Anti-acetylcholine receptor antibodies and myasthenia gravis

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ANTI-ACETYLCHOLINE RECEPTOR ANTIBODIES AND MYASTHENIA GRAVIS

submitted by Andrew James Quinn
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CONTENTS

SUMMARY

The development of a suitable screening assay for the detection of monoclonal antibodies of a required specificity, is essential to the overall success of the technique.

An antibody capture enzyme immunoassay has been developed for the detection of anti-acetylcholine receptor (AChR) antibodies in tissue culture supernatants using biotinylated alpha-bungarotoxin (B-alpha-BGT). Immunoglobulins in culture supernatants were bound indirectly to microtitre plates via an anti-globulin antibody already coupled to polyvinyl plates. Anti-AChR antibodies were then detected by incubation with AChR crude extract. Bound AChR was revealed by incubation with B-alpha-BGT followed by horseradish peroxidase conjugated avidin (HRP-avidin). This latter reagent was subsequently replaced by HRP-Streptavidin, after the discovery that the very basic avidin, interacted with the casein used as a non-specific blocker in the assay buffers. Casein was also replaced for this reason by fish skin gelatin. The assay was shown to be more specific and sensitive than the commonly used double antibody radioimmunoassay (RIA), avoids the use of radioactive material, is practical for large numbers of samples and is particularly suitable for detecting anti-AChR antibodies in tissue culture supernatants. All assay conditions were subsequently optimised by utilising murine monoclonal anti-Torpedo AChR antibodies.

This approach was favoured over an anti-idiotypic assay,

which
was also being developed, due to the fact that the biotin/avidin ELISA was applicable to antibodies raised from any patient, whereas the anti-idiotype approach is only suitable for use in detecting the idiotypes against which the anti-idiotypes were raised. The idiotypes being obtained from one patient.

Even with the development of a highly sensitive assay for the detection of human monoclonal antibodies, directed against the nAChR, little success was achieved in the generation of human monoclonal anti-AChR antibodies. Partial success was achieved in the detection of anti-AChR antibodies, by utilising the direct Torpedo ELISA, and culture supernatants from EBV transformed lymphocytes and heterohybridomas. Observations made during the course of cell manipulations and immortalisations, confirmed previously held reports on the instability of antibody production seen within lymphoblastoid/heterohybridoma cells with antibody production declining unless vigorous and early cloning is carried out. Consequently, the techniques themselves are labour intensive with often little reward.

ABBREVIATIONS

ACh	Acetylcholine
AChE	Acetylcholinesterase
mAChR	muscurinic Acetylcholine receptor
nAChR	nicotinic Acetylcholine receptor
AMLR	Autologous mixed lymphocyte reaction
APC	Antigen presenting cell
α-BGT	α-Bungarotoxin
B-alpha-BGT] B-α-BGT]	Biotinylated-α-bungarotoxin
BSA	Bovine serum albumen
BZQ	Benzoquinonium chloride
cDNA	complementary Deoxy-ribonucleic acid
CMI	cell mediated immunity
CNBr	Cyanogen Bromide
CNS	Central Nervous System
DMPP	1-1-dimethyl-4-phenylpiperazine
DMSO	Dimethylsulphoxide
DNA	Deoxy-ribonucleic acid
EAMG	Experimental autoimmune myasthenia gravis
EBV	Epstein-Barr virus
EDTA	Ethylene diamine tetra-acetic acid
ELISA	Enzyme Linked ImmunoSorbent Assay
EJ	Extrajunctional
EM	electron microscope
EPP	End-plate potential
FCA	Freund's complete adjuvant
FCS	Fetal Calf serum

HAT	Hypoxanthine, aminopterin thymidine
HLA	Human leucocyte antigens
HRP	Horseradish peroxidase
Ig	Immunoglobulin
IgA	Immunoglobulin A
IgD	Immunoglobulin D
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL	Interleukin
LAK	Lymphokine Activated Killer cells
LCL	Lymphoblastoid cell line
Mab	Monoclonal antibody
MEPP	Minature end-plate potential
MG	Myasthenia gravis
MHC	Major histocompatability complex
MIR	Main Immunogenic region
mRNA	messenger Ribonucleic acid
NGS	Normal goat serum
NHS	Normal human serum
NK	natural killer cells
NMJ	Neuromuscular junction
NRS	Normal Rabbit serum
O.D.	Optical Density
PBL	Peripheral Blood Lymphocytes
PBS	Phosphate buffered saline
PEG	Polyethylene glycol

PEI	Polyethyleneimine
PHA	Phytohaemagglutinin
PWM	Pokeweed mitogen
pI	Isoelectric point
PMSF	Phenylmethyl-sulphonyl fluoride
RIA	RadioImmunoAssay
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
* TCA	Trichloroacetic acid
TMB	3,3',5,5'-tetra-methylbenzidine
T_H	Helper T-cells
T_S	Suppressor T-cells
T_{CS}	Contra-suppressor T-cells
T_C	Cytotoxic T-cells
TCR	T-cell receptor
TT	Tetanus Toxoid
* SRBC	Sheep Red Blood Cells

INTRODUCTION

1. THE IMMUNE RESPONSE

Our immune system saves us from certain death by infection. Any child born with a severely defective immune system will die unless the most extraordinary measures are taken to isolate it from a host of infectious agents - bacterial, viral, fungal and parasitic.

The immune response is highly specific. An individual who recovers from measles is protected against future exposure to the virus, but he is not protected against other common viruses, such as mumps and chicken pox. This specificity is a fundamental characteristic of the immune response.

Yet another fundamental characteristic of the immune response is its ability to distinguish between foreign and self molecules. This is essential when it is considered that the majority of the reactions carried out by the immune system are of a destructive nature. Occasionally it fails to make this distinction and reacts against self components, such as in the autoimmune diseases.

There are two classes of immune response:

1) Humoral Antibody Response.

2) Cell Mediated Immune Response.

These responses and their interactions will be briefly considered in an overview of the body's immune responses.

Further discussions are to be found in, Alberts et al (1983), Roitt (1987 & 1989), and Feinberg & Jackson (1983).

1.1 Humoral Antibody Responses

Antibody responses are mediated by B-lymphocytes, which account for 5-15% of the circulating lymphocytes. Each B-cell during its development from haematopoietic stem cells, becomes committed to the recognition of one particular antigenic component, and to the subsequent production of one specific type of antibody towards it.

B cells display characteristic markers on their cell surface, some of which are antigen receptors, predominantly surface bound immunoglobulins. Other cell surface markers include, MHC class II antigens (HLA-DP, DQ and DR) used in cooperation with T-cells: receptors for the complement components C3b (CR1, CD35) and C3d (CR2, CD21), (CR2 is also the receptor used by EBV in gaining entry to the cell), and Fc receptors for IgG (FcRII, CDw32). Other markers, CD19, CD20, and CD22 also occur and it is, in fact, these that are mainly used to identify human B-cells.

Upon stimulation, the B cells develop into plasma cells. These cells are larger than the immature cells and are characterised by large amounts of rough Endoplasmic Reticulum. Despite their name, they are not found in the plasma, but are associated with secondary lymphoid tissues, and only survive for 1-2 days producing vast amounts of immunoglobulin.

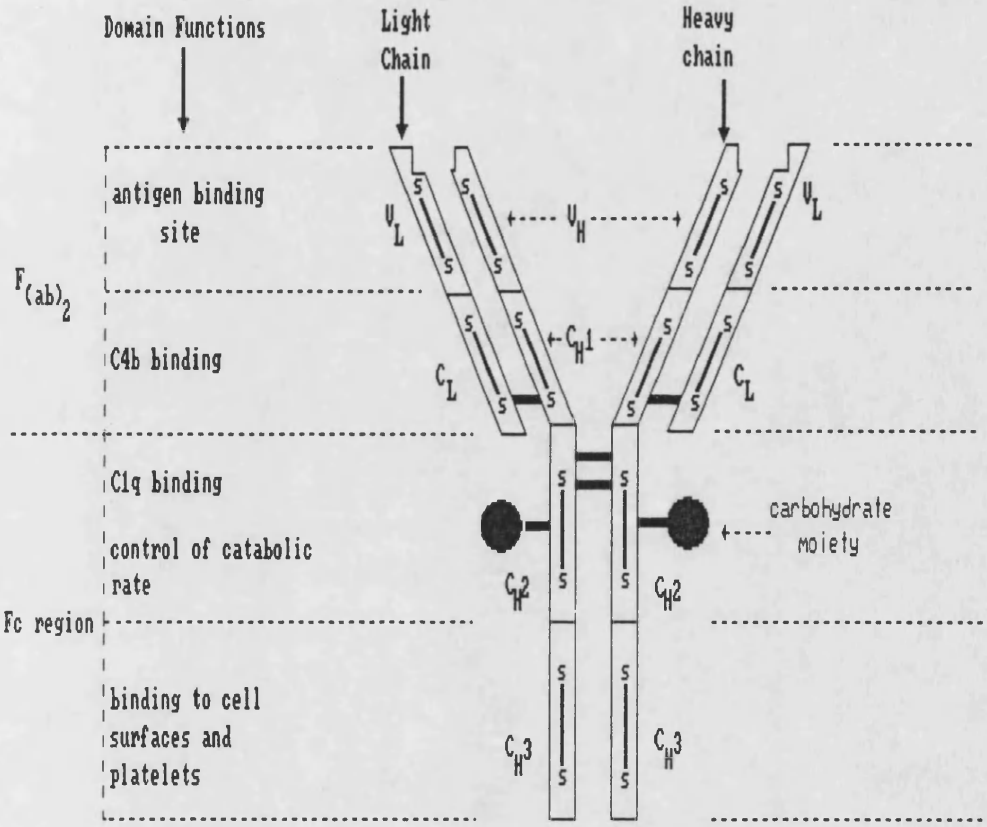
Although B-lymphocytes are capable of producing five different classes of immunoglobulin, during their development they become committed to the production of antibodies of only one class and specificity.

1.1.1 The Immunoglobulins

The immunoglobulins are a group of blood glycoproteins sharing similar structures. The basic structural unit of an immunoglobulin consists of four polypeptide chains, two identical light chains (approx. 220 a.a. units, Mwt approx. 25,000) and two identical heavy chains (approx. 440 a.a. units, Mwt approx. 50,000 - 77,000) associated together to give two antigen binding sites (see Fig. 1 which gives a diagramatic summary of IgG). The two heavy chains are joined together by interchain disulphide bridges, and each light chain is joined to one heavy chain by another disulphide bridge (this was first proposed as a model in 1962 by Porter). There are five different classes of immunoglobulin, known as isotypes, (IgG, IgM, IgA, IgE, IgD), differing in heavy chains (gamma, mu, alpha, epsilon, delta) and biological properties. Within the IgG and IgA classes are differing sub-classes, also isotypes (IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2). They all, however, share the same two light chains (kappa or lambda)

IgG is a monomeric glycoprotein, accounting for 70 - 75% of total serum immunoglobulin, and is the major antibody of the secondary immune response. As can be seen in Fig. 1, it is made up of four polypeptide chains, two heavy chains and two light chains. Amino acid sequencing and protein folding experiments

Fig.1 - Diagramatic Representation of IgG Showing Major Domains Related to Function



— Interchain Disulphide Bond

S—S Intrachain Disulphide Bond

Light Chain - consists of approx. 220 amino acid units
M.wt. of approx. 25,000

Heavy Chain - consists of approx. 440 ammino acid units
M.wt. between 50 - 77,000

have established that both the heavy and the light chains are characterised by domains sealed with intrachain disulphide bridges, and each domain has a particular function (see Fig. 1). Both the heavy and light chains have constant and variable domains, with the variable domains of both the light and heavy chains located at the antigen binding site. Further to this, it has also been shown that there are three hypervariable regions within the variable region of each chain and together, all six form the antigen binding site. Each different binding site specificity is known as an idiotype.

IgM is found as pentamer of the four chain basic structure linked by disulphide bridges, together with a J or joining chain. It accounts for 10% of total serum immunoglobulin, and is the main antibody in the primary immune response. Due to its pentameric structure, which tends to make it more rigid, it is much larger than IgG, has more domains, 10 antigen binding sites, and binds complement with greater affinity than IgG. This is due to the rigid structure, holding the individual units in the correct orientation for complement fixation. IgM, along with IgD, is also found in a monomeric membrane bound form on the cell surface of B lymphocytes, where it acts as an antigen receptor.

IgA and IgE have the same basic structure as IgG, IgA being found in a dimeric form, and each has its own function which will not be considered here (see Roitt, 1989)

1.1.2 Generation of Antibody Diversity

The immune system has to be able to recognise millions of

different antigenic determinants, and produce antibodies towards them. This constitutes a problem of genetics rather than of protein chemistry.

The greatest structural variation in immunoglobulins involves the antigen binding site, and most workers have studied the gene rearrangements in both mouse and man, which bring about these variations. Whereas, most work has been carried out using the mouse system, it is generally believed that the mechanics involved in the mouse, with subtle variations, apply to man.

In man, the genes encoding the heavy chains are located on chromosome 14, and those encoding the light chains are on chromosomes 22 and 2 (lambda and kappa respectively). The genes are a highly complex family consisting of multiple variable (V_{1-n}) and joining (J_{1-n}) genes, and a single constant (C_L) gene, with respect to the light chains (see Fig. 2), with additional diversity (D_{1-n}) genes and five heavy chain genes in the heavy chain gene family (see Fig. 3). The gene rearrangements involved are also highly complex, and apply to both the heavy chain and light chain genes. They are too complex for an adequate review to be given. However, Fig. 2 gives a diagramatic summary of the DNA rearrangements in the light chain gene family, whereas, Fig. 3 gives the same details for the heavy chain genes. Both diagrams show how multiple variable regions are obtained through the joining of genes by DNA deletion. In addition, Fig. 3 shows diagramatically how membrane bound IgM and IgD is obtained, through differential RNA splicing, and how the isotype switch occurs upon antigenic stimulation. The rearrangements, class

Fig.2 - Gene Rearrangements In Light Chain Genes

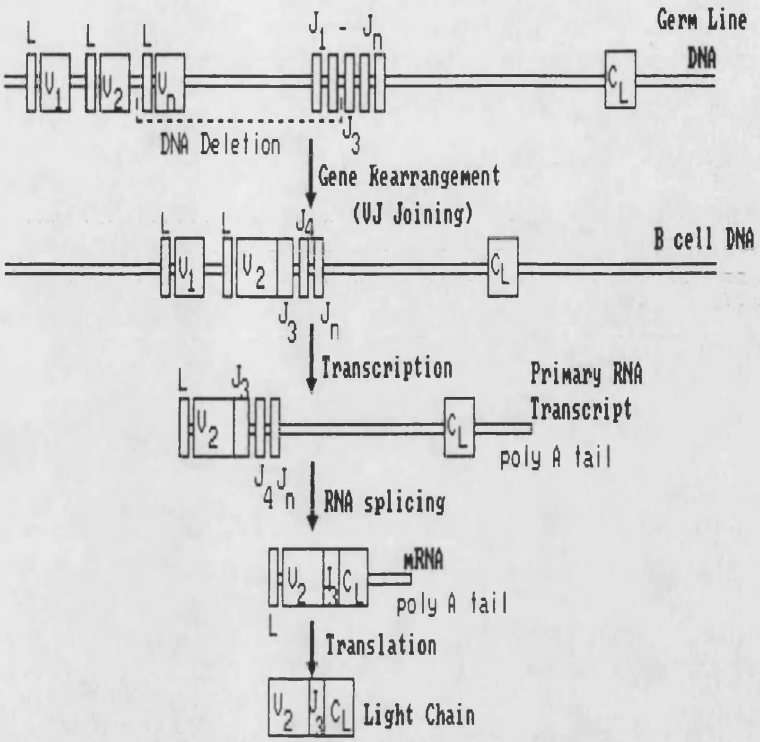


Figure shows gene rearrangements and processes used by the B cell in the formation of Light chain protein. This is a simplified diagram applicable to both the Lambda and Kappa loci, located on chromosomes 22 and 2 respectively, in man. The exact numbers of V and J sequences are not given. The mechanism for the DNA rearrangement is believed to occur through the looping out of the interim DNA using specific sequences located upstream of the J sequence, and downstream of the V sequence.

Fig.3 - Gene Rearrangements In Heavy Chain Genes

Diagram shows production of membrane bound IgM + D by differential RNA splicing from a long RNA primary transcript in non-stimulated B-cells, together with isotype switching upon antigenic stimulation Heavy chain genes are located on chromosome 14. Exact numbers of V, D, J sequence genes are not given.

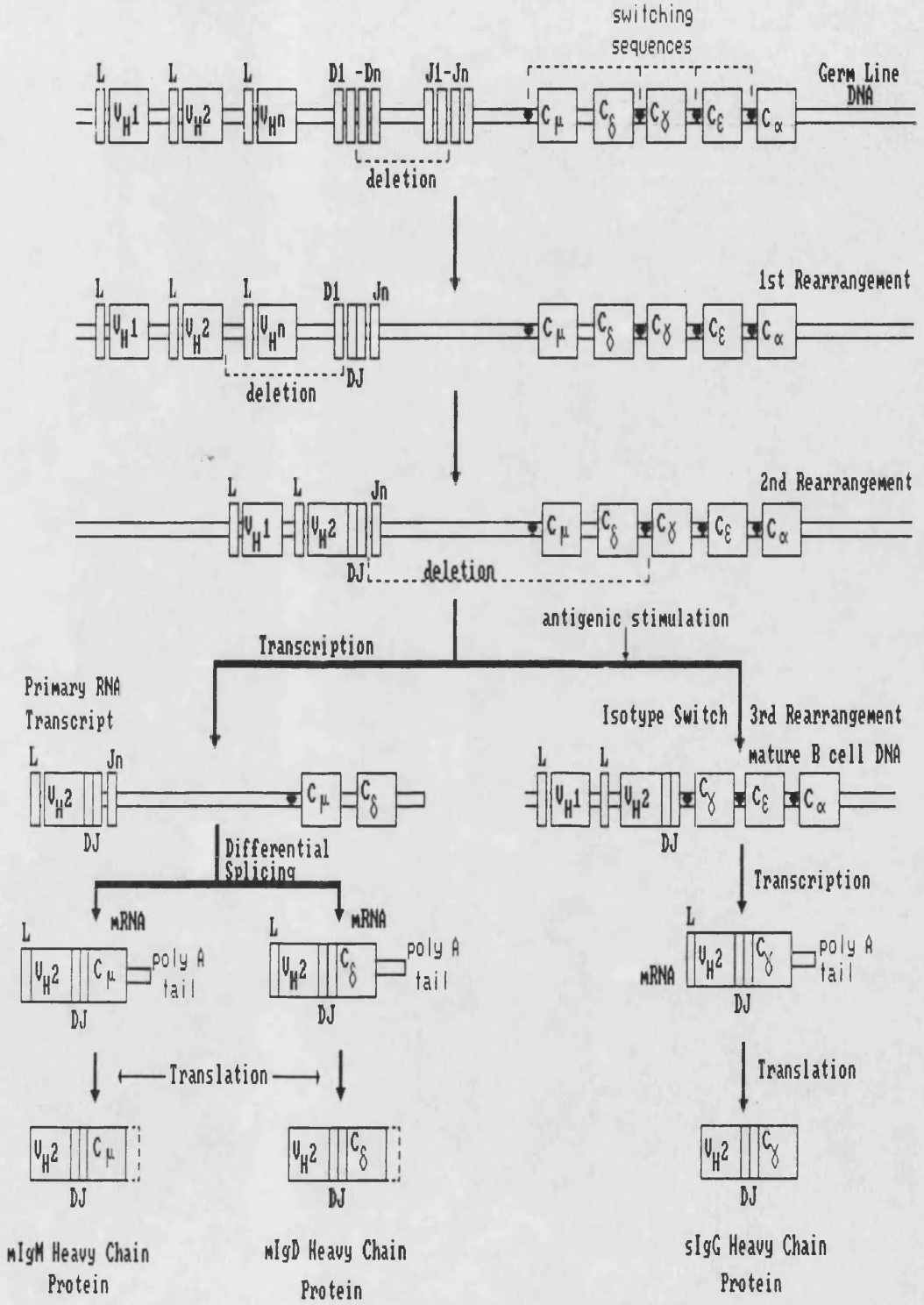
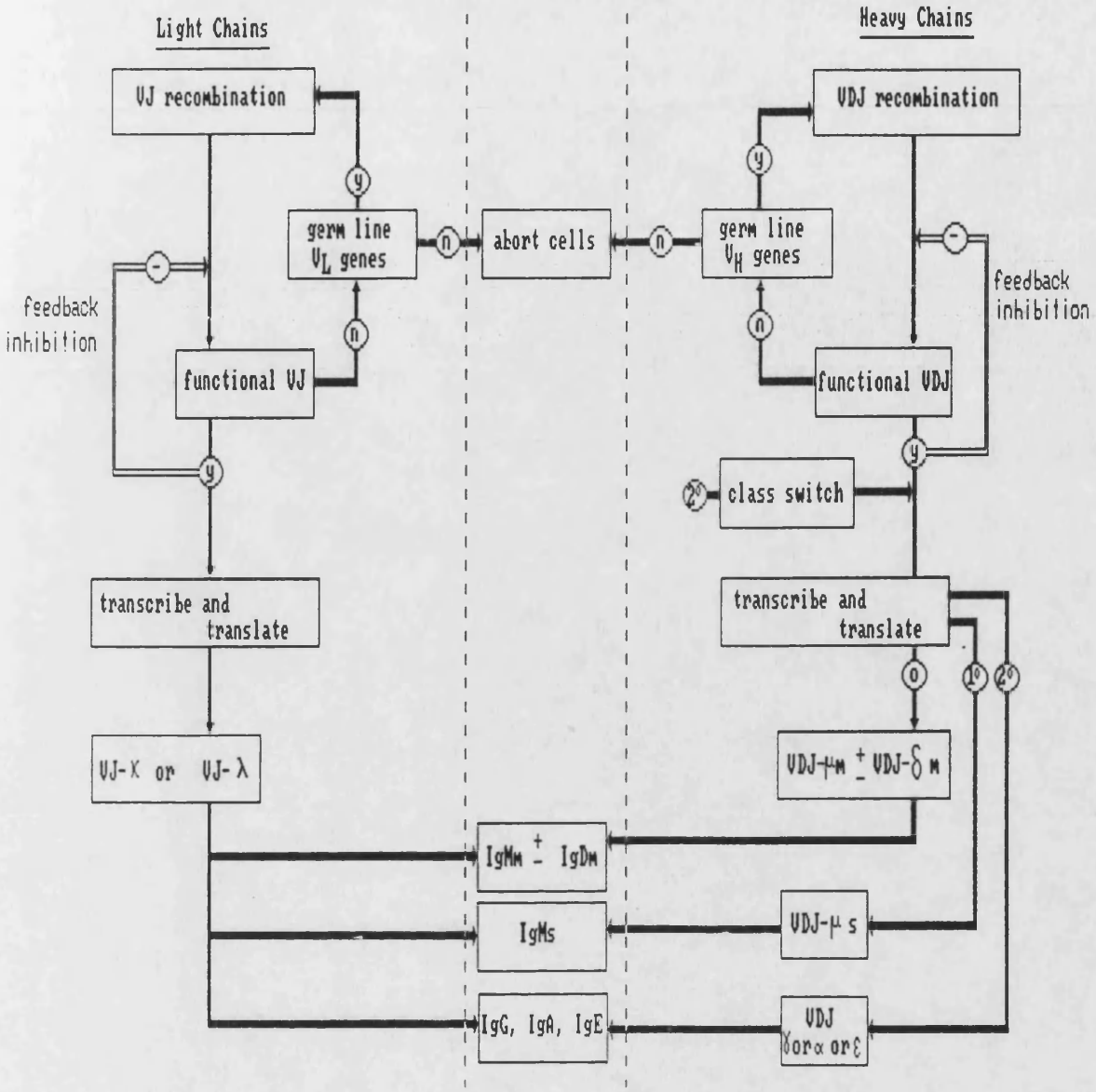


Fig.4 - diagramatic summary of antibody production



y - yes

n - no

o - no antigenic stimulation, producing membrane bound IgM & IgD

1°- primary antigenic stimulation producing secreatory IgM's

2°- secondary antigenic stimulation causing isotype switching

switch and control of antibody production, in relation to primary and secondary antigenic stimulation, are given in Fig. 4.

1.2 Lymphocyte Activation and Intercellular Cooperation in Antibody Responses.

There are two main theories which explain B cell activity by T cell independent and T cell dependent antigens:

1) Critical Matrix Theory - simple cross linking of cell surface receptors.Ig provides the required signal (explains T-ind responses).

2) Signal Theory - binding of antigen at the cell surface provides the primary signal for activation followed by a secondary signal for full activation, provided by soluble factors from helper T cells (explains T-dep responses).

It is probable that both mechanisms operate together, with activation of B-cells by T-dependent antigens requiring a complex series of cellular interactions, together with augmentation of their response by the release of lymphokines and cytokines. The cellular interactions involved in B cell activation are summarised in Fig. 5. Here, Antigen Presenting Cells (APCs) present the

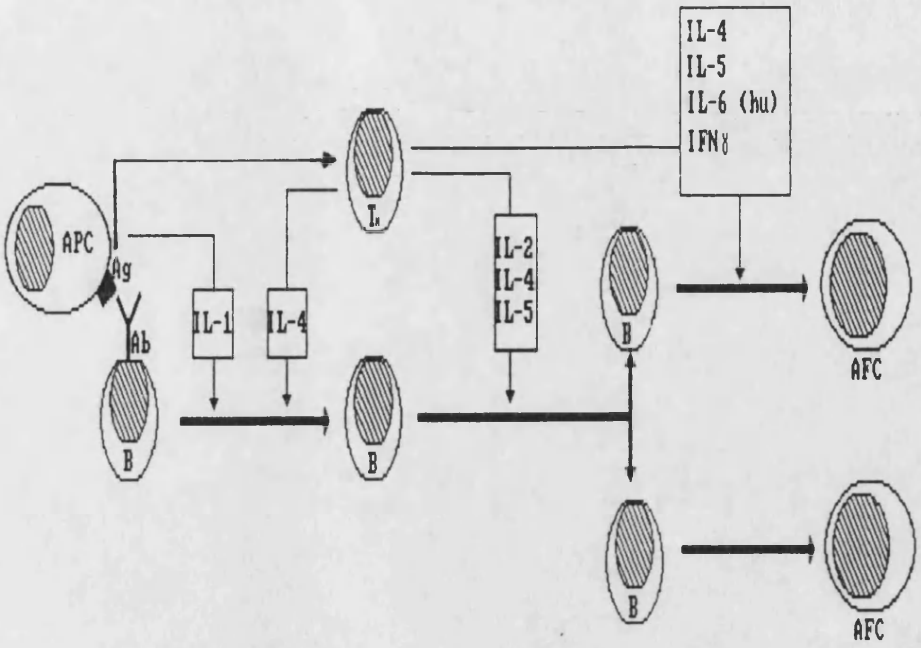


Fig.5 - Summary Of B Cell Activation And Development

Only one cycle of cell division is shown but many will occur

AFC - Antibody Forming Cells

antigen in a more immunogenic form to both T and B cells. Interaction with B cells occurs through membrane bound Ig's, and with T cells through the T cell receptor (Introduction, section 1.2.1). The result is activation of the B cell, and release of soluble factors, interleukins (IL-1 to 6), which further influence the B cells causing them to divide and mature into AFCs.

Control of these interactions is by the expression of certain glycoprotein antigens on the cell surfaces. These antigens, in man, are known as the human leucocyte antigens (HLA) of the major histocompatibility complex (MHC) gene cluster located on chromosome 6 (see Fig. 6 for gene organisation). The MHC codes for several different classes of gene product, each of which has its own role in recognition and control, the most important of which are:

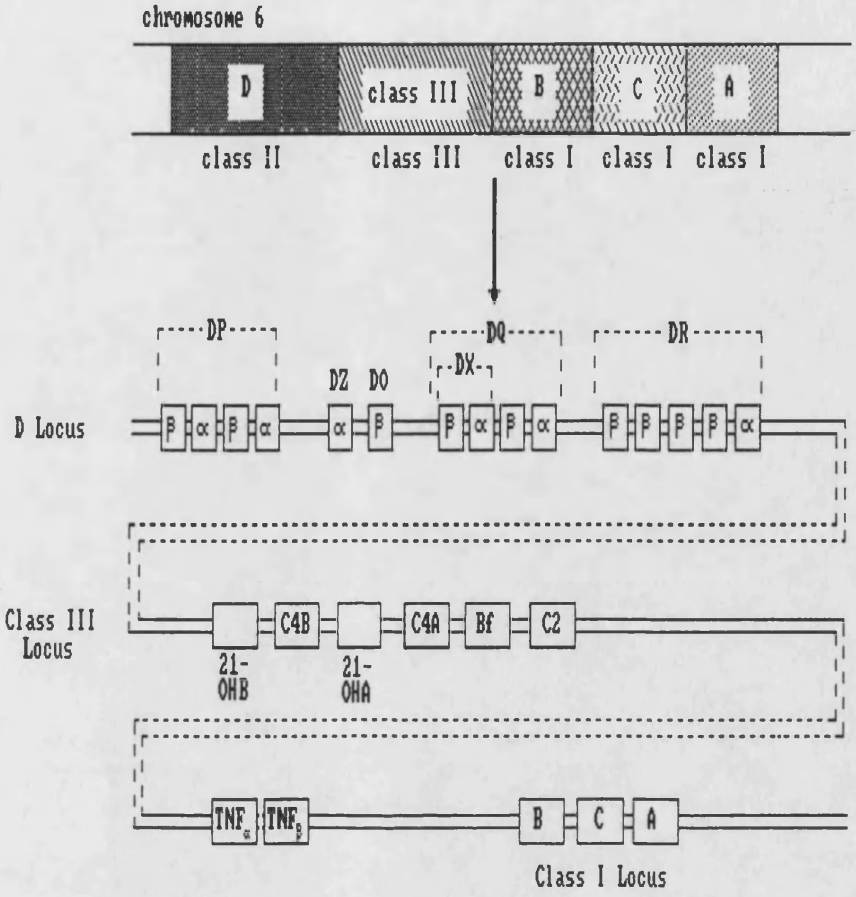
MHC class I antigens are transmembrane glycoproteins, non-covalently associated with beta-2-microglobulin (see Fig. 7 for structure) and are found on all nucleated cells, platelets and erythrocytes. These antigens are particularly important in the control and generation of cell mediated immune responses.

MHC class II antigens are transmembrane glycoproteins consisting of an alpha and a beta chain (see Fig. 8 for structure) and are found on most cells of the immune system. APCs express processed antigen in association with class II antigens when presenting it to helper T cells and B cells during the development of an antibody response.

1.2.1 T-Cell Antigen Receptor

The activation of T_H cells by antigen in association with

Fig.6 - Diagram Showing Organisation Of Human MHC On Chromosome 6



The above gene map shows that the MHC D region is sub-divided into 3 regions, DP, DQ, + DR. Each region codes for a number of α and β chains.

Class III region codes for: C4 (C4A + C4B) and C2 complement factors, together with Factor B (Bf).

Genes for Tumour Necrosis Factor (TNF) lie between the Class III and Class I loci.

21-OHB / 21-OHA - genes coding for Cytochrome P450 21-Hydroxylase

Fig.7 - Diagramatic Representation of Class I MHC Antigens

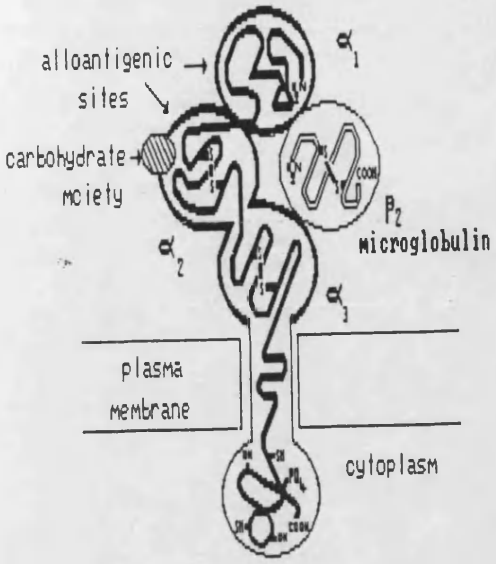
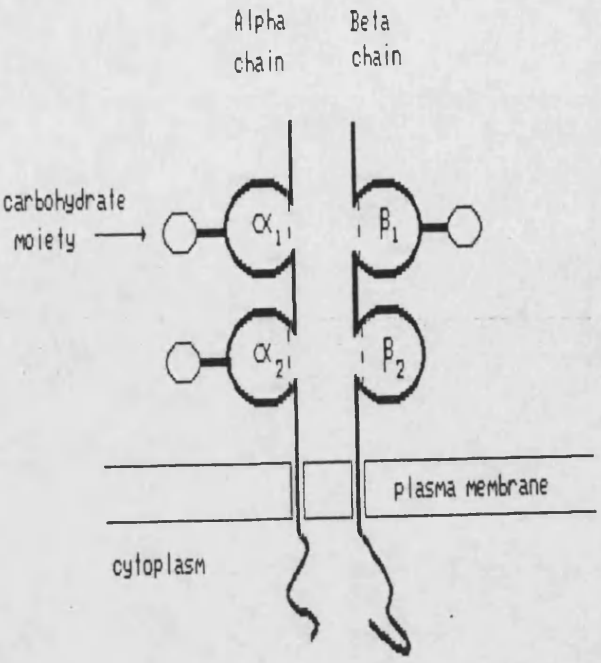


Fig.8 - Schematic Representation Of Class II Antigens



-------- - intrachain disulphide bond

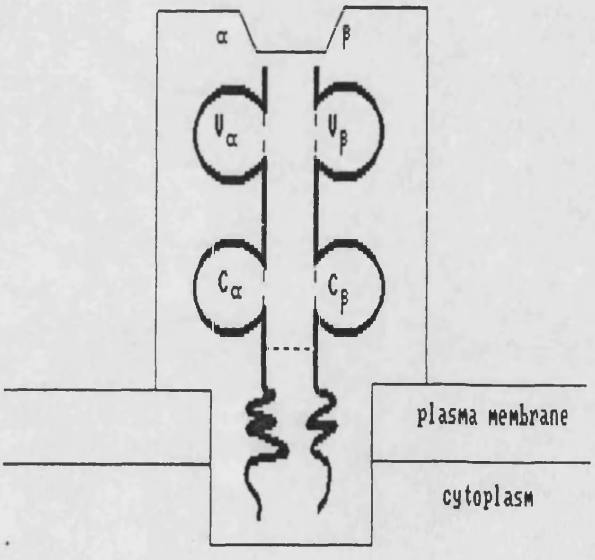
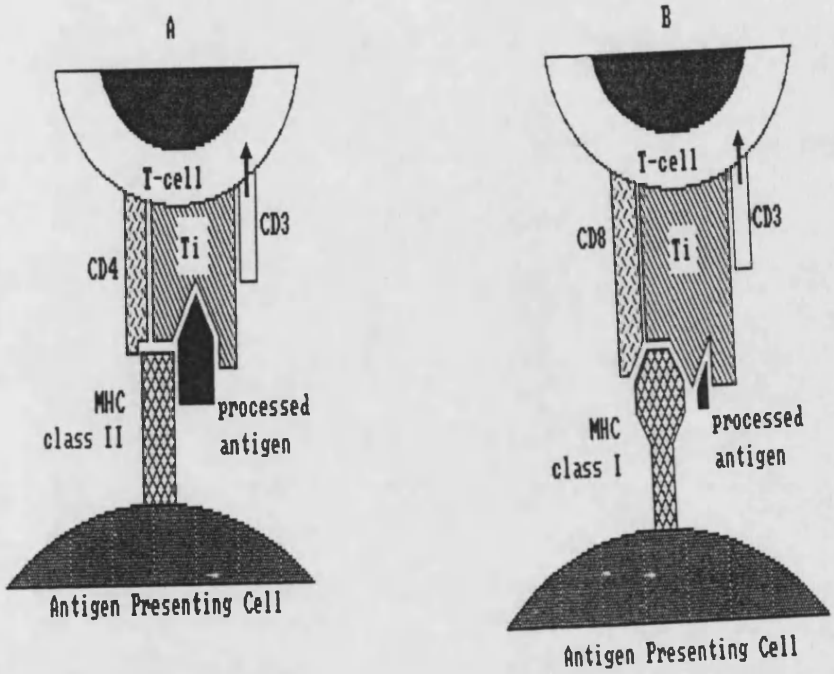


Fig.9 - Diagramatic Representation of TCR2 Receptor

-------- disulphide bonds

Fig.10- Diagram Showing Molecular Interactions Between MHC Antigens Processed Antigen, T-Cell Receptor And CD4/8/3



A) MHC Class II restricted activation of helper T cells
Antigen presented to T cells in association with Class II protein. Interaction occurring through the CD4/3/TCR-2 complex on the T cell.

B) MHC Class I restricted actication of cytotoxic T cells
Antigen presented to T cells in association with Class I protein. Interaction occurring through the CD8/3/TCR-2 complex on the T cell.

class II MHC antigens on APCs, occurs through a T-cell receptor (TCR), of which there are several types. The TCR-2 receptor is the one which has been most closely studied, and comprises of a heterodimer of alpha and beta chains (see Fig. 9), which is associated with another membrane bound antigen, CD3, consisting of three peptide chains. The alpha and beta chains of the TCR-2 have been found to consist of a constant and a variable region, as in the immunoglobulins. Each region forming a closed domain by the use of disulphide bridges.

As with the structure of the TCR, the gene arrangements also bear a striking resemblance to those of the immunoglobulins. It is believed that the same mechanisms of gene rearrangements are used for forming the antigen binding site of the TCR, that are used in forming the variable region of the immunoglobulins.

It has been found that the TCR/CD3 complex is in fact associated with another membrane glycoprotein, CD4. This antigen appears to be crucial to the recognition of class II MHC antigens on APC's and is found on T_H cells (see Fig. 10A). Similarly, association of TCR/CD3 with the glycoprotein CD8 confers on the cell the ability to recognise class I MHC antigens, and is found on T_C cells (see Fig. 10B).

1.3 Cell Mediated Immune Responses

Cell mediated immunity (CMI) involves the specific activation of certain lymphoid cells of the immune system.

The resulting responses being, in the main, cellular based instead of antibody based. It is generally responsible for immunity to micro-organisms, viruses, grafts and tumour cells.

Whereas antibody responses are MHC class II restricted, most cell mediated responses are MHC class I restricted. As class I antigens are found on all nucleated cells, then CMI is dependent on recognising such cells either as foreign by the class I antigens displayed or with foreign antigen associated with self class I antigens.

As with antibody responses, the central cells involved are APCs and T_H cells. Cellular interactions, in this case, occur through antigen and class II recognition. The activation of T_H cells in this way then results in the activation of killer cells by the release of lymphokines.

In the first instance, cytotoxic T cells (T_C cells) are activated which recognise the target cell through a MHC class I restricted interaction, associated with antigen. In the second instance, non-specific MHC unrestricted killer cells are activated, in particular, Natural Killer cells (NK cells), and Lymphokine Activated Killer cells (LAK cells). NK cells recognise determinants expressed on neoplastic cells, and K cells, along with monocytes, interact with antibody via Fc receptors, in what is known as antibody dependent cell mediated cytotoxicity (see Fig. 11) where antibody acts as a marker for antigen. Fig. 11 gives a diagramatic summary of cell mediated immune responses.

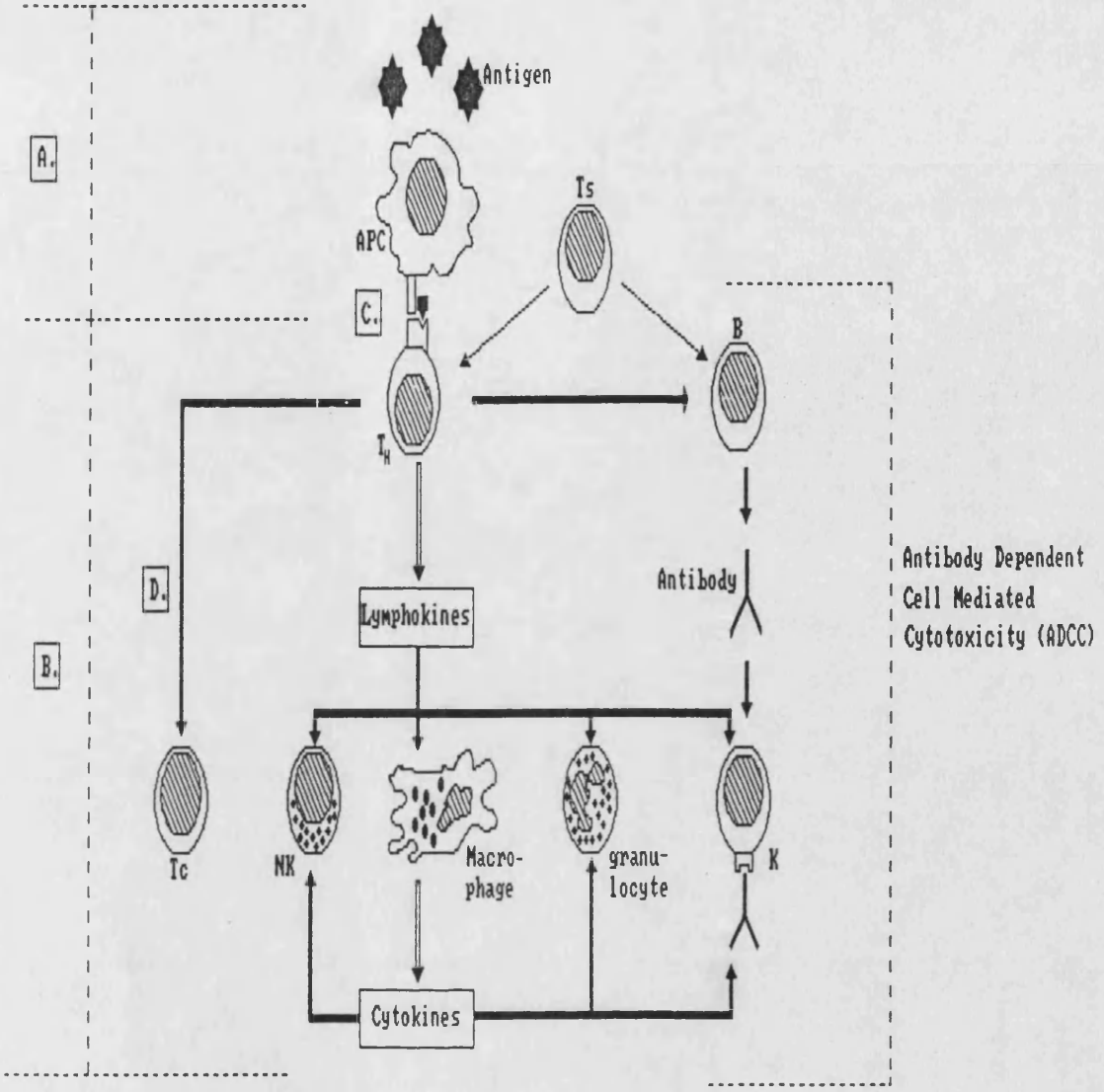


Fig.11- A Summary Of Cell Mediated Immune Responses

A. - Antigen uptake, prossessing and presentation to T helper cells by Antigen Presenting Cells.

B. - Activation of T helper cells by presentation. T helper cells activate further cell mediated immune responses through direct interactions and release of lymphokines.
Modulation of cell mediated immune reponses through release of cytokines.

C. - Presentation is MHC Class II restricted.

D. - T helper / T cytotoxic cell interactions are MHC Class I restricted.

1.4 Immunoregulation

In the individual, the normal immune responses must be subject to strict control. The control of the immune system exists on several levels.

Firstly, the primary regulator is the antigen itself, which can either produce a reaction or generate tolerance.

Secondly, there is the antibody which has been shown to have a regulatory function through feedback mechanisms directly. Along with these feedback mechanisms, is the ability to produce anti-idiotypic antibodies directed against the antigen binding site of an antibody idiotype, thereby neutralising that antibody. As can be seen, this is a self perpetuating system of subtle control and such a network was first postulated by Jerne. If immune complexes formed in this way have a regulatory effect then it follows by the same reasoning that antigen-antibody immune complexes would also have some effect. It has been shown that immune complexes can influence antibody responses by either inhibiting or augmenting B cell reactions.

Thirdly, there are cellular regulatory mechanisms, through suppressor T cells and contrasuppressor T cells. It has been shown that T_S cells suppress the response of normal cells, whereas T_{CS} interfere with the suppression by T_S cells. The exact mechanisms by which these cells bring about their functions, are not completely understood, but they have been shown to be MHC restricted and appear to involve soluble factors (see Fig. 12 for summary).

Fig.12- Summary Of Regulatory Mechanisms Involved In Control Of The Immune Response

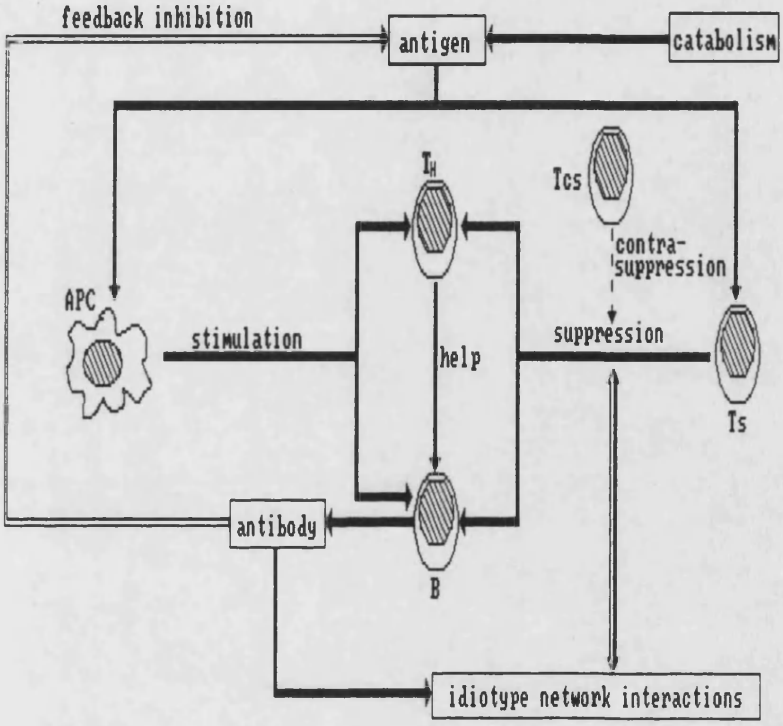


Diagram shows immunoregulation involving :

i) Feedback Inhibition

ii) Idiotype Networks

iii) Immune Complexes

iv) Suppression by Ts cells

v) Contra-suppression by Tcs cells

Underlying all these mechanisms is the genetic control of the immune response by immune response genes which are closely allied with the MHC. However, there are other IR genes which can act at various stages of the immune response.

2. AUTOIMMUNITY

2.1 General

If immune responses towards self components are allowed to occur without the normal control processes being fully operative, then disease states may be caused. Such diseases are known as autoimmune diseases, and are usually characterised by the production of auto-antibodies. Nydegger (1985) has stated that the term, autoimmune, should be confined to diseases where the pathogenic role of the auto antibodies can be shown to be a causal factor in the disease process, and should not be used to describe situations in which harmless auto-antibodies are a secondary phenomenon in tissue damage (eg. anti-heart muscle antibodies produced following Myocardial infarction).

2.2 Classification of Autoimmune Diseases

The wide variety of autoimmune diseases have been classified according to two systems.

The first, and simpler, involves determination of the site of autoimmune attack, and then establising whether or not it is

organ specific attack (see Table 1.) In organ specific diseases the antigen is essentially localised to a given organ, which is the only site of attack, whereas in non-organ specific diseases, the antigen is widespread throughout the body, with the systemic deposition of immune complexes particularly in the kidneys, joints and skin.

Table 1 Spectrum of Autoimmune Diseases
(taken from Roitt (1989))

Organ Specific	Hashimoto's thyroiditis
	Primary myxoedema
	Thyrotoxicosis
	Pernicious anaemia
	Autoimmune atrophic gastritis
	Addison's disease
	Premature menopause (rare)
	Insulin dependent diabetes mellitus
	Goodpasture's syndrome
	Myasthenia gravis
	Male infertility (rare)
	Pemphigus vulgaris
	Pemphigoid
	Sympathetic ophthalmia
	Phacogenic uveitis
	Multiple sclerosis (?)
	Autoimmune haemolytic anaemia
	Idiopathic thrombocytopenic purpura
	Idiopathic leucopenia
	Primary biliary cirrhosis
	Active chronic hepatitis
	Cryptogenic cirrhosis
	Ulcerative colitis
	Sjogren's syndrome
	Rheumatoid arthritis
	Dermatomyositis
	Scleroderma
	Mixed connective tissue disease
	Discoid lupus erythematosus
Non-organ specific	Systemic lupus erythematosus (SLE)

Table 2 Classification of Autoimmune Diseases
(taken from Smith & Steinberg (1983))

Class	Description
A	A defect in the afferent limb of the immune system initiated without requirement for a specific external agent (SLE) With important genetic requirements. Without important genetic requirements.
B	A defect in the afferent limb of the immune system initiated by a specific external agent (acute rheumatic fever). With important genetic requirements. Without important genetic requirements.
C	A defect in effector mechanisms of immunity without a requirement for a specific external agent (hereditary angioedema).
D	A defect in effector mechanisms of immunity initiated by a specific external agent (certain nervous system viral infections).
E	Combinations of the above.

The second system of classification is more complex, and is based on locating the immune defect in either the afferent limb or in the effector mechanisms. This classification is coupled with a suggested possible cause, ie. genetics or external agents. Under this system, classical autoimmune disease fall within Class A, in that they are initiated without requirement for a specific external agent, with the abnormality lying in the self antigen, the immune response to it, or both (see Table 2: Smith & Steinberg (1983)).

Whatever classification is used, it must be stressed that autoimmune diseases are multifactorial, being influenced to a greater or lesser extent, by the genetic background of the individual, the state of the immune system and enviromental factors (Smith & Steinberg (1983)).

2.3 Mechanisms of Autoimmune Attack

Many autoimmune diseases (eg. arthritis or glomerulonephritis) have common features, such as circulating auto-antibodies, inflammation, kidney damage etc. Such features are believed to result from the limited number of immune mechanisms that are operable in autoimmune diseases.

Mechanisms involved include, hypersensitivity reactions: Type I (anaphylactic: Type II (cytotoxic): Type III (antibody-antigen complexes): and Type IV (cell mediated). These specific mechanisms can be augmented by non-specific measures, such as,

cellular (via lymphokines) or humoral (complement, coagulation, kinin, and fibrolytic) amplification systems. The overall interaction of these complex processes leads to the specific outcome of each disease.

The cytotoxic (Type II) mechanism can involve the reaction of circulating autoantibody with antigen bound to cell surfaces. The antigen may be the cell membrane, a receptor on that membrane, or an antigen such as a virus or drug, that has become attached to the cell surface. The pathology of many of the autoimmune diseases is believed to be initiated by this mechanism. The final outcome of the cytotoxic process may be mediated by complment, or by an antibody dependent cell mediated cytotoxic reaction (ADCC). ADCC may, in general, be the most important mechanisms. The final effect, irrespective of mediation, is usually cell lysis, elimination or inactivation (Smith & Steinberg (1983): Coombe & Gell (1977)).

2.4 Aetiology of Autoimmune Diseases

When trying to delineate the aetiology of autoimmunity, it is first necessary to consider it in relation to the mechanisms of tolerance to self antigens.

In 1959 Burnett proposed the clonal selection theory of immune selectivity (see Fig. 13), from which it was proposed that tolerance was generated through clonal deletion or abortion of self reactive lymphocytes, and that immunological memory was obtained by the generation of specific memory cells following primary and secondary exposure to antigen.

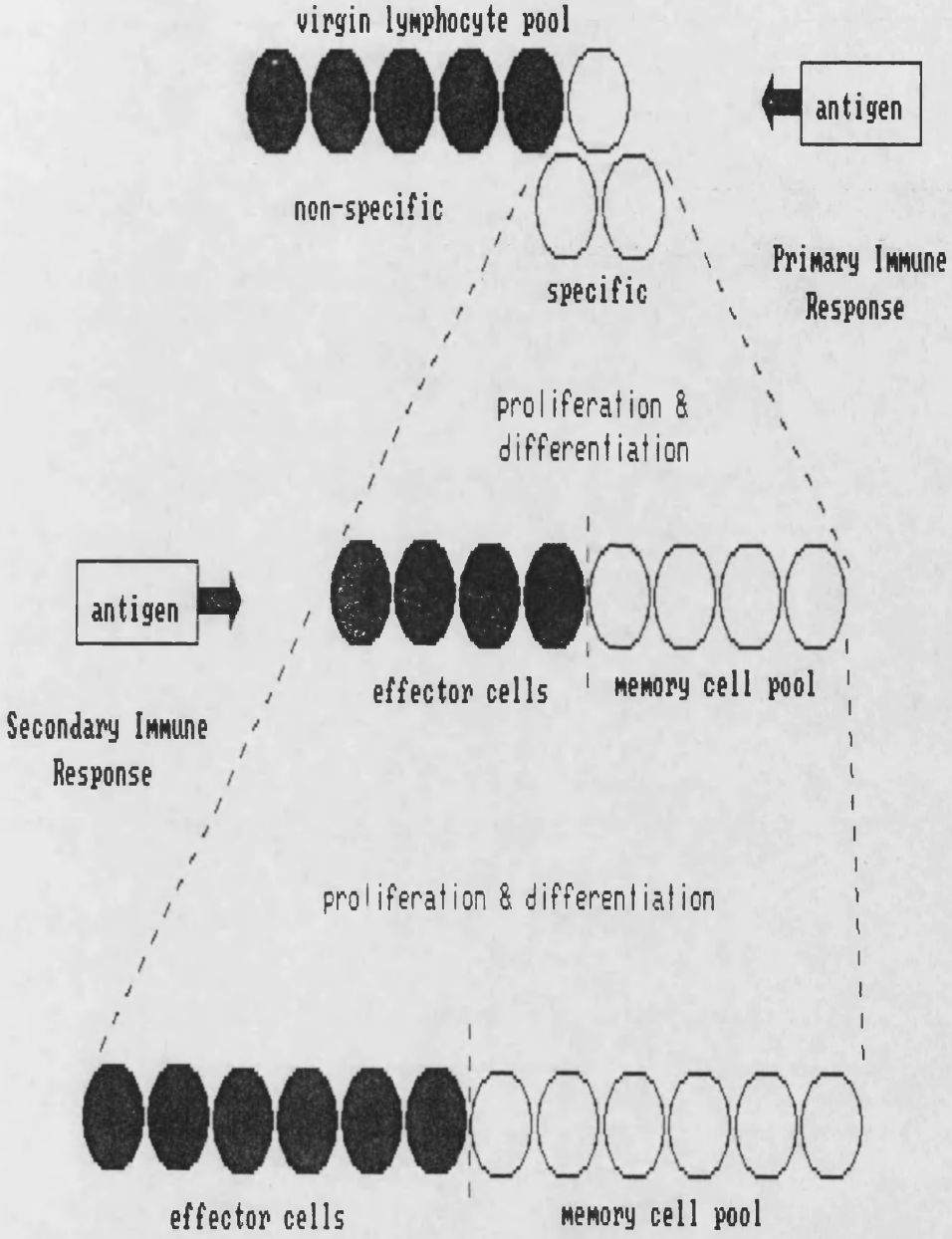


Fig.13- Clonal Expansion of Lymphocytes in Primary and Secondary Immune Responses

During fetal development, none of the cells of the immune system are mature. It is at this stage that the immune system develops the ability to recognise, but not to react to, self antigens. This phenomenon of tolerance is largely developed in the immature cells of the immune system, although mature cells can also develop tolerance depending on dosages of antigen and form of antigen presentation.

