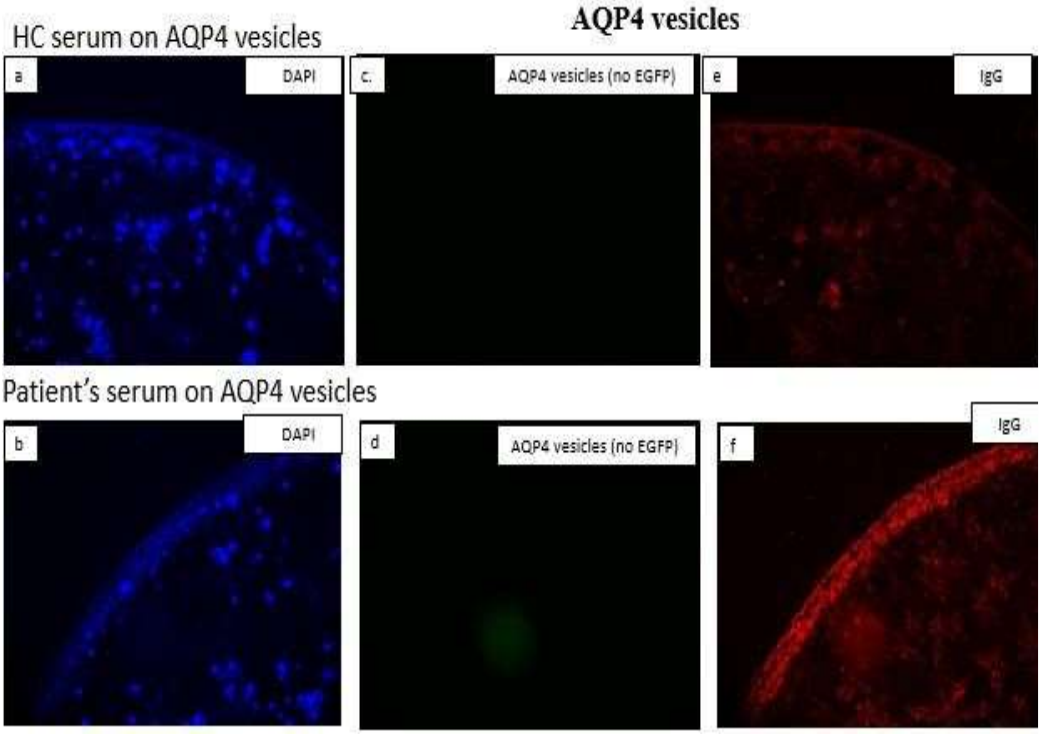


Figure 4.3. AQP4 vesicles stained with healthy control and patients' sera. a, b shows staining DAPI indicating some nuclear material in the vesicle rim. c, d shows vesicles only without serum. e shows HC serum IgG does not bind to AQP4 vesicles, whereas f shows patient's serum binds to vesicles. Binding detected by Alexa flour 568 (red). Pictures are taken at 20x magnification.

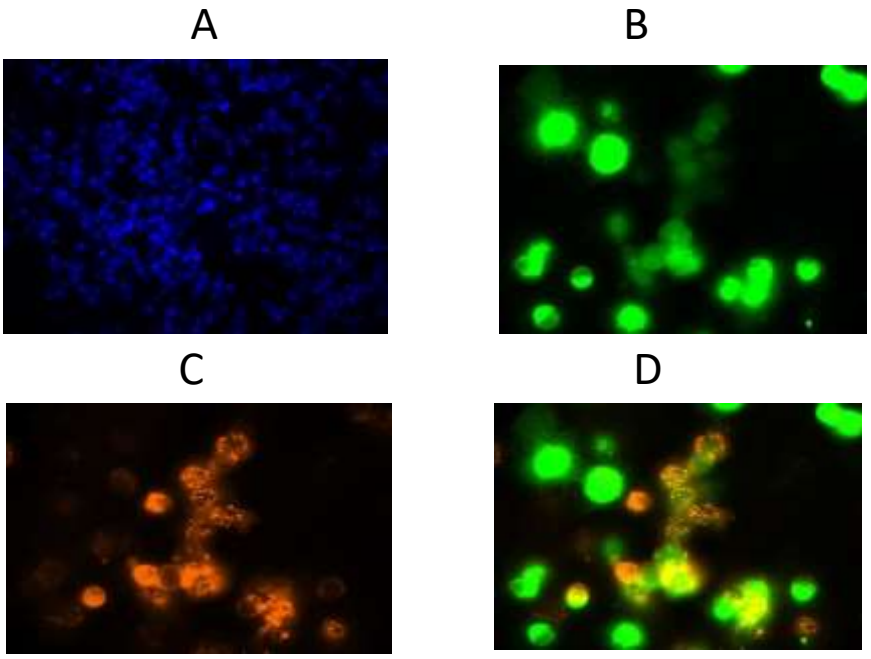


Figure 4.4a. HEK293T cells transfected with AChRs with EGFP tagged rapsyn show clusters on their surface. A. DAPI stained nuclei. B. EGFP expression shows clustering of the rapsyn in the cells. C. MG patient antibodies show strong binding to the AChRs in a clustered pattern. D. Merged image

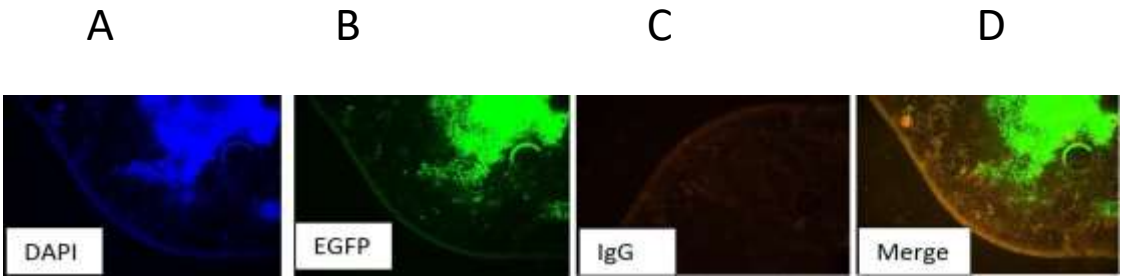


Figure 4.4b Membrane vesicles expressing EGFP co-transfected with adult AChRs. 0.5µl droplet of vesicle suspension on a glass slide covered with a glass coverslip. A. DAPI shows nuclei material present in the vesicle suspension. B. Vesicles contain EGFP but no binding of IgG (C) IgG on the periphery, not clear. D. merge showing vesicle rim and no strong binding.

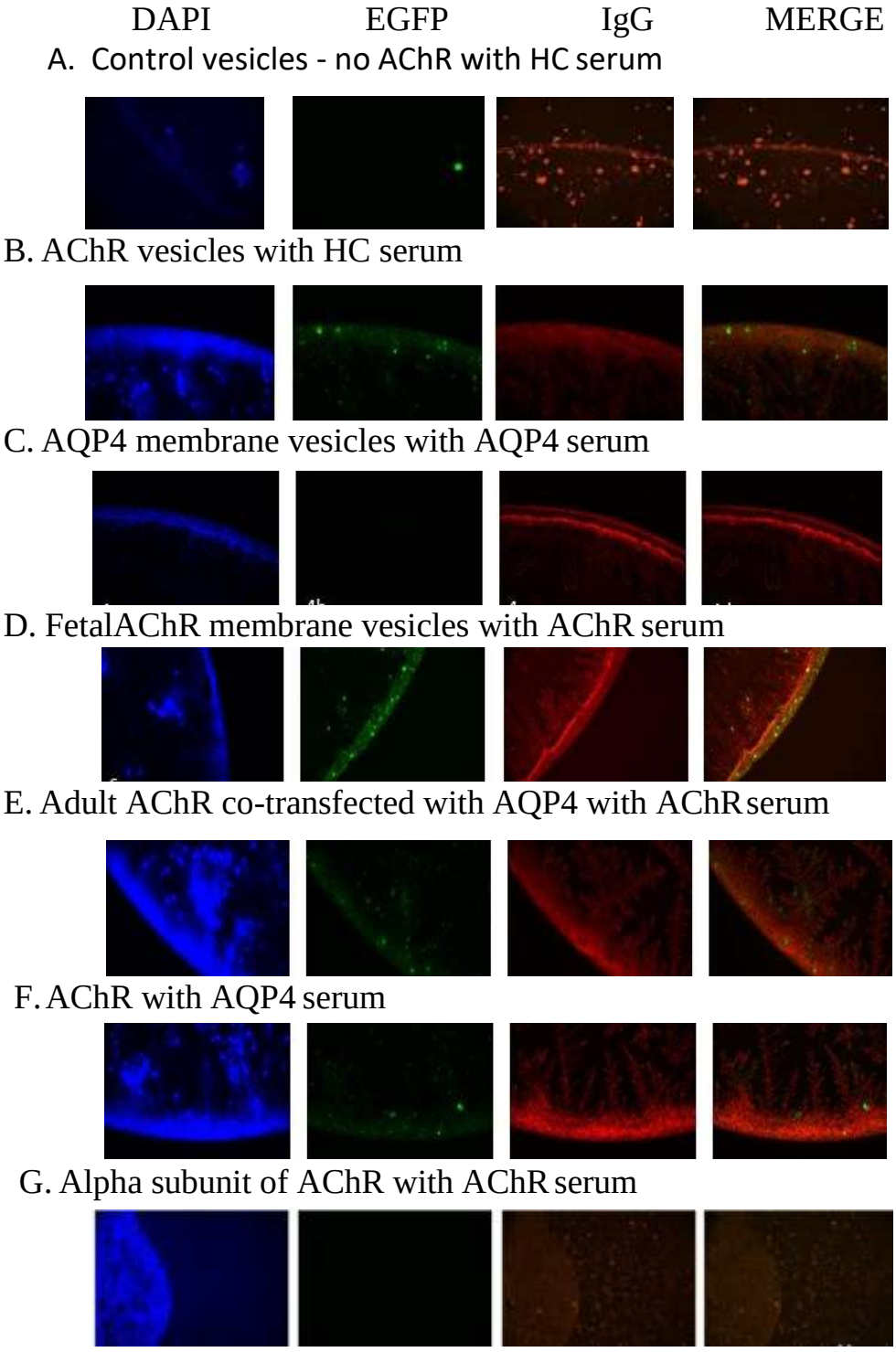


Figure 4.5. The images of parallel experiments performed using different transfections to generate membrane vesicles. A. Control HEK vesicles and control sera provided negative controls. B. AChR vesicles tested with HC serum show no binding. C. AQP4 vesicles incubated with AQP4+ serum show strong binding. D. Membrane vesicles expressing fetalAChRs bound AChR+ patients' serum. E Vesicles from cells transfected with AQP4 andAChR bound AChR+ serum. F. However, AChR vesicle bound AQP4+ serum although not in the same manner as those above. G. Vesicles from cells only transfected with α subunit of AChR (no EGFP) were negative with AChR MG+ serum.

well to fetal AChR vesicles and also to adult AChR co-transfected with AQP4 to increase vesicle formation (Figure 4.5 D,E), but AChR antibodies appeared to also bind to AQP4-transfected vesicles (Figure 4.5F). On the other hand, AChR antibodies did not bind to alpha subunit transfected EMVs (Figure 4.5G).

