IDIOTYPIC ANTIBODIES IN MYASTHENIA GRAVIS

A thesis presented by

ANDREW JAMES ROBERTS

for the degree of Doctor of Philosophy
in the University of London.

Department of Neurological Science,
Royal Free Hospital School of Medicine.

SEPTEMBER 1988

ABSTRACT

Myasthenia gravis is an autoimmune disease characterised by autoantibodies against acetylcholine receptor (AChR). The idiotypic properties of ten monoclonal anti-human AChR antibodies (mabs) have been investigated using anti-idiotypic antibodies.

Using $F(ab')_2$ fragents of each mab to inhibit the binding of intact mab to AChR, five partially overlapping binding regions of human AChR were defined.

Polyclonal anti-idiotypic sera were raised in syngeneic mice against the anti-human AChR mabs. A total of 47 mice were immunised, of which 27 had anti-idiotypic antibodies against antigen binding site idiotopes, and four against non-antigen binding site idiotopes.

Using the anti-idiotypic sera to inhibit the binding of heterologous mab to AChR, both private and cross-reactive idiotypes were detected within the antigen binding site. Three sets of anti-idiotypic sera inhibited the binding to AChR of just the immunising mab. Cross-reactive idiotypes were found in three pairs of mabs, but were always restricted to mabs that bound to the same region of AChR. A cross-reactive idiotype was found outside the antigen binding site, but again this was only shared by two mabs having the same specificity for AChR.

No inhibition by the polyclonal anti-idiotypic sera of the binding of myasthenic antibodies to AChR was found.

Seven IgM monoclonal anti-idiotypes against three different anti-human AChR mabs were produced by fusing the splenocytes of mice immunised with mab by direct intrasplenic injection. Anti-idiotypic activity was measured by direct binding to anti-human AChR mabs in an ELISA. The cross-reactive idiotypes detected by five of the seven monoclonal anti-idiotypes were less restricted than those found with the polyclonal anti-idiotypic sera. The specificity of the anti-idiotypes was confirmed by inhibition of binding by soluble idiotype.

None of the seven monoclonal anti-idiotypes inhibited the binding of mab to AChR, but the small amount of binding to mab-AChR complexes sugggested that they were directed against idiotopes outside the antigen binding site of the anti-human AChR mabs.

TABLE OF CONTENTS

page no.

CHAPTER ONE: INTRODUCTION

1.1 Structure of immunoglobulin, T cell receptor and MHC. 2

1.1.1 Immunoglobulin. 2

1.1.2 T cell receptor. 7

1.1.3 Major Histocompatibility Complex. 9

1.2 Idiotype Network Theory. 11

1.2.1 Idiotype/anti-idiotype interactions. 12

1.2.2 Nomenclature. 12

1.2.3 Cross-reactive Idiotypes. 17

1.3 Tolerance. 17

1.4 Autoimmunity. 21

1.4.1 Environmental factors. 21

1.4.2 Genetic factors. 23

1.4.3 Defects in immune regulation. 24

1.4.4 Idiotypes in autoimmunity. 26

1.4.5 Autoimmune diseases. 31

1.5 Myasthenia Gravis. 34

1.5.1 Myasthenia gravis as an example of an autoimmune disease. 34

1.5.2 Acetylcholine receptor. 39

1.5.3 Anti-AChR antibodies. 42

1.5.4 Idiotypes and anti-idiotypes in myasthenia gravis 45

1.6 Aims. 47

CHAPTER TWO: MATERIALS AND METHODS

Materials. 50

Methods. 52

2.1 Preparation of AChR and monoclonal antibodies. 52

2.1.1 Extraction of acetylcholine receptor. 52

2.1.2 Iodination of α-bungarotoxin. 53

2.1.3 Preparation and purification of monoclonal antibodies. 53

2.1.4 SDS-PAGE analysis of purified monoclonal antibodies. 55

2.1.5 Conjugation of monoclonal antibodies to Sepharose 4B beads 55

2.1.6 Preparation of $F(ab')_2$ fragments of IgG. 56

2.2 Immunisation. 56

2.2.1 Production of polyclonal anti-idiotypic antisera. 56

2.2.2 Production of monoclonal anti-idiotypic antibodies. 57

2.2.3 Fusion (Somatic Cell Hybridisation). 58

2.3 Purification of anti-idiotypic antibodies. 60

2.3.1 Polyclonal anti-idiotypic sera. 60

2.3.2 Monoclonal anti-idiotypic antibodies. 60

2.4 Anti-AChR assays. 61

2.4.1 Anti-AChR radioimmunoassays. 61

2.4.2 Assays of mab - Sepharose 4B beads. 62

2.4.3 Inhibition of mab binding to ^{125}I-BuTx-AChR by $F(ab')_2$ fragments. 63

2.5 Anti-idiotypic assays. 64

2.5.1 Polyclonal anti-idiotypes. 64

2.5.2 Monoclonal anti-idiotypes. 67

CHAPTER THREE: PURIFICATION AND CHARACTERISATION OF ANTI-HUMAN AChR MONOCLONAL ANTIBODIES

3.1 Introduction. 70

3.2 Results. 70

3.2.1 Purification of mabs. 70

3.2.2 Preparation of $F(ab')_2$ fragments. 73

3.2.3 Protection of mab binding-sites by $F(ab')_2$. 79

3.2.4 Conjugation of mab to Sepharose 4B beads. 81

3.3 Discussion. 81

3.4 Summary. 87

CHAPTER FOUR: POLYCLONAL ANTI-IDIOTYPES

4.1 Introduction. 89

4.2 Results. 90

Homologous anti-idiotypes.

4.2.1 Ab2gamma. 90

4.2.2 Ab2alpha. 93

4.2.3 ELISA. 100

Cross-reactive anti-idiotypes.

4.2.4 Ab2gamma. 105

4.2.5 Ab2alpha. 111

4.2.6 ELISA. 111

4.2.7 Inhibition of myasthenic autoantibodies. 113

4.3 Discussion. 115

4.4 Summary. 123

CHAPTER FIVE: MONOCLONAL ANTI-IDIOTYPIC ANTIBODIES

5.1 Introduction. 124

5.2 Results. 125

5.2.1 Mice immunised with anti-AChR mabs with Freund's adjuvant. 125
5.2.2 Intrasplenically-immunised mice. 128
5.2.3 Binding of monoclonal anti-idiotypic antibodies. 130
5.3 Discussion. 155
5.4 Summary. 160
CHAPTER SIX: PURIFIED ANTI-IDIOTYPIC ANTIBODIES
6.1 Introduction. 161
6.2 Results. 161
6.2.1 Purification of polyclonal anti-ids. 161
6.2.2 Purification of monoclonal anti-ids. 164
6.2.3 Assays of purified anti-idiotypic antibodies 169
6.3 Discussion. 177
6.4 Summary. 178
CHAPTER SEVEN: CONCLUDING REMARKS 180
REFERENCES 191
APPENDICES 204

LIST OF FIGURES

Figure page no.

1.1: Genes encoding the light and heavy chains of immunoglobulin. 3

1.2: Diagramatic representation of the immunoglobulin molecule. 5

1.3: Computer generated 3-dimensional structure of IgG. 6

1.4: Structure of the T cell receptor. 8

1.5: Schematic representation of the antigen binding site of the HLA-A2 molecule. 10

1.6: Illustration of the limited degree of branching of the Idiotype Network. 13

1.7: Schematic representation of the Ab2 nomenclature of anti-idiotypes. 15

1.8: Development of autoimmunity by idiotypic interactions. 27

1.9: The neuromuscular junction showing the location of AChR on the post-synaptic folds. 36

1.10: Three ways in which anti-AChR antibodies can cause myasthenia gravis. 38

1.11: Structure of acetylcholine receptor. 41

1.12: Schematic representation of the five partially overlappping binding regions of anti-human AChR mabs. 43

3.1: DEAE- Affigel blue chromatography. 71

3.2: 10% SDS-PAGE gel of DEAE- Affigel blue purified mab F8. 74

3.3: ELISA binding of $F(ab')_2$ fragments of anti-

human AChR mabs. 76

3.4: Precipitation of ^{125}I-BuTx-AChR by mab-Sepharose 4B beads. 82

3.5: Schematic representation of anti-human AChR mab binding regions based on present findings. 88

4.1: Time course of anti-id response to mab G10. 91

4.2: Inhibition of id (mab or F(ab')$_2$) binding to AChR by anti-id. 94

4.3: Inhibition of id binding to AChR by anti-id preincubated with normal mouse serum. 96

4.4: Ab2gamma titres of individual mice. 98

4.5: ELISA absorbance readings of individual mice. 102

4.6a: Cross-reactive inhibition of id binding to AChR by anti-ids to regions 1 and 2 mabs. 106

4.6b: Cross-reactive inhibition of id binding to AChR by anti-ids to regions 3 and 5 mabs. 108

5.1a: ELISA binding of anti-id (6D2, 8D8 & 3G6) to homologous idiotype. 131

5.1b: ELISA binding of anti-id (5C5, 5F9, 10C1 & 10G3) to homologous idiotype. 133

5.2: Cross-reactive ELISA binding of anti-ids. 136

5.3: Titration of anti-G10 anti-ids versus cross-reactive ids. 140

5.4: Titration of anti-G10 anti-ids at different temperatures. 144

5.5: Inhibition of 5F9 binding to id in ELISA by soluble idiotype. 147

5.6: Inhibition of 10G3 binding to id in ELISA by

soluble idiotype. 149

5.7: Inhibition of 5C5 and 10C1 binding to id in ELISA by soluble idiotype. 151

6.1 Inhibition of homologous id binding to AChR by affinity purified polyclonal anti-idiotypes. 162

6.2 ELISA binding of monoclonal anti-idiotypic ascites to idiotype. 165

6.3 ELISA titration of affinity purified monoclonal anti-idiotypes to homologous id. 164

6.4: Indirect binding of purified anti-idiotypes to id-^{125}I-BuTx-AChR measured by RIA. 172

6.5: Binding of purified monoclonal anti-idiotype to ELISA plates. 175

LIST OF TABLES

Table		page no.
1.1:	The spectrum of autoimmune diseases.	32
1.2:	Properties of anti-human AChR mabs.	43
3.1:	Precipitation of ^{125}I-BuTx-AChR by F(ab')$_2$ fragments.	78
3.2:	Inhibition of mab binding to AChR by F(ab')$_2$.	80
3.3:	Precipitation ^{125}I-Protein A by mab - Sepharose 4B beads.	84
4.1:	Comparative titres of the four Ab2alpha - positive anti-ids.	101
4.2:	Results of different anti-ids detected in five mice immunised with mab C7.	104
4.3:	Cross-reaction of Ab2gamma anti-ids with anti-human AChR mabs.	110
4.4:	Summary of polyclonal anti-idiotypic responses measured by different assays.	112
4.5:	Inhibition of MG sera binding to AChR by anti-ids.	114
5.1:	Fusions of splenocytes from mice immunised with idiotype and Freund's adjuvant.	126
5.2:	Fusions of splenocytes from mice immunised intrasplenically.	129
5.3:	Summary of cross-reactive binding of anti-G10 anti-ids.	143
5.4:	Summary of inhibition of of anti-id binding to solid phase id by soluble idiotype.	154
6.1:	Lack of inhibition of homologous idiotype binding to AChR by purified monoclonal anti-id.	170

6.2: Lack of inhibition of heterologous id binding to AChR by purified monoclonal anti-id. 171

ACKNOWLEDGEMENTS

I would like to acknowledge the support of Professor Newsom-Davis and the helpful advice of Dr. Angela Vincent and Dr. Nick Willcox during the course of this study.

I greatly appreciate the expertise of Dr. Andy Jermy and Chris Fisher in the immunising and bleeding of animals, and the generous help of Helen Wood.

I am very grateful to Dr. Bethan Lang for the many hours of conversations, and for all her encouragement and guidance.

Finally, I would like to thank the staff of the Department of Neurological Science for their support thoughout the time I spent at the Royal Free Hospital.

ABBREVIATIONS.

Ab	antibody
Ab2alpha	anti-framework anti-idiotype
Ab2beta	internal image anti-idiotype
Ab2gamma	anti-combining site anti-idiotype
ACh	acetylcholine
AChR	acetylcholine receptor
Ag	antigen
Anti-id	anti-idiotype
BSA	bovine serum albumin
BuTx	α-bungarotoxin
cpm	counts per minute
CRI	cross-reactive idiotype
DMSO	dimethylsulphoxide
EAMG	experimental autoimmune myasthenia gravis
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme linked immunosorbent assay
FCA	Freund's complete adjuvant
FCS	fetal calf serum
FIA	Freund's incomplete adjuvant
HAT	hypoxanthine (100 uM), aminopterin (0.4 uM) & thymidine (16 uM)
HLA	human leucocyte antigen
HT	hypoxanthine (100 uM) & thymidine (16 uM)
HRP	horseradish peroxidase
Id	idiotype
IdI	private idiotype
IdX	public idiotype
Ig	immunoglobulin
LEMS	Lambert-Eaton myasthenic syndrome

mab	monoclonal antibody
MG	myasthenia gravis
MIR	main immunogenic region
MHC	major histocompatibility complex
nms	normal mouse serum
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PA-S4B	Protein A - Sepharose 4B
PBS	phosphate buffered saline
PBST	0.1% (v/v) Tween 20 in PBS
PEG	polyethyleneglycol
PMSF	phenylmethylsulphonylfluoride
PTX	0.1% (v/v) Triton X100 in 0.1M phosphate buffer
ReI	regulatory idiotype
RF	rheumatoid factor
RF_1	1% (v/v) FCS in RPMI
RF_{10}	10% (v/v) FCS in RPMI
RIA	radioimmunoassay
RPMI	Roswell Park Memorial Institute 1640 media
SDS	sodium dodecylsulphate
SLE	systemic lupus erythematosus
TMB	tetramethylbenzidine

CHAPTER ONE

INTRODUCTION

The immune system provides the means by which the body is able to defend itself from infectious agents such as viruses, bacteria, fungi and parasites. The immune system has two components, innate and adaptive immunity with which it can respond to foreign organisms. Innate immunity includes physical and chemical barriers such as the skin and the mucosal lining of the respiratory and gastro-intestinal tracts, and non-specific cells such as phagocytes. The important feature of the adaptive system is its ability to learn and to change its response when re-exposed to the same antigen. The two effector arms of the adaptive immune response are mediated by B and T cells. The antigen-specific, cell surface receptors of B cells are immunoglobulins, which are also expressed in a secreted form as antibodies. The equivalent molecule of T cells is the T cell receptor (TCR), which is specific for a combination of antigen and a product of the major histocompatibility complex (MHC). The humoral response usually requires the help of T cells in the form of specific growth and differentiation factors (lymphokines) produced by activated T cells for B cells to mature into antibody secreting plasma cells.

1.1 Structure of immunoglobulin, T cell receptor and MHC.

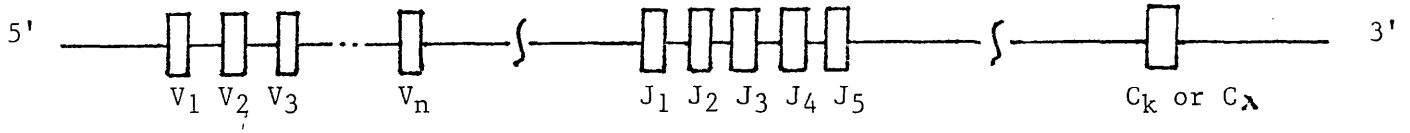
1.1.1 Immunoglobulin.

T and B cells utilize similar mechanisms to generate the diversity required to recognise a whole spectrum of possible antigenic determinants. Antibody molecules consist of two heavy and two light chains covalently linked by disulphide bridges. The heavy and light chains are encoded by separate germline gene clusters (Figure 1.1) that undergo somatic gene rearrangements which are then transcribed to form function antibody molecules (Tonegawa 1983). Each heavy chain consists of one variable (VH) and three constant (CH) domains, and the light chain of one constant (CL) and one variable (VL) domain. The variable regions are formed from multiple variable (V), joining (J) and in the case of the heavy chain, diversity (D) germline gene segments (Figure 1.2). The antigen binding site is formed by the combination of the VH and VL domains (Figure 1.3), and the constant domain provides the link to effector functions such as activation of the complement pathway and of phagocytic cells. In each chain there are three distinct hypervariable regions also termed complementarity determining regions (CDR1, CDR2 and CDR3) (Wu and Kabat 1970), which together contribute to the overall antigen binding site of an antibody molecule. The germline VH and VL gene segments each encode two of the CDRs (CDR1 and 2), and CDR3 arises from the junctional region formed by joining of the VDJ-heavy and VJ-light gene segments. Further diversity of the antibody molecule is provided by somatic mutation of the rearranged

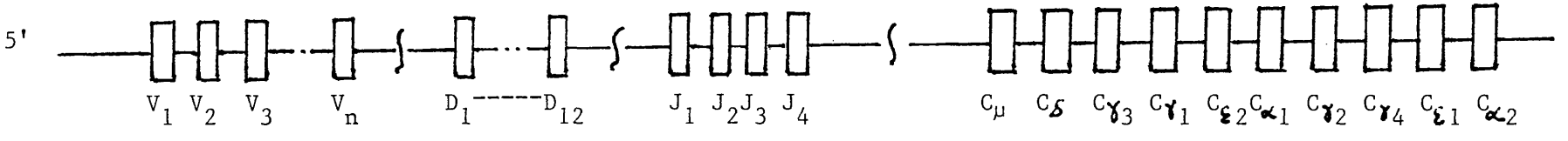
Figure 1.1: Genes encoding the light and heavy chains of immunoglobulin.

In the light chain a V region gene associates with one J region gene to give a functional V region which then associates with a C_{κ} or C_{λ} gene. The heavy chain is formed by the recombination of one V gene, a D gene and a J gene to produce a recombined VDJ gene. This becomes linked to a constant region gene during mRNA splicing.

Light Chain Gene



Heavy Chain Gene



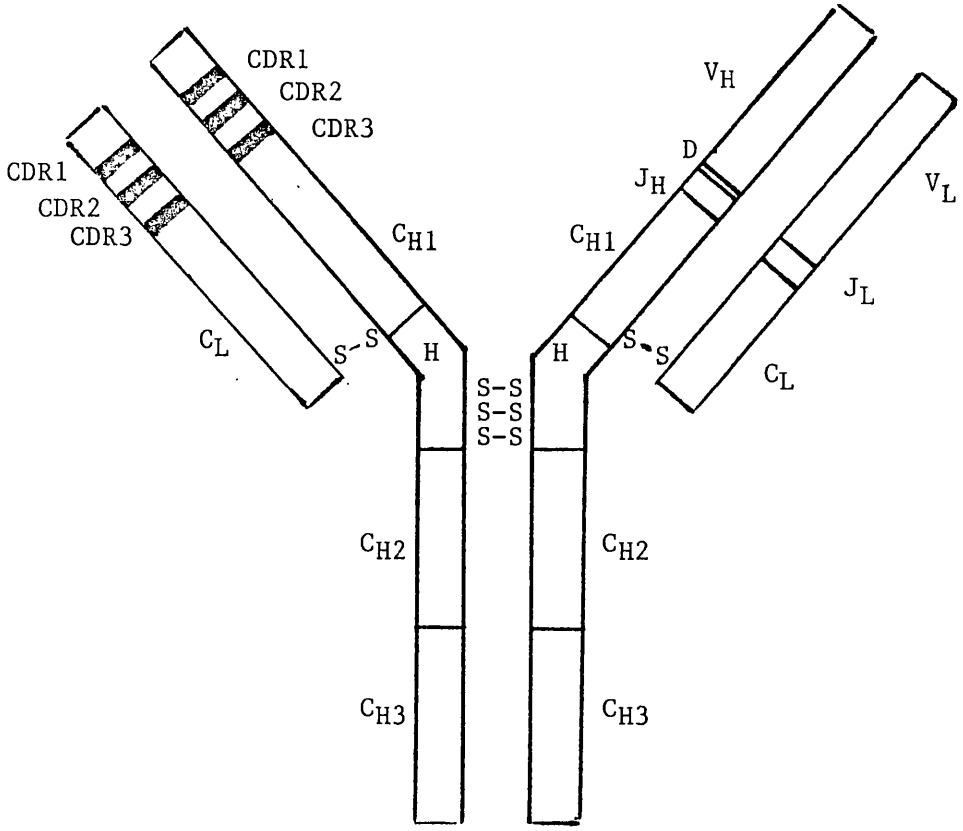


Figure 1.2: Diagramatic representation of the immunoglobulin molecule.

A typical subunit of an immunoglobulin molecule comprising of two identical heavy and two identical light chains linked by disulphide bridges. Variable regions are indicated on the right subunit, CDRs on the left.

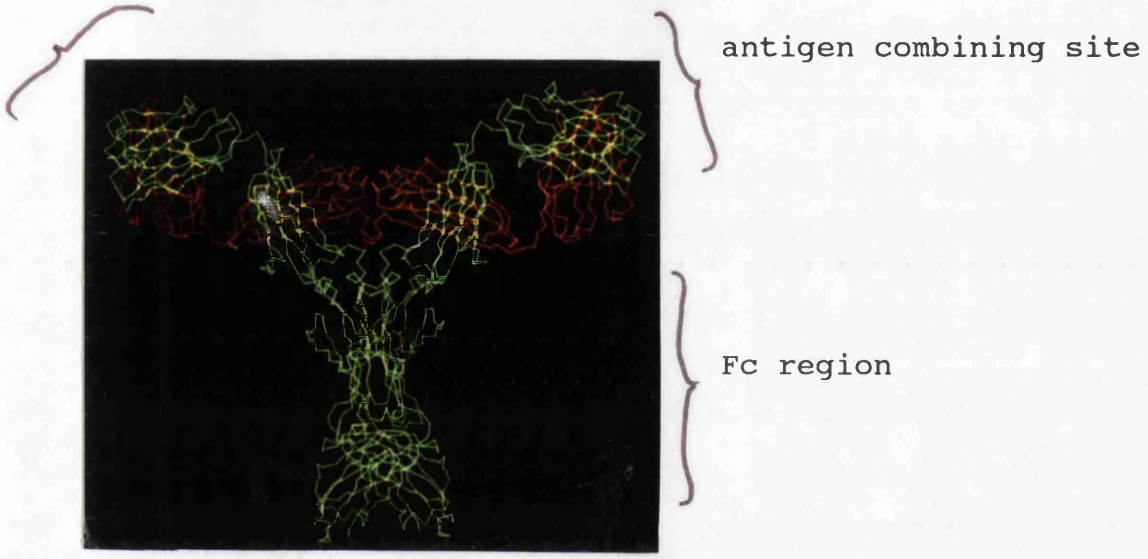


Figure 1.3: Computer generated 3-dimensional structure of IgG.

The heavy chains are shown in green, the light chains in red and yellow showa where light and heavy chains are superimposed. The antigen combining site and the Fc portion of the molecule are as indicated.

(Photograph the kind gift of Dr. J. Singh, Birkbeck College London, and Dr. D Madahevan, Kings College School of Medicine, London).

genes and is found predominantly in the first two hypervariable regions (CDR1 and 2). Overall the total antibody repertoire has been estimated at 10^8 - 10^{10}. Hence the humoral arm of the immune response has the capacity to recognise and combine with an almost infinite number of foreign antigens.

1.1.2 T cell receptor.

The TCR of most T cells is a dimer of two polypeptide chains α and β (although 10% of T cells bear a TCR formed from τ and δ chains), and is closely associated with the invariant molecule CD3 (Figure 1.4) (Marrack and Kappler 1987). Antibodies and the TCR are structurally related molecules and the genes encoding them are members of the 'immunoglobulin super family'. Like antibody genes, the TCR genes have constant and variable domains, the variable domain of each TCR chain comprising of V, J and in the case of the the β chain D region genes which undergo somatic gene rearrangements to form the functional TCR molecule (Davis and Bjorkman 1988). Although the TCR is not thought to somatically mutate (Behlke et al 1985), the potential TCR repertoire is still at least 10^7.

TCR, unlike antibody is not produced in a secreted form. T cells also differ from B cells in that the TCR does not recognise antigen in its native form. Antigen must first be processed by cells termed 'antigen presenting cells' (APC) such as macrophages and dentritic cells. Antigen is taken up by these cells and degraded into peptide fragments.

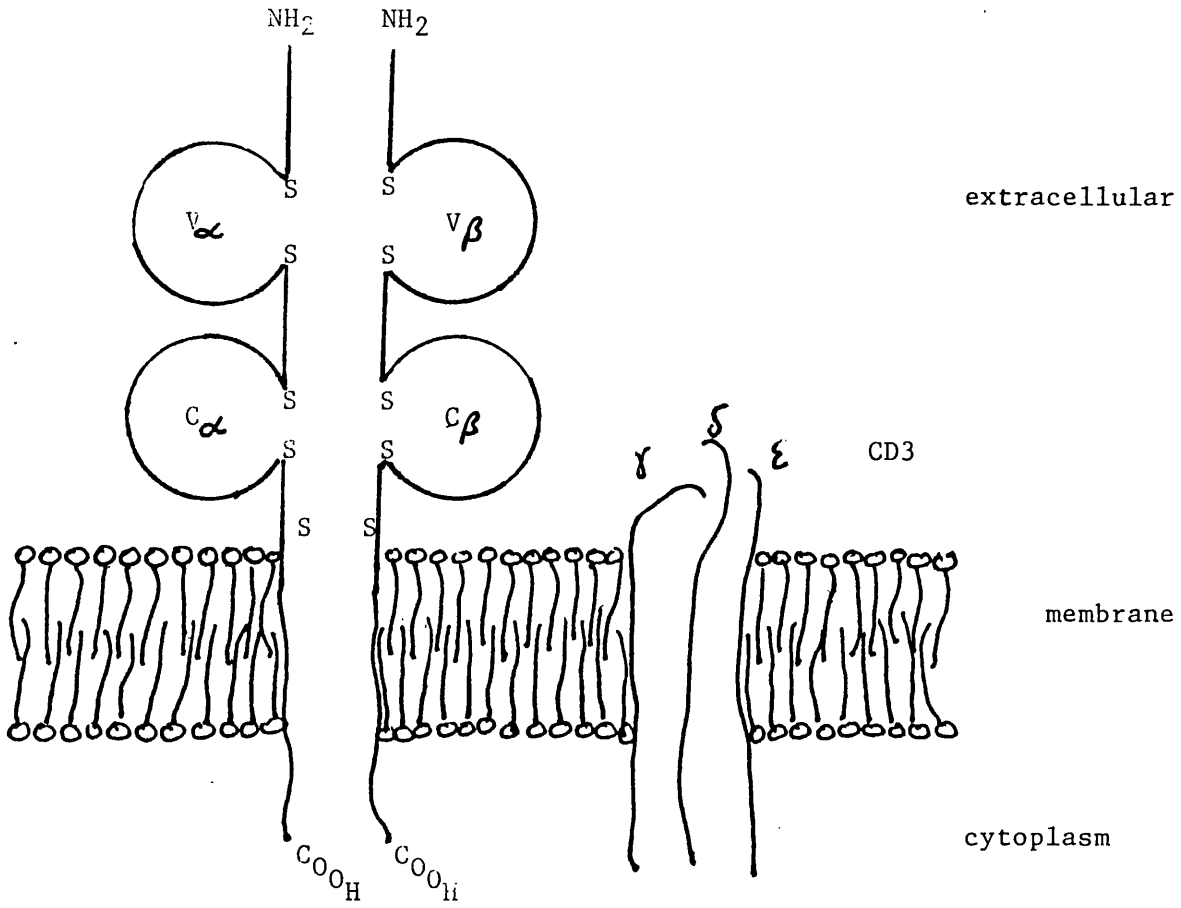


Figure 1.4: Structure of the T cell receptor.

Two covalently linked transmembrane polypeptide chains α and β each having a variable (V) and constant (C) domain are shown associated with the CD3 molecule which consists of three non-covalently linked peptides τ, δ and ϵ.

These fragments then become associated with MHC molecules and expressed as a complex which T cells recognise (Unanue 1984). Mature T cells express either CD4 or CD8 molecules on the cell surface which bind to non-polymorphic portions of class II and class I MHC respectively. These interactions enhance the binding of TCR to its ligand. Engagement of the TCR by ligand (i.e. antigen + MHC) leads to T cell proliferation and expansion, possibly by signalling via the CD3 molecule. In addition, the binding of CD4 or CD8 molecules may also contribute to this signalling.

1.1.3 Major Histocompatibility Complex

Within the MHC are two gene clusters coding for class I and class II histocompatibility antigens, called human leukocyte antigens (HLA) in man. These molecules interact and present peptide fragments of antigen to T cells. In man the class I locus is divided into three sub-regions A, B and C, and the class II locus has five sub-regions DN, DO, DP, DQ and DR. Class I MHC molecules consist of a single chain with three variable domains bound to a β_2 microglobulin molecule. The class II molecules consist of an α and a β chain, the latter being highly polymorphic. The crystal structure of the class I A2 molecule has recently been elucidated (Bjorkman et al. 1987), and has revealed that the antigenic peptide is bound in a pocket on the external surface of the molecule. The sides of the pocket is formed by polymorphic regions of the MHC molecule. (Figure 1.5). Therefore, presumably it would be this part of the MHC-

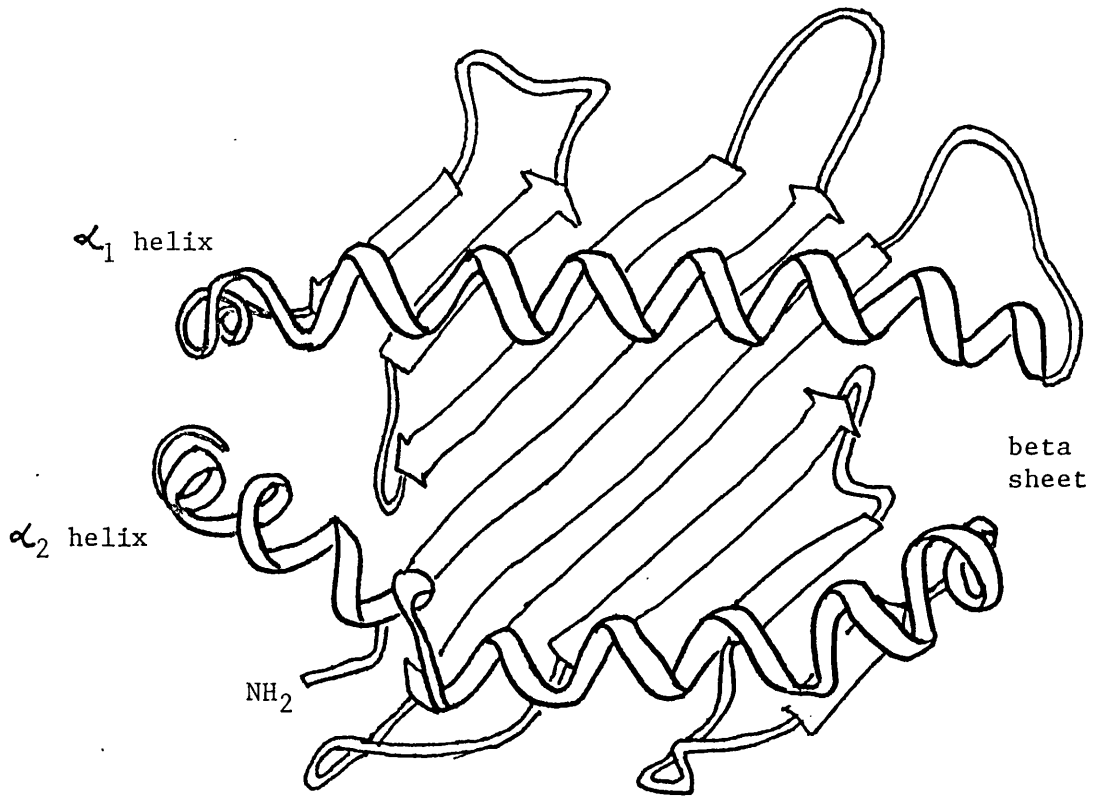


Figure 1.5: Schematic representation of the antigen binding site of the HLA-A2 molecule.

The antigen binding site of the class I molecule is formed from the α_1 and α_2 domains. The upper helix is part of the α_1 domain and the lower helix is from α_2. Each domain also consists of four anti-parallel beta strands followed by a helical region, and the domains pair to form a single eight stranded beta sheet topped by two alpha helices.

(Taken from Bjorkman et al. 1987).

antigen complex that the TCR recognises and interacts. Class I MHC molecules are expressed by all somatic cells, whereas class II molecules are only normally expressed by certain cells such as macrophages and B cells.

1.2 Idiotype Network Theory.

In 1963 two separate groups observed that antibody molecules could be considered as antigens to other antibodies (Oudin and Michel 1963; Kunkel et al. 1963). The variable region of any given antibody molecule has several unique determinants that are antigenic to other antibodies. These determinants are termed idiotopes, and collectively they form the idiotype expressed by that antibody. As shown in figure 1.2 each antibody molecule has six CDRs in the variable region, and it is these CDRs that contribute to an antibody idiotype. Antibodies recognising the idiotype of other antibodies are called anti-idiotypes, although it should be noted that the terms 'idiotypic' and 'anti-idiotypic' are purely operational, as one is considering the interactions of complementary structures of the hypervariable regions of two antibodies. Niels Jerne later formulated the Idiotype Network Theory (Jerne 1974) which simply proposed that antibodies and cells in the immune system could recognise one another via idiotype/anti-idiotype interactions. The Idiotype Network Theory has been extensively investigated as a potential regulator of immune responses. As T cell receptors and immunoblobulin are structurally very similar it is a distinct possibility that

T and B cell responses are co-ordinated by elements within the Idiotypic Network.