The mechanism of self tolerance was originally ascribed to the elimination of anti-self lymphocytes prior to full maturity, however, evidence was subsequently obtained which proved the presence of quiescent self reactive B lymphocytes (Rose et al, (1963); Bankhurst et al, (1973)) and T lymphocytes (Kong et al, (1979)) in the normal individual. It also became increasingly apparent that self reactivity was, indeed essential as shown by MHC Class I/II controlled cellular recognition and idiotype-anti-idiotype interactions. These observations have led immunologists to rethink self tolerance, leading to two models which account for this apparent unreactiveness in the normal individual:

1) T_H cell deletion (Fig. 14B)

   B cell clones are unable to respond due to lack of T cell help resulting from clonal deletion of anti-self T_H cells. Breakdown occurs where non-self T_H cells react to a similar antigen (see Fig. 14)

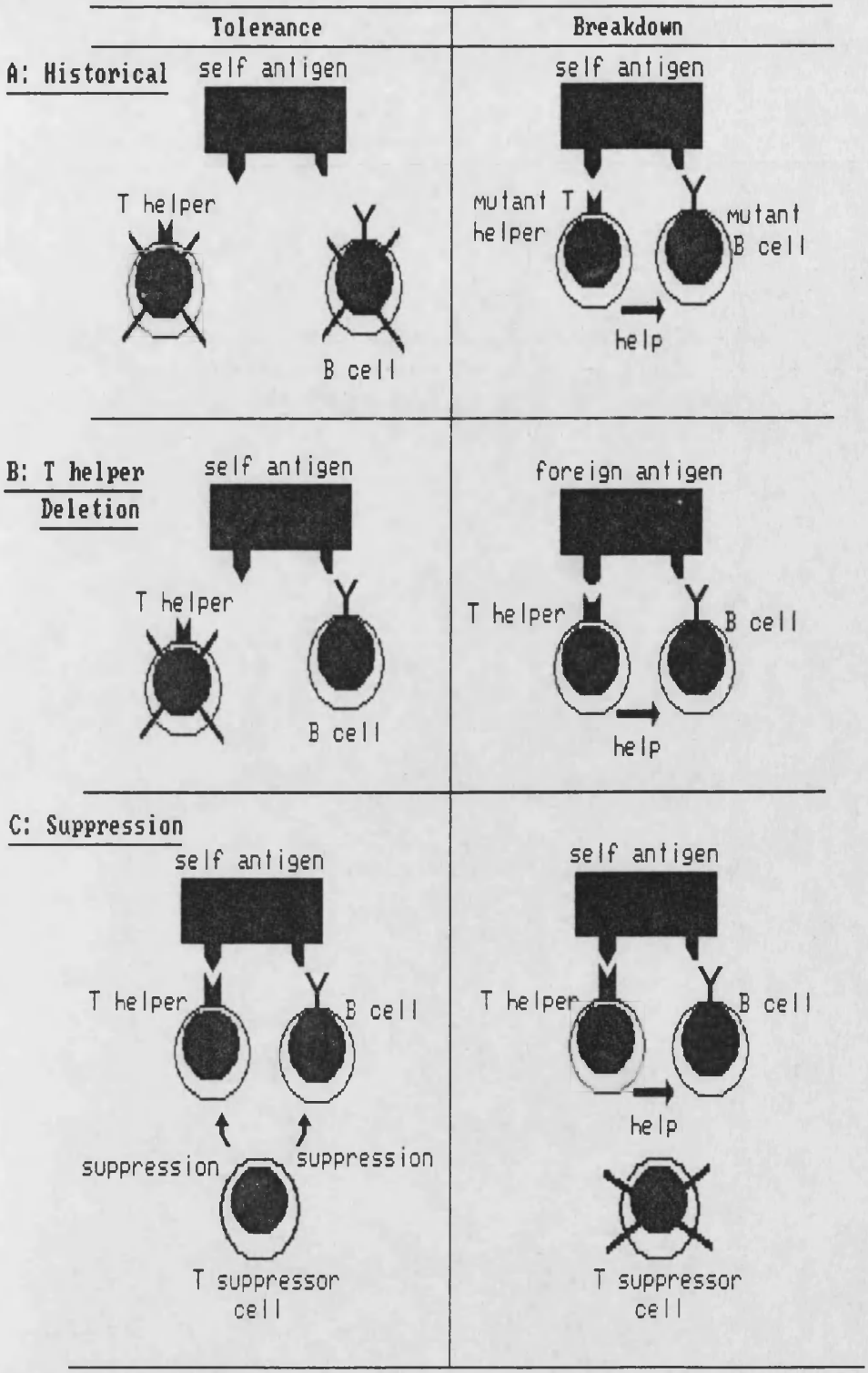


Fig.14- Mechanisms of Self Tolerance

A: Historical - proposed by Burnet & Fenner. Self-antigens induce elimination of self reactive lymphocytes. Autoimmunity occurs through mutation in the adult.

B: T helper deletion - prevents B cells from reacting by removing T cell help. Breakdown occurs through cross-reactivity.

C: Suppression - of T and B cells by T suppressor cells loss of which results in an antoimmune reaction.

2) Suppression (Fig. 14C)

Suppression of T_H/B cells by T_S cells. Breakdown occurs with the loss of T_S cells (see Fig. 14). A similar effect is the veto cell effect, where self reactive T cells are controlled by further T cells.

Both these mechanisms are believed to operate in adult life, but functional deletion of T_H cells is probably the primary mechanism, with suppression acting as a safeguard.

As it is believed that tolerance is due to either helper T cell deletion or through the development of suppressor T cell circuits (see Fig. 14), then these mechanisms themselves, suggest how possible abnormalities may occur, leading to a breakdown of tolerance and uncontrolled autoimmunity. Figs. 15 and 16 summarise the mechanisms which may give rise to auto-immunity through bypass of the control networks. Fig. 15 gives an overall view of how the regulatory mechanisms interact with one another and of the several points which are susceptible to breakdown. Fig. 16 gives a more detailed description of each of the suggested defects. These mechanisms may originate through viral/microbial action, drug modification of antigen, idiotype-anti-idiotype interactions or be genetically induced.

2.5 Auto-antibodies In Autoimmune Diseases

One of the characteristic features of many autoimmune diseases is the production of auto-antibodies. However, it

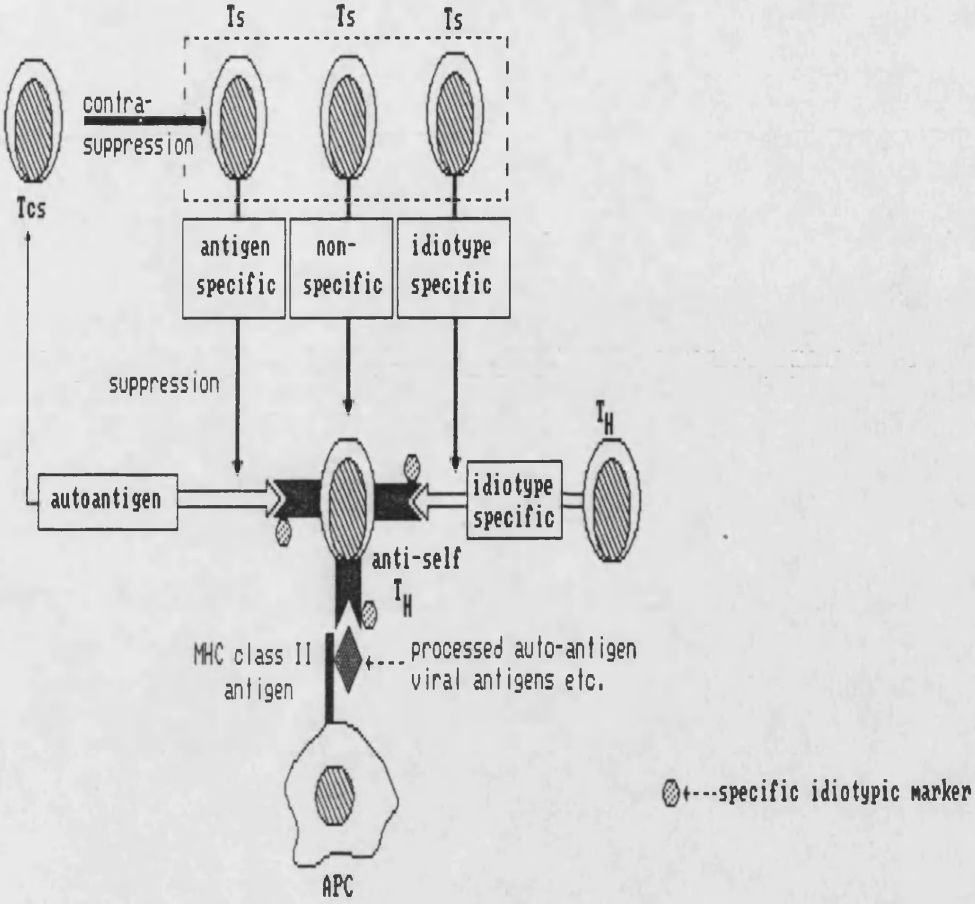


Fig.15: Induction of Autoimmunity through bypass of regulatory mechanisms

Anti-self T helper cells can be activated by :

i) defective T suppressor cells.
ii) Stimulation of contrasuppressor T cells.
iii) inappropriate MHC class II expression on a cell converting it into an APC
iv) Stimulation through an idiotypic helper T cell.

Stimulation of the anti-self T helper cell results in activation of anti-self B and T effector cells, with resultant formation of autoimmune disease.

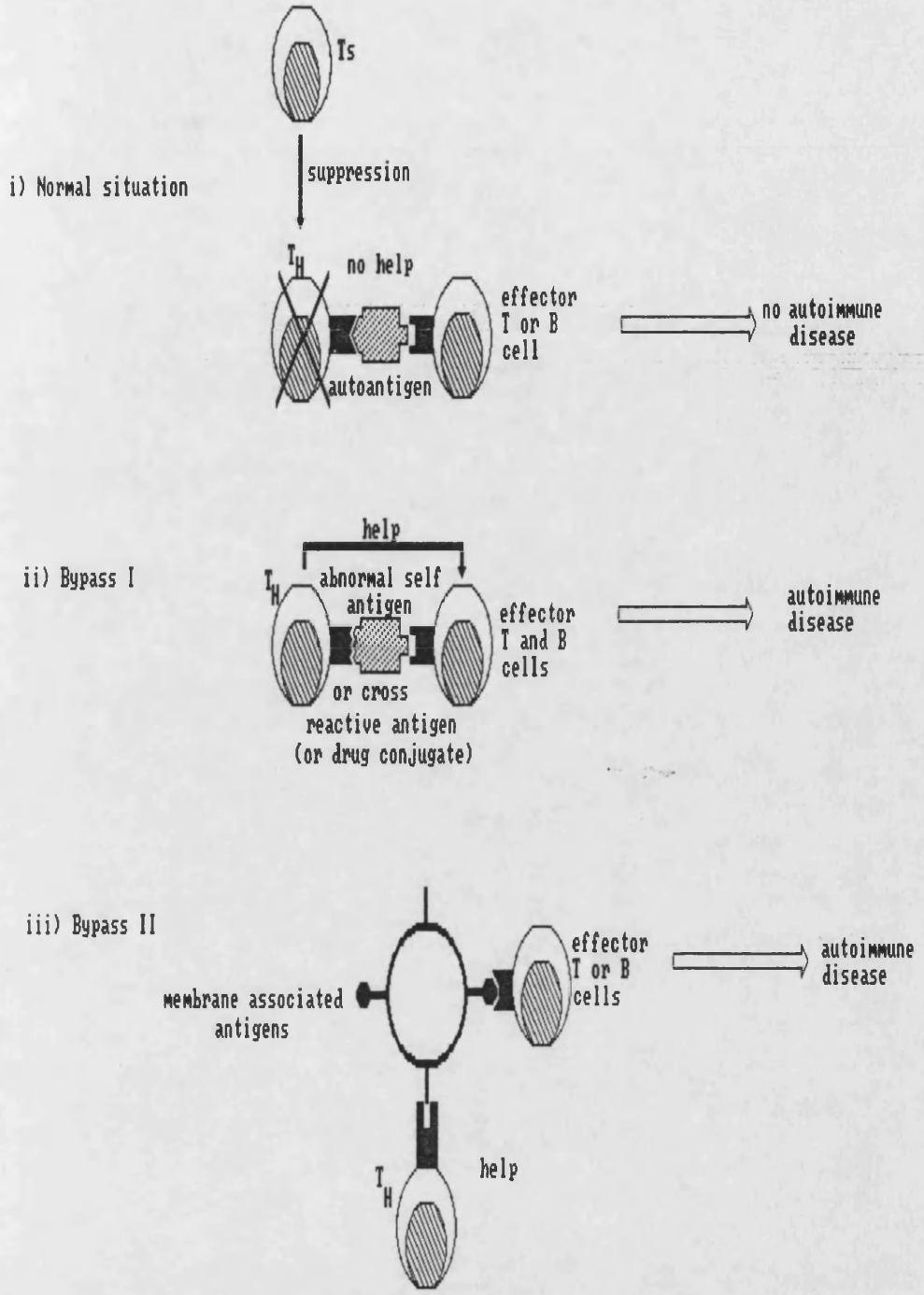


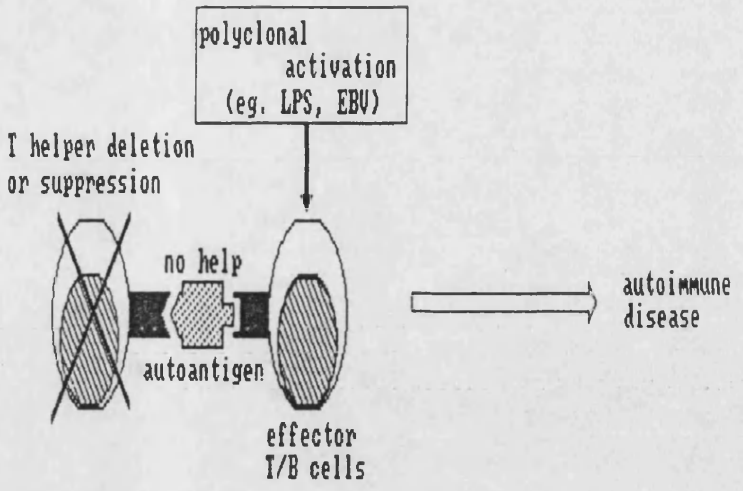
Fig.16: Induction of Autoimmunity by T Cell Bypass (adapted from Roitt (1989))

i) Normal situation - no disease because of either suppression or T helper cell deletion.

ii) Bypass I - abnormal/cross-reactive or drug modified antigen interacts with a cross-reactive helper T cell which provides help to self reactive effector T/B cells.

iii) Bypass II - autoantigen associated on cell membrane with either a modified self antigen or foreign antigen (eg. viral coat protein) interacts with helper T cell specific for the altered/foreign antigen, which provides help for the formation of effector T/B cells.

iv) Bypass III



v) Idiotype Bypass

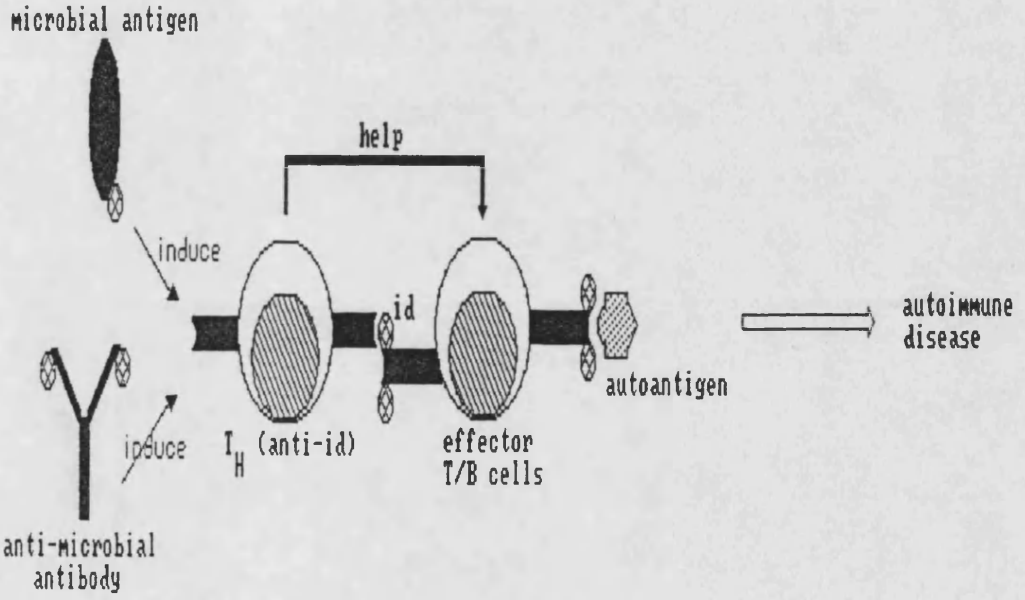


Fig.16(contd)

iv) Bypass III - self reactive T/B cells stimulated directly through polyclonal activation by external agents.

v) Idiotype Bypass - self reactive T/B cells carrying a cross-reactive public idiotype which cross-reacts with an antibody stimulated by a microbial agent results in the generation of auto reactive anti-idiotypic antibodies.

has been shown that there is continuous production of low levels of circulating auto-antibodies in normal healthy individuals. Such antibodies tend to be of the IgM isotype with a short half life (Cohen & Cooke (1986)). As an individual ages there is an increase in the quantity of auto-antibodies directed against nucleoproteins, DNA, immunoglobulins (rheumatoid factors, anti-idiotypes), complement (immunoconglutinins) and various other tissue antigens (Nydegger,(1985): Hallgren et al,(1973): Goodwin et al,(1982): Kunkel,(1982): Bullock et al,(1979)).

These observations give direct evidence for the presence of self reactive B lymphocytes within normal healthy individuals (Schatter (1986/87), and raise the question as to their function and regulation. Grabar (1983) proposed that auto-antibodies act to clear self antigens from sites of tissue damage and from ageing tissues, thus explaining the observed increase in such antibodies during ageing. A role for auto-antibodies in the maintenance of homeostasis, was later supported by Khansari & Fudenberg (1984). However, Cohen & Cooke (1986) argue from Darwinian logic, that the presence of such antibodies must have some intrinsic survival value. They further suggested that auto-antibodies help in the avoidance of autoimmune diseases within the healthy individual, by blinding the immune system to environmental epitopes that are cross reactive with self antigens, but their production is kept under strict control. Holmberg & Coutinho (1985) state that this control is via idiotype-anti-idiotype mechanisms.

If auto-antibodies are produced in normal individuals, and have a physiological role, how are they involved in disease processes? It is not sufficient to postulate that a simple increase in the quantity of auto-antibodies will lead to disease, because in certain of the diseases (eg. Myasthenia gravis) there is little correlation between auto-antibody titre and disease severity. Some individuals show no symptoms but high antibody titres and others display severe disease and low antibody titres. It may be that a specific antibody sub-population, or isotype, is responsible for the disease. An isotype switch may accordingly be important, as natural auto-antibodies tend to be IgM, whereas those associated with the disease process tend to be of the IgG isotype (Schattner,(1986/87): Cohen & Cooke,(1986): Clough & Valenzuela,(1980)).

Apart from changes in the nature of the auto-antibodies, changes in the regulation of their formation may be important. Fig. 16 shows mechanisms by which regulatory systems can be circumvented. As B cell function is regulated by the activities of helper/suppressor T cells, NK cells and idiotype-anti-idiotype interactions, then a dysfunction of any of them may result in B cell activation and unregulated auto-antibody production. However, much of the evidence available points to an important role for diminished suppressor T cell activity, with possible anti-idiotypic involvement (Schattner (1986/87)). Such changes could be brought about by both environmental and genetic factors.

2.6 Genetic Influences In Autoimmunity

A striking feature of autoimmune diseases is their linkage to particular MHC alleles and antigens (Svejgaard et al (1983)). The influence of the MHC in autoimmunity was reviewed by Strominger (1986) who put forward a plausible theory of autoimmunity (Introduction, Section 2.7)

In some cases the linkage is very high. For example, 95% of patients with ankylosing spondylitis carry the HLA-B27 allele, compared to 7% of controls. In others, however, linkage is much lower, as in MS where 59% of patients are HLA-DR2, compared with 26% of controls.

When the disease is tightly linked to an HLA allele, as with ankylosing spondylitis, it is assumed that the HLA gene/protein itself is in some way related to the disease. However, when the degree of association is lower, ie. in MS (DR2), rheumatoid arthritis (DR4), or juvenile onset diabetes (DR3 or 4) several other explanations exist, as eg:-

a) The actual gene associated with the disease is in linkage disequilibrium with the gene being assayed. Linkage disequilibrium (failure of adjacent genes to segregate in the population) is a well known phenomenon of the HLA system (Awdeh et al, (1983)), and the relatively low extent of correlation of MS with HLA-DR2, for example, may be due to the fact that MS is related to a gene in linkage disequilibrium with DR2 itself.

In coeliac disease, in which 45% of patients are HLA-DR7 and 28% DR3, 95% have been shown to carry the DQ2 allele in linkage disequilibrium with DR3 and 7 (Tosi et al, (1983)). Congenital adrenal hyperplasia is known to be caused by deletion of a 21-steroid hydroxylase gene, which is linked to HLA-B47. The gene is actually located between the C4B locus in the Class III region and the HLA-B locus in the Class I region. Thus the disease association may be to a gene in the MHC region that is not an HLA gene.

b) Linkage of a disease with DP genes, which have little or no linkage disequilibrium with DQ or DR loci. There is at least one 'hot spot' for recombination between DP-DX and DQ-DR. Thus a disease with two forms, one linked to DP and the other to DQ or DR, would only show linkage to the latter.

c) HLA alleles are heterogenous within themselves, and a disease may be linked with a particular HLA polymorphism.

d) Promiscuous epitopes may account for lower linkage, as the epitopes of the HLA genes are the functional units of these genes/proteins, but, they are not entirely fixed to one gene/protein and can be shuffled to other genes/proteins within the system.

e) The disease itself may be heterogenous, eg. Type 1 but not Type 2 diabetes is HLA linked.

2.7 A Possible Theory Of Autoimmunity

Several other points have been made concerning autoimmune diseases. These include:

i) At least two genes are involved in the disease process, one of which is an MHC gene (Spielman & Nathanson,(1982): Buse & Eisenbarth,(1985)).

ii) Viruses, or some other environmental agent, may play some role in the genesis of at least some of the diseases (Nathanson & Miller,(1978): Manser et al,(1978)).

iii) The disease process is, in many instances, specific for a particular target tissue.

iv) Concordance is low in monozygotic twins (although higher than in dizygotic twins or families) (Williams et al,(1980): Barnett et al,(1981)).

All these factors, along with HLA linkage, led Strominger (1986) to postulate the following theory with respect to the generation of autoimmune diseases. He has concentrated on cell mediated mechanisms but his arguments apply to antibody mediated autoimmune diseases.

He postulated that infection with a virus initiates development of Class II restricted virus specific cytotoxic T cells, which have broad specificity, and cross react with a determinant on some cell surfaces on normal cells. Equally it is conceivable that another environmental agent may be responsible for provoking the primary immune response. However, unlike viral infections, autoimmunity is rare, and susceptibility to a particular autoimmune disease may be linked to the possession of a second gene (either a polymorphism in the target antigen or T cell receptor). This basic theory explains how possession of certain HLA alleles, environmental agents and possession of certain antigenic polymporphisms, may result in an autoimmune disease, and also explains why monozygotic twins show relatively low concordance. This latter fact arises because during the maturation of the immune system, each individual will undergo a different set of T cell receptor and immunoglobulin gene rearrangements. Consequently, each twin will possess different sets of idiotypes, each with differing potentials for cross reactivity. Thus, chance may play a prominent role in the potential for any individual to develop an autoimmune disease.

3. MYASTHENIA GRAVIS

3.1 General Introduction

It is now well accepted that Myasthenia gravis (MG) is an autoimmune disease, characterised by muscle weakness and fatigue. The primary defect is the loss of nicotinic acetylcholine receptors (nAChR) on the post synaptic membrane of the neuromuscular junction (NMJ), brought about largely by antibody mediated immune responses.

There are several very good general reviews available, covering all aspects of MG, since its discovery nearly 300 years ago (see Lindstrom, 1979: Vincent, 1980: newsom-Davis & Vincent, 1982: Lisak et al, 1985: Drachman, 1981, 1978: Oosterhuis, 1981: Fuchs, 1979: Engel, 1987, 1980, 1979: Harrison & Behan, 1986: Pachner, 1988). Consequently, this section is designed only to summarise the information currently available, including research on the nAChR, synaptic organisation and nerve/muscle physiology.

3.2 NEUROMUSCULAR JUNCTION AND THE NICOTINIC AChR

3.2.1 Neuromuscular Transmission

In the normal sequence of events, muscular contraction begins with the generation of an action potential in the cell body of a motor neurone in the spinal cord. This action

potential travels as a wave of depolarisation along the axonal membrane of the neurone until it reaches the nerve terminal at the muscle endplate or neuromuscular junction (NMJ) and is the result of the sequential opening of sets of Na^+ and K^+ ion channels, with the corresponding passive movement of the ions along their respective electrochemical gradients, polarising the membrane from its normal negative resting potential. As this action potential invades the NMJ it initiates the release of the neurotransmitter, acetylcholine (ACh) stored within synaptic vesicles in the terminal (See Fig. 17). This release is through a process of exocytosis, and is Ca^{2+} dependent, with a net influx of Ca^{2+} ions through voltage dependent cation channels, caused as a direct result of the action potential's invading the nerve terminal (Reichardt & Kelly, 1983). Upon release, ACh diffuses across the synaptic cleft and associates with AChR's located on the fold crests of the post-synaptic membrane (See Fig. 17)

The binding of ACh results in a conformational change in the receptor which is coupled with the transient opening (1-2msec) of a cation selective ion channel. This allows Na^+/K^+ ions to flow down their respective electrochemical gradients with Na^+ flowing through the receptor channel resulting in local depolarisation of the muscle membrane. This local depolarisation produces a miniature end plate potential (MEPP). If sufficient

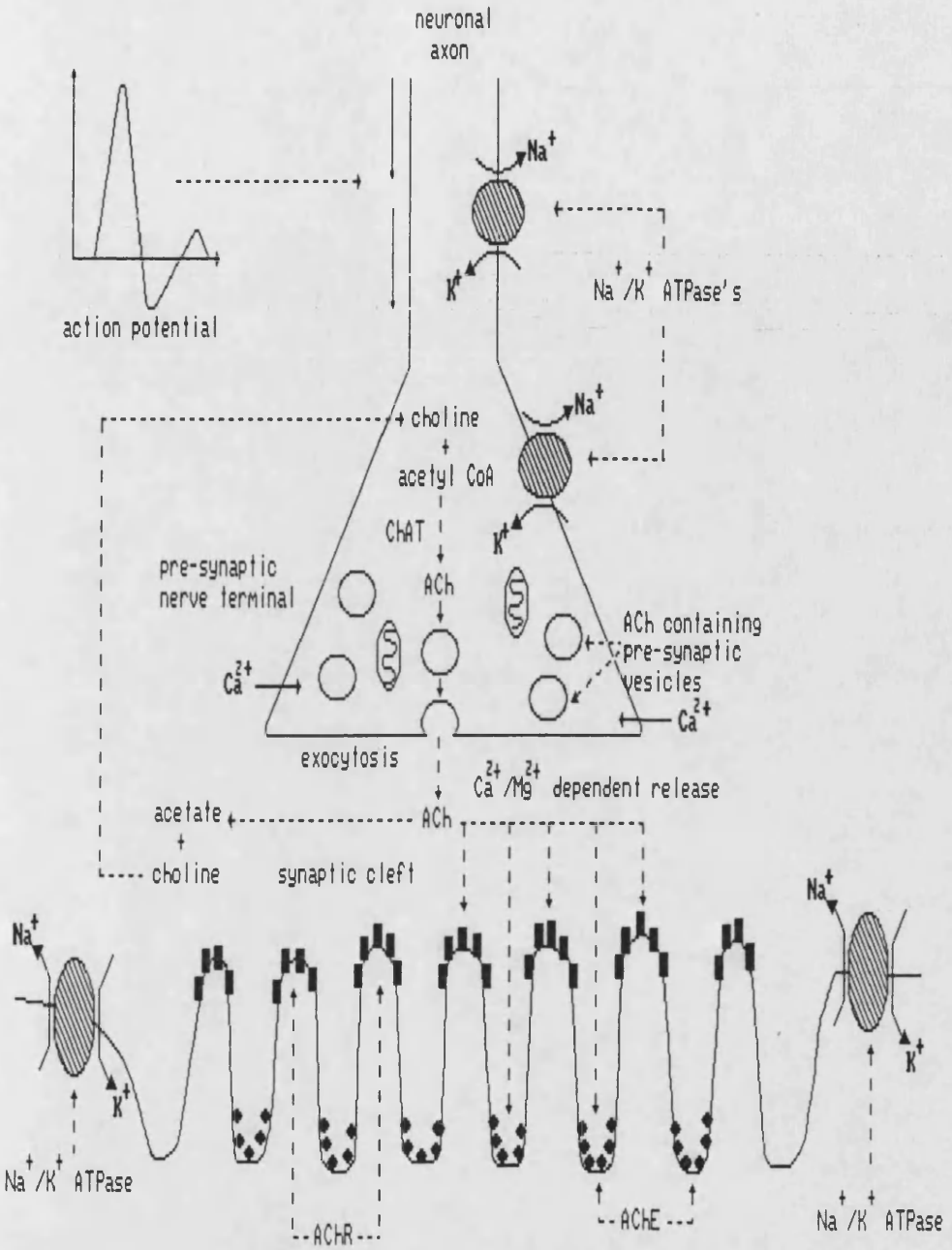


Fig.17: Schematic representation of the neuromuscular junction showing steps in ACh release through Ca^{2+} dependent exocytosis and interaction with post-synaptic AChR & AChE resulting in muscle contraction.

ACh is released then the MEPP's summate to produce an end plate potential (EPP). If the EPP approaches the threshold value (-15mV) then an action potential is generated in the muscle fibre resulting in contraction.

The ACh is not covalently attached to the receptor, and its action is terminated by dissociation from the receptor and subsequent hydrolysis by acetylcholine esterase (AChE). This enzyme is membrane bound and is located in the folds of the post-synaptic membrane (See Fig. 17).

In both the neurone and the muscle fibre, the membranes resting potentials are re-established by membrane bound Na^+/K^+ ATPases.

It has also been established that there is a safety margin in transmission which allows for some fall in either ACh release or AChR numbers, while still maintaining sufficiently high EPP's for contraction to occur. This is because the ACh is released in excess, but very little actually interacts with receptors, which are also present in excess.

3.2.2 Nicotinic Acetylcholine Receptors

There are several good reviews covering the nAChR, see Dolly & Barnard, 1984: Dreyer, 1982: Stroud & Finer-Moore, 1985: Dolly, 1979, Anholt et al, 1984: Hucho, 1986,1987:

McCarthy et al, 1986: Karlin, 1980: Changeux, 1981: Conti-Tronconi & Raferty, 1982, 1983: Wonnacott, 1990.

AChR's can be subdivided into two distinct types, nicotinic AChR's (nAChR) and muscarinic AChR's (mAChR), both of which have different distributions and pharmacologies.

The mAChR is found on smooth muscle, cardiac muscle and in the brain. As well as interacting with ACh, it also responds to muscarine but not to nicotine, and can be blocked by atropine. The nAChR is found in the brain, at autonomic ganglia and at the skeletal muscle NMJ. It responds to nicotine as well as ACh, but not to muscarine, and can be blocked by d-tubocurarine.

Nicotinic AChR's, themselves have been historically divided on the basis of their responses to hexamethonium (C6 receptors) and decamethonium (C10 receptors). Ganglionic nAChR's respond to hexamethonium and NMJ nAChR respond to decamethonium. Such divisions are now superseded by the recognition of multiple subtypes based on molecular cloning data.

Characterisation of mammalian nAChR is hindered by the low levels of receptor present in skeletal muscle and the relatively high levels of proteolytic enzymes (Dolly, 1979). However, the discovery of a rich source of nAChR's from the electic organs of electric fish (Torpedo genus) and eels (Electrophorus electricus), coupled with the isolation of the α-neurotoxins from the venom of certain snakes, which bind specifically and with high affinity to

nAChRs, have led to isolation and structural studies (Chang & Lee, 1962: Lee,1972, 1979: Hunter, 1978: Wang & Schmidt, 1980: Barnard et al,1972: Chang et al,1973: Dolly et al,1981).

The electric organs of both the electric fish and eels are phylogenetically evolved from striated muscle, but they have lost the ability to contract. Instead their function is to generate a charge upon excitation by nerve stimulation. It is generally assumed that the electric organ AChR has all the important properties of vertebrate muscle AChR, with characteristic nicotinic pharmacology, but can be obtained in 1000 fold greater quantities (electric organ approx. 100mg/kg, muscle 0.5mg/kg). For reviews see Harrison & Behan, 1986: Anholt et al,1984: Stroud & Finer-Moore,1985: Hucho,1986: McCarthy et al,1986.

3.2.3 Isolation of nAChRs

Purifications were originally carried out using electric organs and were based on the extraction of the receptor protein from its membrane environment using non-ionic detergents, such as Triton X-100 or Tween-20, or anionic detergents such as sodium deoxycholate. The use of these detergents results in the receptor's being solubilised in detergent micelles, but still retaining pharmacological properties (Changeux et al,1970:

Miledi et al,1971). The solubilised receptor can then be purified further by affinity chromatography using immobilised neurotoxins, cholingeric ligands, lectins or specific monoclonal antibodies (Lennon et al,1980: Anholt et al,1984) followed by biospecific elution using a variety of quaternary ammonium salt analogues of ACh.

Isolation of nAChR from mammalian muscle poses serious problems created by proteolysis of the receptor during purification. An extensive array of anti-proteases are accordingly utilised (Lindstrom et al,1980: Schorr et al,1981: Einarson et al, 1982). In the case of mammalian skeletal muscle, denervation partly overcomes the problem of low density of receptor, causing them to proliferate over the entire muscle fibre membrane. The receptors that develop outside the NMJ are known as extra-junctional receptors, and behave slightly differently to junctional receptors. The purification procedures, themselves, are based on those successfully employed in the isolation of AchR from electric organs.

Throughout the purification of AChR from electric organs or skeletal muscle, various assay procedures are utilised. They are largely based on the separation of AChR-^{125}I-αBGT complexes from unbound receptors and free ^{125}I-αBGT. This has been achieved using Sephadex G50 (Miledi et al,1971): ultrafiltration of the receptor

toxin complex on sucrose density gradients (Lindstrom & Patrick, 1974): ammonium sulphate precipitation (Meunier et al,1972): DEAE cellulose filtration assay (Schmidt & Raftery,1973: Dolly,1979): and more recently a polyethylenimine (PEI) filtration assay (Methods, section 1.5.2). AChR content is usually expressed in terms of α-BGT binding sites per gram of protein or as a function of extract volume, if a crude extract is being used.

3.2.4 Characterisation of the nAChR

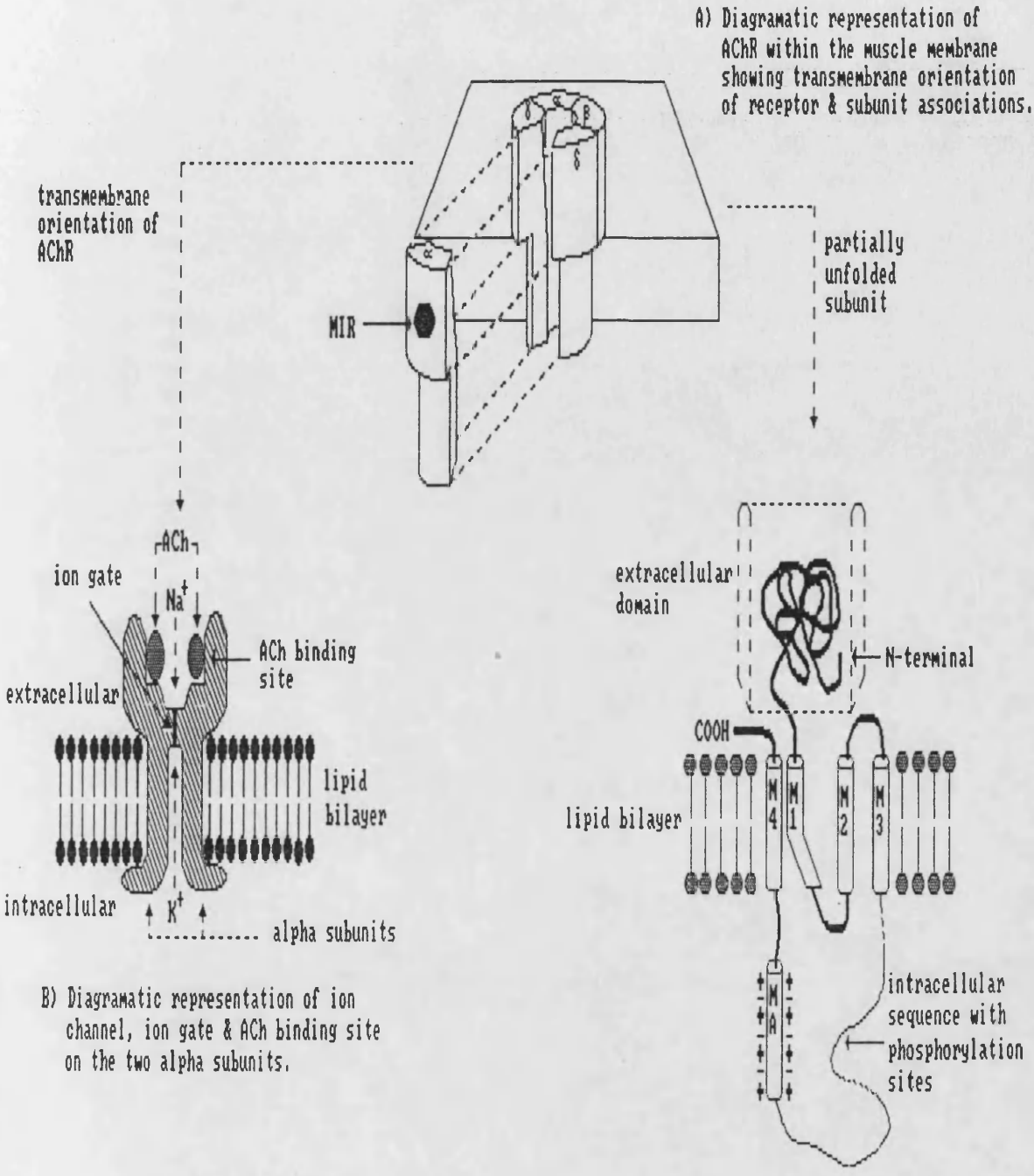
The nAChR from electric organs and skeletal muscle is a transmembrane glycoprotein, exposed both intra and extracellularly, extending approximately 5nm into the extracellular space and 1.5nm into the intracellular space (Ross et al,1977) with an apparent MW of 250,000 (Conti-Tronconi & Raftery, 1984). It contains 4-7% carbohydrate, and is composed of 4 different polypeptide chains arranged as a pentamer with a stoichiometry of $\alpha_2\beta\gamma\delta$. Their apparent M.Wts, from SDS/PAGE, are 40k, 50k, 60k, 65k respectively (Reynolds & Karlin,1978: Lindstrom et al,1979a: Raftery et al,1980). The α-subunits carry the ACh binding site, and, consequently, there are two sites per receptor, as determined by labelled α-BGT binding (Tzartos & Changeux, 1984: Wilson et al, 1984). The AChR is also a phosphoprotein (Vandlen et al,1979) with the phosphate groups attached to the cytoplasmic face of the receptor (Huganir et al,1984).

The four receptor subunits show considerable sequence homology and immunological cross-reactivity (Numa et al,1983: Tzartos & Lindstrom,1980: Mehraban et al,1982). DNA cloning/ translation experiments (Mendez et al,1980: Anderson & Blobel, 1981: Sumikawa et al,1981: Mishina et al,1984), coupled with amino acid sequence and nucleotide sequence analysis on the AChR of Torpedo marmorata (Sumikawa et al,1982a: Deillers-Thiery et al, 1983) and Torpedo californica (Ballvet et al,1982: Noda et al, 1982, 1983a,b), together with EM studies (Kumblek, 1987) have led to a model being proposed for the quaternary structure of the electric organ AChR.

Brisson & Unwin (1985) proposed that the five membrane spanning subunits lie in a pentamer of quasi-fivefold symmetry, around a central ion channel (see Fig. 18a). The ion channel is believed to be lined with alternating regions of positive and negative charges (Stevens,1985) and the extracellular portion of the receptor appears as a funnel. Amino acid sequence analysis and DNA cloning, had led to the proposal that each subunit has four transmembrane hydrophobic helical segments, one of which forms part of the ion channel, with a fifth hydrophilic helical segment found to lie on the intracellular side of subunit (see Fig. 18c: Noda et al,1982: Claudio et al,1983: Devillers-Thiery et al,1983: Stroud & Finer-Moore,1984: Guy & Hucho 1987: Wonnacott,1990). However, this is only one of at

Fig.18: Diagramatic Summary of Current Information on the AChR

(adapted from Stroud & Finer-Moore, 1985; Dolly & Barnard, 1984
Brisson & Unwin, 1985; Stevens, 1985, & Wonnacott et al, 1990)



least 3 models proposed. The precise tertiary structure and the location of the COOH terminal are somewhat controversial (Hucho, 1987). In its natural environment the receptor is associated with several small proteins, whose functions are not fully understood but are believed to be involved in receptor stabilisation and synapse formation (Anholt et al,1984). DNA cloning, amino acid sequence analysis, site directed mutagenesis and monoclonal antibody studies, have indicated that the four subunits have evolved from a single ancestral gene (Conti-Tronconi et al,1983), and have assisted in the location of functional domains within each subunit (Wonnacott,1990). For example, the αBGT binding site has been specifically identified (Mulac-Jenicevic & Atassi, 1987: Wilson & Lentz, 1988). Similarly, phosphorylation sites have been located in homologous positions in all 4 subunits (Huganir & Greengard, 1987), and it has been suggested that phosphorylation may regulate the rate of receptor desensitization (Huganir et al,1986: Hopfield et al,1988).

In Torpedo, the AChR has been found to exist in monomeric and dimeric forms with Sw_{20} values of 9S and 13S respectively (Reynolds & Karlin, 1978). The dimer is believed to be the functional unit in vivo (Schindler et al,1984) and is formed by two monomers, covalently linked by a disulphide bond through the delta subunits (Sobel et al,1977).

The characterisation of the vertebrate skeletal muscle AChR

has been somewhat slower, partly because it is less accessible, and partly due to the presence of two distinct forms of the receptor, juntional (J-AChR) and extrajunctional (EJ-AChR) (Famborough, 1981). EJ receptors differ from J-AChR's in several respects; namely they exhibit lateral mobility, have lower half-lives, longer channel opening times (Anholt et al,1984) and less glycosylation (Turnbull et al,1985). Another, significant difference, is that anti-AChR antibodies from Myasthenic sera, react preferentially with EJ receptors (Weinberg & Hall, 1979: Almon & Appel, 1975: Savage-Marengo et al,1980: Harrison et al, 1983). The same has been seen with rabbit anti-cat AChR anti-sera (Dolly et al,1983) and mouse anti-Torpedo AChR antibodies (Souroujon et al,1983). Similarly, a monoclonal anti-human AChR antibody has been shown to react preferentially with human denervated receptor. These studies and the demonstration that some Myasthenic sera block toxin binding to rat EJ-AChRs (Dwyer et al,1982a,b: Hall et al,1983) have led to the postulate that EJ-AChRs or foetal AChRs may be the autoimmunogen in Myasthenia gravis.

To date, most of the information has shown that the vertebrate receptor bears a remarkable resemblance to the electric organ AChR. Cloning experiments on calf AChR (Tanabe et al,1984) and human SChR (Shibahara et al,(1985) have confirmed this homology, and appear to suggest a common ancestral gene

(Harrison & Behan,1986). Vertebrate AChR shows a Sw_{20} of 9S, which is a monomer, with no detection of a 13S dimer. A smaller 4S toxin binding species has also been detected (Lo et al,1981), and this may be a degradation product. The subunit pattern also appears to be similar with all four subunits being detected. The α-subunit also carries the ACh/α-BGT binding site.

Calf AChR does show an anomaly, however. It has been shown to contain an epsilon subunit, which is homologous with the gamma subunit (Takai et al,1985). It has been suggested that the embryonic form of the receptor possesses the gamma subunit which is replaced by the epsilon subunit during development into the adult (Schuetze, 1986).

3.3 MYASTHENIA GRAVIS

3.3.1 History

Myastenia gravis (MG) is a disease characterised by weakness and fatiguability of voluntary muscles. The first description of the disease was made by Thomas Willis (1672), and, since that time, various researchers and physicians have described patients with symptoms amounting to clinical MG (see Harrison & Behan,1986, for review). However, it was not until 1895 that the term 'Myastenia gravis pseudoparalytica' was coined for the disease by Jolly (1895), the 'pseudoparalytica' being dropped a short while

later. It was Campbell & Brambell (1900) who formally summarised the case histories of over 70 patients, all of whom were displaying symptoms of MG, and first suggested a possible aetiology for the disease involving a circulatory factor, possibly a microbial toxin.

In 1934, Dr. Mary Walker made a connection between the symptoms of MG and those displayed by patients suffering from curare poisoning, which led her to use the antidote for curare poisoning, phytostigmine, as a treatment for MG. This drug blocks the action of anti-acetylcholine esterase (AChE), and was remarkably successful in treating MG patients. It also gave the first direct evidence that the primary lesion in MG was located at the NMJ. However, this was not confirmed until Engel & Santa (1971) described a simplified morphology at the NMJ in Myasthenic patients when compared to normals, and Fambarough, (1973) demonstrated the reduction in the numbers of AChRs at the NMJ.

It was also during the early part of this century that a connection was made between MG patients and patients with thymic abnormalities, with the result that thymectomy was used to treat MG patients (Blalock, 1939; for review see Harrison & Behan,1986).

The first suggestions that MG was possibly an autoimmune disease, came from Smithers (1959), supported by reports from immunological abnormalities in MG patients from Nastuk et al, (1956,1960), and Straus et al,(1960), but it was Simpson,(1960)

who made the most authoritative case for an autoimmune aetiology for MG. However, the identification of AChRs at the NMJ as the site of the autoimmune attack, was not made until Lindstrom & Patrick,(1973) demonstrated the appearance of MG like symptoms in rabbits immunised with purified fish electric organ AChR.

3.3.2 Clinical and Pathological Features of MG

MG is a rare disease affecting approximately 4-6 individuals per 100,000 (Kurtzke & Kurland, 1977). It can attack both males and females at any age, but 60% of patients are female with an onset age between 15-40 yrs (Osserman & Genkins, 1971). Males tend to develop the disease much later in life. This sex and age distribution appears to reflect, to some degree, the distribution of certain HLA antigens. Studies on Caucasian patients show that B8 and DRW3 appear to be associated with females suffering from early onset MG and thymic hyperplasia (Rule, 1973: McDevitt & Bodmer, 1974: Feltkamp et al,1975: Fritze et al,1976: Pirskanen, 1976: Gross et al,1977), and A2 or A3 appear to be associated with late onset MG in males also suffering from thymomas (Feltkamp et al,1975: Fritze et al,1976).

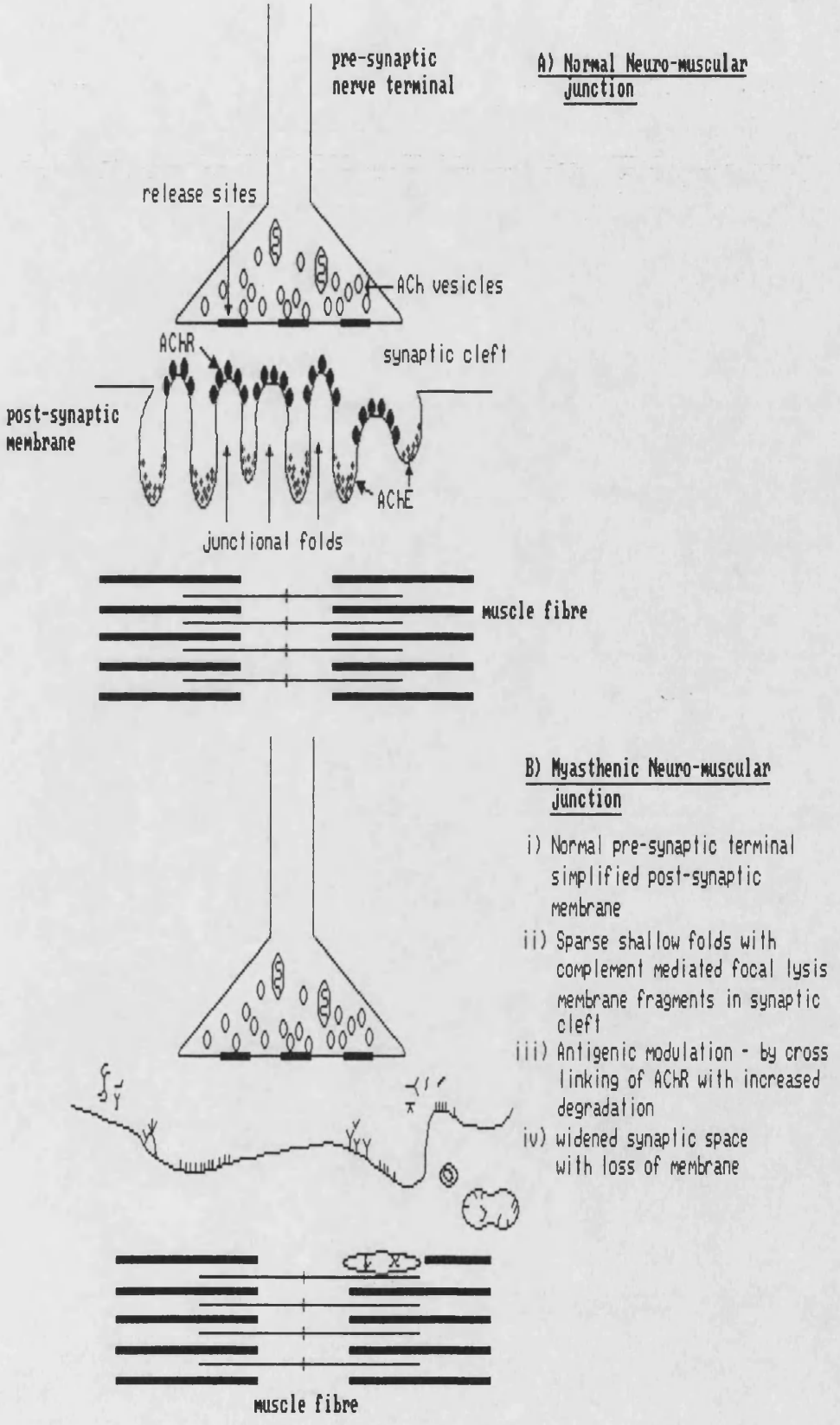
The most characteristic clinical feature of MG is muscle weakness and fatigue. However, the clinical picture is varied, complicating diagnosis. In the mildest forms, extraocular

muscles are affected, resulting in droopy eyelids (ptosis) and double vision (diplopia). Up to 90% of all patients show these symptoms. In more generalised cases, other muscle groups are affected, particularly in the extremeties and trunk. This general weakness has been shown to increase with exercise, with fluctuations in symptoms occuring daily, weekly or even over longer periods, and total remissions occuring in only 20% of patients. A large number of patients have also been shown to suffer from a variety of other diseases, a significant proportion of which are autoimmune (see Harrison & Behan, 1986).

Coupled with these clinical observations are pathological and morphological changes in the thymuses and at the NMJ of patients suffering from MG. About 10-15% of patients have thymoma with thymic hyperplasia or germinal centres observed in 75-85% of patients (Castleman, 1966). Germinal centres contain mainly B lymphocytes with a few T cells and macrophages. They are also found in patients suffering from a variety of other auto-immune diseases.

The NMJ of MG patients have been shown through EM studies (Engel & Santa,(1971)), to have a simplified morphology compared to normal NMJ's. The post-synaptic membrane shows sparse shallow folds with reduced numbers of AChR's (Alberquerque et al,1976: Engel et al,1979b: Fambrough,1973). In contrast, the nerve terminals are slightly reduced in size, but contain normal

Fig.19: Diagramatic representation of normal & myasthenic NMJ's



numbers of synaptic vesicles with twice the concentration of ACh (Ito et al,1976: Cull-Candy et al,1978, 1980). Membrane fragments with complement components have been found in the synaptic cleft, which is also wider due to the loss of membrane (see Fig. 19). These changes at the NMJ result in decreased efficiency of synaptic transmission, which, if severe enough will lead to the reduction of muscle power and hence weakness and fatigue.

Clinical diagnosis of MG purely on observations of symptoms is less than satisfactory, and makes the evaluation of therapies difficult. Consequently, various test conditions are applied. The most regularly used clinical test is the 'Tensilon' test, based on the administration of edrophonium chloride (an acetylcholinesterase inhibitor), followed by observation of improvement in weak muscles. If there is an improvement, then the test is positive.

A test carried out in conjunction with the Tensilon test, and one which specifically diagnoses MG, is the determination of anti-AChR antibodies in the sera (Methods, section 1.6 for details of assay). Although false positives do not occur, false negatives commonly do. Thus a minority, of MG patients have normal titres. These include patients presenting purely ocular symptoms (Vincent, 1984), and some patients on corticosteroids or who have undergone thymectomy (Oosterhuis et al,1983). Even within those patients showing negative titres, correlation between antibody titre and

clinical state is poor (Vincent & Newsom-Davis,1980: Limburg et al,1983).

Other tests include normal electromyographs, single fibre electromyographs, acoustic tests and eye tests. Kelly et al,(1982) compared results, over 3 years, from numerous MG patients, utilising ocular testing, routine EMG and antibody titres. They concluded that if patients with mild MG were tested by all 3 methods, then positive diagnosis could be made in 95% of all cases.

3.3.3 Therapy

The most common form of therapy is treatment with AChE inhibitors, which act by increasing the effective concentration of ACh in the synaptic cleft. It has also been suggested that the effect of anti-AChE treatment is to produce greater numbers of activated AChRs.

Thymectomy is frequently used to treat patients who are not well controlled on antiesterase drugs along. As previously stated, many patients have thymic abnormalities (Thomas et al, 1982: Bofill et al,1985: Castleman,1966). Mulder et al,(1983) reported an overall benefit to 87% of 249 patients who had undergone thymectomy, with female patients faring better. Bartoccioni et al,(1980) showed significant reductions in anti-AChR antibody levels following thymectomy.

Steroid therapy is also used and has been shown to be very effective in many cases (reviewed by Harrison & Behan,1986). Associated with this form of therapy, is the use of immunosuppressive drugs, such as azathioprine and 6-mercaptopurine (Mattell et al,1976: Aertel,1979).

In patients with severe generalised MG, plasmapheresis (plasma exchange) coupled with immunosuppressive drug therapy (Pinching et al,1976: Dau et al,1977, 1979, 1980, 1981, 1982a,b: Newsom-Davis et al,1978, 1979a,b: Behan et al,1979: Kornfeld et al,1979, 1981, 1982: Lisak et al,1979a: Perlo et al,1981: Orlarte et al,1981: Keesey et al,1981: Newson-Davis & Vincent,1982), and thoracic duct drainage (Mattell et al,1976: Bergstrom et al,1973) have been used with success in many cases. Following the use of this technique, an immediate and dramatic drop in anti-AChR antibody levels is often followed by a noted clinical improvement.

Gammaglobulin therapy has also been used (Gajdos et al, 1984) with claimed clinical improvement and reduction of antibody titres. Related to this form of therapy is the suggestion that anti-idiotypic therapy may be of benefit in autoimmune diseases (Fuchs, 1980), either by using polyclonal sera or monoclonal anti-idiotypic antibodies. There is increasing evidence of an operational idiotype-anti-idiotype network in MG (Schwartz et al, 1978: Fuchs,1980: Dwyer et al,1983: Feingold et al,1980). However, the benefit of such therapy may be of limited value.