Membrane vesicles expressing the fetal form of AChR appeared to bind AChR antibodies more strongly than the adult form but a strict comparison was not made.

4.4 Identification of AChR proteins in vesicles on western blots

To try to confirm the presence of AChRs membrane vesicles in the pellet, western blotting and flow cytometry were performed. AChR membrane vesicles in the suspension were denatured and run on the gel electrophoresis. The blotted membrane was incubated with a monoclonal antibody to the AChRβ subunit followed by washing and incubation with secondary anti-mouse IgG antibody. Bands after the exposure to ECL demonstrate the proteins present in the test samples. Figure 4.6 shows binding of the monoclonal antibody to band at around 40KDa in the AChR HEK cell lysate and very strong binding to bands in the range of the AChR subunits, especially the β subunit (45 – 50 KDa) and to other bands in the two lanes containing membrane vesicles (Figure 4.6, lanes 4,5). Figure 4.7 shows the relationship between protein bands and binding of the monoclonal antibody. The Coomassie stain shows many protein bands in the AChR cell lysates, as expected with no clearly specific bands in lysates or the EMV preparations. However, after transfer to nitrocellulose and blotting with the β subunit antibody bands at 45 and 48 KDa were present. However, the presence of other bands detected by the β subunit antibody or perhaps by the anti-mouse IgG secondary antibody, were detected by the β subunit

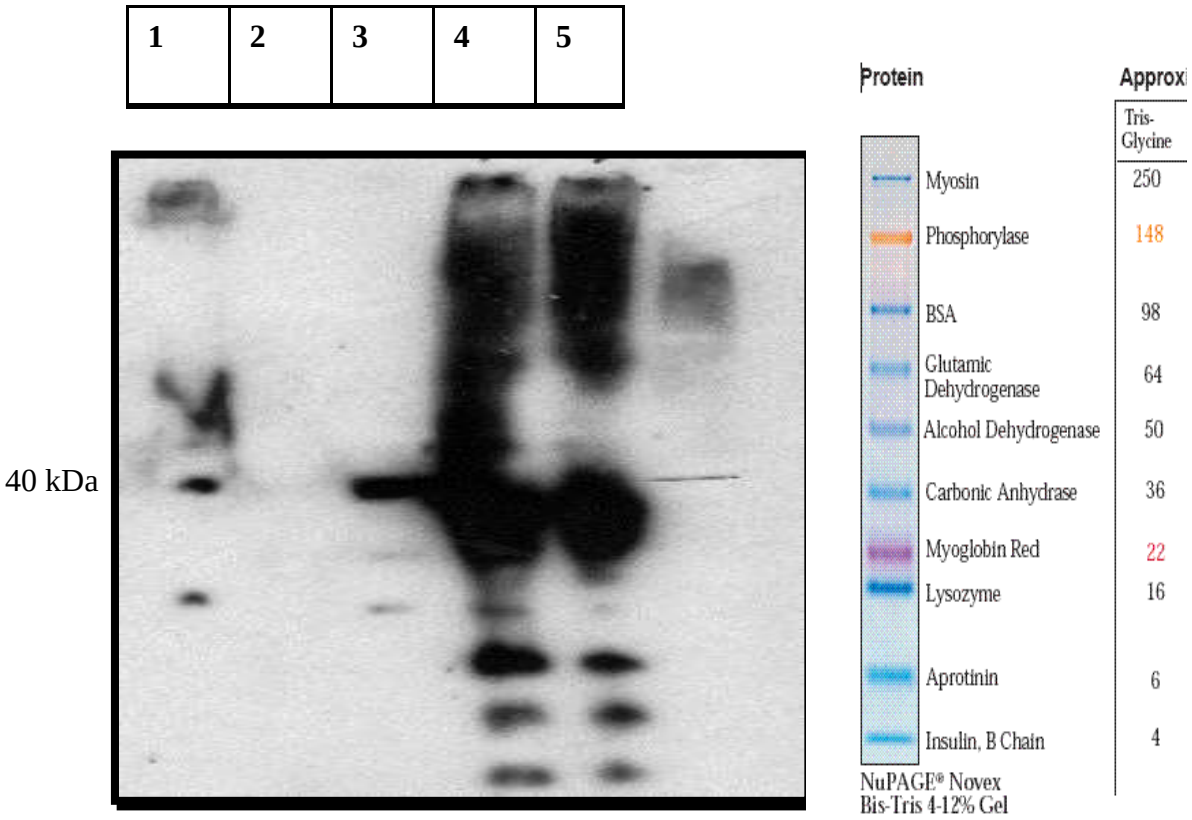


Figure 4.6. Western blot to detect AChR in vesicles. Lane 1 is HEK cell lysate control with no detectable band present. Substantial binding of AChR antibody to lines 3, 4 and 5. The blot was incubated overnight with β cytoplasmic antibody and polyclonal anti-mouse IgG as a secondary to detect the bands. Protein bands prominent at around 40 KDa were expected to be the β subunit of AChRs.

Lane 1, HEK cell lysate, lane 2, blank, lane 3, AChR HEK cell lysate, lanes 4 and 5 membrane vesicles from AChR expressing HEK 293T cells.

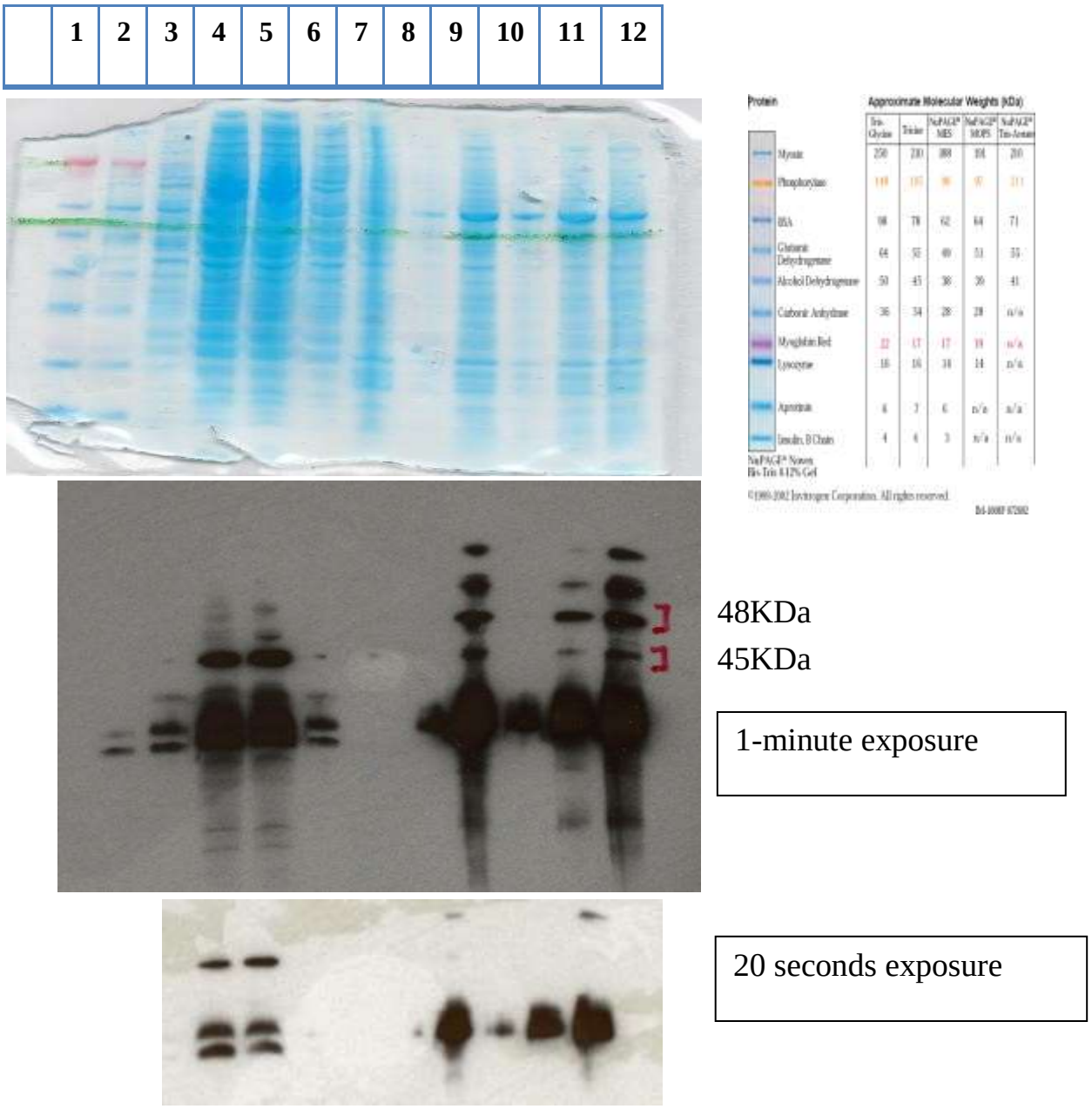


Figure 4.7. Coomassie stain and western blot of AChR vesicles, produced from HEK293T cells. 4 wells were used first two for AChR lysate as a positive control and last three for AChR membrane vesicles. On exposure to ECL on different time scale, blots show bands of AChR proteins in the lysate and vesicles. Control and ladder do not show any band on the blot. Lane 1,2 ladders, lane 3 control, lanes 4,5 AChR cell lysates, lanes 9, 11, 12 AChR vesicles. Lanes 6,7,8,10 nil.

antibody, or perhaps by the anti-mouse IgG secondary antibody, were disappointing. Unfortunately, no control antibodies were used to show the specificity of the beta subunit antibody but the binding to only three bands on AChR lysate, compared with the large number of proteins in the lysate shown on the Coomassie, suggested that the binding could be relatively specific. The 20s exposure of the blot shown expanded below emphasises this point.

On further optimization of the western blotting method, increasing the washing steps and incubation time with the primary antibody, the results were better showing a single band of around 41 kDA in the lysates and slightly stronger in the EMV preparations, with no other bands (Figure 4.8).

4.5 Identification of membrane vesicles by flow cytometry

An alternative technique to detect AChR in vesicles, was FACS. Using freshly prepared vesicles, flow cytometry (Fig 4.9) revealed evidence for EGFP in the vesicle preparations, although the scatter in Figure 4.10 shows very little and mainly in the adult AChR-transfected vesicles.