1.2.1 Idiotype/anti-idiotype interactions.

Work by Urbain and co-workers (Wikler et al. 1979) demonstrated the relationships between idiotypes and anti-idiotypes in the Idiotype Network. A schematic representation of their results are shown in Figure 1.6. Anti-idiotypic antibodies (Ab2) were raised in rabbits to anti-tobacco mosaic virus antibodies (Ab1). Subsequent rabbits were immunised with purified Ab2 to raise Ab3 antibodies and these animals were later given an additional antigen boost that generated Ab1' antibodies. Ab4 antibodies were raised by immunising further rabbits with Ab3. They were able to show that Ab1 and Ab1' were idiotypically similar. Moreover, although Ab3 did not in general react with the original antigen, they shared idiotypic specificities with Ab1 as shown by Ab4 reacting with Ab1. Their experiments demonstrated that the idiotype network does not exist in a simple equilibrium of one idiotype with one complementary anti-idiotype and vice versa. Nor does it consist of an infinite cascade of ever diverging Ab1, Ab2, Ab3, Ab4,... antibodies. The Idiotype Network can be thought of as a complex web of inter-related components linked by one or more interactions.

1.2.2 Nomenclature.

Ab2 anti-idiotypes have been divided into three

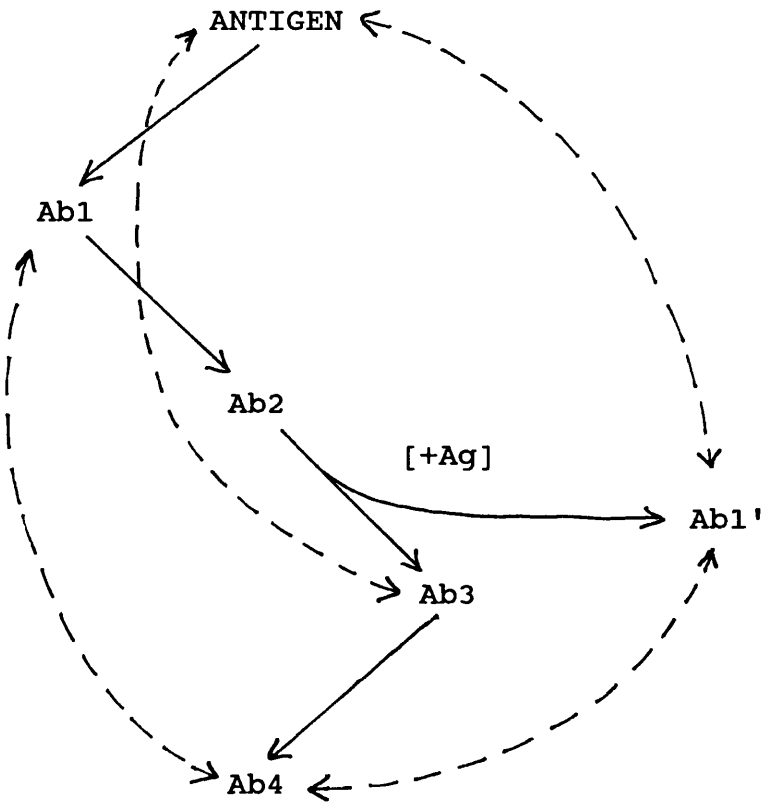


Figure 1.6: Illustration of the limited degree of branching of the Idiotype Network.

Solid line = direct interaction.

Broken line = cross-reactive interaction.

subgroups depending on the location of the idiotope relative to the antigen binding site (paratope) (Jerne et al. 1982; Kieber-Emmons and Kohler 1986).

Ab2alpha. An Ab2alpha anti-idiotype binds to an idiotope away from the paratope. Antigen does not inhibit its binding to Ab1; nor does it inhibit the binding of Ab1 to antigen.

Ab2beta. In the scheme outlined in Fig. 1.6, Ab3 are depicted as being capable of reacting with the nominal antigen, even though the immune system had not been exposed to that antigen. Therefore, the Ab2 used to raise Ab3 must have looked like the antigen to the immune system. Such an Ab2 is termed an internal image, and expressed as Ab2beta. Ab2beta inhibit the binding of antigen to idiotype, and vice versa. If the Ab2 antibody is to be a true internal image of the antigen it should elicit antigen binding Ab3 across a species barrier. A second condition can be applied to an internal image anti-idiotype of an antigen that has a ligand binding site for a drug or toxin. In this case the anti-idiotype should also bind the particular ligand.

Ab2gamma. An Ab2gamma anti-idiotype binds to an idiotope close to, or at the paratope, such that it inhibits the binding of Ab1 to antigen. Antigen also inhibits the binding of Ab2gamma to Ab1. However an Ab2gamma is not an internal image anti-idiotype, and does not bind ligands.

Figure 1.7 shows the three possible types of anti-idiotypes.

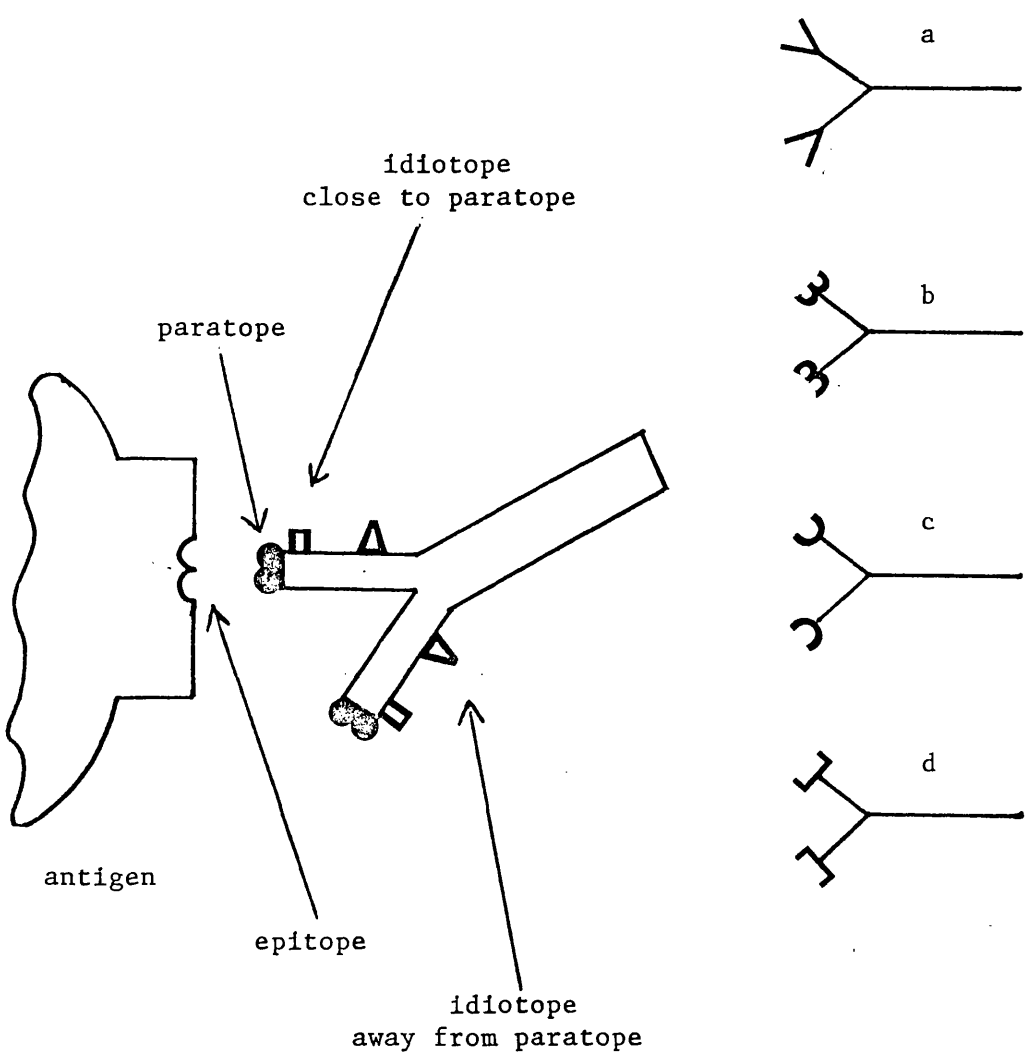
Figure 1.7: Schematic representation of the Ab2 nomenclature of anti-idiotypes.

a) Ab2alpha - binds to an idiotope (△) away from the paratope.

b) Ab2beta - binds to the paratope (●), and also mimics the epitope (ie an internal image of the epitope).

c) Ab2gamma - binds to the paratope but is not an internal image of the epitope.

d) Ab2gamma - binds to an idiotope (□) close to the so that the paratope cannot bind to the epitope.


anti-idiotype
a
b
c
d
idiotope
close to paratope
paratope
antigen
epitope
idiotope
away from paratope

1.2.3 Cross-Reactive Idiotypes.

If an anti-idiotype reacts with an idiotype of a different antibody or individual it is said to recognise a cross-reactive idiotype (CRI). Other terms used to describe these idiotypes are public or shared idiotypes (IdX). Cross-reactive idiotypes that are found in a large number of individuals are called recurrent idiotypes. An anti-idiotype which does not cross-react with any other idiotype is said to recognise a private or individual idiotype (IdI).

Antibodies sharing a cross-reactive idiotype may be against the same antigen, or against two quite unrelated antigens. Also, the anti-idiotype to the CRI may be Ab2alpha to one idiotype and Ab2beta to the other.

1.3 Tolerance

Little is known of the means by which the immune system discriminates between foreign and self-antigens. An example of the tolerance of immune system to self antigen can be found in certain congenic strains of mice. Mice of a strain deficient in the fifth component of complement (C5) are not tolerant to this serum protein whereas normal mice are, indicating that self-reactive cells in some way become tolerant to normal self-antigen (Harris et al. 1983).

Classic experiments established that tolerance to self antigens is acquired during the development of the immune system (Owen 1945; Billingham et al. 1956). These early discoveries led to the 'Clonal Selection Theory' of Burnet (1959), who proposed that self reactive clones of

cells were deleted at a critical stage of development of the immune system. For T cells this event occurs in the thymus, as recent experiments are beginning to show.

During T cell development in the thymus, there are two important maturation events. Precursor cells enter the thymus where they randomly rearrange and express the entire repertoire of T cell receptor sequences. It is at this stage of development (when the cells probably co-express both the CD4 and CD8 molecules) that the positive and negative selection processes occur (Marrack and Kappler 1987). Positive selection allows cells likely to bear receptors able to recognise foreign antigen plus MHC in the periphery to continue to mature. In negative selection, self reactive cells (i.e. cells that may recognise self MHC too well or which recognise self antigens plus MHC) are deleted by the mechanisms of tolerance (Kappler, Roehm and Marrack 1987). Kappler, Marrack and colleagues, and MacDonald and others have shown how tolerance may involve deletion of T cells bearing specific TCR. Both groups of investigators used mice identical at the MHC locus, but mismatched for minor lymphocyte stimulating (Mls) molecules. Cells from mice carrying the Mls^b allele elicit vigorous proliferative responses from MHC identical cells carrying the Mls^a allele, the alloreactive T cells utilizing particular Vβ genes. Mice expressing Mls^a were found to have few or no T cells bearing receptors containing these specific β chain variable region genes demonstrating that T cells using the relevant Vβ genes in the TCR are eliminated from the TCR pool during ontogeny

Kappler et al. 1988; MacDonald et al. 1988). Using a different approach Teh et al. (1988) created mice transgenic for a TCR specific for the H-Y antigen, an antigen only expressed by male mice. They found that female transgenic mice expressed the transgenic TCR on a large fraction of the T cells, whereas in the males the autoreactive (i.e. specific for H-Y) T cells bearing the transgenic TCR were eliminated. These two sets of results clearly show that mature T cells do not bear self-reactive TCRs, and that induction of tolerance involves the elimination of autoreactive T cells during development in the thymus.

However the observation that even mature T cells can be profoundly tolerised by minute amounts of aggregate-free antigen (Dresser 1962) suggests that there are other mechanisms of tolerance induction. The majority of B cell responses are T cell dependent, therefore in theory tolerance need only extend to the T cell compartment. An example of this is the complement C5 experiments described above, and the experiments of the cytoplasmic liver protein F. This protein is found in mice in two forms F1 or F2. In certain strains of mice alloimmunisation with the F protein can lead to an autoantibody response directed against determinants common to F_1 and F_2, implying that self-tolerance is maintained by the T cells (Iverson and Lindenmann 1972).

In an elegant study by Goodnow and others, new light has been shed on the fate of autoreactive B cells. They generated two lines of transgenic mice, one carrying the

gene for hen egg lysozyme (HEL), and one carrying the genes for the corresponding anti-HEL antibodies. In the former, transgenic line the HEL constituted a neo self-antigen, and mice were tolerant to immunisation with HEL. When the two founder lines were mated the double transgenic mice were tolerant to HEL despite the fact that large numbers of anti-HEL specific B cells existed in these mice. These anti-HEL specific cells remained unresponsive in adoptive transfer experiments, indicating that they had been functionally silenced (Goodnow et al. 1988). Nossal had coined the term 'clonal anergy' to describe this state of tolerance. He proposed that two signals are required for the activation of mature B cells to a T-dependent antigen. The first of these signals alone i.e. antigen would induce tolerance in the form of anergy, or non-responsiveness. Both signals, acting in concert, i.e. antigen and lymphokine would activate the B cells (Nossal 1983). Immature and pre-B cells are much more susceptible to tolerance induction than mature B cells. However it should be noted that for some proteins, (for example albumin or transferrin) whose serum concentrations are high, anergic self reactive B cells are not found. One might conclude that in some instances self-reactive B cells are deleted.

Other mechanisms of tolerance have been postulated. One such theory is that a network of suppressor T cells exists that controls self-reactive cells. Many studies have shown that transfer of immune splenic T cells can block a response in a recipient animal. It is thought that these

animals are unresponsive because the transferred T cells are exerting a suppressive effect on otherwise reactive cells. However, the role of suppressor cells is still disputed, (Moeller 1988) and it may be these mechanisms may serve only as a failsafe to buttress tolerance induced by clonal deletion or anergy.

1.4 Autoimmunity.

In most individuals tolerance to self is maintained throughout life. However in some the loss of tolerance results in autoimmunity, which may lead to overt autoimmune disease. Autoimmunity may be a normal immune response to an abnormal self antigen, or conversely an abnormal response to a normal self antigen. Many contributing factors may be involved in precipitating autoimmunity, including genetic, environmental, hormonal as well as defects in the immune system itself.

1.4.1 Environmental factors

As has already been discussed, tolerance is thought to affect mainly the T cell compartment. As a collorary of this, Weigle (1971) and Allison (1971) proposed that autoimmunity arose from the loss of T cell tolerance. For instance, infection by Epstein-Barr virus, a polyclonal B cell activator may bypass T cell tolerance and directly activate autoantibody production. Tolerance could also be broken by antigens bearing epitopes that cross-react with self antigen. In this way T cell help elicited for the antibody response to self antigen would also help self-

reactive B cells. This may account for the autoantibody response to insulin seen in patients who had been maintained on porcine or bovine insulin. Pork and beef insulin differ from human insulin by one and three amino acids respectively. These differences may be sufficient to induce an immune response to the foreign insulin, which would enable the tolerance to human insulin to be bypassed. It was shown in myasthenia gravis that monoclonal antibodies raised against the autoantigen, acetylcholine receptor, cross-reacted with epitopes present on Escherichia coli and Klebsiella pneumoniae membrane proteins (Stefannson et al. 1985). However viral antigens have not been found in association with the disease (Aoki et al. 1985; Klavinskis et al. 1985).

Self antigen may coincidentally be processed with foreign antigen during the course of an infection, resulting in the expression of self antigen in a context to which the immune system is not tolerant. Alternatively, infection could alter the expression of self antigen so that it is presented to the immune system in a different way. Autoimmunity may follow an immune response during which cell lysis of infected cells may release self antigen. Autoimmunity may arise following a viral or bacterial infection possibly through antibodies cross-reacting with similar epitopes on self-antigen. Drugs can also lead to a state of autoimmunity, as in hydrallazine-induced systemic lupus erythematosus (SLE) (Batchelor et al. 1980). Myasthenia gravis can arise following the treatment of

rheumatoid arthritis with D-penicillamine (Bucknell et al. 1975), but withdrawal of D-penicillamine treatment leads to an amelioration of the myasthenic symptoms.

1.4.2 Genetic factors

Suspectibility to autoimmune disease is thought in part to be genetically determined, as shown by identical twin studies. For instance, in insulin dependent diabetes mellitus (IDDM), in 50% of cases the identical twin of an individual with IDDM will also develop the disease (Barnett et al. 1981). Familial incidences of autoimmune diseases are also found which show inherited susceptibility not only to a particular disease but to groups of diseases. Perhaps one of the most interesting aspects of the genetic association with autoimmune disease is that of the MHC. Many autoimmune diseases have strong correlations with particular HLA haplotypes, for example rheumatoid arthritis (DR4), myasthenia gravis (DR3), IDDM (heterozygote DR3/DR4) and Celiacs disease (DR3,DR7) (Batchelor et al. 1982). One possible explanation of these associations is that it actually reflects the increased ability of certain HLA molecules to present self antigen to the immune system. An alternative view is that the HLA genes are part of a more extended haplotype, and are in 'linkage disequilibrium' with the real susceptibility genes. Linkage disequilibrium is the phenomenon in which certain genes tend to co-segregate and are inherited together. This is found in the MHC where the haplotype HLA A1, B8, DR3 would have an expected frequency

of 1% of caucasians but in fact is found in 15% of the population. The MHC contains other gene clusters such as those that code for tumour necrosis factor (TNF), stress proteins and for the C2 and C4 components of complement, and it is possible that other unknown genes may be identified that are more closely associated with the autoimmune diseases.

It has already been mentioned that there is an increased incidence of HLA DR3/DR4 and DR3, DR7 in IDDM and Celiacs disease respectively. However recent studies have shown that there is a much greater disease association with alleles of the DQ locus. In particular Todd and colleagues have argued that an aspartic acid at residue 57 in the β chain of the DQ molecule confers resistance to IDDM whereas any other amino acid (valine, serine or alanine) confers susceptibility (Todd, Bell and McDevitt 1987). However, it has not been proved definitively that this amino acid substitution at position 57 is absolutely necessary for the development of IDDM, and it possible that upon closer scrutiny other residues are as important in defining disease.

1.4.3 Defects in immune regulation

Some self antigens (for example the lens of the eye or sperm) do not induce tolerance either because they appear in the body after tolerance is normally induced or because they are confined to privileged sites inaccessible to the immune system. One such site is the anterior chamber of the

eye, which, following a trauma may be exposed to the immune system for the first time. This may lead to an autoimmune response to a self antigen not encountered before (see Nussenblatt and Silverstein 1985).

In the paraneoplastic autoimmune disease Lambert-Eaton Myasthenic Syndrome (LEMS), often found in association with small cell lung carcinoma (SCLC), it has been suggested that SCLC determinants trigger antibodies which cross-react with similar determinants at the neuromuscular junction. It was shown that the K^+-stimulated influx of Ca^{2+} ions into a cultured SCLC cell line was inhibited when the cells were cultured with the IgG fraction of LEMS patients' plasma (Roberts et al. 1985). It had previously been shown that passive transfer of LEMS IgG to mice impaired the release of acetylcholine by acting on pre-synaptic calcium channels at the neuromuscular junction (Lang et al. 1984). These two observations indicate that the causal phenomenon in LEMS may be antibodies generated against calcium channel determinants of SCLC which then cross-react with similar determinants at the neuromuscular junction.

Alterations in the normal functioning of regulatory circuits (be they idiotype network interactions, suppressor cells etc.) may lead to deleterious results. For example, Bottazzo and colleagues have postulated that inappropiate expression of class II MHC antigens on endocrine cells may lead to autoimmune attack of these cells (Bottazzo et al. 1983). This followed the observation that thyroid cells from patients with Graves' disease expressed class II MHC

antigens (Hanafusa et al. 1983). However, interferon is known to induce class II expression and a lymphocytic infiltration into the gland may produce these factors. Class II expression would therefore be seen as a secondary event following the lymphocytic infiltration (Grubeck-Lebenstein et al. 1988). Bottazzo et al. (1983) had noted that aberrant class II expression alone would not be enough to cause the development of autoimmunity, and other factors would be involved.

If there is a delicate balance between factors that help and those which suppress then the immune system may be susceptible to perturbations that lead to autoimmunity. Although the exact nature of T suppressor cells is not known, it is feasible that they may play a part in maintaining tolerance, and a defect in suppressor function, together with other factors could precipitate autoimmunity. The Idiotype Network may also have an important role in controlling an immune response and a great deal of interest has been shown in the Idiotype Network and autoimmunity.

1.4.4 Idiotypes in autoimmunity.

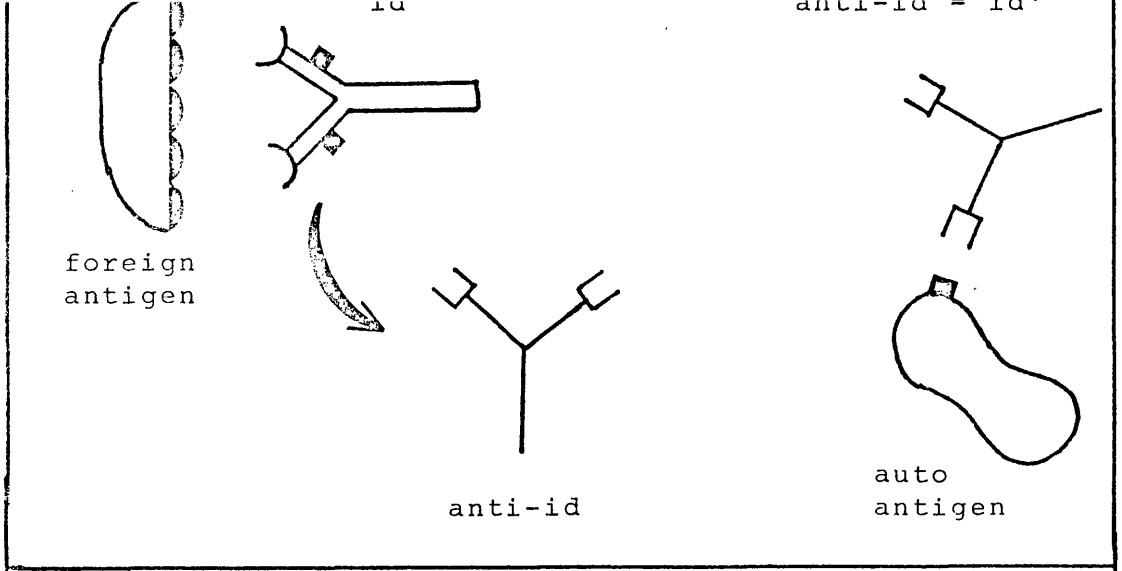
Perturbations in the Idiotype Network could conceivably initiate autoimmunity in a number of ways (Figure 1.8). Firstly, anti-idiotypes generated during the immune response to a foreign antigen could fortuitously react with an autoantigen (Plotz 1983). Secondly, a parallel set of idiotypes (id') reactive with autoantigen could be up-regulated by an anti-idiotype arising from two possible

Figure 1.8: Development of autoimmunity by idiotype interactions.

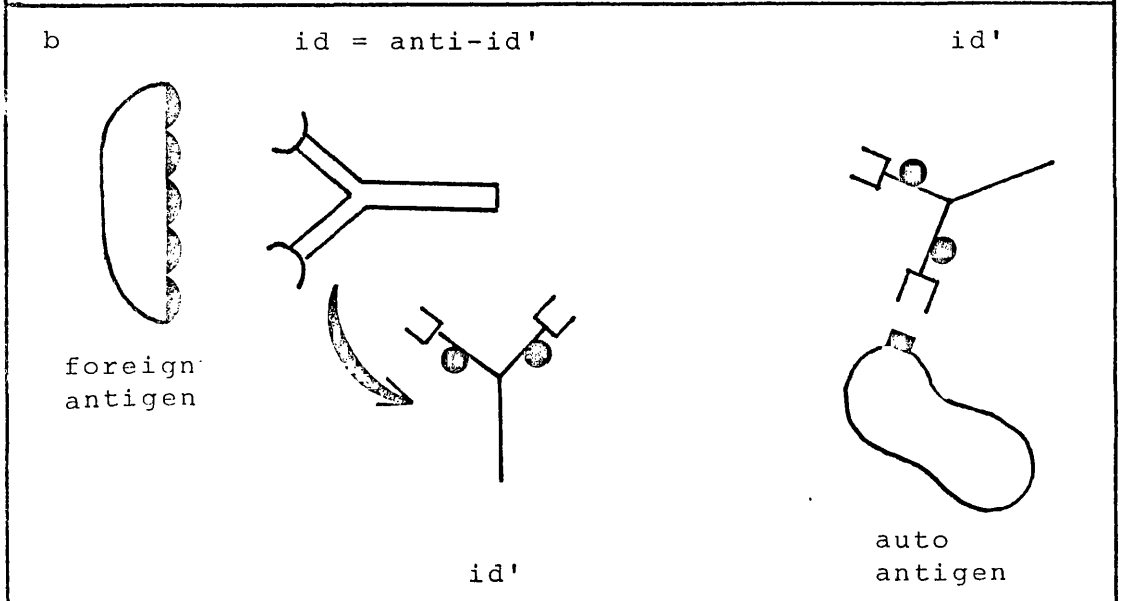
a) An antibody to a foreign antigen bears an idiotope (■) which generates an Ab2alpha anti-id, which happens to be an autoantibody.

b) An antibody to a foreign antigen happens to be an Ab2alpha anti-id to an idiotope (●) on a parallel set autoantibodies.

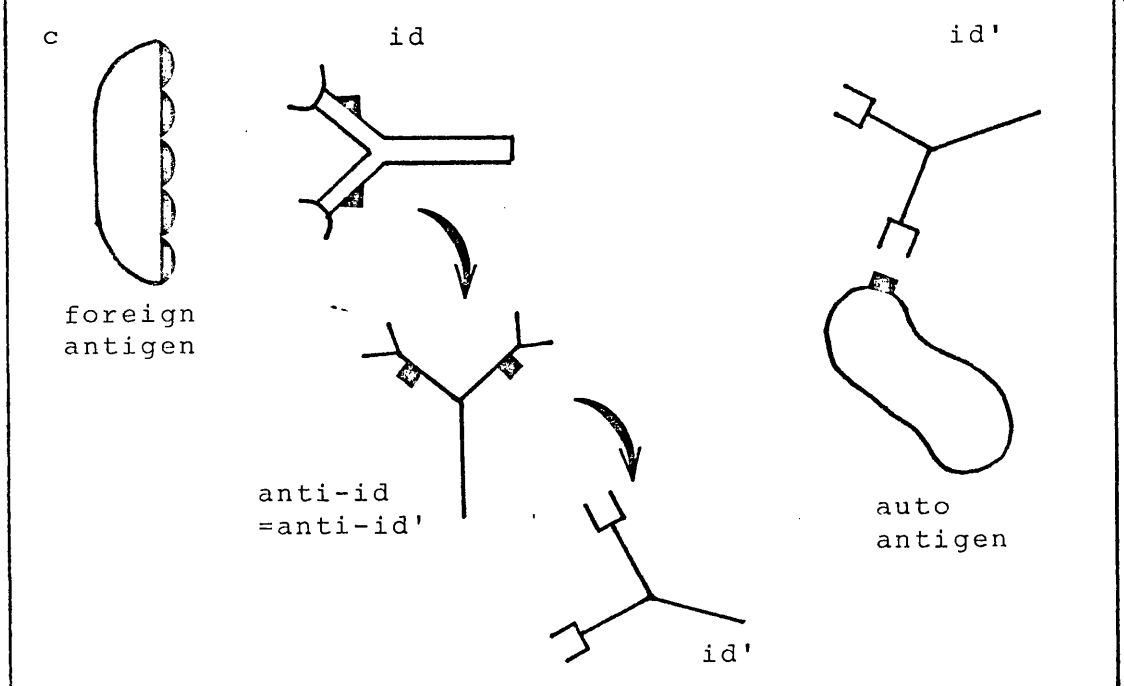
c) An antibody to a foreign antigen bears an idiotope (▲) generates an anti-id. This anti-id is also an anti-id to a parallel set of idiotopes (⊔) on autoantibodies.


id
anti-id = id'
foreign
antigen
anti-id
auto
antigen

b


id = anti-id'
id'
foreign
antigen
id'
auto
antigen

c


id
id'
foreign
antigen
anti-id
=anti-id'
auto
antigen
id'

mechanisms. The idiotype (id) of one antigen system is the anti-idiotype of an autoantigen system; or the anti-idiotype to the initial idiotype (id) is also the anti-id to auto-idiotype. These perturbations would require the generation of spontaneous anti-id during an immune response, and anti-ids have been demonstrated in several systems including anti-insulin (Schechter et al. 1982) and interleukin 1 (Zuberi et al. 1988). Cleveland and colleagues have reported elegant work using a ligand (BisQ) to acetylcholine receptor (AChR) to produce spontaneous anti-anti-BisQ antibodies that also bind to AChR (Cleveland et al. 1983). Using the cross-reactions between idiotypes and anti-idiotypes in AChR and dextran systems, an Idiotype Connectivity theory has been postulated (Dwyer et al. 1986). They proposed that an autoimmune disease like myasthenia gravis can arise from the pertubations in the Idiotype Network caused by (for example) bacterial infection.

There has been much research into the existence of recurrent idiotypes in autoimmunity for two reasons. Firstly, to establish the factors (environmental, genetic etc.) that may be associated with autoimmunity; and secondly, to devise possible ways of therapeutic manipulation of the immune system. Recurrent idiotypes have been reported in many diseases, for example SLE (reviewed by Isenberg and Shoenfeld 1988). The 16/6 idiotype is found in approximately 50% of cases with SLE, but is also present in the normal population where it is not associated with anti-DNA antibodies (Isenberg et al. 1984). In the latter, the

idiotype is found on antibodies generated in response to bacterial antigens such as the Klebsiella polysaccharide K30 (El Roiey et al. 1987). Hence, although infection by foreign antigen can give rise to an antibody response bearing an autoreactive idiotype, the presence of this idiotype does not necessarily lead to an autoimmune disease. Therefore there are obviously many other factors that contribute to the loss of tolerance to autoantigens.

Recently Crowley et al. (1988) have reported two CRIs each found on 30% of randomly selected monoclonal IgMk paraproteins with anti-IgG activity. The two CRIs were found in normal human IgM but only in very small amounts. Their results suggested that an increased frequency of usage of certain V-region gene families among autoantibodies. It has been suggested that autoantibodies may arise from somatic mutation of germline genes that originally encode antibodies that recognise foreign antigens. Idiotypes could presumably be markers of the V-regions of germline genes that undergo somatic mutations. Diamond and Scharff (1984) have shown that a single amino acid mutation in an anti-phosphoryl-choline (PC) binding monoclonal antibody of the T15 id (the dominant idiotype found in the anti-PC response of Balb\c mice) results in a loss of PC binding. They found that instead, the mutated antibody bound to other potential self antigens such as dsDNA and cardiolipin whilst retaining the ability to bind to some, but not all anti-T15 antibodies.

1.4.5 Autoimmune Diseases

The range autoimmune diseases, broadly classified into organ specific and non-organ specific is shown in Table 1.1. Organ specific diseases tend to overlap, for example diseases of the thyroid and the stomach can occur in the same individual or family members, but there is little overlap between the spectra of organ and non-organ specific diseases. For organ specific diseases the autoantigenic targets are localised to certain tissues which include the thyroid (Hashimoto's thyroiditis and Graves' disease), the adrenal gland (Addison's disease, the stomach (pernicious anaemia), and the pancreas (IDDM). In contrast, non-organ specific autoimmune diseases involves reactivity with self-components found throughout the body.

Autoimmune diseases can be mediated both by humoral and cellular mechanisms. An example of the former is pernicious anaemia where the anaemia is caused by autoantibodies to intrinsic factor which blocks the absorption of vitamin B12 from the stomach. Hashimoto's thyroiditis is a cell mediated disease where imflammatory lymphoid cells, including activated T cells are found infiltrating the thyroid gland. This results in the destruction of the gland and leads to a loss of thyroid function. In rheumatoid arthritis the lesion is caused by immune complexes deposited in the joints. These immune complexes are the result of self-reactivity with IgG. In SLE anti-DNA antibodies again leads to immune complex formation the lesions typically being found in the kidneys. Sjogren's syndrome affects the exocrine glands, in

Table 1.1: The spectrum of autoimmune diseases.

organ specific	Hashimoto's thyroiditis
↑	Primary myxoedema
	Thyrotoxicosis
	Pernicious anaemia
	Autoimmune atrophic gastritis
	Addison's disease
	Insulin dependent diabetes mellitus
	Goodpasture's syndrome
	Myasthenia gravis
	Lambert-Eaton myasthenic syndrome
	Pemphigus vulgaris
	Pemphigoid
	Sympathetic ophthalmia
	Phacogenic uveitis
	Multiple sclerosis
	Autoimmune haemolytic anaemia
	Idiopathic leucopenia
	Primary biliary cirrhosis
	Active chronic hepatitis
	Ulcerative colitis
	Sjogren's syndrome
	Rheumatoid arthritis
	Dermatomyositis
↓	Scleroderma
non-organ specific	Systemic lupus erythematosus

particular the lachrimal and the salvary glands.