Heininger et al,(1983) demonstrated that remission phase patients do not contain measurable amounts of blocking anti-idiotypic antibodies, either in a free or immuno-complexed state, and suggested that such a mechanism does not induce remission. Such a therapeutic strategy may also prove difficult, because of the demonstration of the heterogeneity within patients of anti-AChR antibodies (Newsom-Davis & Vincent,1980: Levfert,1981). Vincent (1981) did suggest the presence of a restricted idiotype, possibly corresponding to the main immunogenic region (MIR) of the AChR (Tzartos & Lindstrom,1980). However, Killen et al, (1985) have demonstrated that the MIR does not generate antibodies of a predominant idiotype.

3.3.4 Autoimmune Nature of MG

The possibility that MG might be an autoimmune disease was originally suspected on the basis of indirect evidence, consisting of a high incidence of thymic abnormalities in patients (Castleman, 1966), the association of MG with other diseases of autoimmune aetiology (Simpson,1960), and reduced levels of complement proteins in some patients (Nastuk et al,1960). Two further discoveries also lent weight to this postulate. Firstly, a proportion of myasthenic patients were shown to have serum antibodies that reacted against skeletal muscle (Strauss et al,1960), and secondly, the induction of an experimental animal model

by Lindstrom & Patrick,(1973). The latter workers discovered that immunisation of rabbits with purified electric eel AChR, caused marked muscular weakness and respiratory difficulty, coupled with the production of anti-AChR antibodies. This model, termed 'experimental autoimmune myasthenia gravis' (EAMG), has been extensively used to investigate the pathology and immunology of MG (for reviews see Harrison & Behan, 1986: Vincent, 1980: Fuchs, 1979: Lindstrom, 1979).

The autoimmune origin of MG was finally confirmed by several lines of evidence. It was shown that the binding of α-BGT to human J/EJ-receptors in frozen sections (Bender et al 1976), and of radiolabelled α-BGT to rat EJ-receptors (Almon et al,1974) could be blocked by anti-AChR antibodies found in myasthenic sera This, coupled with the determination by radioimmunoassay (RIA) of these antibodies in some 90% of MG patients (Lindstrom et al, 1976a,b, 1977, 1981: Lindstrom,1979) indicated that the anti-AChR antibodies were involved in the pathogenesis of MG.

It was further shown that anti-AChR antibodies were observed in transient neonatal MG, the titres declining as the patient improved (Lindstrom et al,1976b: Keesey et al,1977), showing that trans-placental transfer of anti-AChR antibodies were responsible. Ultrastructural studies of myasthenic NMJ's also showed the presence of IgG, C9 and C3 (Engel et al,1977a,b,c). Lindstrom & Lambert (1978) were able to show that receptor numbers were

reduced at the NMJ in MG patients, and that the AChR's were complexed with anti-AChR antibodies. They also demonstrated that patients with severe MG had lower numbers of receptors and more of them were complexed with anti-AChR antibodies.

These results gave direct evidence that the primary defect in MG is the reduction in AChR numbers at the NMJ, and is the result of an autoimmune attack involving humoral mechanisms.

3.3.5 Origin Of The Autoimmune Response In MG

A major question arises as to how tolerance to self AChR breaks down. Much attention has been paid to the thymus, especially because of the association between MG and thymic abnormalities (Castleman,1966: Thomas et al,1982: Bofill et al, 1985) and the beneficial effects of thymectomy in some patients (Simpson,1958: Buckingham et al,1976: Oosterhuis, 1981: Hankins et al,1985). Certain thymic cells, known as myoid cells, display characteristics of striated muscle, and cross react with anti-skeletal muscle antibodies (Van der Geld,1966). Cells cultured from the thymuses of rats (Kao & Drachman,1977: Wekerle et al, 1975), mice (Wekerle et al,1981) and humans (Kao & Drachman,1977) appear to be typical of skeletal muscle cells in culture and display surface AChR's. Specific mab's have been used to detect AChR's on thymic cells from both normal and myasthenic patients

(Kirchner et al,1987). AChR's have also been detected in extracts of thymuses from a variety of sources (Lindstrom et al,1976c: Ueno et al,1980: Raimond et al,1984), and anti-AChR antibodies have been shown to interact with a particular constituent of thymuses (Aharanov et al,1975). The strategic location of receptor bearing cells, T cells and B cells in the thymus, has accordingly led to the suggestion that the thymus is the site where the autoimmune response develops, possibly through alteration in the AChR resulting in an interaction leading to a breakdown in tolerance.

It has also been suggested that the auto-immunogen may be located on lymphocytes. It has long been established that lymphocytes bear mAChR (see Harrison & Behan, 1986 for references) but it has only recently been suggested that they also bear nAChR (Richman & Arnason,1979: Richman et al,1981: Menard & Rola-Pleszozynski, 1983: Walsh et al,1986).

Stefansson et al (1985) proposed that a viral or bacterial infection may result in the autoimmune response through cross-reactivity with microbial antigens. However, the spectrum of antibodies produced in an MG patient suggests the immunogen to be intact receptor, rather than a single cross-reacting determinant of microbial origin.

An anti-idiotypic cause has also been suggested especially where there is defective immuno-regulation. At least some

autoimmune diseases develop as a result of an anti-idiotypic response towards antibodies produced against a biologically active molecule, such as insulin or thyrotropin (Wasserman et al, 1982: Shechter et al,1982), and it is possible that this may be the case in MG (Cleveland et al,1983: Erlanger et al,1984).

3.3.6 Humoral Immunity In MG

The role of anti-AChR antibodies in the pathogenesis of MG has been inferred from indirect evidence (Introduction, section 3.3.7). However, most of the work with anti-AChR antibodies in myasthenic patients, has been as a direct result of the development of sensitive assays for their detection.

Almon et al (1974) first demonstrated that serum immunoglobulin from approximately one third of a group of myasthenic patients was capable of blocking α-BGT binding to rat AChR. This was followed by Bender et al (1975) who showed that 68% of myasthenic sera inhibited α-BGT binding to frozen human muscle sections. However, it was not until the development of sensitive RIA procedures, based on the complexing of ^{125}I-α-BGT labelled AChR by antibody, that it was possible to show that nearly 90% of all MG patients had anti-AChR antibodies in their sera (Appel et al,1975: Lindstrom et al,1976, 1981a: Mittag et al,1976, 1984: Lindstrom et al,1977: Monnier & Fulpuis,1977: Levfert et al,1978:

Toyka et al,1979: Bartoccioni et al,1980: Walker et al,1980: Cerrato et al,1981: Morel et al,1982). This assay has become a standard for the clinical diagnosis of MG (Methods, section 1.6) Precise methodical details vary between groups but with little effect on results and data obtained (Carter et al,1981). It has been established that human AChR or primate (McAdams & Roses,1980: Mickle et al,1983) is most useful, with cross-species AChR giving lower titres (Oda et al,1980: Savage-Marengo et al,1979, 1980: McAdams & Roses,1981c: Appel et al,1975: Brenner et al,1978: Konishi et al,1981: Kornfeld et al,1981b: Mittag et al,1981a,b, 1984: Buot et al,1982: Zielinski et al,1982).

Enzyme linked immuno-sorbent assays (ELISA) have been used (Norcross et al,1980: Kawanami et al,1984) with varying degrees of success, but these, in contrast to RIA usually require purified human AChR, which is difficult to obtain (Stephenson et al,1981: Momoi & Lennon,1982: Turnbull et al,1985). A modification, using crude receptor preparations and based on the adsorption of monoclonal antibodies onto a microtitre plate has been described (Dwyer et al,1983a). Two further ELISA's have been described in which crude human receptor extracts were used (Kobayashi et al, 1984: Jailkhani et al,1986) while Hinman et al (1983) described and ELISA in which the plates were coated with α-BGT and then incubated with Torpedo AChR, but unfortunately, this assay relied on cross-reactivity. Other assays include an EIA in which

radiolabelled αBGT is replaced with horseradish peroxidase labelled α-BGT (Furakawa et al,1984), and a haemagglutination assay (Muir & Jehanli,1985).

Even though several ELISA's and EIA's have been described, none has been popular for either clinical or experimental use.

3.3.7 Pathogenic Role of Anti-AChR Antibodies in MG

The concentration of anti-AChR antibodies in MG patients varies from 0.6 - 1000 nM, with an average of 50 nM (Lindstrom et al,1976d: Ito et al,1978: Levfert et al,1978). Although titres do not correlate closely with disease severity (Lindstrom,1979: Cerrato et al,1981), it is clear that anti-AChR antibodies are important in the pathogenesis of MG.

The lack of correlation may arise from a number of reasons. There is good evidence showing that myasthenic anti-AChR antibodies are polyclonal, comprising various immunoglobulin classes and subclasses (Vincent & Newsom-Davis,1980: Lefvert,1981), with many sera containing α-BGT binding site antibodies (Vincent & Newsom-Davis,1982: Mittag et al,1981: Vernet-der-Garabedian et al, 1986). The standard RIA is unable to detect anti-site antibodies, unless modified (Zielinski et al,1982). Sera from different donors also vary in their cross-reactivity with AChR preparations from different species (Lindstrom et al,1978a: Savage-Marengo

et al,1980: Harrison et al,1981), and to different AChR preparations from the same species (Vincent & Newsom-Davis,1979: Almon & Appel,1975: Weinberg & Hall,1979).

There is increasing evidence to suggest that a small antibody sub-population may be the primary pathogenic agent in MG. The main immunogenic region (MIR) of the AChR (Tzartos & Lindstom, 1980) is located on the extracellular side of the α-subunit, and is distinct from the ACh/ α-BGT binding site. Myasthenic sera can be prevented from binding to the MIR by anti-MIR mab's, suggesting that the majority of anti-AChR antibodies in MG are directed towards the MIR, although, Tzartos et al,(1982,1983) have shown that a minority of antibodies of other specificities exist. The fact that these antibodies are pathogenic was demonstrated by their ability to modulate AChR by cross linking, and to fix complement (Tzartos & Lindstrom,1980). Certain anti-MIR mab's were shown to be able to transfer acute EAMG (Lindstrom, 1984).

As previously stated, the primary defect in MG is the reduction in numbers of AChR's at the NMJ. The exact mechanism of how anti-AChR antibodies are involved in this, is not known but several suggestions have been made.

A) Direct Block of AChR

Blocking antibodies have been detected in a large number of sera (Mittag et al,1981: Drachman et al,1982,1977: Lennon et al,

1983: Fulpuis et al,1981). Although in most cases these antibodies account for only a minor fraction of the total antibodies found (Tzartos et al,1982: Vincent et al,1983) their effect could be significant (Gomez & Richamn,1983).

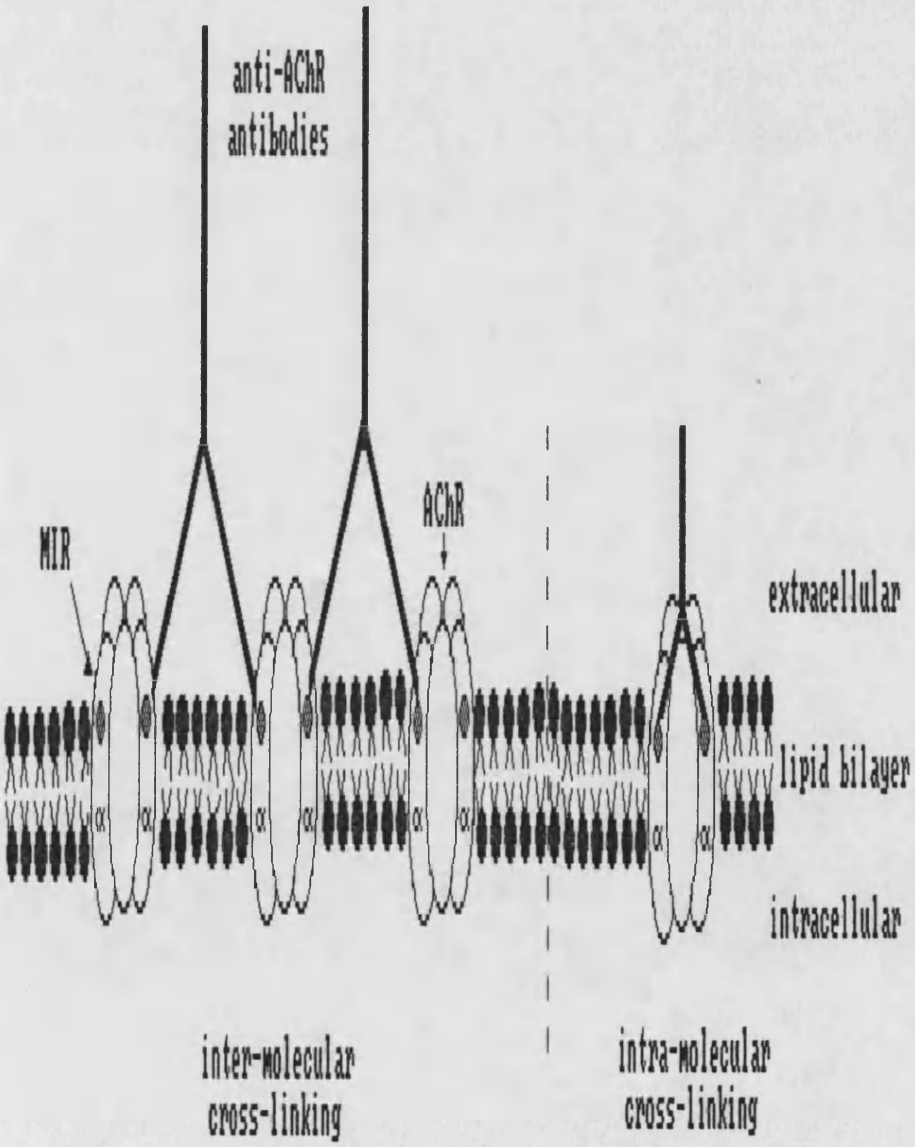
B) **Antigenic Modulation**

This is believed to be an important mechanism in receptor loss. Cross-linking of the receptors at the post-synaptic membrane by anti-AChR antibodies, accelerates AChR internalisation and degradation (Reiness et al,1978: Stanley & Drachman,1978: Merlie et al,1979: Fumagalli et al, 1982a,b) as receptor cross-linking causes changes in the membrane mobility and receptor distribution resulting in endocytosis and lysosomal destruction of receptors. The increase in the rate of receptor degradation is triggered by antibody alone and is dependent upon the ability of the antibodies to cross link receptors (Drachman et al,1978: Conti-Troconi et al,1981b) ie. upon their divalent nature. (See Fig 20).

C) **Complment Mediated Lysis**

The evidence for this mechanism is largely indirect, and results from the localisation of antibodies, C9, and C 3 on the post-synaptic membrane of NMJ from myasthenic patients, together with the visualisation of membrane fragments in the synaptic cleft (Sahashi et al,1978, 1980: Engel et al,1979, 1981b: Engel & Fumagalli,1982). Complement mediated lysis has been demonstrated

Fig.20: Diagram showing inter- & intra- molecular cross-linking of AChR by Anti-AChR antibodies & showing possible involvement of MIR



on cultured rat myotubes mediated by myasthenic sera (Childs et al, 1985, 1987: Ashizawa & Appel,1985). Ashizawa & Appel,(1985) demonstrated that the ability of myasthenic sera to cause lysis was correlated with disease severity.

Neither complement mediated lysis nor accelerated internalisation and degradation could deplete the numbers of AChR's, if the loss was balanced by replacement. It is therefore believed (Engel, 1987) that antigenic modulation and complement mediated lysis operate together. In addition, destruction of the junctional folds by complement reduces available membrane sites left for the insertion of new AChR's, and in turn amplifies the subsequent AChR depleting effect of either modulation or complement.

Other mechanisms may also be involved, for example, anti-idiotypic antibodies have been found in some myasthenic sera (Dwyer et al,1983) and their titres have been shown to be inversely related to the idiotypic titre.

3.3.8 Cellular Immunity in MG

The production of anti-AChR antibodies is the final stage in a complex immune reaction. The demonstration that PBL's and thymic cells from myasthenic patients (Lisal et al,1983a,b, 1984: Vincent,1979), when cultured *in vitro* produce anti-AChR anti-

bodies, and the occurence of other autoimmune diseases in MG patients, have led to the idea of a defect in immuno-regulatory mechanisms in MG (Drachman et al,1982: Penn,1979: Shore et al, 1979).

Numerous workers have attempted to gain evidence of abnormalities in the cellular immune responses of lymphocytes from MG patients. In particular, most work has concentrated on comparisons of B cells, T cells, and T cell subsets, in MG patients compared with normal individuals (extensively reviewed by Lisak et al,1985: Harrison & Behan,1986). However, the results so far obtained do not present a clear picture, and are somewhat conflicting, but, in general, show an increase in B cell and a decrease in T cell populations, in both thymuses and peripheral blood of MG patients.

It is conceivable that the proliferation of B cells, could result from either a decrease in the number of suppressor T cells and/or a defect in suppressor T cell function, or an increase in the numbers and/or function of autologous helper T cells. Indirect evidence supporting changes in regulatory T cells, comes from the fact that susceptibility to MG appears to be MHC linked (Introduction, section 3.3.2, Oosterhuis et al,1981: Harrison & Behan,1986), and that PBL's from MG patients undergo proliferation when incubated with purified AChR from electric eel (Abramsky et al,1975: Richman et al,1976, 1979), Torpedo electric organ

(Conti-Troconi,1977, 1979, 1982: Hohlfeld et al,1984) and human AChR peptides (Harcourt et al,1987: Hohlfeld et al,1987), supporting the concept that AChR specific T cells are important in the aetiology and regulation of MG. This has been further supported by Greenberg et al,(1984), and Richards et al,(1986), both of whom investigated the proliferative responses of purified T cells from MG patients, when co-cultured with autologous non-T cells, in a reaction known as the Autologous Mixed Lymphocyte Reaction (AMLR). Greenberg found an increased AMLR which was abolished by thymectomy, whereas, Richards discovered a decreased AMLR in MG patients. Even though the two results are different, they do indicate a defect in immune cell function in MG. In general, stimulation of lymphocytes in response to a specific antigen, indicates that the cells have previously been sensitised to that antigen. Suppressor T cell numbers have been shown to be decreased in various other autoimmune diseases (Berrih et al,1981 Raeman et al,1981).

There is also increasing evidence of a T cell immuno-dominant recognition site on the AChR, which appears to be on the α-subunit but is distinct from the MIR and ACh/ α-BGT binding site (Hohlfeld et al,1987). Recent studies have shown that there is no single immunodominant T-cell epitope on the AChR alpha-subunit, but that there are a number of distinct antigenic 'hotspots' on which the T-cell response is focused. These

'hotspots' being distinct from the anti-AChR antibody binding site (Hohlfeld,1989: Hohlfeld et al,1988: Zang et al,1988: Fujii et al,1988: Yokoi et al,1987: Melms et al,1989: Harcourt et al, 1988). However, the exact role of cellular mechanisms in the pathogenesis of MG is as yet unclear.

3.3.9 Monoclonal Antibodies and MG

The development of murine monoclonal antibody technology (Kohler & Milstein,1975, 1976: Introduction section 4) has vastly increased the structural and functional knowledge of the nAChR. Thus:

1) Investigation of the structural homologies between subunits indicate that they were probably derived from a common ancestral gene (Tzartos & Lindstrom,1980: Tzartos et al,1981: Fracher et al,1983: Gullick & Lindstrom et al,1985, 1983). This has been confirmed through amino acid sequence and DNA sequence analysis (Raftery et al,1980: Noda et al,1983).

2) Structural homologies have been shown between receptors from different species (Tzartos & Lindstrom,1980: Tzartos et al,1981, 1983: Gomez et al,1981: Mochly-Rosen & Fuchs, 1981: Lindstrom et al,1980).

3) The transmitter binding site and conformational changes

in the receptor during activation and desensitisation have been analysed (Gomez et al,1979: James et al,1980: Mochly-Rosen & Fuchs,1981: Lindstrom et al,1981).

4) The role of glycosylation in the AChR subunit assembly has been studied suggesting that it is important for receptor assembly (Merlie et al,1982).

5) Affinity purification of AChR's has been effected from a variety of sources (Momoi & Lennon,1982, 1984: Lennon et al,1980).

6) The antigenic specificities of myasthenic antibodies have been studied and show that the majority of them are directed towards the MIR on the α-subunit (Tzartos & Lindstrom,1980: Tzartos et al,1981, 1982, 1983). Mab's have also been used to map antigenic determinants on receptors, as model auto-antibodies and as probes for determining the specificities of auto-antibodies in MG patients (Lindstrom,1985).

7) Mab's have been used to passively transfer EAMG into rats, mice and guinea pigs, with subsequent development of myasthenic like characteristics (Tzartos & Lindstrom,1980: Lennon & Lambert,1981: Gomez et al,1981: Gomez & Richman, 1983: Merlie et al,1982: Dwyer et al,1981) with antigenic modulation being detected in some cases (Conti-Tronconi et al,1981: Tzartos & Stazinski-Powitz,1986). Antigenic

modulation by myasthenic sera could be blocked by the use of monoclonal anti-AChR Fab fragments (Tzartos et al,1985).

8) The topography and transmembrane nature of the AChR has also been studied by using specific mab's (Froehner et al, 1983: Lindstrom et al,1984, 1985, 1986: Sargent et al,1984: Young et al,1985: Ratnam et al,1986a,b).

Murine mabs have clearly been widely used to investigate AChR's and MG/EAMG. There are, however, several areas where it would be advantageous to develop human mabs. Firstly, they may allow the development of high specificity antibodies, which could be used to map antigenic determinants on the human AChR which are active during the disease state, and delineate the specificities of pathogenic antibodies in MG and EAMG. Secondly, they may allow the development of human gammaglobulin therapy for MG, either through the administration of large doses of neutralising anti-idiotypic antibodies, manipulation of the idiotype network or possibly by targeting and elimination of self-reactive autologous lymphocytes active in the disease state. Thirdly, human mabs may prove useful in the identification of the immune mechanisms responsible for the development of MG.

At present there have been only two reports of human monoclonal anti-AChR antibodies having been produced from patients suffering from MG. Kawo et al,(1982) described the

production of such antibodies through EBV transformation of PBL's (Introduction section 4.2.3 (2)), and Blair et al,(1985) briefly described the production of monoclonal anti-AChR antibodies through a human x human fusion technique (Introduction, section 4.2.3 (i)A)).

4. MONOCLONAL ANTIBODIES

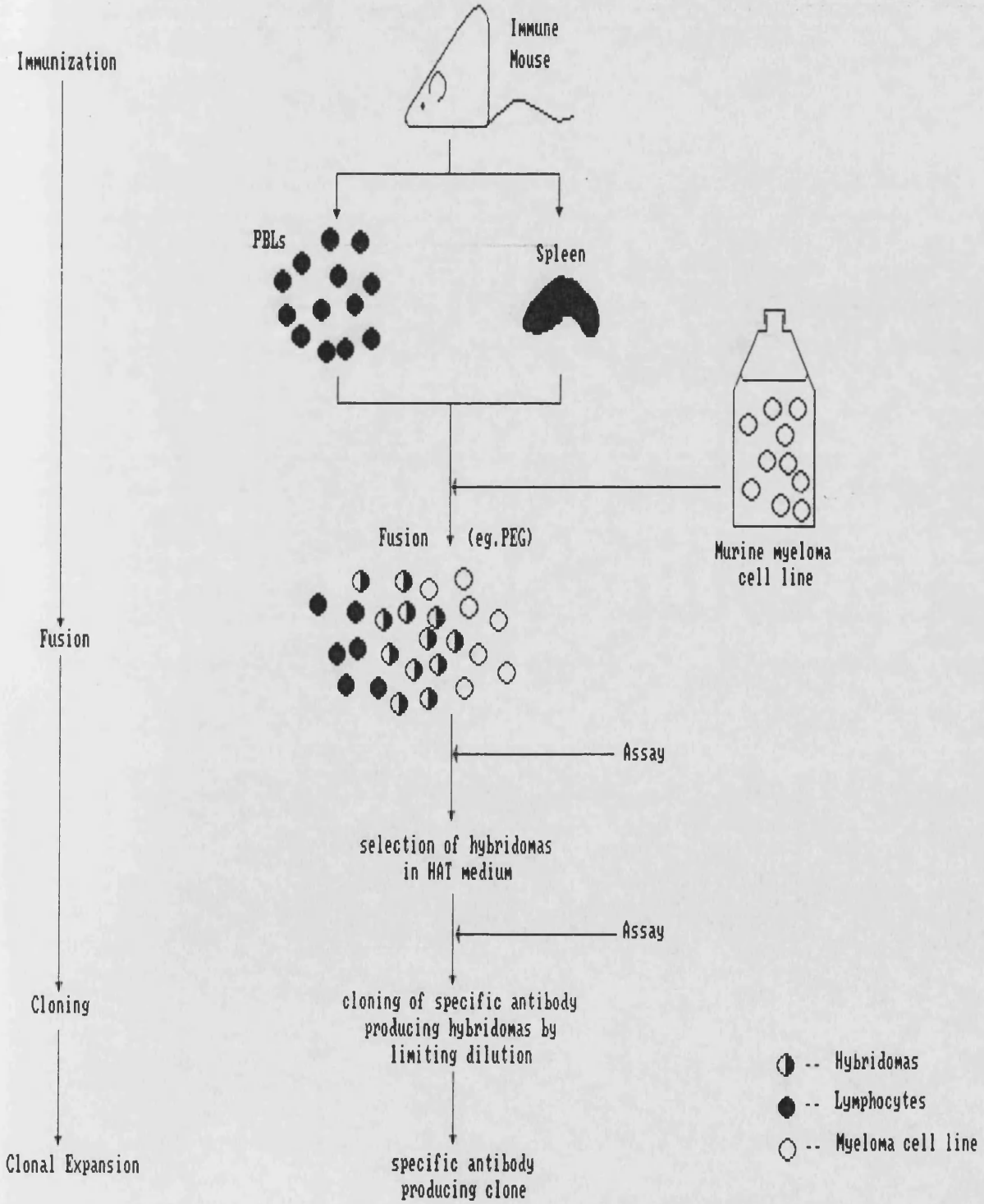
4.1 Murine Monoclonal Antibodies

In 1984 G. Kohler & C. Milstein were awarded the Nobel Prize for Physiology & Medicine for their work in the field of somatic cell hybridization which allowed the production of monoclonal antibodies of predetermined specificity.

The technique described by Kohler & Milstein (1975, 1976: fig. 21), revolutionised the study of the immune system and protein chemistry.

Antibody producing rodent hybridomas are prepared in vitro by the fusion of antibody producing B cells with rodent myeloma cell lines. In the original technique Sendai virus was used to fuse the cells (Kohler & Milstein,1975: 1976) but has largely been replaced by Polyethylene glycol (PEG). A further technique involving electrofusion of cells (Zimmerman and Vienken,1982: Biscoff et al,1982) is also used, and is claimed to be 100,000 times more effective than PEG fusing small numbers of cells

Fig.21- Schematic Of Murine Monoclonal Antibody Production



Cell Line	Cell Type	Secreted Immunoglobulin	Refs.
Murine			
MOPC-21	myeloma	IgG1	2.5
MPC-11	myeloma	IgG2b	2.5
P3/X63-Ag8	myeloma	IgG1	1.3
P3/NS1/1-Ag4-1 (NS-1)	myeloma	kappa light chains	1.4
X63-Ag 8.653	myeloma	none	6
NSO/1	myeloma	none	7
SP2/O-Ag14 (SP2/O)	myeloma	none	1.8
FOX-NY	myeloma	none	9
Rat			
Y3-Ag 1.2.3	myeloma	kappa light chains	1.10.11
YB2/3HL.P2.G11.16Ag20 (YB2/O)	myeloma	none	1.12

References:
1) ATCC (1985)
2) Cotton & Milstein (1973)
3) Kohler & Milstein (1975)
4) Kohler & Milstein (1976)
5) Melchers et al (1978)
6) Kearney et al (1979)
7) Galfre & Milstein (1981)
8) Shulman et al (1978)
9) Taggart & Samloff (1982)
10) Galfre et al (1979)
11) Lane (1985)
12) Kilmartin et al (1982)

TABLE 3: Available Rodent Fusion Partners

(adapted from Galfre & Milstein (1981): Kozbor & Roder (1983): and Samoilovich et al (1987))

(Dorfmann,1985). The hybridomas inherit some characteristics of both parental cells, and lose others eg. they inherit immortality from the myeloma cells and the ability to form antibody from the B cells. Monospecific antibodies of monoclonal origin are maintained by clonal selection of the specific antibody producing clones (see Fig. 21).

The success of this technique since its introduction has been in the main due to the development of a number of very good rodent fusion partners of murine and rat myeloma origin, which do not secrete antibody, undergo fusion readily, and allow the selection of antibody producing hybridomas. Of these the preferred choice is often the murine cell line X63-Ag 8.653 (Kearney et al,1979).

Table 3 gives a summary of the most common murine and rat fusion partners.

4.2 Human Monoclonal Antibodies

Even though rodent mabs are of great use, both to the scientist and clinician, there are a number of problems associated with their use, which makes the construction of human mabs advantageous.

In particular it could be possible to produce abs for: targetting of drugs to specific cells eg. malignant cells,

pathogens, and lymphocytes which are responsible for autoimmune disease or allergic reactions (Blair & Ghose,1983: Levey & Miller, 1983); diagnostic uses in the imageing and localisation of damaged tissues, malignant cells etc (Emanuel et al,1986: Rosen et al,1983: Glassey et al,1983: Cole et al,1984: Olsson et al, 1984: Haspel et al,1985a,b: Matsushita et al,1985: Andreason et al,1986: Kan-Mitchell et al,1986: Smith et al,1987: Strelkaushas, 1987a: Sikora,1983: Sikora et al,1987: Glassey et al,1985: Burnett et al,1987); immunisation procedures, both passive (e.g to prevent heamolytic disease of the newborn, to neutralise toxins, drugs and opsonise bacteria and viruses) and active (e.g immunisation with anti-idiotypic mabs to stimulate abs against pathogens for which there are currently no vaccines) (Atlaw et al,1985: Matsushita et al,1986: Schmidt-Ullrich et al,1986: Hunter et al,1982: Emmanuel et al,1984: Crawford et al,1983: Bron et al,1984); they can also be used in the therapy of autoimmune diseases (Groce et al,1980: Sasaki et al,1984: Chiorazzi,1986), and for tissue typing (Steinitz et al,1977: Hulette et al,1985: Effros et al,1986). In addition to these uses they can also be used to investigate the immune system in both health and disease e.g characterisation of antigens (Smith & Teng,1987: Satoh et al, 1983: Bron et al,1984: Carroll et al,1986) and B cell activity (Glassey et al,1983: Winger et al,1983: Cote et al,1985, 1986: Watson et al,1983). Human mabs are particularly useful in

immunisations and gamma globulin therapy, where the use of xenogenic abs or sera results in the sensitization of the patient, anaphylatic shock, or serum sickness. Almost half of all patients treated with murine mabs developed a response to them, preventing effective therapy (Miller et al,1982: Dillman et al,1982: Sears et al,1982). Naturally, administration of human mabs would circumvent this problem, even though it may result in the development of an anti-idiotypic response. Such a response could be the basis of therapy in auto-immune diseases, where the anti-idiotype could neutralise the effect of auto-antibodies.

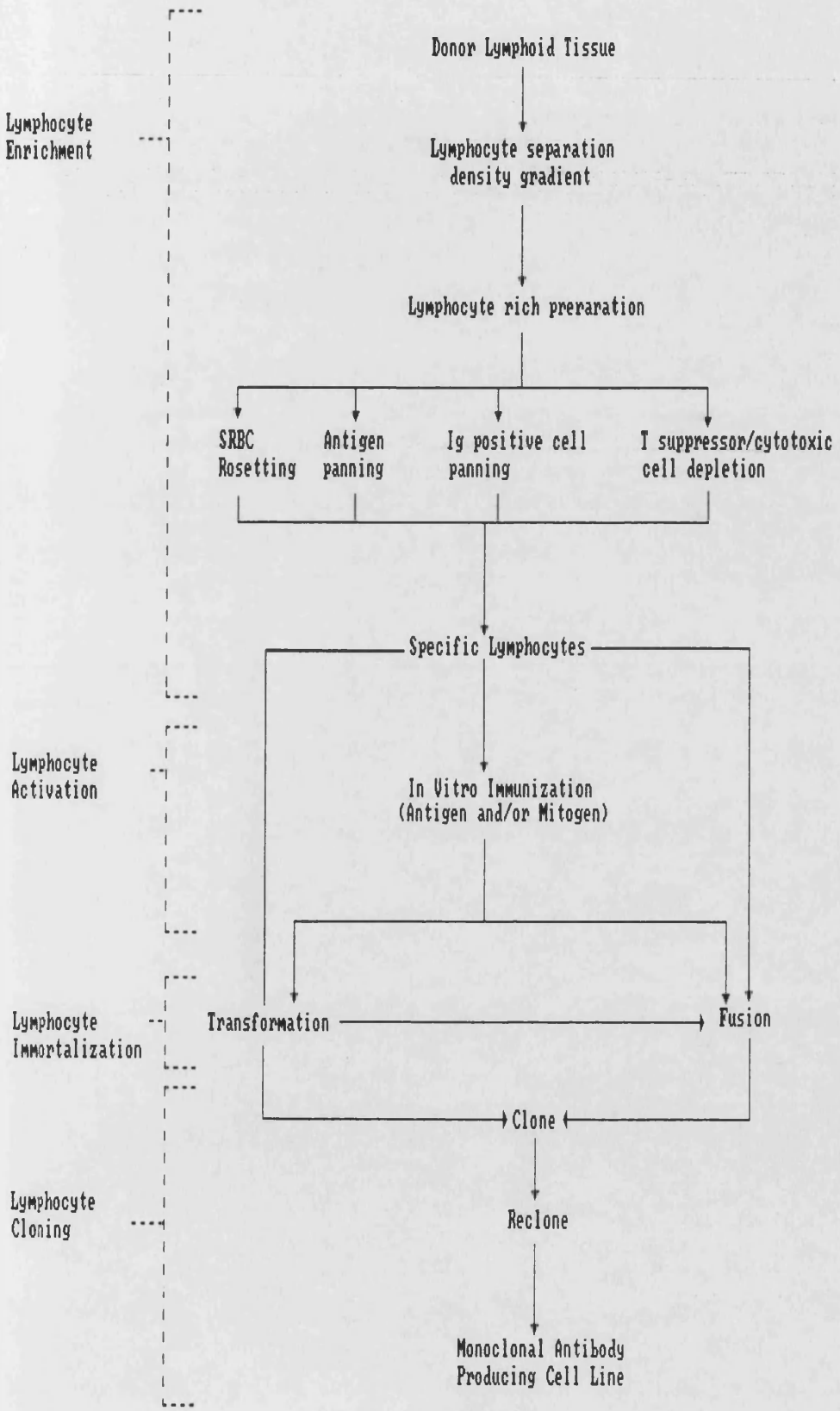
4.2.1 Human Monoclonal Antibodies - Production Strategies

The generation of human mabs has been hindered to some degree by the absence of an appropriate human fusion partner which grows well, produces stable hybrids, does not secrete antibodies of its own, and yet secretes high levels of antibody when fused to an immune lymphocyte (Samoilovich et al,1987). Obtaining sufficient numbers of immune human lymphocytes for fusion is also a problem (James & Bell,1987). It is also believed that lack of knowledge of the ideal state of cell differentiation for successful fusion has also contributed to the slow progress in human mab technology (Schwaber et al,1984).

Nevertheless, human mabs to a wide range of antigens have

Fig.22- Basic Steps For Human Monoclonal Antibody Production

(taken from James & Bell,1987)



been produced (see Fig. 22 for summary of production techniques). The practical techniques can be subdivided into 3 areas, each of which will be considered separately. Before this the source of lymphocytes for fusion will be considered.

4.2.2 Lymphocyte Sources

The successful development of murine mabs has not been limited by the availability of immune lymphocytes, for provided that the antigen is available in sufficient amounts, is non-toxic and immunogenic, then appropriate immunisation procedures can be used to ensure sufficient immune cells for fusion. This, however, is not the case with human mab production, where the range of immunogens which can be injected is limited by ethical and other considerations. Furthermore, the source of immune lymphocytes is usually restricted to peripheral blood (PBL's) which is not an ideal choice. James & Bell (1987) compared the results of numerous workers who produced human mabs to a variety of antigens utilizing immune lymphocytes from different sources. They were able to show that lymphocytes derived from spleens and tonsils provided better results than PBL's. However, spleens and tonsils, are not in general, readily available from immune subjects.

The poor performance of PBL's has been attributed to various

reasons, including, insufficient B cells (Cote & Houghton,1985: Ho et al,1986); insufficient memory cells or antigen specific B cells (Seigneurin et al,1983: Olsson et al,1983: Ho et al,1986); transient appearance of antigen specific B cells in blood following immunisation (Burnett et al,1985); inappropriate state of B cell differentiation (Schwaber et al,1984); low mitotic activity of B cells (Burnett et al,1985); lack of Ia positive macrophages (Teng et al,1985); excess of cytotoxic T cells (Cote & Houghton,1985); presence of suppressor T cells (Cote & Houghton, 1985).

Several techniques have been used to increase the number of specific B cell clones either in or derived from peripheral blood. Where ethics allow, in vivo immunisation is used. Peripheral blood is collected from donors who have been immunised following approved schedules or as a result of disease processes or accidental exposure to immunogens. While most described mabs are directed against viruses or bacteria or autoantigens in patients, others have been deliberately induced (see Table 4). Numbers of specific B cells may also be increased by in vitro immunisation (see Table 5).

In addition to these approaches, various lymphocyte manipulations have been used to increase the numbers of B cells and deplete the numbers of suppressor/cytotoxic T cells in order to provide a more favourable ratio of B cells to helper T cells

Table 4 : Human Monoclonal Antibodies Produced By In Vivo Immunisation Procedures

(adapted from James & Bell 1987)

Immunization Strategy	Antigens	Refs
Natural infection/ inadvertant exposure	Capsular polysaccaride	Gigliotti et al (1984)
	Influenza virus	Hunter et al (1982) Crawford & Callard (1983)
	PPC	Yoshie et al (1980)
	P. aeruginosa	Siadek & Lostrom (1985)
	Tuberculin	Garzelli et al (1986)
	Chlamydia	Rosen et al (1983)
	Cytomegalovirus	Emanuel et al (1984) Redmund et al (1986)
	EBV. Rabies & Rubella	Koizumi et al (1986)
	Hepatitis A	Van Meel et al (1985)
	Hepatitis B	Stricker et al (1985)
	HTLV-1	Matsushita et al (1986)
	P. falciparum	Schmidt-Ullrich (1986) Udomasangpetch et al (1986)
	Blood group A	Raubitschek et al (1985)
	Rhesus D	Bron et al (1984)
	Rhesus G	Foung et al (1986)
	HDNPA	Steinitz et al (1977/79)
Active immunisation	Diptheria toxoid	Gigliotti et al (1984)
	Gram neg. endotoxin	Bogard et al (1985) Teng et al (1985a)
	Influenza virus	Gigliotti et al (1984)
	Pneumococcal polysaccharide	Schwaber et al (1984)

Table 4 (cont)

Immunisation Strategy	Antigens	Refs
Active immunisation (cont)	Tetanus toxoid	Zurawski et al (1978) Kozbor & Roder (1981) Chiorozzi et al (1982) Gigliotti & Insel (1982) Larrick et al (1983) Gigliotti et al (1984) Boyd et al (1984b) Tiebout et al (1984/85) Burnett et al (1985)
	Hepatitis B	Burnett et al (1985)
	Colorectal cancer	Haspel et al (1985a,b)
	Rhesus D	Boylston et al (1980) Crawford et al (1983) Doyle et al (1985) Paire et al (1986) Thompson et al (1986)
	HLA	Hulette et al (1985) Effros et al (1986)
	DNCB	Ollson & Kaplan (1980)
	KLH	Lane et al (1982)
Active disease	M. leprae	Atlaw et al (1985)
	EBV	Koizumi et al (1986)
	Rubella	Croce et al (1980)
	Bladder tumour	Paulie et al (1984)
	Brain glioma	Sikora et al (1982/83)
	Breast tumour	Wunderlich et al (1981) Cote et al (1983/84/85/86) Campbell et al (1986) Strelkauskas et al (1987a)
	Colorectal cancer	Hirohashi et al (1982b) Borup-Christensen et al (1986)

Table 4 (cont)

Immunization Strategy	Antigens	Refs
Active disease (cont)	Gastric tumour	Hirohashi et al (1982b)
	Lung cancer	Hirohashi et al (1982a) Cote et al (1983/84/85/86) Murakami et al (1985)
	Lymphoid system tumours	Abrams et al (1984) Andreason & Olsson (1986) Carroll et al (1986)
	Melanoma	Trie et al (1982) Warenius et al (1983) Watson et al (1983) Kan-Mitchell et al (1986)
	Renal cancer	Cote et al (1983/84/85/86)
	Autoantigens	
	Astrocytes	Simpson et al (1986)
	Cytoskeletal	Cote et al (1986)
	ss/ds DNA	Shoenfield et al (1982) Massicotte et al (1984) Sasaki et al (1984)
	Erythrocytes Platelets	Shoenfield et al (1982)
	Golgi	Cote et al (1986)
	Islet cells	Eisenbarth et al (1982)
	Neurofibrillary plaques/tangles	Simpson et al (1986)
	Nucleus & Nucleolus	Simpson et al (1986) Cote et al (1986) Someya et al (1986)
	Sperm	Kyurchiev et al (1986)

Table 4 (cont)

Immunization	Antigens	Refs
No disease or immunization	Autoantigens	
	Astrocytes Neurofibrillary plaques/tangles Neurons	Simpson et al (1986)
	Cytoskeletal Golgi	Cote et al (1983/86)
	ss/ds DNA Erythrocytes	Winger et al (1983)
	Multiple organs	Garzelli et al (1986)
	Nucleus & Nucleolus	Simpson et al (1986) Cote et al (1986)

Abbreviations:

PPC - Phosphorylcholine
P.aeruginosa - Pseudomonas aeruginosa
EBV - Epstein-Barr virus
HTLV-1 - Human T cell Lymphotrophic Virus 1
P.falciparum - Plasmodium falciparum
HDNPA - 4-hydroxy-3,5-dinitro-phenacetic acid
HLA - Human Leucocyte Antigens
DNCB - 2,4-dinitrochlorobenzene
KLH - Keyhold Limpet Haemocyanin
M.leprae - Mycobacterium leprae
ss/ds DNA - single and double stranded DNA

Table 5 : Human Monoclonal Antibodies Produced By In Vitro Immunization Procedures

(adapted from James & Bell 1987)

Immunogen	Antigen	Refs
Bacterial	Tetanus toxoid	Kozbor & Roder (1984) Ho et al (1985/87) Ichimori et al (1985)
	H.influenzae N.meningitides	Brodeur et al (1987)
	Streptococcus	Wasserman et al (1986)
Viral	Influenza	Crawford & Callard (1983)
	Rubella	Ritts et al (1983) Hilfenhaus et al (1986)
	Hepatitis B	Ichimori et al (1985)
	Herpes simplex	Masuho et al (1986)
	Cytomegalovirus	Matsumoto et al (1986)
Erythrocytes	Sheep	Strike et al (1984)
	Blood group A	Larrick et al (1985)
	Forssman antigen	Nowinski et al (1980)
	Rhesus D	Astaldi et al (1982)
Other	DNP	Teng et al (1985b)
	DNP,KLH & sperm whale myoglobin	Bieber et al (1987)
	PAP	Yamaura et al (1985)
	Bombesin	Ho et al (1985/87)
	klh	Wasserman et al (1986)

Abbreviations:

H.influenzae - Haemophilus influenzae
N.meningitides - Neisseria meningtides
DNP - Dinitrophenol
KLH - Keyhold Limpet Haemocyanin
PAP - Prostatic Acid Phosphatase

for fusion. These include, SRBC rosetting (Teng et al,1985a); separation on nylon wool (Gazzelli et al,1984: Ho et al,1985: Yamoura et al,1985) or Sephadex G-10 columns (Hoffman & Heist, 1985a,b) specific lysis with anti-suppressor T cells mabs (Lagrace et al,1985: Brodeur et al,1987); reconstitution of non-adherent and adherent cells (Ho et al,1985) and polyclonal activation of lymphocytes with PWM, PHA, LPS, Staphylocus aureas Cowan I (B cell stimulant) or cyclosporin A.

4.2.3 Immortalisation Procedures

1) Fusions

In general, antibody producing B cells are fused, using PEG, to an immortal cell line which is sensitive to a selecting agent [usually hypoxanthine-aminopterin-thymidine (HAT)], and which is capable of supporting antibody secretion in culture.

The mechanism by which PEG brings about fusion is not fully understood, but it appears that close contact between the cells being fused is necessary (Knutton & Pasternak,1979). PEG appears to promote the close apposition of cell membranes while additives actually stimulate the fusion process (Wocjcieszym et al,1983). The use of DMSO has also been shown to modify the cell membranes and enhance fusion (Fazekas de StGroth & Scheidegger,1980).

All fusion protocols used can be further subdivided

depending on the nature of the immortal cell line used.

A) Human x Murine Fusions

Production of heterohybridomas by fusion was the first technique used in attempts to produce human mabs (Schwaber & Cohen,1973: Levy & Dilley,1978) and was the direct result of applying murine hybridoma technology to human cells. To date this method has been used with considerable success, with mabs being produced to a wide variety of antigens (see Table 6).

The advantage of this system is the wide availability of non-secreting rodent cell lines, which grow well and support antibody production (see Table 3) eg. X63 (Kearney et al,1979). Unfortunately the main disadvantage of the system is the inherent genetic instability of interspecies hybrids, with preferential loss of human chromosomes, in particular chromosome 2, which carries the kappa light chain locus. Surprisingly, chromosomes 14 and 22 appear to be retained, coding for the human heavy chain locus and the lambda light chain locus respectively (Croce et al,1980: Koskimies et al,1979). However, Thompson et al,(1986) were able to show that genetic instability was no worse than with murine hybrids, and could be minimised by early cloning.

Table 6: Human Monoclonal Antibodies Produced By Fusion Of Lymphocytes To A Murine Cell Line

(adapted from James & Bell 1987)

Antigen	Cell Line	Refs
Tetanus toxoid	X63 x PBL	Giolotti et al (1982)
" "	NS-1 x SP/TON	Ho et al (1985)
" "	NS-1 x PBL	Tiebout et al (1985)
" "	SP1 x PBL	Butler et al (1983)
" "		Kozbor et al (1982)
Bombesin	NS-1 x SP/TON	Ho et al (1985)
Forssman ag	NS-1 x SP	Nowinski et al (1980=
Influenza virus	X63 x PBL	Gigliotti et al (1984)
Keyhold limpet haemocyanin	SP-1 x PBL	Lane et al (1982)
Pseudomonas aeruginosa LPS	P3/X63-AgU1 x PBL/TON	Sawada et al (1985)
Hepatitis B surface ag	NS-1 x PBL	Tiebout et al (1985)
" "	P3/HT x PBL	Burnett et al (1985)
Cytomegalovirus	P3/X63-AgU1 x SP	Matsumoto et al (1986)
Herpes simplex virus	P3/X63-AgU1 x TON	Masuho et al (1986)
Phesus D ag	SP2/0 x PBL	Astaldi et al (1982)
DNA	X63 x PBL	Thompson (1986) Ph.D Thesis

Table 6: (cont)

Colorectal tumour	NS-1 x PBL	Haspel et al 1985a,b)
Mammary carcinoma	NS-1 x All poss	Wunderlich et al (1981) Cote et al (1983;1984; 1985;1986) Schlom et al (1980)
Lung adeno-carcinoma	X63 x LN	Hirohashi et al (1986)
Lymphocytic leukaemia	NS-1 x PBL	Abrams et al (1984)
Melanoma	NS-1 x LN	Kan-Mitchell et al (1987)
Sperm coating	X63 x PBL	Kyurkchiev et al (1986)

Abbreviations:

PBL - peripheral blood lymphocytes
SP - splenic lymphocytes
TON - tonsular lymphocytes
LN - lymph node lymphocytes
ag - antigen
LPS - lipopolysaccharide

B) Human x Human Fusions

The first report came from Ollson & Kaplan (1980) who obtained an anti-2,4-dinitrophenyl mab by fusing splenic lymphocytes with a human myeloma cell line. This report was followed by that of Croce et al,(1980a) who fused PBL's with a human myeloma, to give hybridomas which secreted anti-measles virus mabs. But, since 1980, progress has been slow, largely because of the lack of any really satisfactory human cell line. Many laboratories have developed their own, but, as yet, none has become routinely used. Table 7 lists some of the human cell lines currently available. Of these lines, very few are myelomas and the rest are human lymphoblastoid cell lines (LCL), derived from EBV transformation of human lymphocytes. LCL's are easier to handle in culture than myelomas, but, like myelomas, have low fusion frequencies, support only low mab secretion, and secrete antibody themselves.
No completely satisfactory line is currently available, but a number of useful human mabs have nevertheless been produced by this technique (see Table 8).

C) Human x Human Heterohybridoma Fusions

In order to improve the fusion rates and growth characteristics of the existing LCL's, several groups fused them

Table 7 : Human Cell Lines Available For Fusion

(adapted from James & Bell 1987; Samoilovich et al 1987)

Cell Line	Cell Type	Ig Secreted	Refs
SKO-007	myeloma	IgE	Olsson & Kaplan (1980)
RPMI 8226	myeloma	lambda light chains	Abrams et al (1983)
HFB 1	myeloma	none	Hunter et al (1982)
KMMI	myeloma	IgG	Togawa et al (1982)
RH-L4	lymphoma	none	Olsson et al (1983)
NAT-30	lymphoma	N/K	Murakami et al (1985)
GM 1500-6TG-AL	LCL	IgG2	Croce et al (1980)
GM 1500-6TG-OA	LCL	N/K	Hulette et al (1985)
KR4	LCL	IgG2	Kozbor et al (1982)
GM 467	LCL	IgG2	Sato et al (1972)
GM 4672	LCL	IgG	Croce et al (1980)
ARH-yy	LCL	IgG	Burk et al (1978)
LICR-LON-Hmy2	LCL	IgG1	Edwards et al (1982)
W1-L2	LCL	N/K	Emanuel et al (1984)
H35-1-1	LCL	IgM	Chiorazzi et al (1982)
W1-L2-729 HF2	LCL	IgM	Strike et al (1984)
W1-L2-727	LCL	IgG	Emanuel et al (1984)
UC 729-6	LCL	IgM	Glassy et al (1983)
MC/CAR	LCL	none	Ritts et al (1983)
MC/MNS-2	LCL	IgG1	Ritts et al (1983)

Table 7 : (cont)

LTR 288	LCL	IgM	Larrick et al (1983)
LSM 2-7	LCL	N/K	Schwaber et al (1984)
HS Sultan	LCL	none	Lazarus et al (1982)
GK-5	LCL	kappa light chains	Satoh et al (1983)
HO-323	LCL	N/K	Ohashi et al (1986)
KR-12	h/h hybrid myeloma	IgG2	Kozbor et al (1984)
3 HL	m/h/h hybrid myeloma	IgM	Teng et al (1985a)
HuNS1	LCL	-	Blair et al (1985)

Abbreviations:

LCL - Lymphoblastoid Cell Ine
N/K - Not Known
h/h - human/human
m/h/h - murine/human/human

Table 8 : Human Monoclonal Antibodies Produced By Fusion With A Human Fusion Partner
(adapted from James & Bell 1987)

Cell Line	Lymphocyte Source	Antigen	Refs
HFB-1	SP	Influenza virus	Hunter et al (1982)
LSM2-7	PBL & SP	Pneumococcal polysaccharide	Schwaber et al (1984)
GM4672	PBL	Plasmodium falciparum	Schmidt-Ullrich et al (1986)
	All	Breast tumour	Cote et al (1983)
	PBL	ds/ss DNA Rheumatoid factor	Rauch et al (1986)
	PBL & SP	ds/ss DNA Platelets, Erythrocytes	Shoenfeld et al (1982)
Hmy2	LN	Colorectal tumour	Borup-Christensen et al (1986)
	All	Breast tumour	Cote et al (1986)
	ITL	Glioma	Sikora et al (1982; 1983)
	SP & TON	Streptococcus KLH	Wasserman et al (1986)
HF-2	SP & TON	Streptococcus KLH	Wasserman et al (1986)
	PBL & TON	Sheep Erythrocytes	Strike et al (1984)
UC729-6	All	Breast tumour	Cote et al (1984)
	LN,SP & PBL	Misc tumours	Glassy et al (1985)
SKO-007	All	Breast tumour	Cote et al (1985)
	PBL	Blood group B	Brodin et al (1983)
RH-L4	PBL	Leukaemia	Olsson et al (1984)
FU266	SP	DNCB	Olsson et al (1980)
GM1500	PBL	Measles virus	Croce et al (1980)
KR4	PBL	TT	Kozbor & roder (1984)
HuNS1	PBL	AChR	Blair et al (1985)

Abbreviations:

TON - tonsular lymphocytes
PBL - peripheral blood lymphocytes
SP - splenic lymphocytes
LN - lymph node lymphocytes
TT - Tetanus toxoid
ds/ss DNA - double stranded & single stranded DNA
KLH - Keyhold limpet haemocyanin
DNCB- Dinitro-chlorobenzene
ITL - Intra-tumoural lymphocytes

Table 9 : Human/Murine Heteromyelomas Available For Fusion

(adapted from James & Bell 1987)

Cell Line	Parental Cell Lines		Refs
	Murine	Human	
PSV2.Neo	X63	FU266	Teng et al (1983)
SHM-D33	X63	FU266	Bron et al (1984) Yamaura et al (1985) Carroll et al (1986) Teng et al (1985b)
HM/5	X63	Hmy2	Ichimori et al (1985)
OrgM HH1	X63	normal B cell	Van Meel et al (1985)
SBC/H20	SP2/0	FU266	Carroll et al (1986) Foung et al (1985)
SP2/SP	SP2/0	Spleen cells	Brodeur et al (1987)
SP2/HPT	SP2/0	normal PBL & TON	Brodeur et al (1987)
K6H6/B5	NS-1	B cell lymphoma	Carroll et al (1986)
K6H9/G12	NS-1	B cell lymphoma	Carroll et al (1986)

Abbreviations:

PBL - peripheral blood lymphocytes
TON - tonsular lymphocytes

Table 10 : Human Monoclonal Antibodies Produced Using Heteromyeloma Fusion Partners

(adapted from James & Bell 1987)

Cell Line	Lymphocyte Source	Antigen	Refs
PSV2.Neo	PBL & LCL	2.4-DNP ds/ss DNA J5 endotoxin RhD. TT. rRNA	Teng et al (1983)
SHM-D33	PBL SP LN SP	RhD PAP Lymphoma J5 endotoxin	Bron et al (1984) Yamaura et al (1985) Carroll et al (1986) Teng et al (1985b)
HM-5	PBL	Hepatitis B surface ag, TT	Tchimori et al (1985)
OrgM	PBL	Rubella, Rabies, Hepatitis A & B	Van Meel et al (1985)
SBC/H20	LN PBL & SP	Lymphoma Varicella zoster, blood group A, EBV, early ag, M.leprae	Carrol et al (1986) Foung et al (1985b)
SP2/SP	TON	Influenza virus	Brodeur et al (1987)
K6H6/B5	LN	Lymphoma	Carrol et al (1986)
K6H9/G12	LN	Lymphoma	Carroll et al (1986)

Abbreviations:

PBL - peripheral blood lymphocytes
TON - tonsular lymphocytes
LN - lymph node lymphocytes
SP - splenic lymphocytes
M.leprae - Mycobacterium leprae
EBV - Epstein Barr Virus
TT - Tetanus Toxoid
PAP - Prostatic acid phosphatase
ds/ssDNA - double stranded & single stranded Deoxyribonucleic acid
2,4-DNP - 2,4-dinitrophenol
rRNA - ribosomal Ribonucleic acid

with a murine myeloma (or occasionally a human myeloma) thereby obtaining a heteromyeloma which could then be used as a fusion partner. It has been claimed that these heteromyelomas perform better than their original parent cell lines in fusion experiments, as they tend to be a compromise between the two systems. Several useful cell lines (see Table 9) and human mabs (see Table 10) have been produced by this approach (Teng et al,1983, 1985a: Foung et al,1985a: Kozbor et al,1984).