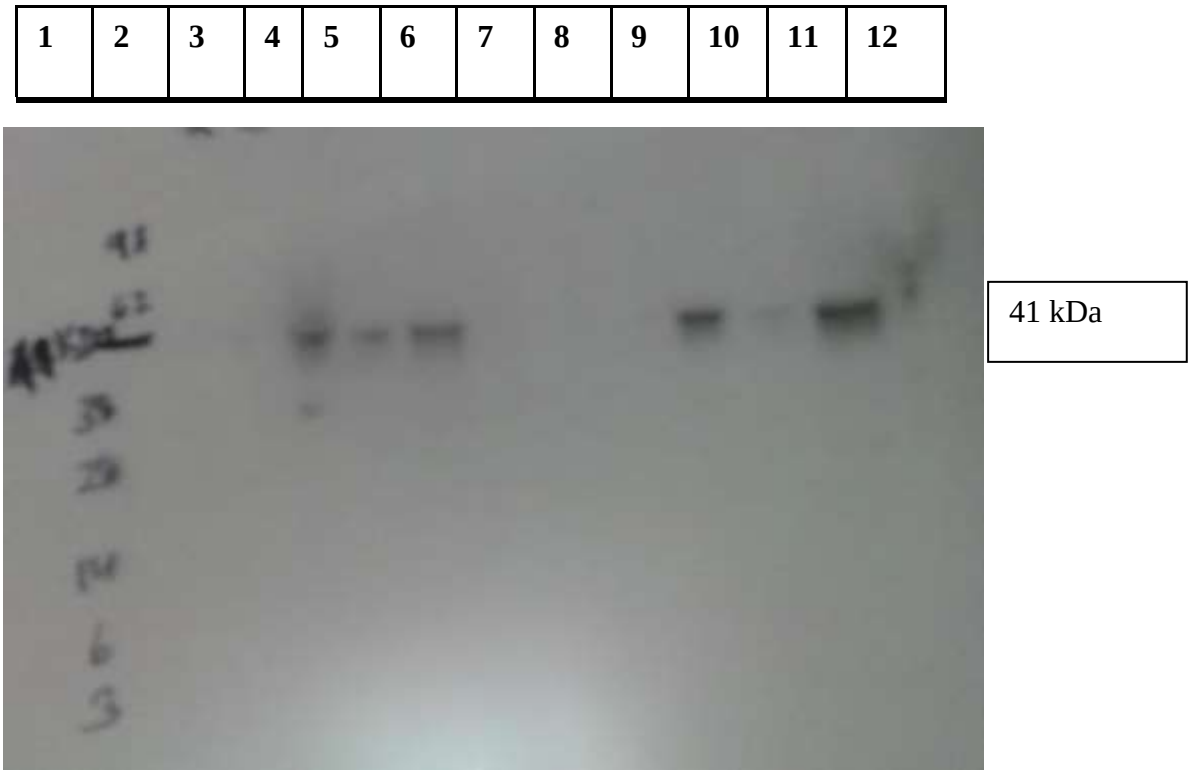


Figure 4.8 Improved western blot of AChR. 41 kDa bands consistent with AChR beta (β) subunit are seen in lanes 4, 5, 6, 9 and 11. Proteins were incubated overnight with β cytoplasmic antibody and anti-mouse as a secondary ab to detect the antibody binding. Lane 1 blank, lane 2 ladder, lane 3 control vesicles no AChR protein, lanes 4,5, 6 AChR cell lysate, lane 9, 11 AChR membrane vesicles, lanes 7,8, 10 and 12 were blank.

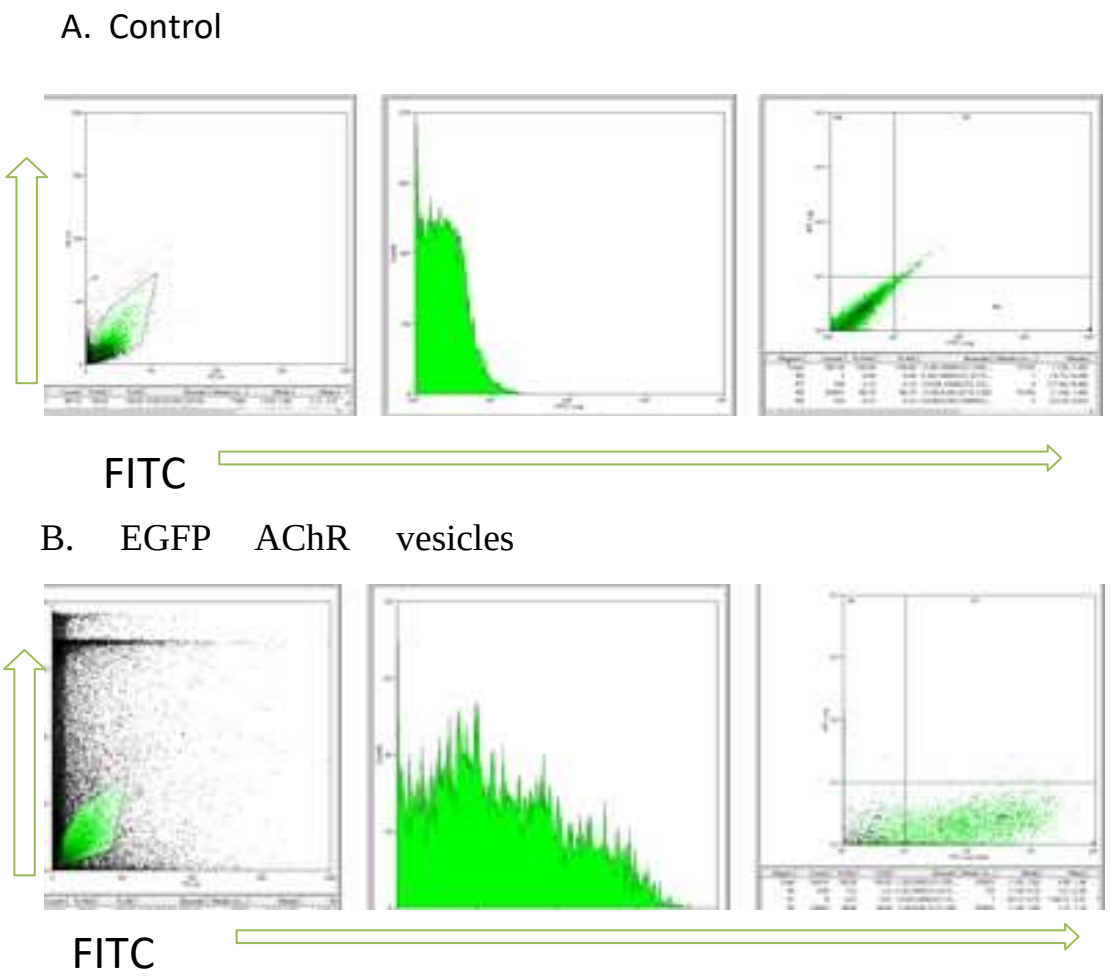
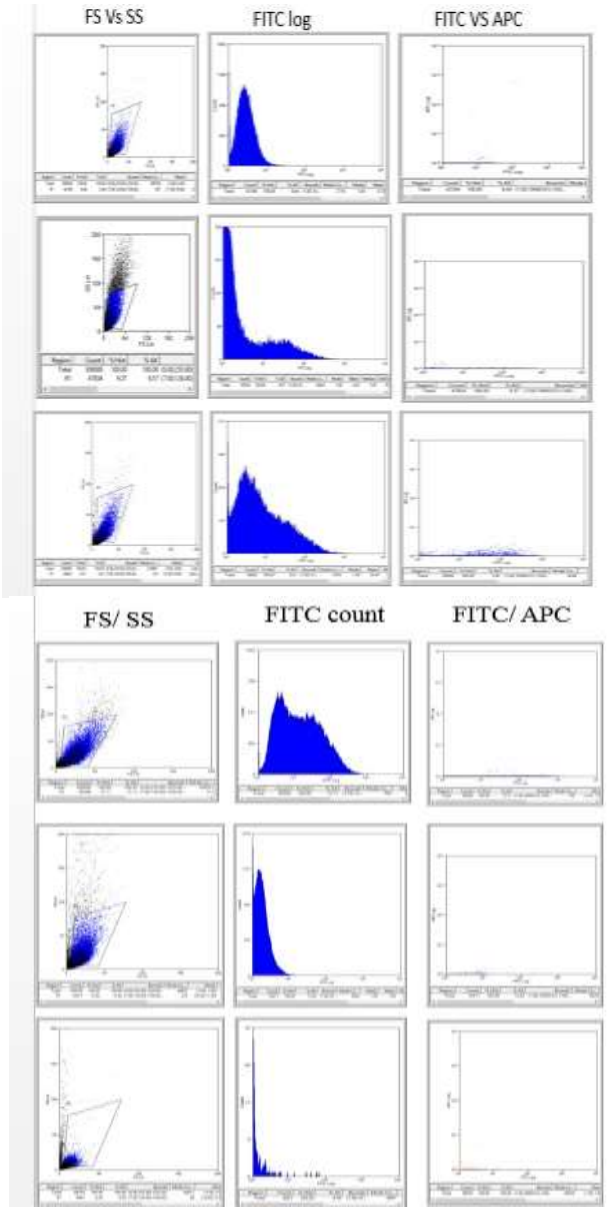


Figure 4.9. Flow cytometric analysis of EGFP AChR vesicles. X- axis is FITC channel and y-axis is counts per test. A. Control vesicles do not contain significant EGFP expressing vesicles. B. EGFP positive vesicles shown by right shift increased FITC expression on X-axis.



Control

AChR (fetal) EMVs with EGFP

AChR (adult) EMVs with EGFP

AChR (adult) + AQP4 EMVs

Non-clustered AChR EMVs no

AQP4 EMVs as control

Figure 4.10. Flow cytometric analysis of membrane vesicles showing EGFP. R1 gate shows no colour. Autofluorescence is compensated. Surprisingly, only the adult AChR EMVs with and without AQP4 co-expression showed a faint signal in the FITC panels. In this experiment fetal AChR showed less detectable vesicles compared with adult AChRs, not very different from the controls.

Discussion

The aim here was to prepare AChR membrane vesicles for use as a source of intact AChR antigen for detecting AChR-antibody specific B cells. The immunostaining results were variable and western blotting did not always show a band of the appropriate size for the beta subunit. However, there were some encouraging results.

While optimizing the technique for preparation of the vesicles, there were important aspects to consider such as the centrifugation speeds used for pelleting the nuclei, and then for pelleting the vesicles. Preparation started with AQP4 because it is a single gene product and is expressed strongly on the plasma membrane and the cells shed off EMVs into the medium within 3-4 days after transfection. In fact, some vesicles were apparently present around the cells (as shown in Figure 3.2). The AQP4-vesicles gave convincing staining with AQP4 antibody NMO serum. A single α subunit of the AChR was a suitable negative control as it is not expressed on the surface of the cells or on any vesicles.as shown here. It is not suitable, however, for B cell identification in future studies because, although it contains the "main immunogenic region", this is a very conformational epitope and is not present on the alpha subunit alone which is, in addition.

The question of whether there is sufficient AChR expressed on vesicles, unlike AQP4, is still not clear. Overall, the results were variable for clustered and non-clustered AChRs (ie without rapsyn-EGFP) and for fetal and adult AChRs, although fetal AChRs tended to give more encouraging results in some WB and immunostaining tests. These fetal specific clustered AChR vesicles showed positive staining by immunofluorescence and apparently specific antibody binding of the monoclonal β subunit antibody on western blots, but adult AChR vesicles were also convincing in some experiments. Vesicles are

micro to nano particles which are shred off by the plasma membrane of large cells. PBS proved to be a suitable solution to preserve vesicles and no azide or similar preservatives were added as those had been shown to be toxic to the vesicles. From that study, membrane vesicles preparations should be used within a week. Alternatively, it might be possible to use the vesicles if frozen at -80 ^{o}C for up to one month.