Animal models are important aids to the understanding of autoimmunity. For example in experimental autoimmune thyroiditis rats or mice immunised with thyroglobulin provide a model of Hashimoto's thyroiditis. In experimental autoimmune myasthenia gravis animals may become severely weak upon immunisation with AChR. Models of spontaneously arising autoimmune disease in certain inbred strains also exist. For example the NZW/B F1 strain of mouse develop SLE, whereas the MRL/lpr strain acts as models for both SLE and rheumatoid arthritis. The obese strain of chicken and Buffalo rats develop thyroiditis which resembles human Hashimoto's thyroiditis in many respects.

Although some diseases are thought undoubtedly to be autoimmune the autoantigens have eluded all attempts of identification. In IDDM it is known that the insulin-secreting beta cells of the pancreas are destroyed. Multiple sclerosis (MS) is a disease of the central nervous system and it is the myelin sheaths surrounding the nerve fibres that are the target of attack. However animal models of these two diseases do exist which may help our understanding of these diseases. Both the BB strain of rat and the NOD strain of mice spontaneously develop diabetes, and the current model of MS is experimental autoimmune encephelomyelitis (EAE) in which animals immunised with myelin basic protein (MBP) in Freunds adjuvant, or T cell clones specific for MBP develop symptoms consistant with the primary stages of the disease (Zamvil et al. 1985).

Humphrey and White (1970) postulated three conditions which should be met for a disease to be defined as autoimmune. Firstly there should be evidence for immune involvement at some stage of the disease. Secondly the lesions characteristic of the disease should be inducible in experimental animals by the suspected antigen; and thirdly these lesions should then be reproduced in syngeneic recipients by transferring the potent cells or antibodies.

1.5 Myasthenia Gravis.

1.5.1 Myasthenia Gravis as an example of an autoimmune disease.

A paradigm of an organ specific autoimmune disease is myasthenia gravis (MG), a disease of neuromuscular transmission characterised by weakness of voluntary muscle (reviewed by Willcox and Vincent 1988). In its mildest form MG only affects the eyes, but more severe symptoms include difficulties in speech and swallowing, loss of movement of limbs and in some cases extreme difficulties in breathing. MG has been extensively studied by immunochemical and cellular means, and the evidence for its autoimmune aetiology is described below.

MG has a well defined target antigen, AChR which is located on the crests of the post-synaptic folds of the neuromuscular junction. Acetylcholine released from vesicles at the pre-synaptic nerve terminal in response to a nerve impulse, cross the synaptic cleft and bind to the post-synaptic AChR. This causes a conformational change in the

AChR, opening a cation channel through which mainly Na^+ ions flow, generating an action potential (Figure 1.9)

The evidence accumulated over the past 15 years has supported the original hypothesis of Simpson (1960) and has established myasthenia gravis as a model organ specific autoimmune disease. It was in 1973 that AChR was recognised as the probable autoantigenic target in myasthenia gravis. Whilst trying to elicit antibodies to purified electric eel AChR Patrick and Lindstrom (1973) observed that the immunised rabbits became weak and paralysed, consistent with the symptoms of MG. Subsequent treatment of the rabbits with acetylcholine esterase inhibitors (thereby prolonging the effect of ACh) lead to a temporary abatement of the symptoms. Patrick and Lindstrom concluded that the myasthenic symptoms may have arisen in response to AChR. It was also reported in that year that muscle biopsies from myasthenia gravis patients had much reduced levels of AChR (Fambrough et al. 1973).

Following the induction of myasthenic symptoms in rabbits, experimental autoimmune myasthenia gravis (EAMG) has been induced in rats (Lennon et al. 1975), and in mice (Berman and Patrick 1980) by immunisation with purified AChR. EAMG has also been induced by passive transfer of anti-AChR antibodies (Gomez et al. 1985; Tzartos et al. 1987). EAMG has also been successfully transferred to mice using the MG patients autoantibodies (Toyka et al. 1975; Wilson et al. 1983).

The passive transfer of antibodies from an MG mother to

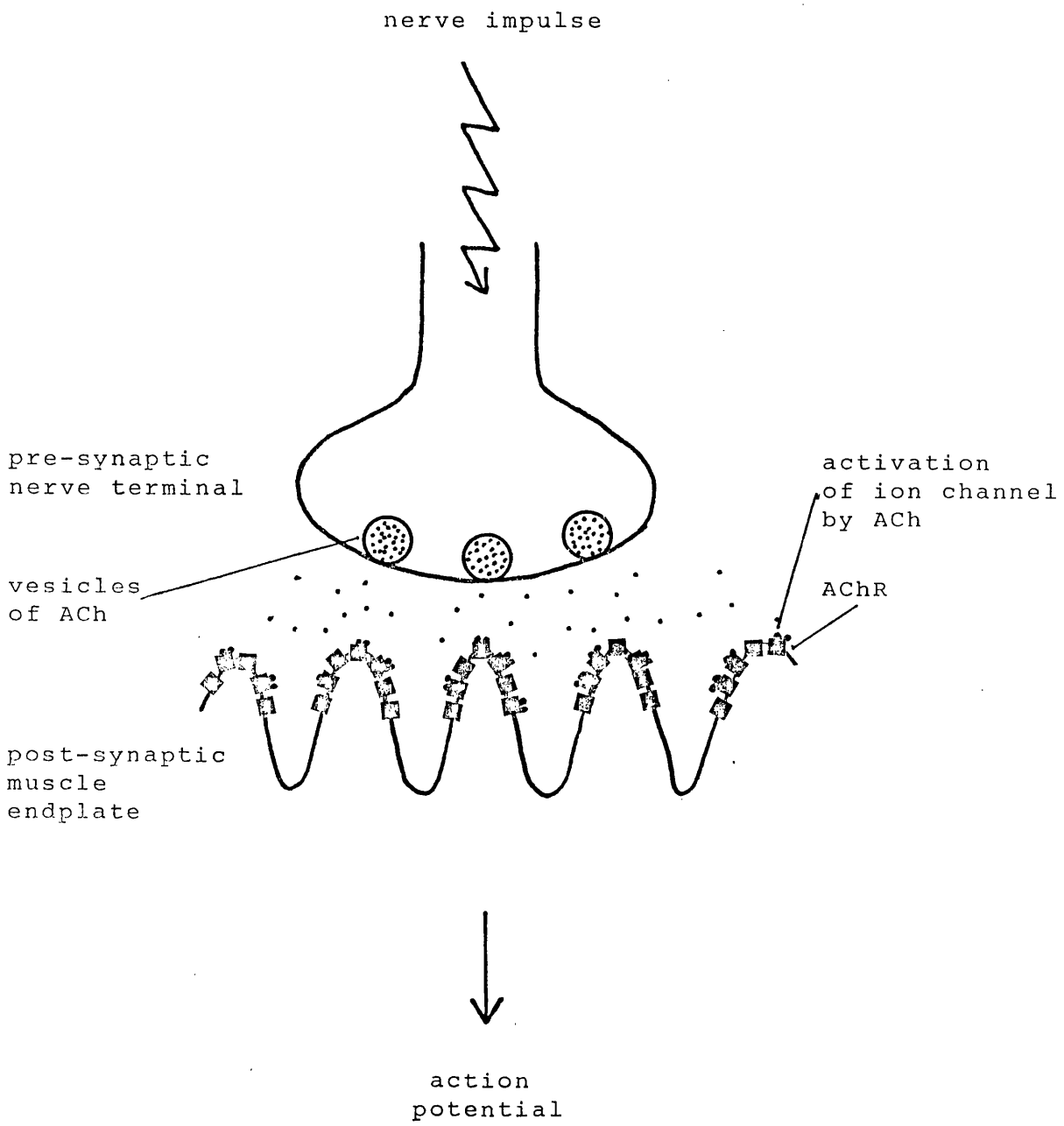


Figure 1.9: The neuromuscular junction showing the location of AChRs on the post-synaptic folds.

her unborn child is often observed (Keesey et al. 1977). The transient MG symptoms gradually disappear as the new-born baby catabolises the transferred antibodies. Fetal transfer of autoimmunity is a phenomenon seen in other autoimmune diseases such as Graves' disease.

The disease can be transiently ameliorated by the removal of circulating anti-AChR antibodies by plasma exchange (Pinching et al. 1976; Newsom-Davis et al. 1978).

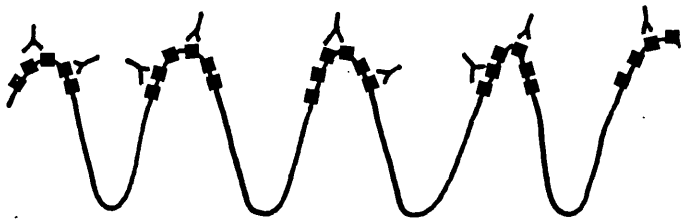
Further evidence indicative of an autoimmune aetiology for MG is the occurrence of MG with other organ specific autoimmune diseases such as pernicious anaemia. MG is also found in association with certain HLA haplotypes (Compston et al. 1980; Dawkins et al. 1983), especially A1 B8 DR3.

It is now accepted that MG is an IgG antibody-mediated disease leading to a loss of functional AChR at the neuromuscular junction. Figure 1.10 shows the three possible modes of action of the autoantibodies:

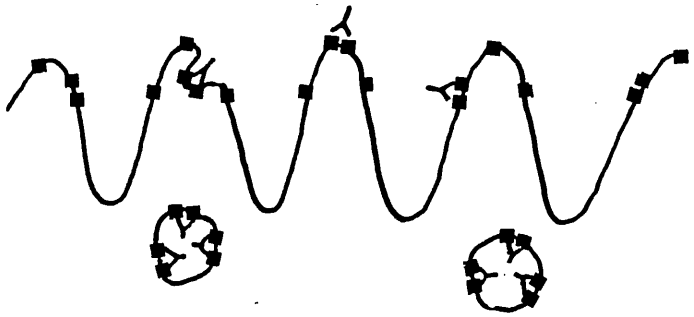
1. Direct blockade of acetylcholine (ACh) action by antibody. Antibodies that can either prevent ACh from binding directly to the ACh binding site, or inactivate the ion channel either sterically or conformationally are not thought to be important in many patients (see Willcox and Vincent 1988).

2. Accelerated degradation of AChR. Binding of antibody to AChR can cause cross-linking of AChR and an apparently consequential increase in the rate at which AChR is internalised. This leads to a loss of AChR at the membrane surface (Kao and Drachman 1977).

a



b



c

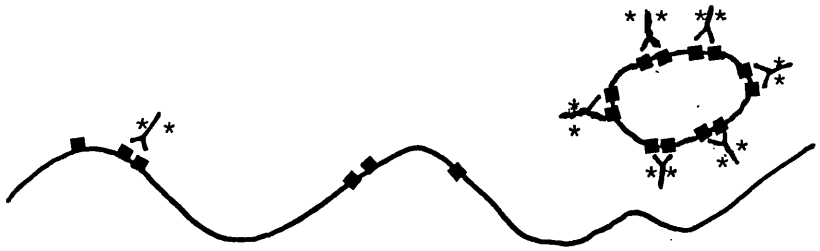


Figure 1.10: Three ways in which anti-AChR antibodies can cause myasthenia gravis.

a). Direct blockade of AChR (■) by antibodies(≻)

b). Increased rate of internalisation of AChR by cross-linkage of antibodies.

c). Complement (*) mediated lysis.

3. Complement-mediated lysis. The regular folds of the post-synaptic membrane can be destroyed by complement-dependent attack via anti-AChR antibodies at the membrane surface (Engel and Arahata 1987).

The thymus has been recognised as being intimately involved in MG for many years, although the role it plays is still not known. Thymectomy very often has a beneficial effect in alleviating the course of the disease. Myasthenic thymuses contain germinal centres, and when cultured *in vitro* thymic cells are capable of spontaneously producing anti-AChR antibodies (Scadding *et al*. 1983). Myoid (muscle like) cells in the thymus have been shown to express AChR (Schluep *et al*. 1987; Kirchner *et al*. 1987), suggesting that autosensitisation to AChR may occur in the thymus.

Although myasthenia gravis is predominantly an antibody mediated disease, AChR-responsive T cell lines have been isolated from human peripheral blood lymphocytes (Hohlfeld *et al*. 1984; Harcourt *et al*. 1988). AChR specific T cells seem to be specifically enriched in the myasthenic thymus (Sommer *et al*. 1988).

1.5.2 Acetylcholine Receptor.

The acetylcholine receptor is perhaps one of the best characterised transmitter receptor molecules. AChR is a large transmembrane protein approximately 255 kD, consisting of five subunits (stoichiometry α_2,β,τ or ϵ,δ) which together form a cation channel. The fetal form of AChR

consists of a ϵ-subunit which is replaced during maturation by an τ-subunit. The electric organs of electric fish such as Torpedo and Electrophorus are very rich sources of AChR although AChR can also be obtained in smaller amounts from mammalian muscle (Conti-Tronconi and Raftery 1982). The genes encoding the subunits of various species of AChR have been cloned, including the α- and δ-subunits of human AChR (Noda et al. 1983; Shibahara et al. 1985), and the amino acid sequences determined. Figure 1.11 presents a prediction of the 3D structure of AChR (based on the model of Finer-Moore and Stroud (1984), Harcourt and Jermy (1987)), showing the positions on the α-subunit of two important sites, the "main immunogenic region" (MIR) and the α-bungarotoxin-binding site. The MIR is a region defined by Lindstrom and Tzartos to which the majority of anti-AChR antibodies are directed (Tzartos and Lindstrom 1980; Tzartos et al. 1982). α-bungarotoxin is a neurotoxin component of the venom of the snake Bungarus multicinctus which binds specifically and essentially irreversibly to AChR (Chang and Lee 1962), and its binding site is thought to be closely associated with the transmitter binding site. The positions of the MIR and the α-bungarotoxin binding site have recently been assigned using electron image analysis (Kubalek et al. 1987).

1.5.3 Anti-AChR antibodies.

Myasthenic antibodies form a heterogeneous population, comprising all IgG subclasses (Vincent and Bilkhu 1982), however it is possible that IgG1 and IgG3 predominate

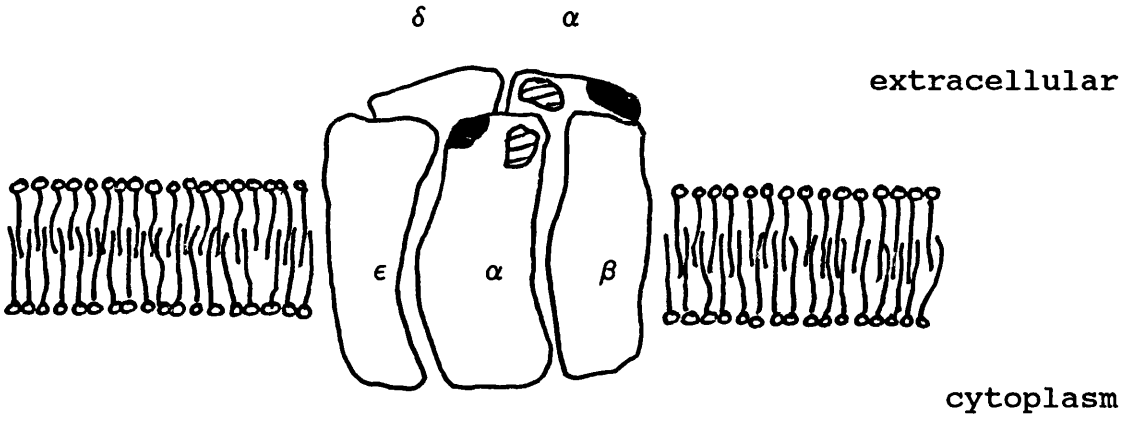


Figure 1.11: Structure of acetylcholine receptor.

The five subunits form a transmembrane ion channel.

The alpha subunit has two important sites.

a) the α-bungarotoxin binding site (solid shading), and

b) the main immunogenic region (cross-hatch shading).

(Taken from Harcourt & Jermy 1987).

(Nielsen et al. 1985). Serum antibody levels do not correlate with disease severity, although within an individual remission can be associated with a decline in specific anti-AChR antibodies (see Willcox and Vincent 1988).

Monoclonal antibodies (mabs) have been produced against AChR from the electric organ of various electric fish, as well as mammalian sources of AChR such as human, rat, and bovine muscle. Most of the mabs have been found to bind to the α-subunit and in particular to the MIR. The MIR was originally defined as the region to which the majority of anti-electric eel mabs were directed (Tzartos and Lindstrom 1980). The MIR is a highly conserved region and antibodies to it cross-react with AChR from a variety of species. Over 60% of the antibodies of an individual with MG are directed against MIR (Tzartos et al. 1982), and recent work has shown that there are some differences in the antibodies between the patient subgroups in terms of the epitopes on AChR that they recognise (Whiting et al. 1986a; Heidenreich et al. 1988). Mabs against Torpedo and human AChR have previously been raised and characterised in this laboratory (Whiting et al. 1985, 1986b). The anti-human AChR mabs have been used to define five partially overlapping regions on the α-subunit (Table 1.2; Figure 1.12), two of which (regions 3 & 4) appear to coincide with the MIR (Heidenreich et al. 1988). Four of these mabs (region 1) bind only to the denervated form of AChR, the others bind to both denervated and normal AChR.

Table 1.2: Properties of anti-human AChR mabs[a].

mab	IgG subclass	Group[b]	AChR specificity[c,d]
B8	1	1	D
C2	1	1	D
C9	1	1	D
F8	1	1	D
B3	1	2	D, N
C3	1	3	D, N, MIR
G10	1	3	D, N, MIR
D6	2b	4	D, N, MIR
C7	2a	5	D, N
G3	2b	5	D, N

[a] all mabs bind to the α-subunit of AChR

[b] defined by competition experiments using ^{125}I-mabs (Whiting et al. 1986b).

[c] D = denervated AChR, N = normal AChR (Whiting et al. 1986b)

[d] MIR = main immunogenic region, defined by competition experiments (Heidenreich et al. 1988).

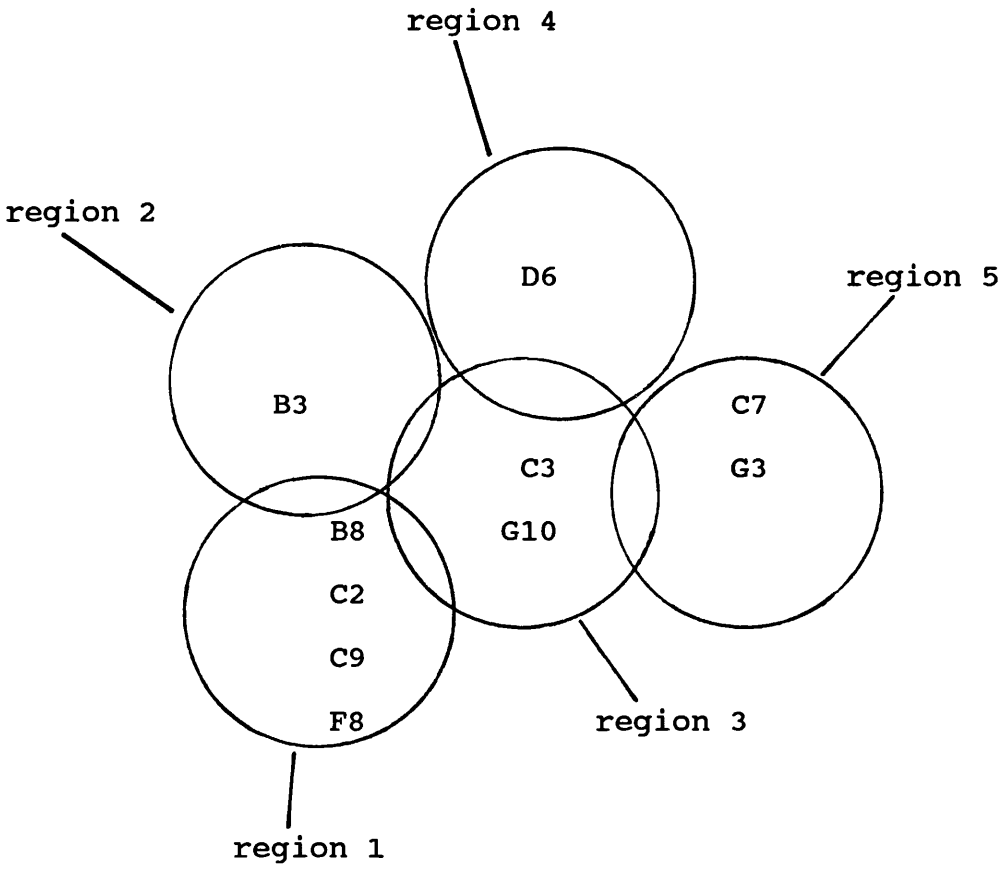


Figure 1.12: Schematic representation of the five partially overlapping binding regions of anti-human AChR mabs (defined by P. Whiting 1984).

1.5.4 Idiotypes and anti-idiotypes in myasthenia gravis.

The Idiotype Network Theory may be particularly relevant in an antibody-mediated autoimmune disease such as myasthenia gravis. Jerne's original concept can be expanded not only to explore possible reasons for the break in self-tolerance but also to find a potentially beneficial way of specifically manipulating the immune system.

The existence or otherwise of cross-reactive idiotypes in MG is a contentious subject. Dwyer and Lefvert have both reported CRI in human sera detected by antibodies to mouse and human idiotypes respectively (Dwyer et al. 1983; Lefvert 1988). Elsewhere, Lang reported that rabbit antibodies raised against idiotypes purified from the plasma of three MG patients did not detect any significant CRI (Lang et al. 1985). This difference may be due to the assays used in each case, or a difference in interpretation of results. The former two studies measured direct binding of idiotype to monoclonal anti-idiotype by ELISA allowing the detection of low levels of anti-idiotypes. Lang et al. used rabbit antisera to inhibit the total anti-AChR repertoire thereby only detecting CRI if they made a significant contribution to the overall anti-AChR response. In fact one rabbit antiserum did inhibit other patients' antibodies but only when large amounts of anti-idiotype were used. In a separate report using polyclonal rabbit anti-idiotypic antisera Lefvert (1981) presented similar results to those of Lang et al. but interpreted them as demonstrating CRI. In a study of idiotypes expressed by anti-MIR mabs Killen and co-workers

(1985) did not find a dominant CRI; and Souroujon et al. (1985) were unable to detect a CRI between three mabs that bound to different Torpedo AChR epitopes. However, using rabbit anti-idiotypic sera raised by Lang et al. (1985) Whiting was able to inhibit the binding of some murine monoclonal anti-human AChR antibodies to AChR (Whiting 1984).

The existence of naturally occurring anti-idiotypes is another controversial point. Again Lefvert et al. (1986) and Dwyer et al. (1983) have both reported antibodies that bind to mouse anti-AChR idiotypes, but Vincent (1988) has failed to detect anti-idiotypes using a number of techniques, including binding to mouse anti-AChR mabs by ELISA, indirect precipitation of ^{125}I-mabs to AChR, and inhibition of mab binding to AChR by putative anti-idiotype purified by mab-sepharose columns. These results support those of Heininger et al. (1983) who were unable to show an inhibitory effect of sera from patients in remission on autologous anti-AChR antibodies collected during the acute phase of the disease. However, Lefvert et al. (1985) have reported anti-AChR anti-ids in the Ig fraction of healthy first-degree relatives of MG patients. These anti-ids where able to inhibit the binding of the anti-AChR antibodies of the myasthenic relative, and the possible indication is that natural anti-ids in some way prevent normal individuals from developing MG.

Experimental autoimmune myasthenia gravis (EAMG) has

been modulated using idiotypic and anti-idiotypic antibodies in a variety of ways. Lymph nodes cells from AChR-sensitized animals have been treated *in vitro* with ricin conjugates of AChR (Killen and Lindstrom 1984) or anti-idiotype (DeShambo and Krolick 1986). Both T cell proliferation and antibody production following the addition of AChR was greatly diminished. Priming animals with anti-idiotypes has been shown to protect the induction of EAMG either by immunisation with AChR (Agius and Richman 1986) or by passive transfer of anti-AChR antibodies (Souroujon *et al*. 1986). Elsewhere animals primed with idiotype to elicit anti-idiotypic antibodies and then immunised with AChR subsequently produced a greatly reduced anti-AChR response that did not express the original idiotype (Lennon and Lambert 1981; Souroujon *et al*. 1985). Finally, passively transferred EAMG was abrogated by the administration of anti-idiotype (Souroujon *et al*. 1986).

1.6 Aims.

The first aim of the this study was to investigate further the mab binding regions of human AChR using the panel of mabs previously raised in this laboratory. Initially, the binding regions were defined on the basis of data from three sources: the binding to AChR from different species, the inhibition of binding of patients' antibodies to AChR by the mabs, and competition experiments using five ^{125}iodinated-mabs and unlabelled mabs. In this study the five binding regions were re-evaluated using $F(ab')_2$

fragments of each mab to prevent the binding of each intact mab to AChR. Furthermore, an anti-MIR mab was included in the study to provide a point of reference. The anti-MIR mab was M35, (a kind gift of Dr. J. Lindstrom) a rat anti-electric eel AChR mab used by Tzartos and others to define the MIR. More information about the precise epitopes recognised by the mabs may help in understanding the idiotypic cross-reactions found between the mabs.

The second aim was to raise polyclonal and monoclonal anti-idiotypic antibodies to these mabs to investigate the extent of idiotypic sharing between the panel of mabs. There have been many studies using anti-AChR anti-idiotypes since Schwartz et al. (1978) first produced anti-idiotypic antibodies in mice. Two studies have failed to detected much sharing of ids between anti-AChR mabs (Killen et al. 1985; Souroujon et al. 1986), and in a previous study in which CRIs were found, the precise binding specificities of the mabs was not reported (Lennon and Lambert. 1981). In this study information about the binding specificities comes from four sources which should enable firm conclusions to be drawn about the nature of any cross-reactive idiotypes.

Once anti-idiotypic antibodies have been produced, it may be possible to use them to investigate the idiotypes expressed by MG patients' anti-AChR antibodies. Using the rabbit polyclonal anti-idiotypic sera raised by Lang et al. (1985), Whiting (1984) found inhibition of binding to AChR of the anti-AChR mabs, particularly mabs in groups 3, 4 and 5. This demonstrated that the murine mabs shared idiotypes

with the patients' antibodies, and hence it should be feasible to perform the reverse assay and look for shared idiotypes in anti-AChR MG sera using anti-murine anti-idiotypes.

Anti-idiotypic antibodies have the capacity to be more than serological markers for antibodies, and might in the long term provide a means of manipulating the Idiotype Network.

CHAPTER TWO

MATERIALS AND METHODS

Suppliers.

Chemicals.

All chemicals were AnalaR grade or equivalent, and were purchased from BDH or Sigma, Poole, Dorset, except for the following:-

Pristane : Aldrich, Gillingham, Dorset.

Freund's Adjuvant : Difco Laboratories, East Molesey, Surrey.

PEG 1500 : Koch Light, Haverhill, Suffolk.

PBS tablets : Oxoid, Basingstoke, Hampshire.

Immunochemicals.

Goat anti-mouse, human or rat Ig antisera were purchased from Lawrance Laboratories, Australia.

All class-specific and horseradish peroxidase conjugated antibodies were purchased from Jackson Immunochemicals, New Jersey, USA.

Tissue Culture Reagents.

RPMI, L-glutamine, HAT & HT : GIBCO, Uxbridge, Middlesex.

(L-glutamine (100X), and HAT & HT (50X) were obtained as concentrated supplements and diluted to 1X).

Fetal Calf Serum : SeraLab, Sussex.

Plastics : Nunc, Uxbridge, Middlesex.

The NS1 mouse myeloma cell line P3-NS1-Ag4-1, an azaguanine

resistant variant of P3X63 Ag8 was obtained from Dr. A. Goodall, Royal Free Hospital.

Other Reagents.

Protein A- Sepharose 4B beads : Pharmacia, Milton Keynes, Buckinghamshire.

DEAE- Affigel blue beads : Biorad, Watford, Hertfordshire.

Ultrathimbles : Anderman, Kingston, Surrey.

Acetylcholine receptor was extracted from denervated muscle from amputated legs obtained from hospitals in the London area.

α-bungarotoxin : Biotoxins Inc. Florida, USA.

Monoclonal Antibodies.

Anti-human and anti-Torpedo AChR mabs had previously been raised by Mr. Whiting, Dept. of Neurological Science, Royal Free Hospital.

The anti-electric eel mab M35 was the gift of Dr. J. Lindstrom, Salk Institute, San Diego, USA.

The control mabs RFT-1, RFT-2, and T-34 were gift of Dept. of Immunology, Royal Free Hospital; and the anti-pig von willebrand factor and anti-hepatitis B mabs were the gift of Dr. A. Goodall, Haemophilia Unit, Royal Free Hospital.

Equipment.

Gamma counter : Packard Instruments, Pangbourne, Berkshire

ELISA Plate reader (MR 590) : Dynatech, Billingshurst, Sussex.

Spectrophotometer (CE 292) : Cecil Instruments.

Buffers.

Phosphate buffer : Na_2PO_4 (20mM), $NaHPO_4$ (20mM), pH 7.2

Phosphate Buffered Saline, pH 7.2 was prepared from tablets (Oxoid).

PBST : 0.1% (v/v) Tween 20 in PBS.

PTX : 0.1% (v/v) Triton X100 in phosphate buffer.

Final Sample Buffer : 2-mercaptoethanol (6%), SDS (4%), TRIS-HCl pH 6.8 (120 mM), bromophenolblue (0.02%), glycerol (20%).

Tissue Culture Media

RPMI 1640 media was supplemented with L-glutamine (2mM) and used with the addition of penicillin (100IU/ml) and streptomycin (100ug/ml) as antibiotics.

Preservatives.

The protease inhibitor phenylmethylsulphonylfluoride, (0.01M) and as bactericide NaN_3 (0.02%) were added to all antibody and acetylcholine receptor preparations prior to storage.

METHODS

2.1 Preparation of AChR and monoclonal antibodies.

2.1.1 Extraction of acetylcholine receptor.

AChR was extracted from human leg muscle using Triton X100 as described by Lang et al. (1982). Briefly, a piece of muscle weighing approximately 50g was minced up and

homogenised in 250 ml phosphate buffer. After centrifugation (24000g, 45 minutes at 4^{o}C) the supernatant was decanted and an equal volume of 2% (v/v) Triton X100 in phosphate buffer added to the tissue pellet. The mixture was shaken for 2 hours at room temperature and then centrifuged (24000g, 45 minutes). The supernatant was collected, filtered to remove excess fat, and stored at -70^{o}C.

2.1.2 Iodination of α-bungarotoxin.

BuTx was labelled with ^{125}I to a specific activity of 300 cpm/fmole (200 Ci/mmol) using the iodine monochloride method (Vogel et al. 1972) (performed by Dr. A. Vincent). Free iodine was separated from ^{125}I-BuTx by gel filtration using a 1 ml column of Sephadex G25 (Pharmacia). AChR was labelled with ^{125}I-BuTx at 1-2x10^{6} cpm/ml.

2.1.3 Preparation and purification of monoclonal antibodies.

a) Production of ascites fluid.

Large quantities of monoclonal antibodies were obtained as ascites fluid by propagating hybridoma cells in the peritoneum of Balb\c mice. Balb\c mice (3 - 6 months of age), primed 7 days earlier with Pristane (0.5 ml) were injected with 10^{6} cells resuspended in RPMI media (0.2 ml). 10 - 20 days later the peritoneum was drained of the ascites fluid that had accumulated and the mice killed. The ascites fluid was collected in heparinised tubes, centrifuged (10000g, 15 minutes) in a MSE microcentrifuge and stored at -70^{o}C.

b). Protein A- Sepharose 4B affinity chromatography.

Monoclonal antibodies of all IgG subclasses were purified from ascites fluid by Protein A - Sepharose 4B (PA-S4B) affinity chromatography. PA-S4B beads were prepared as per the manufacturer's recommendations, and packed into a column (0.5 x 10 cm, 5 ml total bed volume). Ascites fluid was diluted in PBS (4 ml) and recirculated through the PA-S4B for 3 hours. Unbound protein was washed from the column using PBS (10-20 volumes) until the absorbance at 280 nm was less than 0.03 units. Bound protein was eluted from the column using $NaClO_4$ (2M in PBS), and 1 ml fractions of eluent were collected and the absorbance at 280 nm recorded. $NaClO_4$ was used as the chaotropic salt to elute bound protein as it had been reported that the more frequently used salt, potassium thiocyanate interfered with the [125I] iodination of proteins (George and Schenck 1983), (Dr. Bill Tampion, RFH, personal communication). The peak fractions were pooled and dialysed against PBS (3 x 2 litres).

c) DEAE- Affigel blue column chromatography.

Initially mabs were purified using protein A- Sepharose 4B beads, including mabs of subclass IgG1. However, when a new batch of PA-S4B beads gave poor yields of pure IgG1 mabs, DEAE- Affigel blue column chromatography was used instead. DEAE- Affigel blue beads were packed into a column (0.5 x 30 cm, 10 ml total bed volume) and washed with 3 bed volumes of propan-2-ol / 0.1M acetic acid (pH 3) (40% v/v containing 2M NaCl). The column was then equilibrated with

column buffer (20mM TRIS, pH 7.2) and ascites fluid (1 ml pre-dialysed against column buffer) was applied. A salt gradient (0-100 mM NaCl in 200 ml column buffer) was then applied and 3 ml fractions collected. The absorbance of each fraction at 280 nm was recorded and samples of the peak fractions were analysed by SDS-PAGE. Fractions of the IgG peak were pooled and concentrated by vacuum dialysis using ultrathimbles which retained proteins with a relative molecular weight greater than 75kD.