2) Transformation

Viral cell transformation is potentially an extremely valuable aid to the production of human mabs, but, at the present time, it is limited to the use of viruses known to transform B cells, in particular the Epstein-Barr Virus (EBV) which is the most commonly used, and preferentially infects human B cells (Johdal & Klein,1973). Table 11 lists some of the human mabs produced by this technique.

EBV is obtained by culturing a marmoset cell line B95-8, which secretes live virus into the surrounding culture medium (Miller & Lipman,1973). The virus transforms B cells by binding to their C3D(CR2) complement receptor (Frade et al,1985). However, only a small proportion of the infected cells become transformed, and there is some debate as to the identity

Table 11 : Human Monoclonal Antibodies Produced By EBV Cell Transformation
(adapted from James & Bell 1987)

Antigen	Lymphocyte Source	Refs
Tetanus toxoid	PBL	Zurawski et al (1978) Tiebout et al (1984) Boyd et al (1984a,b) Kozbor et al (1981)
Pneumococcal polysaccharide	TON	Yoshie & Ono (1980)
Chlamydia salpingitis	PBL	Rosen et al (1983)
Herpes Simplex II	PBL	Evans et al (1984)
Herpes Simplex glycoprotein D	BM	Seigneurin et al (1983)
Hepatitis B	PBL	Furuya et al (1982)
Influenza virus	PBL	Crawford & Callard (1983)
Cytomegalovirus	PBL	Emanuel et al (1984) Redmond et al (1986)
EBV	All	Koizumi et al (1986)
Plasmodium falciparum	PBL	Udomsangpetch et al (1986)
RhD	PBL	Boylston et al (1980) Crawford et al (1983) Doyle et al (1985) Melamed et al (1985) Paire et al (1986) Koskimies et al (1979)
	SP & PBL	Goossens et al (1987)
Blood group A	SP	Raubitschek et al (1985) Steinitz et al (1979)
	SP & PBL	Goossens et al (1987)
Melanoma	ITL	Watson et al (1983)
Transitional cell carcinoma	PBL	Paulie et al (1985)
Lung tumour	LN	Hirobashi et al (1982a)
Gastric tumour	LN	Hirobashi et al (1982b)

Table 11 : (cont)

Antigen	Lymphocyte Source	Refs
DNA	PBL	Sasaki et al (1984) Sasaki et al (1985)
DNA, Sperm, Erythrocytes	PBL	Winger et al (1983)
Thyroid ags	PBL	Garzelli et al (1984)
Neurofibillary plaques & tangles	PBL	Simpson et al (1986)
NNP	PBL	Steinitz et al (1979)
Rheumatoid factor	PBL	Steinitz et al (1982) Steinitz et al (1980)
Human IgG/M/A	PBL	Brown et al (1982) Steinitz et al (1980)
Trinitrophenol	PBL	Kozbor et al (1979)
T cell leukaemia	LN	Matsushita et al (1986)
AChR	TH	Kamo et al (1982)

Abbreviations:

PBL - peripheral blood lymphocytes
TON - tonsular lymphocytes
SP - splenic lymphocytes
LN - lymph node lymphocytes
BM - bone marrow cells
NNP - 4-hydroxy-3,5-dinitrophenacetic acid
ITL - intra tumoural lymphocytes
EBV - Epstein Barr virus
RhD - Rhesus D antigen
TH - Thymic lymphocytes

of this specific population of B cells (Aman et al,1985: Chan et al,1986).

The advantage of this system is the ability to immortalise a large proportion of B cells. However, in a mixed lymphocyte population, as found with PBL's, the presence of viral coat proteins on the cell surface of infected B cells does result in the sensitization of T cells with subsequent formation of cytotoxic T cells, which will kill the infected B cells (Tsoukas et al,1981). It is therefore necessary either to preselect the B cells by removal of the T cells prior to transformation, or to incorporate PHA or cyclosporine A into the system, which polyclonally activates all the T cells before they become sensitized (Bird et al,1981).

Apart from the above disadvantage, there are others, not least of which is the handling of EBV. This virus is a herpes type virus, first isolated by Epstein et al,(1964), and is known to be involved in the development of Burkitts Lymphoma in parts of Africa, and nasopharyngeal carcinoma in Southern China (Keiff et al,1984). In the majority of populations of both developed and underdeveloped countries, it is usually found as a subclinical infection known as infectious mononucleosis (glandular fever) (Henle & Henle,1966, 1969). As can be seen, precautions must be taken when handling the virus.

Coupled with these problems are those associated with the

transformed cell lines themselves. It is not uncommon for the cell lines to grow well for 1-2 months, before antibody titre suddenly declines. This titre, even before decline, tends to be only a fraction of that seen when using hybridomas (Boylston et al,1980: Melamed et al,1985). Although early cloning has, in some cases, rescued antibody secretion, many cell lines have been lost because of the extremely poor cloning efficiency of LCL's (Zurawski et al,1978: Kozbor & Roder,1981, 1983).

3) Transformation Followed By Back Fusion (EBV/Hybridoma Technique)

This technique combines those of fusion and transformation. In essence it involves the EBV transformation of B cells from a donor, followed by either vigorous cloning to obtain a LCL which is then fused to a fusion partner, or direct fusion of the transformed B cells following a period of growth and partial cloning (this has the effect of expanding the B cell population to provide sufficient numbers of cells for fusion).

Kozbor et al,(1982) first successfully combined the two techniques. It has been reported that transformation prior to fusion actually increases the fusion frequencies (Kozbor & Roder,1984). This has been explained by Burnett et al,(1985), who reasoned that EBV transformation activates B cells, allowing

them to fuse more efficiently.

The hybrids produced by this method have the same instability problems as any other hybrid, but this can be overcome by early cloning. It has also been shown to increase antibody production over the original LCL, and in some cases, actually rescue antibody production (Kozbor et al,1982: Foung et al,1984: Levy & Dilley,1978).

Disadvantages of this system include the requirement to carry out two immortalization steps, with an increased risk of contaminantion and infection. Secondly, as the procedure involves the fusion of two immortal cell lines, then a second selection step is required to select non-fused LCL's, so ouabain is usually incorporated to kill non-fused LCL's.

As the original fusion technique was subdivided according to the nature of the fusion partner (Introduction, section 4.2.3.(i)), then so can the EBV/hybridoma method.

A) LCL x Murine Fusions

This was the obvious choice, due to the ready availability and ease of handling of non-secretor murine myeloma cell lines (see Table 3). However, as with the human/murine fusions, interspecies hybrids are inherently unstable (Introduction, section 4.2.3.(i)A), even though some groups state they can be stabilised through early cloning, and are easier and safer to handle than human/human fusions (Cote et al,1983: Thompson et al,1986).

A further advantage of this system, is the natural resistance of murine cells to ouabain, 10,000 times more resistant than human cells. As a consequence of this, the double selection procedure is very effective. (see Table 12 which lists some of the mabs raised by this technique).

B) LCL x Human Fusions

Due to the reported instability of interspecies hybrids, several groups fused their antibody secreting LCL to a human partner (Table 7 lists some available partners).

Kozber et al,(1982), designed their own human fusion partner, KR4, which is ouabain resistant, and derived from a 6-thioguanine resistant LCL (GM1500-6TG-A-11). But unfortunately KR4, like its parent secretes IgG. However, they were able to obtain IgM anti-TT mabs. Yet, again, the main disadvantage of this technique is the non-availability of a fusion partner with the required characteristics (Introduction, section 4.2.3 (i)B). (See Table 12 which lists some of the mabs raised by this method)

C) LCL x Heterohybridoma Fusions

Heterohybridomas represent a compromise between human and murine fusion partners, and have been used successfully as fusion partners in this technique (see Table 12 which lists some of the mabs raised by this method). This procedure is not widely used even though it may prove very useful.

Table 12 : Human Monoclonal Antibodies Produced By The EBV/Hybridoma Technique

(adapted from James & Bell 1987)

Technique	Antigen	Fusion Lines	Refs
LCL x Human	TT	KR4	Kozbor et al (1982)
	TT	KR-12	Kozbor et al (1984)
	P.aeruginosa	LTR228	Siadek & Lostrom (1985)
	M.leprae	KR4	Atlaw et al (1985)
	CMV	W1-L2	Emanuel et al (1984)
	Rubella, Rabies, Hepatitis B	OrgMHH1	Van Meel et al (1985)
	HLA	GM 1500	Hulette et al (1985)
	Lung carcinoma	KR4	Cole et al (1984)
	Breast carcinoma	KR4	Campbell et al (1985)
	Rheumatoid factor	KR4	Haskard et al (1984)
	Rhesus D	KR4	Goossens et al (1987)
LCL x Murine	Exotoxin Blood group A	SP2/0	Larrick et al (1985)
	Rhesus D	X63	Thompson et al (1986) Goossens et al (1987)
	PAP	SP2/0	Yamaura et al (1985)

Table 12 : (cont)

Technique	Antigen	Fusion Lines	Refs
LCL x Hetero-myelomas	P.aeruginosa	F3B6	Siadek & Lostrom (1985)
	Gram negative	SHM-D33	Teng et al (1985a)
	Rhesus D	SHM-D33	Brom et al (1984)
	Rhesus G	SBC-H20	Foung et al (1985)
	Blood group A	SBC-H20	Foung et al (1985a)
	PAP	SHM-D33	Yamaura et al (1985)

Abbreviations:

P.aeruginosa - Pseudomonas aeruginosa
M.leprae - Mycobacterium leprae
TT - Tetanus toxoid
CMV - Cytomegalovirus
PAP - Prostatic acid phosphatase
HLA - Human leucocyte antigens
LCL - Lymphoblastoid cell line

As can be seen all these methods have had some success, some more than others, but an essential ingredient to all of them is the incorporation of feeder cells or conditioned media, when cloning or expanding cells at low densities, in order to try and improve growth characteristics (James & Bell,1987).

The feeder cells generally used are of murine origin, either peritoneal macrophages (Fazekas de StGroth & Scheidegger, 1980), splenocytes (Goding,1986) or thymocytes (Oi & Herzenberg, 1980). They all provide factors which enhance the growth of hybrids and LCL's, but their use does have certain disadvantages, namely the possibility of infections, competition for metabolites and increased accumulation of waste products which may be inhibitory (Samoilovich et al,1987). An alternative to feeder cells, is the use of conditioned media which has been shown to support the growth of cell lines and hybridomas (Nordan & Potter, 1986: Rathjen & Greczy,1986: Van Snick et al,1986).

PROJECT AIMS

Polyclonal myasthenic sera and murine monoclonal antibodies have been invaluable tools in delineating the autoimmune response and in studying the structure of the nAChR in relation to the autoimmune disease, Myasthenia gravis. However, future understanding of specific antigenic sites on the human NMJ nAChR, which are active in the disease state, coupled with prospective new therapies, have led various research groups into the field of human monoclonal antibodies.

The development of human anti-AChR monoclonal antibodies from myasthenic patients will allow characterisation of the active antigenic sites on the nAChR and the origin of the autoimmune response in Myasthenia gravis, and may lead to the use of human anti-idiotypic monoclonal antibodies or manipulation of the immune system regulatory mechanisms, as possible new therapies for patients.

Thus, this study, is the first attempt by this group to develop human monoclonal antibodies directed against human nAChR from patients suffering from Myasthenia gravis. In addition to this aim, work was also undertaken to develop a suitable screening assay for the detection of such antibodies in culture supernatants.

MATERIALS

1) Sources of AChR

Torpedo marmorata electric organs were purchased from Institut de Biologie Marine, Arcachon, France and stored at -80°C

Bovine fetal calves were obtained fresh from a local slaughter house. Muscle from the fore and hind limbs was removed and frozen in liquid nitrogen before being stored at -80°C for up to 3 months.

Adult human muscle was supplied by local hospitals and was obtained by dissecting calf muscle from lower limb amputations, where the amputation was carried out as a result of severe vascular disorders. The muscle so obtained, free from fat, tendons and skin, was frozen immediately in liquid nitrogen and stored at -80°C for up to 2 months.

2) Immunoreagents & ELISA Reagents

Myasthenic blood samples were obtained from local hospitals, in particular from the Bristol Eye Hospital, under a collaboration with consultant eye specialist Dr Campbell.

Normal blood samples were obtained from volunteers within the Biochemistry Department.

All blood samples were collected with 10IU heparin per ml of blood, and the myasthenic blood was collected in sterile evacuated tubes to minimise contaminations.

Goat anti-(human IgG) and anti-(mouseIgG) antiseras were gifts from colleagues, as were murine monoclonal anti-neurofilament protein antibodies and murine monoclonal anti-AChR antibodies (C7, C11, B11, E8).

Normal mouse and goat antisera were obtained from normal animals kept at the University of Bath animal house.

Freund's complete and incomplete adjuvants were from Miles Laborataries, Stoke Poges, Slough, UK.

Enzyme conjugated second antibodies and substrates for ELISA studies were obtained from Sigma Chemical Co., Poole, Dorset, UK, as were avidin and streptavidin HRP conjugates, and biotin-N-hydroxy-succinimide in dimethylformamide.

ELISA microtitre plates were obtained from NUNC, Uxbridge, UK.

3) **Tissue Culture Reagents**

RPMI-1640, fetal calf serum, antibiotics, selection media for mab production and Ficoll-Hypaque seperation medium were obtained from Flow Laboratories, Irvine, UK.

Tissue culture flasks, plates, wells and other disposable apparatus was obtained from NUNC, Uxbridge, UK.

Mouse myeloma cell line, X63 and EBV producing marmoset cell line, B95-8, were both gifts from Dr A Jehanli.

Ouabain and PEG were obtained from Sigma Co., Poole, Dorset, UK.

4) **Radiochemicals**

All radiochemicals were obtained from Radiochemical Centre, Amersham International, Amersham, Bucks, UK. This included $Na^{125}I$ and [^{3}H]-biotin-N-hydroxy-succinimide.

5) **Ligands**

α-BGT from Bungarus multicinctus was obtained from Boehringer Corp., Mannheim, W. Germany, as a lyphilised powder.

Benzoquinonium chloride was a gift from Stirling Winthrop Inc., Rensselaer, N.Y., U.S.A.

Decamethonium chloride and nicotine were gifts from colleagues.

6) **Chemicals**

All standard laboratory chemicals were obtained from Sigma Co., Poole, Dorset, UK. BDH Chemicals, Poole, Dorset, UK, or Aldrich Chemical Co. Ltd., Gillingham, UK.

Gel filtration/ion exchange equipment was supplied by Pharmacia Ltd., Hounslow, UK.

Ion exchange resins, DEAE cellulose filters, GFB & GFC glass fibre filters were all obtained from Whatman Laboratories Ltd., Maidstone, Kent.

Polyethyleneimine was obtained from Sigma Co., Poole, Dorset, UK. As was fish skin gruatin.

7) **Instrumentation**

[^{125}I] was counted using an LKB Ultragamma counter.

[^{3}H] was counted using a Packard Tri-carb scintillation counter (model 3255).

All ELISA's were read at 450nm using a Dynatech mini-Scan II EIA reader.

METHODS

1. GENERAL PROCEDURES

1.1 Iodination Of Alpha-BGT

αBGT was labelled to a high specific activity with ^{125}I by the Chloramine-T method (Hunter, 1967) as modified by Urbaniak et al,(1973).

Carrier free Na ^{125}I (100mCi/ml, 20μl)in dilute sodium hydroxide was added to αBGT (2.5nmols) in 50mM potassium phosphate buffer, pH 7.5 (20μl). The reaction was started by the addition of Chloramine-T (10μl 5mg/ml) in 50mM potassium phosphate buffer, pH 7.5, and the mixture was stirred for 1 min at room temperature. The reaction was then terminated by the addition of sodium metabisulphite (750μl, 0.16mg/ml) in 50mM potassium phosphate buffer, pH 7.5, followed by the addition of carrier potassium iodide (200μl,10mg/ml) in 50mM potassium phosphate buffer, pH 7.5.

Labelled αBGT was then separated from unreacted ^{125}I by passage through a Sephadex G25 column (25 x 1cm) which had been previously equilibrated with 10mM potassium phosphate buffer, pH 7.5, containing 1% (w/v) BSA. The^{125}IαBGT was then eluted by using the same buffer.

Fractions (1ml) were collected from the column, and aliquots (5μl) from each fraction were counted for radioactivity.

The peak fractions, containing ^{125}I-αBGT were pooled and stored for no longer than 3 weeks at 4°C. The specific radioactivity of the ^{125}I-αBGT was calculated assuming 100% recovery of the protein.

1.2 Biological Activity Of ^{125}I-αBGT

The biological activity of radiolabelled αBGT was determined by measuring the proportion of ^{125}I-αBGT bound by a large molar excess of purified Torpedo AChR by using a DEAE filtration assay.

Triplicate samples of Torpedo AChR (20 pmol, 100μl) were incubated with radiolabelled αBGT (0.2 pmol, 50μl) for 90 mins at room temperature in the presence and absence of 2.5mM (final concentration) Benzoquinonium chloride.

Bound and free ^{125}I-αBGT were then separated by ion-exchange chromatography on DEAE cellulose filters, by filtering the reaction mixture through two DE81 cellulose filters in a millipore scintered glass filtration system. The filters before being counted, were washed with assay buffer (3 x 1ml). The biological activity of the radiolabelled αBGT was then expressed as a percentage of the total radioactivity added.

The buffer used throughout this procedure was the toxin binding assay buffer which consisted of 10mM potassium phosphate buffer, pH 7.4, 0.1% (w/v) BSA, and 1% (v/v) Triton X-100.

$$\text{Biological Activity} = \frac{^{125}\text{I Bound Specifically}}{\text{Total } ^{125}\text{I added}} \times 100\%$$

1.3 Preparation Of AChR Extracts From Various Sources

Crude AChR extracts were prepared from Torpedo marmorata electric organ; foetal calf muscle and human skeletal muscle. The procedure used was essentially the same for all tissues and was based on the methods described by Wonnacott et al,(1980): Sinigaglia & Gotti,(1983): Stephenson et al,(1981) and Turnbull et al,(1985). A schematic representation of the basic method is shown in Fig. 23.

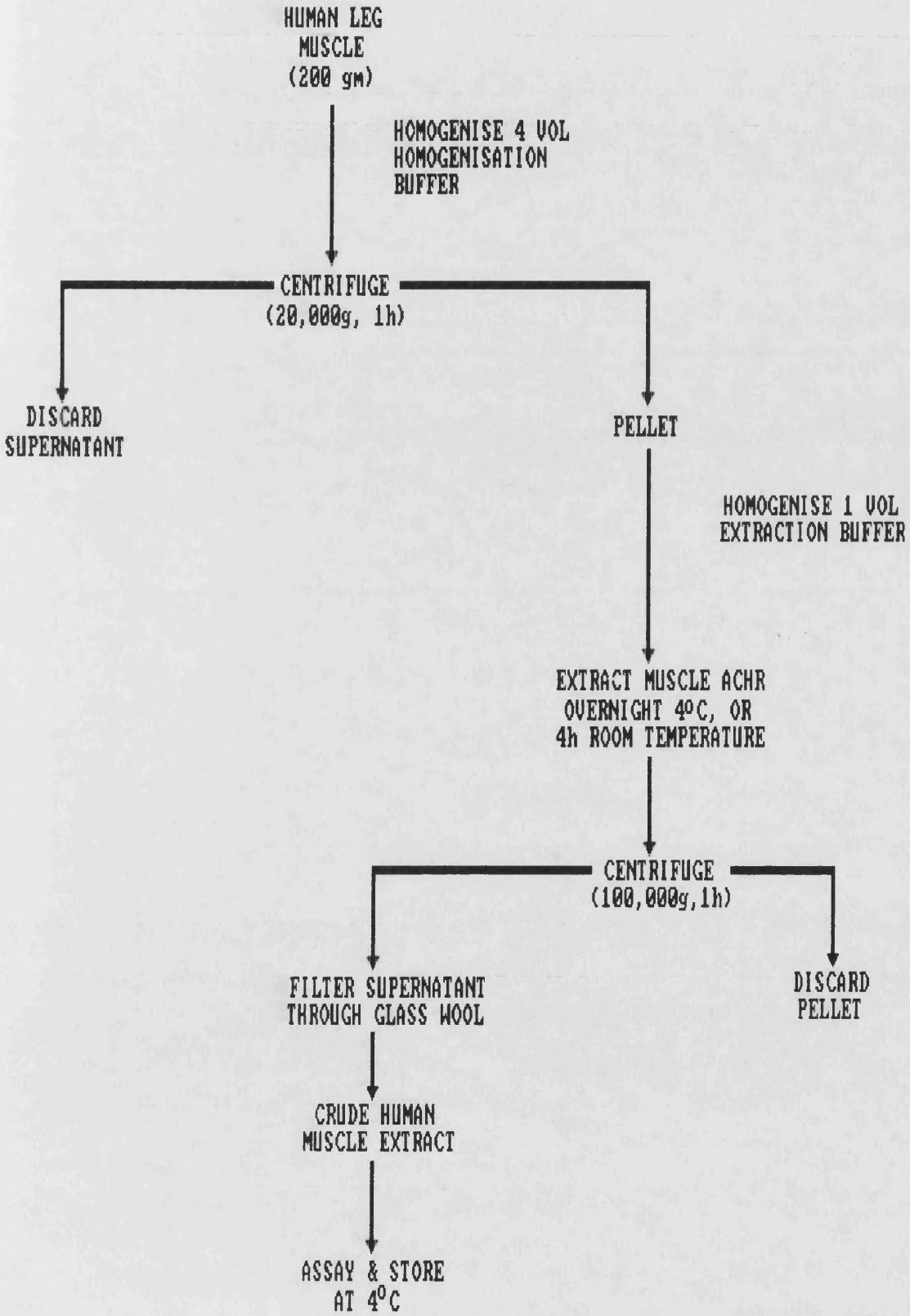
Frozen tissue (200-300g) was coarsely chopped, and homogenised in a Waring blender at full speed for 2 min in 10mM potassium phosphate buffer, pH 7.4 (4 vol), containing various protease inhibitors (1mM EDTA, 0.1mM PMSF, 0.1mM Benzethonium chloride and 500 g/ml Bacitracin). The resultant homogenate was centrifuged (10,000g, 1h, 4°C) and the supernatant was discarded. The pellet was then homogenised as before, in one volume of the same buffer, but this time containing 2% (w/v) Triton X-100. Following homogenisation the mixture was extracted by paddle stirring for 2-4h, over ice, at 4°C. The resulting extract was then centrifuged (100,000g, 1h, 4°C), and the supernatant (crude extract) filtered through glass wool before assaying for AChR activity (Methods, section 1.5).

1.3.1 Variation Of Procedures Depending On Tissue Type

i) Torpedo marmorata Electric Organ

Electric organs were readily available, having been freshly

Fig.23 - Diagramatic Summary Of nAChR Extraction From Human Leg Muscle



dissected and frozen immediately. The tissue could be stored at -80°C for periods of up to a year without appreciable loss in receptor yield. The AChR appears to be relatively resistant to intrinsic proteolytic degradation during purification procedures, and so tissue solubilisation and AChR purification, by affinity chromatography, were carried out at 23°C.

ii) Foetal Calf Muscle

Foetal calves are inexpensive and easily obtainable from local slaughterhouses. Muscle was removed by dissection from freshly slaughtered calves, frozen immediately and stored at -80°C for up to 3 months. Foetal calf AChR is known to be susceptible to proteolytic degradation (Einarson et al,(1982). For this reason, the preparation of the crude extract was completed within as short a time as possible, and all steps were carried out on ice or at 4°C.

iii) Human Skeletal Muscle

Adult human leg muscle was obtained from patients undergoing amputations necessitated by either vascular abnormalities, or diabetic gangrene. The muscle (normally gastronemius) was coarsely dissected and frozen immediately in liquid nitrogen. It was then stored for periods of up to 6 months at -80°C. Due to the fibrous nature of human skeletal muscle, and the relative stability of the adult AChR (Turnbull et al,(1985), solubilisation times were increased.

1.4 Purification Of Torpedo marmorata AChR by Affinity Chromatography

The crude AChR extract obtained from the electric organs of Torpedo marmorata, was processed further to obtain a pure preparation of Torpedo AChR. This procedure involved separating the AChR from the other constituents of the crude extract by the use of an affinity column. The affinity resin consisted of Sepharose 4B beads that had been activated by cyanogen bromide (March et al, 1974), to which αBGT purified from Naja naja siamensis, had been coupled according to the method of Lindstrom et al,(1981). The resin was stored at 4°C, and has been shown to remain active for over one year, without appreciable loss of efficiency (Nickless, PhD Thesis, 1985).

A crude preparation of Torpedo AChR (Methods, section 1.3) was filtered through glass wool and stirred for 2-4 h at room temperature with the Sepharose 4B resin (25ml packed volume). The affinity beads were then filter-washed with 10mM potassium phosphate buffer, pH 7.4, containing 0.1% (v/v) Triton X-100, protease inhibitors, and 0.5M sodium chloride (500ml), and then with the same buffer without sodium chloride (500ml).

Following washing, the affinity beads were packed into a column (2cm x 8cm) and the AChR was eluted from the beads, by passing 25mM benzoquinonium chloride (BZQ) in 10mM potassium phosphate buffer, pH 7.4, containing 0.1% (v/v) Triton X-100,

and protease inhibitors, through the column, and then directly onto a DE 52 ion exchange column (1cm x 5cm). The BZQ was recirculated through the two columns overnight at 4°C, following which the DE 52 column was washed free of BZQ using 10mM potassium phosphate buffer, pH 7.4, containing 0.1% (v/v) Triton X-100, and proteae inhibitors. The AChR was then eluted off the DE 52 column by using 0.5M sodium chloride in 10mM potassium phosphate buffer, pH 7.4, containing 0.1% (v/v) Triton X-100 and protease inhibitors. 1-2 ml fractions were usually collected, and the fractions were assayed for [^{125}I]-αBGT binding activity (Methods, section 1.5). The peak fractions containing the AChR were pooled and assayed again for [^{125}I] -αBGT binding activity (Methods, section 1.5). The purified AChR preparation was then usually dialysed to remove the sodium chloride, against 10mM potassium phosphate buffer, pH 7.4, containing 0.1% (v/v) Triton X-100, and protease inhibitors, and the purified preparation stored at 4°C. The affinity beads were regenerated after use by washing, firstly, with 10mM potassium phosphate buffer, pH 7.4, containing 0.1% (v/v) Triton X-100, and 0.5M sodium chloride (500ml) followed by the same buffer without sodium chloride (500ml). The beads were then stored in 10mM potassium phosphate buffer, pH 7.4 containing 0.1% (v/v) Triton X-100, and 0.02% (w/v) sodium azide as an anti-bacterial agent.

1.5 Assay Of AChR Activity In Crude And Purified Extracts

The assays involved the addition of an excess of [^{125}I]-αBGT to a preparation containing AChR. Non-specific binding was measured by carrying out parallel incubations in the presence of a large excess of the competitive antagonist benzoquinonium chloride (BZQ).

1.5.1 Ammonium Sulphate Precipitation Assay

The AChR content of crude receptor preparations was measured by using a method adapted from that of Meunier et al, (1972).

Triplicate samples of serially diluted receptor extracts (100μl) were incubated with an excess of [^{125}I]-αBGT (2.5 - 5 nM final concentration) diluted in 10mM potassium phosphate buffer, pH 7.4, containing 2% (v/v) Triton X-100, for 45 min at room temperature, before the addition of saturated ammonium sulphate solution (133μl) to give a final concentration of 40% (v/v). The mixtures were then incubated overnight at 4°C, and the resulting precipitates were collected on Whatman GF/C glass fibre filters. The filters were washed with 40% (v/v) saturated ammonium sulphate (3 x 1 ml) by vacuum filtration on a Millipore filter unit, and counted for radioactivity.

Specific binding of [^{125}I]-αBGT was blocked in parallel incubations performed as above, but containing, additionally, 2.5mM BZQ (50μl, 10mM). A value for the specifically bound

radioactivity in the sample was obtained by subtraction. One picomole of AChR was defined as the amount of receptor that binds one picamole of [^{125}I]-αBGT under saturating conditions. Thus:

$$[\text{AChR}]_{(\text{pmol/ml})} = \frac{\text{cpm (sample)} - \text{cpm(+BZQ)}}{\text{cpm (total)}} \times \text{pmol toxin added} \times \text{dilution of sample} \times 10$$

1.5.2 Polyethylenimine (PEI) Filtration Assay

In this assay, triplicate samples of serially diluted crude receptor extracts (100μl) were incubated with an excess of [^{125}I]-αBGT (Methods, section 1.5.1) diluted in 10mM potassium phosphate buffer, pH 7.4, containing 2% (v/v) Triton X-100, for 45 min at room temperature before the addition of PBS (1ml). The samples were then filtered through a GF/B glass fibre filter, which had previously been soaked for either 3h at room temperature or overnight at 4°C, in PBS containing 0.33% (v/v) polyethylenimine. The filters were washed (2 x 1ml) with PBS under vacuum on a Millipore filter unit, and allowed to dry before being counted for radioactivity.

Specific binding of [^{125}I]-αBGT was blocked by carrying out parallel incubations, performed as above, but containing 2.5mM BZQ (50μl, 10mM). The specifically bound radioactivity in the sample was then calculated as before.

1.5.3 DEAE-Cellulose Filtration Method For Purified AChR

The content of AChR in the purified extract was measured by

using a DEAE-cellulose filtration assay similar to that described by Schmidt and Raftery, (1973).

Triplicate dilutions of receptor samples (100μl), in 10mM potassium phosphate buffer, pH 7.4, containing 1% (v/v) Triton X-100 and 0.1% (w/v) BSA, were incubated with [^{125}I]-αBGT (1/100 dilution of stock solution, equivalent to 1.0-2.5 nM, 50μl) for 90 min at room temperature. The samples were then filtered through 2xDE-81 cellulose filter discs, pre-moistened with the potassium phosphate buffer. The discs were washed (3 x 1ml) by vacuum filtration of a Millipore filter unit, using the potassium phosphate buffer. Following washing, the discs were then counted for radioactivity. Non-specific binding was measured in parallel incubations performed as above, but containing in addition 2.5mM (final concentration) BZQ (50μl, 10mM). Determination of the specifically bound [^{125}I]-αBGT gives a value for the AChR content in the purified extract in pmol/ml as described previously.

1.6 Assay Of Anti-AChR Antibodies In Myasthenic Sera

A conventional radioimmuno-assay (RIA) procedure, similar to that described by Lindstrom et al,(1981), was used to detect anti-AChR antibodies in Myasthenic sera. The procedure involved the immunoprecipitation of the ^{125}I- α BGT/AChR-antibody complexes by using a second anti-globulin antiserum.

AChR (0.5nM) was incubated (45min. 23°C) with an excess of

^{125}I-αBGT (5.0nM) in the presence and absence of BZQ (2.5mM). The toxin-AChR complex (100μl) was incubated (overnight, 4°C) with the antiserum (5μl), appropriately diluted in normal human serum. The complex was then precipitated by the addition of the second antibody, goat anti-human IgG antiserum, and incubated for 2h at room temperature, or overnight at 4°C. The precipitate so formed, was pelleted by centrifugation (15000rpm, 10 min, 4°C), washed (3 x 1 ml) with RIA buffer (10mM potassium phosphate buffer, pH 7.4; 0.15M sodium chloride; 1%(v/v) Triton X-100; and 0.01%(w/v) sodium azide), and then counted for radioactivity. All tests were carried out in triplicate at differing dilutions, and subtraction of the counts obtained in the presence of BZQ from the counts obtained in its absence, gave specifically bound radioactivity in the test sample, with the antibody titre being expressed as moles of specific α-BGT binding sites precipitated per litre of serum.

2. PROCEDURES RELATING TO CELL CULTURE

2.1 Maintenance Of EBV Marmoset Cell Lines And Production Of EB Virus

Epstein-Barr virus (EBV) containing culture supernatant was prepared from a B958 Marmoset cell line, which actively secretes the live virus into the culture medium (Miller et al,1973). The Marmoset cells are normally stored frozen in liquid nitrogen. On

being thawed for use, the cells (10 x 10^6 cells/ml, 2 ml) were transferred to complete tissue culture medium (20ml), in order to dilute out the freezing mixture, and then washed by centrifugation (2300 rpm, 10 min) using culture medium (20mls). The cells were then resuspended in a small volume of tissue culture medium and a viability count was carried out. They were then seeded at 1 x 10^5 viable cells/ml in complete tissue culture medium in tissue culture flasks (25ml), and maintained in an atmosphere of 95% relative humidity containing 3% carbon dioxide at 37°C. When the culture medium was depleted (approx. 10 days), as indicated by a colour change in the medium from pink to yellow, (added pH indicator), the supernatant was collected by centrifugation (2500 rpm, 10 min) in an MSE bench centrifuge, and either filtered by positive pressure through a 0.45μm Millipore membrane and stored in 20ml aliquots at -20°C, or stored immediately at -20°C in 100ml aliquots and filtered just prior to use. The cells at this stage could either be frozen down and stored, resuspended to produce more EBV containing supernatant, or discarded totally. If they were being stored, the pelleted cells were suspended in freezing media (RPMI containing 2mM Glutamine, 50%(v/v) FCS, and 10%(v/v) DMSO) at a cell density of 10 x 10^6 cells/ml. The cells were then transferred to freezing ampoules and placed in a slow freezer, suspended in the vapour phase of liquid nitrogen overnight, before transferring the ampoules to liquid nitrogen containers.

2.2 Separation Of Peripheral Blood Lymphocytes

Peripheral blood lymphocytes (PBL's) were separated from whole blood, using a modification of the procedures described by Boyum (1968).

Blood samples (15-20ml) were obtained from patients suffering from Myasthenia gravis. The blood samples were usually collected and processed the same day with all manipulations being carried out under strictly sterile conditions.

The blood samples were first diluted (1:1) with PBS containing heparin (10 units/ml), and mixed thoroughly. Aliquots of the diluted blood (7ml) were then layered carefully onto the top of Ficol lymphocyte preparation medium (3ml) in sterile siliconised polycarbonate centrifuge tubes (12ml). The Ficol/ blood gradients were then centrifuged (2000 rpm, 25 min) in an MSE bench centrifuge.

The separated lymphocytes, located at the Ficol/serum interface (see Fig. 24, which gives a diagramatic summary of the separation of lymphocytes and their transformation) were removed by using a sterile disposable plastic pipette, with circular/ criss-cross movements, being careful not to disturb the pelleted red cells, and placed in a sterile tube. The cells were then washed twice with fresh sterile PBS (20ml), by centrifugation (2300 rpm, 10 mins) in a bench centrifuge, in order to remove any traces of the Ficol. After the final wash, the PBS was decanted

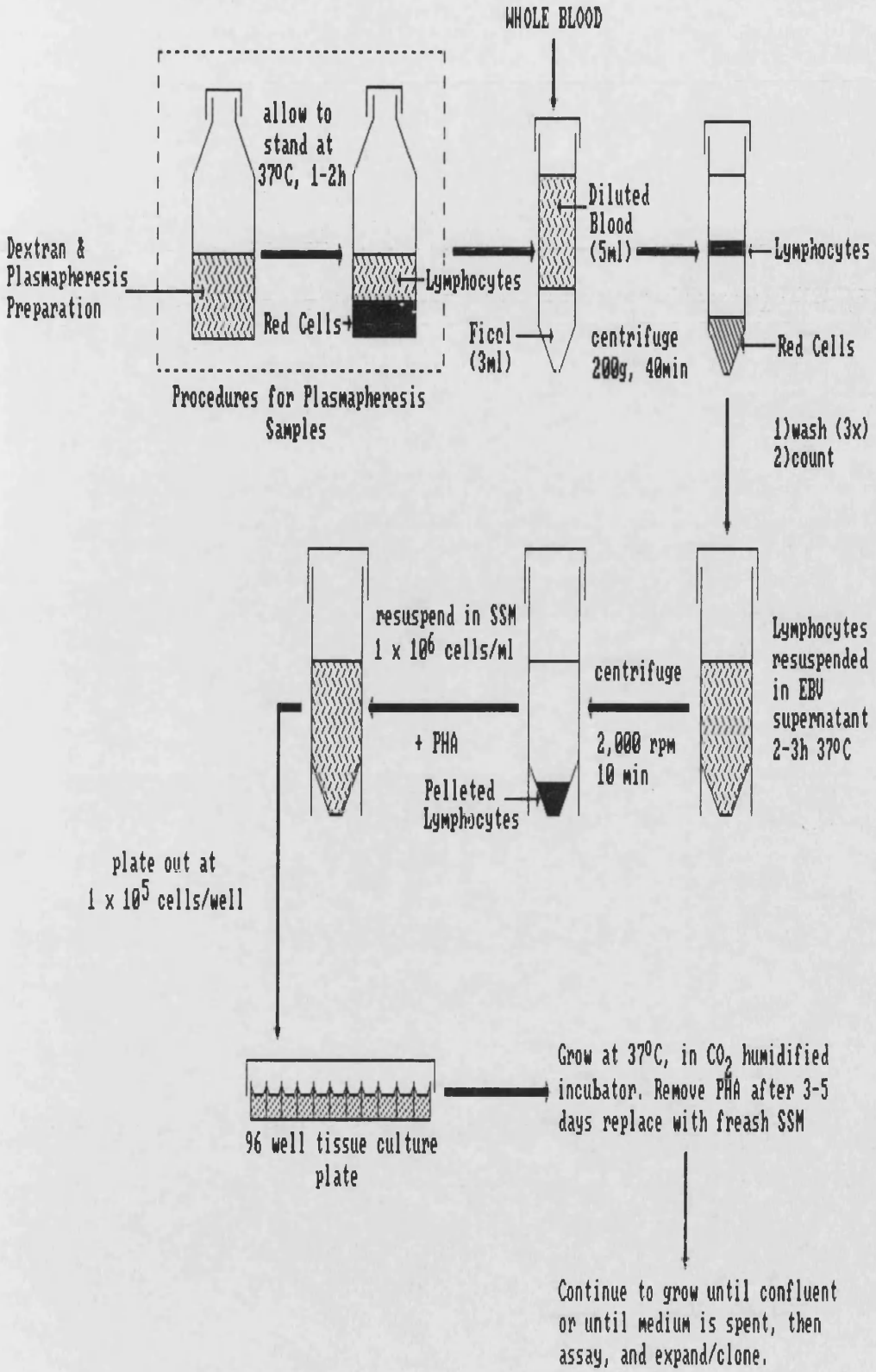


Fig.24 - Diagramatic Summary of Procedures Employed In Lymphocyte Separation & Transformation

off, and the cells resuspended in PBS (20ml), by gentle agitation using a sterile pipette. A small sample of the suspension was taken for counting (Methods, section 2.6), prior to transformation.

Occasionally, a leucocyte enriched plasma fraction (approx. 50-100ml) was available from plasmapheresis. In this case a crude cell separation was initially carried out prior to Ficol fractionation.

To the lymphocyte rich plasma (75ml), a solution of sterile Dextran in PBS was added [25ml, 2% sol.(w/v)] in sterile tissue culture bottle. Following thorough mixing, the cell suspension was left to stand for 1-2 h at 37°C, until most of the contaminating red cells had settled, leaving a turbid white supernatant containing lymphocytes. This layer was removed by pipette and washed twice with PBS by centrifugation (2300 rpm, 10 min) in order to remove the Dextran. The pelleted cells were then suspended in PBS and applied to Fical gradients as described above.

2.3 Transformation Of PBL's Using EB Virus

PBL's separated on Ficol gradients, were resuspended in EBV containing culture supernatant (Methods, section2.1) at a density of 1-2 x 10^6 cells/ml. The cells were incubated with the EBV containing supernatant, for 2-3 hr at 37°C, before being pelleted by centrifugation (2300 rpm, 10 min). The EBV containing supernatant was decanted off, and the cells resuspended in

complete tissue culture medium, at a density of 1 x 10^6 cells/ml . They were then plated out into 96 well flatbottomed tissue culture plates (100μl/well, 1 x 10^5 cells/well) which already contained 100μl/well complete tissue culture medium with added phytohaemagglutinin [1%(v/v) final concentration, final well volume 200μl]. The cells were grown in the presence of PHA for between 3-5 days, in a carbon dioxide/humidified incubator at 37°C, after which time the supernatant was removed by way of gentle aspiration using a sterile glass pipette attached to a vacuum line. The medium was then replaced with fresh complete tissue culture medium not containing PHA, and this medium changed every seven days, or when the old media changed colour from orange to yellow, whichever was the sooner.

The cells were grown in this way until they were near confluency, at which time the supernatant from individual wells was collected and assayed for anti-AChR activity (Methods, section 3).

2.4 Pokeweed Mitogen Stimulated PBL's

PBL's separated by Ficol gradient centrifugation were resuspended in complete tissue culture medium at a density of 2 x 10^6 cells/ml. The medium contained, in addition to the normal supplements, Pokeweed mitogen (25μg/10^6 cells). The cells were then incubated in a tissue culture flask (25ml) for five days at 37°C in a carbon dioxide/humidified incubator. The

enhanced B cell population was harvested by centrifugation (2300 rpm, 10 min), and washed twice with PBS by centrifugation (2300 rpm, 10 min) in order to remove the Pokeweed mitogen. The pelleted cells were resuspended in EBV containing supernatant (Methods, section 2.1) at a density of 1-2 x 10^6 cells/ml. After 2-3 h at 37°C, the cells were harvested by centrifugation (2300 rpm, 10 min) and the EBV containing supernatant decanted off. The cells were resuspended in complete tissue culture medium at a density of 1 x 10^6 cells/ml, and plated out into 96 well flatbottomed tissue culture plates (100μl/well, 1 x 10^5 cells/well) which already contained irradiated murine thymocyte feeder cells (Methods, section 2.5.1). The final well volume was made up to 200μl with complete tissue culture medium and the cells were grown at 37°C in a carbon dioxide/humidified incubator. The medium was replaced every seven days with fresh, or when it changed colour from orange to yellow, whichever was the sooner.

2.5 Fusion Of Lymphoblastoid Cell Lines

2.5.1 Preparation Of Feeder Cells

The day prior to the fusion, feeder cells were prepared from one of three sources, and plated out into 96 well flat bottomed tissue culture plates.

Preferred feeder cells were isolated human mononuclear lymphocytes. These were isolated from either normal blood samples (40ml) or lymphocyte enriched plasma fractions (obtained

from volunteers from a local hospital), by the Ficol density gradient centrifugation technique (Methods, section 2.2). Once isolated, the cells were washed twice with PBS, by centrifugation (2300 rpm, 10 min), resuspended in PBS (20ml), and a small fraction was retained for counting (Methods, section 2.6). The cells were then irradiated for 250 sec by using a gamma source (Co^{60} isotope). Following irradiation, the cells were pelleted (2300 rpm, 10 min), and resuspended in complete tissue culture medium at a density of 1×10^6 cells/ml. They were then plated out at 1×10^5 cells/well, into 96 well flatbottomed tissue culture plates, and incubated, until use, in a carbon dioxide/ humidified incubator at 37°C.

If human mononuclear lymphocytes were not available, which was usually the case, then murine macrophages or thymocytes were used as alternatives. These were prepared from either adult balb/C mice, in the case of macrophages, or 6 week old balb/C mice, in the case of thymocytes. In the preparation of macrophages, the adult mice were killed by way of cervical dislocation, whereas in the preparation of thymocytes, the mice were killed by asphyxiation, as cervical dislocation normally contaminated the thymus with blood.

The macrophages were obtained from the abdomens of freshly killed mice, by injecting PBS (5-10ml) into the abdominal cavity with a syringe. The mice were then gently massaged, sprayed with

alcohol, and a small incision was made in the side of the abdominal wall. Through this incision the PBS, containing the macrophages, could be extracted with a sterile disposable pipette. After collection, the cells were washed with PBS, by centrifugation (2300 rpm, 10 min), resuspended in PBS (20ml), and counted (Methods, section 2.6). They were then irradiated for 250 sec using a gamma source, harvested by centrifugation (2300 rpm, 10 min), resuspended in complete tissue culture medium at a density of 1×10^6 cells/ml, and plated out into 96 well flat-bottomed tissue culture plates at 1×10^5 cells/well. The cells were then incubated at 37°C in a carbon dioxide/humidified incubator until use.

Thymocyte feeder cells were prepared from the dissected thymuses of 6 week old mice. The thymuses were placed into sterile PBS, so that they could be washed, and the larger blood vessels were dissected from them. After this, they were transferred to fresh PBS and crushed by using blunt forceps to release the cells. A single cell suspension was obtained by passing crushed organs repeatedly through a syringe needle. The cells were then washed twice with PBS, by centrifugation, before being counted and irradiated. They were then treated in exactly the same way as the macrophages.

2.5.2 Maintenance of X63 Murine Myeloma Cell Line

The murine myeloma cell line, X63, was normally stored

frozen in freezing ampoules (2ml), in liquid nitrogen, at a density of 5-10 x 10^6 cells/ml in freezing mixture (Methods, section 2.1 for details of cryopreservation).

On thawing, the cells were transferred immediately to complete tissue culture medium (10ml), followed by washing, with the same medium, by centrifugation (2300 rpm, 10 min) so as to remove most of the DMSO. The cells were then resuspended in complete tissue culture medium and counted for viability (Methods section 2.6). The cells were then resuspended in complete tissue culture medium at a density of 1 x 10^5 viable cells/ml, in a tissue culture flask (25ml), and grown in a carbon dioxide/ humidified incubator at 37°C. They were grown under these conditions until the medium changed colour from orange to yellow. At this time, the cells were gently dislodged from the bottom of the flask, using a sterile plastic pipette, and a further viability count was carried out. They were then reseeded in fresh medium in a tissue culture flask (25ml), at a density of 1 x 10^5 viable cells/ml, and grown as before. This procedure was repeated several times until the viability of the cells reached 95%, at which time they were ready for fusion.

2.5.3 Ouabain Sensitivity Of X63 Cells

X63 cells grown up to 95% viability, were harvested by centrifugation (2300 rpm, 10 min) and resuspended at a density of 4 x 10^5 cells/ml, in complete tissue culture medium. The cells

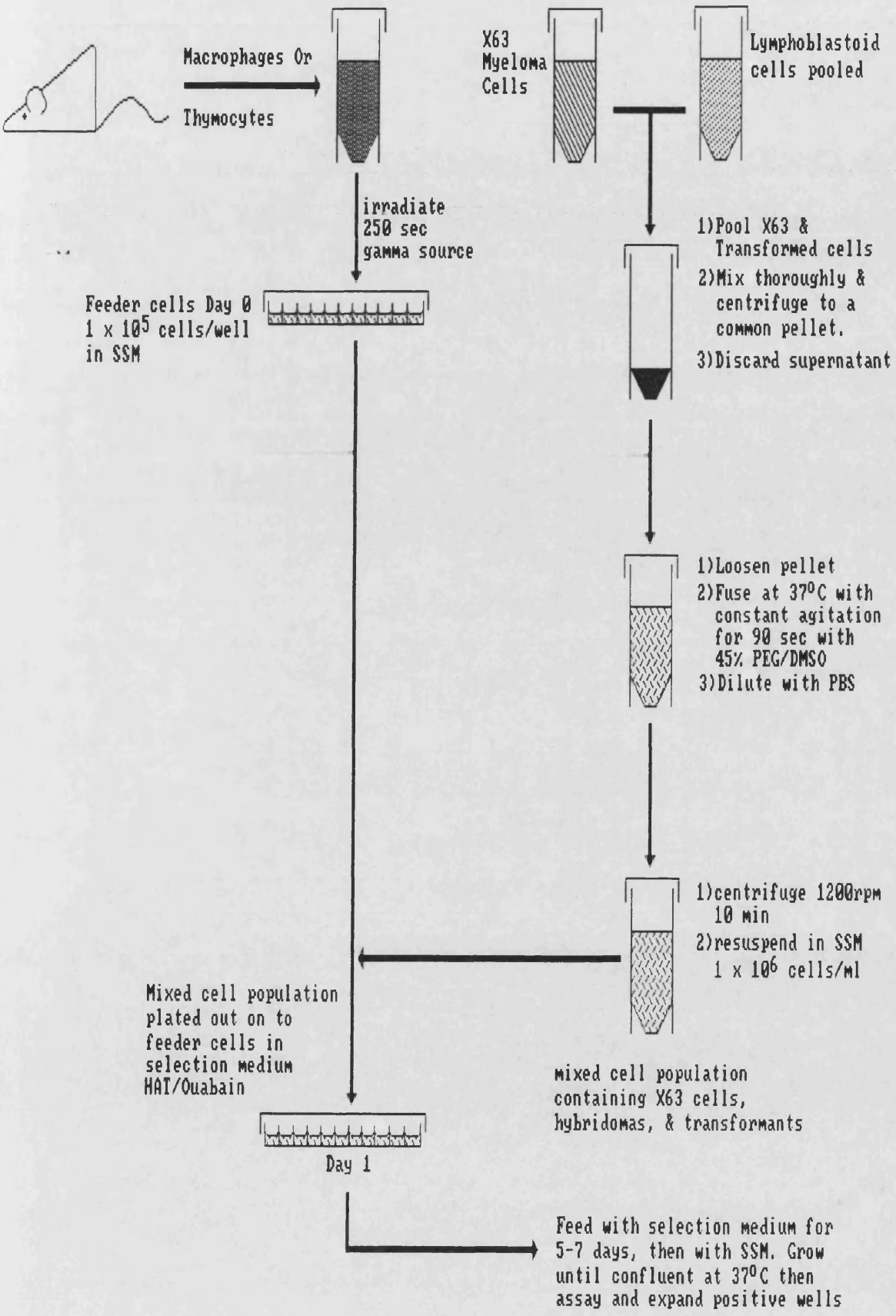
were then plated out into 24 well flatbottomed tissue culture plates (0.5ml suspension/well, 2 x 10^5 viable cells/well). To each well was added further complete tissue culture medium (0.5ml) which contained appropriate dilutions of Ouabain (ranging from 5mM to 1μM final concentrations). The cells were grown for 6 days at 37°C in a carbon dioxide/humidified incubator, after which time the cells from individual wells were harvested and counted by using a Trypan blue exclusion assay (Methods, section 2.6). Triplicate test wells were used for each Ouabain concentration.

2.5.4 Fusion of Lymphoblastoid Cells with X63

Lymphoblastoid cells, previously shown to produce anti-AChR antibodies, growing in 24 well expansion plates, were harvested and pooled. A cell count (Methods, section 2.6), showed between 5-10 x 10^6 cells. These cells were then mixed with an equal number of X63 cells, and the resulting mixed cell population was centrifuged to a common pellet (2300 rpm, 10 min). The cells were washed in Mg^{2+}/Ca^{2+}-free PBS, by centrifugation (2000 rpm, 10 min), and the PBS was carefully aspirated off from the pellet, which was loosened from the bottom of the tube by gentle agitation. Sterile PEG 1500/DMSO (1ml, 45% sol) was added drop-wise to the cells, over 1 min at toom temperature with constant agitation. The fusion mixture was then incubated for 90 secs in a waterbath at 37°C, again with constant agitation. PBS was

added at room temperature, with agitation over the first 2 mins, at a steadily increasing rate (1ml over 1 min, 3ml over next min, up to 20ml total volume). The cells were pelleted by centrifugation (1200 rpm, 10 min), and resuspended, by gentle agitation, in complete tissue culture medium, over a period of 5-10 mins. The cell density of the suspension was then adjusted to 1 x 10^6 cells/ml, and the cells were plated out into 96 well flat bottomed tissue culture plates (100μl/well, 1 x 10^5 cells/well) already containing feeder cells (Methods, section 2.5.1). The plates were incubated overnight at 37°C in a carbon dioxide/humidified incubator, and on the following day, complete tissue culture medium containing 4%(v/v) HAT and 2μM Ouabain was added to the wells (100μl/well, final HAT conc. 2%(v/v), final Ouabain conc. 1μM). The Ouabain was kept as a stock solution of 200μM in distilled water, and was filter-sterilised by passage through a 0.2μm Millipore filter. Following the addition of HAT/Ouabain medium, the cells were grown at 37°C in a carbon dioxide/humidified incubator, and were fed every 7 days with fresh medium containing HAT/Ouabain. The hybridomas so produced, were grown in this way for approximately 5-6 weeks, when supernatants were removed from the wells and assayed for anti-AchR activity (Methods, section 3). Fig. 25 gives a diagramatic summary of fusion procedures.

Fig.25 - Diagramatic Summary Of Procedures Employed In The Production Of Human Hetero-Hybridomas



2.6 Cell Counting And Cell Viability

Cell counting was carried out by using a haemocytometer counting chamber (improved Neubauer ruling).

Both the cell chamber and the coverslip were cleaned with water and alcohol, before the coverslip was placed over the counting area.

A sample of the cell suspension (100μl) was mixed with an equal volume of 0.01% methylene blue in 1% acetic acid. The sample was then introduced into the counting area by capillary action, and the cells were allowed to settle for 1 min before counting. Only the blue-stained cells are counted, and in order to obtain an accurate count, over 200 cells were counted in either one or more large squares.

$$\text{cells/ml} = \frac{2 \times N \times 10^4}{X}$$

N = Nos. Cells Counted
X = Nos. Squares

Cell viability was determined by using the Trypan blue dye exclusion test.

A sample of the cell suspension under study (100μl) was removed and mixed with an equal volume of Trypan Blue solution (0.2% (v/v) in PBS) prior to counting. Counting was carried out within 10 min of the addition of the dye, as cell viability decreases with time owing to the toxic nature of the dye. This also avoids inaccuracies which may arise because of non-specific adsorption of the dye by membrane proteins (Kruse et al 1973).

$$\text{Viability} = \frac{\text{Nos. Viable Cells}}{\text{Total Nos. Cells}} \times 100$$

3. ASSAY OF CULTURE SUPERNATANTS FOR ANTI-AChR ANTIBODIES

3.1 Direct Enzyme-Linked-Immunosorbent Assay

A direct ELISA was used to assay culture supernatants obtained from EBV-transformed cells, and from heterohybridomas.

A 96 well microtitre ELISA plate was incubated overnight with a diluted solution of purified Torpedo AChR (Methods, section 1.4; 5μg/ml, 100μl/well, diluted in 50 mM sodium carbonate/bicarbonate buffer, pH 9.6), at 4°C. Following coating, the plate was washed (3 x 10 min) with PBS containing 1% casein, and 0.05% Tween-20, and blocked by using the same buffer (200μl/well) by incubation at room temperature for 30-40 min.

The wells were then incubated overnight at 4°C with culture supernatant from the test plates (100μl/well), before being washed (3 x 10 min) with the PBS/casein buffer, and incubated with goat anti-human (gamma & mu specific) IgG horseradish peroxidase conjugate in PBS/casein buffer (100μl/well, 1/500 dil. of each specific conjugate) for 3 h at room temperature. The wells were then washed (3 x 10 min) with buffer and incubated with 100μl of substrate solution [tetramethyl benzidine (100μg/ml) in 1M acetate/citrate buffer, pH 6.0 (10ml), and hydrogen peroxide (1.5 μl/10 ml)] for 45-60 min at room temperature. The reaction was quenched by the addition of 2M sulphuric acid (25μl/well). The optical density of the wells was then measured in an ELISA reader at 450nm.

3.2 Modified Radio Immuno Assay

A modified RIA procedure was used to assay culture supernatant from expanded EBV transformed cells and from expanded heterohybridomas.