Staining of vesicles followed CBA methodology where washing of spotted vesicles was considered an important step to get rid of the non-specific binding of patients' sera and IgG. Unlike AQP4 vesicles, AChR preparations did not show specific binding on immunofluorescence staining. Flow cytometric results demonstrated the presence of EGFP in the preparations but not the AChRs as clusters. Western blotting with the beta subunit antibody but to a certain extent it proved that the membrane vesicles should have AChRs, as hypothesized and a bit of work was done in the past on this specific monoclonal antibody against β. Highly specific technique to prove AChR membrane vesicles or other antigen in the suspension is CBA where AChR antibody should bind to IgG. From these results the best preparation and methodology was set as the protocol for future preparations.

Although it was not that clear membrane vesicle staining with antibodies was entirely specific, considering the western blotting, the membrane vesicles were taken forward for the antigen presentation as described in the next chapter.

CHAPTER 5

Characterization of antigen specific B cells in MG thymus.

The aim of this chapter was to identify antigen-specific B cells in the thymic culture supernatants.

The normal role of the thymus is to develop mature T cells that are not self-reactive. However, in myasthenia gravis, plasma cells are present in germinal centres in the thymus and produce antibodies against AChRs, which are then circulated to the neuromuscular junction. AChR antibodies lead to complement mediated lysis and internalisation of the receptors, as mentoned in the Introduction. Depletion of AChR-specific B cells, or AChR-antibody producing plasma cells, is a major goal for treatment. B cells pass through various developmental stages before and after exposure to antigen. These stages are defined by differential expression of surface and intracellular markers, as well as their immunoglobulin secretion. After antigen presentation B cells become antibody secreting cells (ASCs) called as plasmablasts or plasma cells. The aim of this work was to use AChR-containing membrane vesicles to try to isolate the B cells that will become the AChR antibody secreting plasma cells.

5.1 Identification of B cells and its subsets in Raji cells

To identify B cell subsets, especially memory B cells and plasma cells, first Raji cells were used to establish flow cytometric analysis. The Raji cell line is a B cell expressing cancer line which is easy to maintain and was particularly suitable for the initial experiments.

The Raji cells are grown in suspension and take time to reach 80%confluency. The cells were cultured from 7 days to 14 days but usually the cells could be used on the seventh day. 50,000 cells were used for each test. Figure 5.1A-D shows the presence of APC-labelled CD19+ B cells, but not CD3+ T cells or CD138+ plasma cells. Figure 5.1E-F shows the presence of CD27+ B cells and thei co-expression of CD19 and CD27 on these cells. These results demonstrated that the FACS methodology was suitable and that B cells could be identified in preparations of PBMCs and thymic cultures.

5.2 Analysis of B cells in thymic cells and peripheral blood mononuclear cells

In all analyses, the control cells were unstained cells, If multiple types of cells were used then the controls were adjusted accordingly. The compensation of multiple fluorochromes at the start of each analysis was done on the controls followed by sample tests. The first experiment compared thymic cells with PBMCs.

In Figure 5.2 A the controls were unstained thymic cells or PBMCs shown in separate windows. The PBMCs clearly contained two populations of cells expressing CD3 and CD19 respectively. Thymic cells were fewer in number than freshly isolated PBMCs as expected and did not appear to contain two populations but some that co-expressed both markers. This could be because they were freshly thawed (only 12 hours post thaw). The thymic cells were subsequently cultured for at least 2 days before testing.

In Figure 5.2 B the cells were incubated with EGFP vesicles. There were two peaks of vesicles in the PBMC preparation, and the added vesicles were also seen in the thymic lymphocyte preparation along with the CD19 cells.

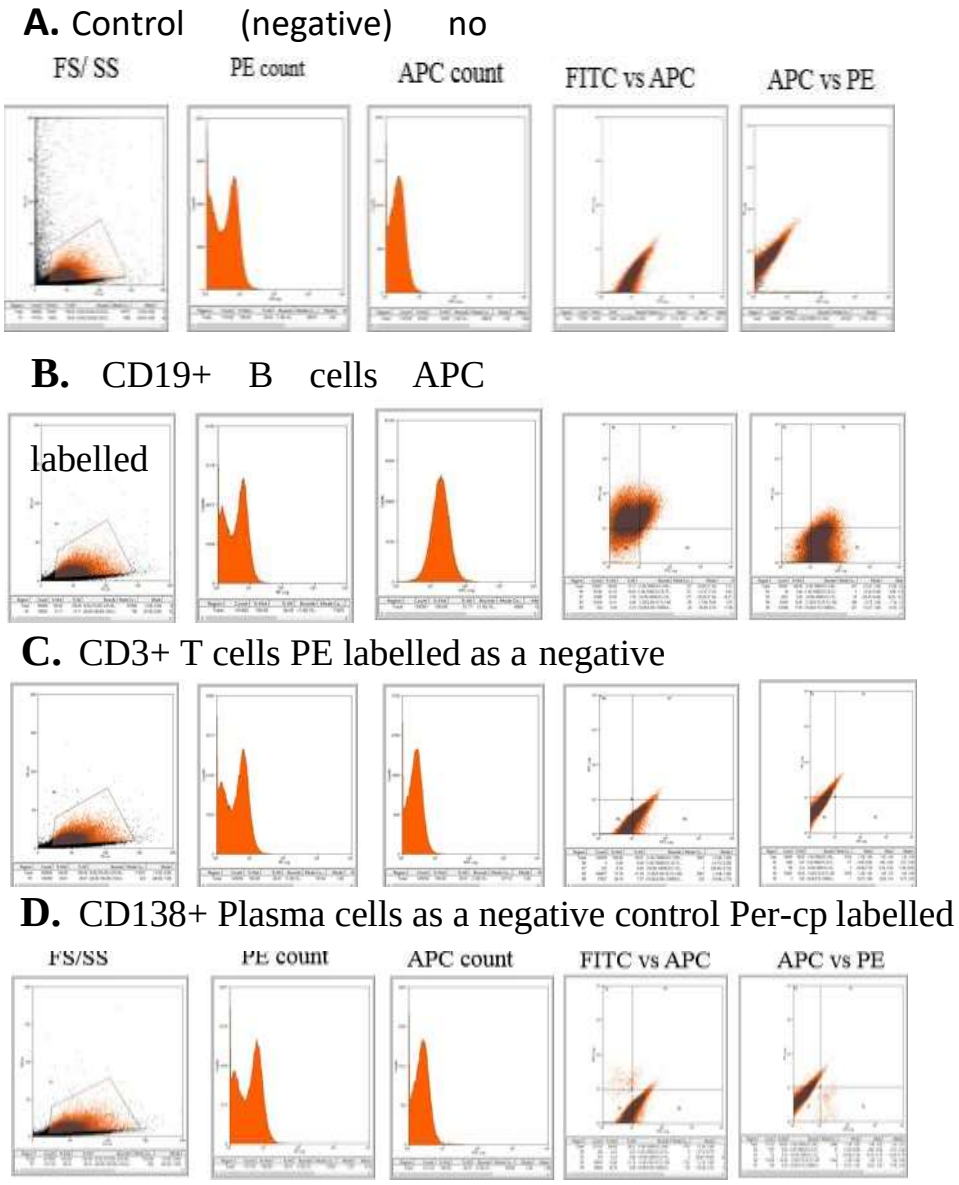
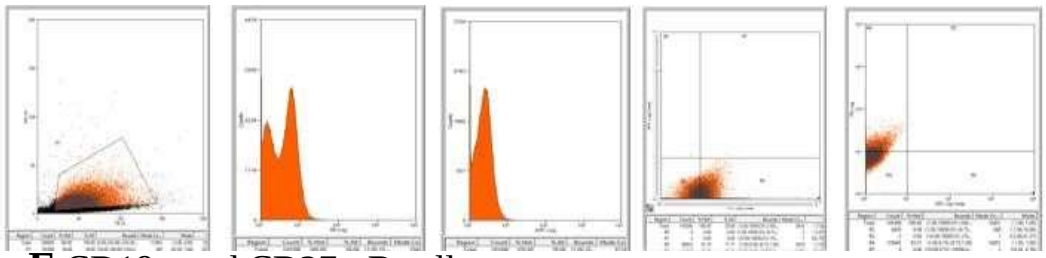
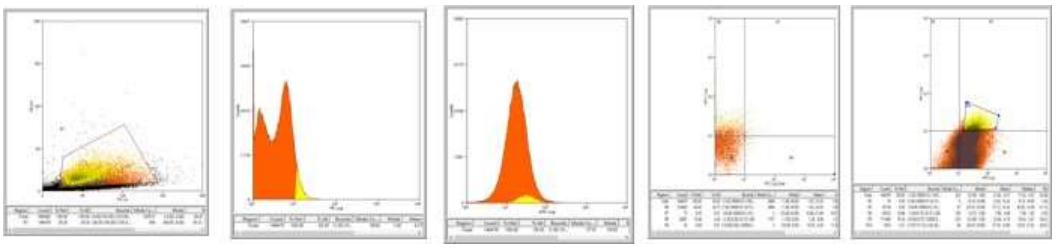


Figure 5.1 Shows presence of CD19 and CD27 expression as markers of B cells on Raji cells. Anti-human-CD3, a T cell marker and CD138, a plasma cell marker, were used as negative controls. Non-specific binding for fluorochromes were compensated for APC, PE and PerCP in control tests. A. Gated population on unstained cells on FS/SS shows no evidence of fluorescence in control sample. B. APC count shows high proportion of CD 19+ B cells in the Raji cells suspension as expected. C. Negative control. Showing no CD3+ T cells in the suspension. D. Shows lack of CD138- plasma cells in the suspension, as a negative control. Cont. overleaf.

E. CD27+ B cells as a positive control PE labelled



F.CD19+ and CD27+ B cells



G. CD19+ CD27+ and CD138-

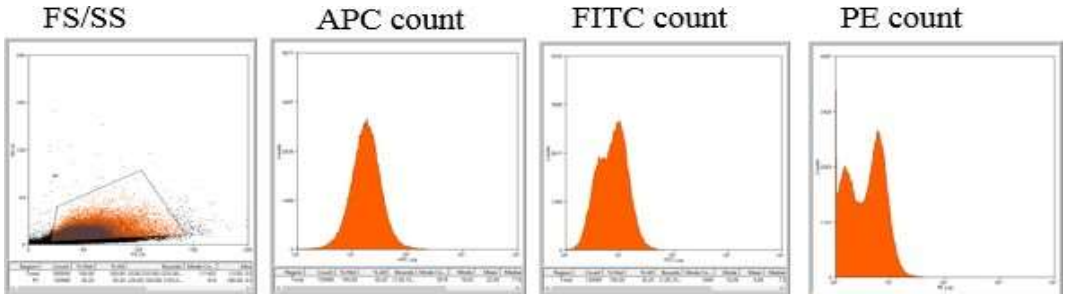


Figure 5.1 continued E. CD27+ staining showing a shift of the B cells in the suspension F. CD 19 and CD 27, B cell markers shows positive population. G. Shows that the CD19 and CD27 positive cells and negative for CD138, as expected.