2.1.4 SDS-PAGE analysis of purified monoclonal antibodies.

Aliquots (10 ul) of purified samples were denatured by heating for 5 minutes at 90^{o}C with an equal volume of final sample buffer. The samples were then run on a 10% SDS-PAGE gel at 200 volts for 3 hours. The gels were fixed in 10% acetic acid and stained with PAGE 83 stain (0.25% PAGE 83 in 20% methanol, 5% acetic acid) for 20 minutes. The gel was destained with 5% methanol, 10% acetic acid (1 hour) and dried.

2.1.5 Conjugation of monoclonal antibodies to Sepharose 4B beads.

CNBr-activated Sepharose 4B beads (1.5g) were swollen in HCl (1mM) according to the manufacturer's recommendations. Purified monoclonal antibody (3 - 4 mg) previously dialysed against coupling buffer (0.1M $NaHCO_3$ pH 8.3, 0.5M NaCl) was added to the swollen beads (approximately 5 ml bed volume) and mixed overnight at 4^{o}C.

Unbound mab was washed away with coupling buffer (20 volumes) and the remaining active groups on the Sepharose beads blocked by incubating with ethanolamine (1M pH 9) for 2 hours at room temperature. The beads were then washed with three cycles of alternating sodium acetate buffer (0.1M pH 4, 0.5M NaCl) and TRIS buffer (0.1M pH 8, 0.5M NaCl). The conjugated beads were stored at $4^{o}C$.

2.1.6 Preparation of $F(ab')_2$ fragments of IgG.

$F(ab')_2$ fragments of each of the anti-human AChR mabs were prepared by pepsin digestion. Protein A purified mabs (approximately 5 mg) were dialysed against sodium acetate buffer (0.1M, pH 3.5). Pepsin was dissolved in acetate buffer (2 mg/ml) and added to the mab (2mg pepsin per 100 mg mab). The mixture was incubated (16 hours at $37^{o}C$), centrifuged (10000g, 10 minutes) and dialysed against PBS. Undigested IgG was removed by adsorption onto a PA-S4B column and the effluent concentrated and stored at $4^{o}C$.

2.2 Immunisations.

2.2.1 Production of polyclonal anti-idiotypic antisera.

Purified monoclonal anti-human AChR antibodies were used as the immunising idiotypes (id). Ten experimental groups of five 6-8 week old Balb/c mice were each immunised with 50 ug of purified id emulsified in Freund's Complete Adjuvant (FCA) injected at multiple subcutaneous and intramuscular sites. Similar injections of id in Freund's Incomplete Adjuvant (FIA) were given 4 and 9 weeks later.

Two weeks following the third injection each mouse was bled by cardiac puncture and the sera assayed for anti-idiotypic activity. All sera were stored at -20^{o}C. Those sets of mice without detectable anti-idiotypic response (those injected with B8, F8 or D6), were injected 13 weeks after the initial immunisation with id and FIA in an attempt to elicit a response. After a further four weeks all sets of mice received a final injection of id and FIA and bled two weeks later; the sera were assayed and stored as before.

2.2.2 Production of monoclonal anti-idiotypic antibodies.

a) Freund's Adjuvant.

6 - 8 week old Balb/c mice were immunised with id emulsified in FCA as above and given booster injections of 50 ug id in FIA 4 and 9 weeks later. Slight variations in the immunising protocol are noted in the results. 10 days after the second boost a serum sample was taken by cardiac puncture, and the anti-idiotypic activity measured by the inhibition of binding of id to ^{125}I-BuTx-AChR. Mice with anti-idiotypic antibodies were given an intraperitoneal injection of id (50 ug) in saline 2- 12 weeks after the second boost; three days later the animals were killed by cervical dislocation and the spleen removed for fusion.

b) Intrasplenic Injection.

The " single shot" immunisation technique described by Spitz et al. (1984) was used. A six week old mouse was anaesthetised and a small incision made along the left hand

side of the abdomen. Care was taken not to cut through into the peritoneum. Idiotype (20 - 125 ug in PBS, 100ul) was injected directly into the spleen. The tissue at the site of the injection blanched and this indicated a successful injection. The incision was sutured, and the mouse allowed to recover. This procedure was performed by Dr. Andy Jermy and Mr Chris Fisher (Dept. of Neurological Science, Royal Free Hospital). Three or four days after immunisation the mouse was killed and the spleen was removed for fusion.

2.2.3 Fusion (Somatic Cell Hybridisation).

A modification of the method first described Kohler and Milstein (1975) was followed using polyethylene glycol (PEG) to mediate fusion between mouse lymphocytes and the HAT-sensitive myeloma cell line NS1.

a). Preparation of spleen cells.

The spleen from an immunised mouse was placed in sterile RPMI media, and the tissue teased apart. The cells were washed twice and resuspended in 10ml RPMI. The number of lymphocytes was estimated using the Trypan blue exclusion method.

b). Preparation of myeloma cells.

NS1 cells were grown in RF_{10} media in 80 cm^2 tissue culture flasks. Cells were collected during log phase growth, centrifuged and washed once with RPMI. The cell pellet was resuspended in 5ml RPMI.

c). Fusion.

Splenocytes and myeloma cells were mixed in a ratio of 5 or 10 : 1, centrifuged (1100rpm, 7 minutes) and the supernatant aspirated. 1ml of 40% (w/v) PEG solution pre-warmed at 37^{o}C was added to the cell pellet slowly over a period of one minute with continuous agitation. The mixture was gently pipetted up and down for a further 30 seconds before being placed at 37^{o}C for 7 minutes. 20 ml RPMI (also pre-warmed at 37^{o}C) was gradually added to the fusion mixture, doubling the volume every 60 seconds. The mixture was centrifuged, and the cell pellet resuspended in 2 ml RF_{10} media. The cells were placed at 37^{o}C for one hour and then dispensed into 96-well microtitre plates, 100 ul per well at a lymphocyte concentration of 1-2 x10^{5} cells per well. The plates were placed in a 5% CO_2 incubator at 37^{o}C.

d). HAT selection.

24 hours after fusion 100 ul of 2x HAT media in RF_{10} was added to each well. Every four days the media was replaced with fresh 1x HAT in RF_{10} media.

e). Growth and Screening of hybrids.

Wells containing growing colonies were screened for antibody production when about 60% confluent. Hybrids of interest were cloned by limiting dilution into microwells containing mouse macrophages as feeders. On cloning the growth media was changed to RF_{10} containing 1x HT supplement. When confluent cells were screened for antibody

production the positive clones were expanded first into 24-well tissue culture plates, and eventually grown in bulk in 80cm^2 flasks. Thereafter HT supplement was discontinued from the growth media. Cells were frozen in 10^7 aliquots in Fetal Calf Serum containing 10% DMSO, and stored at -70^{o}C in a vapour phase liquid nitrogen tank.

2.3 Purification of anti-idiotypic antibodies.

2.3.1 Polyclonal anti-idiotypic sera.

Polyclonal anti-idiotypic sera (5 ul in 100 ul PTX buffer) were mixed with homologous mab-Sepharose 4B beads (250 ul) for 2 hours at room temperature. The beads were pelleted (10000g, 5 minutes) and the supernatant removed and stored at 4^{o}C. The beads were washed twice with PTX and then mixed with 2M $NaClO_4$ (500 ul) in order to elute the bound anti-idiotypic antibodies. After 5 minutes the beads were pelleted and the supernatant dialysed immediately against PBS (3 x 1 litre). The purified anti-idiotypic antibodies were stored at 4^{o}C.

2.3.2 Monoclonal anti-idiotypic antibodies.

a) Affinity chromatography.

Mab-Sepharose 4B beads were packed into a column (0.5 x 10 cm, 2.5 ml bed volume) and equilibrated with PBS. Ascites fluid of the monoclonal anti-idiotypic antibodies (0.5 ml diluted in 3 ml PBS) was applied to the column and recirculated for 3 hours. Unbound protein was washed from the column with PBS (20 - 30 volumes) until the absorbance

at 280 nm was below 0.03 units. Bound anti-idiotypic antibodies were eluted from the column with either 2M $NaClO_4$ or citrate/phosphate buffer (0.1M citric acid / 0.1M sodium phosphate buffer, pH 3.5, 1M NaCl). 1 ml fractions were monitored for absorbance at 280 nm and the peak fractions pooled and dialysed against PBS (3 x 1 litre). The purified antibodies were stored at $4^{o}C$.

b). Ammonium sulphate precipitation of culture supernatant.

Cells producing monoclonal anti-idiotypic antibodies were seeded at a cell concentration of 10^5 per ml of RPMI media containing 1% FCS and allowed to overgrow. Supernatants were removed after 7-10 days and centrifuged (2000rpm, 7 minutes) to remove the cell debris. Immunoglobulin was precipitated from the supernatant by adding ammonium sulphate to give a 50% saturated solution. After stirring for two hours the mixture was centrifuged (14000g, 45 minutes) and the pellet resuspended in PBS. Ammonium sulphate was added to give a 45% saturated solution and the mixture stirred for two hours. After centrifugation the pellet was resuspended in PBS in a volume one tenth of the starting volume and dialysed against PBS (3 x 1 litre). The purified anti-idiotypes were stored at $4^{o}C$.

2.4 Anti-AChR Assays.

2.4.1 Anti-AChR radioimmunoassay.

^{125}I-BuTx-AChR (40 fmoles) was incubated with varying amounts of anti-AChR antibodies diluted in PTX for 2 hours

at room temperature. Antibody- ^{125}I-BuTx-AChR complexes were precipitated with anti-species-Ig, depending on the nature of the anti-AChR antibody (either human, mouse or rat). Carrier serum was added where necessary to bring the total volume of serum in each assay up to 1 ul. After overnight incubation at 4^{o}C the precipitates were centrifuged, the pellets washed with PTX, and counted in a gamma counter.

When a particular assay required less than saturating amounts of anti-AChR antibody, the antibody was titrated against ^{125}I-BuTx-AChR. The amount of antibody that precipitated 50-70% of the ^{125}I-BuTx-AChR was determined and called the "limiting amount".

The titre of each antibody was expressed as the number of moles of BuTx-binding sites of AChR precipitated per litre of antibody.

2.4.2 Assays of mab-Sepharose 4B beads.

a). Precipitation of ^{125}I-BuTx-AChR.

Varying amounts of mab-Sepharose beads (0.2 - 5 ul) were mixed with ^{125}I-BuTx-AChR (40 fmoles) for 2 hours at room temperature in a total volume of 0.5 ml of PTX buffer. Unconjugated Sepharose 4B beads were added (to a total volume of 5 ul of beads) and the beads pelleted by centrifugation (10000g, 5 minutes). The pellets were washed twice with PTX buffer and bound ^{125}I-BuTx-AChR counted in a gamma counter.

b). Precipitation of ^{125}I-Protein A.

Increasing amounts of ^{125}I-Protein A (the kind gift of Helen Wood, Royal Free Hospital) was mixed with 5 ul of mab-Sepharose 4B beads for 1 hour at room temperature. The beads were pelleted, washed twice with PTX buffer and bound ^{125}I-Protein A counted in a gamma counter.

2.4.3 Inhibition of mab binding to ^{125}I-BuTx-AChR by F(ab')$_2$ fragments.

^{125}I-BuTx-AChR was incubated (4 hours at room temperature) with saturating amounts of F(ab')$_2$ fragments of each anti-human AChR mab. Limiting amounts of each mab diluted in PTX were then added and incubated for two hours at room temperature. Intact mab bound to ^{125}I-BuTx-AChR was precipitated using goat-anti-mouse IgG (specific for Fc), or in the case of the rat monoclonal antibody, goat-anti-rat IgG. 1 ul normal mouse or rat serum was added as carrier. Following overnight incubation at 4^{o}C the precipitates were centrifuged, and the pellets washed and counted as before. The percentage inhibition of each mab binding to AChR by F(ab')$_2$ was expressed as

$$\% \text{ inhibition} = \frac{cpm_{cf} - cpm_{if}}{cpm_{cf}}$$

where cpm_{if} and cpm_{cf} are the cpm precipitated of ^{125}I-BuTx-AChR pre-incubated with anti-AChR F(ab')$_2$ and control F(ab')$_2$ respectively.

2.5 Anti-idiotypic assays.

2.5.1 Polyclonal anti-idiotypes.

a). Anti-combining site anti-idiotypes.

Anti-ids were measured by their ability to inhibit the binding of id to AChR in a radioimmunoassay. Increasing amounts of anti-id sera (0.001-5.0 ul) in PTX buffer (100 ul) were incubated with limiting amounts of id for 2 hours at room temperature. The final amount of mouse sera in the assay was always brought up to at least 1 ul using non-immune Balb/c sera. ^{125}I-BuTx-AChR was then added and incubated for a further two hours at room temperature. The ^{125}I-BuTx-AChR-id complexes were precipitated by goat-anti-mouse Ig at 4^{o}C overnight. The precipitates were centrifuged, washed in PTX buffer, and counted in a gamma counter. Each serum sample was assayed at least twice, and the mean result taken.

To ensure that the mouse antisera themselves did not contain any anti-AChR activity, aliquots (1 ul) of each sample were incubated with ^{125}I-BuTx-AChR (two hours at room temperature) before being precipitated and counted as above.

F(ab')$_2$ fragments were also used in the inhibition assay. Dilutions of anti-ids were incubated with limiting amounts of F(ab')$_2$ fragments instead of intact mab and the assay continued as above.

To ensure that the inhibition observed was specific, anti-id sera were pre-incubated with 1 ul non-immune Balb/c sera prior to the addition of id to absorb any antibodies raised against determinants present in normal serum. The

assay was then continued as above.

The precipitation of ^{125}I-BuTx-AChR by mab in the presence of anti-id was expressed as a percentage of that precipitated in non-immune Balb/c mouse serum (nms) alone, i.e.

$$\% \text{ inhibition} = 100 \times \frac{CPM_{nms} - CPM_{anti\text{-}id}}{CPM_{nms}}$$

and the titre (moles/litre) expressed as

$$\frac{\% \text{ inhibition} \times \text{mab (moles)}}{\text{anti-id (litres)}}$$

b). Anti-framework anti-idiotypes.

Limiting amounts of $F(ab')_2$ fragments of each mab were incubated with ^{125}I-a-BuTx-AChR (4 hours at room temperature). Increasing amounts of anti-id sera (0.01-5 ul) were added and incubated for a further 2 hours. ^{125}I-BuTx-AChR- $F(ab')_2$- anti-id complexes were precipitated by goat-anti-mouse IgG (Fc specific) antisera overnight at 4^{o}C. The precipitates were centrifuged, washed and counted as before. It was assumed in this assay that idiotopes close to, or at the antigen binding site would be occluded by AChR and therefore anti-combining site anti-ids would not interfere with the assay.

The results were expressed as moles ^{125}I-BuTx-AChR-$F(ab')_2$ precipitated per litre anti-id.

c). ELISA.

Direct binding of anti-id to $F(ab')_2$ fragments of the mabs was measured in this assay. 96-well ELISA plates were coated with $F(ab')_2$ (100 ul of 10 ug/ml in 0.1M TRIS buffer pH 10) overnight at 4^oC. Between each step the wells were washed three times with PBST (0.1% Tween 20 in PBS). After washing the unreactive binding sites on the plastic were blocked using gelatin (0.1%) in PBS (1 hour, room temperature). Serial dilutions of the anti-id sera (50 ul in PBST containing 0.1% gelatin, 0.1M EDTA) were applied to the wells and incubated at 37^oC for 2 hours. The plates were then incubated at 37^oC with 50 ul horseradish peroxidase (HRP) conjugated goat-anti-mouse IgG (Fc specific), diluted 1/5000 in PBS. 100 ul of substrate, tetramethyl benzidine (TMB) (6 mg/ml in DMSO) was diluted 1/100 in sodium acetate buffer (0.1M, pH 5) containing 0.003% H_2O_2. The reaction was stopped after 15 minutes at room temperature by the addition of 0.5M sulphuric acid, and the absorbance read at 450 nm. The background binding of anti-id to wells which had been coated with $F(ab')_2$ prepared from normal mouse IgG was determined and subtracted from the readings obtained.

d). Cross-reactive anti-ids.

Anti-idiotypic sera positive in each of the above assays were used to determine whether common idiotopes existed in the panel of anti-human AChR mabs. In each assay, the homologous id was substituted by each of the other ids, and the assays performed as before. The cross-reactive

titres against the heterologous ids were calculated and the percentage cross-reaction expressed as

$$100 \times \frac{\text{heterologous anti-id titre}}{\text{homologous anti-id titre}}$$

2.5.2 Monoclonal anti-idiotypes.

a). ELISA.

96-well ELISA plates were coated with goat anti-mouse Ig (1/1000) or target idiotype (10 ug/ml) in TRIS buffer (0.1M, pH 10) by overnight incubation at 4°C. Unreactive binding sites on the plastic were blocked with 1% BSA in PBS (w/v) (1% BSA-PBS) for one hour at room temperature. Plates were washed three times with PBST between each subsequent step. Test anti-id (50 ul) was added to the wells followed 1-2 hours later by HRP conjugated goat-anti-mouse antibodies (diluted 1/5000 in PBS). The conjugated antibodies were specific for IgG(Fc), IgG (heavy & light chains) or IgM. After one hour substrate (100 ul, prepared as above) was added and the reaction at room temperature stopped by the addition of 50 ul of 0.5M H_2SO_4. The OD values are the absorption at 450 nm minus the relevant background values.

b). Inhibition ELISA.

Test anti-ids were titrated against target id to determine the amount required to give 70% of the maximum OD at 450 nm. This amount of anti-id was then incubated (3

hours, 4°C) with varying amounts of inhibiting id (0 - 2.5 ug) diluted in 0.1% BSA in PBST w/v (BSA-PBST). The mixtures were then added to id-coated ELISA plates and the assay continued as above. The degree of inhibition of binding of anti-id to the target idiotype by mab was expressed as the fraction of the OD recorded for anti-id pre-incubated with BSA-PBST alone.

$$\% \text{ inhibition} = \frac{OD_{\text{anti-id alone}} - OD_{\text{anti-id + mab}}}{OD_{\text{anti-id alone}}}$$

c). Reverse ELISA.

Monoclonal anti-ids purified from culture supernatant by ammonium sulphate precipitation were coated onto ELISA plates as previously described. After blocking varying amounts of mab anti-AChR or control mabs diluted in 0.1%-BSA-PBST were added to the wells and incubated for 2-4 hours at room temperature. HRP-goat anti-mouse IgG(Fc specific) was added to each well (one hour at room temperature), followed by substrate and H_2SO_4 as above. The OD at 450 nm was measured and compared to the background values obtained using the control IgG mabs.

Alternatively goat-anti-mouse IgM-coated plates were used in order to "capture" the anti-ids. After blocking with 1%-BSA-PBS, anti-idiotypes diluted in BSA-PBST were added and incubated for 1 hour at room temperature. Varying dilutions of idiotypes were added to the wells and the assay continued as above.

d). Inhibition Radioimmunoassay.

Culture supernatants, ascites fluid or purified anti-ids were incubated (4 hours at room temperature) with limiting amounts of id. ^{125}I-BuTx-AChR was then added and the assay continued exactly as for the polyclonal anti-ids (section 2.4.3a). The degree of inhibition of binding of idiotype to AChR by culture supernatant was calculated in relation to its binding in the presence of spent NS1 culture media, control ascites or a control monoclonal IgM antibody.

$$\% \text{ inhibition} = \frac{cpm_{con} - cpm_{anti\text{-}id}}{cpm_{con}}$$

where cpm_{con} and $cpm_{anti\text{-}id}$ are the cpms of ^{125}I-BuTx-AChR precipitated in the presence of the controls and anti-ids respectively.

e). Framework-binding assay.

^{125}I-BuTx-AChR was incubated with limiting amounts of idiotype for 2 hours at room temperature. Varying amounts of anti-id diluted in PTX was added and incubated for 8 hours at 4^{o}C. The anti-id-id-^{125}I-BuTx-AChR complexes were then precipitated overnight at 4^{o}C using goat-anti-mouse IgM and normal mouse sera as carrier. The precipitates were centrifuged and the pellets washed and counted.

CHAPTER THREE

PURIFICATION AND CHARACTERISATION OF ANTI-HUMAN AChR MONOCLONAL ANTIBODIES

3.1 Introduction.

The ten anti-human AChR monoclonal antibodies had previously been divided into five groups based on competition experiments with ^{125}I-mab, using five of the ten mabs labelled with ^{125}Iodine (Whiting et al. 1986b). Recently, the mabs have been characterised with respect to the α-bungarotoxin binding site (Heidenreich et al. 1988). It was shown that binding regions 1, 2 & 5 are probably all close to the BuTx binding site as these mabs inhibited the binding of a MG plasma sample that contained antibodies to the toxin binding site. Region 1 & 2 mabs did not have a cumulative effect whereas regions 1 & 5 did; suggesting that regions 1 & 2 are on the same α-subunit, and region 5 on the other.

To investigate fully the binding regions of our panel of anti-human AChR mabs, $F(ab')_2$ fragments were used to protect AChR from binding by intact mab.

3.2 Results.

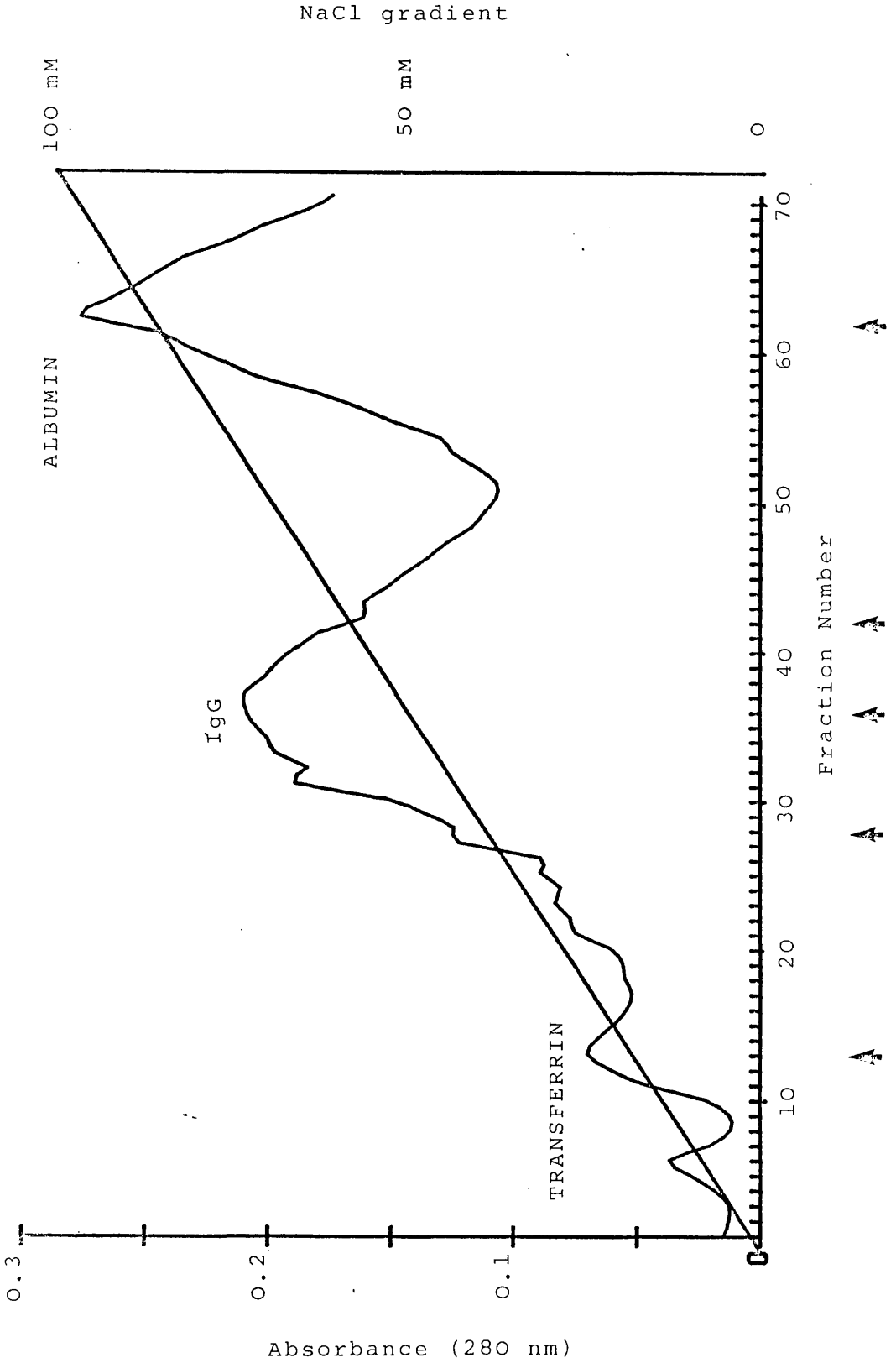
3.2.1 Purification of mabs.

Monoclonal antibodies of all IgG subclasses were purified by Protein A- Sepharose 4B affinity chromatography or by DEAE- Affigel blue chromatography. Figure 3.1 shows a typical elution profile for a mab purified by the latter method, using a linear NaCl gradient (0 - 100 mM). The three

Figure 3.1: DEAE- Affigel blue chromatography.

Mab F8 (1 ml ascites fluid) was eluted from a DEAE- Affigel blue column using a linear salt gradient (0 - 100 mM NaCl in 20 mM TRIS buffer, pH 7.2; right hand ordinate), and the absorbance at 280 nm of serial 3 ml fractions measured (left hand ordinate).

Samples of indicated fractions were run down a 10% SDS-PAGE gel (Fig. 3.2).


NaCl gradient
100 mM
50 mM
0
ALBUMIN
IgG
TRANSFERRIN
0.3
0.2
0.1
0
10
20
30
40
50
60
70
Fraction Number
Absorbance (280 nm)

protein fractions, transferrin, IgG and albumin were eluted as predicted (Bruck et al. 1986) at 25, 30 - 50 & 65 mM NaCl respectively. Samples from each peak were subjected to SDS-polyacrylamide gel electropheresis in order to confirm the identity of each peak by their relative molecular weights, and to assess the purity of the IgG fraction (Figure 3.2). Fractions around the IgG peak were pooled and concentrated by vacuum dialysis. Fig 3.2 demonstrates the purity of F8 prepared by DEAE- Affigel blue (lanes G, H & I) compared to conventional Protein A- Sepharose 4B chromatography (lane J).

3.2.2 Preparation of $F(ab')_2$ fragments.

$F(ab')_2$ fragments of each mab were prepared by pepsin digestion and purified by passing over a Protein A-Sepharose 4B column. After concentrating the $F(ab')_2$ effluent to approximately 0.2 - 0.5 mg/ml, the purified fragments were assayed for IgG(Fc) contamination by ELISA, and for anti-AChR activity by RIA.

a) ELISA.

Figure 3.3 shows results of increasing amounts of $F(ab')_2$ added to anti-Ig coated ELISA plates followed by HRP-anti-IgG(Fc) or HRP-anti-mouse IgG($F(ab')_2$). The purity of each $F(ab')_2$ preparation was > 95%

b) RIA.

Table 3.1 shows the cpm of ^{125}I-BuTx-AChR precipitated by saturating amounts of $F(ab')_2$ with anti-Ig or anti-IgG(Fc). The precipitation of $F(ab')_2$-^{125}I-BuTx-AChR

Figure 3.2: 10% SDS-PAGE gel of DEAE- Affigel blue purified mab F8.

Samples of the eluted fractions from the DEAE- Affigel blue column (Fig. 3.1) were analysed by SDS-PAGE, staining with PAGE 83 stain.

Lane A: Ascites fluid containing mab F8.

Lane B: Fraction 13 (transferrin peak).

Lane C, D & E: Fractions 28, 36 & 42 (IgG peak).

Lane F: Fraction 62 (albumin peak).

Lanes G, H & I: Pooled and concentrated fractions 28 - 32; 33 - 37; 38 - 42.

Lane J: Protein A - Sepharose 4B purified mab F8.

Lane K: Molecular weight markers.

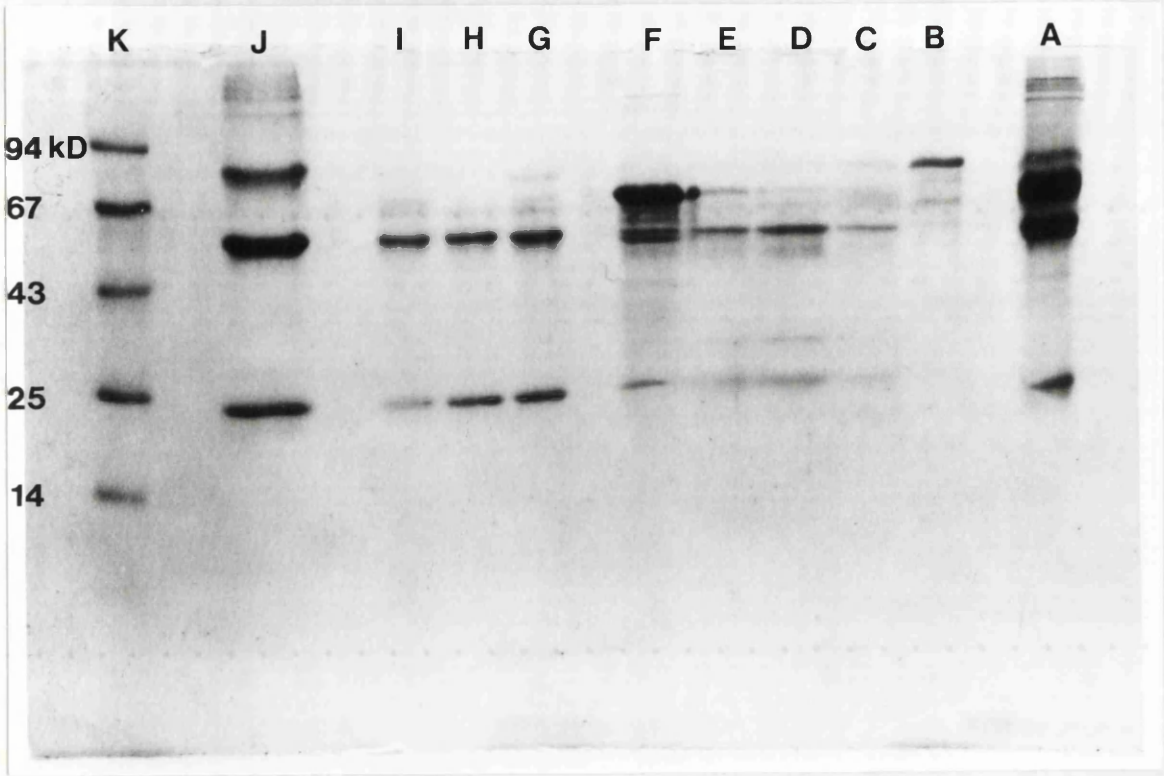

K
J
I
H
G
F
E
D
C
B
A
94 kD
67
43
25
14

Figure 3.3: ELISA binding of $F(ab')_2$ fragments of anti-human AChR mabs.

Dilutions of $F(ab')_2$ fragments (initial concentration 200 - 500 ug/ml) of mabs F8, C3, C7 & G3 were added to anti-Ig coated ELISA plates, followed by HRP-anti-IgG($(Fab')_2$) (▨) or HRP-anti-IgG(Fc) (□). After the addition of substrate and H_2SO_4, the absorbance at 450 nm was read.

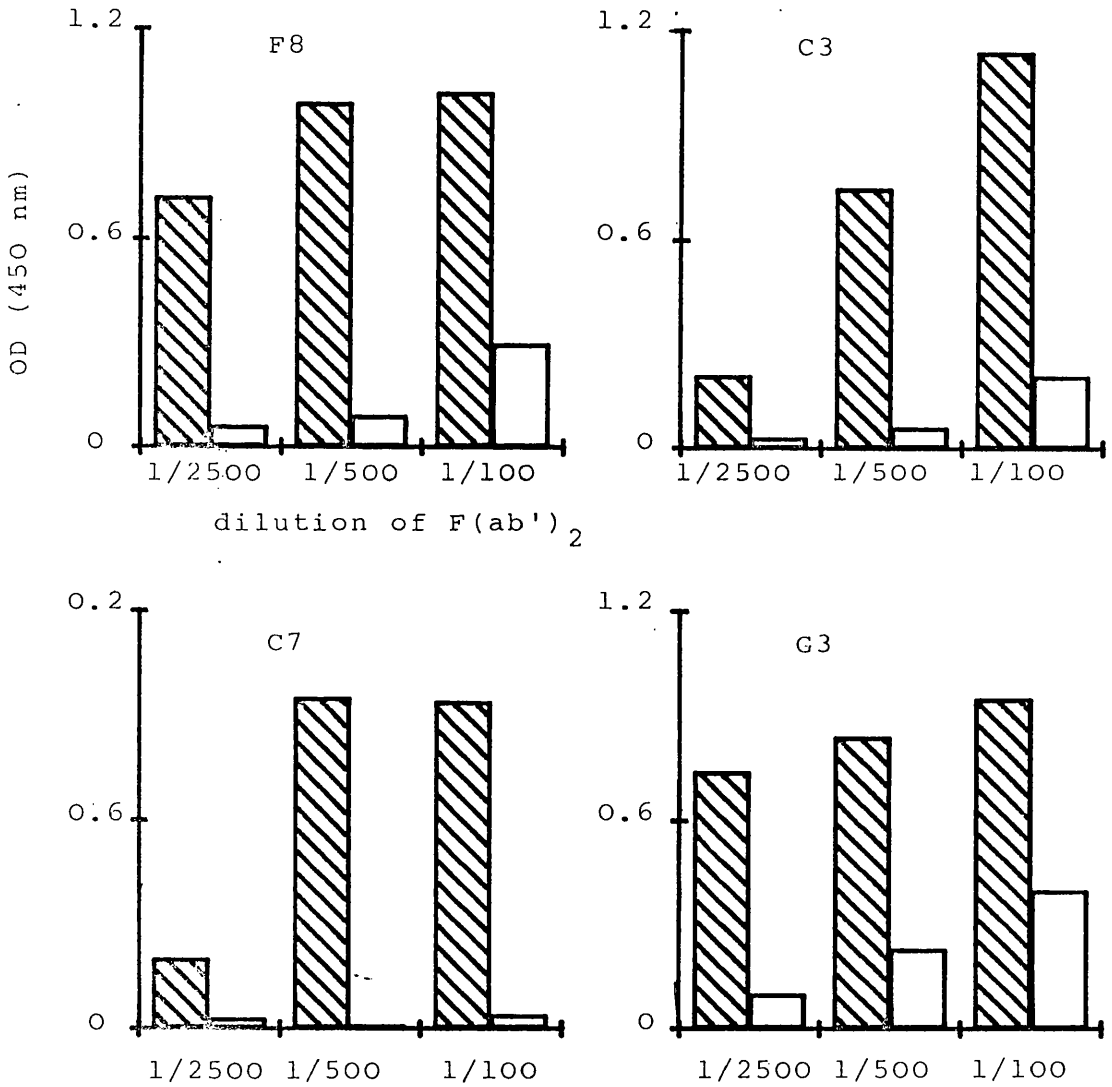

OD (450 nm)
1.2
0.6
0
F8
1/2500
1/500
1/100
dilution of F(ab')2
C3
0.2
C7
G3

Table 3.1: Precipitation of ^{125}I-BuTx-AChR by F(ab')$_2$ fragments.