Culture supernatants (25μl) were incubated with human AChR (200μl), which had previously been radiolabelled with ^{125}I-αBGT (Methods, sections 1.1, 1.3), overnight at 4°C. In order to increase pellet size, pooled normal human serum (5μl) was also added.

Following incubation, precipitating antibody was added (25 - 100μl goat anti-human IgG, depending on batch), and precipitation was allowed to occur overnight at 4°C, following which, the precipitate was pelleted by centrifugation (2500 rpm, 10 min, 4°C), washed (2 x 1 ml) by centrifugation (2500 rpm, 10 min, 4°C) with radioimmunoassay buffer (10mM potassium phosphate buffer, pH 7.4, containing 1% (v/v) Triton X-100, 0.1% (w/v) sodium azide and 0.1% (w/v) BSA). The supernatant was then discarded and the pellets were counted for radioactivity.

Specific binding was measured by carrying out all assays in the presence and absence of BZQ, and all assays were carried out in triplicate.

Antibody titre was then expressed as the amount of radiolabelled receptor bound in the pellet.

$$\text{Titre (nmols/ml)} = \frac{\text{Specific cpms.}}{\text{Spec. Act. Toxin}} \times 1000/25$$

3.3 Antibody Capture ELISA For The Detection Of Anti-AChR Antibodies Using Biotinylated Alpha-BGT (BαBGT)

3.3.1 Biotinylation Of Alpha-BGT

Alpha-BGT was dialysed against 0.1M sodium hydrogen carbonate (2L, overnight at 4°C), and its concentration was adjusted to 5mg.ml. To this solution (1ml) a freshly prepared solution of biotin-N-hydroxsuccinimide in dimethylformamide (0.1ml, 40mg/ml) was added dropwise with gentle stirring at room temperature, before either dialysis or desalting on a G25 Sephadex column.

If dialysis was used, then it was carried out for 48h at 4°C, against phosphate-buffered saline, pH 7.3 (2L). The concentration of the BαBGT was then adjusted to 0.5mg/ml in PBS containing BSA (10mg/ml). To this, an equal volume of glycerol was added [50% (v/v) final concentration], and aliquots (300μl) were stored at -20°C.

If desalting was used then BSA in PBS (10mg/ml, 100μl) was added to the reaction mixture, and the whole was applied to a Sephadex G25 column (5ml), that had been previously equilibrated with PBS containing sodium azide (0.01% (w/v). The BαBGT was eluted by using the same buffer, with 0.5ml fractions being collected. The optical density of the fractions was then measured at 280nm. The BαBGT peak was pooled and aliquots (300μl) were stored at -20°C in the absence of glycerol.

3.3.2 Characterisation of BαBGT

i) Sensitivity Of BαBGT Binding To Purified AChR

The binding of BαBGT to AChR was characterised by means of a solid phase enzyme assay.

Microwell module strips were coated by incubation with purified Torpedo AChR (Methods, section 1.3) in 50mM sodium carbonate/bicarbonate buffer, pH 9.6 (100μl/ml) overnight at 4°C. The strips were then washed (3 x 5 min) with PBS containing 0.05% (v/v) Tween-20 and 1% (w/v) casein (Assay Buffer), and blocked by incubation with the same buffer (200μl/well) for 30 min at room temperature. At this stage the strips can be used immediately or stored at 4°C for at least two weeks without loss of activity.

After coating and blocking, the wells were incubated with serial fold dilutions of BαBGT, diluted in assay buffer (100μl/well) for 2-3h at room temperature, or overnight at 4°C. The wells were then washed (3 x 5 min) with Assay Buffer, and then incubated with appropriate dilutions of Avidin-horseradish peroxidase conjugate (HRP-Avidin) in Assay Buffer (100μl/well) for 3h at room temperature. After washing with assay buffer (3 x 5min), the wells were incubated with enzyme substrate solution (Bos et al,1981). This comprises 0.1M sodium acetate/acetic acid buffer, pH 6.0 (10ml), containing tetramethylbenzidine in dimethylsulphoxide (100μl, 10mg/ml), 0.05% (v/v) Tween-20, and hydrogen peroxide (30% solution, 1.5μl). Incubation was continued

at room temperature, until a colour reaction was observed (between 45min and 2h). The colour reaction was terminated by the addition of 2M sulphuric acid (25μl/well), and the optical density of the wells was read at 450nm.

The sensitivity of BαBGT binding to purified AChR was further shown by the comparison of BαBGT binding and ^{125}I-αBGT binding to AChR coated wells.

Polyvinyl 96 well EIA plates were coated with serial fold dilution of purified Torpedo AChR (5000 - 39 ng/ml), diluted in 50mM sodium carbonate/bicarbonate buffer, pH 9.6 (100μl/well) overnight at 4°C. After washing with Assay Buffer (3 x 5 min) and blocking with the same buffer (200μl/well, 30min at room temperature), the wells were incubated with either BαBGT (1/500 dilution, 100μl/well) diluted in assay buffer, or with an equivalent amount of ^{125}I-αBGT (0.56pmol/μl) also diluted in Assay Buffer, for 3h at room temperature.

The wells that had been incubated with BαBGT were then washed (3 x 5min) and incubated with HRP-avidin (1/500 dilution, 3h at room temperature) followed by washing and incubation with substrate solution (1h, room temperature). The reaction was terminated by the addition of 2M sulphuric acid (25μl/well) and the optical density of the wells was read at 450nm. The wells that had been incubated with ^{125}I-αBGT were washed (3 x 5min), dried and individually counted in an LKB ultrogamma counter.

ii) Specificity Of BαBGT Binding To Purified AChR

The specificity of BαBGT binding for AChR was assessed by the ability of unlabelled αBGT, and the cholinergic ligands, benzoquinonium chloride, decamethonium bromide, and nicotine, to inhibit the binding.

AChR coated wells (Methods, section 3.1) were preincubated with the inhibitor solution (50μl/well) for 2h at room temperature before the addition of the BαBGT (1/500 dilution, 50μl/well), diluted in assay buffer. The wells were then incubated overnight at 4°C, washed (3 x 5min) with assay buffer, and incubated with HRP-avidin (1/500 dilution, 100μl/well), diluted in Assay Buffer, for 3h at room temperature. After the final wash (3 x 5min) with assay buffer, the wells were incubated with the enzyme substrate solution (100μl/well) for 1h at room temperature, before the reaction was terminated by the addition of 2M sulphuric acid (25μl/well). The optical density of the wells were then measured at 450nm.

iii) Determination Of Number Of Biotin Molecules Bound Per αBGT Molecule

The number of biotin molecules bound per αBGT molecule was determined by employing a colourimetric assay developed by Green (1965).

The procedure involved the setting up a standard curve, using a known standard biotin solution.

The spectrophotometer was zeroed at 500nm by using Avidin in 20mM potassium phosphate buffer, pH 7.0 (0.9ml, 0.65mg/ml, 5 M). To this solution, 5mM 4-hydroxyazobenzene benzoic acid (20μl) in 20mM potassium phosphate buffer, pH 7.0, was added and mixed thoroughly. The new optical density (falling between 0.6 and 1.0 optical density units) at 500nm, was noted before continuing. Sequential additions of a standard biotin solution (1μl, 0.2mg/ml in 1mM sodium hydroxide) were made to the same curvette, and the decrease in the optical density was noted. this gave rise to a standard curve from which unknowns could be determined.

Unknown biotin solutions were assayed in a similar way. Cuvettes were set up with the Avidin solution and the 4-hydroxyazobenzene benzoic acid, as before, but this time, additions of the BαBGT solutions (10μl) were made up to a maximum of 40μl. Again the decrease in the optical density was noted.

From this decease, by using the standard curve, a measure of the number of biotin molecules per αBGT molecule was made.

3.3.3 Antibody Capture Enzyme Linked Immunosorbent Assay (ELISA) For Detection Of Anti-AChR Antibodies In Culture Supernatants From Murine Hybridomas

i) Binding Of The Monoclonal Anti-AChR Antibodies to Purified AChR

This was investigated by a direct ELISA system, so as to determine which monoclonal antibodies would be of use for further study.

Microtitre module strips were coated with purified Torpedo AChR (5μg/ml, 100μl/well, (Methods, section 1.4) in 50mM sodium carbonate/bicarbonate buffer, pH 9.6, overnight at 4°C. The wells were then washed (3 x 5min) and blocked (30 min, 200μl/well, room temperature) with Assay Buffer. They were then incubated with increasing dilutions of the several monoclonal antibodies available for study (an anti-neurofilament monoclonal antibody was also included as a control), for 2h at room temperature, the antibodies being diluted in assay buffer.

After washing (3 x 5min), the wells were incubated with horseradish peroxidase-goat anti-mouse IgG (gamma specific) conjugate (1/500 dilution, 100μl/well) for 3h at room temperature. After washing (3 x 5min) the wells were finally incubated with enzyme substrate solution (Methods, section 3.1, 100μl/well) for 45min at room temperature, before the reaction was terminated by the addition of 2M sulphuric acid (25μl/well). The optical density of the wells were then measured at 450nm.

ii) Antibody Capture ELISA

Having determined which monoclonal antibodies were to be used for further study, the antibody capture ELISA was investigated.

Microtitre module strips were coated with goat anti-mouse IgG (5μg/ml, 100μl/well) in 50mM sodium carbonate/bicarbonate buffer, pH 9.6 overnight at 4°C. After washing (3 x 5min) and

blocking (30min, 200μl/well, room temperature) with Assay Buffer, the wells were incubated with serial fold dilutions of the antibody-containing culture supernatant (100μl/well) for 2-3hrs at room temperature, the supernatants being diluted in assay buffer.

After washing (3 x 5min), the wells were incubated with either crude Torpedo receptor preparation (2pmol), or crude foetal calf receptor preparation (0.2pmol), the former appropriately diluted, overnight at 4°C (100μl/well, Methods, section 1.3). Following receptor incubation, the wells were incubated with BαBGT (1/500 dilution, 100μl/well), HRP-Avidin (1/500 dilution, 100μl/well), and finally, with enzyme substrate solution (100μl/well, 1h, at room temperature), as described previously (Methods, section 3.3). Between each step the wells were washed (3 x 5min) with Assay Buffer.

After incubation with substrate (Methods, section 3.1), the reaction was terminated by the addition of 2M sulphuric acid (25μl/well), and the optical density of the wells was measured at 450nm.

3.3.4 Antibody Capture ELISA to Detect Anti-AChR Antibodies In Myasthenic Sera

Microtitre module strips were coated with either goat anti-human IgG (gamma specific) or goat anti-human IgG (mu specific) antibodies (5 g/ml, 100μl/well) in 50mM sodium

carbonate/bicarbonate buffer, pH 9.6, overnight at 4°C.

Wells were washed (3 x 5min) and blocked (30min, at room temperature) with Assay Buffer (PBS, pH 7.3, containing 0.05%(v/v) Tween-20, and 0.2%(v/v) Fish Skin Gelatin, as blocking agent), before being incubated with serial fold dilutions of various Myasthenic sera (neat - 1/5120 dilution), including pooled normal human sera, for 3 hrs at room temperature.

Sera chosen for study, covered a wide range of antibody titres (high, medium and low) as indicated by conventional radioimmunoassay (Methods, section 1.6).

After washing (3 x 5min), wells were incubated with undiluted crude human muscle receptor preparation (1pmol/ml, 100μl/well) overnight at 4°C. This was then followed by washing (3 x 5min), incubation with BαBGT (1/500 dilution, 100μl/well) for 2h at room temperature; washing (3 x 5min); incubation with Streptavidin peroxidase (1/200 dilution, 100μl/well) for 2h at room temperature; washing (3 x 5min); and finally, incubation with enzyme substrate solution (100μl/well) for 20 min at room temperature. The reaction was terminated by the addition of 2M sulphuric acid (25μl/well) and the optical density of the wells was read at 450nm.

4. PRODUCTION OF ANTI-IDIOTYPIC ANTIBODIES DIRECTED TOWARDS ANTI-AChR ANTIBODIES

The basic procedure for the production of anti-idiotypic antibodies involved the immunization of a laboratory bred rabbit

with an $F(ab)_2$ fraction prepared from IgG purified from a Myasthenic patient. After immunization and subsequent boosting, blood samples were taken from the rabbit and processed to give an anti-idiotypic fraction. This was then tested for anti-idiotypic activity.

4.1 Purification of Human IgG from a Myasthenic Patient (P.)

Myasthenic serum (5 ml) was dialysed overnight against 30mM potassium phosphate buffer (7.3 pH, 500 ml) and applied to a DE52 cellulose ion exchange column (1 x 30cm) which had been equilibrated in 30mM potassium phosphate buffer, pH 7.3. The serum was eluted by using the same buffer with a flow rate of 36ml/h, and fractions (4ml) were collected. The optical density (A_{280}) of each fraction was measured and the fractions containing IgG were pooled.

The column was washed with 30mM potassium phosphate buffer, pH 7.3, containing 0.5M sodium chloride, re-equilibrated with 30mM potassium phosphate buffer, pH 7.3 and stored at 4°C.

4.2 Pepsin Digestion Of Purified IgG And Purification Of $F(ab)_2$ Fragments

The Myasthenic IgG was concentrated (x10) in a Minicon Concentration Unit (B15), to give an IgG concentration of approximately 6mg/ml.

The concentrated IgG fraction was then dialysed against 0.1M sodium acetate buffer, pH 4.5 for 3h at room temperature,

with constant stirring. The pH of the fraction was monitored until it had dropped to 4.5 at which point a solution of pepsin (20μl, 30mg/ml) was added and the digestion mixture was incubated overnight at 37°C.

The digest was then centrifuged (4000rpm, 15min), and the pellet was discarded. The pH of the supernatant was adjusted to 7.4 by dropwise addition of 2M Tris-(hydroxymethyl)-methylamine, in order to inhibit the action of the pepsin.

The digest was then applied to a DE52 cellulose ion exchange column (1cm x 5cm), that had been previously equilibrated in 10mM potassium phosphate buffer, pH 8.0. The $F(ab)_2$ fragments were eluted in the void volume with 10mM potassium phosphate buffer, pH 8.0. Fractions (2ml) were collected, their optical density (A_{280}) was monitored. Fractions containing $F(ab)_2$ fragments were pooled.

4.3 Identification Of $F(ab)_2$ Fragments In Purified Fraction

4.3.1 Determination of Anti-AChR Binding Sites

A standard radioimmunoassay was carried out, similar to that described for the assay of culture supernatants (Methods, section 3.2).

Samples of the $F(ab)_2$ fraction were diluted 1:1 to 1:64) in radioimmunoassay buffer (RIA buffer). Triplicate samples of the diluted $F(ab)_2$ fraction (25μl) were then incubated with radiolabeled AChR (100μl) with or without added benzoquinonium, for 2hrs at room temperature. The assay was then carried out as previously described (Methods, section 3.2).

4.3.2 Immunodiffusion

Immunodiffusion gel (4.4ml) comprising Agar (0.5g), distilled water (25ml), PBS (25ml), 0.1%(w/v) sodium azide, and 3%(w/v) polyethylene glycol 6000, was poured on to a level plastic microscope slide, and allowed to solidify for 15min at 4°C.

Following solidification, a symmetrical arrangement of wells was cut into the agar gel. This arrangement consisted of one central well with six wells arranged equidistant from the central well in a hexagonal array (see Fig. 48). Two such arrangements can be placed on the same slide.

In the central well of the first array was placed anti-human gamma chain IgG (20μl), and in the other one was placed anti-human light chain IgG (20μl). The remaining wells contained samples (20μl) of the $F(ab)_2$ fractions (as obtained from the column), samples of wash fractions from the column and a sample of Fc gamma.

The plate was then incubated at 4°C for 24hr, after which it was washed overnight in PBS and distilled water. It was then stained in 0.5% (v/v) Ponceau-S / 5% (v/v) TCA for 15min. Destaining was carried out using 5% (v/v) acetic acid. The plate could then be dried completely at 37°C.

4.3.3 SDS PAGE of $F(ab)_2$ Fragments

A 7.5% acrylamide gel was prepared by mixing 25% stock acrylamide (12ml), 1M Tris/1M Bicine (4ml) and distilled water (22.7ml). The acrylamide solution was degassed for 5min prior

to the addition of 10% (w/v) SDS (0.4ml), TEMED (0.025mls) and ammonium persulphate (0.9mls, 1.5% (w/v)) were added just prior to pouring. The gel was poured with the comb in position, allowed to polymerize, and then clamped into the electrophoresis tank.

Samples were prepared in reduced and non-reduced forms. Reduced samples were prepared by boiling protein (10μg) with SDS/mercaptoethanol (10μl, 10%/10%) and bromophenol blue (2.5μl, 1mg/ml BPB in 50% (v/v) glycerol) for 5min. The non-reduced samples were prepared in exactly the same manner but without the added mercaptoethanol. Sample volumes were increased to 20μl with elution buffer, which contained 10% (w/v) SDS (20ml) and 1M Tris/1M Bicine (40ml) in distilled water (2l).

The samples were then loaded onto the gel and a loading voltage of 50 volts applied. After loading is complete, which can be seen visually, the voltage was increased to 150 volts (or 10-15 v/cm). The gel was allowed to run until the bromophenol blue had reached the bottom of the gel. The gel was then removed from the apparatus and stained overnight, with constant shaking, in 10% (v/v) TCA containing 1% (v/v) Coomasie Blue R250 (10% solution in methanol). The gel was then destained in 10% (v/v) TCA.

Standard proteins were phosphorylase B (3μg); -Galactosidase (3μg); human IgM (2μg); human IgG (2μg); BSA (2μg); Aldolase (4μg) and Carbonic Anhydrase (4μg).

4.4 Immunization of Rabbit

A mature laboratory bred rabbit was immunized with a cocktail consisting of $F(ab)_2$ fragments (100μg in 250μl distilled water), and complete Freunds adjuvant (750μl). This was split into two equal fractions and injected intramuscularly into two sites on the rabbit.

After six weeks the rabbit received a booster injection of the same cocktail. After five days a bleed was taken from an ear vein (20mls). A further bleed was taken after another four days. All bleeds were taken in non-heparinised tubes. Any further boosts were carried out using the same quantity of protein but in non-complete Freunds adjuvant.

4.5 Preparation of Anti-Idiotypic Fraction From Rabbit Serum

The basic procedure involves absorbing the rabbit anti-human light chain antibodies from the rabbit serum on a Sepharose 4B / Human IgG column, followed by sodium sulphate precipitation of the remaining immunoglobulins. The concentrated immunoglobulin fraction was then tested for the presence of anti-idiotypic antibodies directed against anti-AChR antibodies.

4.5.1 Linkage of Human IgG to Sepharose 4B Beads Using Cyanogen Bromide

Human IgG from normal human serum (NHS) was prepared as previously described (Methods, section 4.1).

The IgG fraction obtained from NHS (10ml) was concentrated

by ammonium sulphate precipitation. An equal volume of a saturated ammonium sulphate solution was added dropwise, and the whole stirred for 1h at room temperature. After stirring the precipitate was separated out by centrifugation in a bench centrifuge (6000rpm, 15min). The precipitate was dissolved in distilled water (10ml) and dialysed overnight against 50mM sodium carbonate/bicarbonate buffer, pH 9.5.

Packed Sepharose 4B (10ml) was washed (3 x 40ml) with distilled water, by centrifugation, before being suspended in distilled water (10ml) and 2M sodium carbonate solution (20ml). The slurry was cooled to 4°C with constant stirring. With increased stirring, cyanogen bromide in acetonitrile (2.12ml, 0.94 g/ml) was added dropwise over 15 sec. followed by vigorous stirring (3min) on ice. The slurry was then filtered through a scintered glass filter and washed immediately with 0.1M sodium carbonate/bicarbonate buffer, pH 9.5 (50ml), distilled water (50ml), and 0.2M sodium carbonate/bicarbonate buffer, pH 9.5 (50ml). All the washing buffers had been previously cooled on ice. The gel was resuspended in the antibody containing solution and stirred continuously overnight at 4°C.

The gel was then washed by filtration, using 0.1M sodium acetate buffer, pH 4.5 (160ml), containing 0.5M sodium chloride, and 0.1M sodium carbonate/bicarbonate buffer, pH 9.5 (160ml), containing 0.5M sodium chloride. It was at this stage that the

degree of coupling was calculated based on the amount of protein added to the gel and that which was calculated to be still remaining in the first filtrate.

Unbound groups on the Sepharose beads were blocked by stirring the gel with 1M ethanolamine/HCl buffer, pH 9.5 for 1hr at room temperature. The beads were then washed with PBS and then packed into a column.

4.5.2 Preparation of Anti-Idiotypic Fraction from Rabbit Serum

Blood (20ml) collected from the rabbit was allowed to coagulate by incubation at 37°C for 30min, followed by an overnight incubation at 4°C. The serum was then separated by centrifugation (4000rpm, 20min).

The serum was then applied to the Sepharose 4B/human IgG column in order to absorb out the rabbit anti-human light chain antibodies. The column was eluted with PBS (20ml/hr) and fractions (2ml) were collected. The optical density of the fractions was measured at 280nm, and the void volume peak was pooled to give the anti-idiotypic fraction. The column was cleaned by using a solution of 0.5M ammonium hydroxide. The anti-human light chain antibodies so produced were dialysed against PBS (4°C overnight), and stored at -20°C.

The anti-idiotypic antibody fraction was concentrated by ammonium sulphate precipitation, involving the dropwise addition of a saturated ammonium sulphate solution to the antibody

fraction until a ratio of 10:6 (antibody : ammonium sulphate) was achieved. The whole was stirred for 1h at room temperature to maximise precipitation. The precipitate was separated by centrifugation (4000rpm, 20min), following which it was dissolved in distilled water (1ml), and dialysed against PBS (overnight at 4°C).

A similar concentration procedure was carried out on normal rabbit serum, which was then used as a control in the assay for anti-idiotypic antibody activity.

4.6 Assay of Immune Rabbit Serum for Anti-Idiotypic Activity

4.6.1 Competitive RadioImmunoAssay

Samples of Myasthenic serum (5μl, 1/10 dilution in NHS) were incubated with varying dilutions of the anti-idiotypic fraction, or dilutions of the concentrated NRS Ig fraction (1/1; 3/4; 1/2; 1/4; 0) for 2h at 37°C, prior to the addition of radiolabelled AChR (200μl, as previously described. Methods, section 1.6) with or without added benzoquinonium. The samples were left to react overnight at 4°C, before the addition of the second precipitating antibody (35μl). Precipitation was allowed to occur overnight at 4°C, before centrifugation and washing. The pellets were then counted for radioactivity and the results calculated as before.

4.6.2 ELISA For Anti-Idiotypic Antibodies

A 96 well microtitre plate was coated with either myasthenic

$F(ab)_2$ fragments (100μl/well, 5μg/ml) or normal human $F(ab)_2$ fragments (100μl/well, 5μg/ml) in 50mM carbonate buffer, pH 9.5 overnight at 4°C. The wells were then washed (3 x 10min) with PBS containing 0.05% (v/v) Tween-20, and 1% (w/v) casein, and then blocked for 40min by using the same buffer, at room temperature. The wells were then incubated with increasing dilutions of the anti-idiotypic fraction or normal rabbit IgG preparation (2h, 37°C, or overnight at 4°C). The wells were then washed (3 x 10min) with the PBS/casein buffer, and incubated with goat anti-rabbit gamma chain horseradish peroxidase conjugate (100μl/well, 1/500 dil. 2h, room temperature). Following washing substrate solution was added (Methods, section 3.1) and incubated for 40min at room temperature. The reaction was quenched by the addition of 2M sulphuric acid (25μl/well). The optical density of the wells was measured at 450nm.

4.6.3 Adsorption of Anti-AChr Antibodies onto a Sepharose 4B / Anti-Idiotypic Column

i) Purification of IgG from Anti-Idiotypic Serum

A sodium sulphate precipitation was carried out on the anti-idiotypic fraction as prepared previously (Methods, section 4.5).

Solid anhydrous sodium sulphate was added slowly to the anti-idiotypic fraction, until a concentration of 18% (w/v) was reached. The whole was stirred slowly at room temperature for

1h, before the precipitate was separated out by centrifugation (4000rpm, 15min). The pellet was then washed in 0.1M sodium bicarbonate containing 18% (w/v) sodium sulphate, and dissolved in PBS (10ml). The solution was dialysed against PBS (overnight 4°C), and then against 50mM sodium carbonate/bicarbonate buffer, pH 9.5 (3h, room temperature).

ii) **Cyanogen Bromide Coupling of Anti-Idiotypic IgG to Sepharose 4B**

The anti-idiotypic IgG prepared by sodium sulphate precipitation (Methods, section 4.6.3.i) was attached to Sepharose 4B affinity chromatography gel beads by means of cyanogen bromide coupling (Methods, section 4.5.1) and packed into an affinity column (1cm x 10cm).

iii) **Adsorption of Myasthenic Serum onto Affinity Column**

The Sepharose 4B/anti-idiotypic IgG affinity column was equilibrated with PBS containing 0.5M sodium chloride. Following this, Myasthenic serum (100μl, containing added myoglobin and Dextran blue) was applied to the column. The column was eluted with the PBS/sodium chloride buffer, and fractions collected (0.5ml). The optical density of the fractions was measured at 280nm, and the void volume peak pooled.

The column was washed with 0.5M ammonium hydroxide and re-equilibrated with PBS containing 0.2% (w/v) sodium azide. The column was then stored at 4°C.

The adsorbed serum was then examined by radioimmunoassay (Methods, sections 3.2 & 4.3.1) against an equivalent dilution of the original myasthenic serum used.

RESULTS

1. GENERAL PROCEDURES

1.1 Iodination of Alpha-BGT With $Na^{125}I$

The iodination of commercially available BGT was routinely carried out by using $Na[^{125}I]$ and the Chloramine-T method (Methods, section 1.1).

In general, this procedure iodinated the toxin to a very high specific activity, ranging from 745-767 Ci/mmol [751±7.8 (6)] with incorporations of 89 - 97% [94±1.8% (6)] [mean± SEM(n)].

A typical elution profile obtained from the Sephadex G25 column from one of these preparations is shown in Fig. 26. This particular preparation had a specific activity of 763 Ci/mmol, 96% incorporation, 0.55 pmol toxin/μl, and a biological activity of 51%.

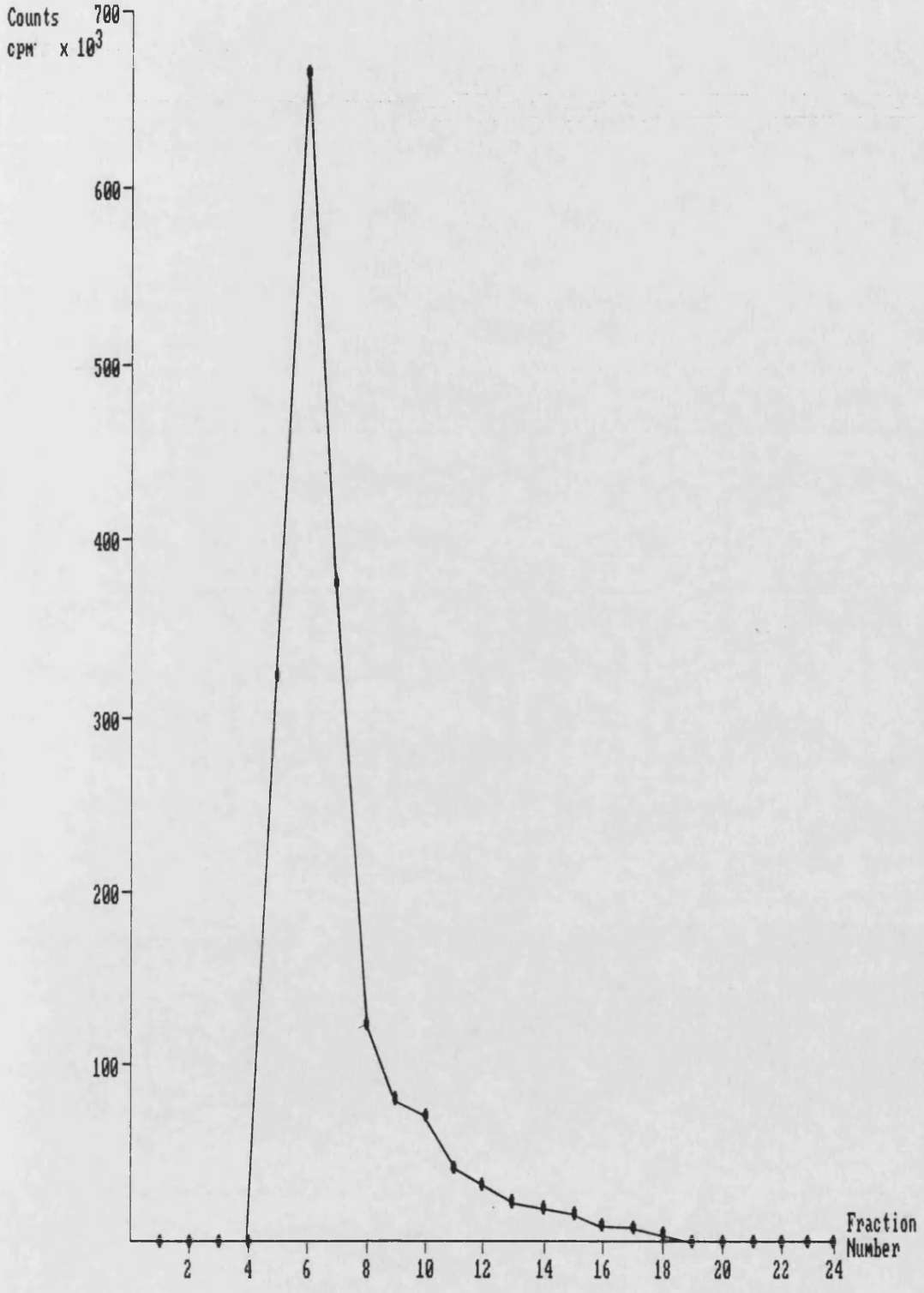
1.2 Determination Of The Biological Activity of $[^{125}I$ - BGT]

The biological activity of the iodinated BGT was assessed by its ability to bind large molar excesses of Torpedo AChR. Toxin receptor complexes were separated from unbound species by ion-exchange chromatography on DEAE-cellulose filters. The biological activities of the $[^{125}I$- BGT] species prepared ranged from 48 - 52% [51 ± 1.3% (6)] as defined by:

$$\text{Biological Activity} = \frac{[^{125}I] \text{ bound}}{\text{Total } [^{125}I] \text{ added}} \times 100\%$$

Fig.26 - Gel Filtration Profile Of The Iodination Of Alpha - BGT

1ml fractions collected from a G25 Sephadex gel filtration column and counted for radioactivity



1.3 Preparation Of AChR Extracts From Various Sources

Receptor extracts were prepared from three sources, human gastronemious muscle, foetal calf muscle, and Torpedo marmorata electric organs. In addition to the crude extracts prepared from all three, a purified extract was obtained from the Torpedo marmorata electric organs (see Fig. 27, for elution profile).

A difficulty inherent in the purification of AChR from mammalian, particularly human, sources, is the susceptibility of the receptor to proteolytic degradation. For this reason, the procedures are largely carried out at 4°C in the presence of protease inhibitors.

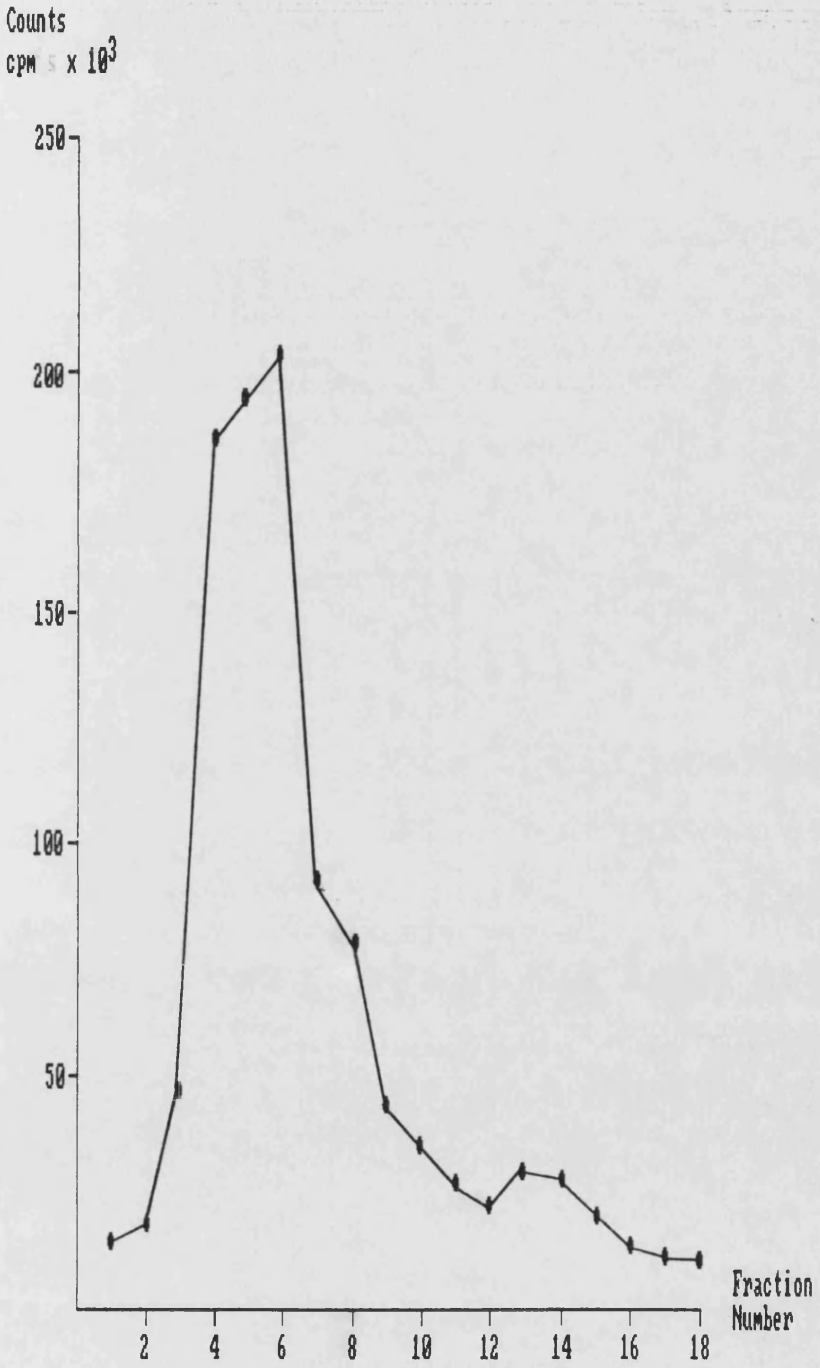
The purified Torpedo AChR was stored at 4°C in the presence of 0.02% (w/v) sodium azide, and was used for periods of longer than 3 months without appreciable losses in [^{125}I]-αBGT binding activity. The purified Torpedo AChR extract was assayed by the DF-81 cellulose filtration assay, shortly after initial preparation, and then at regular intervals, until it was either discarded or completely used. Upon initial assay, the purified receptor extract contained 291 pmol/ml of AChR as determined by the DEAE-cellulose filtration assay, this dropped to 263 pmol/ml after 3 months.

The foetal calf and the human muscle crude extracts were stored at 4°C in the presence of 0.02% (w/v) sodium azide.

Initially all the crude receptor extracts were assayed by

Fig.27 - Elution Profile Of The Purification Of Torpedo AChR

The elution profile of Torpedo AChR from DE 52 column. The AChR was identified by its ability to bind ^{125}I - alpha - BGT using the DEAE cellulose filtration assay



* Specific Activities of AChR preps

From a variety of sources

Source		Specific Activities (pmol ^{125}I-αBGT binding sites/μg protein)	
Torpedo	Crude	0.13 ± 0.01	(1)
	Purified	4.30 ± 0.05	(1)
Human	Crude	1.75×10^{-4} $\pm 6.2 \times 10^{-5}$	(6)
Feotal Calf	Crude	2.65×10^{-4} $\pm 1.4 \times 10^{-5}$	(2)

(MEAN ± S.E. (n))

the ammonium sulphate precipitation assay (Methods, section 1.5.1) but this tended to give variable results with high non-specific binding. The PEI assay (Methods, section 1.5.2) on the other hand, gave more consistent results with low non-specific binding, and was preferred for assaying crude receptor extracts.

From six human muscle AChR preparations the AChR content of the crude extracts ranged between 0.5 pmol/ml and 1.5 pmol/ml.* Of two foetal calf muscle extracts, the AChR contents were 2.4 and 2.7 pmol/ml.* Both the human and the foetal calf extracts were assayed immediately after preparation, and later after storage for approximately 1 month. It was found that there was a slight but not significant drop in the AChR content of the extracts. It was usual for the human extract to have been completely used within the month following its preparation.

The crude Torpedo AChR extract prepared was also stored at 4°C in the presence of 0.02% (w/v) sodium azide. Upon initial assay, using the PEI assay, the AChR content was 1446 pmol/ml.* This value, again, decreased slightly upon storage but not to any significant degree.

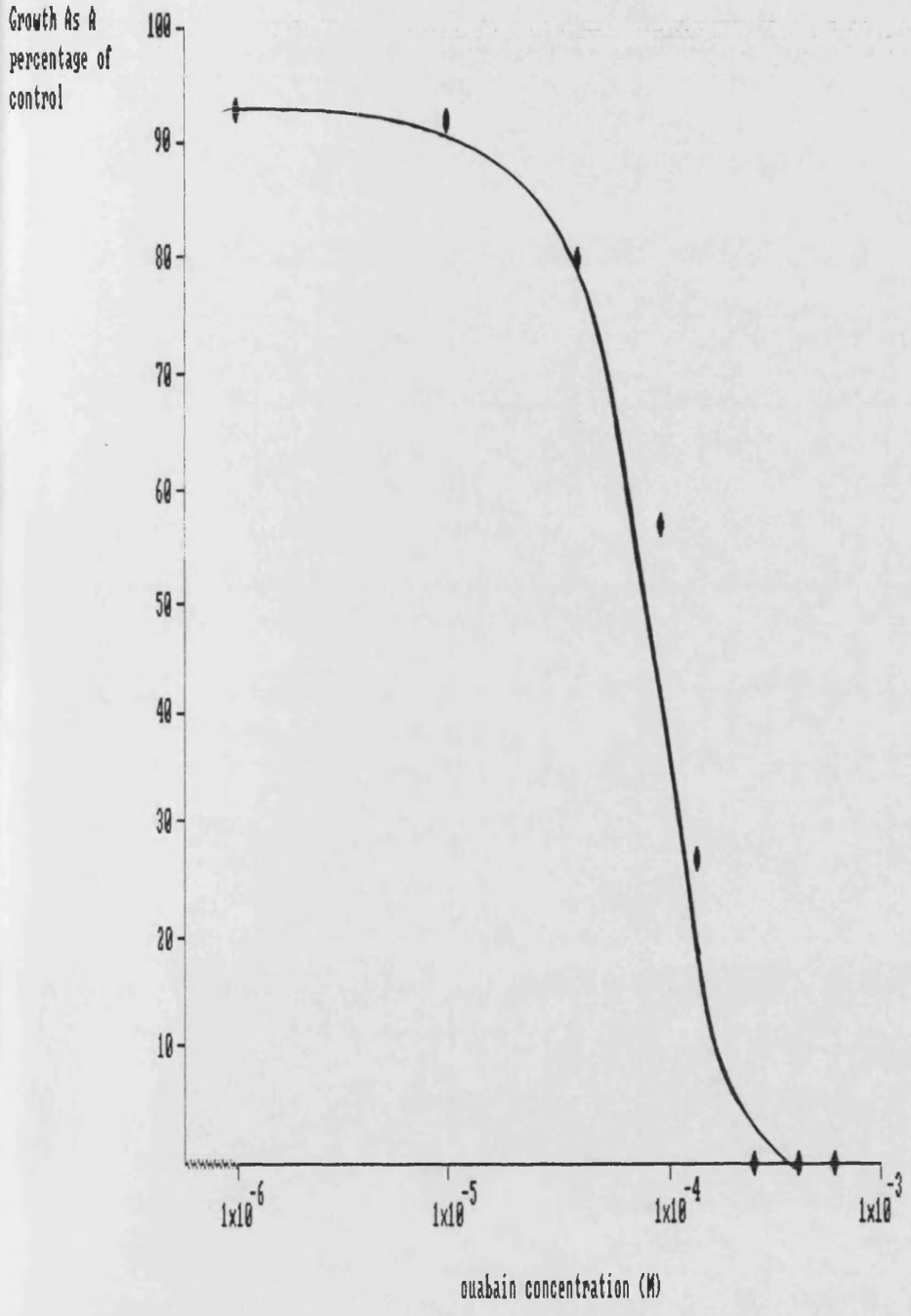
2. CELL MANIPULATIONS AND TRANSFORMATIONS

2.1 Ouabain Sensitivity Of X63 Myeloma Cell Line

X63 cells were grown for 6 days in complete tissue culture medium with added ouabain, in a carbon dioxide/humidified incubator at 37°C (Methods, section 2.5.3). After this period

Fig.28 - Ouabain Sensitivity Of X63 Myeloma Cells

Growth is expressed as a percentage of the growth of control wells, as determined by the counting of viable cells.



the number of viable cells per well were counted, together with the total number of cells per well, using the Trypan blue dye exclusion assay (Methods, section 2.6).

For each concentration of ouabain, ranging from 1μM to 5mM, triplicate test wells had been seeded. The number of viable cells per well was then expressed as a percentage of the total number of viable cells in the control wells (i.e. wells with no added ouabain).

Fig. 28 shows that the X63 myeloma cells are capable of withstanding concentrations of ouabain up to 10μM without loss of viability, 50% inhibition of growth was shown at a final ouabain concentration of approximately 100μM. Consequently, when carrying out fusions, a concentration of 2μM Ouabain was used.

2.2 PBL Transformations/Fusions

Blood samples from patients suffering from Myasthenia gravis, were used to obtain human peripheral blood lymphocytes for transformation/fusion experiments. Approximately 76 heparinised blood samples (15-40ml) were processed. The number of cells obtained from a sample varied greatly, but was approximately 1.6×10^6 cells/ml of blood.

In addition to blood samples obtained by this method, lymphocytes were also prepared from lymphocyte-enriched plasma obtained from patients who were undergoing plasmapharesis therapy.

However, there were only 6 of these. They gave approx. 16×10^6 cells/ml of lymphocyte enriched plasma [$18 \times 10^6 \pm 0.6 \times 10^6$ (n=6)].

For separation techniques, see Methods, section 2.2.

Of the samples transformed, only 3 gave rise to plates that were assayable. Of these only one plate of cells were used in a fusion experiment from one patient.

In the case of this patient (code BB) used for the fusion, venous blood (15ml) was collected, and contained 13×10^6 cells (0.8×10^6 cells/ml blood). The cells were transformed on day 0 (Methods, section 2.3), and plated into a 96 well flatbottomed tissue culture plate, where they were grown for 13 days in complete tissue culture medium. They were then tested for anti-AChR antibody production by direct ELISA with Torpedo AChR (Methods, section 3.1; Table 15).

By this method, it was determined that wells C7, F8, G5, G10, and H7 contained antibodies against Torpedo AChR (see Table 15), and these cells were subsequently expanded into 3ml tissue culture plates. The cells were grown almost to confluence for the next 9 days in the 3ml plates. At this time, the cells were harvested and pooled (total cell count 16×10^6 cells) and fused with an equal number of X63 cells (Methods, section 2.5.4). The fused cell products were plated out onto thymocyte feeder cells (Methods, section 2.5.1) at a cell density of 1.6×10^5 cells/well, and grown in selection media containing HAT and

Table 15

Assay Results Of Direct Torpedo ELISA Performed On Culture Supernatants Obtained From EBV Transformed Lymphocytes

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.14	0.11	0.13	0.12	0.17	0.13	0.18	0.12	0.13	0.16	0.19	0.12
B	0.15	0.15	0.18	0.16	0.15	0.19	0.13	0.14	0.17	0.16	0.15	0.15
C	0.16	0.17	0.15	0.15	0.17	0.13	0.27	0.13	0.15	0.19	0.14	0.18
D	0.18	0.15	0.18	0.16	0.19	0.17	0.20	0.24	0.16	0.17	0.15	0.21
E	0.13	0.23	0.14	0.16	0.17	0.17	0.20	0.19	0.14	0.16	0.14	0.19
F	0.17	0.16	0.16	0.14	0.16	0.13	0.20	0.49	0.16	0.14	0.16	0.16
G	0.18	0.21	0.18	0.21	0.35	0.16	0.23	0.19	0.21	0.28	0.16	0.16
H	0.16	0.17	0.23	0.20	0.19	0.14	0.25	0.18	0.18	0.17	0.15	0.14

All marked wells were pooled and the pooled cells fused with an equal number X63 murine myeloma cells using PEG.

Table 16

Results Of Direct Torpedo ELISA On Culture Supernatants From Heterohybridomas

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.08	0.06	0.06	0.07	0.07	0.06	0.07	0.20	0.07	0.06	0.07	0.06
B	0.21	0.08	0.07	0.07	0.30	0.34	0.07	0.08	0.06	0.07	0.06	0.07
C	0.63	0.17	0.08	0.30	2.07	0.44	0.06	0.33	0.07	0.19	0.30	0.07
D	0.13	0.47	0.48	0.41	0.44	0.07	0.07	0.06	0.25	0.07	0.80	0.09
E	0.40	0.06	0.07	0.07	0.07	0.36	0.07	0.41	0.75	0.33	0.65	0.06
F	0.30	0.07	0.06	0.06	0.07	0.07	0.09	0.45	0.56	0.63	0.91	0.07
G	0.07	0.07	1.55	0.58	0.08	0.08	0.49	0.47	0.31	0.66	0.61	0.07
H	0.10	0.08	0.08	0.07	0.06	0.07	0.08	0.85	0.09	0.08	0.06	0.07

All marked wells were expanded into larger wells (1ml x 24) and re-tested after 9 days of growth.

Table 17

Assay Of Heterohybridoma Culture Supernatants Using The Direct Torpedo ELISA Following Expansion Into 1ml Wells

	1	2	3	4	5	6
A	0.03 B5	0.04 B6	0.28 C1	0.05 C4	0.64 C5	0.02 C6
B	0.07 C8	0.06 C11	0.06 D2	0.07 D3	0.17 D4	0.21 D5
C	0.06 D9	0.50 D11	0.20 E1	0.02 E6	0.12 E8	0.42 E9
D	0.05 E10	0.08 E11	0.20 F1	0.19 F8	0.19 F9	0.20 F10

	1'	2'	3'
A'	0.52 F11	0.22 G8	0.11 H8
B'	0.11 G3	0.03 G9	
C'	0.25 G4	0.32 G10	
D'	0.10 G7	0.02 G11	

All marked wells were expanded into 3ml tissue culture dishes prior to cloning. However, all dishes became infected.

Ouabain (Methods, sections 2.5.3 & 2.5.4) for 34 days. At this time, the supernatants from the wells were collected and assayed for anti-AChR antibody production, using the direct ELISA with Torpedo AChR (Methods, section 3.1). Of the 96 wells tested, 33 were positive (see Table 16) all of which were expanded into 24 well tissue culture plates. The hybridomas were grown for 9 days in these plates before being re-assayed both by the direct ELISA (Methods, section 3.1), using Torpedo AChR, and by RIA using human AChR (Methods, section 3.2). Of the original 33 positive wells assayed prior to expansion, only 12 of them reassayed as positive (see Table 17). When tested for reaction against human AChR, by RIA, none of the wells were positive. Of the 12 positive wells assayed 6 of them were expanded into 3ml tissue culture plates (A3, A5, B2, B6, C1, and BB1). At this stage, all of the expanded plates were lost to infection.

3. ANTIBODY CAPTURE ELISA FOR THE DETECTION OF ANTI-AChR ANTIBODIES USING BIOTINYLATED ALPHA-BGT

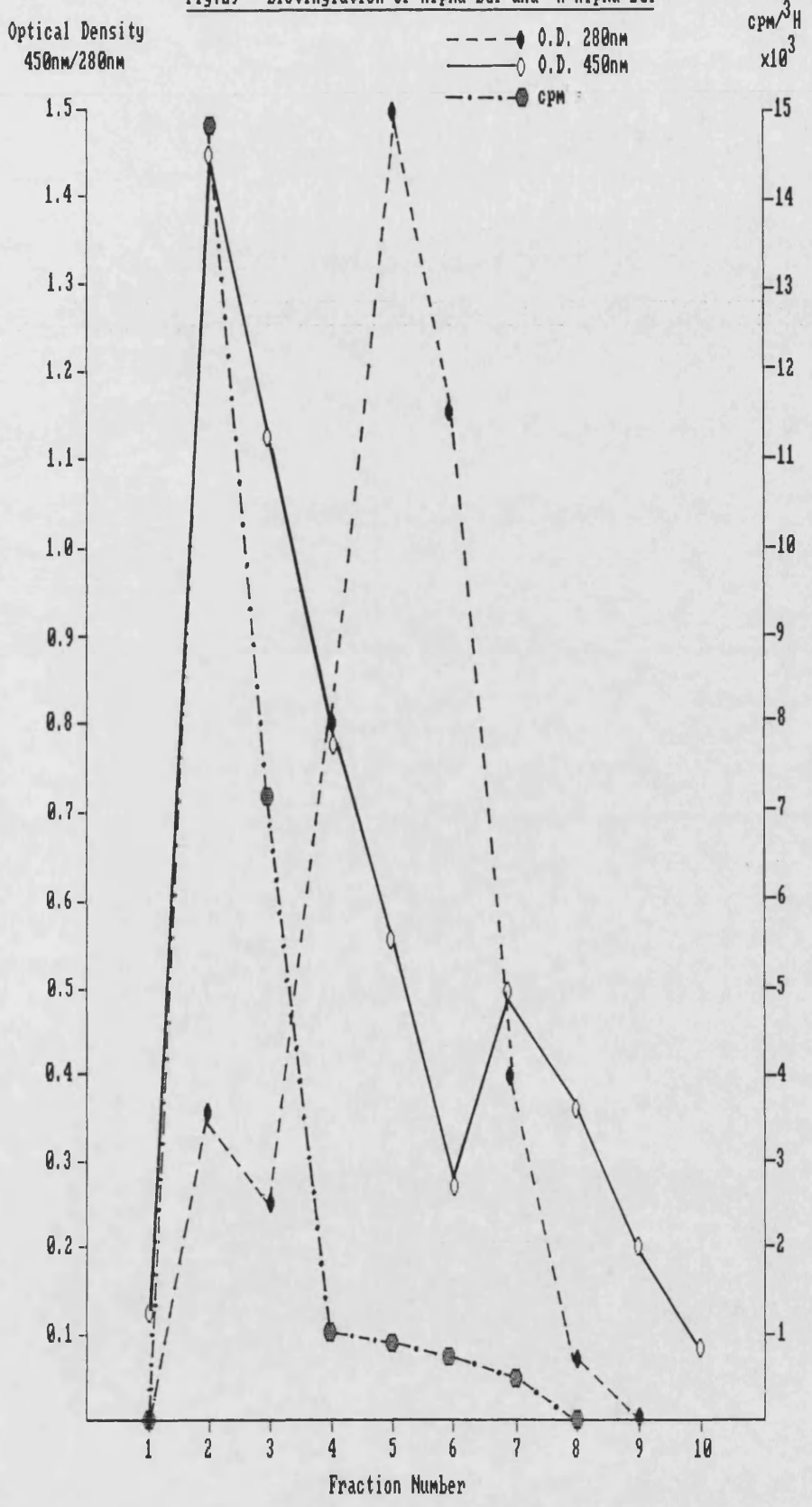
3.1 Biotinylation of Alpha-BGT

The B BGT prepared was stable at -20°C for at least 11 months, without loss of activity.

Six lots of conjugate were prepared (Methods, section 3.3.1) two by using dialysis, and four by using a Sephadex G25 column.

The optical density profile, at 280nm, of the fractions obtained from a typical G25 column (Fig. 29), shows two distinct peaks. In order to determine which peak contained BαBGT, ^{3}H-αBGT (50μl, 0.05 Ci/μl) was added to the biotinylation mixture before

Fig.29 - Biotinylation of Alpha-BGT and ^{3}H-Alpha-BGT



application to the G25 column. Fractions obtained from the column were then counted for radioactivity and tested for BαBGT activity by ELISA, using purified Torpedo AChR (Methods, section 3.3.2). The profiles obtained are shown in Fig. 29, in which it can be seen that the BαBGT is located entirely in the first of the two peaks. Fractions corresponding to this peak were accordingly collected in subsequent preparations.

3.2 Characterisation of BαBGT Species And Its Binding To Purified AChR

3.2.1 Sensitivity Of BαBGT Binding To Purified AChR

The binding of BαBGT to purified Torpedo AChR was examined by a solid phase assay as described in the Methods, section 3.3.2.i).

Concentrations of BαBGT as low as 0.6pmol/ml (1/51,200 dilution) can readily be detected by this assay, a feature of which is the low background (OD $<$ 0.05) observed in the absence of BαBGT (Fig. 30). For subsequent studies, dilutions of 1/500 were chosen for both the BαBGT and the HRP-avidin, representing concentrations of 0.5μg/ml and 1μg/ml respectively.

Comparison of BαBGT with ^{125}I-αBGT in a radioimmunometric AChR binding assay (Methods, section 3.3.2.1) shows that the BαBGT is more sensitive at detecting lower AChR concentrations than equivalent amounts of ^{125}I-αBGT (Fig. 31).

Fig. 30 - Interaction Of B-alpha-BGT With Torpedo AChR Coated Mictrotitre Wells

Checkerboard titration of B-alpha-BGT concentration and HRP-avidin concentration: 1:300 (●), 1:900 (■), 1:2700 (◆) and 1:8100 (▲). Coating antigen concentration used 5μg/ml.

Concentrations of stock solutions of HRP-avidin and B-alpha-BGT were 500μg/ml and 250μg/ml respectively.