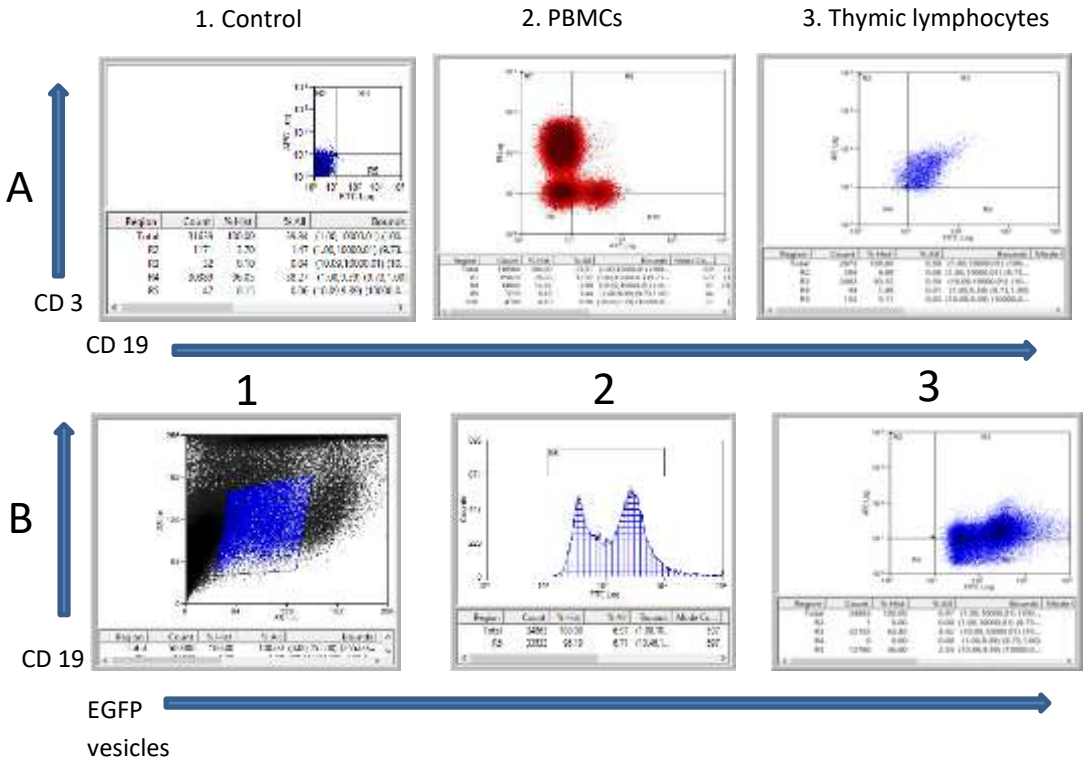


Figure 5.2. Identification of cells in PBMCs and thymic lymphocyte preparations. A1 Unstained thymic cells on forward and side scatter. A2 Shows anti-CD19+ B cells on x axis and anti-CD3+ T cells on Y-axis in PBMCs. A3 shows that there are insufficient CD3+ and CD19+ cells in the thymic suspension. B1. Unstained thymic cells with EGFP vesicles. The cells were first gated. B2. Two significant peaks on the graph showed CD19+ B cells and EGFP vesicles in the PBMC suspension. B3. Shows EGFP vesicles on the x-axis but few APC-labelled anti-CD19+ B cells in the same thymic suspension. The R3 gates show that some EGFP vesicles were present with the CD19 B cells.

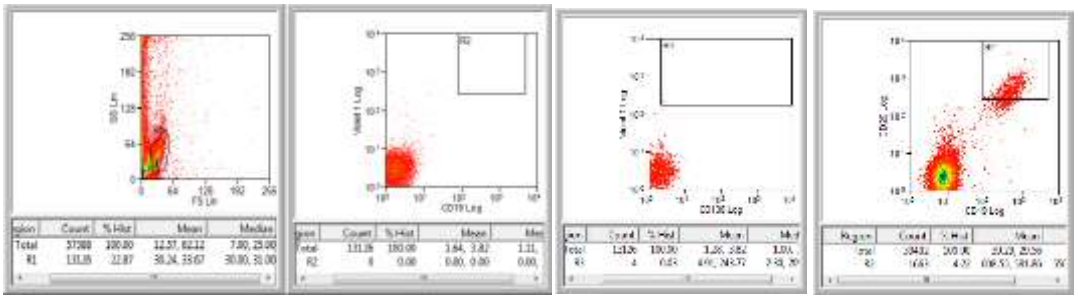
5.3 Flow cytometric analysis of CD19+ B cells and CD138+ plasma cells in thymic lymphocytes.

After optimization of the method the B cell subsets were first identified in the thymic pool of cells from selected patients that had high levels of AChR antibodies in their sera. The cells of two patients (nos 3 and 7 in Chapter 3) were stained with CD20 and CD138 markers to sort B cells and plasmablasts. CD20 is present on B cells but not plasma cells, whereas CD19 is more specific to memory B cells. These patients' thymic populations showed the presence of CD19 B cells but no CD138 plasma cells (Fig 5.3). CD138+ cells were not expected to be produced in the thymic culture within 12 hours and without any re-stimulation by antigens. The results for patient 3 were expected as the cultures had produced some AChR antibody but were surprising for patient 2. However, the presence of B cells does not necessarily correlate with specific antibody production.

A more detailed analysis of CD19+ B cells on PBMCs is shown in Figure 5.4. Figure 5.4A shows the controls, 5.4B shows the CD19+ cells and C shows the CD3 cells. These experiments used 10^6 cells for each test (per test tube). These experiments helped to set up the tests on thymic cells. As thymic cells were precious and were not available in abundance, use of PBMC and other cell lines saved the extra usage and developed a protocol which was ideally suited on thymic cells, will be described further in the text and experiments. As PBMC and thymic cells shared the T cell and B cell development stages, it was beneficial to start experiments with human PBMC and then moving on to precious archived preparations from thymic cells.

PBMCs were easier to handle and cell subsets were easily identified with the help of surface markers whereas thymic cells were clearly more difficult to deal with due to the limited number, thawing and growing. To improve the chances of seeing the cells in the

A Patient 2 CD19+



B Patient 3 CD19+

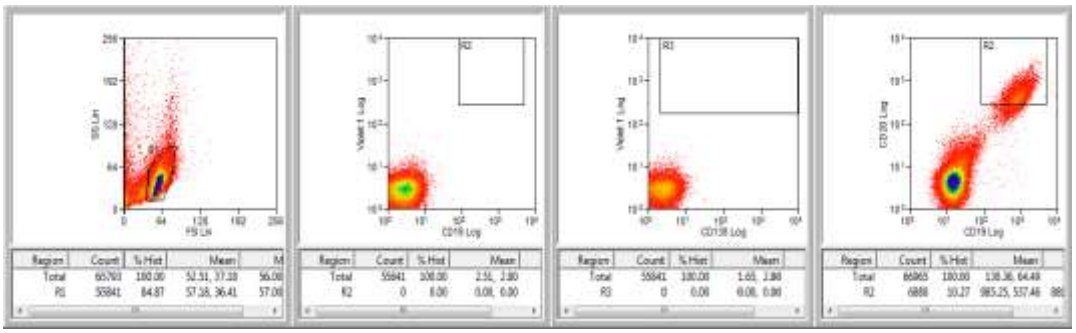


Figure 5.3. Flow cytometric analysis of thymic cells in suspension. Thymic cells of patient 2 and 3 (from table 2 in chapter 3) labelled with anti-CD19, anti-CD20 and anti-CD138 to look for plasma cells. . CD19 positive population were significant present in R2 , but there were no apparent CD138 plasma cells. These samples were tested 12 hours after thawing.

A. Control

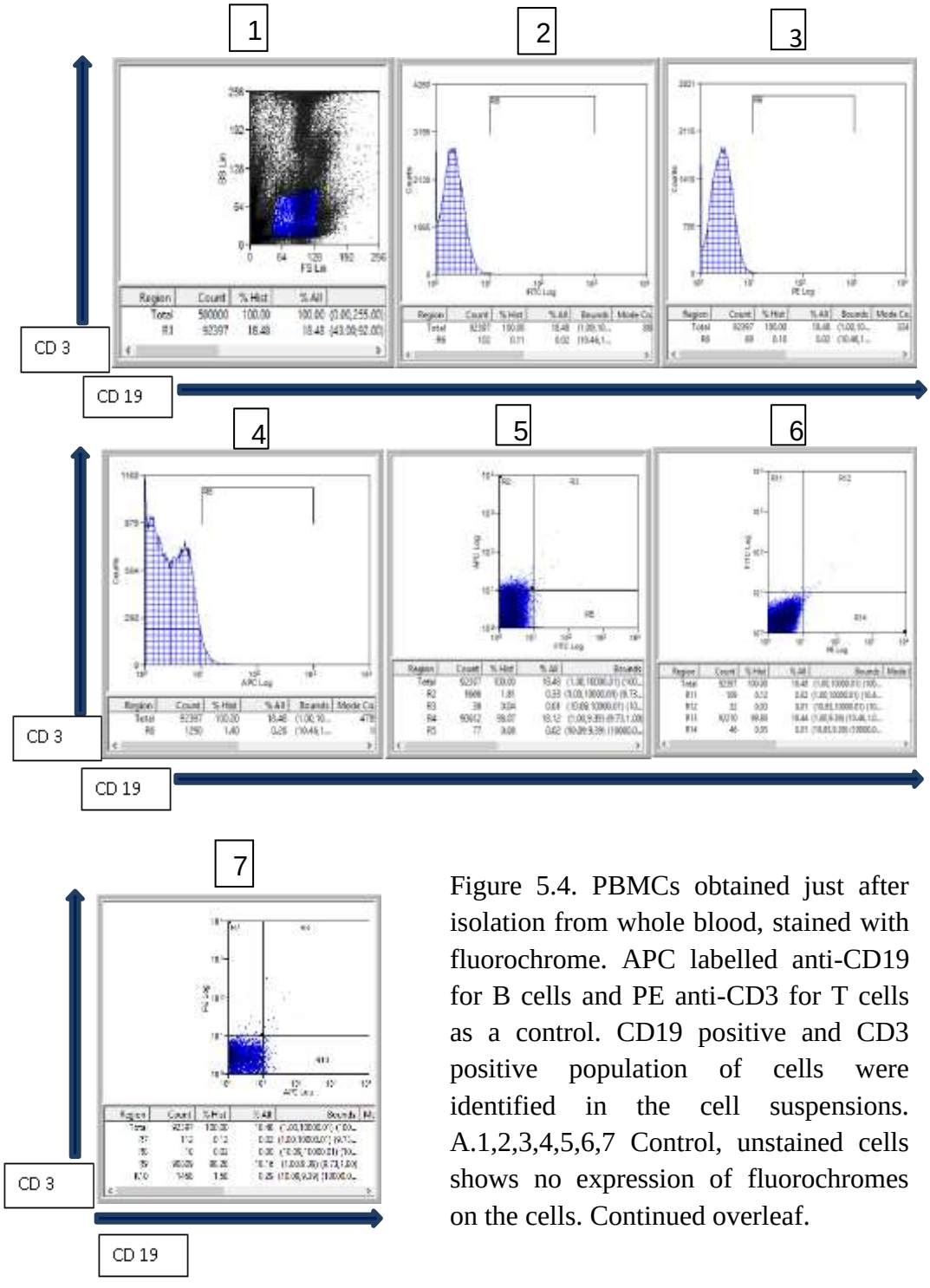
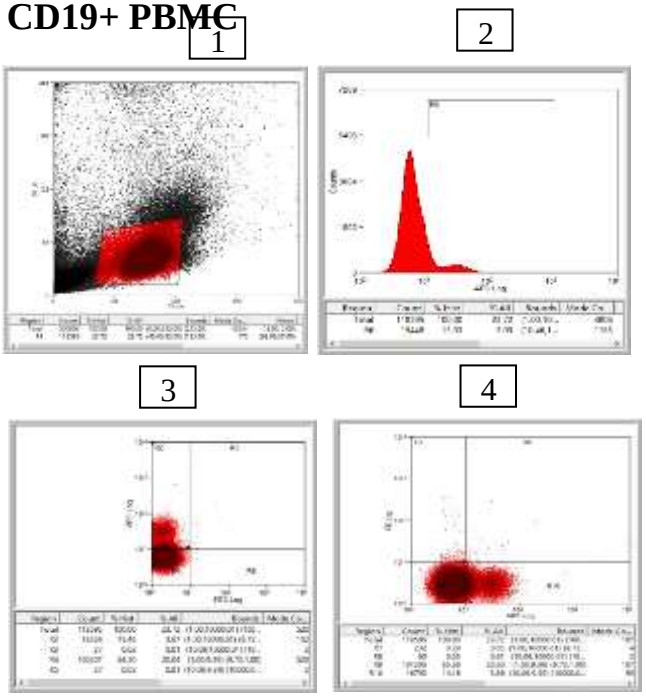