	Anti-AChR mab/F(ab')$_2$									
	B8	C2	C9	F8	B3	C3	G10	D6	C7	G3
F(ab')$_2$+anti-Ig[a]	5968	6947	6052	6132	6761	8074	9360	6830	7485	6260
F(ab')$_2$+anti-Fc[b]	480	241	469	427	567	634	872	385	542	660
Mab + anti-Fc[c]	2835	3616	4561	3190	3035	3976	2838	4713	4884	3803

NMS + anti-Ig = 776 cpm

NMS + anti-Fc = 550 cpm

positive anti-AChR + anti-Ig = 8840 cpm

results expressed as total cpm of ^{125}I-BuTx-AChR precipitated without substracting the background values of NMS.

[a] saturating amount of F(ab')$_2$ + ^{125}I-BuTx-AChR precipitated by goat anti-Ig.

[b] saturating amount of F(ab')$_2$ + ^{125}I-BuTx-AChR precipitated by goat anti-IgG(Fc).

[c] limiting amount of mab + ^{125}I-BuTx-AChR precipitated with goat anti-IgG(Fc).

complexes by anti-IgG(Fc) was less than 10% of that by anti-IgG $F(ab)_2$. In contrast, the cpm precipitated by limiting amounts of intact mab with anti-IgG(Fc) was 30 - 60% of the total cpm in the assay.

3.2.3 Protection of mab-binding sites by $F(ab')_2$.

The mab binding sites of AChR were protected by preincubation with saturating amounts of each $F(ab')_2$ prior to the addition of limiting amounts of intact mab. Table 3.2 shows the percentage inhibition of binding of whole mab to ^{125}I-BuTx-AChR in the presence of $F(ab')_2$.

The binding regions of human AChR defined by competition experiments using ^{125}I-labelled and unlabelled mabs (Whiting et al. 1986b) were confirmed by these results. In addition, inhibition of a rat anti-electric eel AChR mab M35 (a mab used to define the MIR) was measured.

Region 1: All the mabs in region 1 were inhibited by each $F(ab')_2$ from region 1 but no other region.

Region 2: B3 was only inhibited by its own $F(ab')_2$.

Region 3: Both mabs were inhibited by $F(ab')_2$ from regions 3 & 4; but only by G3 $F(ab')_2$ of group 5.

Region 4: D6 was inhibited by $F(ab')_2$ from regions 3, 4 and to a lesser degree region 5.

Region 5: Both mabs were inhibited by $F(ab')_2$ from regions 4 & 5 but not region 3.

MIR: M35 was inhibited by $F(ab')_2$ from regions 3, 4 and to some extent region 5.

Table 3.2: Inhibition of mab binding to AChR by $F(ab')_2$.

Inhibiting $F(ab')_2$	Region[a]	Anti-AChR mab										
		B8	C2	C9	F8	B3	C3	G10	D6	C7	G3	M35
B8	1	92[b]	92	71	64	0	6	7	13	2	5	0
C2	1	78	93	85	81	0	0	0	8	1	0	ND
C9	1	100	100	100	100	0	0	0	4	0	0	ND
F8	1	73	87	75	80	0	0	0	4	0	0	ND
B3	2	0	0	11	0	85	0	2	19	15	1	ND
C3	3	0	11	16	0	0	94	89	14	0	0	90
G10	3	0	0	0	0	0	100	99	26	0	0	ND
D6	4	2	0	14	16	4	70	76	100	82	78	81
C7	5	3	6	5	0	4	8	0	69	100	100	11
G3	5	10	0	1	19	0	37	45	73	99	99	33

[a] AChR mab binding regions defined by Whiting et al (1986).

[b] inhibition expressed as percentage of cpm ^{125}I-BuTx-AChR precipitated by mab in the presence/absence of $F(ab')_2$.

3.2.4 Conjugation of mab to Sepharose 4B beads.

Five monoclonal anti-human AChR antibodies (C2, C9, G10, D6 & C7) were conjugated to CNBr-activated Sepharose 4B beads at a concentration of 0.5 - 1 mg/ml. The mab-Sepharose 4B beads were assayed by direct precipitation of ^{125}I-BuTx-AChR (Figure 3.4) and ^{125}I-Protein A (Table 3.3). These results demonstrate that neither anti-AChR activity nor Protein A binding was lost by conjugation to Sepharose.

3.3 Discussion.

Anti-human AChR mabs were purified from ascites fluid by Protein A- Sepharose 4B affinity chromatography, or by DEAE- Affigel blue chromatography. Both methods yielded purified antibodies essentially free of albumin although some preparations had residual transferrin contamination.

Pepsin cleavage of IgG mab to produce F(ab')$_2$ fragments did not affect the ability of fragments binding to AChR. The degree of purity of the F(ab')$_2$ ensured that they could be used to protect the binding sites of AChR without being precipitated by anti-IgG(Fc). Therefore, when ^{125}I-BuTx-AChR was precipitated it could safely be attributed to whole mab not protected by F(ab')$_2$. The inability of anti-IgG(Fc) to bind to the F(ab')$_2$ fragments in ELISA proved to be an invaluable property when assaying the syngeneic anti-idiotypic antibodies (Chapter 4).

Mabs conjugated to Sepharose 4B beads were still able to bind to ^{125}I-BuTx-AChR.

Figure 3.4: Precipitation of ^{125}I-BuTx-AChR by mab-Sepharose 4B beads.

^{125}I-BuTx-AChR (40 fmoles) was precipitated by increasing amounts of the indicated mab-Sepharose 4B beads. Results are expressed minus the background cpm precipitated by IgG(F(ab')$_2$)-Sepharose 4B beads.

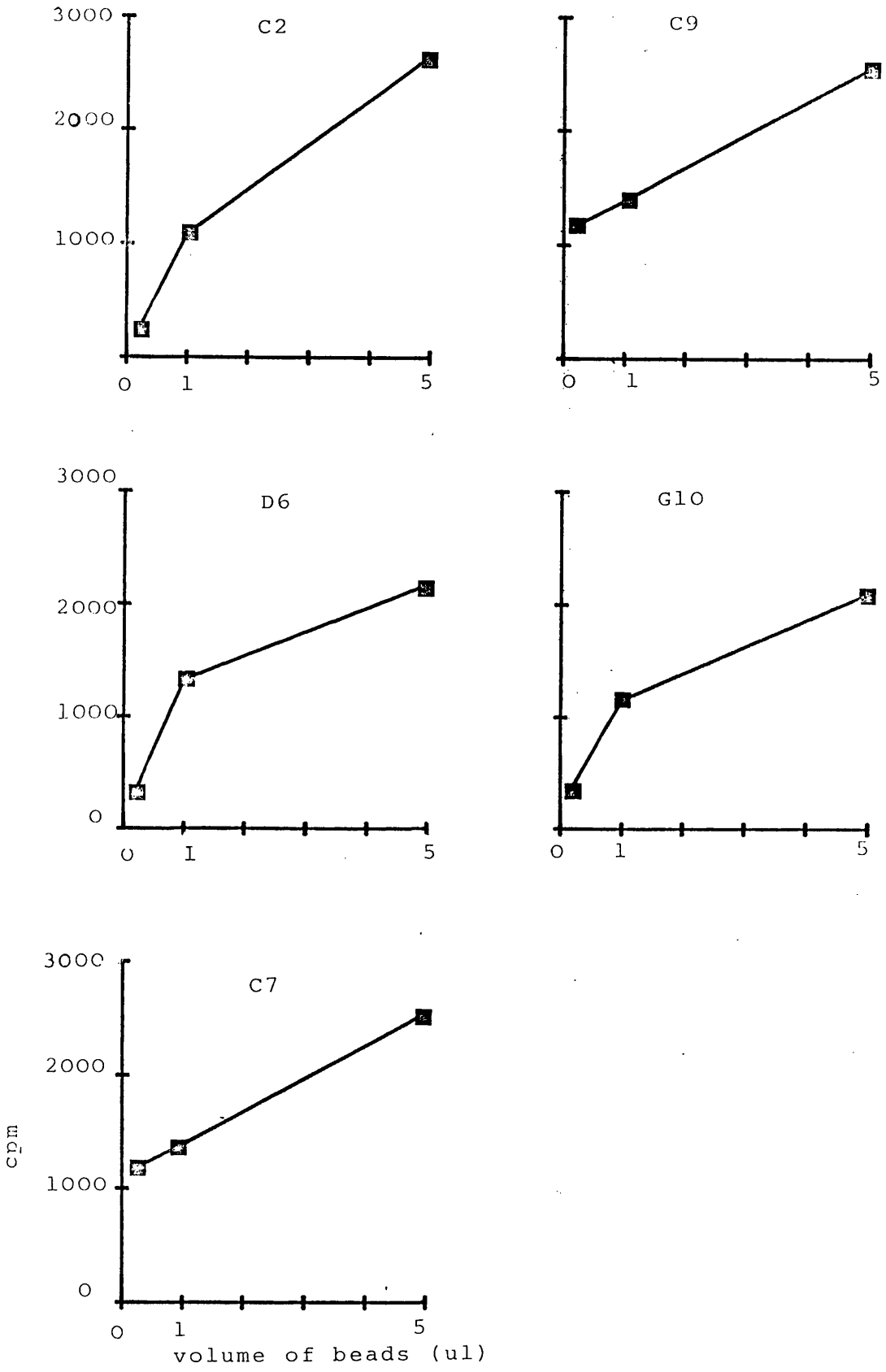

C2
C9
D6
G10
C7
3000
2000
1000
0
1
5
cpm
volume of beads (ul)

Table 3.3: Precipitation of ^{125}I-Protein A by mab-Sepharose 4B beads.

^{125}I-Protein A (ul)	mab-Sepharose 4B beads		
	C7	D6	G10
0.04	12023	9909	8198
0.2	59245	44174	44975
1.0	311124	91771	226747

Results expressed as cpm of ^{125}I-Protein A corrected for non-specific precipitation (approximately 30%) by IgG(F(ab')$_2$)-Sepharose 4B beads.

The groupings of the mabs by Whiting et al. (1986b) have been confirmed using $F(ab')_2$ to protect the binding regions of AChR. Binding regions 1 and 2 are thought to be on one α-subunit, and region 5 on the other. There is no evidence to suggest that there are any amino acid differences between the two α-subunits, but the environments of the two are different. One α-subunit is bordered by a β and a δ subunit whereas the other is bordered by a β and a τ/ϵ. This may induce subtle conformational differences in the two subunits which may affect the binding of the anti-AChR mabs (Dr. Angela Vincent, RFH, personal communication). Although it is thought that region 1 (mabs B8, C2, C9 & F8) and region 2 (mab B3) are both close to the BuTx binding site of the same α-subunit, I was unable to detect any overlap between the two. However, it is perfectly possible for mabs to two non-overlapping regions to independently inhibit the binding of the anti-toxin site antibody. The group 5 mabs (C7 & G3) did not behave exactly the same indicating that they probably recognise slightly different determinants in region 5. The observation that the region 4 mab (D6) inhibited the binding of the anti-MIR mab, M35, confirms the indirect evidence that D6 is an anti-MIR mab (Heidenreich et al. 1988). There, a strong correlation was found between D6 and M35 inhibiting the binding of MG sera to AChR, but not between C3 and M35.

Antibodies to the MIR are considered important not only because they are the majority of all anti-AChR antibodies (Tzartos et al. 1980), but also because they have been shown

to be pathogenic (Tzartos et al. 1985, 1987). Indeed, those mabs corresponding to the MIR (mabs D6, C3 & G10) bind murine AChR in vitro and in vivo (Whiting et al. 1986b) and also cause in vivo receptor loss in mice (Jermy, unpublished observations). The position of the MIR on the α-subunit was initially mapped to residues 6 - 85 using recombinant DNA fusion proteins (Barkas et al. 1987). These workers have further restricted the MIR to residues 61 - 76 using the same approach (Barkas et al. 1988). Studies using synthetic peptides to localise the MIR have been more difficult as the anti-MIR mabs do not bind to linear peptides particularly well (Ralston et al. 1987). Nevertheless, Tzartos et al. (1988) using overlapping peptide sequences (14 - 20 residues long) were able to detect ELISA binding of anti-MIR mabs to a peptide spanning residues 67 - 76 of the α-subunit. In an independent study Wood et al. (1988), using a similar method to that of Tzartos, found binding of the anti-MIR mab D6 to a peptide containing residues 65 - 78. Together, these two studies confirm the observations of Barkas et al.

However, although residues 61 - 76 appear to be important for B cell recognition, peptides corresponding to the MIR were unable to stimulate the uptake of [^{3}H]-thymidine by T cells taken from the peripheral blood of MG patients. T cells did proliferate in response to peptides corresponding to residues 125 - 143, and 257 - 269 of the α-subunit, but this response was MHC-restricted, the accessory (antigen presenting) cells being matched for the DR loci of HLA (Harcourt et al. 1988).

3.4 Summary.

Monoclonal antibodies of the IgG isotype were purified from ascites fluid using Protein A- Sepharose 4B or DEAE-Affigel blue column chromatography. $F(ab')_2$ fragments of each mab were prepared by pepsin digestion and assessed to be greater than 90% free of IgG(Fc) contamination by both ELISA and RIA.

The five AChR binding regions defined by Whiting et al. (1986b) were confirmed using $F(ab')_2$ to protect the binding sites of AChR. Regions 1 and 2 are both distinct, and there is little overlap between regions 3 and 5 although region 4 overlaps both. The MIR coincides with regions 3 and 4, and overlaps region 5.

Figure 3.5 shows schematically a modified version of the five binding regions of the α-subunit of AChR defined by the panel of mabs in relation to the α-bungarotoxin binding site and the MIR.

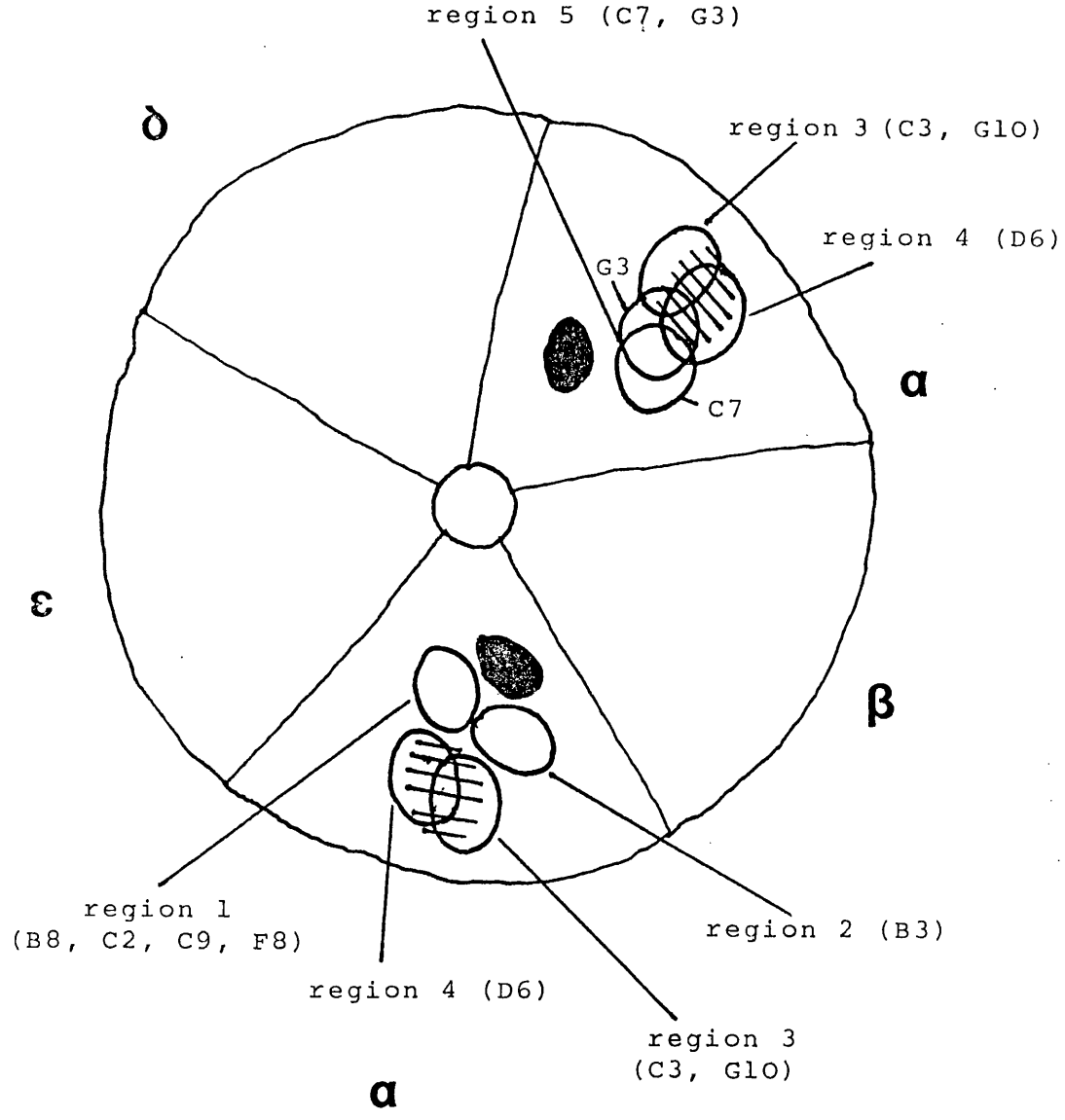


Figure 3.5: Schematical representation of anti-human AChR mab binding regions based on present findings.

Regions 1 & 2 are on the same α-subunit, region 5 on the other. Region 5 is divided into two, showing G3 but not C7 overlapping region 3.

Solid shading = BuTx binding site, cross-hatch shading = MIR.

CHAPTER FOUR

POLYCLONAL ANTI-IDIOTYPES

4.1 Introduction.

The panel of anti-human AChR mabs used in our laboratory have been well characterised. They have been used to define five binding regions of the α-subunit of AChR, and these regions are also target epitopes of MG patients' autoantibodies (Whiting et al. 1986a; Heidenreich et al. 1988). In this chapter the idiotypes of the mabs were investigated using anti-idiotypic sera raised in syngeneic Balb/c mice.

A possible disadvantage of using the anti-human AChR mabs as the source of idiotypes was that these mabs were originally derived from fusions using the myeloma cell line NS1. This cell line synthesises, but does not secrete immunoglobulin light chains. These light chains may however be incorporated into the mabs which were then secreted. Anti-ids to such hybridoma products would of course detect a great deal of idiotypic sharing, but would be meaningless. However, the mobilities of of the denatured mab light chains on SDS-PAGE were quite heterologous, indicating that the light chains were not NS1 in origin.

Several factors have to be considered when raising the anti-idiotypic sera in mice, not least the small quantities of sera obtainable (0.5 ml) from mice compared to that from rats (5 ml) or rabbits (20 - 100 ml). Also, the conventional method of detecting xenogeneic anti-idiotypes, by species-

specific antisera can not be used. However such considerations are far outweighed by the advantages listed below.

a). Absorption of the antisera to remove antibodies to isotypic or allotypic determinants should be unnecessary. Such absorption steps would be time consuming to perform, especially when antibodies to 10 mabs are involved.

b). Anti-idiotypic antibodies raised syngeneically would bear greater relevance to the Idiotype Network. The Idiotype Network is concerned with interactions of the immune system within an individual, and not across species.

c). It may be possible to produce monoclonal anti-idiotypes by the fusing of splenocytes of mice that gave a good anti-idiotypic response.

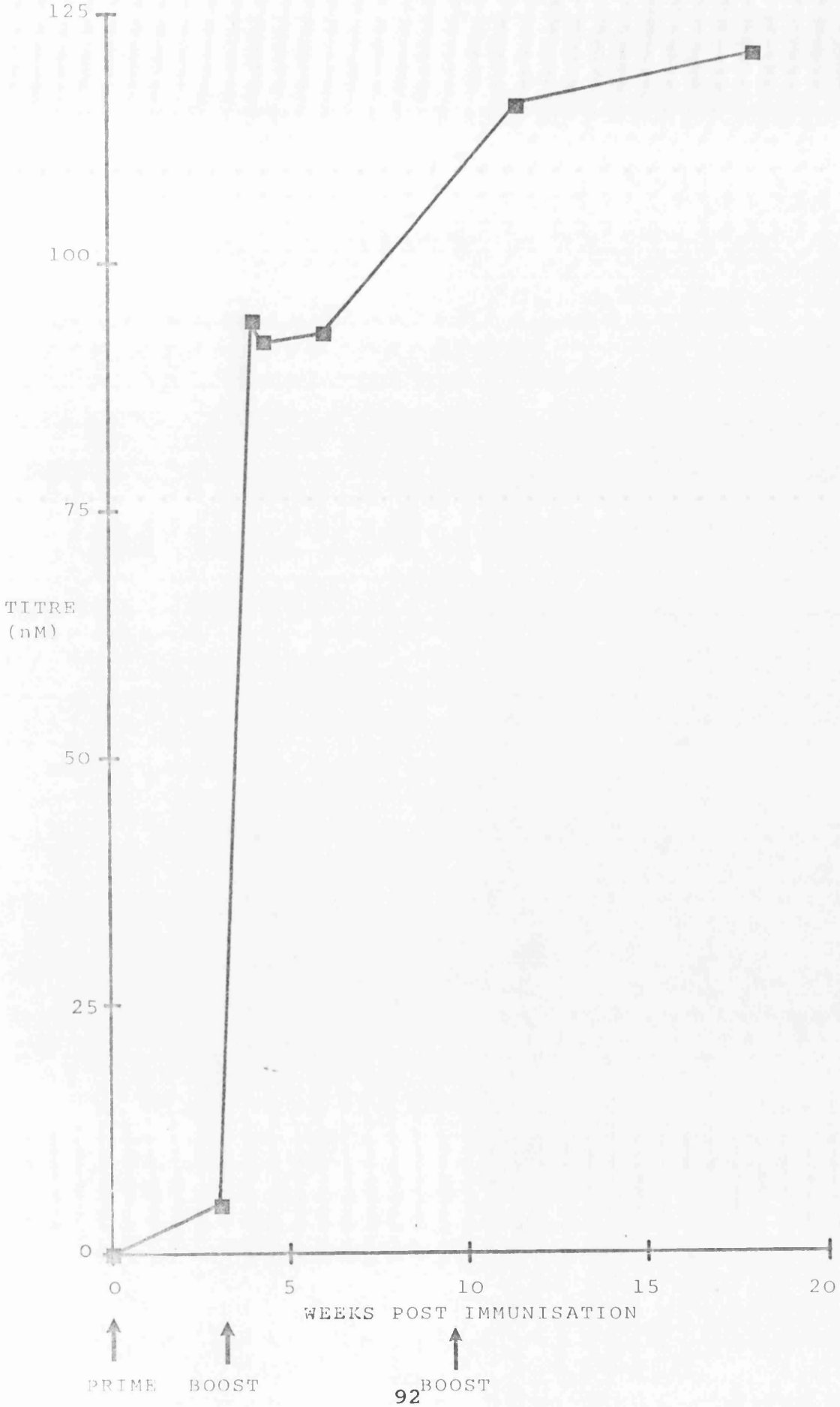
4.2 Results.

4.2.1 Ab2gamma.

Anti-ids to the antigen binding site of the anti-human AChR mabs were detected by the inhibition of mab binding to AChR, and the titre expressed as the moles of mab inhibited per litre anti-id. The results of a preliminary experiment (Figure 4.1), show the anti-id response over a period of time. The inhibition profile with respect to the immunisation schedule demonstrates that a good anti-idiotypic response (100 nM) was achieved following the first boost of id, which was then enhanced by the second boost.

Of the 47 mice immunised, 27 had produced an anti-idiotypic response. Mice challenged with mabs B8 or D6

Figure 4.1: Time course of anti-id response to mab G10. Serial serum samples were taken from a mouse immunised with G10 emulsified in Freund's adjuvant boosted 3 and 9 weeks after priming. Serum samples were taken immediately before the first two injections, and then at five time points over 18 weeks. The Ab2gamma titre was calculated for each sample and expressed as moles of mab G10 inhibited per litre antisera.


125
100
75
50
25
0
TITRE
(nM)
0
5
10
15
20
WEEKS POST IMMUNISATION
PRIME
BOOST
BOOST

failed to elicit a response, even after multiple boosts. Anti-AChR activity caused by residual mab or potential Ab3 antibodies was not found in any serum sample (data not shown).

Figures 4.2 and 4.3 show the inhibition of id binding to AChR by increasing amounts of anti-id. A representative anti-id is shown for each group. The inhibition was determined to be anti-idiotypic as two conditions were satisfied.

a) The inhibition of $F(ab')_2$ binding to AChR was similar to that of whole mab (Fig. 4.2), demonstrating the target idiotopes were on the $F(ab')_2$ part of antibody molecule.

b) The inhibition was not affected by preincubation of anti-id with normal non-immune mouse sera (Fig. 4.3), demonstrating that the anti-idiotypic activity could not be absorbed by determinants present in control sera.

The Ab2gamma titres of each individual serum sample are shown in Figure 4.4. The greatest anti-id responses were to mabs C9 (4/5 sera, titre > 1000 nM), C2 (5/5 sera, titre 100 - 1000 nM), and B3 (4/5 sera, titre 100 - 500 nM). The other mabs elicited variable Ab2gamma titres in the range 10 - 1000 nM.

4.2.2 Ab2alpha.

Ab2alpha anti-ids were detected by their ability to indirectly precipitate $F(ab')_2$-AChR complexes. As discussed in chapter 3, it was important that anti-IgG(Fc) did not precipitate the $F(ab')_2$-AChR complexes directly (see Table

Figure 4.2: Inhibition of id (mab or $F(ab')_2$) binding to AChR by anti-id.

Limiting amounts of mab (▲) or $F(ab')_2$ (●) were preincubated with anti-id (anti-C2, anti-C9, anti-F8, anti-B3, anti-C3 or anti-C7) before addition of ^{125}I-BuTx-AChR.

Ordinate = % inhibition of id binding to AChR.

Abscissa = volume of anti-id (ul).

For derivation of values see Appendix One.

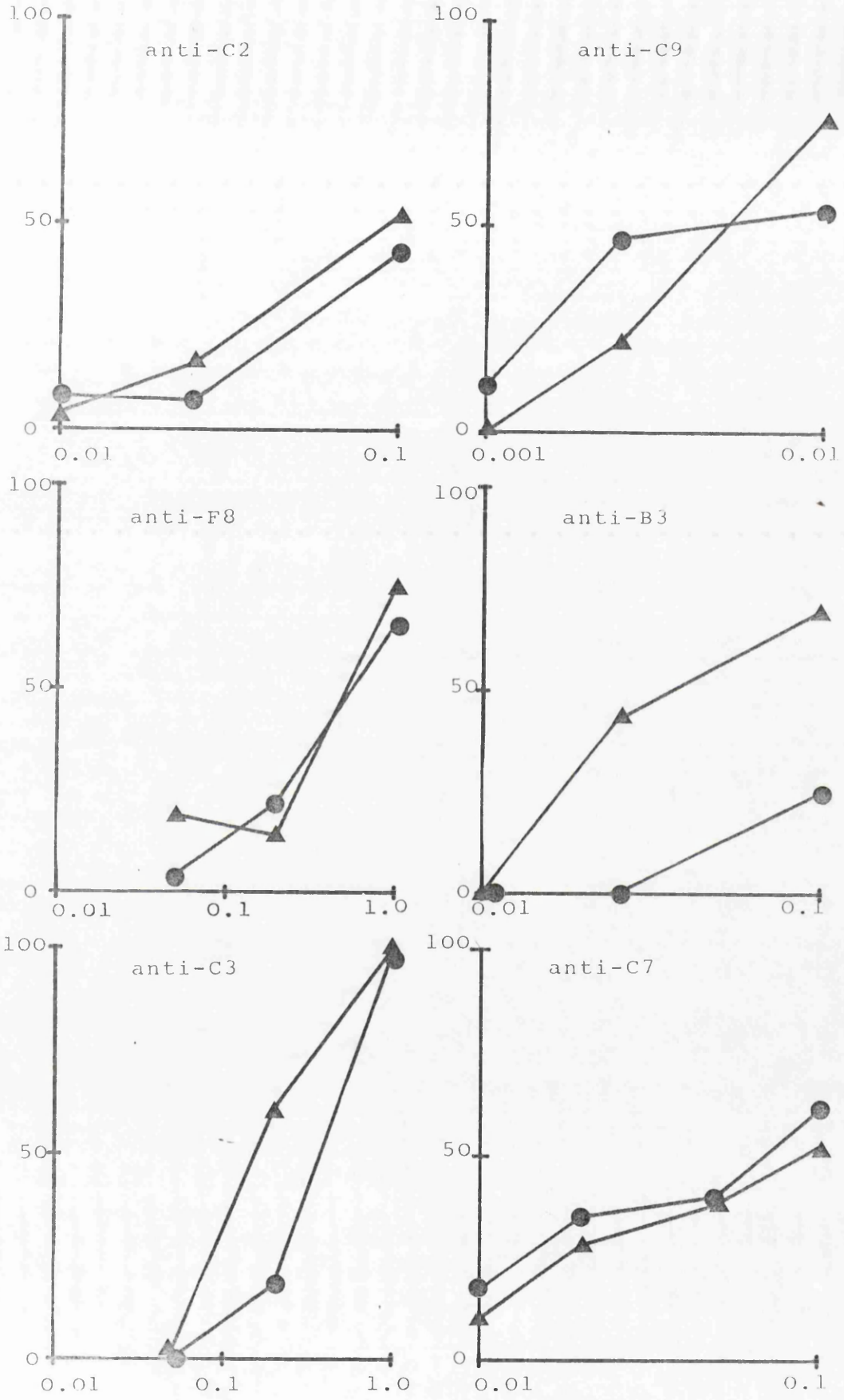

anti-C2
100
50
0
0.01
0.1
anti-C9
100
50
0
0.001
0.01
anti-F8
100
50
0
0.01
0.1
1.0
anti-B3
100
50
0
0.01
0.1
anti-C3
100
50
0
0.01
0.1
1.0
anti-C7
100
50
0
0.01
0.1

Figure 4.3: Inhibition of id binding to AChR by anti-id preincubated with normal mouse serum.

Anti-id (anti-C2, anti-C9, anti-F8, anti-B3, anti-C3 or anti-C7) were preincubated with normal mouse sera (■) or PTX buffer (▲) before adding to limiting amounts of id.

Ordinate = % inhibition of id binding to AChR.

Abscissa = volume of anti-id (ul).

For derivation of values see Appendix Two.