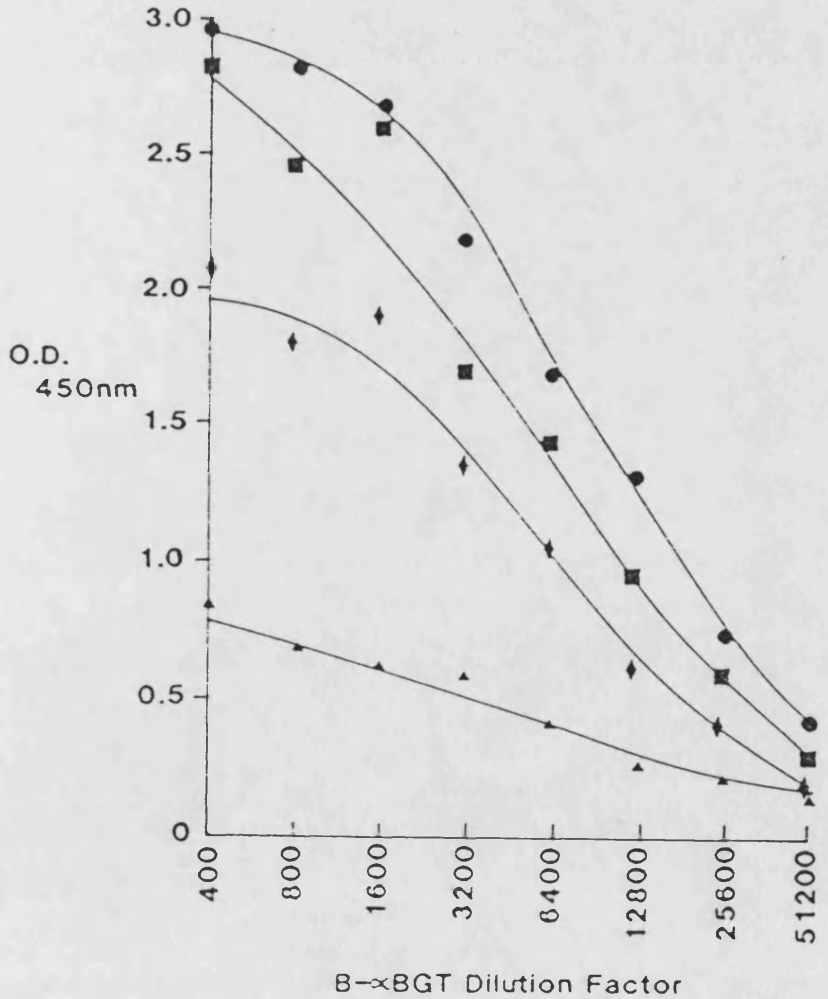
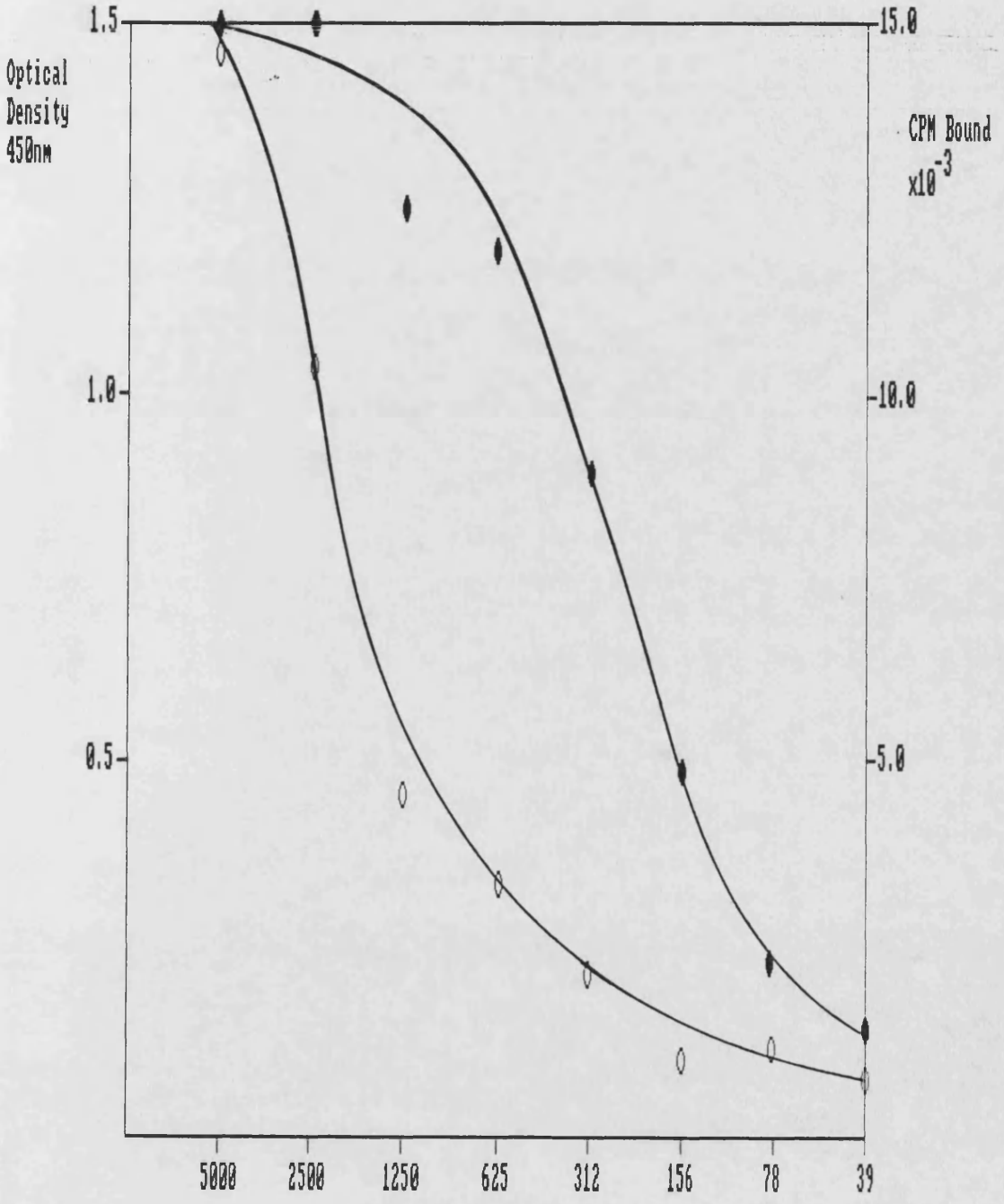


Fig.31 - Comparison Of Binding Of B-Alpha-BGT (1:500 dil.)(♦) And ^{125}I-Alpha-BGT (0,56 pmol)(○) to microtitre wells Coated With Varying Concentrations Of AChR.



3.2.2 Specificity Of BαBGT Binding To Purified AChR

Specificity of BαBGT binding was investigated by the use of a solid phase assay, using purified Torpedo AChR, in the presence of various inhibitor ligands (Methods, section 3.3.2.ii).

The binding of BαBGT to AChR was specific, as shown by its inhibition with unlabelled αBGT and the cholinergic ligands, nicotine, benzoquinonium and decamethonium (Fig. 32).

3.2.3 Determination Of The Number Of Biotin Molecules Per αBGT Molecule

The number of biotin molecules bound per αBGT molecule was determined by employing a colorimetric assay developed by Green (1965, Methods, section 3.3.2.iii). This involved using a standard biotin solution to obtain a standard curve from which unknowns could be determined (Fig.33).

All the conjugates produced in this study contained 4-5 biotin molecules per molecule of αBGT as determined by this assay. The biological activity and affinity of the BαBGT for Torpedo AChR was not determined in this study but they were taken to be 70% and 25% respectively, to that of the native toxin, as shown by Lukasiewicz et al (1978). This will be discussed in greater detail in the discussion.

3.3 Antibody Capture Enzyme Linked Immunosorbent Assay (ELISA)

3.3.1 Binding Of Murine Anti-AChR Monoclonal Antibodies To Purified Torpedo AChR

The binding of the various monoclonal antibodies available, was investigated by means of a direct ELISA, so as to determine

Fig.32 - Inhibition Of B-Alpha-BGT To AChR By Various Ligands

B-Alpha-BGT - 1:500 dil. HRP-avidin - 1:500 dil.
unlabelled Alpha-BGT - ♦
Benzoquinonium - ○
Decamethonium - ■
Nicotine - □

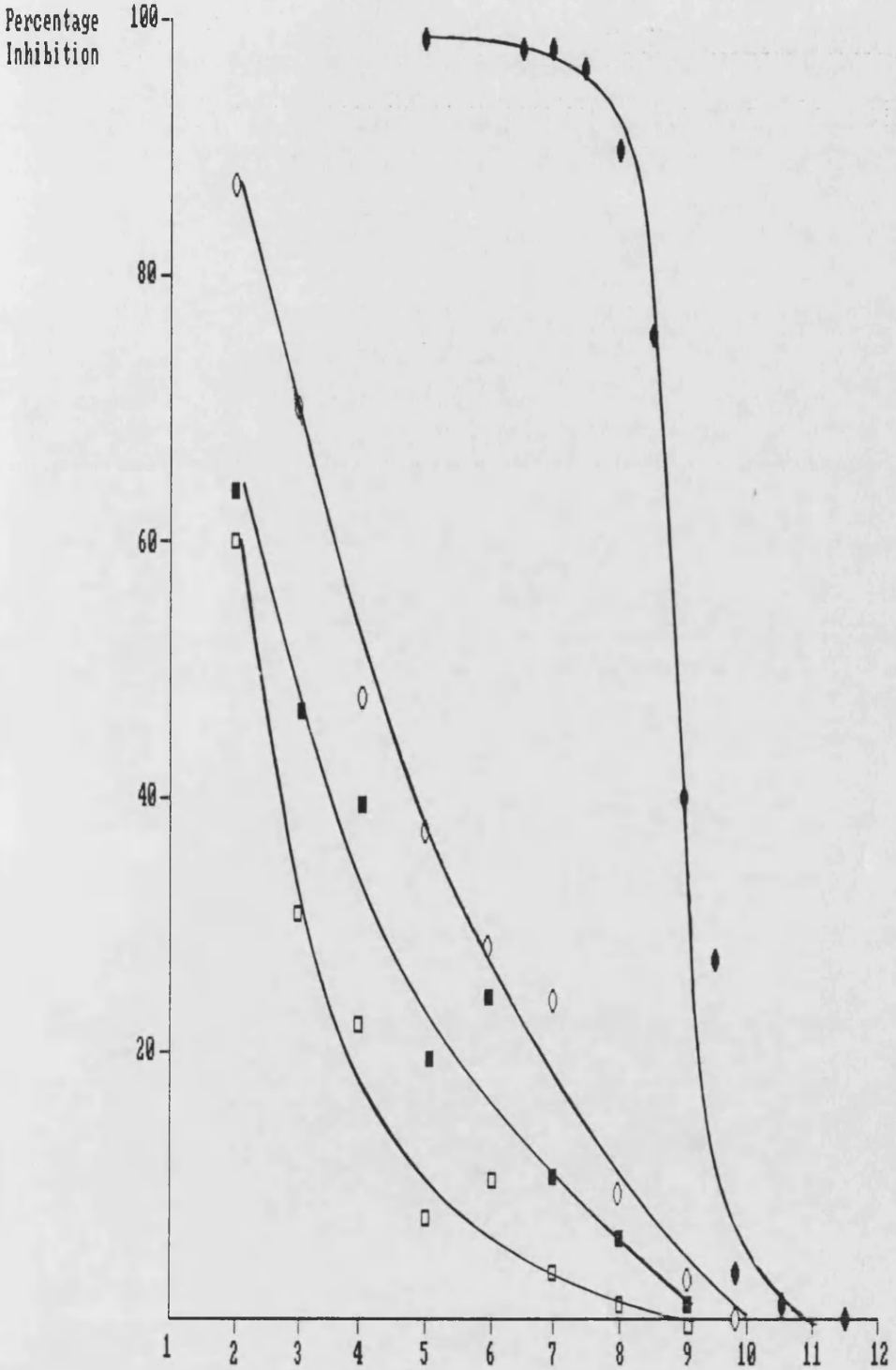
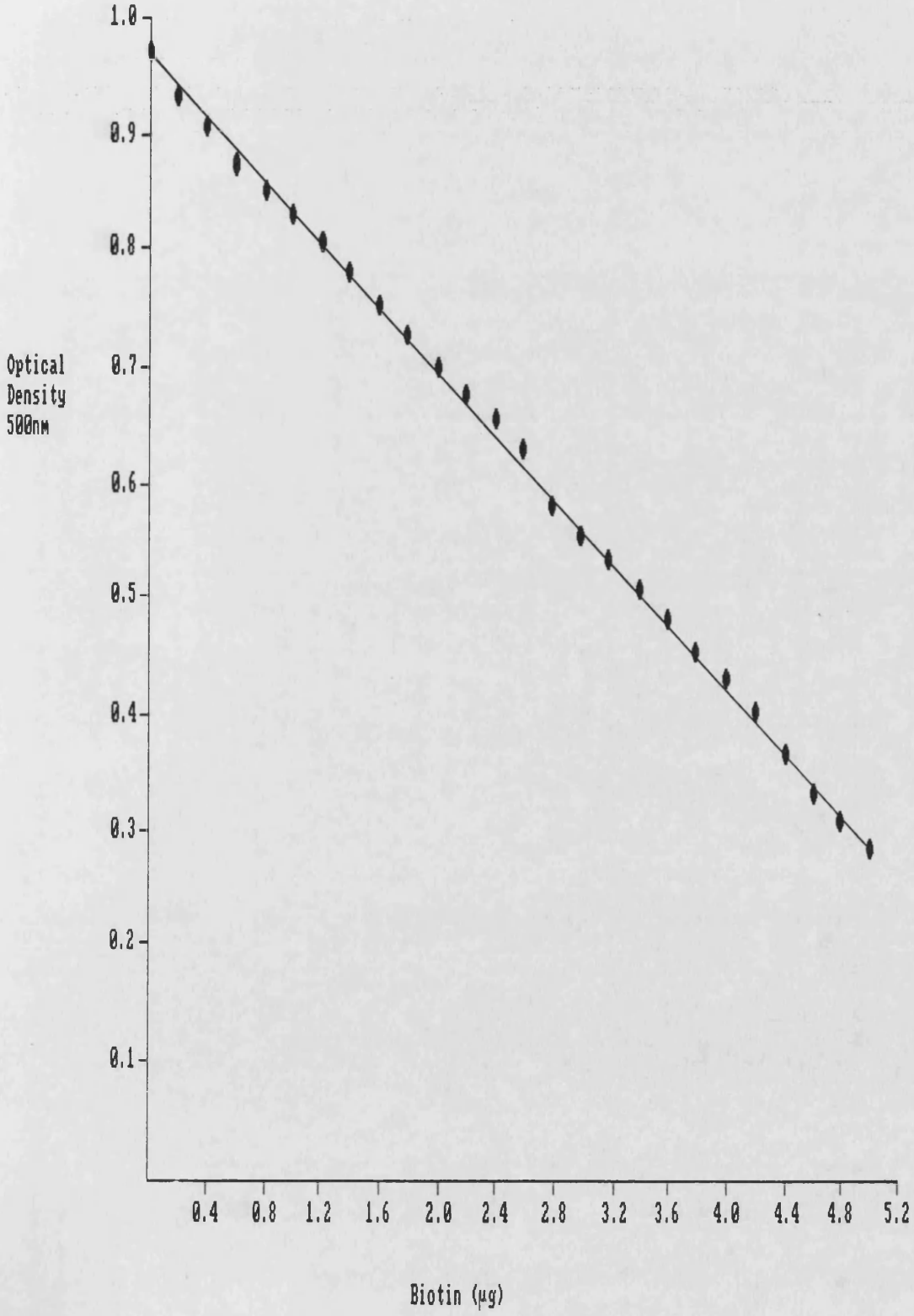


Fig.33 - Standard Curve Used For The Determination Of The Number Of Biotin Molecules Attached Per Alpha-BGT Molecule



which monoclonal antibodies would be suitable for further study (Methods, section 3.3.3.i).

Six monoclonal antibodies (C7, C11, B11, E8, A3 and E11) were investigated, together with an anti-neurofilament monoclonal antibody, used as a control. From the ELISA results (Fig. 34) it was decided to use monoclonals C7, C11, B11, and E8, for further study. The properties of these monoclonals are also shown in Table 18, from which it can be seen that all four monoclonals are of the IgG-2b subclass, and have IgG concentrations, in the undiluted supernatant, ranging from 7.5 - 15μg/ml. They all reacted against Torpedo AChR, but only monoclonals C11, B11, and E8, reacted against fetal calf AChR.

3.3.2 Antibody Capture ELISA

The antibody capture assay for anti-AChR antibodies was assessed in terms of the detection of monoclonal antibodies in supernatants from the four murine hybridomas C7, C11, B11 and E8.

The ELISA procedure allowed reproducible detection of anti-AChR antibodies against Torpedo AChR (Fig. 35) and fetal calf AChR (Fig. 36), over a wide range of antibody dilutions from 1/100 to 1/5120, for all four monoclonals. This represents detection of antibody levels from 15 - 0.2ng/ml IgG (Table 19 gives an indication of the minimun detection levels).

Direct comparison of the antibody capture ELISA with the conventional double antibody radioimmunoassay, using the

Fig.34 - Direct Torpedo ELISA For Anti-AChR Monoclonal Antibodies

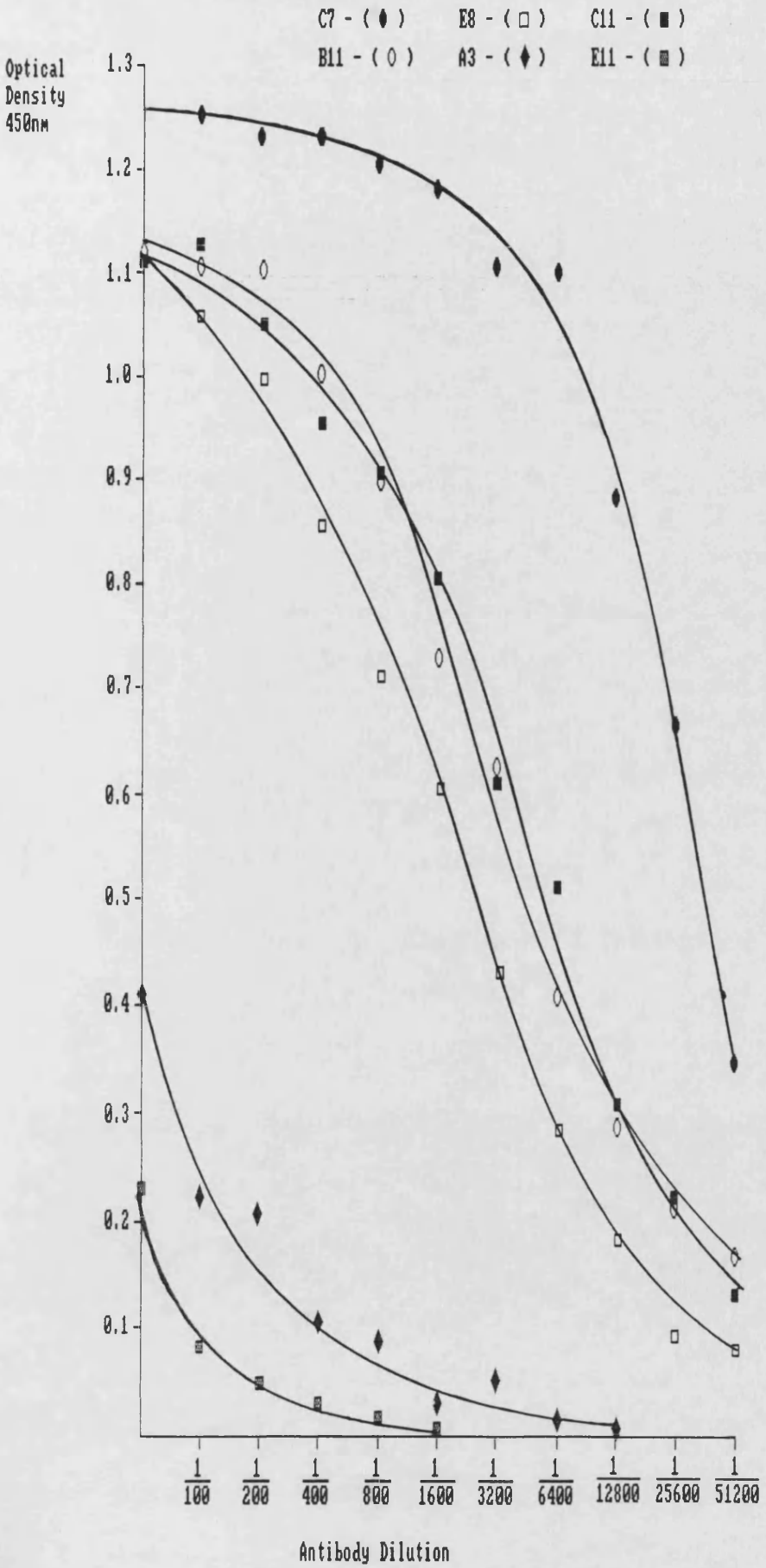


TABLE 18

PROPERTIES OF MOUSE MONOCLONAL ANTI-AChR ANTIBODIES

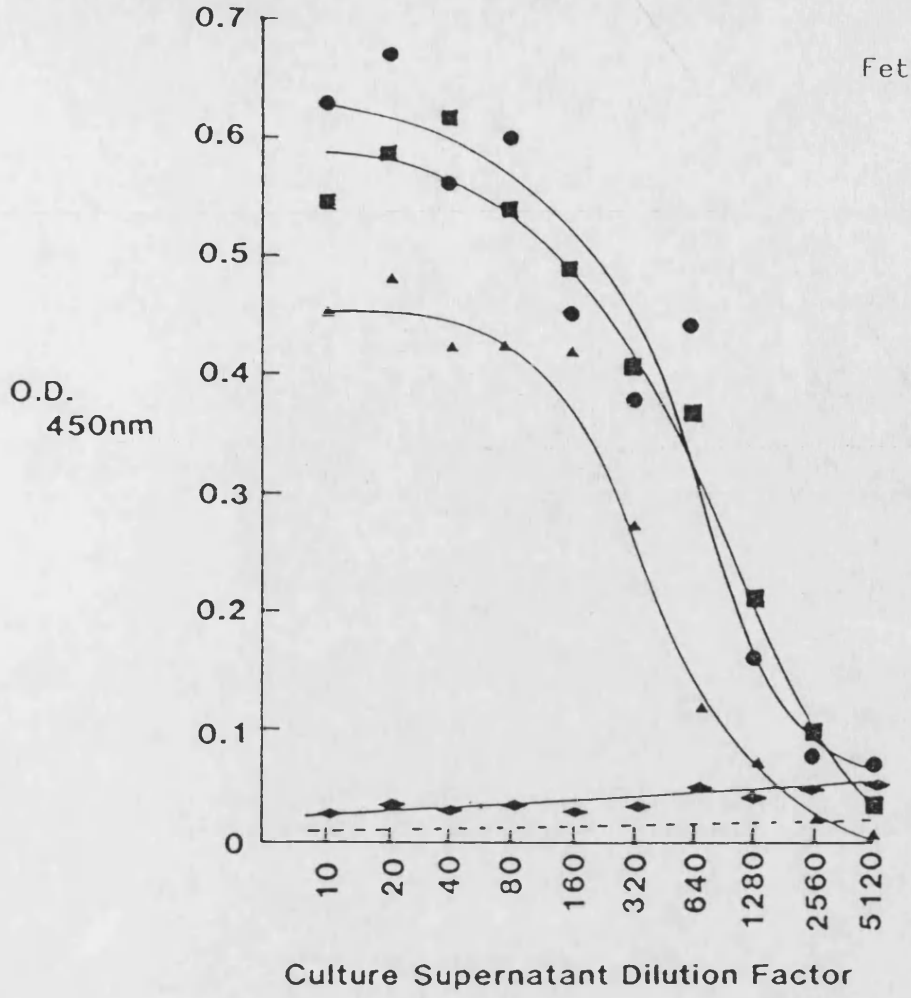
Antibody	Subclass	IgG concentration (μg/ml)	Anti-AChR antibody titre of supernatant (nM)[a]	
			Torpedo AChR	Fetal calf AhR
C7	1gG2b	10.0	110	ND[b]
C11	"	8.0	139	0.75
B11	"	7.5	180	4.50
E8	"	15.0	301	2.32

a) titre detected by immunoprecipitation radioimmunoassay (Carter et al, 1981) and expressed in terms of αBGT binding sites.

b) ND = not detected

Fig. 35 & 36 - Titration Of Anti-AChR Antibodies

Fig. 35 and 36 Antibody-capture ELISA for the detection of antibody binding to Torpedo AChR and Fetal Calf AChR, respectively. Monoclonal antibody culture supernatants used were C7 (♦), C11 (●), B11 (■), E8 (▲), and anti-neurofilament (----). Values are the average of triplicate samples. Percentage standard deviation for each reading was less than 20%.


Fetal Calf AChR
O.D.
450nm
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
10
20
40
80
160
320
640
1280
2560
5120
Culture Supernatant Dilution Factor

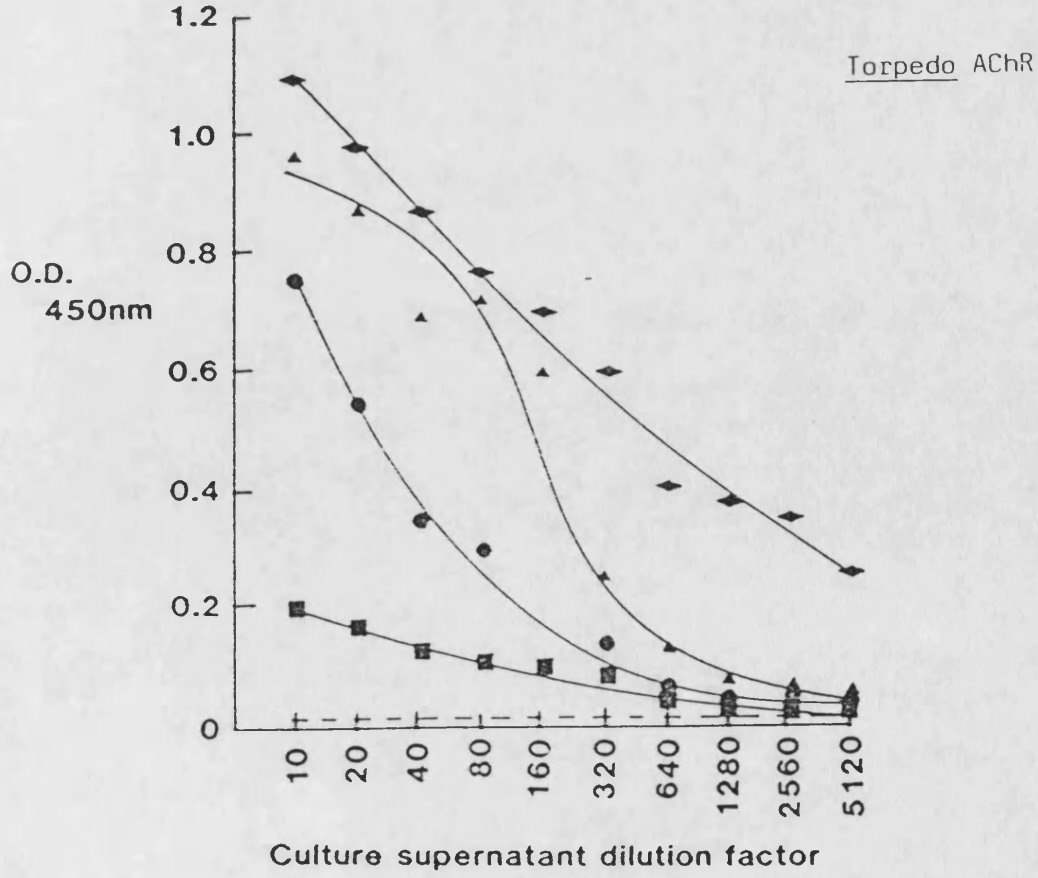

Torpedo AChR
O.D.
450nm
1.2
1.0
0.8
0.6
0.4
0.2
0
10
20
40
80
160
320
640
1280
2560
5120
Culture supernatant dilution factor

TABLE 19

TITRATION OF MOUSE MONOCLONAL ANTI-AChR ANTIBODIES BY ELISA

Antibody	Minimum detection levels			
	Torpedo AChR		Fetal calf AChR	
	Supernatant dilution	IgG conc. (ng/ml)	Supernatant dilution	IgG conc. (ng/ml)
C7	1/5120	1.95	N.D. (a)	N.D.
C11	1/320	25.0	1/2560	3.1
B11	1/80	94.0	1/2560	2.9
E8	1/640	23.4	1/1280	11.7

(a) N.D. not detected

monoclonal antibody C7, showed the ELISA to be considerably more sensitive, detecting minimum levels of IgG of 0.2 ng/ml compared with 25 ng/ml for the radioimmunoassay (Fig. 37).

The ELISA also confirmed the lack of cross reactivity of monoclonal antibody C7 against fetal calf AChR, which was initially indicated by the radioimmunoassay (see Table 18).

The specificity of BαBGT binding to AChR was further demonstrated by assaying a monoclonal anti-neurofilament antibody, as a control, which showed no response against either type of AChR, and was further shown, by the fact that responses in the assay to anti-AChR antibodies were completely abolished in the presence of 20mM decamethonium bromide, as in the inhibition assay already described (see Methods, section 3.3.3.ii).

3.3.3 Optimisation Of Assay Conditions

In order to develop the antibody capture ELISA further for use in detecting human anti-AChR antibodies produced either by Epstein-Barr virus transformed human lymphocytes, or human/murine heterohybridomas in culture, the various assay conditions were optimised by using C7 monoclonal antibody.

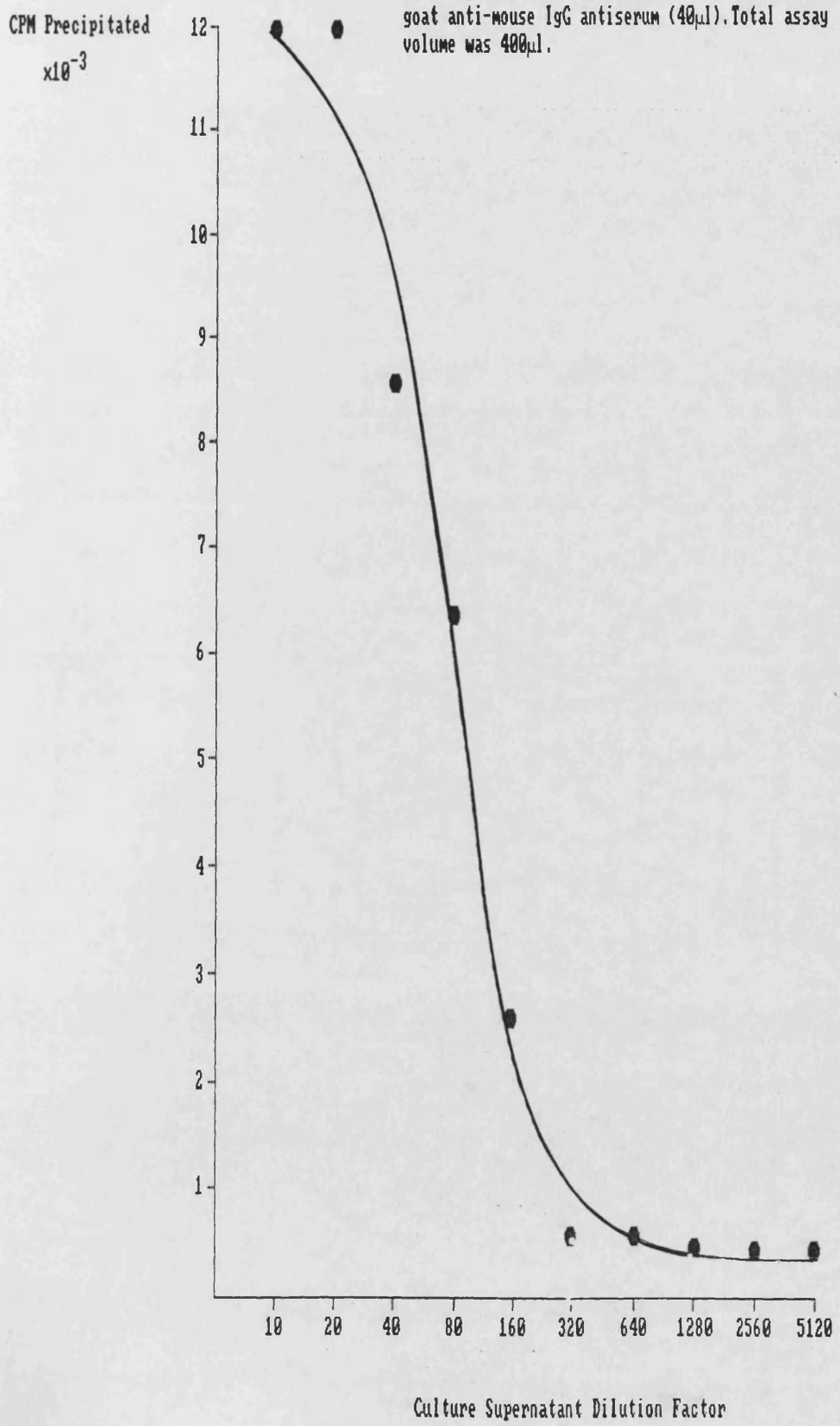
i) Optimisation Of Coating Antibody Concentration And Monoclonal Antibody Dilution

Concentration of coating antibody and monoclonal antibody dilution were both examined in a checkerboard assay.

In this assay, microtitre module strips were coated with

Fig.37 - Double Antibody RIA For The Detection Of Monoclonal Antibody, C7 (●) Binding to Torpedo AChR (0.1 pmol)

Varying amounts of C7 supernatant and mouse normal serum (5µl) were added and the ^{125}I-alpha-BGT-AChR-antibody complex was precipitated by the addition of goat anti-mouse IgG antiserum (40µl). Total assay volume was 400µl.



increasing amounts of goat anti-mouse IgG (gamma specific) antibodies, from 0 - 10μg/ml, in coating buffer overnight at 4°C. The wells were then incubated with varying dilutions of C7 monoclonal antibody for 2-3h at room temperature, and processed as before, except that the HRP-avidin was made up in PBS, 0.05% Tween-20 buffer without casein (see Discussion for explanation).

It was found that a coating antibody concentration of 5μg/ml was sufficient to saturate the binding capacity of the wells (Fig. 38). From the same assay a monoclonal antibody dilution of 1/400 was adopted for use in successive assays.

It was observed whilst carrying out these assays that in certain wells set up as negative and positive controls that very high background optical densities were recorded. This was something not normally seen, as one of the main advantages of the assay is the low background levels ($<$ 0.05 O.D. units). This observation was attributed to the presence of casein as a blocking agent (see Discussion)

These wells had been blocked with the casein buffer, but did not have any coating antibody attached, nor were they incubated with the monoclonal antibody, or Torpedo AChR. They were, however, incubated with BαBGT and/or HRP-avidin. Both these test wells gave equivalent O.D. values, a fact attributed to an interaction of the HRP-avidin with the casein bound to the wells (see Discussion).

Fig.38 - Effect Of Coating Antibody Concentration Varied With Regard To Monoclonal Antibody (C7) Dilution

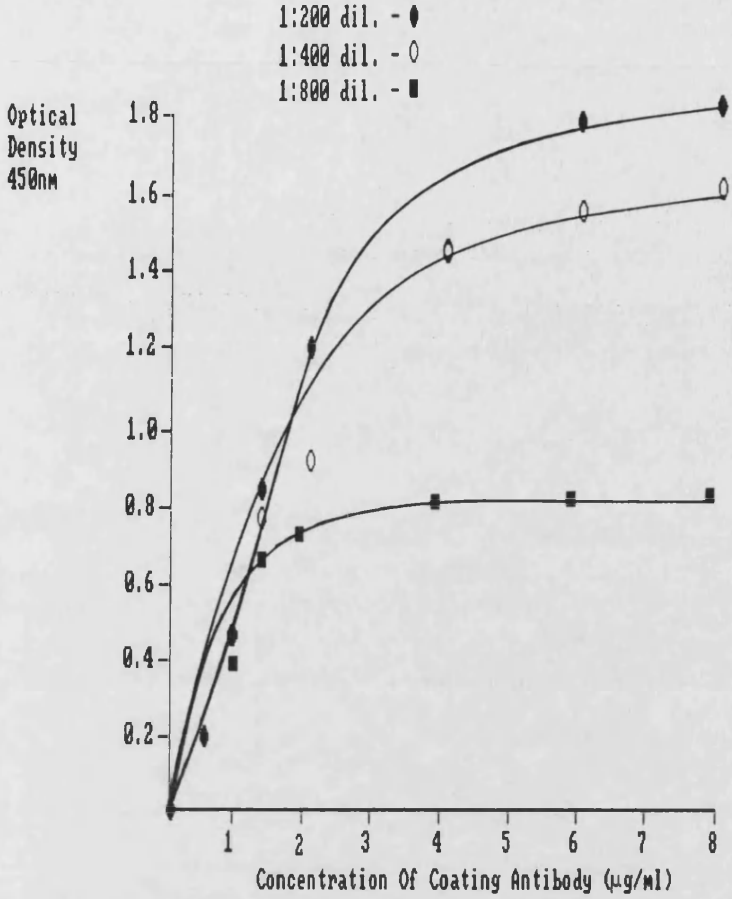
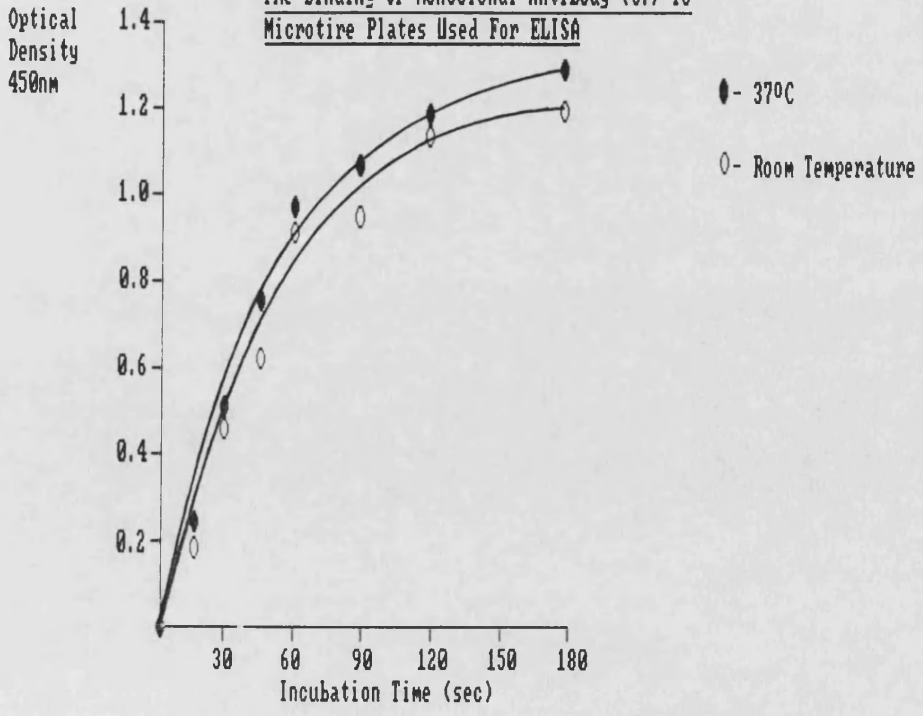


Fig.39 - Effect Of Incubation Time And Temperature On The Binding Of Monoclonal Antibody (C7) To Microtire Plates Used For ELISA



As it appeared that the casein used in the buffers was interfering with the assay, steps were taken to alter the assay/ blocking buffer. Various systems were investigated, all were PBS based, but differed in the nature of the component used to reduce the non-specific binding. These components included 1% (v/v) Tween-20, 1% BSA, 5% fetal calf serum, or 5% normal goat serum. However, they were all found to be ineffective blockers, again giving high background levels.

The final buffer system examined was PBS containing 0.05% (v/v) Tween-20, and 0.1 - 0.2% (v/v) fish skin gelatin as the non-specific blocker. This was found to be a very effective blocker, especially when used in conjunction with Streptavidin horseradish peroxidase conjugate (see Discussion). A combination of both these changes resulted in a reduction of the background levels down to the levels previously seen ($<$ 0.05 O.D. units), and at the same time the sensitivity of the assay appeared to increase. These changes were therefore adopted for subsequent assays.

ii) <u>Optimal Incubation Times And Temperatures For C7 Monoclonal Antibody</u>

Incubation times and temperatures were altered in order to determine conditions for maximum binding of the C7 monoclonal antibody to the ELISA plates.

In the assay, wells were incubated with culture supernatant containing C7 monoclonal antibody (1/400 dilution) from between

0 - 3h, at two different temperatures (room temperature (23°C and 4°C).

It was found that there was no significant difference between carrying the incubation out at toom temperature or at 4°C (Fig. 39). Maximum binding of the monoclonal antibody to the coated wells was achieved after approximately 2h incubation.

It was therefore decided to carry out the incubation for 2-3h at room temperature, for subsequent assays.

iii) **Optimal Binding Of Torpedo AChR**

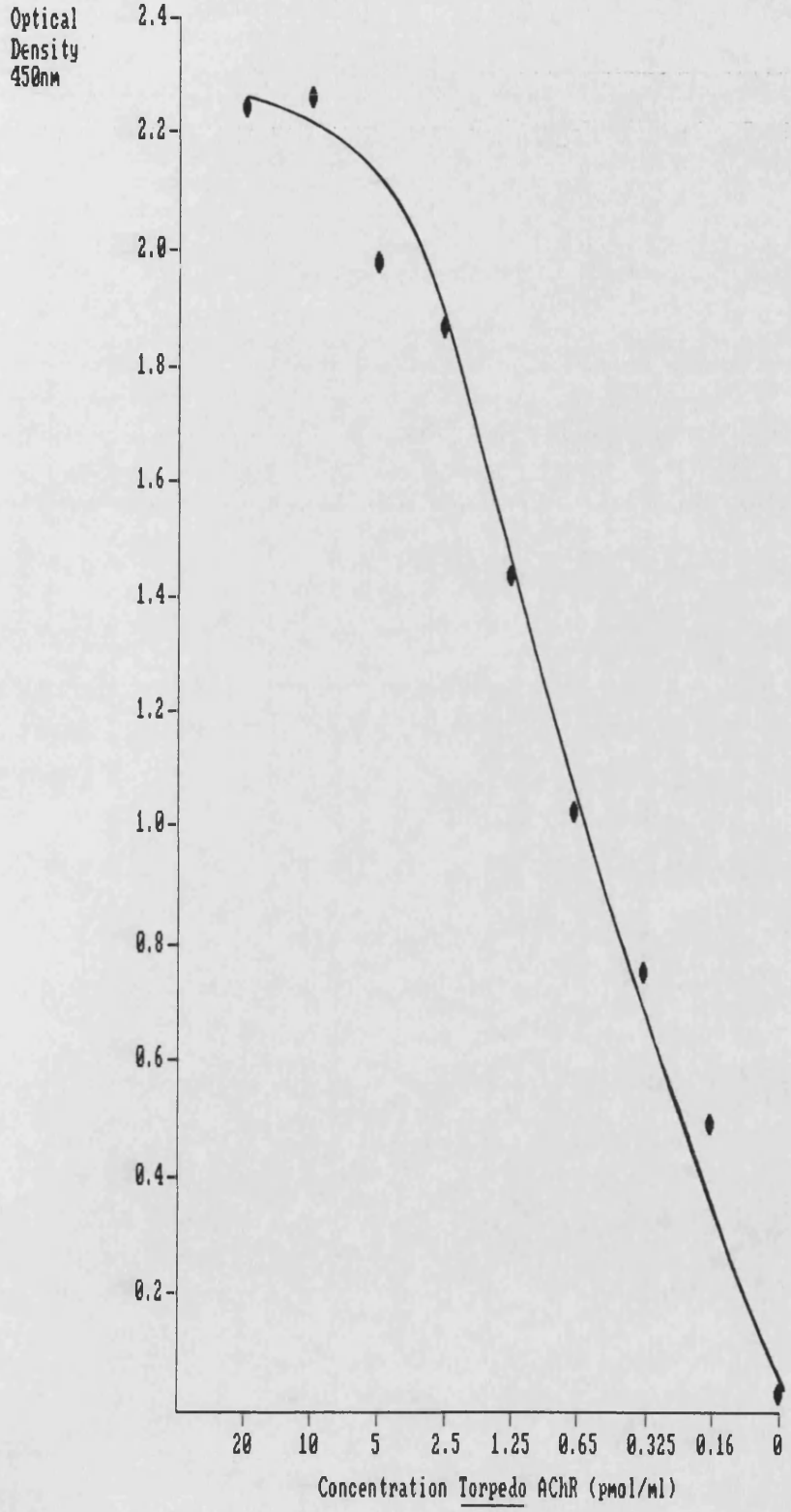
Receptor concentration was examined along with incubation times and temperatures.

In the first assay, after the wells had been incubated with the monoclonal antibody, they were incubated with increasing amounts of a crude Torpedo AChR (0-20pmol/ml, 100μl/well) overnight at 4°C, before being processed as already described. A dilution profile was obtained (Fig. 40), which can be seen to be linear over the range of AChR concentrations, 0-5pmol/ml, with saturation occuring at a receptor concentration of 5 pmol/ml.

Of note from the profile, is that receptor levels as low as 0.1 pmol/ml give measurable O.D. values above background, and that a receptor concentration of 1 pmol/ml gives O.D. readings 1.0 optical density units, indicating that the assay has a high sensitivity at low receptor concentrations. This becomes important if mammalian/human receptor preparations are to

Fig.40 - Dilution Profile For Torpedo AChR Binding In ELISA

Assay carried out using C7 mab (1:400 dil.), Streptavidin-HRP conjugate (1:500 dil.), and B-alpha-BGT (1:500 dil.).



be used in the assay, as they frequently only have receptor levels of approximately 1.0 pmol/ml.

In the second assay, receptor binding was examined with a view to optimising incubation times and temperatures.

After incubation of the wells with monoclonal antibody, they were incubated with a crude preparation of Torpedo AChR 5 pmol/ml, 100μl/well) at either room temperature over a time period of 0-3h, or at 4°C over a time period of 0-20h.

Figure 41 indicates that binding at room temperature is complete after approximately 3h, whereas at 4°C it is complete only after the overnight incubation. It would also appear that the incubation at 4°C results in a slightly higher final optical density than the incubation at room temperature.

From both these assays, it was decided to carry out the incubation with a receptor concentration of 5 pmol/ml, and overnight at 4°C.

iv) Optimal Concentrations Of BαBGT And Streptavidin Peroxidase

Several assays were carried out for both species, so as to determine optimal concentrations to use in the assay, and to determine incubation times and temperatures.

With regard to BαBGT, once the wells had been incubated with receptor, they were incubated with varying dilutions of BαBGT (1/50 - 1/1638400 dilution, 100μl/well) for 2h at room temperature. They were then incubated with Streptavidin peroxidase (1/200 dilution, 100μl/well) for a further 2h at room temperature, prior to development with substrate. From this, a dilution profile for BαBGT was obtained (Fig. 42), from which it

Fig.41 - Effect Of Incubation Times And Temperature On The Binding Of Torpedo AChR To Microtitre Plates In The Antibody Capture ELISA For Anti-AChR Antibodies

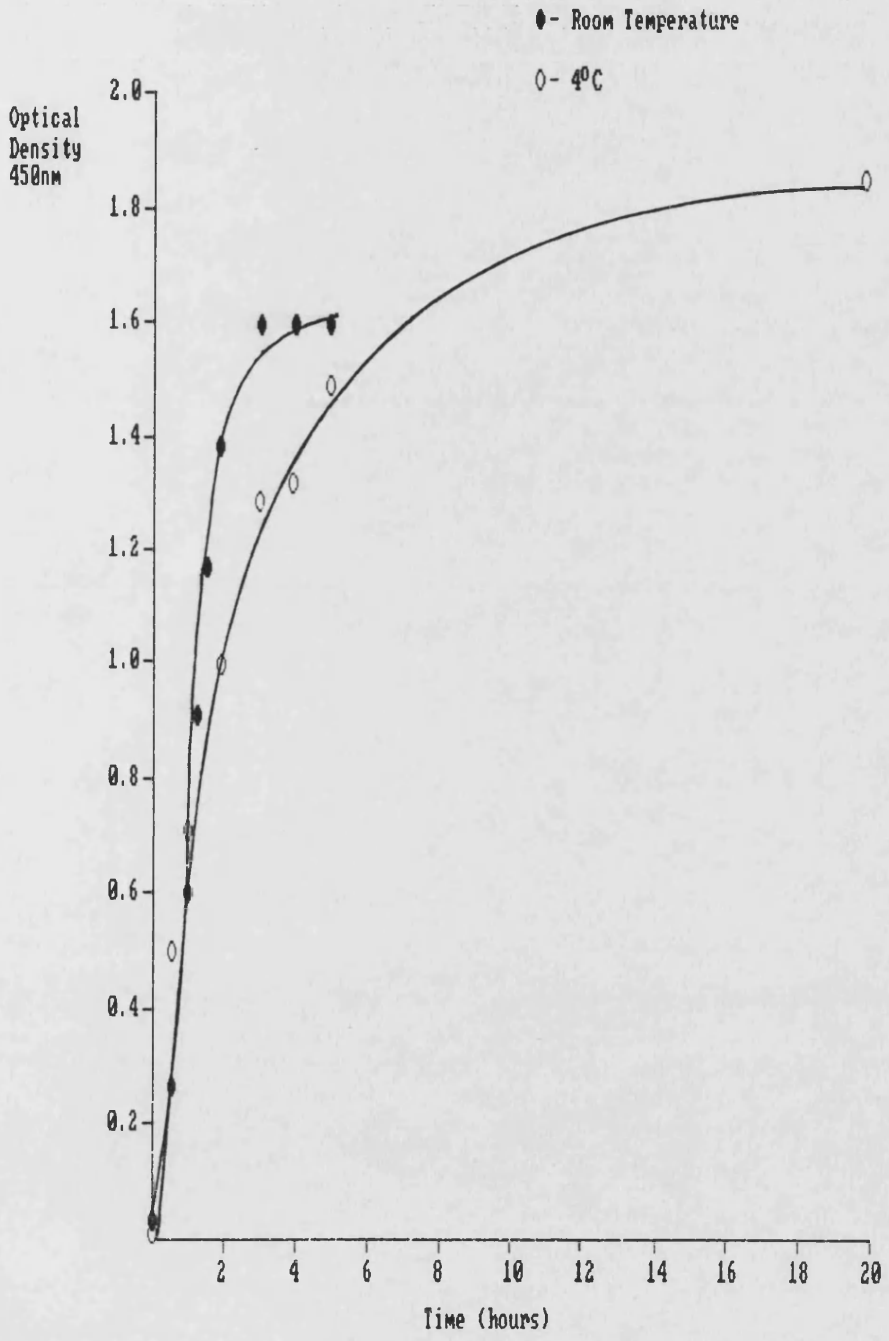
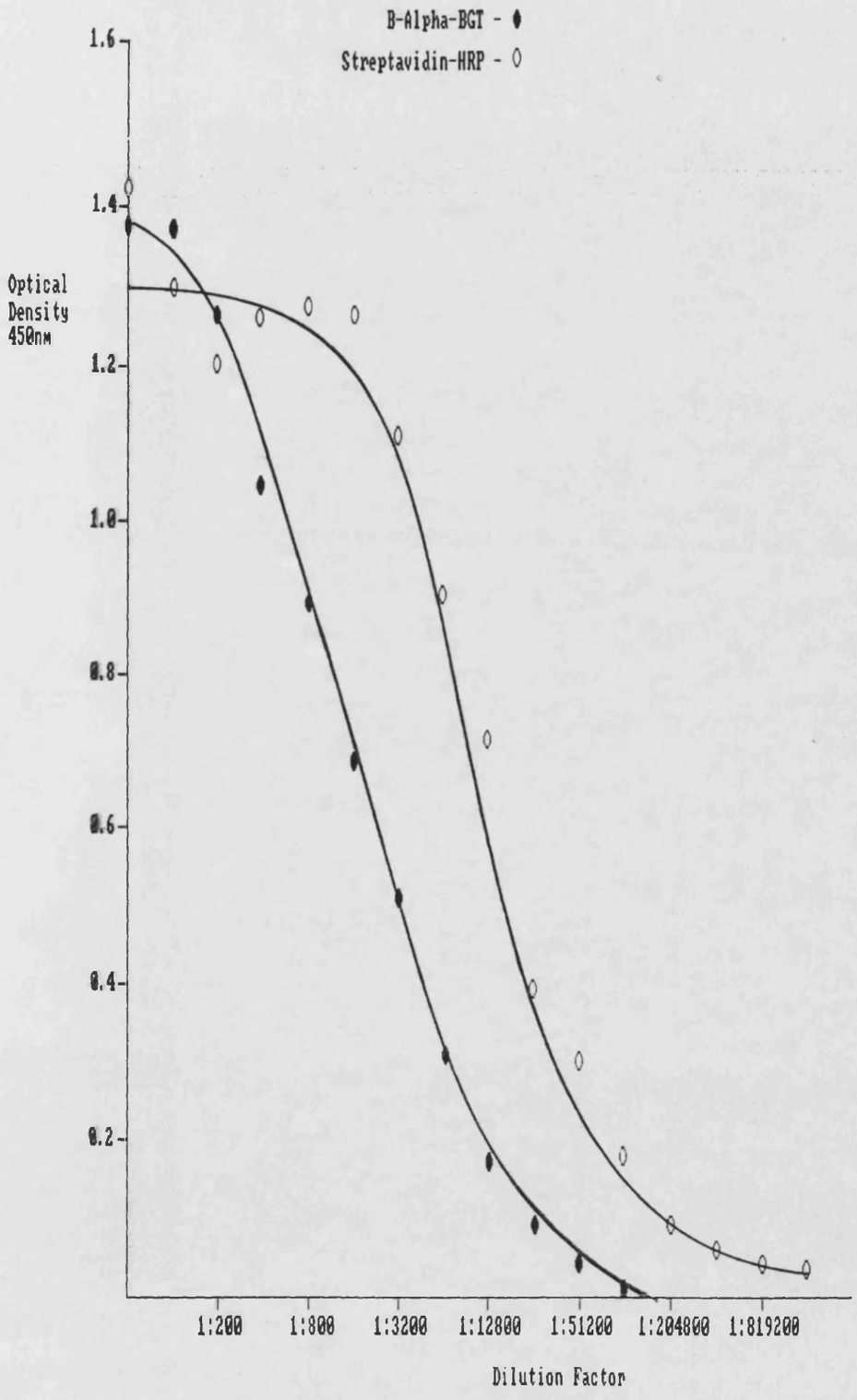


Fig.42 - Dilution Profiles For Both B-Alpha-BGT And Streptavidin-HRP In The Antibody Capture ELISA Used For The Detection Of Ant-AChR Antibodies



can be seen that the maximum response is obtained by using a BαBGT dilution of 1/800, but to ensure a slight excess when carrying out the assay, a 1/500 dilution was used in further assays.

A similar dilution profile (Fig. 42) was obtained for the use of Streptavidin peroxidase, but in this case, after the wells had been incubated with AChR, they were incubated with a 1/500 dilution of BαBGT for 2h at room temperature, prior to the addition of varying dilutions of Streptavidin peroxidase (1/50 - 1/102400, 100μl/well, 2h at room temperature). From this profile it was decided to continue to use a 1/200 dilution of Streptavidin peroxidase in conjunction with a 1/500 dilution of BαBGT.

Having determined optimum incubation concentrations for both species, assays were carried out to determine optimal incubation times and temperatures.

In both cases the assays were carried out at 37°C and at room temperature, over the time period of 0-3h, using the concentrations already mentioned above. With regard to the BαBGT (Fig. 43), there appears to be no difference between binding at 37°C and at room temperature. However, with Streptavidin peroxidase (Fig. 44) there does appear to be a temperature effect, in that the assay carried out at 37°C gives lower optical density values than those obtained in the assay carried out at room temperature. In both cases, maximum response

Fig.43 - Effect Of Incubation Time And Temperature On The Binding Of B-Alpha-BGT

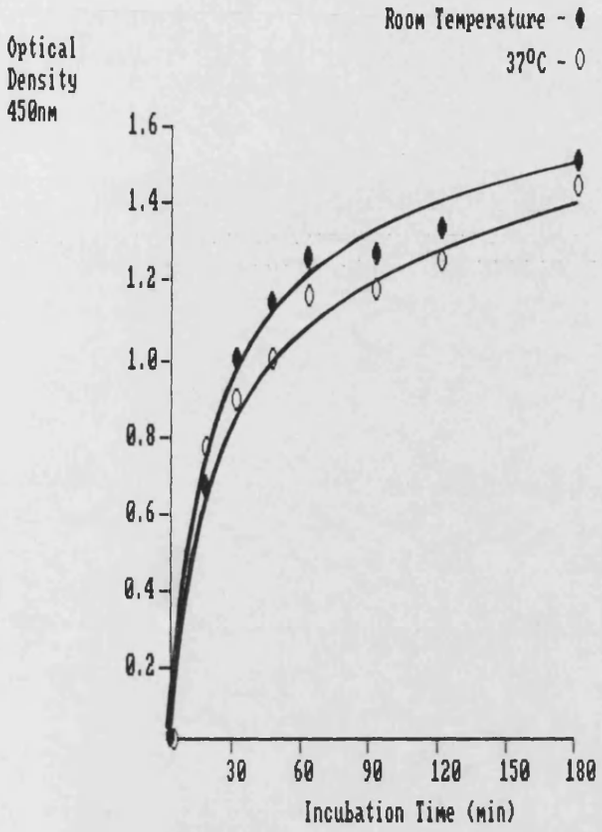
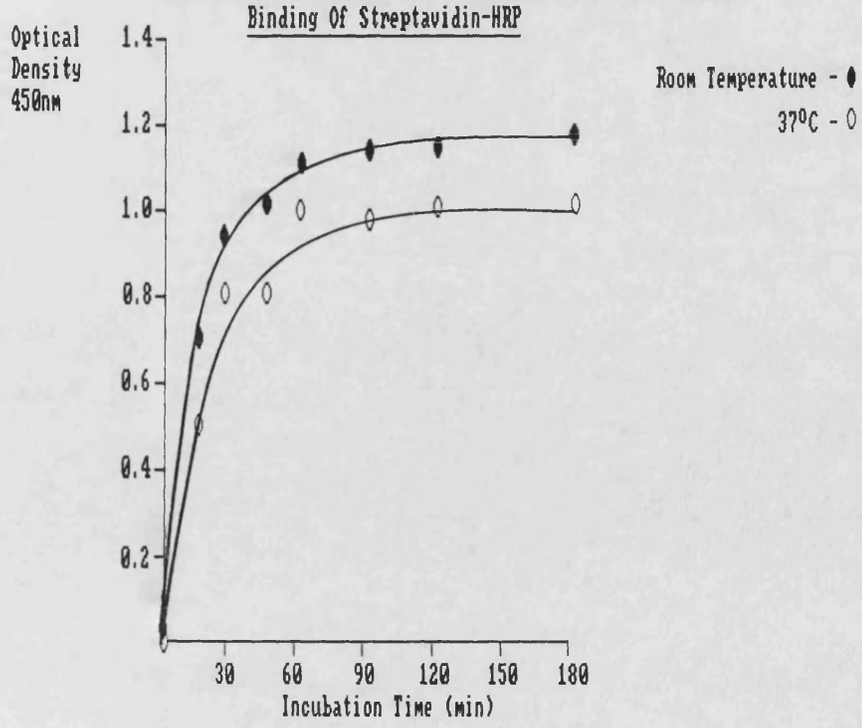


Fig.44 - Effect Of Incubation Time And Temperature On The Binding Of Streptavidin-HRP



is obtained after a 2-3h incubation with either species.