Figure 5.4. PBMCs obtained just after isolation from whole blood, stained with fluorochrome. APC labelled anti-CD19 for B cells and PE anti-CD3 for T cells as a control. CD19 positive and CD3 positive population of cells were identified in the cell suspensions. A.1,2,3,4,5,6,7 Control, unstained cells shows no expression of fluorochromes on the cells. Continued overleaf.

B. **APC CD19+ PBMC**



C. **PE CD3+ PBMC**

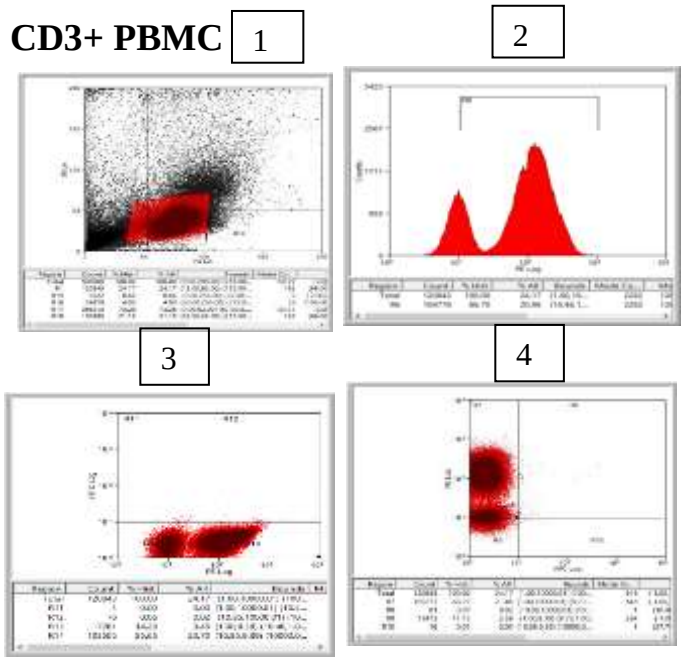


Figure 5.4 continued. B. 1,2,3,4, APC labelled anti-CD19 antibody shows CD19+ population of B cells as expected. C. 1.2.3.4 PE labelled anti-CD3 control shows CD3+ T cells in the pool of PBMCs.

D. APC CD19+ and PE CD3+ PBMCs

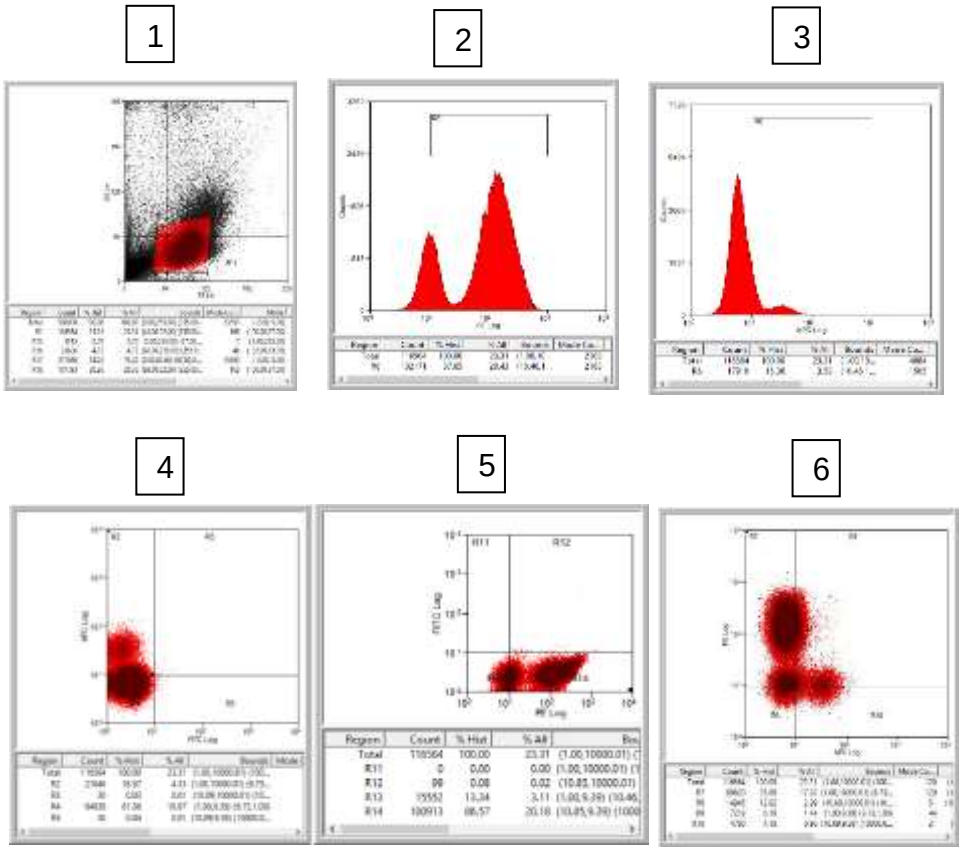


Figure 5.4 continued. D. 1,2,3,4,5,6 shows CD19+ B cells and CD3+ T cells in the PBMCs. D6. clearly identifies CD19+ B cells and CD3+ population T cells in the suspension.

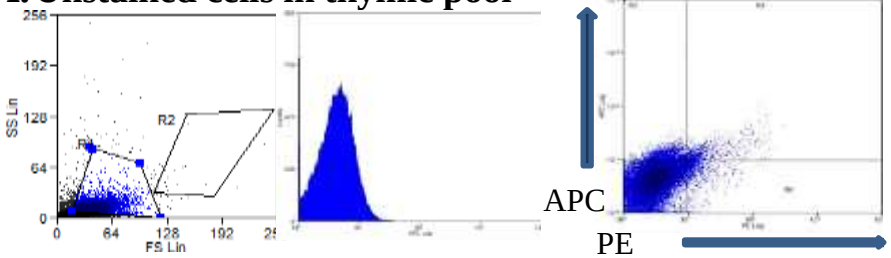
thymic cultures, the experiments were performed after 1 day of culture. In this case, CD3+ and CD19+ cells were identified as two populations (Figure 5.5). 36 hours after thawing, a small population of CD138 plasma cells were detected in the R11 and R12 gates. (Figure 5.6).

5.4 Selection of antigen re-stimulated B cells from the thymic lymphocytes of MG patients producing antibodies at high titres in sera

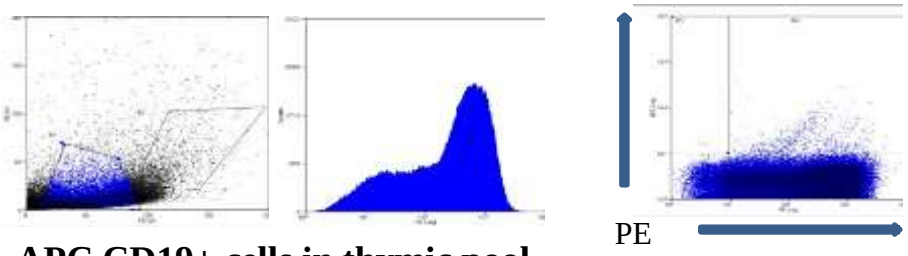
Thymic cells were incubated with AChR EGFP vesicles to try to identify antigen specific B cells in the pool. The cells were stained with anti-CD19, anti-CD3, ant-CD27 and anti-CD138 as above. In this case the patient whose cells were tested had high titres of AChR antibody scoring 3 at 1:1320. The results showed B cells in the thymic cultures as before with no detectable change in the presence of control vesicles. The thymic cells were incubated with AChR-EGFP vesicles for 30 minutes at 4ºC, to try to identify antigen specific B cells. The results (Figure 5.7F) show some co-staining of the APC CD19+ positive cells with the vesicles (Fig 5.7). CD3 T cells were not tested in last experiment mentioned above as it is a negative control in all the experiments. CD3+T cell marker helps to sort the T cell population from the pool which makes it easier to identify B cell population and specifically antigen specific B cells when restimulated with the specific antigen, in this case AChR membrane vesicles.