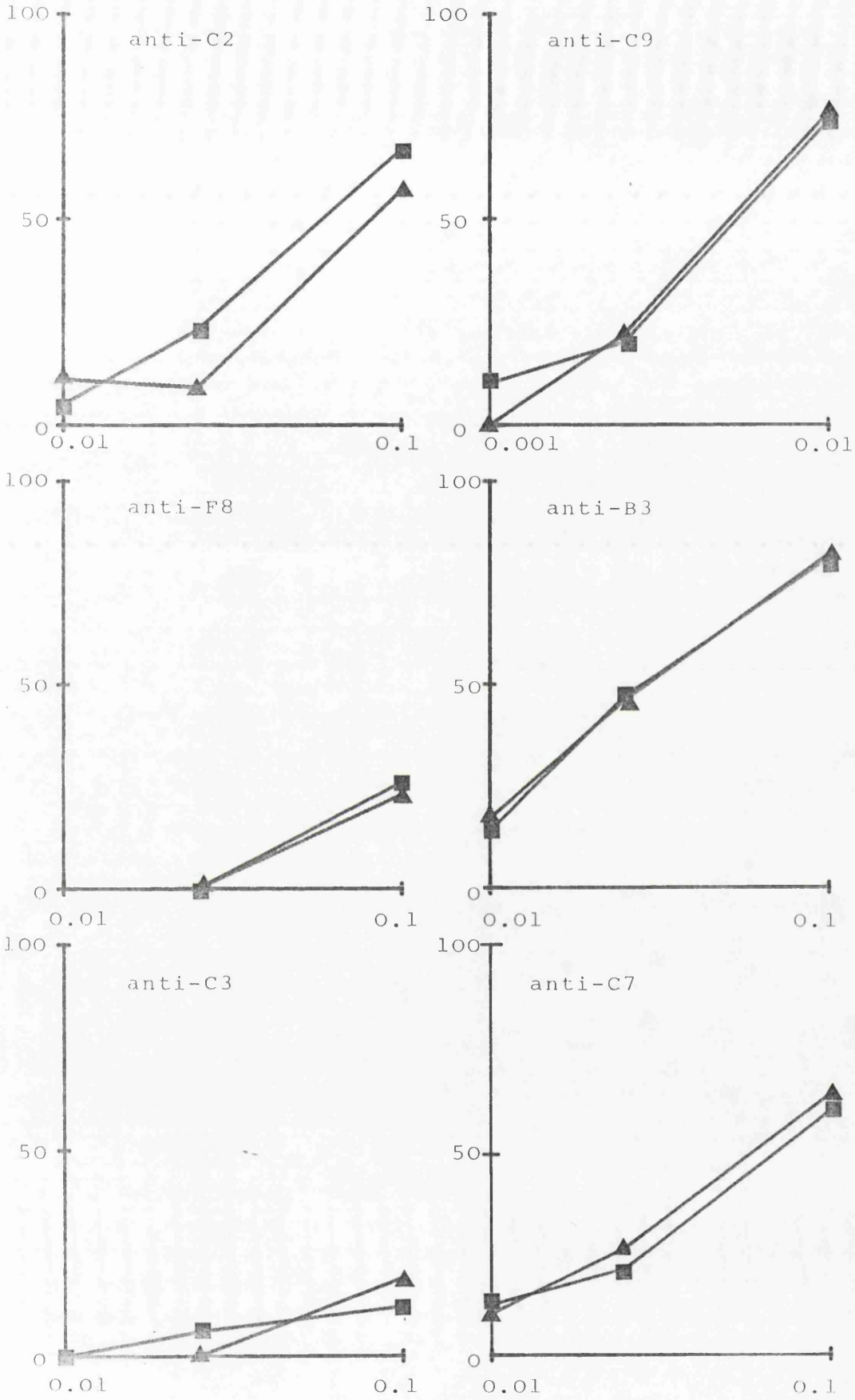
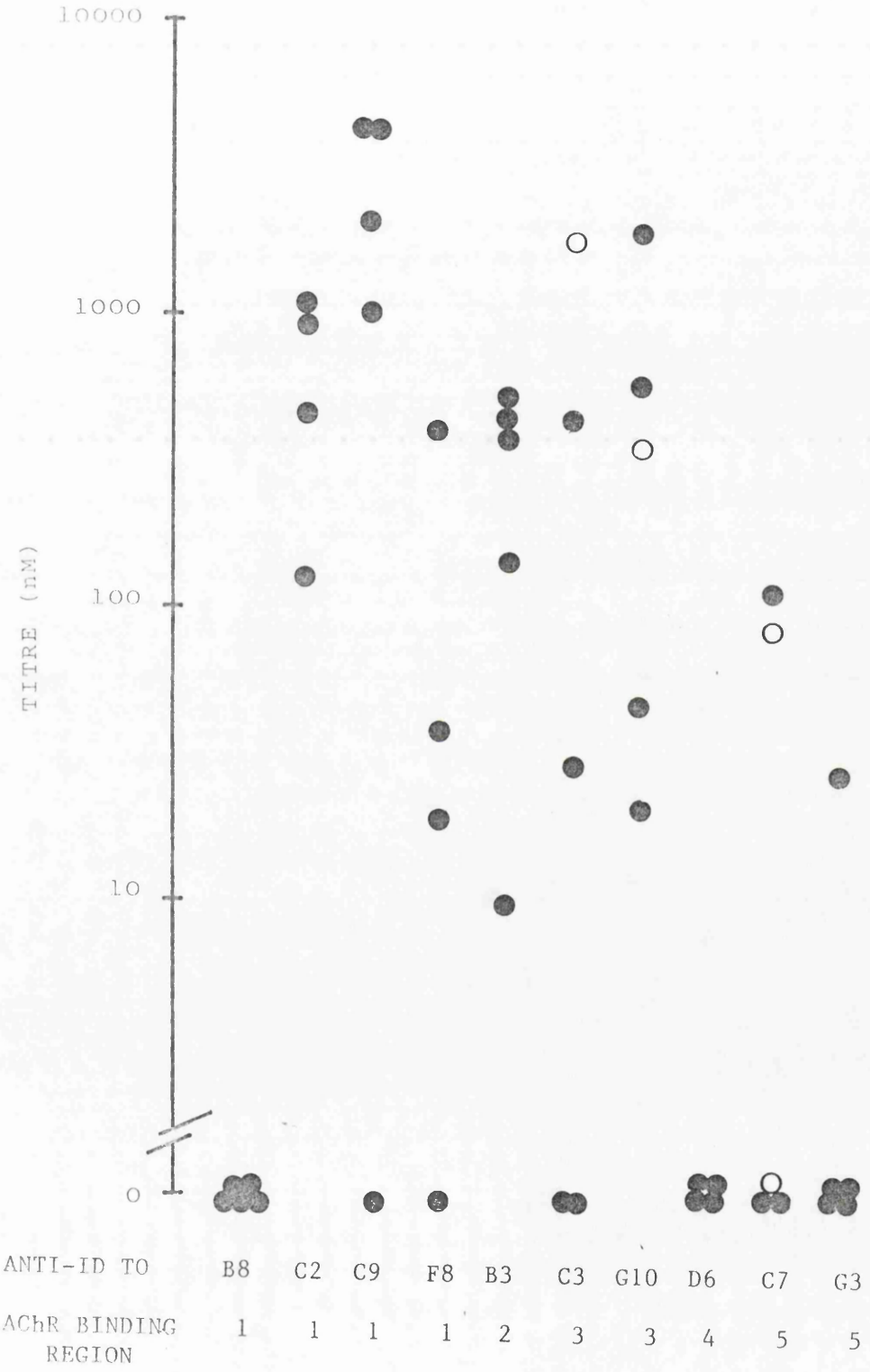

100
anti-C2
50
0
0.01
0.1
100
anti-C9
50
0
0.001
0.01
100
anti-F8
50
0
0.01
0.1
100
anti-B3
50
0
0.01
0.1
100
anti-C3
50
0
0.01
0.1
100
anti-C7
50
0
0.01
0.1

Figure 4.4: Ab2gamma titres of individual mice.

Ab2gamma titres (moles of id inhibited per litre anti-id) were calculated for each serum sample. The open symbols denote samples in which Ab2alpha activity was also detected.


10000
1000
100
10
0
TITRE (nM)
ANTI-ID TO
B8
C2
C9
F8
B3
C3
G10
D6
C7
G3
AChR BINDING REGION
1
1
1
1
2
3
3
4
5
5

3.1), so that the precipitation could be attributed to the Ab2alpha anti-ids. Ab2alpha anti-ids were found in only four serum samples, 3 of which also had Ab2gamma anti-ids (Table 4.1). Titres of Ab2alpha were low, in each case < 5% of the corresponding Ab2gamma titre of the same anti-id.

4.2.3 ELISA.

Because only four of the $F(ab')_2$ preparations bound to the ELISA plates, sera raised against mabs B3, B8, C2, C3, F8 and G3 could not be assayed by direct means. However all sera positive for Ab2gamma or Ab2alpha anti-ids against mabs C7, C9, and G10 also bound directly to the respective $F(ab')_2$ in the ELISA (Fig. 4.5). One serum sample (anti-$C7_B$) bound in the ELISA but was not detected in either of the RIAs.

Table 4.2 summarises the results of the group of five mice immunised with mab C7. All five mice responded differently. Mouse A did not have anti-ids detectable by any of the three assays, whereas mouse B only had anti-ids revealed by the ELISA. Of the three remaining mice, all had anti-ids detected by ELISA; C being Ab2alpha only, D Ab2gamma only, and E which was both.

Table 4.1: Comparative titres of the four Ab2alpha-positive anti-ids.

	Ab2alpha titre[a]	Ab2gamma titre[b]
anti-C3	100	1905
anti-G10	2	144
anti-$C7_C$[c]	1	0
anti-$C7_E$	4	90

[a] moles ^{125}I-BuTx-AChR-F(ab')$_2$ precipitated per litre anti-id.

[b] moles mab inhibited per litre anti-id.

[c] these two anti-ids to C7 (C and E) are also shown in Table 4.2.

Figure 4.5: ELISA absorbance readings of individual mice. The OD readings at 450 nm were measured of anti-ids (1/100 dilution) binding to $F(ab)_2$ of mabs C9, G10, D6 and C7. Open symbols denote samples with Ab2alpha activity. nd = not done.

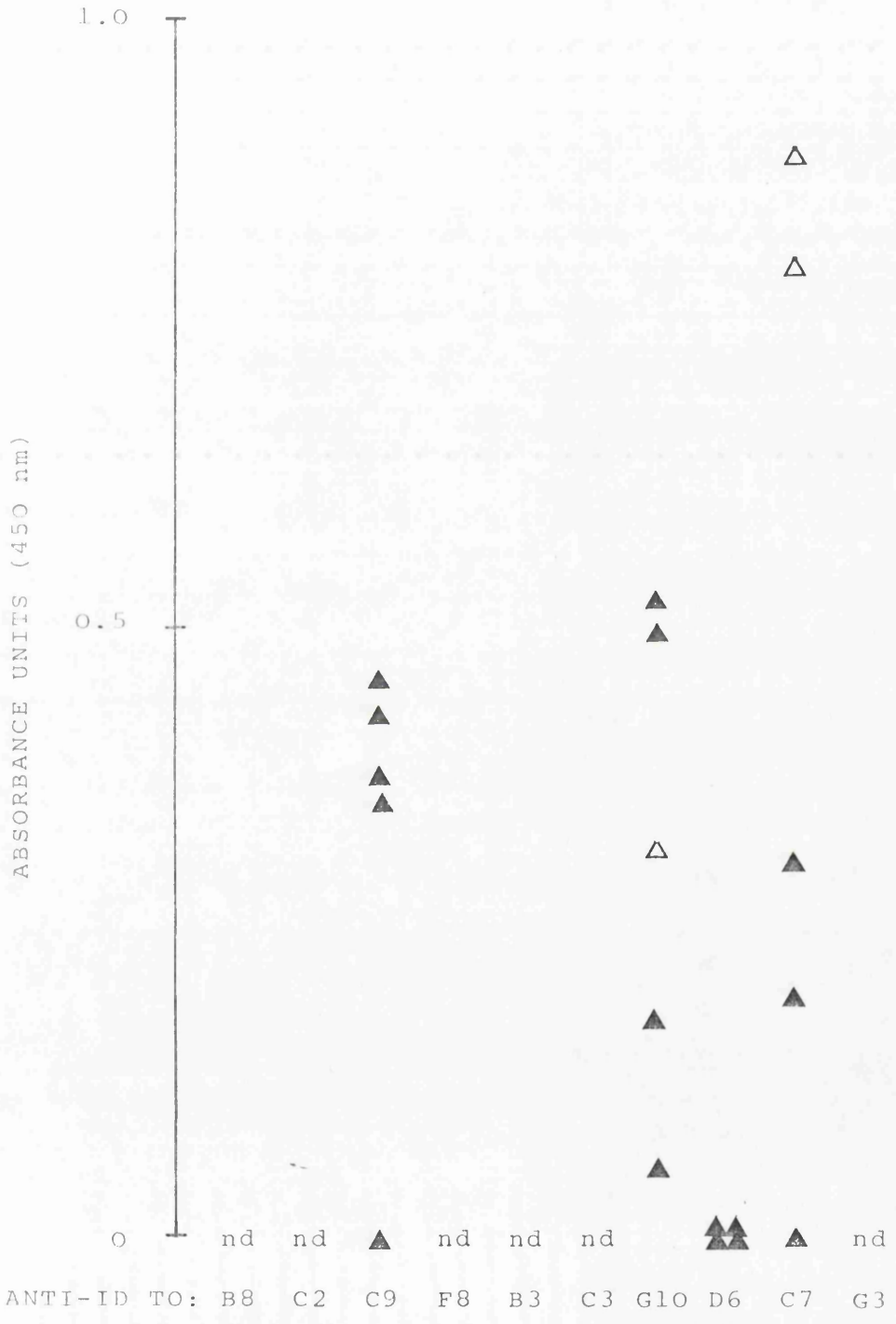

1.0
0.5
0
ABSORBANCE UNITS (450 nm)
nd nd nd nd nd nd nd
ANTI-ID TO: B8 C2 C9 F8 B3 C3 G10 D6 C7 G3

Table 4.2: Results of different anti-ids detected in five mice immunised with mab C7.

	Assay		
mouse	ELISA	RIA-Ab2alpha	RIA-Ab2gamma
A	-	-	-
B	+	-	-
C	+	+	-
D	+	-	+
E	+	+	+

anti-idiotypic antibodies detected (+) or not detected (-) in assays as reported in sections 4.2.1, 4.2.2 and 4.2.3.

Cross-reactive anti-idiotypes.

Each anti-idiotypic serum sample was used to test for shared ids amongst the panel of anti-human AChR mabs by substituting the homologous mab in each assay with heterologous mab.

4.2.4 Ab2gamma.

Cross-reactive inhibition of mabs by anti-ids is shown in Figure 4.6. A representative anti-id is shown for each group. Broad cross-reaction between the anti-ids and the panel of mabs was not found. Anti-ids to C2, C9 & F8 (region 1 mabs) each cross-reacted with one other region 1 mab but not with mabs that bind to other regions (Fig. 4.6a i, ii, iii). Likewise anti-ids to G10 and C3 (region 3) only cross-reacted with one another (Fig 4.6b i, ii). Anti-ids against B3 (region 2), and G3 & C7 (region 5) showed no cross-reaction at all (Fig. 4.6a iv; 4.6b iii, iv).

The degree of cross-reaction found in the inhibition assay is expressed as a percentage of the homologous titre (Table 4.3). In general the cross-reaction was 5-30%, although interestingly the inhibition of G10 by anti-C3 anti-ids was always 2-4 times greater than the homologous inhibition of C3.

Figure 4.6a: Cross-reactive inhibition of id binding to AChR by anti-ids to regions 1 & 2 mabs.

Limiting amounts of id (◆ = B8, ■ = C2, ● = C9, ▲ = F8, ▼ = B3, o = C7) were preincubated with anti-id (anti-C2, anti-C9, anti-F8, or anti-B3) before the addition of ^{125}I-BuTx-AChR. Solid line = homologous inhibition, broken line = cross-reactive inhibition.

Ordinate = % inhibition

Abscissa = volume of anti-id (ul).

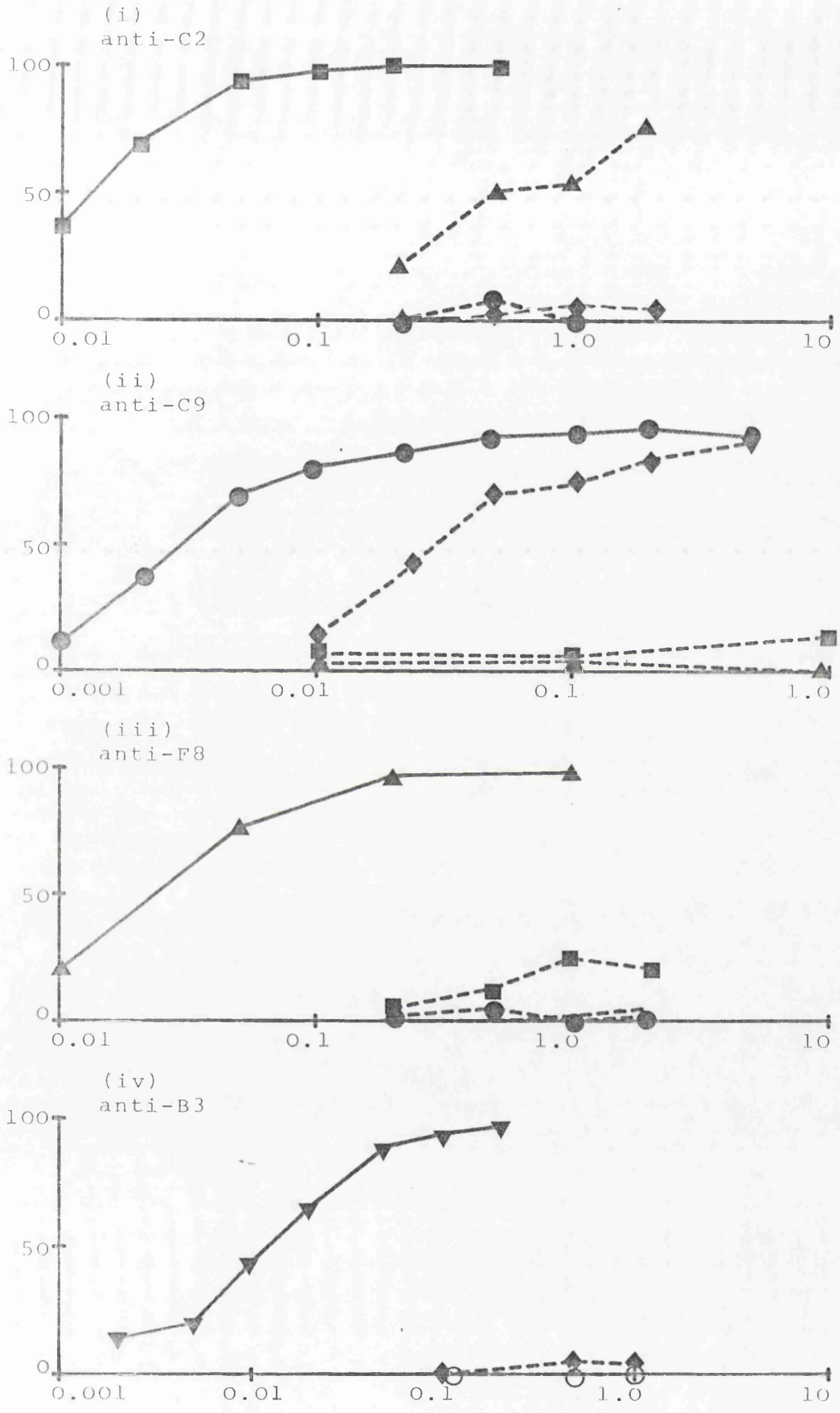
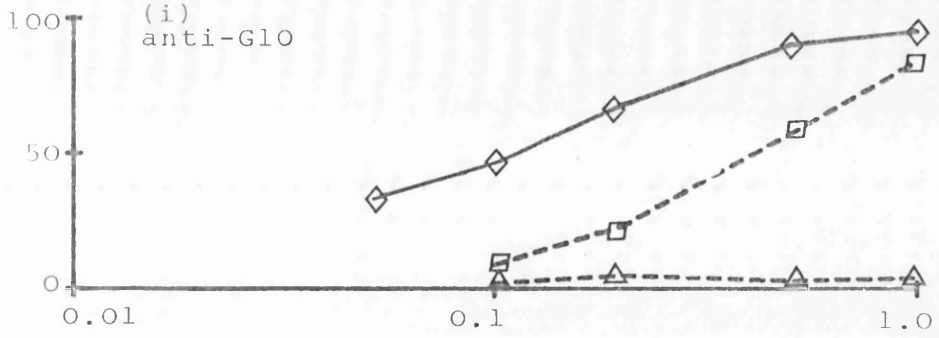

(i)
anti-C2
100
50
0
0.01
0.1
1.0
10
(ii)
anti-C9
100
50
0
0.001
0.01
0.1
1.0
(iii)
anti-F8
100
50
0
0.01
0.1
1.0
10
(iv)
anti-B3
100
50
0
0.001
0.01
0.1
1.0
10

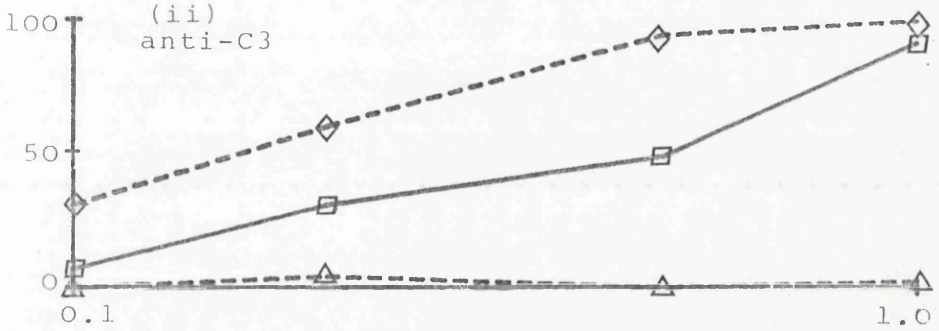
Figure 4.6b: Cross-reactive inhibition of id binding to AChR by anti-ids to regions 3 & 5 mabs.

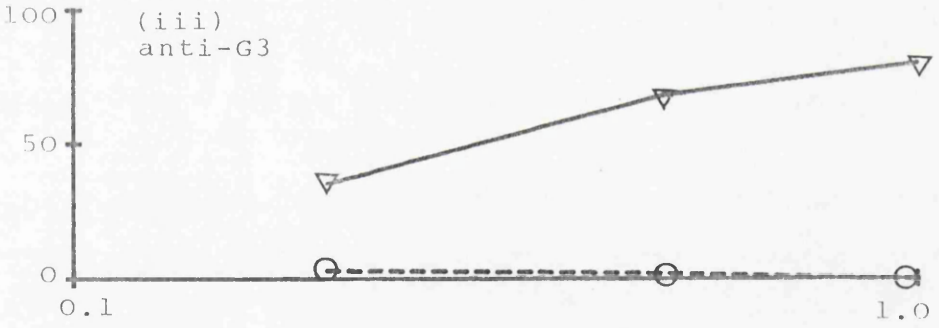
Limiting amounts of id (◇ = G10 □ = C3, ▽ = G3, o = C7, △ = D6) were preincubated with anti-id (anti-G10, anti-C3, anti-G3, or anti-C7) before the addition of ^{125}I-BuTx-AChR. Solid line = homologous inhibition, broken line = cross-reactive inhibition.

Ordinate = % inhibition

Abscissa = volume of anti-id (ul).


(i)
anti-G1O
100
50
0
0.01
0.1
1.0


(ii)
anti-C3
100
50
0
0.1
1.0


(iii)
anti-G3
100
50
0
0.1
1.0

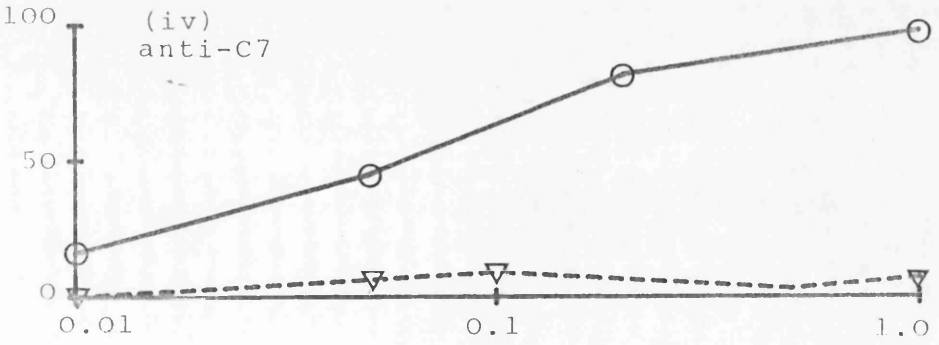

(iv)
anti-C7
100
50
0
0.01
0.1
1.0

Table 4.3: Cross-reaction of Ab2gamma anti-ids with anti-human AChR mabs.

	mab									
	B8	C2	C9	F8	B3	C3	G10	D6	C7	G3
anti-C2 (4)[a]	0	100	0	8±12[b]	0	0	0	0	0	0
anti-C9 (4)	18±12	0	100	0	0	0	0	0	0	0
anti-F8 (3)	0	8±14	0	100	0	0	0	0	0	0
anti-B3 (5)	0	0	0	0	100	0	0	0	0	0
anti-C3 (3)	0	0	0	0	0	100	313±144	0	0	0
anti-G10 (5)	0	0	0	0	0	25±11	100	0	0	0
anti-C7 (2)	0	0	0	0	0	0	0	0	100	0
anti-G3 (1)	0	0	0	0	0	0	0	0	0	100

[a](n) = number of anti-ids.

[b]Expressed as percentage of the titre of anti-id against heterologous mab compared to homologous mab (mean ± standard error).

4.2.5 Ab2alpha.

A small degree of cross-reaction was found with these anti-ids although once again restricted to mabs within an AChR binding region group. Anti-C3 anti-id had an equal ability to precipitate C3 and G10-$F(ab')_2$ AChR complexes, as did the anti-G10 anti-id. However the Ab2alpha anti-C7 anti-ids did not bind to ^{125}I-BuTx-AChR-G3($F(ab')_2$) or any other cross-reactive $F(ab')_2$-complex (data not shown).

4.2.6 ELISA.

Unfortunately, owing to the limited binding of the $F(ab')_2$ to ELISA plates it was difficult to demonstrate cross-reaction by ELISA. However all 47 serum samples were tested against the $F(ab')_2$ that did bind to the ELISA plates. No cross-reactive binding to C9, D6 or C7 was found, but further evidence of the cross-reaction of anti-C3 with G10 was provided by anti-C3 anti-ids binding to G10($F(ab')_2$) (data not shown).

Table 4.4 summarises the results obtained using the three assays developed. 47 mice were immunised with id; 4 produced anti-ids detected using the Ab2alpha RIA, 27 produced anti-ids detected using the Ab2gamma RIA, and 13 of the 19 assayed were positive in the $F(ab')_2$ ELISA.

Table 4.4: Summary of polyclonal anti-idiotypic responses measured by different assays.

anti-id		Ab2alpha$^+$	Ab2gamma$^+$	ELISA$^+$	Cross-reaction
anti-B8	(5)[a]	0	0	nd[b]	-
anti-C2	(4)	0	4	nd	F8
anti-C9	(5)	0	4	4	B8
anti-F8	(4)	0	3	nd	C2
anti-B3	(5)	0	5	nd	none
anti-C3	(5)	1	3	nd	G10
anti-G10	(5)	1	5	5	C3
anti-D6	(4)	0	0	0	-
anti-C7	(5)	2	2	4	none
anti-G3	(5)	0	1	nd	none

[a] (n) = number of mice immunised.

[b] nd = not done.

4.2.7 Inhibition of myasthenic autoantibodies.

Heidenreich et al. (1988) found that MG sera could be partially inhibited from binding to human AChR by preincubating AChR with one of the mabs to protect a particular binding region. Thirteen serum samples from that study were used to ascertain whether the autoantibodies and the mabs that bound to the same AChR region had CRI. Each sample was titrated against ^{125}I-BuTx-AChR by overnight incubation at 4^{o}C to determine the limiting amount of sera to use. This amount was then incubated for 6 hours at 4^{o}C with 5 ul of each anti-id, or a pool of the anti-ids. ^{125}I-BuTx-AChR was then added and incubated overnight at 4^{o}C. MG anti-AChR antibodies bound to ^{125}I-BuTx-AChR were precipitated and the inhibition of MG sera binding to AChR measured relative to the non-specific inhibition by normal mouse serum (nms) (Table 4.5). No significant inhibition (i.e. more than 2 standard deviations) compared to the overall mean of each MG serum sample was found for any anti-id.

Table 4.5: Inhibition of MG sera binding to AChR by anti-ids.

patient	titre[a] (nM)	inhibiting anti-id									
		none	nms	anti-B3	anti-C2	anti-C3	anti-C7	anti-C9	anti-F8	anti-G10	pool[b]
1	10	2208[c]	2100	2199	2143	2066	1961	2015	2380	2013	2057
2	3	2929	2999	2816	2793	2707	2858	2814	2906	2735	2779
3	15	2905	3013	2859	2672	2701	2730	2819	2929	2848	2564
4	1500	3652	3708	3650	3535	3648	3592	3315	3551	3544	3659
5	4	3699	3935	3636	3326	3408	3478	nd[d]	3417	3612	3630
6	90	1818	1812	1897	1860	1755	2265	1931	1715	1868	1807
7	6	2364	2251	2194	2261	2244	2381	2252	2258	2209	2161
8	3	2542	2522	2534	2419	2619	2273	2202	2198	2587	2288
9	8	1177	1151	1141	1281	1138	1103	1417	1235	1091	1224
10	45	1827	1800	1820	2035	1911	2107	1854	1863	1845	1793
11	14	1459	1521	1661	1431	1482	1530	1485	1479	1382	1537
12	10	1031	1109	1016	1079	1055	970	997	1043	980	1180
13	230	4567	4576	4670	5182	5008	4916	4941	4878	4979	4934

[a] titre expressed as moles of AChR precipitated per litre serum.
[b] pool = pool of anti-ids.
[c] results expressed as cpm of ^{125}I-BuTx-AChR precipitated by limiting amounts of MG sera preincubated with anti-id.
[d] nd = not done.

4.3 Discussion.

The advantage of using anti-idiotypes produced in syngeneic mice is that, in theory, only the idiotype is recognised as foreign, and as a result time consuming absorption steps to remove antibodies to isotypic determinants should be unnecessary. On the debit side one has the problem of assaying, as species specific antisera cannot be used.

Several alternatives have been employed. Firstly, a "sandwich" ELISA has been used in which idiotype is coated onto ELISA plates, followed by the anti-id, and then a second layer of idiotype, suitably labelled (Thanavala et al. 1985; Agius & Richman 1988). A second approach is to use subclass specific antisera that will detect anti-ids of a different subclass to the original idiotype. However this forfeits the ability to detect anti-ids of the same isotype as the idiotype (Streicher et al. 1986).

In this study radioimmunoassays have been used to measure firstly, the inhibition of binding of id to AChR, and secondly the binding of anti-id to id-AChR complexes. These assays detect Ab2gamma and Ab2alpha anti-ids respectively. In addition an ELISA was used to measure the direct binding of anti-id to $F(ab')_2$ fragments of the id (Uytdehaag and Osterhaus 1985). However due to the poor binding of some $F(ab')_2$ fragments to the ELISA plates only anti-ids to 4 mabs could be assayed directly.

Of the ten mabs used to immunise Balb/c mice all but

two elicited an anti-idiotype response, the two non-responders being B8 and D6. This lack of response could not be attributed to IgG subclass as other mabs of IgG1 (eg C9) and IgG2b (eg G3) elicited anti-idiotypes. There have been other reports of a lack of an anti-idiotype response to some monoclonal antibodies. Killen and colleagues (1985) found a better anti-id response when the idiotypes were conjugated to lipopolysaccharide, yet still some failed to elicit a response. Hemmi and co-workers (1985a) noted that some mabs to major histocompatibility antigens failed to elicit anti-idiotypic antibodies, and rabbits failed to respond to mabs specific for thyroglobulin (Male et al. 1983).

The original mabs had been produced using the NS1 myeloma cell line as the fusion partner. This cell line synthesises but does not secrete its own light chains, which following fusion may become assembled into the resulting monoclonal antibody. It is possible that all the cross-reactivity seen was a consequence of such products, i.e. all cross-reactivities were against NS1 light chain determinants. However, as the V_H genes are thought to contribute most to the CDRs of immunoglobulins, and the mobilities of the different anti-human AChR mabs under SDS-PAGE reducing conditions were different, this would suggest that this was not a substantial problem.

The majority of anti-ids were Ab2gamma (Table 4.4). Such a ratio in favour of Ab2gamma was perhaps surprising, although the assays may have been biased towards the detection of Ab2gamma over Ab2alpha. The Ab2alpha assay

involves the binding of anti-id to $F(ab')_2$-AChR complexes. The binding of AChR to mab may cause allosteric changes in the antibody reducing its capacity to bind anti-id. Alternatively, the binding of anti-id to id may have been impaired when id was used in the form of $F(ab')_2$ rather than intact antibody. One cannot exclude the possibility that the inhibition seen was due to remote allosteric effects of anti-id binding to framework idiotopes preventing the paratope from binding to AChR. However, even if this was the case it should not diminish the significance of the anti-id, but rather enhance it, as inhibition of antigen binding through an anti-id binding to a framework idiotope would be a extremely potent regulator of the immune system.

The observation that the inhibition was not affected by preincubation of anti-id with normal mouse serum meant that it was unlikely that the antisera were directed against non-idiotypic determinants (Figure 4.3). Further proof was provided by the same inhibition obtained when $F(ab')_2$ fragments were used instead of intact mab (Figure 4.2).

The ELISA results (Figure 4.5), where applicable, confirmed those obtained using the two RIAs, apart from one anomaly, an anti-C7 anti-id which bound in the ELISA but was not detected by either of the two RIAs. However, as already mentioned, the binding of $F(ab')_2$ to AChR in the Ab2alpha assay may induce conformational changes making the idiotope unrecognisable to the anti-id. It is possible that the anomalous anti-C7 anti-id might have been directed to an idiotope changed by the binding to AChR.

In total, only four mice had detectable Ab2alpha anti-ids, of which three occurred with Ab2gamma (Table 4.1). The Ab2alpha titres were very low, being less than 5% of the corresponding Ab2gamma titre. In contrast, the Ab2gamma titres ranged from 5 - 5000 nM, and in general reached a maximum after three injections of id. Others have found it necessary to immunise four or five times with mab anti-AChR to achieve comparable levels of anti-id (Killen et al. 1985; Souroujon et al. 1985; Agius et al. 1988).