From these assays it was decided to carry out further studies using an incubation time of 2-3h at room temperature for both species, and to use a 1/500 dilution of BαBGT, and a 1/200 dilution of Streptavidin peroxidase.

3.3.4 Antibody Capture ELISA To Detect Anti-AChR Antibodies In Myasthenic Sera

The antibody capture ELISA, developed to detect anti-AChR antibodies in culture supernatants, was used to detect human anti-AChR antibodies found in the sera of patients suffering from Myasthenia gravis. These antibodies are normally detected by the use of the conventional double antibody radioimmunoassay procedure (Table 20).

The sera from five Myasthenic patients were chosen for study, as they represented a typical cross section of antibody titres(Table 20), as detected by radioimmunoassay. Pooled normal human sera was also included as a control. Titres are normally compared to this serum which has a titre of 0 - 3.3 x 10^{-10}M.

In assays for anti-AChR IgG antibodies, (i.e. plates coated with goat anti-human IgG (gamma specific antibodies) there was no detectable reaction from any of the sera tested, even from the high titre IgG sera.

However, this was not the case with the assays attempting to detect IgM anti-AChR antibodies (i.e. plates coated with

TABLE 20

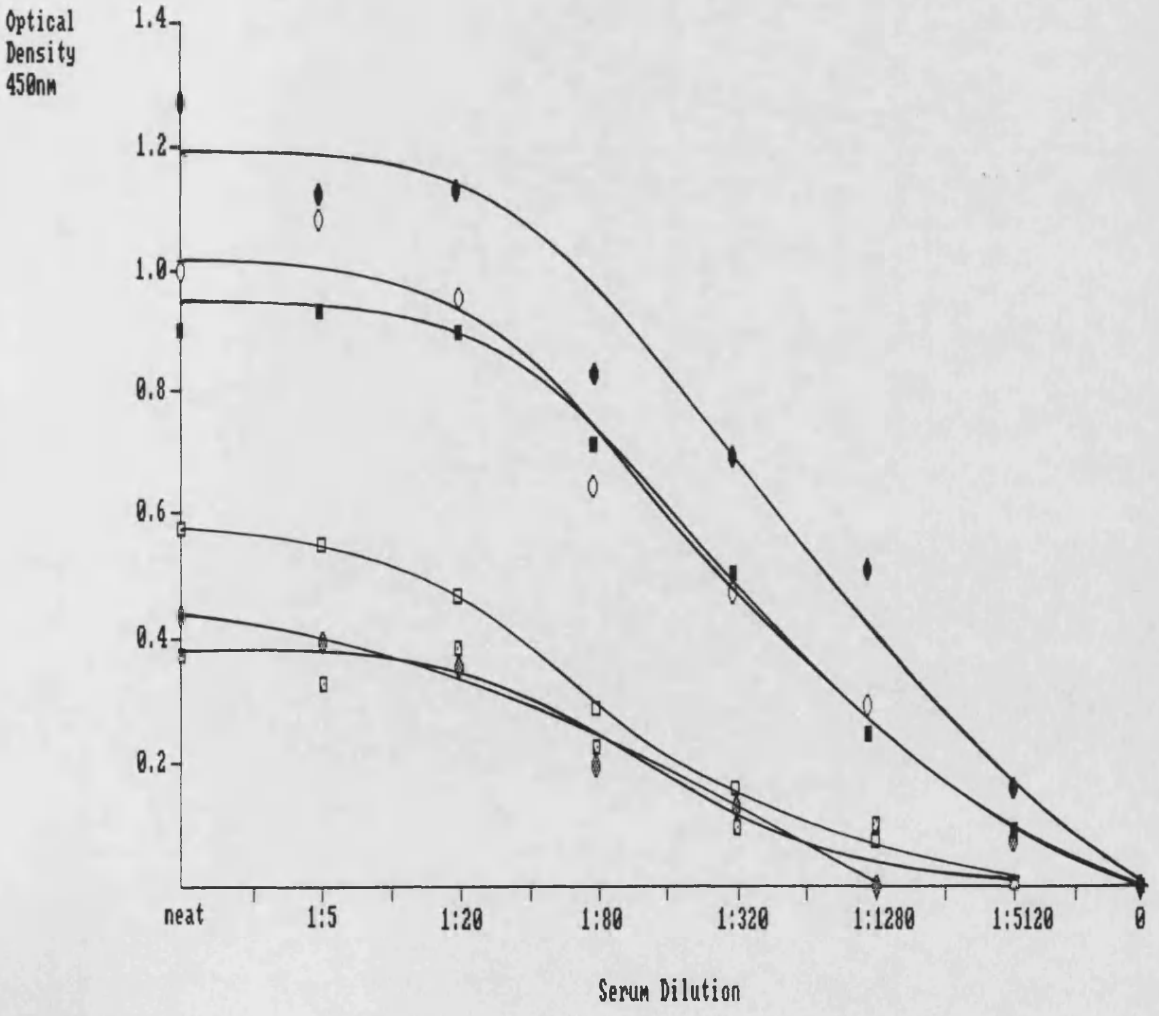
COMPARISON OF ANTI-AChR ANTIBODY TITRES OF DIFFERENT PATIENTS AS DETERMINED BY CONVENTIONAL RADIOIMMUNOASSAY

Patient	Titre x 10^{-10}M	Duration of Disease	Myotoxicity % lysis
N.H.S	0 - 3	N/A	10 - 20%
RATHBONE	35	5yrs	ND
SHAW	22 - 25	8yrs	ND
HARWOOD	199	8yrs	30%
ROSS	100-1000(713)	8yrs	30%
PETLEY	100 (420)	8yrs	50%

Fig.45 - Antibody Capture ELISA Using Sera From Myasthenic Patients

Assay carried out using goat anti-human (μ) coating antibodies. All sera not heat inactivated.

Patients - a) NHS - □
b) RA - ◊
c) RS - ♦
d) SH - ♦
e) PT - ■
f) HW - ⧈



goat anti-human IgG (mu specific) antibodies (Fig. 45)). It can be seen that there is a reaction with all the sera, including the normal human sera. With two of the sera (HN and RS), the reaction was comparable to that obtained with normal human sera. What is of note is that both these sera are high titre sera with the conventional radioimmunoassay. Of the other three sera, one is a high titre sera (PT), and the other two are low/medium titre sera (RA and SH).

4 PRODUCTION OF ANTI-IDIOTYPIC ANTIBODIES

4.1 Purification Of Anti-AChR Antibodies From Myasthenic Serum

4.1.1 Purification Of IgG Fraction

IgG was prepared by ion exchange chromatography (Methods, section 4.1) from a batch (50ml) of plasmapheresis serum that showed a high titre (100.7nmol/ml) in the conventional radioimmunoassay.

The purified IgG (36ml) contained 30mg total protein. Fig. 46 gives the elution profile.

4.1.2 Pepsin Digestion Of Purified IgG

$F(ab)_2$ fragments were prepared from myasthenic IgG (30mg protein) by pepsin digestion (Methods, section 4.2), and purified by ion exchange chromatography when they were eluted in the void volume (see Fig. 47 for elution profile) to give pooled fractions containing 14.2mg total protein (10ml, 1.42mg protein/ml).

Fig.46 - Elution Profile Of Total Serum IgG From A Myasthenic Patient Using Ion Exchange Chromatography

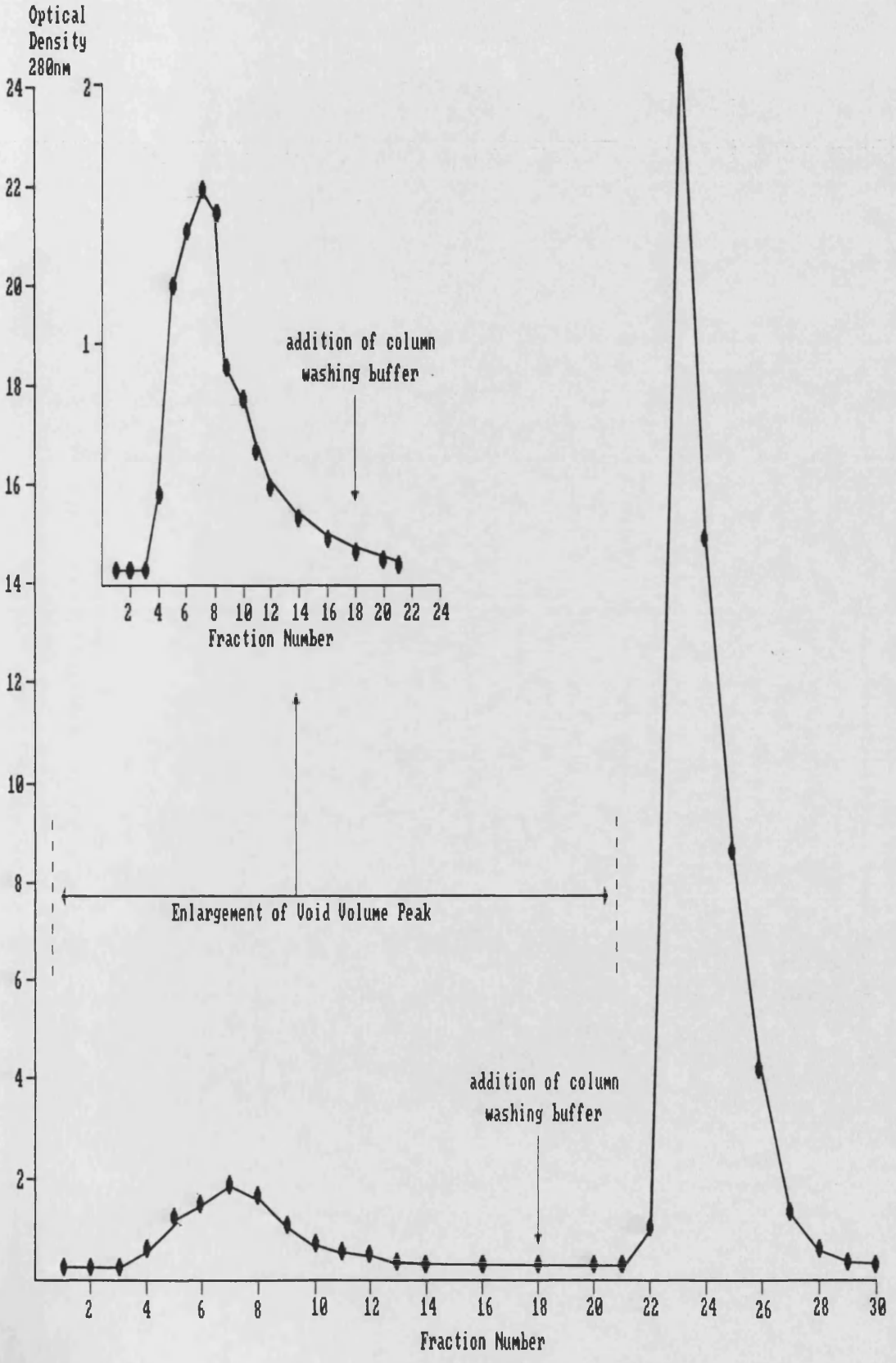
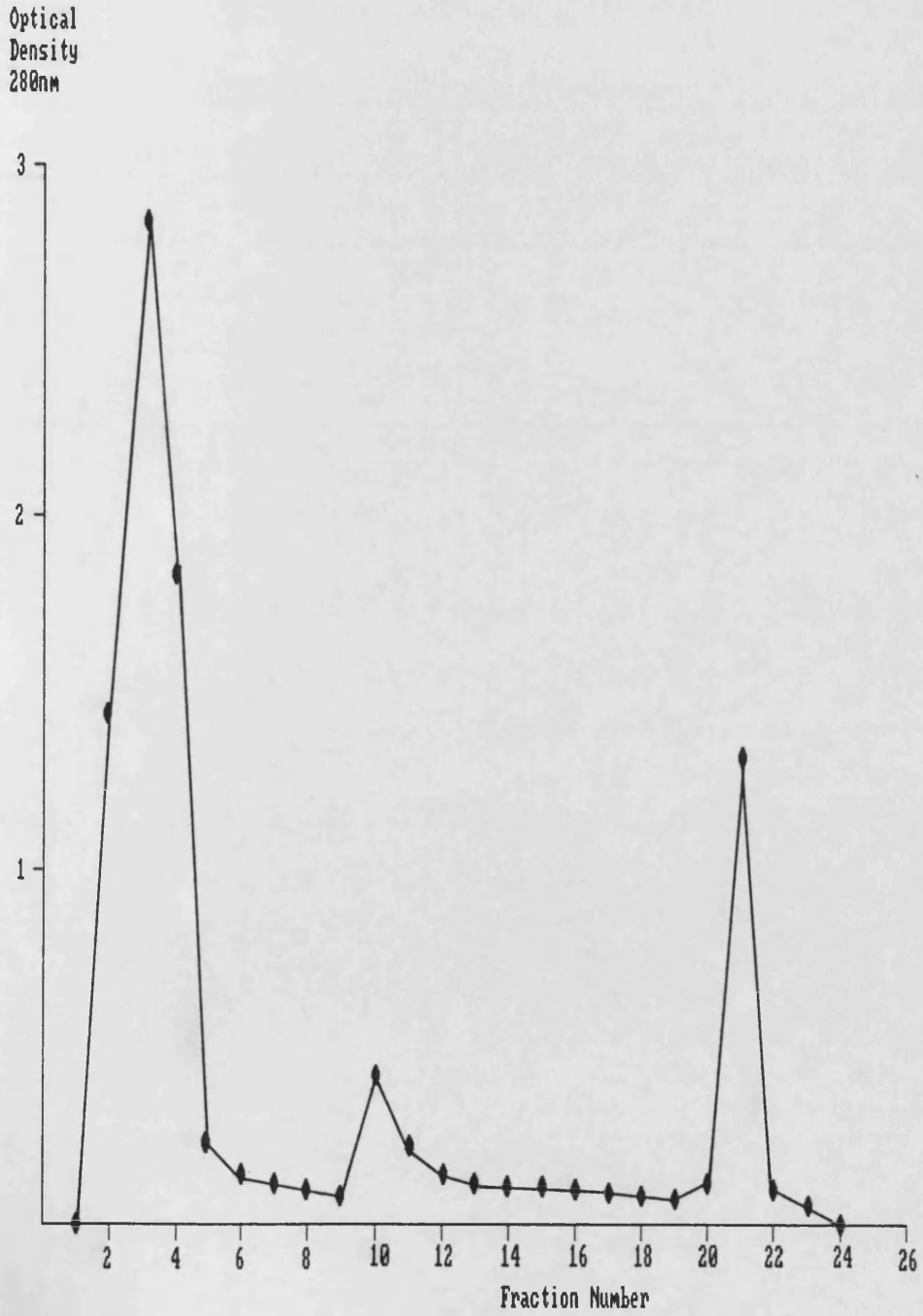


Fig.47 - Elution Profile Of $F(ab)_2$ Fragments Prepared From IgG Purified From Myasthenic Serum Seperated By Ion Exchange Chromatography



4.1.3 Characterization of $F(ab)_2$ Fraction

i) RadioImmunoAssay

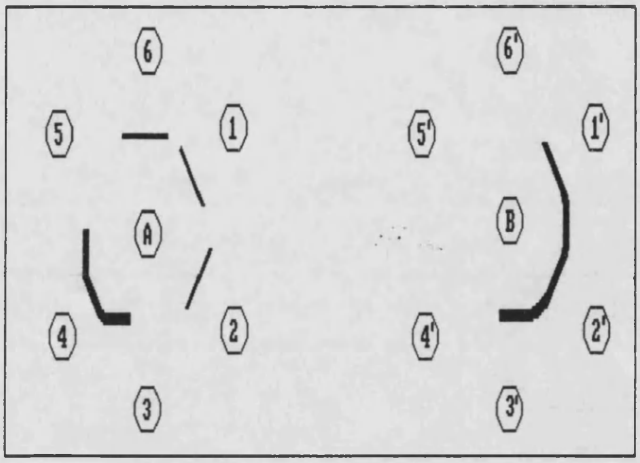
The purified $F(ab)_2$ fraction was assayed by the standard clinical radioimmunoassay (Methods, section 1.6) for its ability to bind to AChR. It was then compared to the anti-AChR antibody titre, seen in the serum donor patient (P). The radioactivity seen to bind in the pooled fraction was also compared to that which bound in each of the other two peaks obtained in ion-exchange chromatography (see Fig. 47).

The $F(ab)_2$ pooled fraction (10ml) had a titre of 19.01 nmols AChR binding sites per ml. This represents a yield of 19% of the binding sites present in the original batch of serum. In addition to the pooled fractions from peak 1 (see Fig. 47), fractions 10 and 21, from peaks 2 and 3 respectively, were assayed by RIA. Peak 2 (fraction 10) contained 1.0 nmols AChR binding sites per ml, and peak 3 (fraction 21) contained 0.78 nmols AChR binding sites per ml, each fraction had a total volume of 2ml.

ii) Immunodiffusion

The three peak fractions from ion exchange chromatography, were further investigated by immunodiffusion (see Fig. 48 which gives a diagramatic representation of the result of the immunodiffusion). It can be seen that only the void volume fractions were precipitated by the goat anti-human light chain IgG.

Fig.48 - Characterisation Of $F(ab)_2$ Fragments By Immunodiffusion Using An Ouchterlony Plate



Key To Contents Of Wells

Well (n = n')	
A	- anti-human (gamma) IgG
B	- anti-human light chain IgG
n = 1	- Fraction 2
2	- Fraction 3
3	- Fraction 4
4	- Human Fc(gamma)
5	- Fraction 10
6	- Fraction 21

iii) SDS PAGE

The purity of the F(ab)2 fraction was investigated by SDS polyacrylamide electrophoresis, using reduced and non-reduced samples (see Fig. 49). This clearly demonstrates that the $F(ab)_2$ fragments were located in the void volume peak. The non-reduced sample gave rise to one clearly defined band, whereas the reduced sample gave rise to two bands.

4.2 Preparation Of Anti-Idiotypes Towards Anti-AChR Antibodies

The purified $F(ab)_2$ fragments were used to immunise a laboratory bred rabbit. Following the immunisation procedure (Methods, section 4.4), anti-idiotypic antibodies were prepared from whole rabbit blood. This involved the removal from the serum of rabbit anti-human light chain antibodies by affinity chromatography.

IgG prepared from pooled normal human serum by ion exchange chromatography (see Fig. 50 for elution profile, pooled fraction 32ml, 3.9mg protein/ml giving total IgG of 124.8mg) and ammonium sulphate precipitation, was linked to Sepharose 4B by cyanogen bromide. Optical density measurements of the antibody solution before and after linkage showed that 89% of the total NHS IgG used (approx. 25mg) bound to the beads. This column was then used to adsorb out the rabbit anti-human light chain antibodies in the immune rabbit serum (see Fig. 51 for elution profile). Anti-idiotypic antibodies were then eluted from the column with

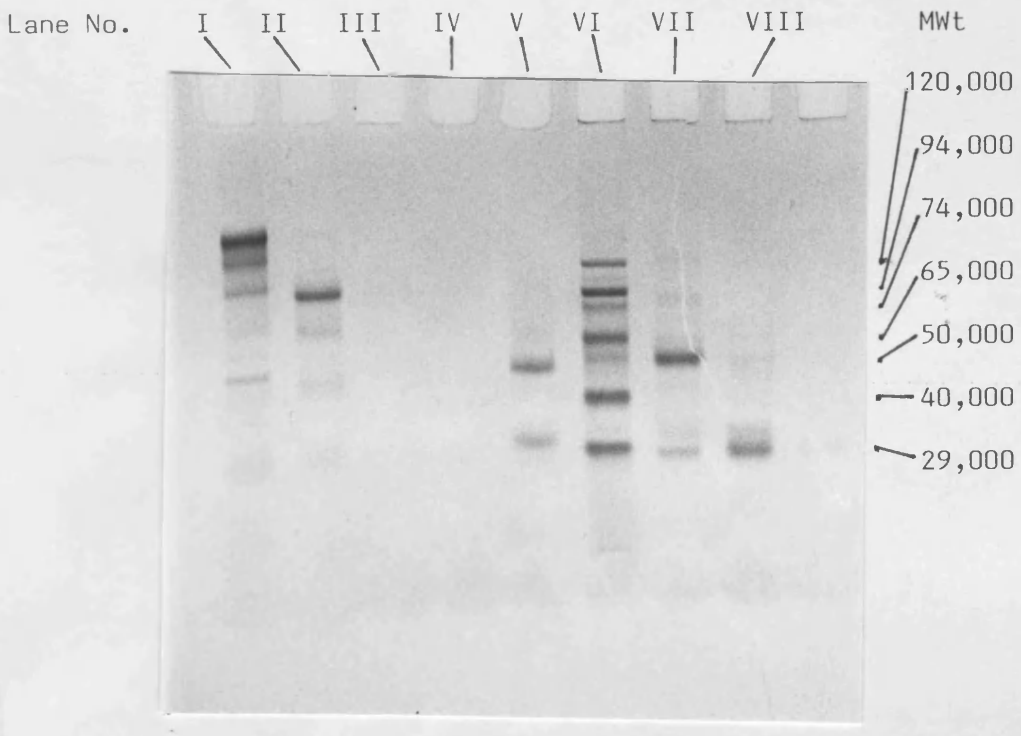


Fig. 49 - SDS PAGE Of Fractions Obtained From The Ion-Echange Chromatography Of The Pepsin Digestion Of MG IgG

Lane I - Unreduced Human IgG

II - Unreduced Myasthenic $F(ab)_2$ Fragments

III - Unreduced Fraction 10

IV - Unreduced Fraction 21

V - Reduced Myasthenic $F(ab)_2$ Fragments

VI - Reduced Standards
(β-galactosidase (120 K);
phosphyrolyase B (94 K);
IgM (mu) (74 K);
BSA (65 K);
IgG (gamma) (50 K);
Aldolase (40 K);
Carbonic anhydrase (29 K))

VII - Reduced IgG

VIII - Reduced Fc Fragments

Fig.50 - Elution Profile Of Normal Human IgG Purified By Ion Exchange Chromatography

Peak Fractions (6 - 13) Pooled

Total Volume = 32 ml
IgG conc. = 3.9 mg IgG/ml
Total IgG = 124.5 mg

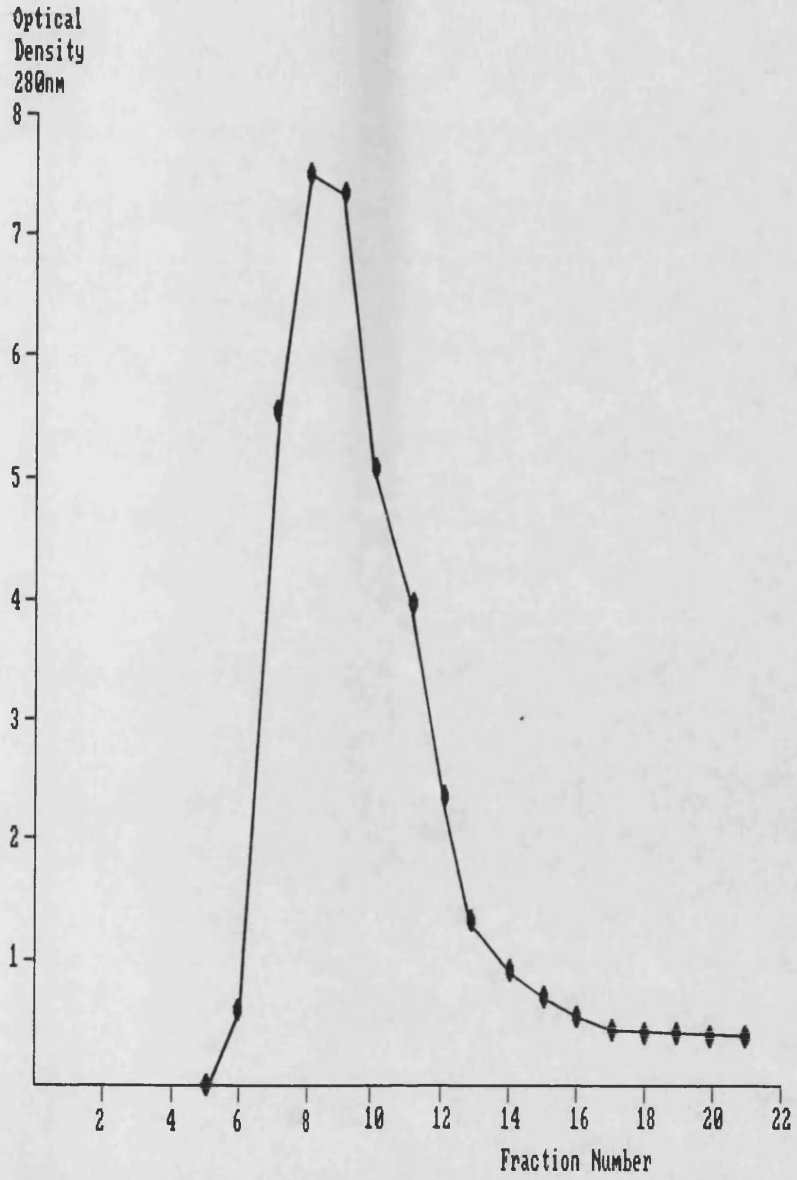
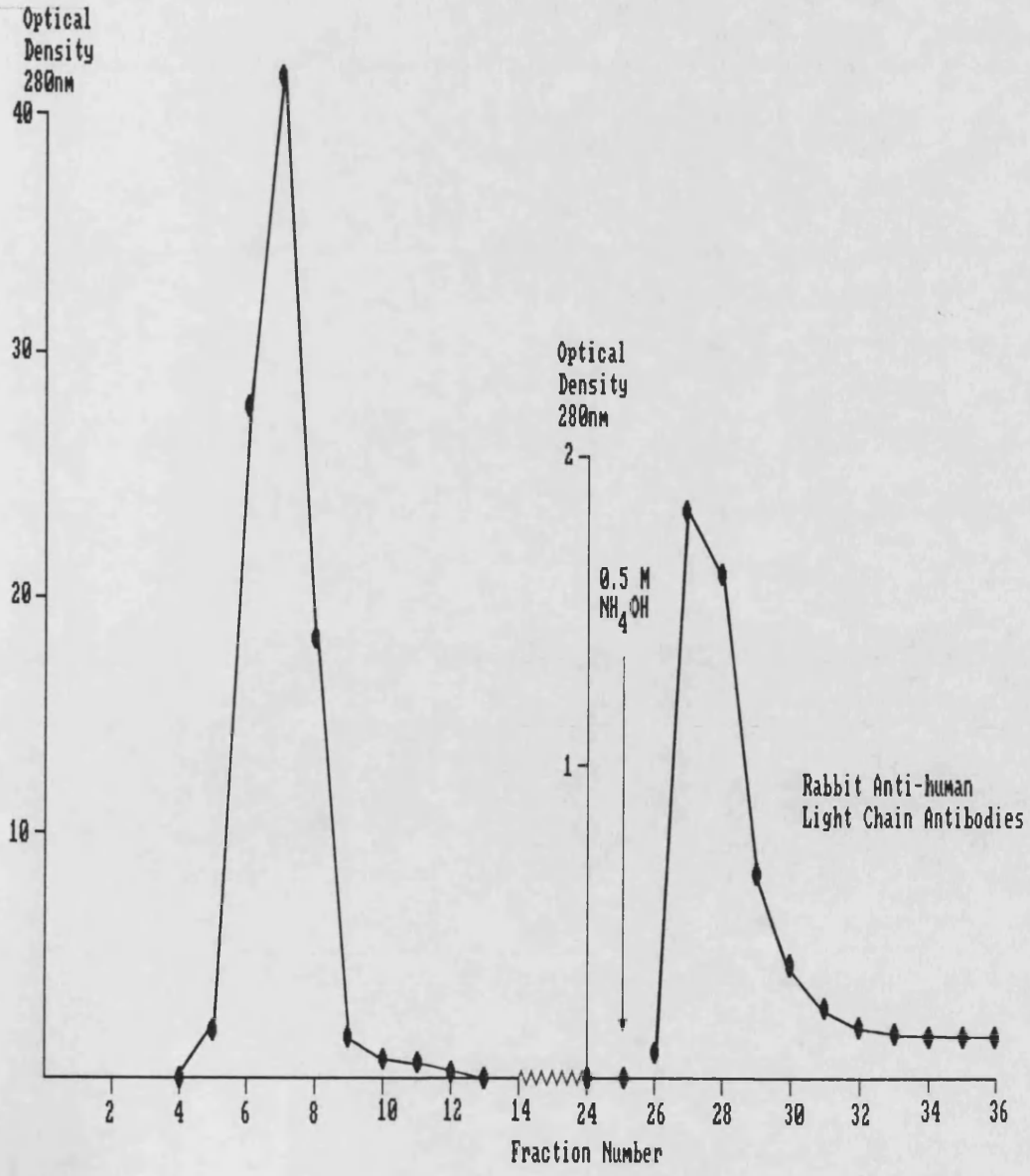


Fig.51 - Elution Profile Of Immune Rabbit Serum Absorbed Onto Sepharose 4B/Human IgG Affinity Column



0.5M NH_4OH. This elute was concentrated by ammonium sulphate precipitation, and the resultant pellet dissolved in PBS (10ml). Optical density measurements at 280nm, gave a protein concentration of 6.9 mg/ml. This gives a total yield of 69mg of protein from 20ml of immune rabbit serum. With 10-15mg of IgG/ml of serum there is a percentage recovery of between 23-34.5%. A similar antibody preparation was made from norman rabbit serum.

4.2.1 Assay Of Anti-Idiotypic Activity

i) Competitive RadioImmunoAssay

The blocking effect of the anti-idiotypic antibodies was investigated by a modification of the standard radioimmunoassay, utilising two Myasthenic sera, and the concentrated NRS IgG as a control. No blockage of radiolabeled AChR binding to anti-AChR antibodies from patient J. was observed with any concentration of anti-idiotype. However, a certain amount of blockage was seen with patient P. against whom the anti-idiotypes were raised (see Fig. 52).

ii) Enzyme Linked Immunosorbent Assay

The ELISA used, investigated the ability of the anti-idiotypes to bind to the myasthenic $F(ab)_2$ fragments, and normal human $F(ab)_2$ fragments, when compared to the control NRS IgG. The results show that the anti-idiotypic antibodies bind to the myasthenic $F(ab)_2$ fragments, but not to the normal human $F(ab)_2$ fragments. However, the concentrated NRS IgG used as a control

Fig.52 - Competative Radioimmuno-Assay Utilising Myasthenic Sera

Anti-idiotypic fraction - ○
Purified normal rabbit IgG (control) - ●

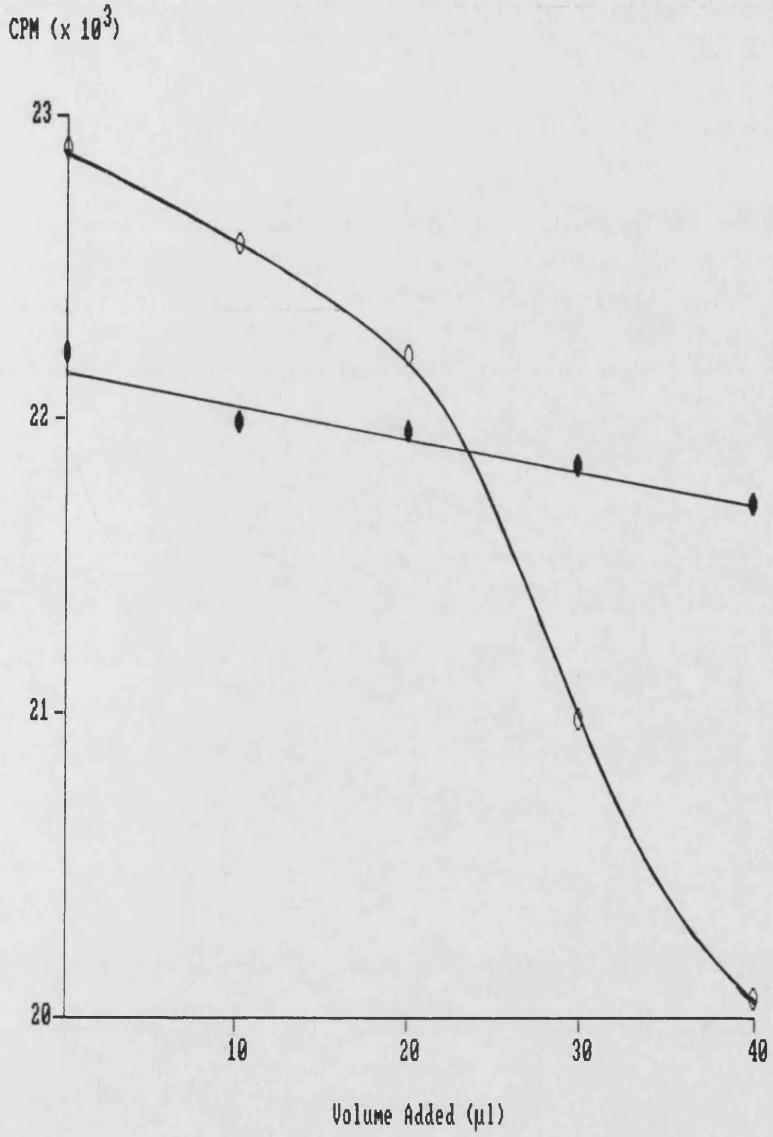


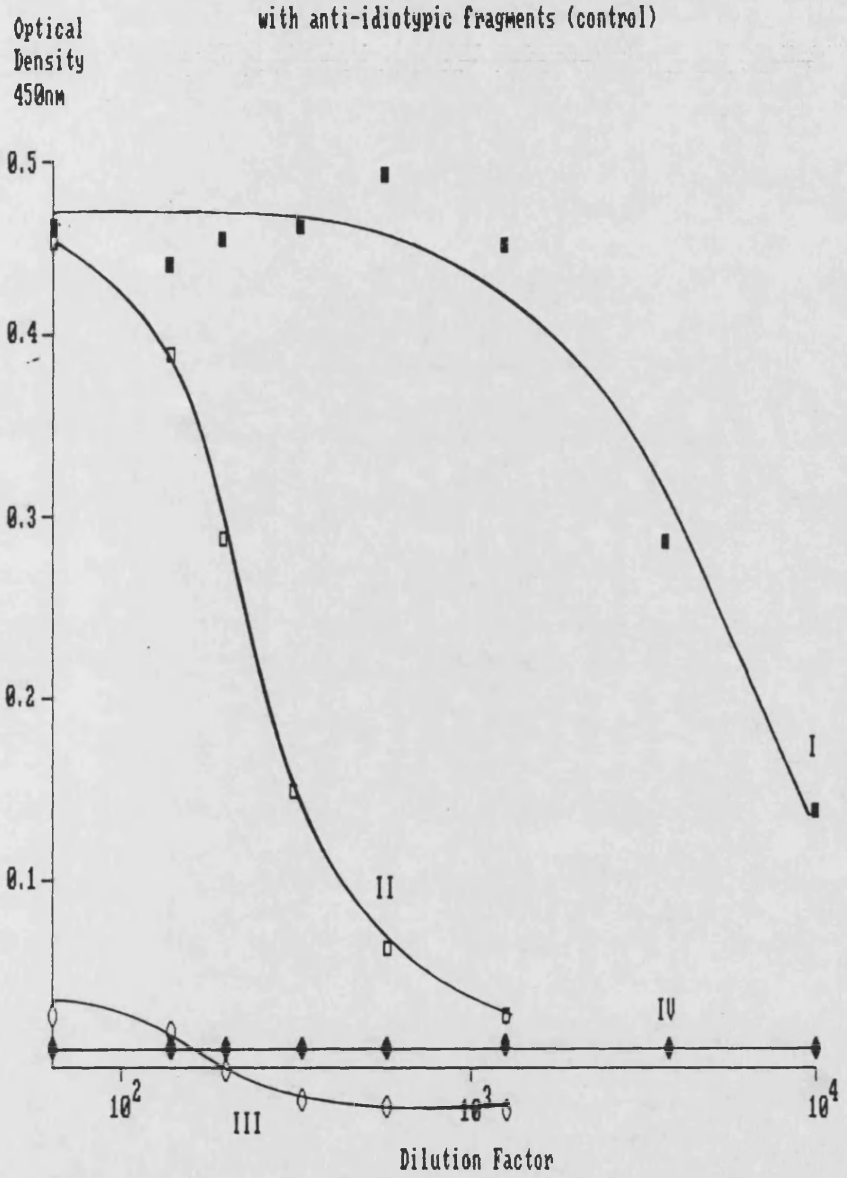
Fig.53 - Enzyme Linked Immuno-Sorbent Assay Of Anti-Idiotypic Fraction

I - Plates coated with Myasthenic $F(ab)_2$ fragments, incubated with anti-idiotypic fraction

II - Plates coated with Myasthenic $F(ab)_2$ fragments, incubated with Normal Rabbit Serum (non-specific binding)

III - Plates coated with Normal Human $F(ab)_2$ fragments, incubated with Normal Rabbit Serum (control)

IV - Plates coated with Normal Human $F(ab)_2$ fragments, incubated with anti-idiotypic fragments (control)



also binds to the myasthenic $F(ab)_2$ fragments but not to the same extent (see Fig. 53, shows the binding ability of anti-idiotypic antibodies).

iii) **Adsorption Of Myasthenic Sera Onto An Anti-Idiotype Affinity Column**

A crude anti-idiotypic IgG preparation obtained by sodium sulphate precipitation of the immune rabbit serum, was linked to Sepharose 4B beads by cyanogen bromide treatment. Optical density measurements of the antibody solution before and after linkage, gave the extent of coupling as approximately 85% (65mg of protein was used, 10.6mg remained in the solution).

A sample of Myasthenic serum (patient P) was applied to the column and the void volume peak collected. The adsorbed fraction was then compared to a similarly undiluted serum sample, by the radioimmunoassay procedure already described (Methods, section 1.6). The results showed a 22% decrease in anti-AChR antibody titre in the adsorbed serum (unadsorbed, 116nmols AChR binding sites/ml: adsorbed, 90nmols AChR binding sites/ml).

DISCUSSION

1. Preparation Of Radiolabelled Alpha-BGT & AChR Extracts

The nAChR is the most extensively characterised neurotransmitter receptor in biochemical terms, with advances being largely attributed to the discovery of the α-neurotoxins, and a rich source of receptor seen in the electric organs of the electric fish and eels (Introduction, section 3.2.2).

In particular, αBGT has been used as an affinity ligand in the purification of nAChRs from a variety of sources, including mammalian, and when radiolabelled in a quantitative assay for nAChR. For a radiolabelled ligand to be of use in detecting small quantities of receptor, it must possess high specific radioactivity and biological activity. This is seen with iodinated derivatives of αBGT, namely ^{125}I-αBGT (Methods, section 1.1; Results, section 1.1) prepared using the Chloramine-T method of Hunter et al (1967), as modified by Urbaniak et al (1973). The procedure is relatively simple to carry out, as is the detection of the radioactive ligands, thus making it ideally suited for such studies. Thus, preparation of ^{125}I-αBGT with high specific radioactivity and biological activity, is carried out as a matter of routine.

The radiolabelled αBGT's prepared during the course of this work had specific activities and biological activities (Results,

section 1.1) which compared favourably with similar preparations made by colleagues (Walsh, (1990); Nickless, (1985). The biological activities decrease notably after a period of approximately three weeks, and consequently, each preparation was discarded after this time. The loss of activity has been attributed to the presence of Chloramine-T, a powerful oxidising agent, and/or by radiation damage caused by the decay products of [^{125}I] (Walsh, (1989)).

The solubilisation, isolation and purification of nAChR's from a whole range of tissues have been carried out in this department for a number of years (Stephenson et al, (1981); Turnbull et al, (1985); Lotwick, (1985); Nickless, (1985); Walsh, (1990))consequently procedures for solubilisation and purification have been optimised and standardised, to such an extent, that it was considered that it was not necessary to undertake these studies again, especially as this was not the immediate aim of this work. Therefore, all procedures described (Methods, section 1.3 & 1.4) are those which are used as a matter of routine in the preparation of nAChR extracts. However, one or two points are worthy of note.

Firstly, Triton-X100 has been shown to be the most effective solubilising agent (Walsh, (1990); Nickless, (1985)) including the solubilisation of nAChR from human muscle (Stephenson et al, (1981). It is a non-ionic detergent which

does not interfere with the biochemical and pharmacological characterisation of the receptor which occurs if ionic detergents are used (Sugujama & Changeax, (1975)), and keeps the receptor in solution by forming mixed micelles with the receptor.

Secondly, mammalian sources of AChR tend to have high levels of proteolytic enzymes present, which does not occur in the electric organs of the electric fish and eels. Consequently, in addition to the low levels of receptor, proteolytic degradation becomes a problem. In order to overcome this a series of additional modifications to the procedure are employed, namely, the introduction of protease inhibitors, shortened extraction/ purification times, and all procedures carried out at 4°C where possible (Lindstrom et al, (1979); Anholt et al, (1984); Schorr et al, (1981); Stephenson et al, (1981)).

All receptor preparations so prepared during the course of this study, were assayed at frequent intervals (Methods, section 1.5; Results, section 1.3), to ensure the AChR content was known before use, and to ensure that it did not change appreciably. A slight drop in levels was seen but this was not appreciable and can be attributed to some proteolytic degradation occuring. It is also possible that some of the receptors present in the extract denature over time producing free subunits. This would not be reflected in the assays carried out using ^{125}I-αBGT as this ligand will still bind to free α-subunits of the receptor. Lindstrom et al, (1980), and Einarson et al, (1982), have shown that, beta,

gamma and delta subunits of the receptor are more sensitive to proteolytic degradation than the relatively protease resistant alpha subunit. AChR content of all extracts, Torpedo, human and fetal calf, compared favourably with similar extracts prepared by colleagues (Walsh, (1990); Nickless, (1985)) and with levels reported in current literature (Stephenson et al, (1981); Momoi & Lennon, (1982); Lotwick, (1985); Turnbull et al, (1985); Einarson et al , (1982); Gotti et al (1982)) Yields do vary between preparations, especially between human muscle extracts. This has largely been attributed to the quality of the material used for extraction. Most extracts are prepared from muscle obtained from lower leg amputations. The amputations are from patients who were suffering from diabetic gangrene, or other ischemic vascular diseases. Ischemia may result in partial denervation of the muscle which in turn may lead to proliferation of EJR (Introduction, section 3) thus providing a richer source of AChR. Alternatively, if the patient has been inactive for long periods because of vascular disease, then wasting of the muscle may occur thus reducing the overall quality of the tissue for extraction purposes. Only one crude fetal calf extract was prepared, along with only one crude Torpedo extract, consequently we can't discuss variability between preparations. In addition some of the crude Torpedo extract was used to obtain purified Torpedo AChR. Even though variability cannot be discussed with respect to these results, it is of interest to note, that in a much more detailed study by Walsh, (1990), fetal age plays an important part in the

yield of nAChR obtained. In fact, the older the fetal calves used, the lower the nAChR content of the extract produced. This was reasoned to be due to the fact that in young fetal calves most of the nAChR is probably EJ in form (Introduction, section 3) on relatively small muscle fibres, consequently, extraction is that much easier compared to fetal calves which are older. In older fetal calves innervation occurs, with a consequent decrease in the AChR content of the muscle fibres, and concentration of the nAChR's at the NMJ. In addition, as the fetal calves become older, the muscle becomes more fibrous in nature, making extraction more difficult.

As previously stated, ^{125}I-αBGT is used in a variety of assays to determine AChR levels in extracts. Throughout this work, three assays were used for this purpose.

The first, was used to determine levels in purified AChR extracts, and involved the separation of the relatively acidic toxin-receptor complex from non-bound toxin by filtration through ion exchange filters (DE-81), the complex binding to the filters, whereas the free toxin washes through. The amount of radio-activity bound to the filter disc was directly proportional to the amount of AChR in the test sample (Methods, section 1.5). In order to prevent the binding capacity of the discs from being exceeded, two discs are routinely used. This assay is more sensitive and reproducible than that normally used to assay crude

receptor extracts, with low non-specific binding principally due to the purified nature of the extract with all other proteins having been removed. Consequently, it was this assay which was used to assay purified AChR preparations.

The second, was used to assay crude receptor preparations, and involves the precipitation of the total protein present in the extract, including the labelled AChR, by addition of ammonium sulphate. The ammonium sulphate assay was first described by Meunier et al, (1972). However, because of the precipitation of the total protein content, this assay tends to give very high non-specific binding with very poor replicates. Consequently, an alternative assay was looked at which is normally used to assay receptor content in crude rat brain homogenates (personal communication, Dr. S. Wonnacott). The PEI assay, as it is known, involves the separation of the receptor/toxin complex from free ^{125}I-αBGT on glass fibre discs which have been preincubated in PEI. The discs so treated retain the acidic receptor/toxin complex because they are negatively charged. This assay when compared to the ammonium sulphate precipitation assay, gives results which are quantitatively comparable, but with greater reproducibility, lower non-specific binding, and is easier to carry out than the ammonium sulphate precipitation assay. A detailed comparison of the two assays was carried out by Walsh (1990). Details of the observations made during this study are

not shown, however, they do confirm the conclusions made by Walsh (1990). Consequently, the PEI assay was used in preference to the ammonium sulphate precipitation assay for the assay of crude receptor extracts.

2. Human Monoclonal Antibodies

Since the development of murine monoclonal antibodies (Kohler & Milstein, 1975; 1976), their uses and applications have become extensive. In particular, they have been used in the immunological characterisation of the nAChR, with particular reference to the autoimmune disease, Myasthenia gravis (Introduction, section 3.3.9). However, even though their uses have been widespread, certain applications make it advantageous to develop human equivalents. For example, in the further characterisation of the immunological responses operative in Myasthenia gravis, and in the development of possible theraputic strategies for patients (Introduction, section 4.2).

The introduction of murine monoclonal antibodies have been in the main successful, however, the development of human equivalents have been somewhat less so. This has been attributed to a variety of reasons (Introduction, section 4) but in particular to the non-availability of a suitable human fusion partner which satisfies all of the desired criteria (Introduction section 4.2.1), and of sufficient numbers of immune lymphocytes for immortalisation. With regard to this latter point, the most readily available tissue is peripheral blood, however, PBL's tend to perform poorly (Introduction, section 4.2.2). Several

procedures have been used to overcome this problem and to increase the relative numbers of specific immune lymphocytes present in peripheral blood. These largely consist of in vivo/in vitro immunisation procedures or preselection techniques (Introduction, section 4.2.2). The problem of a suitable fusion partner has been solved by various researchers, through the development of their own fusion partners for their own particular needs. This has led to a wide variety of techniques and fusion partners becoming available, with no particular one having any real advantage over any of the others (the techniques and fusion partners available are reviewed in the Introduction, section 4).

During this work, only one technique was used for the immortalisation of PBL's. This is the EBV/hybridoma technique (Introduction, section 4.2.3.iii) during which separated PBL's are transformed using EBV and the resultant lymphoblastoid cells fused with the mouse myeloma cell line, X63 (Kearney et al, (1979)).

This procedure has several advantages over the rest. The use of cell transformation, means that a relatively large number of lymphocytes can be transformed at the same time. However, not all of the cells will be transformed. For a cell to be transformed it must be at a particular stage of differentiation, even though there is some debate as to the specific stage when B cells become susceptible to transformation (Introduction, section 4.2.3.ii). Consequently, if cellular differentiation is

important for transformation then this partly explains the poor performance of PBL's, as a large number of cells present in peripheral blood are dormant awaiting activation, and are therefore not at a stage of differentiation for transformation to take place. In general it will be a mixed lymphocyte population that will be transformed, with cells in various stages of cell differentiation. Prior to the separation of PBL's, in vivo/in vitro immunisation or preselection techniques would have been carried out in order to increase the numbers of specific lymphocytes present. A similar situation exists to in vivo immunisation in patients suffering from autoimmune diseases, where clonal expansion of specific immune lymphocytes has already occured. The association of transformation with the stage of cellular activation/differentiation also explains the failure during this work to transform B cells that have been stimulated with the polyclonal B cell activator PWM (Methods, section 2.4) which is used to enrich the B cell population of separated PBL's. The PWM stimulates the cells beyond the stage when they are able to be transformed through infection by EBV. Therefore, PWM was not used to polyclonally expand the B cell population prior to transformation. By not polyclonally expanding the B cell population before transformation another problem is caused, whereby cytotoxic T cells develop because they become sensitized to the presence of viral coat proteins on the cell surface of the

transformed B cells. These would not normally be present if PWM stimulation was used prior to transformation, as the T cells would die in culture. This problem is circumvented by addition of the polyclonal T cell activator, PHA. Following transformation, PHA is included in the culture medium (Methods, section 2.3) where it polyclonally stimulates all of the T cells present before they become sensitized. As they are not immortal cells then they die within a few days of culture, leaving only the transformed cells.

PHA was, however, found to be cytotoxic to the transformed cells if they were left in its presence for up to 7 days. This was discovered through visual monitoring of the cultured cells through an inverted microscope. If the culture cells were examined around 7 days following transformation, it was noted that there was a large amount of cell debris and dead cells in the wells. This was initially attributed to the cell death of the T cells, and untransformed B cells, caused by the presence of the PHA, but the debris appeared too excessive to be explained by this reason alone. In addition, there were very few colonies of transformed cells and the ones which continued to grow did so at a very slow rate, so much so that they could only be assayed after approximately 3-4 weeks (Discussion, section 2). When they were assayed, none were found to be secreting detectable amounts of anti-AChR antibodies. This was in contrast to the situation seen by Thompson et al, (1986, personal communication) in which

colonies grew well and were assayable after only 1-2 weeks, following transformation. It became clear that the only change to the system was the addition of PHA, but its presence was essential, as already described above, so the time period over which the cells were exposed to it was reduced from 7 to 3 days. This simple step alleviated the problem, with the result that the transformed cells grew well and in accordance with the growth seen by Thompson et al, (1986).

Having obtained a satisfactory system for transformation, and identified the culture wells containing the particular antibody of study, two lines can now be followed. The first involves expanding and cloning the lymphoblastoid cells, in order to obtain a cloned lymphoblastoid cell line (LCL), which secretes the antibody of interest. LCL, however, produce antibody titres which are lower than those seen in hybridomas, and have a tendancy to loose antibody production over a period of a few months (Introduction, section 4.2.3.ii). Antibody production can be rescued by back fusion of the LCL to a suitable fusion partner (Introduction, section 4.2.3.iii) and this is the basis of the second line which can be followed. However, it is not necessary to clone a LCL before fusion. In fact, as the cloning procedure is time consuming and labour intensive, to clone a LCL and then clone hybridomas is wasteful both of time and resources. The first cloning step can be eliminated. To do this, following assay

of the transformants, all the positive wells are pooled. This pooled cell population, a large proportion of which are secreting the antibody of interest, are fused immediately with the selected fusion partner. In this study, the fusion technique used was the heterohybridoma fusion (Introduction, section 4.2.3.i) & 4.2.3 iii)) and the fusion partner chosen was the murine myeloma cell line, X63 (Kearney et al , (1979)).

This technique and cell line was chosen because the cell line was readily available, grows well in culture, fuses with high frequency and efficiency, even with human cells, is HAT sensitive and ouabain resistant (Introduction, section 4), in addition there were no suitable human fusion partners available. Ouabain resistance becomes an important factor where two immortal cell lines are being fused together. In the majority of hybridoma techniques, only one selection step is required, as only one immortal cell line is being produced. However, in the EBV/hybridoma technique, two such lines will be present, the unfused lymphoblastoid cells and the hybridomas. If both are left in culture, both will continue to grow, the lymphoblastoid cells contaminating the hybridomas. Therefore, it is necessary to select against the unfused lymphoblastoid cells. The advantage with using X63 cells, is that they are murine in origin and murine cells are 10,000 times more resistant to ouabain than human cells. Consequently, ouabain can be incorporated into the system to kill

the unfused lymphoblastoid cells but leave the hybridoma cells intact. The X63 cells used in this work can withstand a ouabain concentration of 2×10^{-6} M (Results, section 2.1). Ouabain is therefore incorporated into the selection medium, along with HAT, at this concentration.

The fusion was carried out using PEG, and although it is very toxic to cells, it promotes the close apposition of cell membranes, by reducing membrane charges, while the presence of DMSO helps to modify the cell membranes to enhance fusion. By using a short fusion time (1 min) the toxicity to the cells was minimised. In addition, cell transformation prior to fusion, actually increases fusion frequencies by activating the B cells (Introduction, section 4.2.3.ii).

The major disadvantage of the heterohybridoma technique, is the inherant genetic instability of the heterohybridomas (Introduction, section 4.2.3.i) with antibody producing hybridomas rapidly losing antibody production. Such non-producing cells tend to grow faster than antibody producing hybridomas and overgrow them, so clearly cloning is essential when trying to retain antibody production. Thompson et al, (1986), upon whose techniques this work was based, found no such instability in the heterohybridomas produced.

Extensive problems were encountered throughout this work in addition to those already described above. The primary problem

with both the growth of lymphoblastoid cells and hybridomas, was chance infections, mainly bacterial in origin, even though some fungal contaminations did occur. As a consequence, the whole set of procedures used, and asceptic techniques, were re-evaluated on several occasions, especially where recurrent infections occured. However, even with the introduction of slightly different procedures, for example, installation of a sterile vacuum line into the flow cabinets, together with blood samples being collected directly into sterile evacuated tubes, infections continued to play a major role in the lack of success of this technique. With regards to this latter point, a change in the procedure was made as a result of noting that blood samples when taken normally, are done using a syringe and the blood is then transfered into sterile tubes. Unfortunately, this involves opening the tubes to the air, thereby increasing the possibility of infection. So it was requested that blood samples be taken by using evacuated tubes, equiped with a rubber self sealing cap. In addition to this possible cause of infection, it is possible that some infection results from the blood directly, even though this will not be the major cause.