1. stained **2. log graph** **3. scattering**

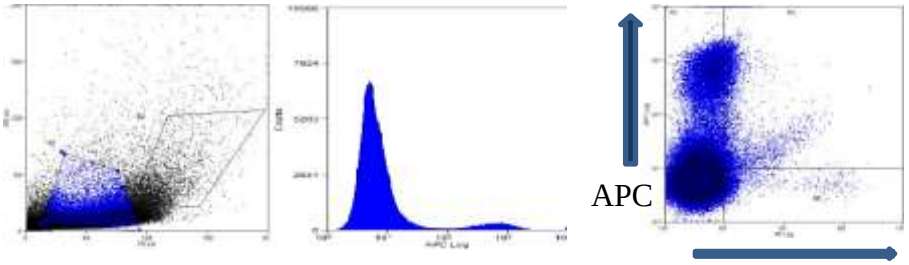
A. Unstained cells in thymic pool



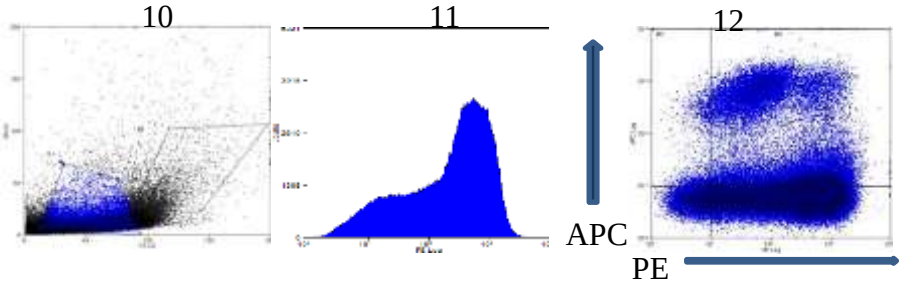
B. PE CD3+ cells in thymic pool



C. APC CD19+ cells in thymic pool



D. CD19+ B cells and CD3+ T cells



APC = CD19 B cells PE = CD3 T cells

Figure 5.5. Anti-CD3 and anti-CD19 detect T and B cells in the suspension of thymic lymphocytes after culture for 1 day. A.1,2,3 Control shows unstained thymic cells. B. 4.5.6. PE CD3 Show CD3+ T cells in thymic culture. C. 7,8,9, Shows APC CD19+ B cells. C9 shows Y-axis has a number of CD19+ B cells on its axis. D. 10,11,12, Combined plots of two different populations of the cells. D 12. CD 19 + cells in the culture suspension were in significant quantity.

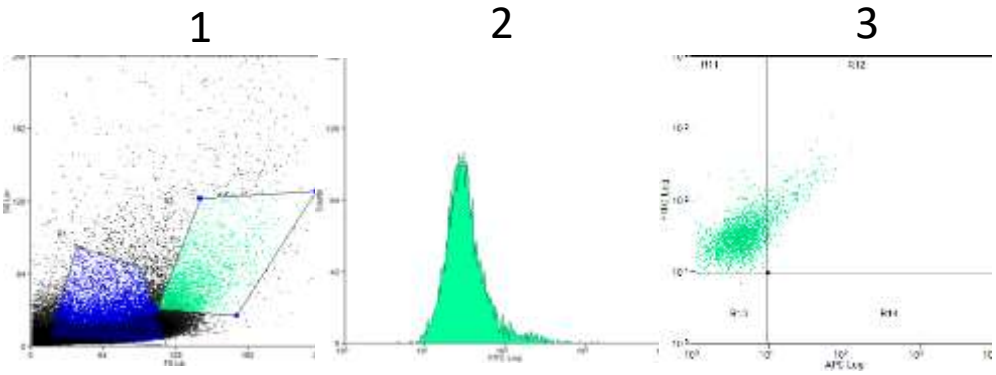


Figure 5.6 Gated population shows CD138+ cells in the thymic suspension. On FITC log CD138+ cells are visible. This test was conducted after 36 hours of thawing of cells and after incubating with AChR vesicles (no colour) for 30 minutes. On plots 2 and 3 CD138+ plasma cells are shown in lesser number, as expected. The peak on plot 2 shows nearly 100 CD138+ plasma cells in the gate, and on plot 3 in the R11 and R 12 gates, 102 CD138 positive plasma cells were present.

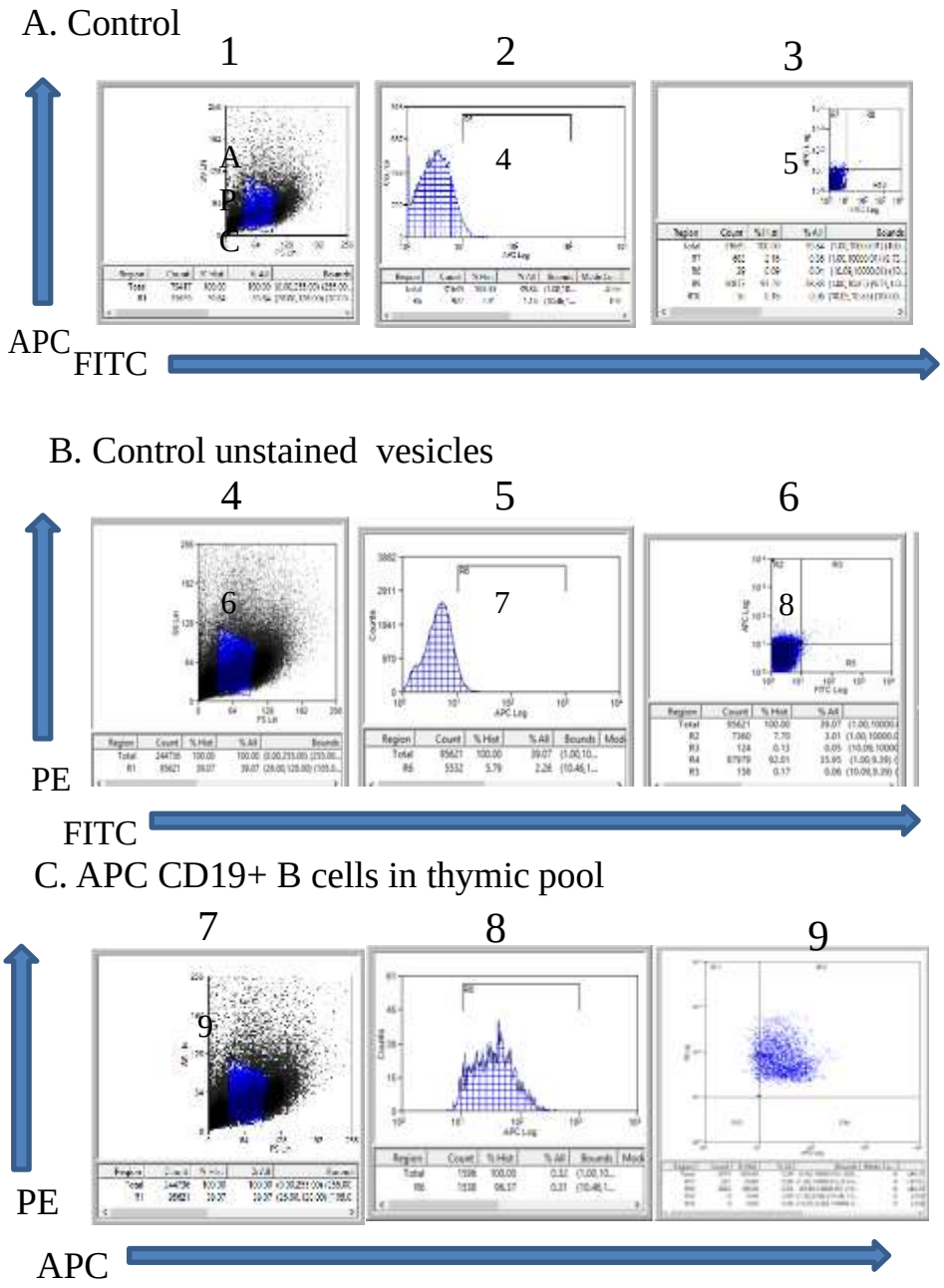


Figure 5.7. Thymic cells incubated with AChR EGFP vesicles. Anti-CD19 was used to identify antigen specific B cells. Anti-CD3 was used to sort T cell population from the pool as a negative control. A.1,2,3 Control compensates the auto fluorescence for all the fluorochromes used in each test. B.4,5,6, Unstained membrane vesicles were used to compensate for the autofluorescence, cells unstained. C.7,8,9 APC anti-CD!9 marker was used to sort the CD19+ B cells from the suspension, C9 plot shows some CD19+ cells on the x-axis. Cont. overleaf.

Discussion

A successful B-cell response to an antigen involves the development of two distinct B-cell types, memory B cells and plasma cells. Memory B cells are long-lived and rapidly mount a secondary antibody response upon re-exposure to the corresponding antigen. Plasma cells secrete large amounts of antibodies spontaneously and die after a few weeks. However, an exceptionally long-lived subpopulation of plasma cells migrates into the bone marrow where it produces high-affinity Immunoglobulins for years. (Archelos & Hartung, 2000). In the hope of identifying AChR antibody specific B cells in the thymic lymphocyte populations, that had previously been shown to produce AChR antibodies (e. g Scadding *et al*., 1981), the AChR-containing vesicles produced in Chapter 4 were tested. The methodology and approach followed was reproducible hence proving hypothesis right however, the results were satisfactory and need further development.

To establish the techniques for identifying the thymic lymphocyte subsets, initial studies focussed on Raji cells and PBMCs. No AChR vesicles could be detected binding to healthy PBMCs but there was some evidence of AChR vesicles binding to B cells in the thymic pool. Due to the uncertain results partial of AChR membrane vesicles, the sorted B cells could not be characterized at a single cell level. but further studies improving the expression of AChR in the vesicles could lead to a way of specifically identifying the cells responsible for producing AChR antibodies in MG. It might be advantageous to try first with AQP4 B cells and plasma-blasts since the AQP4 is very well expressed in the vesicles and B cells producing antibodies have been cultured from NMO PBMCs (Wilson *et al*.,2018).

Raji cells were the easiest to set the standard protocols. On further study, PBMCs of controls were used before moving to the very precious thymic cells which were frozen by Prof Willcox in the past. The hope was that culturing thymic cells and then presenting them with the prepared AChR membrane vesicles at 4^{o}C would demonstrate the binding of the EGFP-labelled vesicles binding to B cells and plasma cells. However, plasma cells were very rare even in cultures with (previously) high synthesis of AChR antibodies. The absence of fresh prepared thymic cells, as were used for the original studies of Prof Willcox, was a limitation of the study.

The hope was that AChR-vesicle-identified B cells could be isolated by FACS, restimulated with the AChR-vesicles and antibody genes amplified using RT-PCR/nested PCR so that the variable H+L chains could be cloned into expression vectors for production of highly specific, patient derived antibodies. Another aspect of the work potentially would be to find ways of specifically deleting the AChR-specific B cells for treatment.

CHAPTER 6

General discussion

MG is an autoimmune syndrome caused by the failure of neuromuscular transmission, which results from the binding of autoantibodies to proteins involved in signaling at the NMJ. The endplate potential is insufficient to reach the crucial firing threshold, resulting in weakness at rest and/or increasing fatigue during sustained efforts, affecting any muscle group (Vincent., 2008). Generalized MG patients can be further subdivided on the basis of their thymic pathology and age at onset (Compston*et.al*., 1980). Nevertheless, despite these clearly different pathological and genetic associations, the AChR antibodies themselves do not differ significantly between the subgroups, suggesting that the antibodies are the common final pathway that can be reached by several different aetiological routes (Vincent *et.al*., 1998).