Cross-reactive idiotypes have been widely reported not only in MG but also in other autoimmune diseases, and in other systems. Using the eight sets of polyclonal anti-idiotypes generated in this study, three pairs of mabs were shown to have a common idiotope. CRI were determined by anti-ids inhibiting the binding of heterologous mab to AChR. Such inhibition was clearly demonstrated using anti-ids to mabs of binding regions 1(C2, C9 & F8) and 3 (C3 & G10), but anti-ids to B3, C7 & G3 recognised purely private idiotypes.

Cross-reaction was restricted to mabs binding to the same region of AChR. In some instances cross-reactive inhibition was not observed even though 100 - 1000 times more anti-id than required to fully inhibit the binding of homologous id was used. This was the amount of excess heterologous anti-id that Morgan et al. (1985) needed to demonstrate cross-reaction between anti-DNA specific idiotypes. The titre of anti-id against heterologous mab was calculated and degree of cross-reaction expressed as a percentage of the homologous titre. The percentage cross-

reaction varied but was mainly in the range 5 - 30%, the one exception being that of anti-C3 with G10.

Each of the three anti-C3 anti-ids had the surprising property of inhibiting the binding of G10 better than C3. These two mabs are different as demonstrated by their differing binding properties to AChR prepared from other species, especially rat AChR, to which C3 does not bind at all (Whiting et al. 1986b). One might speculate that both C3 and G10 share a common idiotope, which on G10 is so positioned that inhibition of AChR binding is more readily achieved. Alternatively, anti-id binding to the G10 idiotope affects a greater change in the AChR binding than it does for C3. A more prosaic argument is that C3 simply has a greater affinity for AChR and therefore is more difficult to inhibit. Further evidence for the close idiotypic identity of C3 and G10 was provided by the cross-reaction of the anti-C3 and the anti-G10 Ab2alpha anti-ids.

Each Ab2gamma anti-id was tested for indirect precipitation of $F(ab')_2$-AChR complexes of each mab, but none was found. The four Ab2alpha anti-ids, apart from the anti-C3 and anti-G10 binding to $F(ab')_2$-AChR complexes of region 3 mabs, did not bind to heterologous $F(ab')_2$-AChR complexes.

Once again the ELISA could only be used in a limited way. However the cross-reaction of the anti-C3 anti-ids with G10 and the lack of cross-reaction with C9, D6 and C7 was confirmed.

The results of the restricted idiotypic sharing of the anti-human AChR mabs fit very well with those using $F(ab')_2$ to protect the AChR binding sites (see Table 3.2). In the latter experiments a difference between C7 and G3 was found as demonstrated by the ability of the $F(ab')_2$ fragments of G3 but not C7 to inhibit mabs C3 and G10. This indicates that C7 and G3 probably recognised different epitopes and the lack of a shared idiotope between the two mabs tends to confirm this.

The Idiotype Network Theory has stimulated many studies concerned with the presence or absence of IdXs or CRIs in autoimmune disease. In myasthenia gravis there is no consensus on this issue. In experimentally derived anti-AChR mabs, others have observed that only mabs having the same specificity for AChR share a CRI. For example, idiotypes were not widely shared between a panel of 9 anti-MIR mabs studied by Killen et al. (1985), although the anti-M35 anti-id did cross-react with another mab, coincidentally obtained from the same fusion as M35. Lennon and Lambert (1981) reported a dominant idiotype between four of their panel of five rat anti-Torpedo AChR mabs. Although not stated, it seems reasonable to suppose that these four mabs were directed against the same region of AChR, as in a previous study these investigators had shown that the mabs had similar binding specificity for AChR from various species (Lennon and Lambert 1980). Elsewhere, using syngeneic anti-id antibodies to three different murine anti-AChR mabs

Souroujon et al. (1985) did not detect any sharing of idiotypes. Taken together the evidence suggests that firstly, any given AChR epitope/region can give rise to more than one idiotype; and secondly, CRI are more likely to be found between mabs having the same or similar binding specificity for AChR. It might be argued that this second conclusion is spurious arising as a result of testing anti-ids by their ability to inhibit the binding of mab to AChR. However, binding of anti-ids to pre-formed $F(ab')_2$-AChR complexes did not reveal a significant CRI, suggesting that this conclusion is valid.

Studies of anti-ids raised against mabs in other antigen systems have also found this restrictive sharing of idiotypes. For example restricted CRIs were found using rabbit anti-ids to anti-rye I allergens (Mourad et al. 1988), and murine anti-ids to anti-MHC mabs (Hemmi et al 1985a; Streicher et al. 1986). However CRIs have been demonstrated on mabs with different specificities for myoglobin (Kohno et al. 1982), thyroglobulin (Zanetti et al. 1983) and haemoglobin (Gorzynski et al. 1985).

The lack of inhibition of MG sera by the polyclonal anti-idiotypes was disappointing but not surprising. MG autoantibodies have been shown to bind to the same regions of AChR as defined by the panel of anti-human AChR mabs used in this study (Whiting et al. 1986a; Heidenreich et al. 1988). However, to show that the human and murine antibodies had shared idiotypes several problems would have to be

faced. Firstly, the anti-ids would have to cross a potential species barrier by reacting with human antibodies, although this has been shown to be possible both for anti-AChR idiotypes (Dwyer et al. 1983) and idiotypes in other diseases (Morgan et al. 1985). Secondly, antibodies in MG are heterogeneous; thus for inhibition to be observed would probably need to be limited, or a single idiotype would need to be dominant in the MG anti-AChR response. As already shown for myasthenia gravis, a majority of autoantibodies are directed against the MIR (Tzartos et al. 1982) but anti-MIR mabs do not bear a dominant idiotype (Killen et al. 1985). In an attempt to detect inhibition of the heterogeneous antibody population in MG sera a pool of anti-ids was used. This approach proved no better than using rabbit antisera to human idiotypes (Lang et al. 1985). However using large amounts of rabbit anti-idiotypic sera Whiting (1984) was able to demonstrate some degree of idiotypic sharing between human and murine monoclonal anti-AChR antibodies. Lefvert (1981) has also reported low levels of CRI in human MG sera using polyclonal rabbit anti-idiotypic antibodies, and has recently pointed out that CRI are probably more readily detected by direct binding of idiotype with anti-idiotype in an ELISA rather than by inhibition of binding of anti-AChR antibodies (Lefvert 1988). Reasons for this could include id having a greater affinity for AChR than anti-id has for id; and the ids detected by anti-ids not being site-related.

Lefvert (1988) has reported findings of CRI in MG sera

using a panel of monoclonal antibodies to human idiotypes, and CRI were present in 14-60% of cases. Autologous anti-idiotypic antibodies have also been reported, both in patients and their first degree relatives (Lefvert et al. 1985). Dwyer et al. (1983) found that 40% (recently revised to 20%, Dwyer 1987) of MG sera tested contained naturally occurring anti-idiotypic antibodies to an anti-AChR mab (ACR 24), and that two patients had antibodies bearing the ACR 24 id when their levels of anti-id were low. A tendency for levels of anti-id to decline with the onset of disease has been noted in MG (Lefvert 1988) and also in SLE (Abdou et al. 1981) supporting the notion that auto anti-idiotypes are involved in regulation of autoantibodies. However, in an extensive study using our ten anti-human AChR mabs as idiotypes, Vincent (1988) was unable to reveal any spontaneous anti-ids in MG sera either by direct ELISA binding or by using mab-Sepharose 4B columns to bind and elute anti-ids.

4.4 Summary.

Polyclonal anti-idiotypic antibodies were raised in syngeneic mice to eight anti-human AChR mabs. Three assays were used to detect anti-idiotypes in the sera. The majority of anti-ids were Ab2gamma, i.e. they inhibited the binding of id to AChR. The anti-idiotypes detected three CRI and three IdI, but sharing was only found between mabs binding to the same region of AChR. None of the anti-ids was able to inhibit the binding of MG sera to AChR.

CHAPTER FIVE

MONOCLONAL ANTI-IDIOTYPIC ANTIBODIES

5.1 Introduction.

In chapter 4 polyclonal anti-idiotypes were used to investigate the idiotypes expressed by the panel of anti-human AChR mabs. The purpose of the work presented in this chapter was to raise and characterise monoclonal anti-idiotypic antibodies to these mabs. As demonstrated in the previous chapter, mice immunised with idiotype emulsified with Freund's Adjuvant ("Freund's immunised") produced good levels of anti-idiotypic antibodies in the sera. It had been shown elsewhere (Thanavala et al. 1985) that it was possible to produce monoclonal anti-idiotypes from the fusions of splenocytes of mice immunised with id in Freund's adjuvant. Therefore fusions using the spleens of ten mice immunised with id in Freund's adjuvant were undertaken in an attempt to produce monoclonal anti-idiotypic antibodies.

Spitz et al. (1984) had described a "single shot" immunisation method for raising monoclonal anti-idiotypic antibodies against human IgM id. This technique is quick, as fusion is performed 3 days post immunisation, and has the added advantage of producing hybrids secreting IgM. This means that hybrids can be assayed for anti-id by ELISA as id and anti-id will be of differing immunoglobulin isotype.

5.2 Results.

5.2.1 Mice immunised with anti-AChR mabs with Freund's Adjuvant.

Immunisation Regime.

Table 5.1 sets out the results of the ten fusions performed on spleen cells from mice immunised with idiotype with Freund's Adjuvant. The serum Ab2gamma titre of each mouse was monitored throughout, and the levels of anti-idiotypes expressed in Table 5.1 refers to the titre three days before fusion. In fusion 1 following a single initial injection of id with FCA, the serum had an Ab2gamma titre of 20 nM 25 weeks after immunisation. Of the remaining nine mice titres ranged from 20 nM (fusion 9) to 4900 nM (fusions 7a and 7b). Fusion 2 was from a mouse which had received only one boost of id with FIA 4 weeks after immunisation. Fusions 3-7 were of spleens from mice that had been immunised and boosted using the standard protocol described in the methods. Fusions 8, 9 and 10 were of spleens from mice that had been immunised more often and over a longer period of time, as indicated in Table 5.1. An intraperitoneal injection of id in saline was always administered three days prior to fusion.

Fusion.

Each spleen yielded approximately 60 x 10^6 lymphocytes which were fused with the mouse myeloma line NS1 in a ratio of 5 or 10 : 1, using 40% (w/v) PEG solution. Cells were then plated into 96 well microtitre plates as indicated in

Table 5.1: Fusions of splenocytes of mice immunised with idiotype and Freund's adjuvant.

Fusion No.	Idiotype	Date of Boosts (weeks)[a]	Date of Fusion (weeks)[a]	Titre[b] (nM)	Seeding density[c] (10^5)	Hybrid rate[d]	Ig posit
1	C2	none	27	20	2.0	155/252 (62%)	47 (30%
2	C9	4	9	40	2.0	232/480 (48%)	86 (37%
3	C9	4, 9	11	35	1.5	81/360 (23%)	22 (27%
4	C9	4, 9	16	25	1.2	131/240 (55%)	121 (92%
5a	B3	4, 9	20	540	1.0	30/180 (17%)	6 (20%
5b	B3	4, 9	20	540	2.0	38/180 (21%)	23 (61%
6a	C2	4, 9	21	75	1.0	45/360 (13%)	3 (7%
6b	C2	4, 9	21	75	1.0	78/180 (22%)	29 (37%
7a	C9	4, 9	21	4900	1.0	31/360 (9%)	4 (13%
7b	C9	4, 9	21	4900	2.0	74/180 (20%)	7 (9%
8	G10	2, 8, 17	25	110	1.0	219/285 (77%)	12 (16%
9	G10	3, 5, 16, 44, 68	72	20	1.7	35/260 (14%)	13 (35%
10	C9	3, 5, 16, 36, 44, 68	72	450	1.5	68/190 (36%)	27 (40%

[a] weeks after priming.
[b] Ab2gamma titre expressed as inhibition of id binding to AChR.
[c] seeding density = number of cells per well.
[d] hybrid rate = number of wells with hybrid growth / total number of wells seeded.
[e] fusion 8 only screened for IgG.

Table 5.1

Cell concentration.

In fusions 5, 6 and 7, cells were seeded at $1x10^5$ or $2x10^5$ cells per well in order to determine the optimal seeding density. In fusion 5 there was no difference in the hybrid success rate (expressed as a percentage of the total wells plated with growing colonies) between the two seeding densities. In fusions 6 and 7, however, the fusion success rates of wells seeded with $2x10^5$ cells (22 and 20%) were twice that of wells seeded at $1x10^5$ cells (13 and 9% respectively) The overall average fusion rate was 32% (range 9 - 77%).

Screening.

a) Immunoglobulin secretion.

On average, 34% of all hybrids were secreting immunoglobulin (Ig^+). In fusions 1, 2, 3, 4, 9 and 10, culture supernatants were assayed for both IgG and IgM antibodies, and were found to be predominantly secretors of IgM. In fusions 5, 6 and 7 culture supernatants were assayed only for total immunoglobulin. Fusion 8 was only assayed for IgG, and a total of 12 out of the 219 hybrids were secretors of IgG.

b) Anti-idiotypic antibodies.

IgG^+ supernatants from fusions 1, 2, 3, 4, 8, 9 and 10 and Ig^+ supernatants from fusions 5, 6 and 7 were assayed

for Ab2gamma by inhibition of mab anti-AChR binding to AChR. IgM^+ supernatants from fusions 1 - 4, and 8 - 10 were tested for anti-id activity by direct binding to id in an ELISA. None of the 93 supernatants assayed for Ab2gamma inhibited the binding of id to AChR; and none of the 309 IgM^+ hybrids assayed were found to bind to id in the ELISA.

5.2.2 Intrasplenically-immunised mice.

Mice were immunised with a single intrasplenic injection of idiotype in saline. The splenocytes were fused in the same way as the Freund's immunised mice three or four days later.

Fusion success rate.

Cells were seeded into microtitre plates at around $2x10^5$ cells per well. The percentage of wells with growing hybrids was on average 25% (range 9 - 60%) (Table 5.2). This rate was similar to that obtained from the fusions from conventionally immunised mice.

Immunoglobulin secretion.

Again, as with the fusions from the Freund's immunised mice, about 40% of hybrids were secreting immunoglobulin. As expected, the immunoglobulin was almost exclusively of the IgM class, with only 3 IgG^+ out of a total number of 201 hybrids.

Table 5.2: Fusions of splenocytes from mice immunised intrasplenically.

Fusion Number	Idiotype	Seeding density[a] (10^5)	Hybrid rate[b]	Ig positive[c]	IgG[d]	IgM	anti-id clones[e]
11	C9	2.0	145/240 (60%)	47 (32%)	0	47	0
12	C7	2.0	104/360 (29%)	33 (32%)	1	32	1
13	C7	2.0	97/600 (16%)	32 (33%)	1	31	1
14	D6	1.3	69/300 (23%)	19 (28%)	0	18	1
15	D6	1.7	22/240 (9%)	10 (45%)	0	10	0
16	G10	2.0	82/618 (13%)	61 (74%)	1	60	4

[a] number of cells per well.

[b] number of wells with hybrid growth / total number seeded.

[c] number of hybrids secreting Ig (% of wells with hybrid growth).

[d] IgG , IgM = number of hybrids secreting IgG or IgM.

[e] number of stable hybrid lines cloned by limiting dilution.

Anti-idiotypic antibodies.

The 3 IgG^+ supernatants were tested for their inhibition of id binding to AChR, but none of them did so. The IgM^+ supernatants were assayed by ELISA for direct binding to idiotype and anti-id^+ were assayed for specific inhibition of id binding to AChR. Specific ELISA binding to id was found for 7 of the IgM^+ supernatants, representing anti-ids against three of the ids. Of these, 4 were to G10, 1 to D6, and 2 to C7. These anti-ids were cloned by limiting dilution and expanded in tissue culture.

5.2.3 Binding of monoclonal anti-idiotypic antibodies.

The monoclonal anti-idiotypic antibodies produced from fusions of spleen cells derived from intrasplenically-injected mice were tested for the specificity of their binding by ELISA.

a). ELISA against homologous id.

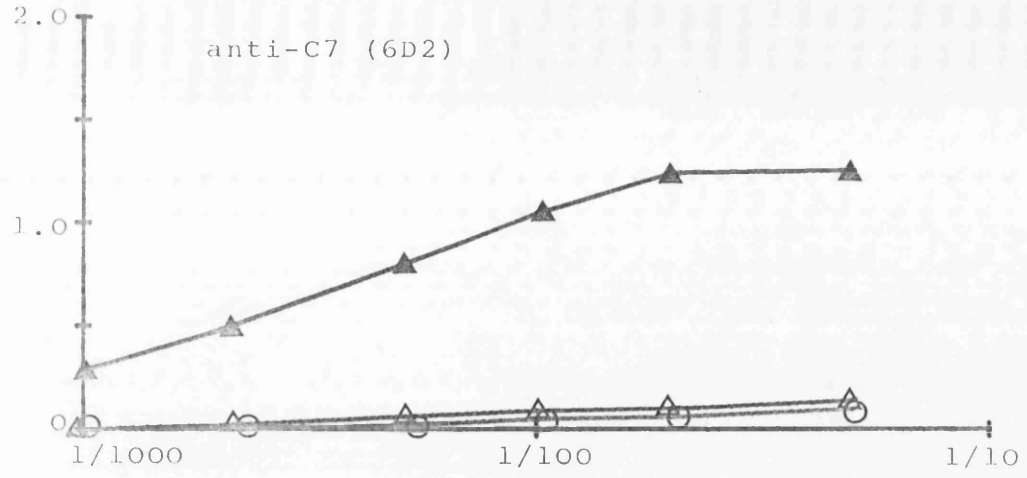
ELISA plates were coated with the immunising id, a control mab of the same IgG subclass, or, to measure the absolute background binding of anti-id to the polystyrene wells, TRIS buffer alone. Figure 5.1 shows the binding of anti-id supernatants (diluted 1/20 to 1/1000 in PBST) to homologous id, control id and to plastic. In this experiment OD reading of up to 1.5 were obtained, although in later assays these readings were generally lower. Figure 5.1 also demonstrates that a small amount of background binding was found, especially at the higher concentrations of anti-id

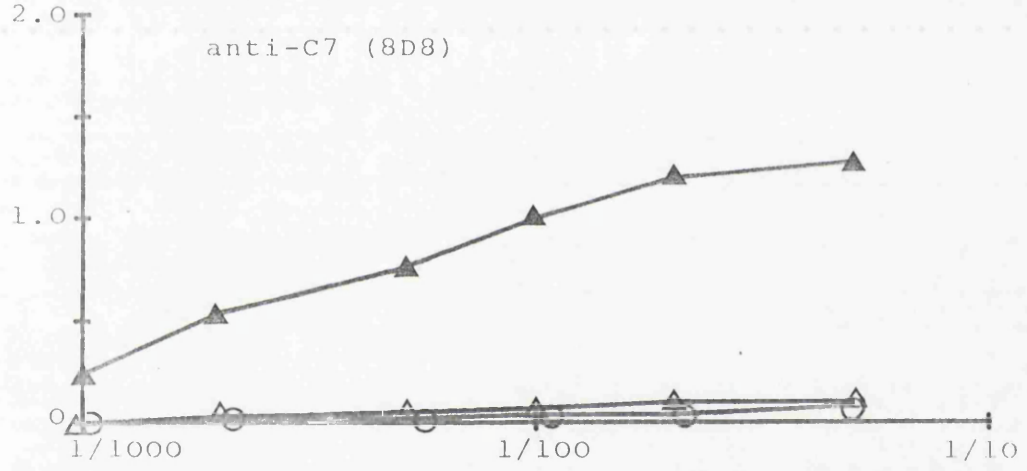
Figure 5.1a: ELISA binding of anti-id (6D2, 8D8 & 3G6) to homologous idiotype.

The binding of anti-ids to id-coated wells (solid symbols = homologous id, open symbols = control id, open circles = uncoated plates) was measured by absorbance at 450 nm.

▲ = id C7, ◆ = id D6, △ = control id R2 (IgG2a), ◇ = control id T34 (IgG2b).

Ordinate = Absorbance units, abscissa = dilution of anti-id.


2.0
anti-C7 (6D2)
1.0
0
1/1000
1/100
1/10


2.0
anti-C7 (8D8)
1.0
0
1/1000
1/100
1/10

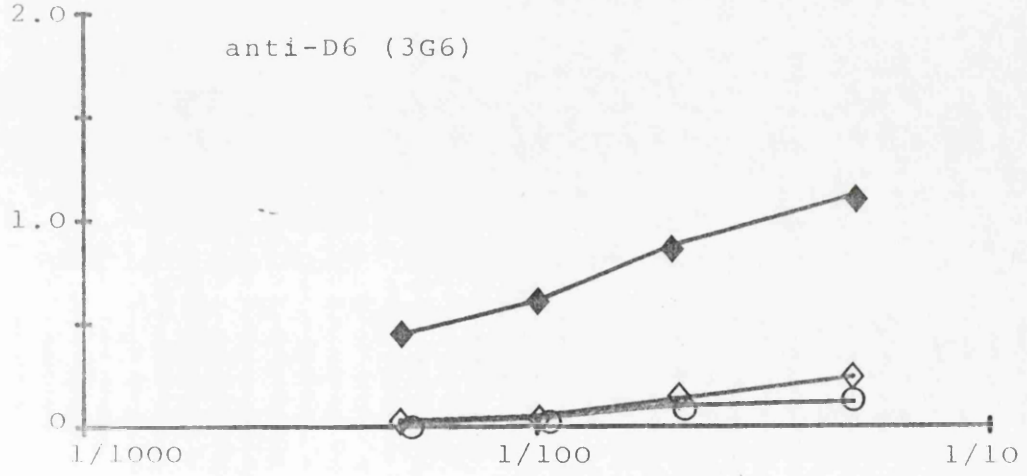
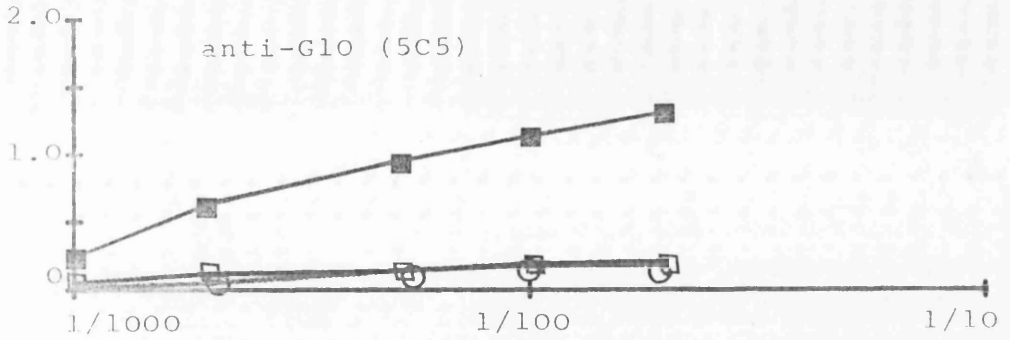

2.0
anti-D6 (3G6)
1.0
0
1/1000
1/100
1/10

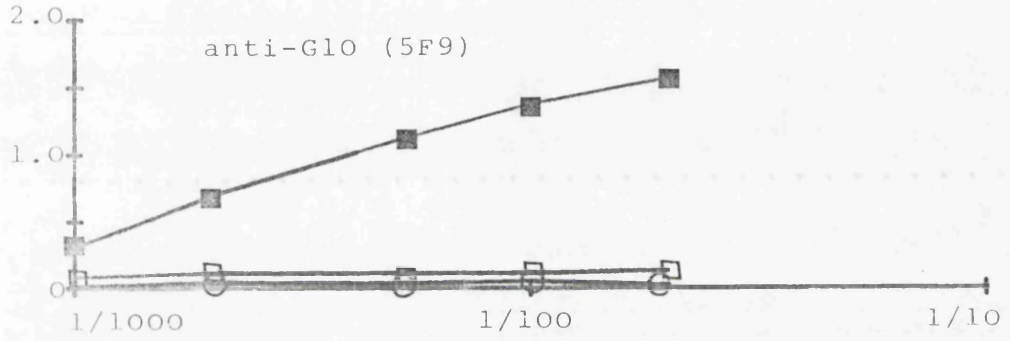
Figure 5.1b: ELISA binding of anti-id (5C5, 5F9, 10C1 & 10G3) to homologous idiotype.

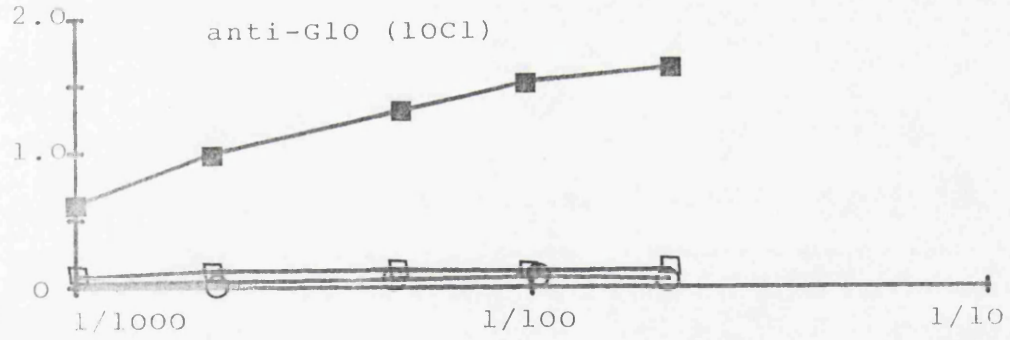
The binding of anti-ids to id-coated wells (solid symbols = homologous id, open symbols = control id, open circles = uncoated plates) was measured by absorbance at 450 nm.

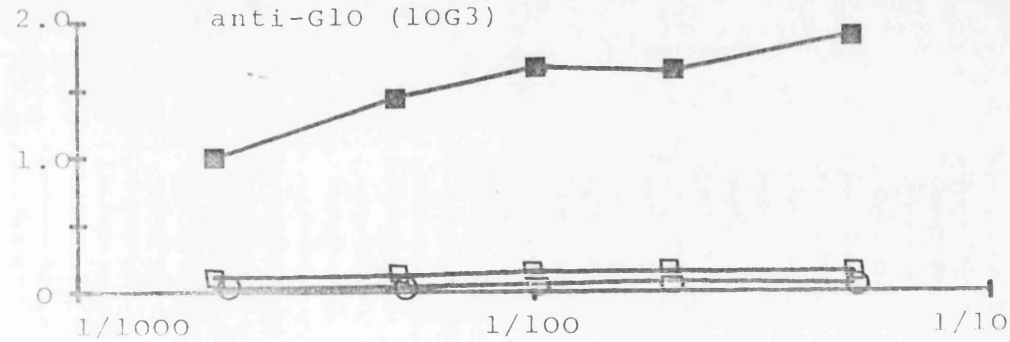
■ = id G10, □ = control id R1 (IgG1).

Ordinate = Absorbance units, abscissa = dilution of anti-id.


2.0
anti-Glo (5C5)
1.0
0
1/1000
1/100
1/10


2.0
anti-Glo (5F9)
1.0
0
1/1000
1/100
1/10


2.0
anti-Glo (10C1)
1.0
0
1/1000
1/100
1/10


2.0
anti-Glo (10G3)
1.0
0
1/1000
1/100
1/10

used. In the following experiments net OD values were expressed by subtracting these background readings from the total OD of anti-id binding to id. This accounts for the negative values sometimes found with the binding of anti-id to control mabs.

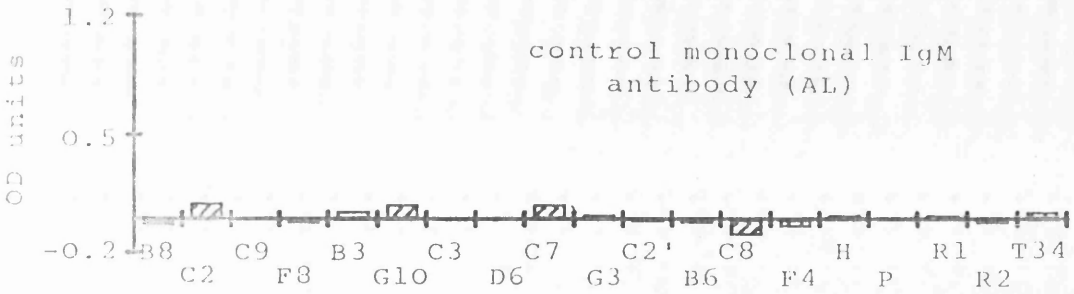
b). Cross-reactive ELISA.

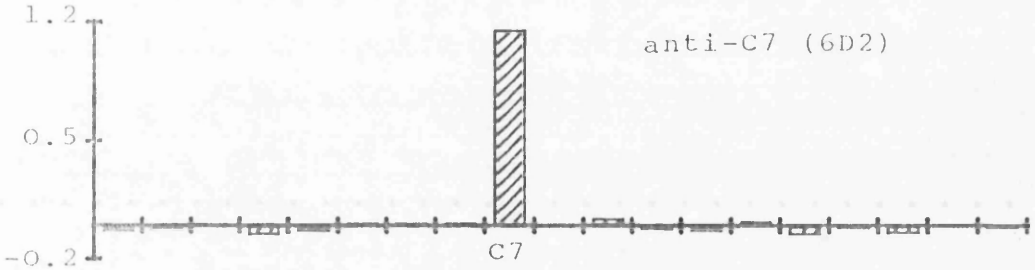
Limiting amounts of each anti-id (as determined from the titration curves against the homologous id in Fig. 5.1) were used to evaluate the cross-reactive binding against the complete panel of IgG mabs. The properties of the panel of mabs are summarised in the legend to Fig. 5.2. The panel included all ten anti-human AChR mabs, four anti-Torpedo AChR mabs (Whiting et al. 1985) and five mabs against irrelevant antigens. The binding of a control IgM monoclonal antibody (AL) raised against a synthetic peptide sequence (amino acid residues 125 - 143) of the α-subunit AChR (Jermy, unpublished results) was used to demonstrate non-specific binding to the IgG mabs (Fig. 5.2a).

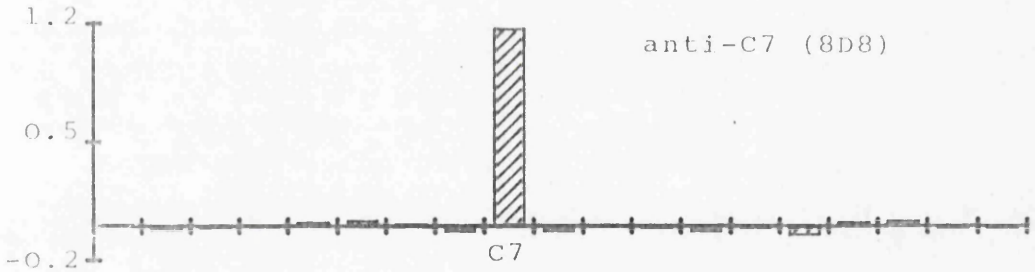
Figure 5.2a demonstrates the lack of binding of the control IgM antibody AL to the panel of IgG mabs. The two anti-C7 anti-ids (6D2 and 8D8) (Figs. 5.2b & c) showed exclusive binding to homologous id C7, whereas the anti-D6 anti-id (3G6) bound to C7 in addition to D6 (Fig. 5.2d).