Freshly taken blood samples were not the only samples used in this study. A large number of samples were received by post from various parts of the country, however, these samples were anything from 7 to 14 days old before they were received. These

samples, generally, gave a very poor lymphocyte preparation when separated on Ficol (Methods, section 2.2), with notable haemoglobin contamination of the lymphocytes, in addition to low viability. These lymphocytes tend to transform poorly, giving rise to very few lymphoblastoid cell lines. The poor performance of such lymphocytes can be explained with regard to their age. In general, the older the cells are the poorer they perform in such studies, consequently, fresh blood samples were used wherever possible.

Even considering all of these problems, some of which were overcome, partial success was achieved, in which one patient successfully gave rise to heterohybridomas following initial transformation of PBL's with EBV. In this case, both the transformants and heterohybridomas were assayed utilising the direct Torpedo ELISA (Methods, section 3.1; Results, section 2.2). A decrease in the assayable antibody titres was noted (Results, section 2.2) on assaying the transformants through to the heterohybridomas, which were finally lost through infection. Only 6 positive wells were detected on initial transformation, however, these increased to 33 on back fusion, before finally decreasing to 6 on expansion before being lost. These results tend to support the view that back fusion can rescue antibody production even though the resultant heterohybridomas are genetically unstable with rapid loss of antibody production (Introduction, section 4.2.3). Assay of the expanded positive wells by modified

RIA (Methods, section 3.2), failed to detect any antibodies directed against human AChR. This result can be explained by a number of possible causes. Firstly, the RIA may not be sensitive enough to detect the small concentration of specific antibodies found in the culture supernatant obtained from transformants or heterohybridomas, whereas ELISA's are (Discussion, section 3). Secondly, the wells chosen for expansion and cloning were done so on the basis of their reaction with Torpedo AChR. Cross-reactivity is not the ideal method of detecting antibodies directed against a similar antigen found in another species, for example, in this case Torpedo AChR is used to detect antibodies directed against human AChR. For this to be successful, there must be some degree of similarity between the two antigens. This is seen in the nAChR's derived from different species (Introduction, section 3.2), however, it is more than probable that certain epitopes on the Torpedo AChR, which are not found on the human AChR, have greater antigenicity than others which are, thus the majority of antibodies produced will be directed against these particular determinants. So it is of no great surprise that the antibodies detected do not react with human AChR.

In addition, the lack of reactivity with human AChR in the RIA, can be further explained, by antigen orientation/presentation in the ELISA. Brennand et al (1986) has suggested that immobilisation of the antigen on ELISA plates in direct ELISA's,

is in such a way that a variety of antigenic determinants are lost either through direct binding to the polyvinyl, or through steric hinderance. Thus if Torpedo AChR binds to the plates in such a way that crossreactive determinants are masked or hidden, then the antibodies detected will be specific for the Torpedo receptor and will not react with the human receptor. Consequently, antibodies detected by the direct Torpedo ELISA will not react with the human receptor.

Without the development of a suitable screening assay, the use of the phenomenon of cross-reactivity in this manner will always pose a problem, as will the inherant problems in the tissue culture techniques themselves (see above).

3. Assaying Anti-AChR Antibodies In Culture Supernatants

3.1 General Discussion

The success of any monoclonal antibody technique, whether human or murine, is wholly dependant on the screening assay used to detect the particular antibody of interest. As can be seen from Figs. 24 & 25, assay steps are crucial in the general procedures employed, and are repeated several times throughout the generation of any monoclonal antibody.

As a consequence of this, several advantageous criteria are sought, when deciding upon the assay of choice. These include, high sensitivity and specificity, the ability to handle a large number of test samples (96 per test plate) with relative ease,

and to give reliable results. In addition, it must be carried out in as short a time as possible. This latter point is particularly important when early cloning is essential to the success of the technique.

3.2 RadioImmunoAssay

This assay is routinely used to clinically assess the anti-AChR antibody levels in the blood sera of patients suffering from the disease, Myasthenia gravis (Introduction, section 3.3.6). Consequently, it was the first assay which was investigated closely with regards to its suitability for the detection of anti-AChR antibodies in culture supernatants. However, it soon became apparent that this assay did not fulfil the required criteria (Discussion, section 3.1).

It is an extremely labour intensive assay, with procedures (Methods, sections 1.6 & 3.2) that make the handling of large numbers of test samples almost impossible. In fact if the basic assay is carried out on just one culture plate of transformed lymphocytes or hybridomas, with the required triplicate repeats, then 96 x 6 sample tubes will be required, in addition to 600 μl of supernatant for each assay and a large quantity of both human AChR extract and radiolabelled αBGT. If dilutions and repeats are then considered in order to increase the accuracy of the assay, then this puts a further strain on the amount of test material available, in addition to the assay reagents (human AChR and ^{125}I-αBGT). The use of radioactive reagents also requires special

handling and safety procedures, in addition to detection equipment. A modification of the basic procedure was carried out by Walsh (1990), in which only one dilution, instead of several, was used, when looking at murine hybridomas producing monoclonal anti-Torpedo AChR antibodies, however, this does not improve the assays desirability for detecting anti-AChR antibodies in culture supernatants as the logistics of both the basic and modified assay are the same. Therefore, this assay was deemed to be unsuitable for screening large numbers of culture supernatants which would be generated during the course of this study. Consequently, a direct ELISA assay (Methods, section 3.1), using purified Torpedo AChR (Methods, section 1.4) was used as a screening assay during the early stages of this study, while a suitable alternative was being investigated.

3.3 Direct Torpedo ELISA

Even though this assay fulfils the requirements already listed (Discussion, section 3.1), it is based on the use of the immunological phenomenon of cross-reactivity. Where cross-reactivity is used in this situation, it is essential that the antigen used in the assay bears a close similarity to the antigen of interest, and that the antigen of interest possesses epitopes also present on the assay antigen. However, as already discussed (Discussion, section 2) if the assay antigen is only weakly cross-reactive, then the majority of antibodies detected will be

directed against this antigen and not the one of interest. It is also possible, when considering Myastenia gravis, that the antibodies detected, whether or not they react with human AChR, are not of idiotypes which are active in the disease process. Consequently, doubt will always exist where cross-reactivity is being used as the basis for detecting antibodies directed against a particular antigen. In this case, though, the direct Torpedo ELISA was used with some success (Results, section 2.2) in the detection of human anti-AChR antibodies in the culture supernatants from transformed lymphocytes and heterohybridomas, and was used, due to the unsuitability of the RIA and the non-availability of any other suitable assay.

3.4 Available ELISA Systems For The Detection Of Anti-AChR Antibodies

Current literature describes several ELISA and EIA systems for the assay of anti-AChR antibodies, all of which have been used with varying degrees of success (Introduction, section 3.3.6), in differing situations. However, of these, several use purified AChR (Norcross et al, (1980); Kawanami et al, (1984); Muir & Jehanli, (1985)). This is a problem in itself, if human receptor is being used (Stephenson et al, (1982); Momoi & Lennon, (1982); Turnbull et al, (1985)).the ELISA putting a great strain on the amount of purified receptor available. A modification was made by Dwyer et al, (1983b), in which assay wells were pre-coated

with a murine monoclonal anti-AChR antibody, prior to incubation with a crude receptor extract. The effect of the pre-coating procedure, is to increase the amount of AChR bound to the well. However, it is wholly dependent on the availability of a suitable coating antibody, and in this case, one was not available. Similarly Furakawa et al, (1984) used HRP conjugated αBGT in an EIA. This assay is similar to the RIA in procedure, and requires the monitoring of an enzyme/substrate time course, making the assay as labour intensive as the RIA, if not more so. Two further assays have been described, both of which used crude receptor extracts. The first, described by Jailkhani et al, (1986), was a direct ELISA, in which crude human receptor was used to coat assay wells directly prior to assaying anti-AChR antibodies in diluted myasthenic sera. The second, described by Hinman et al, (1983), used αBGT to pre-coat the assay wells prior to incubation with crude Torpedo receptor extract. In theory, the αBGT will increase the binding of receptor to the wells, similar to the use of a monoclonal antibody (Dwyer et al, (1983b)). The disadvantage of this assay is the fact that anti-site antibodies will not be detected, however, they are not detected in the RIA either. Both of these assays were investigated with regards to their possible use in the screening of cultured lymphoblastoid cells or heterohybridomas, as they use crude receptor extracts. However, anti-AChR antibodies were not detected in myasthenic sera nor

culture supernatants, by either assay. This supported similar findings by Walsh (1990, unpublished), and is almost entirely due to the low receptor concentration in extracts.

3.5 Idiotypes And Anti-Idiotypes

An alternative approach to the development of an ELISA system was investigated, based on idiotype/anti-idiotype interactions.

Jerne (1958) first postulated the existence of anti-idiotypic antibodies, however, it was some years before their existence was finally proven. Jerne originally proposed their existence, on the basis, that idiotypic determinants on antibodies will themselves be antigenic with the subsequent formation of antibodies directed against them. These secondary antibodies being termed anti-idiotypic antibodies. It will also be noted that these secondary antibodies will themselves be antigenic with formation of anti-idiotypic antibodies. This is a continuing self perpetuating system, forming a subtle regulatory network. The generation of anti-idiotypic response can be utilised in the development of an ELISA for the detection of the original antibody idiotype.

In this case, a purified IgG fraction prepared from the serum of a Myasthenic patient was first used to immunise an experimental animal, to show that anti-idiotypic antibodies can be so raised. Having shown that anti-idiotypic antibodies can be

raised, the same purified IgG can be used to generate murine monoclonal anti-idiotypic antibodies which can then be used in a double antibody sandwich ELISA for the detection of human anti-AChR antibodies in culture supernatants derived from transformed lymphocytes or heterohybridomas. In practise, a whole array of murine anti-idiotypic monoclonal antibodies will be generated for the whole range of IgG idiotypes used in the immunisation, most with differing antigenic specifities and afinities, and all of which will require characterisation. However, the main disadvantage of this approach is the fact that the anti-idiotypes will be specific for one patient and cannot be used for others, unless there is shared idiotypy between patients. Consequently, monoclonal anti-idiotypic antibodies will have to be developed for each individual patient.

The effectiveness of the ELISA for detecting anti-AChR antibodies from one particular patient, using one particular monoclonal anti-idiotypic antibody, will largely be dependent on the extent of the idiotypic restriction present in the anti-AChR antibodies. Otherwise, a pooled anti-idiotypic antibody population will be required to coat assay wells, in order to detect the whole range of idiotypes present within a particular patients myasthenic antibodies, with the resultant loss of sensitivity. The demonstration, that a high proportion of the anti-AChR antibodies found in patients suffering from myasthenic

gravis are directed against one particular region on the α-subunit of the AChR (Vincent, (1981); Tzartos & Lindstrom, (1980); Introduction, section 3.3.7) and are therefore of restricted idiotypy, means that the anti-idiotypic approach should be more effective. There are, however, anti-AChR antibodies present which are directed against other determinants on the AChR, a situation which is not wholly unexpected when considering the size of the receptor, but these are not believed to form the majority of antibodies present and are not thought to be pathogenic (Introduction, section 3.3.7).

In this study, one patient was chosen for investigation, who consistently showed high serum anti-AChR titres (100.7 nmol/ml) on assay with the conventional RIA, and from whom large amounts of serum were available in addition to lymphocytes used for transformation. The large amount of serum available, made the IgG purification relatively easy (Fig. 46). The whole IgG fraction was not however, used for immunisation. In order to increase the likelihood of anti-idiotypic antibodies being generated, a pepsin digest was carried out on the purified IgG fraction. This has the effect of removing the Fc region of the antibodies, leaving $F(ab)_2$ fragments which can be separated from the digest by ion-exchange chromatography, the fragments being located in the void volume (Methods, section 4; Results, section 4.1.2). The removal of the Fc region effectively reduces the

number of dominant antigenic determinants present, increasing the chance of anti-idiotypic antibodies being formed. Unfortunately, light chains and the first constant region of the heavy chains will still be present, and it is to be expected that these will be more antigenic than the idiotype itself. The $F(ab)_2$ fragments so formed were characterised by RIA, Immunodiffusion, and SDS PAGE (Methods, section 4.3; Results, section 4.1.3), which showed their $F(ab)_2$ nature and the fact that they retained AChR binding ability.

The $F(ab)_2$ fragments were used to immunise an experimental rabbit (Methods, section 4.4), the serum of which was tested for the presence of anti-idiotypic antibodies (Methods, section 4). A rabbit was used instead of a balb/C mouse as the test bleeds would give rise to a greater amount of serum for testing, even though a balb/C mouse would be used for monoclonal anti-idiotypic production.

Immunisation and subsequent boosting with the $F(ab)_2$ fragments will give rise to a mixed population of antibodies, some of which will be anti-idiotypic in nature, but not all directed against anti-AChR idiotypes as a mixed IgG population was originally used. The majority of the antibodies so generated will be directed against the light chains and other determinants on the heavy chain. These antibodies were absorbed out of the serum by passing the serum over a Sepharose 4B column which had

normal human IgG attached to it (Methods, section 4.5; Results, section 4.2). The theory being, that antibodies directed against the light chains and other heavy chain determinants will bind to the column leaving the anti-idiotypic antibodies to pass through. In addition, by using normal human IgG, anti-idiotypes other than those directed against anti-AChR antibodies will also be absorbed out, thus enriching the anti-AChR anti-idiotypic antibody content of the eluate. It is obvious that this procedure will not remove all of the unwanted antibodies present and that some of the anti-AChR anti-idiotypic antibodies will also be lost, especially when it is considered that autoimmune antibodies are present in normal healthy persons and are believed to be essential for health. However, these antibodies are not believed to form a significant part of an individuals antibody repertoire (Introduction, section 2.5).

The anti-idiotypic nature of this fraction was investigated by three methods (Methods, section 4.6; Results, section 4.2.1).

Firstly, by competitive RIA, in which the binding of the anti-idiotypes to two different myasthenic sera was observed (Methods, section 4.6.1; Results, section 4.2.1.i) along with NHS used as a control. The numbers of cpm's precipitated decrease on increased addition of the anti-idiotypic fraction to the myasthenic serum from patient P, this was over and above the decrease seen with addition to NHS. The decrease seen with

patient J matched that seen with the control, indicating that the existence of anti-idiotypic antibodies in the fraction and that they are specific for patient P.

Secondly, the anti-idiotypic nature of the fraction was further confirmed by an ELISA, which investigated the binding ability of the anti-idiotypic fraction to the myasthenic $F(ab)_2$ fragments, compared to normal human $F(ab)_2$ fragments (Methods, section 4.6.2; Results, section 4.2.1.ii)). The results showed little binding of the anti-idiotypic fraction to normal human $F(ab)_2$ fragments, but significantly higher binding of the anti-idiotypes to the myasthenic $F(ab)_2$ fragments from patient P. The reaction with myasthenic $F(ab)_2$ fragments gives good supporting evidence to the results obtained from the competitive RIA. Binding of the anti-idiotypes to myasthenic $F(ab)_2$ fragments obtained from patient J was not investigated.

In addition to these two assays, a third was carried out in which a crude IgG preparation of a serum sample obtained from the immunised rabbit, was linked to Sepharose 4B, forming an affinity column, down which a sample of myasthenic serum from patient P, was passed. The anti-AChR antibody titres of the myasthenic serum before and after adsorption were then compared by use of the conventional RIA (Methods, sections 4.6.3 & 1.6; Results, section 4.2.1.iii)). A 22% decrease was found on adsorption suggesting that some of the anti-AChR antibodies were bound to

the column. The disadvantage of this assay, is the fact that the immune rabbit serum did not have the anti-light/heavy chain antibodies adsorbed out of it before it was coupled to the Sepharose, therefore it is entirely probable that the decrease in antibody titre is due to binding through the light chains and not the idiotype. Thus making this assay somewhat inconclusive.

It has therefore been shown that anti-idiotypic antibodies directed against anti-AChR antibodies can be raised in an experimental animal with relative ease. What remained was the development of a murine monoclonal anti-idiotypic antibody which could be used in an assay. However, this approach was abandoned in favour of the more promising avidin/biotin ELISA which was also being developed. But the ease by which anti-idiotypes can be raised has wide implications for their use as biochemical and immunological tools. In particular, anti-idiotypes can be used to purify specific anti-AChR antibodies from sera, which in turn can be used to affinity purify human receptor, something which is normally very difficult to do (Stephenson et al, (1982); Momoi & Lennon, (1982); Turnbull et al, (1985)). By purifying anti-AChR antibodies, their binding specificities and characteristics can be further investigated. Additionally, if Jerne's theory of the operation of regulatory anti-idiotypic networks in an individual is correct, then it follows that purified anti-idiotypic antibodies and idiotypic antibodies, can be used to investigate

an individual's anti-idiotypic antibody repertoire, with subsequent development of better therapeutic strategies, through human monoclonal antibody technology. Several workers have demonstrated the existence of idiotype-anti-idiotype networks in MG (Schwartz et al, (1978); Fuchs, (1980)), even though others have not been able to detect blocking anti-idiotypic antibodies in remission phase MG patients (Heininger et al, (1983), or have suggested that there may be an anti-idiotypic cause to MG (Cleveland et al, (1983); Erlanger et al, (1984)). Thus there is enormous scope for the development and investigation of anti-idiotypes.

3.6 Avidin/Biotin System And Detection Of Anti-AChR Antibodies In Culture Supernatants

The avidin/biotin system consists of the omnipresent vitamin biotin, and the egg white glycoprotein, avidin, which is of restricted distribution in nature. Of similar characteristics with regards to its ability to bind biotin is, Streptavidin, found in the bacterium, Streptomyces avidinii. These two proteins are characterised by the strongest known biological recognition and subsequent non-covalent interaction which has made it one of the most useful systems for a wide variety of biological studies (reviewed by Wilchek & Bayer, (1980;1983), Titssen (1985)) in particular isolation, localisation and immunological studies. Avidin and Streptavidin, both have four binding sites for biotin.

The interaction was first noted by the observation that

avidin, can interact with the so called 'biotin requiring enzymes' where biotin is a natural prosthetic group. Subsequent studies identified the precise nature of the interaction, and the appropriate means of introducing biotin artificially into biological molecules followed.

Only the ureido ring is required for recognition of the biotin molecule. Consequently, the carboxyl group of the valeric acid side chain can be modified chemically to obtain reactive derivatives, which can introduce the biotin moiety into other molecules. The two basic derivatives used are biotinyl-N-hydroxy-succinimide ester, used to biotinylate proteins/amines, and biotin hydrazide, used to biotinylate aldehydes/sugars (Fig. 54). The effective coupling capacity of biotin to proteins is about 70%, and about 5 biotin molecules per protein is usually required for subsequent recognition by avidin probes (Wilchek & Bayer, (1980; 1983)). Avidin can be adapted into a number of probes, namely, fluorescent, radioactive, electron dense, immobilised and enzyme conjugated. Of particular interest, with regards to this present study, is the use of the system in immunological reactions, especially immunoassays, and in the localisation and visualisation of AChR's from Torpedo (Holtzman et al, (1982)) and on cultured myotubes (Axelrod, (1980)) by using biotinylated αBGT.

The first step in the development of an assay, involved labelling of the AChR with biotin. It became obvious that as

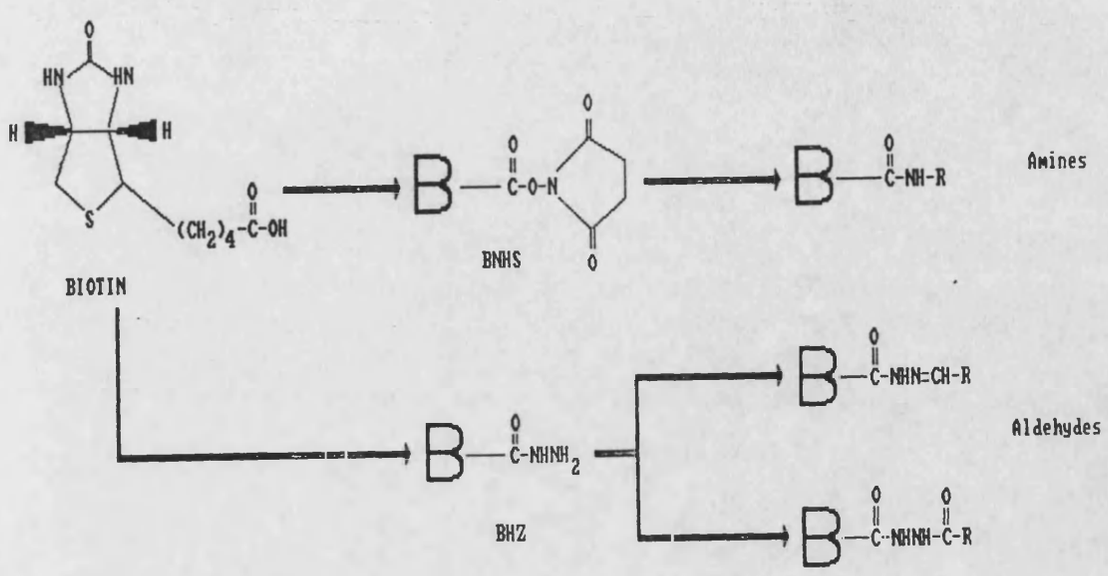
Fig.54 - Biotin/Avidin System : Reactions & Use

A) - shows how the biotinyl group is attached to proteins or sugars through the generation of highly reactive intermediates (BNHS - biotinyl-N-hydroxy succinimide ester; BHZ -biotin hydrazide).
B - biotin group; R - biological molecule (eg. protein).

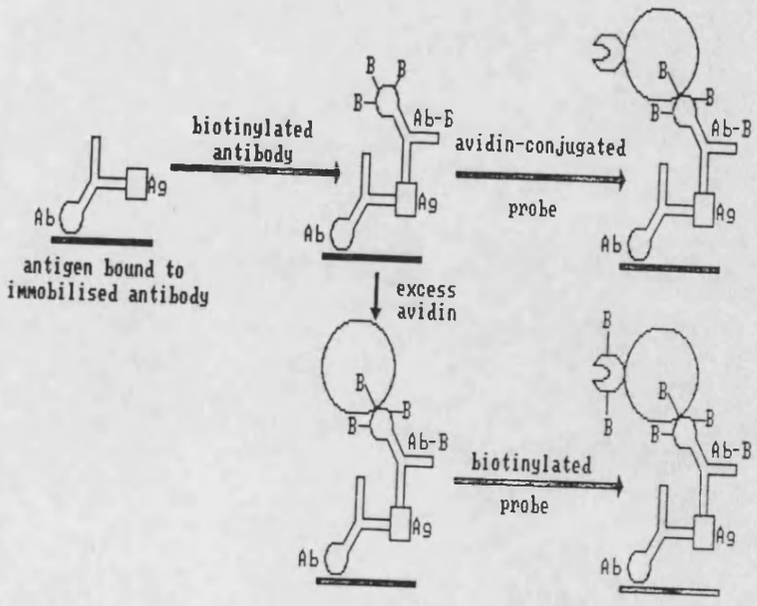
B) - shows use of avidin/biotin system in immunoassays, with two procedures being available ie. utilising an avidin conjugated probe, or a biotinylated probe.

Ag - antigen
Ab - antibody
B - biotin group
Ab-B - biotin labelled antibody

A)



B)



AChR's bind with great specificity and affinity to αBGT that it would be more appropriate to label the αBGT with biotin, a modification which has been used to investigate AChR's on cultured myotubes (Axelrod, (1980)). In addition αBGT is readily obtainable in a reasonably pure form, unlike AChR's, especially mammalian. The αBGT was able to be labelled with biotin very easily (Methods, section 3.3.1), and separated from non-labelled αBGT by Gel filtration. The early preparations of biotinylated αBGT, utilised overnight dialysis to remove the biotinylation reagents. However, the molecular weight cut off point for dialysis tubing is 8000 MW, which is the molecular weight of αBGT (Introduction, section 3.2.2). It is therefore possible that some of the BαBGT could be lost through the tubing. In order to prevent this, the subsequent preparations of BαBGT were separated on a Sephadex G25 column. This is a much easier method than dialysis when handling small volumes as none of it is lost, and can be carried out in a much shorter time period.

The BαBGT so prepared throughout this study was stable for al least 6 months, when stored at -20°C, a shelf life which far exceeds that for the equivalent ^{125}I-αBGT (effectively only 3 weeks), and does not require any special procedural equipment or safety precautions, unlike the preparation of radiolabelled αBGT. Shelf life becomes important where consistency is required. With the conventional RIA, antibody titres in sera, are dependent on

the quality and biological activity of the AChR and ^{125}I-αBGT preparations. Thus repeat assays of the same sera utilising different preparations may result in different titres being obtained.

In addition to its stability, BαBGT when compared to its radioactive counterpart in the detection of Torpedo AChR in a solid phase assay (Methods, section 3.3.2.i; Results, section 3.2.1) is found to be more sensitive, with low background. The binding of BαBGT to Torpedo AChR was, moreover, shown to be specific, through the demonstration that cholinergic ligands inhibit its binding, in a manner which exactly parallels that of αBGT itself (Methods, section 3.3.2.ii; Results, section 3.2.2). An assay based on that developed by Green (1965), determined that each αBGT molecule had 4-5 biotin moieties attached (Methods, section 3.3.2.ii; Results, section 3.2.3) which matches the number required for effective recognition by avidin probes (Wilchek & Bayer, (1983)).

A checkerboard ELISA utilising varying concentrations of both BαBGT and avidin peroxidase conjugate, with excess bound Torpedo AChR, established that 1/500 dilutions of both BαBGT and avidin peroxidase (representing concentrations of 0.5μg/ml and 1μg/ml respectively) achieved maximum sensitivity.

Thus is was possible to show that BαBGT binds with great specificity and selectivity to Torpedo AChR, with low background

levels. Having established this is could then be used in an ELISA for the detection of human anti-AChR antibodies in culture supernatants. Human monoclonal anti-AChR antibodies were not available for use in the development of the assay as this would be the ultimate use for the assay. However, several murine monoclonal anti-Torpedo AChR antibodies were, as they had been previously developed in the department by Dr. S. Walsh, together with a murine monoclonal anti-neurofilament monoclonal antibody developed by Dr. A. Rogers, which was available as a control. These antibodies were therefore used to develop and optimise the assay.

The antibodies available were firstly investigated by use of a direct Torpedo ELISA, and by RIA using Torpedo and fetal calf AChR, with regards to their binding abilities (Methods, section 3.3.3.i; Results, section 3.3.1). From these results, four monoclonal antibodies were chosen for further study. Three of these (C11, B11, & E8) cross-reacted with fetal calf AChR, and one (C7) did not (Tables 18 &19 give the biochemical properties of these antibodies).

The antibody capture ELISA was assessed in terms of its ability to detect these murine monoclonal anti-AchR antibodies (Methods, section 3.3.3.ii; Results, section 3.3.2). It proved to be both reliable and sensitive in the detection of anti-AChR antibodies from culture supernatants, with an ability to be able

to readily detect antibody levels of 2 ng/ml (approximately 2×10^{-11} M αBGT binding sites; Results, section 3.3.2), and even as low as 0.2 ng/ml. This represents a 30 fold increase in sensitivity over the RIA, when one of the monoclonal antibodies, C7, was used. The specificity of the assay was demonstrated by the lack of response seen by antibody C7, with fetal calf, and of anti-neurofilament antibodies with either Torpedo or fetal calf AChR. This agreed with the results obtained by RIA. In addition, specificity was further shown by the responses being totally abolished in the presence of the cholinergic ligand, decamethonium bromide (Results, section 3.3.2).

Having established the essential specificity and sensitivity of the assay, the assay conditions were optimised and standardised using one of the monoclonal antibodies, C7 (Results, section 3.3.3). However, it was observed during the course of these assays, that high background readings were obtained in blank wells which had HRP-avidin added, after being blocked with casein. This was something not normally seen as the low background levels are a major advantage of the assay. Further studies suggested that the HRP-avidin was interacting with the casein present in the assay buffers, and used to block the wells. This was attributed to the highly basic nature of the avidin, pI = 10 (Wilchek & Bayer, (1980)). If the avidin interacts with the casein incorporated into the assay buffers, then this could effectively

reduce the concentration of HRP-avidin in the assay, and thereby reduce the sensitivity of the assay. Thus it is conceivable that the sensitivity of the assay has been underestimated. In order to overcome this problem, several alternatives to casein were investigated (Results, section 3.3.3.i)).

Casein is recognised to be the most effective blocker of non-specific binding. This ability being attributed to it forming a mono-molecular film over the plastic assay wells, however, it has been suggested that in certain assay systems casein may not be the blocker of choice, depending on the requirements of the assay (Vogt et al, (1987)). This appears to be the case with this particular ELISA.

Tween-20, BSA, FCS, and NGS were all found to be ineffective blockers of non-specific binding, supporting similar conclusions by other workers (Vogt et al, (1987)). Vogt et al, (1987), suggested that an alternative to casein, in systems where it is not suitable, is fish skin gelatin. It has been shown to be an excellent blocker of nitrocellulose Western blots (Sarais (1984)), and in addition it does not solidify even under refrigeration at high concentrations. It was found to be an excellent blocker in this particular ELISA system, especially when used in conjunction with Streptavidin-HRP.

Streptavidin-HRP was used as an alternative to avidin-HRP as it is almost neutral, pI = 7.2-7.5, compared to the highly

basic avidin, consequently, it has been found to be more useful in certain systems (Hurt & Taafe, (1987); Wilchek & Bayer, (1980)).

The combination of Streptavidin-HRP with the use of fish skin gelatin as a non-specific blocker, reduced the non-specific binding to the levels previously seen, and a slight increase in sensitivity was noted. Consequently, both these modifications were adopted as a matter of course in subsequent experiments.

Whilst investigating optimal binding of AChR in the assay, it was noted that an AChR concentration of between 0-5 pmol/ml achieved saturation, and that a concentration as low as 0.1 pmol/ml gave measureable readings above background. This level of sensitivity becomes important when mammalian AChR extracts are being used, in particular, human, where concentrations around 1 pmol/ml are frequently encountered (Results, section 3.3.3.iii).

Due to the lack of success of the human monoclonal antibodies, the antibody capture ELISA was used to detect anti-AChR antibodies in myasthenic sera (Methods, section 3.3.4). Five myasthenic sera were chosen for study, which represented a typical cross section of antibody titres, as indicated by RIA. The RIA utilises an anti-human IgG antiserum to precipitate anti-AChR antibodies (Methods, section 1.6), consequently only the anti-AChR IgG antibodies are detected, whereas anti-AChR IgM antibodies are not. In general, it is the anti-AChR IgG antibodies which are deemed to be pathogenic in MG (Introduction, section 3.3.7)

even though myasthenic sera have been shown to be polyclonal in nature, with a variety of isotypes being present. However, if a subpopulation of antibodies is responsible for MG, then considering that complement mediated lysis is believed to be the mechanism responsible for the loss of AChR from the post synaptic membrane (Introduction, section 3.3.7), it would not be inconceivable that these antibodies are of an IgM isotype, as IgM's have a greater complement binding ability than IgG's.

The antibody capture ELISA failed to detect IgG antibodies in any of the myasthenic sera, even in high titred sera as shown by RIA (Results, section 3.3.4), but human anti-AChR IgM antibodies were detected. This could initially be explained by the fact that immobilised IgM could in theory bind up to 5 fold more AChR than IgG, introducing a type of response amplification. The assay showed that all sera tested, including NHS, had detectable levels of anti-AChR IgM antibodies. This gave supporting evidence to the suggestion that even normal healthy individuals produce small amounts of autoimmune antibodies which tend to be IgM in nature and can be equated to a primary immune response (Introduction, section 2). In addition, the ELISA showed that two patients (HN & RS), both of whom have high titres by RIA, have IgM titres equivalent to those seen in NHS, whereas another patient (PT), which also had high titres, had high IgM titres above normal, as did two other patients (RA & SH), who had low

RIA titres (Results, section 3.3.4). The range of IgM titres, when compared with those obtained by conventional RIA for IgG, tends to lend weight to the suggestion that an isotype switch may be important in the generation of an autoimmune response, or at least in the generation of a secondary immune response (Introduction, section 2). Thus, the patients tested by RIA and ELISA, may well be at differing stages in the generation of a secondary immune response, as IgG titres tend to increase on the development of a secondary immune response, whereas IgM titres decrease. In order to provide fairly conclusive evidence of this, it will be necessary to follow a patient's titres as they change with time, from first diagnosis, through to the development of the full blown disease.

It is quite clear that there is scope for further work to be carried out on the use of the ELISA in the detection of anti-AChR antibodies in myasthenic sera. It is unfortunate that the assay could not be tried out on the culture supernatants obtained from cultured lymphocytes or heterohybridomas for the reasons already discussed (Discussion, section 2).

FURTHER AIMS

The inability to utilise the biotin/avidin ELISA, in the detection of human monoclonal anti-AChR antibodies, means that this must be the primary objective of any further study in the development of human cell lines secreating antibodies of this specificity. Theoretically, the sensitivity of the ELISA in detecting anti-AChR antibodies, could be increased through an amplification step (Wilchek & Bayer, (1983); fig. 54), and could be repeated several times. However, steric hinderance and interference between bound components of the system, will limit the number of times the amplification will be effective. Such a step would be particularly useful if human extracts with low levels of AChR activity are being used. However, such a refinement was not carried out during the course of this present study, and would lend itself to further investigation. In addition, such an amplification step may have allowed the detection of human anti-AChR IgG antibodies in myasthenic sera (Methods, section 3.3.4; Results, section 3.3.4), even though it was not used. Coupled with this, is the assaying of anti-AChR IgG and IgM antibodies in the sera of myasthenic patients where changes in titres are monitored with respect to time. These results may then be compared to changes in disease severity within the particular patient in an attempt to obtain some sort of correlation which is lacking at present (Introduction, section 3.3.6 & 3.3.7), and may also be related to the development of a secondary immune response.

Human monoclonal antibody technology itself requires further study and refinement. Even up to the present time, very little headway has been made in developing a general widely applicable technique and a good human fusion partner. Consequently, several different techniques are used to generate human monoclonal antibodies, and unless there is a major advance in the technology, this diversity is likely to continue (Thompson, (1988)). Thus, further work is needed in this area, in particular in the development of a suitable fusion partner which displays the favourable characteristics seen in the murine myelomas, in particular X63 (Kearney et al, (1979)). The development of such a cell line would make the fusion technique particularly attactive. However, the choice of tissue for obtaining lymphocytes will remain a problem. In general, peripheral blood is the only tissue available even though lymph nodes, spleen and bone marrow cells have been shown to be richer in B cells (Thompson, (1988)). Thus, lymphocyte enrichment, in vitro stimulation, and EBV transformation, will continue to be used in order to improve the performance of PBL's.

Having demonstrated that anti-idiotypes can be generated in experimental animals (Methods, section 4; Results, section 4), then it should be relatively easy to generate murine monoclonal anti-idiotypic antibodies. Such antibodies will undoubtedly find numerous immunological and biochemical applications in isolation and characterisation studies, as well as in assays. For example,

in the isolation and purification of specific idiotypes, which may be involved in disease processes with subsequent purification of and characterisation of the antigen; labelling of lymphocytes carrying the specific idiotype of interest on their cell surface, and subsequent separation by FACS, from which monoclonal antibodies can be produced; and, in assaying specific idiotypes of interest.

Even though this particular study has only had limited success, several follow up investigations with regard to the use of the biotin/avidin ELISA, development of human monoclonal antibodies, and investigation and exploitation of the idiotype-anti-idiotype system, are now required.

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APPENDIX - PUBLICATIONS

AUTHORS : A. QUINN, R. HARRISON, A.M.T. JEHANLI, G.G. LUNT & S. WALSH (1988)

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An ELISA for the detection of anti-acetylcholine receptor antibodies using biotinylated α-bungarotoxin

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An antibody-capture enzyme immunoassay has been developed for the detection of anti-acetylcholine receptor (AChR) antibodies in tissue culture supernatants using biotinylated α-bungarotoxin (BαBGT). Immunoglobulins in culture supernatants were bound indirectly to microtitre plates via an anti-globulin antibody already coupled to polyvinyl plates. Anti-AChR antibodies were then detected by incubation with AChR crude extract. Bound AChR was revealed by incubation with BαBGT followed by horseradish peroxidase-conjugated avidin. This assay is specific, more sensitive than the commonly used double antibody radioimmunoassay, avoids the use of radioactive material, is practical for large numbers of samples and is particularly suitable for detecting anti-AChR antibodies in tissue culture supernatants.

Key words: Acetylcholine receptor; α-Bungarotoxin, biotinylated; Monoclonal antibody; ELISA

Introduction

Over the past 10 years there has been considerable interest in the production and characterisation of antibodies to the nicotinic acetylcholine receptor (AChR). This interest largely stems from the discovery of such autoantibodies in the neuromuscular disease, myasthenia gravis (for review, see Harrison and Behan, 1986), and experimentally induced antibodies, both polyclonal and monoclonal, have proved useful in clarifying, not only the pathogenesis of myasthenia, but also structure-function relationships of the receptor itself (Lindstrom, 1984).

Anti-AChR antibodies in serum are most commonly measured by a radioimmunoassay, first described by Lindstrom (1977), which depends upon the precipitation of ^{125}I-α-bungarotoxin-labelled AChR-antibody complex by second antibody (Carter et al., 1981) or by *Staphylococcus aureus* cells (Tindall et al., 1981). A variation of this procedure, using horseradish peroxidase-labelled AChR and enzyme immunoassay, has been described by Furukawa et al. (1984).

Screening of large numbers of cell supernatants during the course of monoclonal antibody production is most conveniently carried out by solid-phase assay using multiwell plates, as in the ELISA and IRMA procedures (Voller et al., 1981); of these, the ELISAs have the advantage of avoiding the use of radioisotopes. A number of ELISAs for anti-AChR antibodies have been reported but are open to various objections. Thus, those described by Norcross et al. (1980), Kawanami et al. (1984) and Kobayashi et al. (1984) all use purified AChR, which is adsorbed onto the solid phase, prior to exposure to test serum and second antibodies. While suitable for the readily available AChR

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from electric fish, this is much less practicable for assays using mammalian receptor since the latter is present in only very low amounts in skeletal muscle (Dolly and Barnard, 1984). The procedures of Hinman et al. (1983), and of Dwyer et al. (1983), on the other hand, do not depend upon purification of AChR. That of Hinman et al. (1983) relies upon the ability of plates, pre-coated with α-bungarotoxin, to adsorb specifically AChR from crude extracts, while that of Dwyer et al. (1983) depends upon pre-coating with a monoclonal antibody to achieve the same result. In our hands (S. Walsh, unpublished data) the former assay was successful with *Torpedo* receptor but less so with AChR from mammalian sources. The latter assay suffers from the obvious disadvantage of requiring a suitable monoclonal antibody.

A passive haemagglutination assay previously described by one of us (Muir and Jehanli, 1985) also requires pure receptor.

We now describe an antibody capture ELISA, based on the biotin-avidin system (Bayer and Wilchek, 1980), that can be used with crude AChR and is particularly suitable for the detection of anti-AChR antibodies in tissue culture supernatants.

Materials and methods

Biotinylation of α-bungarotoxin (αBGT)

αBGT was biotinylated using a modification of the procedure employed for labelling immunoglobulins (Kendall et al., 1983). αBGT was thoroughly dialysed against 0.1 M $NaHCO_3$ and its concentration adjusted to 5 mg/ml. To this solution (1 ml), a freshly prepared solution of biotin-*N*-hydroxy-succinimide (Sigma Co., Poole, England) in dimethylformamide (0.1 ml, 40 mg/ml) was added dropwise with gentle stirring. The mixture was incubated, with stirring, for 2 h at room temperature and then dialysed against phosphate-buffered saline (PBS), pH 7.3 (2 litres) for 48 h at 4° C. The concentration of biotin-conjugated αBGT (BαBGT) was adjusted to 0.5 mg/ml in PBS containing bovine serum albumin (10 mg/ml), and an equal volume of glycerol was added. Aliquots (0.2 ml) were stored at −20° C. For the purpose of this study, five lots of conjugate were made, containing an average of 4–5 biotin molecules per molecule of αBGT, as determined colourimetrically by the method of Green (1965). The biological activity of BαBGT and its affinity for *Torpedo* AChR were 70% and 25%, respectively, compared to those of the native toxin (Lukasiewicz et al., 1978).

Purification of AChR

AChR was purified from the electric organs of *Torpedo marmorata* by detergent extraction and affinity chromatography as previously described (Muir and Jehanli, 1985). The purified receptor had a protein concentration 0.3–0.5 mg/ml and bound 6–8 nmol αBGT per mg protein as determined by the procedure of Schmidt and Raftery (1973). Crude preparations of fetal calf skeletal muscle AChR and *Torpedo* AChR were made by detergent extraction only and showed toxin binding activities of 0.8–2.5 pmol/ml, and 2500–3000 pmol/ml, respectively.

Characterisation of binding of BαBGT to AChR

The binding of BαBGT to AChR was characterised by means of a solid-phase enzyme assay. Microwell Module Strips (Nunc, Uxbridge, U.K.) were coated by incubation with purified *Torpedo* AChR in 50 mM sodium carbonate/bicarbonate buffer, pH 9.6 (100 μl/well, 5 μg/ml) overnight at 4° C. The strips were then washed three times with 50 mM Tris/HCl buffer, pH 7.4, containing 0.15 M NaCl, 0.05% (w/v) Tween 20 and 1% (w/v) casein (assay buffer), and blocked by incubation with the same buffer (200 μl) for 30 min. At this stage, strips can be used immediately or stored at 4° C for at least 2 weeks without loss of activity. Prior to use, the wells were washed again with assay buffer and then incubated with serial dilutions of BαBGT in assay buffer (100 μl/well) for 3 h at room temperature or overnight at 4° C. The wells were washed three times (5 min each) with assay buffer and then incubated with appropriate dilutions of avidin-horseradish peroxidase conjugate (HRP-avidin, 0.5 mg/ml Sigma) in assay buffer (100 μl/well) for 3 h at room temperature. In some experiments, a biotin-avidin-HRP complex (Radiochemical Centre, Amersham, Bucks, U.K.) was used in place of the HRP-avidin.

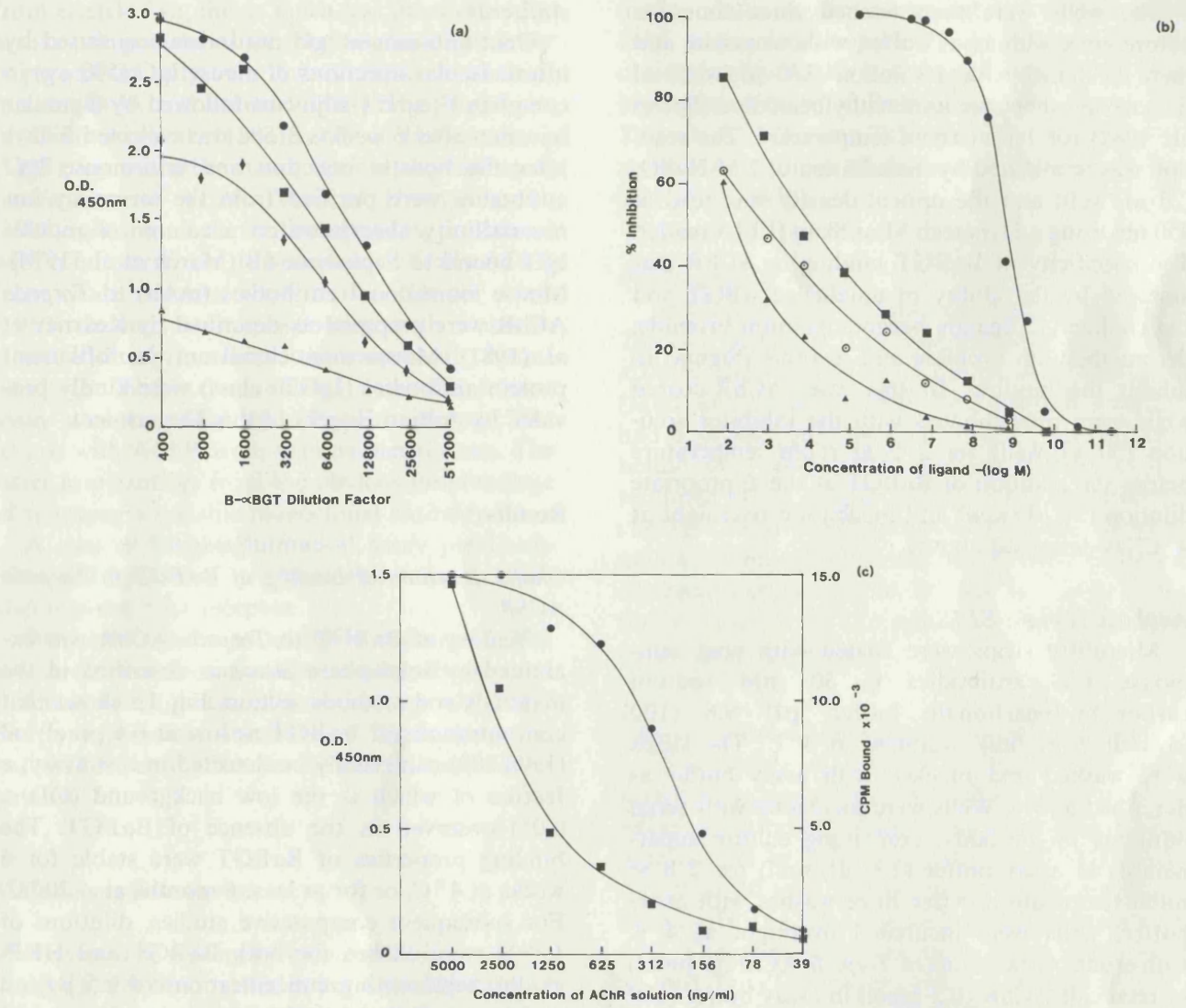

Fig. 1. Interaction of BαBGT with *Torpedo* AChR-coated microtitre wells. *a*: Checkerboard titration of BαBGT concentration and HRP-avidin concentration: 1/300 (●), 1/900 (■), 1/2700 (◆) and 1/8100 (▲). Coating antigen concentration: 5 μg/ml. Absorbance in the absence of BαBGT was < 0.05 with all dilutions of HRP-avidin. *b*: Inhibition of binding of BαBGT (1/500 dil.) to AChR by unlabelled αBGT (●), benzoquinonium (■), decamethonium (⊙) and nicotine (▲). HRP-avidin dilution: 1/500. *c*: Comparison of binding of BαBGT (1/500 dil.) (●), and ^{125}I-αBGT (0.56 pmol) (■) to microtitre wells coated with various concentrations of AChR.

TABLE I

PROPERTIES OF MOUSE MONOCLONAL ANTI-AChR ANTIBODIES

Antibody	Subclass	IgG concentration (μg/ml)	Anti-AChR antibody titre of supernatant (nM) [a]	
			Torpedo AChR	Fetal calf AChR
C7	IgG2b	10.0	110	ND [b]
C11	IgG2b	8.0	139	0.75
B11	IgG2b	7.5	180	4.50
E8	IgG2b	15.0	301	2.32

[a] Titre detected by immunoprecipitation radioimmunoassay (Carter et al., 1981) and expressed in terms of αBGT binding sites.
[b] Not detected.

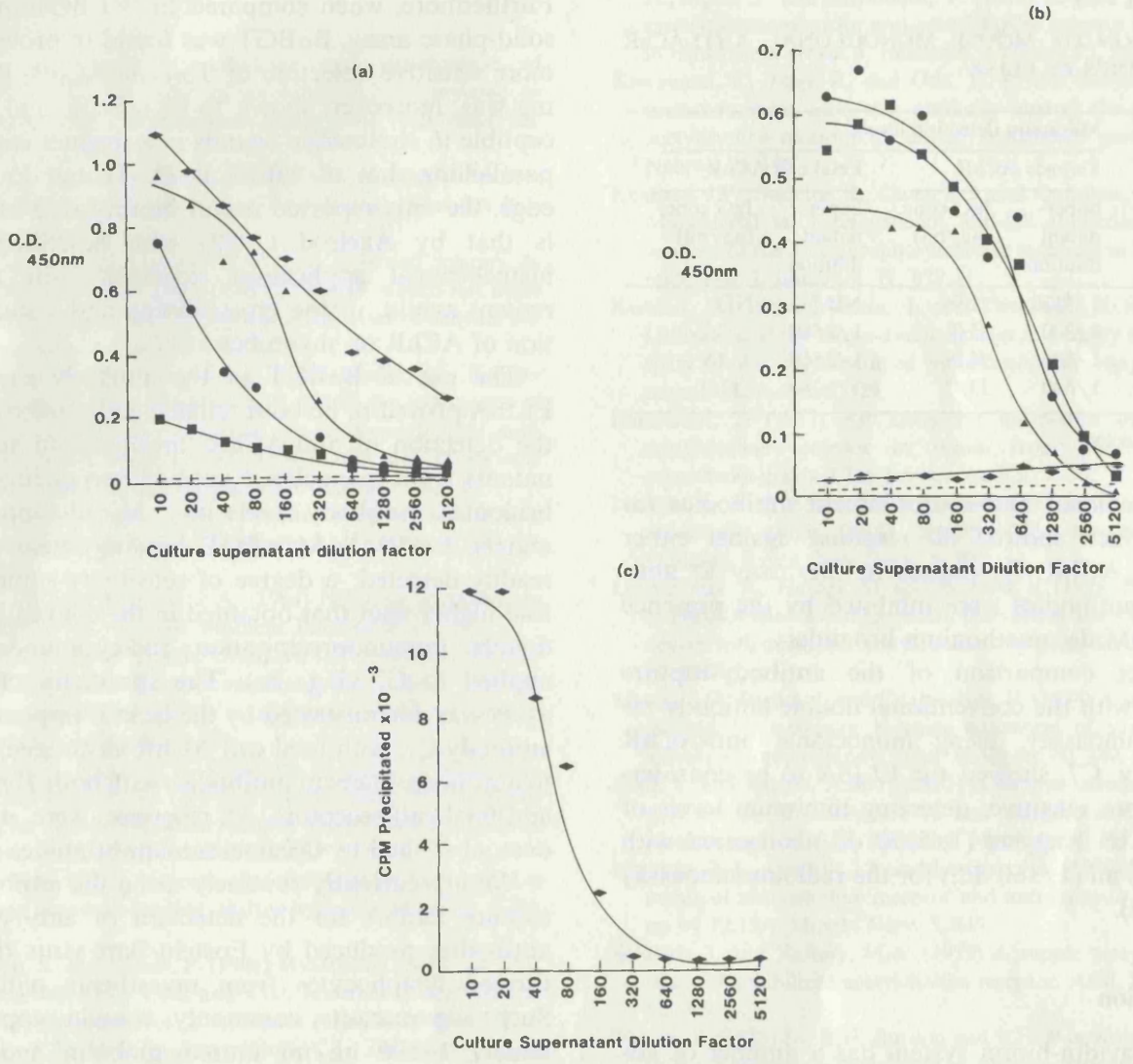

Fig. 2. Titration of anti-AChR antibodies. *a*, *b*: Antibody-capture ELISA for the detection of antibody binding to *Torpedo* AChR and fetal calf AChR, respectively. Monoclonal antibody culture supernatants used were C7 (◆), C11 (●), B11 (■), E8 (▲) and anti-neurofilament (- - - - -). Values are the average of triplicate samples. Percentage standard deviation was $\leqslant 20\%$. *c*: Double antibody RIA for the detection of monoclonal antibody, C7 (◆) binding to *Torpedo* AChR (0.1 pmol). Various amounts of C7 supernatant and normal mouse serum (5 μl) were added and the ^{125}I-αBGT-AChR-antibody complex was precipitated by the addition of goat anti-mouse IgG antiserum (40 μl). Total assay volume was 400 μl.

molecule of αBGT (M_r approx. 8000) fails to accommodate the bulky complexes necessary for amplification of enzyme activity.

Detection of anti-AChR antibodies

The antibody-capture assay for anti-AChR antibodies was assessed in terms of detection of monoclonal antibodies in supernatants from four murine hybridomas. Table I shows the properties of the respective monoclonal antibodies.

The ELISA procedure permitted reproducible detection of anti-AChR antibodies against *Torpedo* AChR (Fig. 2*a*) and fetal calf AChR (Fig. 2*b*). The minimum detection levels of the different antibodies are shown in Table II. The variation in titre between the different monoclonals is most likely a reflection of differences in affinity.

The ELISA confirmed the lack of cross-reactivity of monoclonal antibody C7 against fetal calf AChR which was initially indicated by radioimmunoassay (Table I). The specificity of BαBGT in the ELISA was further demonstrated by the assay

TABLE II

TITRATION OF MOUSE MONOCLONAL ANTI-AChR ANTIBODIES BY ELISA

Antibody	Minimum detection levels			
	Torpedo AChR		Fetal calf AChR	
	Super-natant dilution	IgG conc. (ng/ml)	Super-natant dilution	IgG conc. (ng/ml)
C7	1/5120	1.95	ND [a]	ND
C11	1/320	25.0	1/2560	3.1
B11	1/80	94.0	1/2560	2.9
E8	1/640	23.4	1/1280	11.7

[a] Not detected.

of monoclonal anti-neurofilament antibodies (a-NF), which showed no response against either type of AChR. Responses in the assay to anti-AChR antibodies were inhibited by the presence of 20 mM decamethonium bromide.

Direct comparison of the antibody-capture ELISA with the conventional double-antibody radioimmunoassay, using monoclonal anti-AChR antibody, C7, showed the ELISA to be considerably more sensitive, detecting minimum levels of protein of 2 ng/ml (1/5120 dil.) compared with 62.5 ng/ml (1/160 dil.) for the radioimmunoassay (Fig. 2*c*).

Discussion

The avidin-biotin system has a number of advantages in immunoassay procedures. Biotin can, in general, be readily coupled to proteins with minimal loss of biological activity while avidin, which binds with very high affinity (K_d, 10^{-15}M) to biotin, can be labelled in many ways, e.g., with fluorescent, radioisotope or enzyme probes (Tijssen, 1985). This means that, once characterised, the protein-biotin conjugate can be detected by a range of techniques, requiring little further development. The presence of multiple binding sites for biotin on the avidin molecule offers the possibility of response amplification using avidin-biotin probe complexes in place of avidin-probe conjugates (Hsu and Raine, 1981).

BαBGT prepared in this study was stable for at least 6 months when stored at $-20\,^{\circ}$C, which far exceeds the shelf-life of ^{125}I-αBGT (about 4 weeks). Furthermore, when compared to ^{125}I-BGT in the solid-phase assay, BαBGT was found to provide a more sensitive detection of *Torpedo* AChR. Binding was, moreover, shown to be specific and susceptible to cholinergic ligands in a manner exactly parallelling that of αBGT itself. To our knowledge, the only reported use of biotinylated αBGT is that by Axelrod (1980) who described its histochemical application, together with fluorescent avidin, in the cross-linking and visualisation of AChR on myotubes.

The use of BαBGT in the antibody capture ELISA proved to be both reliable and sensitive for the detection of anti-AChR antibodies in supernatants from monoclonal antibody-producing hybridomas. Antibody levels of 2 ng/ml (approximately 2×10^{-11} M αBGT binding sites) were readily detected; a degree of sensitivity some 30-fold higher than that obtained in the conventional double immunoprecipitation radioimmunoassay applied to C7 (Fig. 2*c*). The specificity of the assay was demonstrated by the lack of response to antibody C7, with fetal calf AChR as antigen, and to anti-neurofilament antibodies with both *Torpedo* and fetal calf receptors. All responses were, moreover, abolished by decamethonium bromide.

We are currently routinely using the antibody-capture ELISA for the detection of anti-AChR antibodies produced by Epstein-Barr virus transformed lymphocytes from myasthenic patients. Such supernatants commonly contain approximately 1–100 ng/ml immunoglobulin and are readily amenable to anti-AChR antibody assay by this method using either fetal calf or human muscle AChR.

The sensitivity and convenience of the antibody-capture ELISA are particularly appropriate for the assay of anti-AChR antibodies in preparations where the relevant antibody fraction is mostly of the desired specificity, e.g., cell supernatants. The binding properties and detection levels of BαBGT itself strongly suggest its extended use in histochemical analysis of AChR.

Acknowledgements

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