In most EOMG patients, the thymic infiltrates include numerous germinal centers (Willcox *et.al.,* 1984), many of them contain autoreactive T- and B-cells along with terminal plasma cells that produce AChR antibodies (Shiono*et.al*.,2003). Several lines of evidence indicate that B cells, plasma cells, and, in particular, antibodies contribute to development and progression of MG. The aim of this thesis was to develop a novel approach to identify the B cells and perhaps early plasmablasts in patients with myasthenia gravis, by using AChR-specific EMVs. Unfortunately, despite some evidence that the technique could work, the cultures available and the results were not yet sufficiently robust to provide the basis for further development.

This study had both advantages and limitations. First, the presence of archived sera and of thymic cultures stored in liquid nitrogen made it possible to do the project on samples that had been shown previously to synthesis AChR antibodies. Howevver, the lack of

fresh samples were a considerable disadvantage as the production of AChR antibodies by the cultures tested here was very poor (and also found by an Oxford DPhil student, ZZ). The amount of thymic cells obtained after thawing was very limited in number compared with the numbers frozen at the time of storing. When comparing with fresh PBMCs, it was very clear that use of fresh thymic cells would have given better yields of cells and made it easier to identify and target B cells and plasma cells in the cultures. Nevertheless, it was hoped that archived preparations could still be used to establish the approach and some preliminary results.

Most of the sera were positive for AChR antibodies by RIPA and by CBA in the serum and there was a wide range of antibody levels in the cohort, as expected. Clustered AChR antibodies were also detected on CBAs as is typical (Leite *et.al.,* 2008, Rodriguez-Cruz *et.al.,* 2015) and it was disappointing that the same did not apply to the thymic cultures; there is previous evidence from competition studies with monoclonal antibodies to human AChR, that the characteristics of thymic and serum antibodies are similar (Heidenreich *et.al.,* 1998). Higher levels of the antibodies in the thymic cultures would have provided more robust results.

After screening sera and supernatants of the MG patients, this study further proceeded to develop a method of obtaining membrane vesicles from the transfected cells. The idea was to use membrane vesicles as a source of antigen for identifying the specific B cells and also potentially as a source for antigen presentation. Harvested AChR membrane vesicles after testing were presented with subsets of B cells to try to sort the antigen specific B cells from the pool of lymphocytes. It was first necessary to try different methods to obtain CD19 specific memory B cells and to sort the B cells and its subsets in PBMCs using 4 healthy individuals, one in each experiment, to optimize the method

and technique of presenting B cells to antigens. Unfortunately, it was not appropriate to make comparisons between them or with MG patients.

In neuromyelitis optica (NMO), an autoantibody specific for aquaporin-4 (AQP-4), a water channel protein, was identified (Lennon *et.al.,* 2005). There is accumulating evidence that NMO is pathologically different from MS and represents a distinct disease entity with antibody-mediated astrocytopathy. Transfer experiments have demonstrated that the Aqp-4 antibody can induce astrocyte pathology on transfer to the brain in animals after disruption of the blood–brain barrier or by intra-cerebral injection with complement (Saadoun*et.al*., 2010). In addition, the AQP4 antibody titer seems to correlate with disease activity, further supporting the concept that AQP-4 is a major target of the autoimmune response in NMO. Although some cloning of B cells has already been performed by other groups (Wilson *et.al.,* 2018), the identification of B cells specific for AQP4 antibodies would be a major advance.

Currently one of the most popular treatments for B cell mediated diseases is therapeutic depletion of CD20-positive B cells. Anti-CD20 (rituximab, Rituxan®) was initially developed to treat non-Hodgkins B-cell lymphoma and has shown promising results in treatment of CNS demyelinating diseases. Although rituximab is not cell specific, since it kills all the B cell subsets, use of the drug in lymphoma led to trials of rituximab and subsequent anti-CD20 specific antibodies in MG and NMO. For example, in a small open-label pilot study evaluating B-cell depletion in neuromyelitis optica (NMO), seven out of eight patients receiving rituximab experienced substantial improvement of neurologic function (Jarius*et.al.,* 2008). It appears that rituximab immediately abolishes cellular B-cell functions, such as B-cell antigen presentation, whereas antibody titres appear to decrease with a substantial delay because antibody-secreting plasma cells no

longer express CD20. Based on this assumption, it seems that that the immediate benefit of B-cell depletion in T-cell-mediated autoimmune disease may relate to an impaired activation of T cells by the B cells rather than a direct effect on antibody production.

Another disease that could be approached in a similar way is multiple sclerosis. Evidence for a role of antibodies in MS derives from histopathological studies in which B cells, as well as B-cell-derived plasma cells and antibodies, are found in the central nervous system (CNS). Antibodies are frequently observed in acute lesions of MS patients and in newly diagnosed patients, histopathological studies demonstrate heterogeneity of acute lesions between individual patients suggesting at least four distinct patterns of acute demyelinating lesions (Keegan *et.al.,* 2005). The most frequent pattern is characterized by significant antibody deposits and complement activation suggesting B cell involvement. Of importance, molecular analyses of B cells in brain lesions demonstrate an accumulation of clonotypic B cells with preferential use of particular variable (V) heavy (H) chain (VH) genes suggesting a restricted local immune response. Furthermore, investigated sequences showed signs of hypermutations reflecting an ongoing and maturating B-cell response to the target antigen (Colombo *et.al.,* 2000). However, other studies suggest that the immunoglobulin G heavy chain repertoire in multiple sclerosis plaques is not entirely distinct from the heavy chain repertoire in peripheral blood lymphocytes (Bankoti *et.al*., 2014) making the peripheral nervous system worth studying further.

If the B cells could have been identified more convincingly in this study, the next step would be fractionation of the cells and amplification of immunoglobulins to create reactive antibody. Combinatorial libraries created by RT–PCR of pooled lymphocytes or tissues provide a majority of immunoglobulin sequences, but cannot be relied upon to

provide correct pairings of heavy (VH) and light (VL) chain V regions. A single cell-based methodology is necessary to yield correctly paired VH + VL Fab. A simple method for the amplification of correctly paired IgG κ or λ Fab from single human B cells or plasma cells that combines the best features of related methods, and improves them by optimization and simplification. This method has the advantage of versatility, sensitivity and reliability, allowing amplification of correctly paired Fab from different cell types and sources. The method almost invariably provides pure mRNA extracts and has built-in controls for the detection of template contamination. Sequence analysis of immunoglobulin (Ig) heavy and light chain transcripts can refine categorization of B cell subpopulations and defines the selective forces that act during immune responses or immune dysregulation, such as autoimmunity, allergy, and B cell malignancy (Rogosch *et.al*., 2012).

A novel and user-friendly tool was described for easier analysis of cloned IgG sequence for the extensive analysis and graphical presentation of a very large collections of Ig transcripts which have been pre-analyzed by IMGT/HighV-QUEST. (Rogosch *et.al.*,2012) .

Ig AT additionally calculates the probability of antigen-driven selection within Ig repertoires and predicts structural properties of the antigen-binding site. IgAT can be used to analyze up to 150 kDa human or murine heavy or light chain transcripts in a single run of the application and automatically generates 25 Microsoft® PowerPoint® graphics files illustrating key characteristics of the Ig repertoire, such as VDJ gene utilization, amino acid use. IgAT readily yields the necessary data to allow statistical and graphical comparisons between various repertoires. (Rogosch et.al., 2012).

However, this technique was not used in this project.

In autoimmune diseases in which pathogenic or excess IgG antibodies are the etiological agents, such as myasthenia gravis, bullous pemphigoid, idiopathic thrombocytopenic purpura (ITP), and systemic lupus erythematosus (SLE), it is sometimes advantageous to reduce endogenous serum IgG levels by interfering with FcRn function. One possible way to interfere with the function of FcRn is to overload it with "innocuous" IgG. As FcRn functions as the IgG homeostatic receptor, the level of FcRn expression determines the serum concentration of IgG. Administering large quantities of exogenous IgG raises the serum concentration above this equilibrium set point and saturates FcRn (Yu & Lennon, 1999). As a result, the excess IgG that does not bind to FcRn enters the degradative pathway. This results in a shortening of the serum IgG half-life. High-dose IVIG treatment is thought to exert an immunomodulatory effect by numerous mechanisms, including engagement of the inhibitory FcγRIIb receptor (Samuelsson *et.al*., 2001) and by FcRn saturation.

Advances in our understanding of the pathophysiology of MG have led to more effective treatments for this condition and have implications for other antibody-mediated disorders. Earlier disease recognition combined with prompt appropriate treatment are key factors in improving the prognosis of MG. Therefore, the need to educate and improve disease awareness is important in the diagnosis and management of this eminently treatable neurological disorder.

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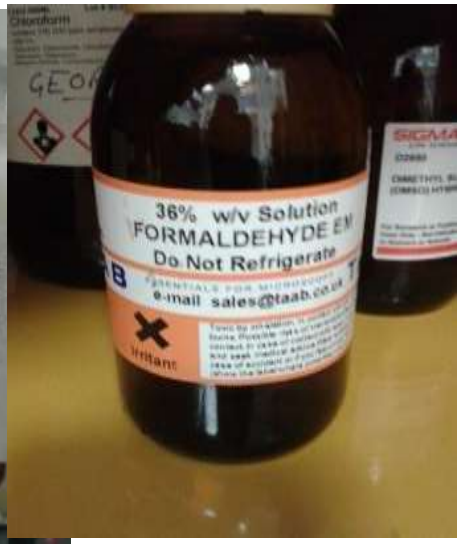
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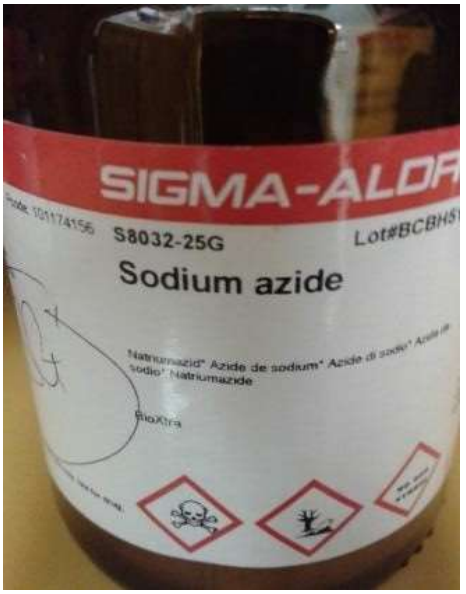
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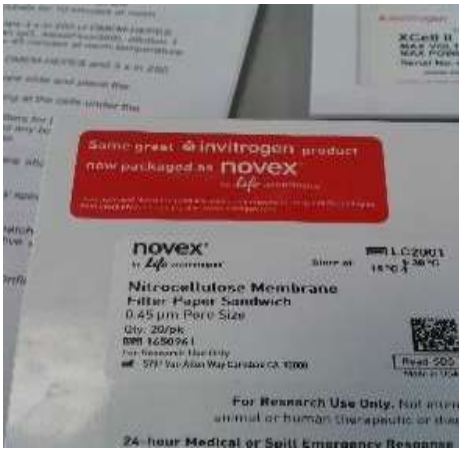
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