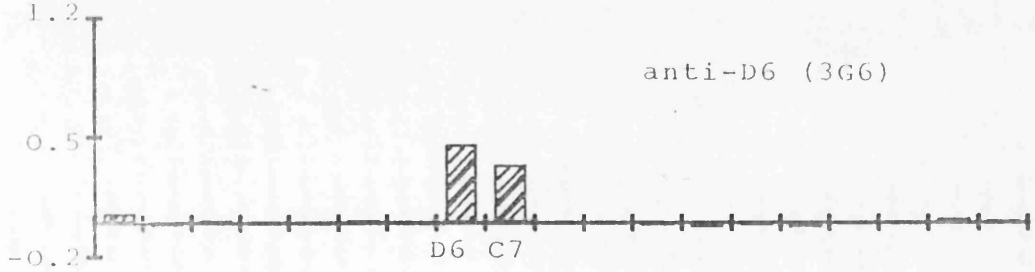
Figure 5.2e - h shows the more varied cross-reaction of the anti-G10 anti-ids. All four anti-ids bound to G10, with 5F9 and 10G3 giving better OD values than 5C5 and 10C1. 5C5 was the least cross-reactive, binding only to C7 with an OD

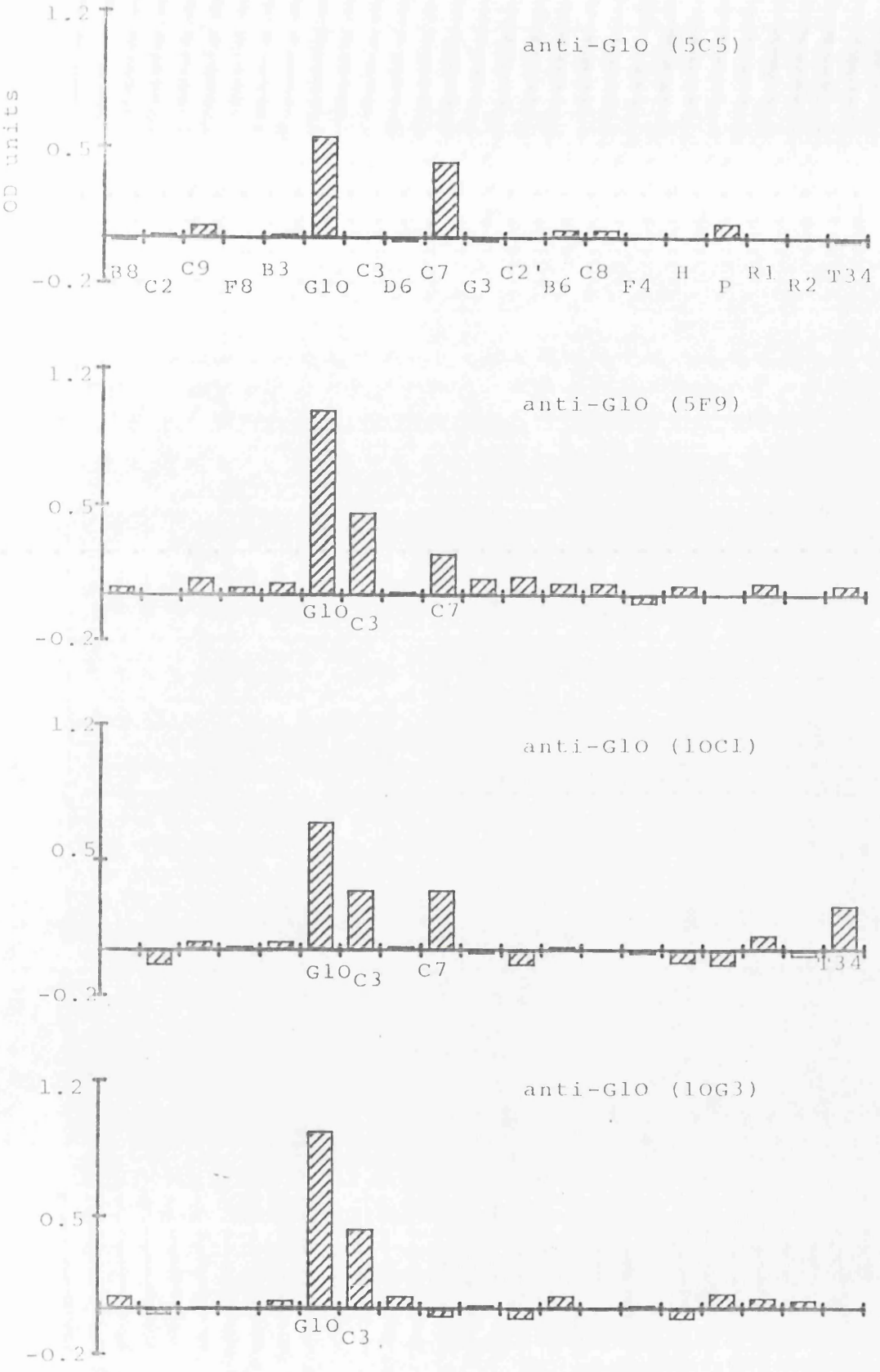
Figure 5.2: Cross-reactive ELISA binding of anti-ids. Limiting amounts of anti-id (against homologous id) were added to ELISA plates coated with the ten anti-human AChR mabs (as defined in Table 3.2); four anti-Torpedo AChR mabs (C2' = IgG2a, B6 = IgG1, C8 = IgG1, F4 = IgG2a); and five control mabs (H = anti-Hepatitis B surface antigen, IgG1; P = anti-pig von Willebrand factor, IgG1; R1 = anti-CD5, IgG1; R2 = anti-CD7, IgG2a; T34 = CD1, IgG2b). The binding was expressed as the absorbance at 450 nm minus non-specific binding to uncoated wells.


OD units
1.2
0.5
-0.2
control monoclonal IgM
antibody (AL)
B8 C9 B3 C3 C7 C2' C8 H R1 T34
C2 F8 G10 D6 G3 B6 F4 P R2


1.2
0.5
-0.2
anti-C7 (6D2)
C7


1.2
0.5
-0.2
anti-C7 (8D8)
C7


1.2
0.5
-0.2
anti-D6 (3G6)
D6 C7

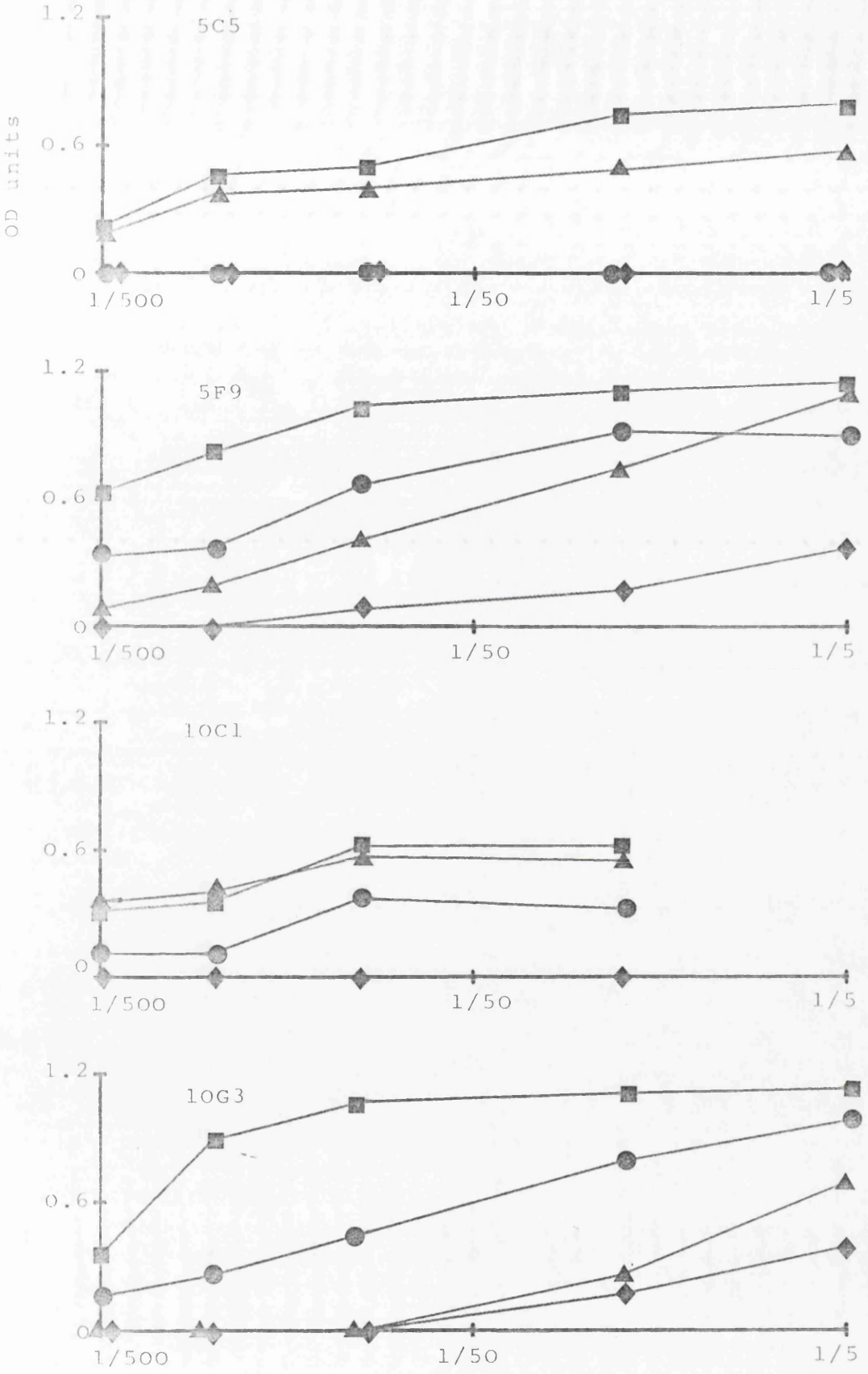

anti-G10 (5C5)
OD units
1.2
0.5
-0.2
B8 C2 C9 F8 B3 G10 C3 D6 C7 G3 C2' B6 C8 F4 H P R1 R2 T34
anti-G10 (5F9)
G10 C3 C7
anti-G10 (10C1)
G10 C3 C7 T34
anti-G10 (10G3)
G10 C3

70% of that for G10 (Fig. 5.2e). An important feature of 5C5 was the lack of cross-reaction with C3. 5F9 and 10C1 both cross-reacted with mab C3 to the same extent, i.e. they gave an OD reading 50% of that for G10. Both also cross-reacted with mab C7, 10C1 (Fig. 5.2f) better than 5F9 (Fig. 5.2g). 10C1 also bound to one of the irrelevant mabs T34, although this binding was variable and difficult to reproduce. Finally, anti-id 10G3 cross-reacted with C3 giving an OD 40% of that against G10 (Fig. 5.2h).

The anti-ids were further investigated for cross-reactive binding by titrating the anti-ids against mabs C3, G10, D6, and C7. The two anti-C7 anti-ids (6D2 and 8D8) again bound only to C7, even at high concentrations of anti-ids; and anti-D6 (3G6) showed the same binding as before (data not shown). However, the anti-G10 anti-ids showed greater cross-reaction when higher concentrations of supernatant were used (Fig. 5.3). The results for 5C5 confirm those found above i.e. 5C5 bound equally well to mabs G10 and C7, and not at all to mab C3 (Fig. 5.3a). By titrating 10C1 it was found that the binding to C7 was better than at first shown using a single dilution of anti-id, as the ODs against G10 and C7 are very similar (Fig. 5.3c). The cross-reaction with C3 was slightly less, at 20% than that found using a single dilution of 10C1 (50%). Striking results were found for 5F9 and 10G3. The titration curves showed the cross-reaction with C3 to be 25% for 5F9 and 35% for 10G3. Using the limiting amounts of these two anti-ids no cross-reaction was found with mab D6, and only

Figure 5.3: Titration of anti-G10 anti-ids versus cross-reactive ids.

Each anti-G10 anti-id was titrated against ids G10 (■), C3 (●), D6 (◆) and C7 (▲). Non-specific binding to plastic was substrated and the absorbance at 450 nm read (ordinate). The dilutions of anti-id are given by the abscissa.


5C5
5F9
10C1
10G3
OD units
1.2
0.6
0
1/500
1/50
1/5

5F9 cross-reacted with C7. However, a more extensive titration against these mabs demonstrated that both did bind to D6, but only to a small degree. The OD values were about 0.35 at the highest concentration of anti-id (1/5), and the corresponding cross-reaction was 0.5% for 5F9 (Fig. 5.3b), and 1% for 10G3 (Fig.5.3d). Binding of 10G3 to C7 was also revealed at the highest concentrations, and the cross-reaction was 2% The cross-reaction of 5F9 with C7 was calculated to be 10%

The degree of cross-reaction of the four anti-G10 anti-ids are summarised in Table 5.3.

c). Temperature dependence.

IgM antibodies are generally found to be of low affinity. Therefore to test whether binding of anti-id to id in the ELISA could be affected by temperature the ELISA plates were incubated at $4^{o}C$, room temperature ($20^{o}C$), or $37^{o}C$. Figure 5.4 shows the results obtained for anti-ids 5C5, 5F9, 10C1 and 10G3 binding to mabs G10 and C3. In each case the binding of anti-id to both G10 and C3 was unaffected by the temperature at which the assay was incubated.

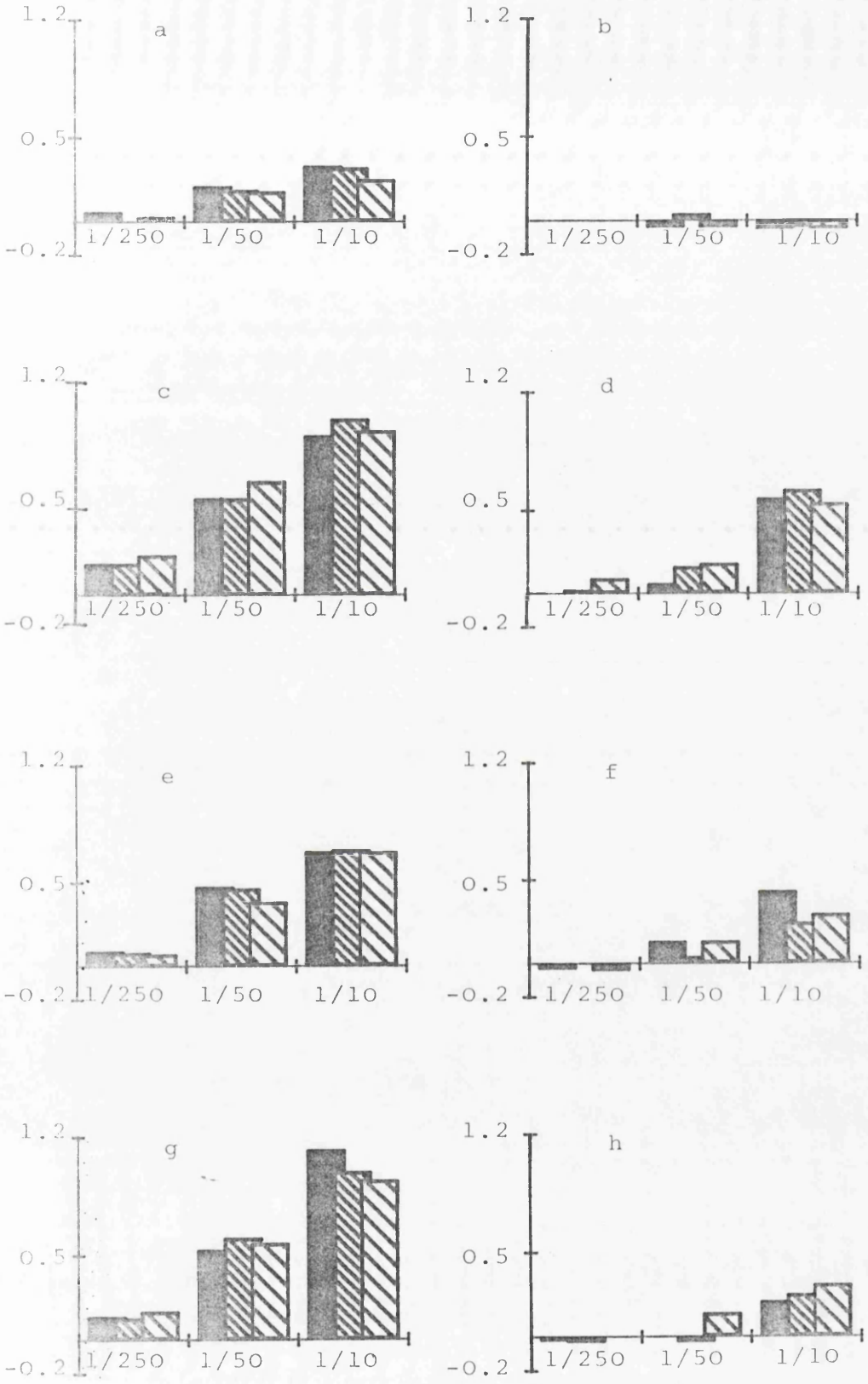
Table 5.3: Summary of cross-reactive binding of anti-G10 anti-ids.

	idiotype			
	G10	C3	D6	C7
anti-id				
5C5	+++	-	-	+++
5F9	+++	++	+	++
10C1	+++	++	-	+++
10G3	+++	++	+	+

- \- no cross-reactive binding.
- \+ cross-reactive binding < 5% relative to homologous binding.
- ++ cross-reactive binding 5 - 100% relative to homologous binding.
- +++ cross-reactive binding = homologous binding.

Figure 5.4: Titration of anti-G10 anti-ids at different temperatures.

Various dilutions of anti-id were added to id coated plates (a, c, e & g = G10; b, d, f & h = C3) and the assay incubated at 4°C (■), room temperature (▨) , or 37°C (▧). a & b = 5C5, c & d = 5F9, e & f = 10C1, g & h = 10G3. Ordinate = absorbance at 450 nm. Abscissa = dilutions of anti-ids.


a
b
c
d
e
f
g
h
1.2
0.5
-0.2
1/250
1/50
1/10

d). Inhibition ELISA.

Binding of anti-id to both homologous and cross-reactive id was inhibited by preincubating anti-id with soluble id before adding to the id-coated ELISA plates. Limiting amounts of anti-id (determined for each id from the cross-reactive titration curves in Fig. 5.3), were preincubated with soluble id (0.2 - 5 ug/well). Mab D6 was excluded from this particular experiment, firstly because the OD readings against D6 were usually low (< 0.4), and secondly because solid phase D6 on its own gave a high background value with HRP-conjugated goat anti-mouse IgM. The results of the OD reading of anti-id with inhibitor were expressed as a percentage of the OD without an inhibitor. Non-specific inhibition was measured by incubating anti-id with a control mab of the same IgG subclass as the target solid phase id. This non-specific inhibition was always less than 10% (data not shown).

Figure 5.5 shows the inhibition of 5F9 binding to G10 (a), C3 (b) and C7 (c). In each case inhibition was found using soluble id, although the binding to C3 was not easy to inhibit (Fig. 5.5b). For 10G3 the inhibition by soluble id was not as good (Figure 5.6), with C3 being a relatively poor inhibitor. The binding of 10C1 to immobilised id was inhibited less by C3 than by G10 or C7 (Figure 5.7a & b). The results of 10C1 binding to C3 were omitted because, even in the absence of inhibitor, the OD reading was so low that the degree of inhibition would have been exaggerated. The binding of 5C5 to solid phase G10 and C7 was inhibited by

Figure 5.5: Inhibition of 5F9 binding to id in ELISA by soluble idiotype.

Increasing amounts of soluble id (■= G10, ▨= C3, ▧= C7) were added to limiting amounts of 5F9 before adding to id coated plates (a = G10, b = C3, c = C7).

The inhibition by soluble id was measured as the percentage of binding without inhibitor.

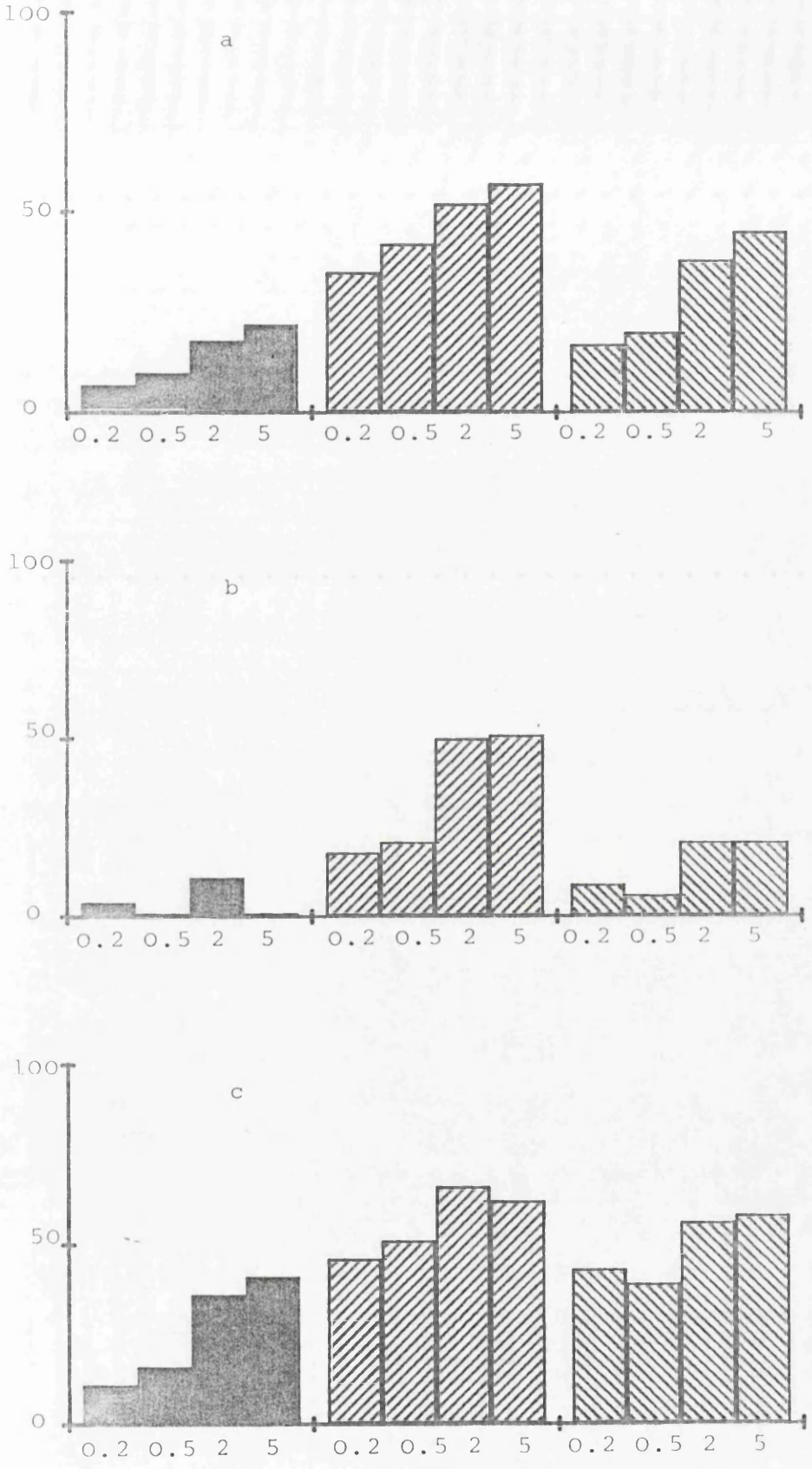

100
a
50
0
0.2 0.5 2 5 0.2 0.5 2 5 0.2 0.5 2 5
100
b
50
0
0.2 0.5 2 5 0.2 0.5 2 5 0.2 0.5 2 5
100
c
50
0
0.2 0.5 2 5 0.2 0.5 2 5 0.2 0.5 2 5

Figure 5.6: Inhibition of 10G3 binding to id in ELISA by soluble idiotype.

Inhibition of 10G3 binding to a) G10, b) C3 c) C7 by increasing amounts of G10 (■), C3 (▨) or C7 (▧), expressed as the percentage of binding without inhibitor.

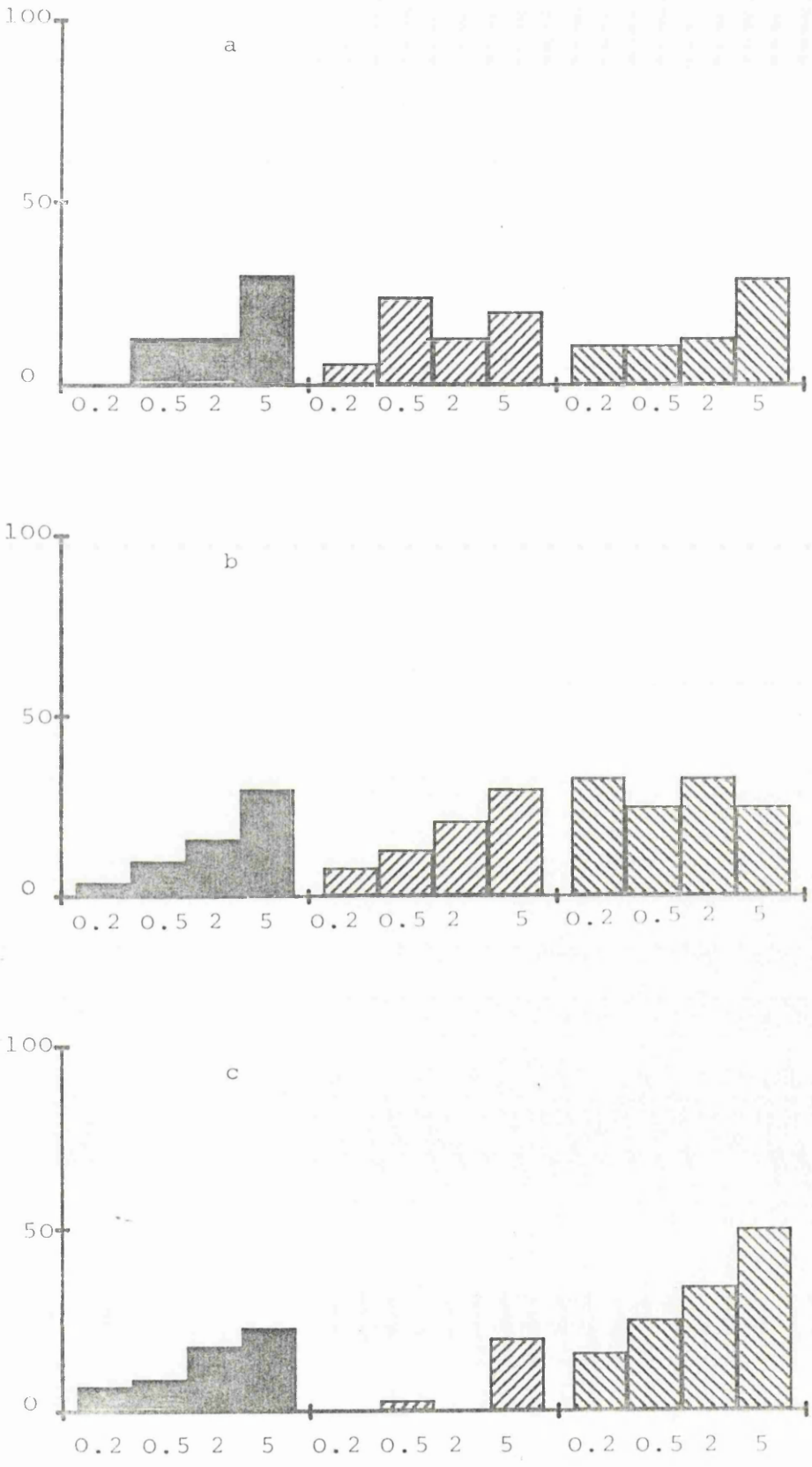
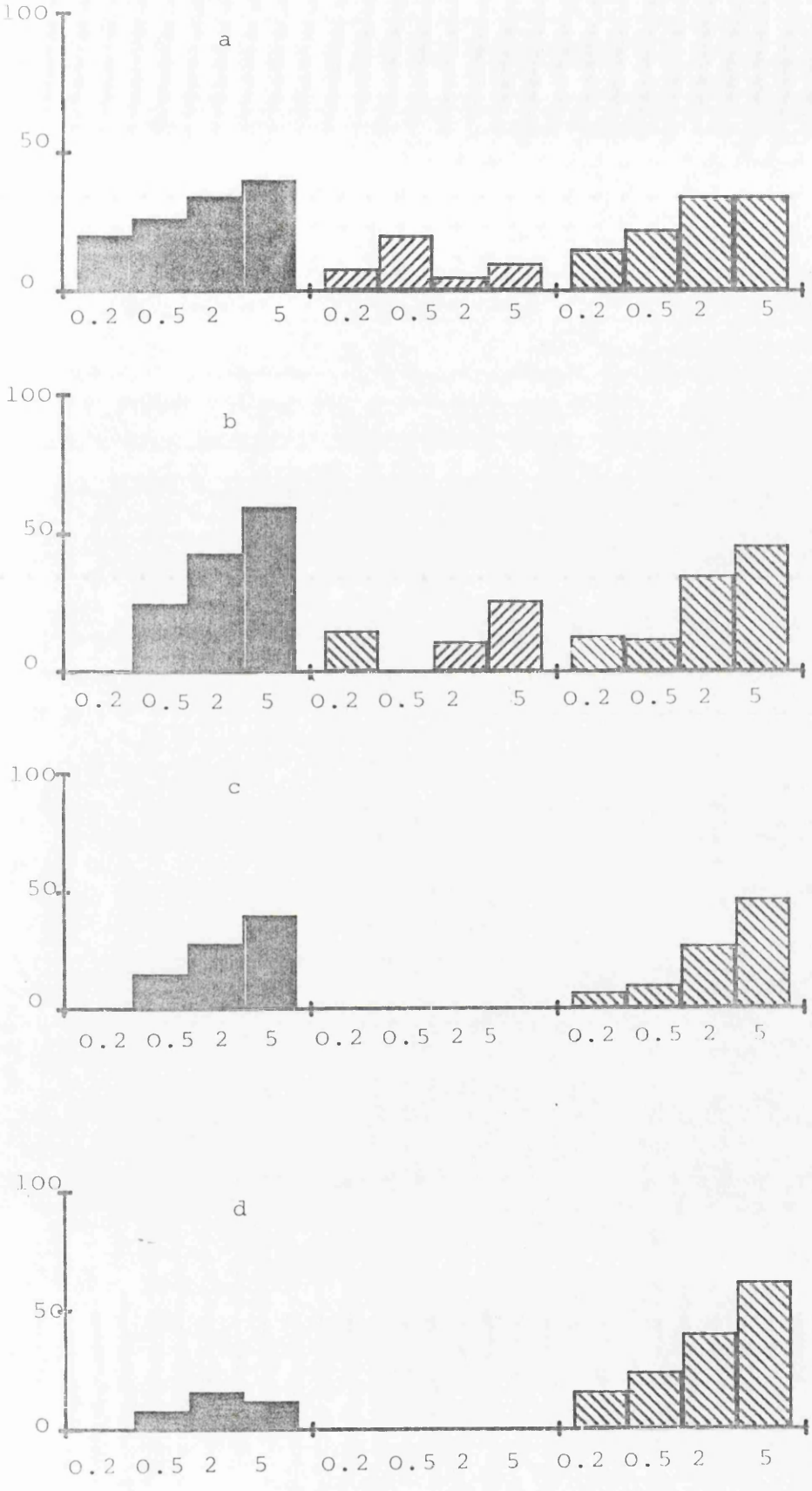

100
50
0
a
0.2 0.5 2 5 0.2 0.5 2 5 0.2 0.5 2 5
100
50
0
b
0.2 0.5 2 5 0.2 0.5 2 5 0.2 0.5 2 5
100
50
0
c
0.2 0.5 2 5 0.2 0.5 2 5 0.2 0.5 2 5

Figure 5.7: Inhibition of 10C1 and 5C5 binding to id in ELISA by soluble idiotype.

Inhibition of 10C1 binding to a) G10, or b) C7; and of 5C5 binding to c) G10, or d) C7 using soluble id G10 (■), C3 (▨) or C7 (▧).

Inhibition expressed as in Figs. 5.5 and 5.6.


a
100
50
0
0.2 0.5 2 5 0.2 0.5 2 5 0.2 0.5 2 5
b
100
50
0
0.2 0.5 2 5 0.2 0.5 2 5 0.2 0.5 2 5
c
100
50
0
0.2 0.5 2 5 0.2 0.5 2 5 0.2 0.5 2 5
d
100
50
0
0.2 0.5 2 5 0.2 0.5 2 5 0.2 0.5 2 5

soluble G10 and C7 (Figure 5.7c & d). No inhibition was found by mab C3 confirming that 5C5 does not react with this mab.

The binding of 6D2 and 8D8 to C7 was not inhibited by soluble C7 even at the highest concentration of free C7 used (data not shown).

Table 5.4 summarises the inhibition by soluble id for each anti-id - solid phase id reaction.

Table 5.4: Summary of inhibition of anti-id binding to solid-phase id by soluble idiotype.

anti-id	solid phase id	soluble idiotype		
		G10	C3	C7
6D2	C7	-	-	-
8D8	C7	-	-	-
5C5	G10	+	-	+
5C5	C7	+/-	-	+
5F9	G10	+	+	+
5F9	C3	+/-	+	+/-
5F9	C7	+	+	+
10C1	G10	+	+/-	+
10C1	C7	+	+	+
10G3	G10	+	+	+
10G3	C3	+	+	+
10G3	C7	+	+/-	+

+ = inhibition > 20%

+/- = inhibition < 20%

- = no inhibition.

5.3 Discussion.

Great care was taken but about six fusions were lost due to infection of the tissue culture plates. Also some batches of fetal calf sera appeared to be non-permissive to fusion.

Two methods of immunising Balb/c mice in preparation for fusion of splenocytes with an immortal partner were used. Firstly, ten fusions were performed following a standard immunisation regime of id with Freund's Adjuvant, usually administered at monthly intervals. Such a protocol proved very successful at producing good serum levels of anti-id. On average, about 40% of the microwells had hybrid growth, but although 1/3 of these hybrids were producing Ig, none of the ten fusions yielded a specific anti-id producing clone, as measured by inhibition of id binding to AChR. Other workers have reported difficulties in producing monoclonal anti-idiotypes. Streicher et al. (1986) found it necessary to conjugate their id to keyhole limpet haemocyanin to obtain 16 anti-id clones from 251 growing hybrids, and other reported anti-id success rates are 0.008% (Phillips et al. 1984) and 0.05% (Hemmi et al. 1985b). In the latter case their first 15 fusions were all negative for anti-idiotype. Agius et al. (1988) obtained a much better yield of anti-ids (0.8 - 2.6%) using the draining lymph nodes as the lymphocyte source, but lymph nodes cells of mice used for fusions 9 and 10 were fused, without success. Incidentally, Streicher also noted the problems associated with screening by an inhibition assay, and used an assay

selecting for anti-ids of a different isotype to the idiotype.

The second approach was to immunise by a direct injection of id intrasplenically, followed by fusing 3 or 4 days later. This technique was first described by Spitz et al. (1984) who immunised with B cell lymphoma derived Ig. Keay et al. (1988) have recently used the technique to raise monoclonal anti-ids to anti-human cytomegalovirus mabs, and established nine stable IgM clones.

In this study monoclonal anti-ids have been raised to three different anti-AChR mabs using this technique. Overall, the fusion success rate (approximately 40%), was no greater than for the Freund's immunised fusions, but following screening for IgM secreting hybrids 7 stable anti-id producing clones were eventually isolated.

IgM antibodies are generally held to be of low affinity but of high avidity. The anti-id ELISAs were incubated at varying temperatures to see if a difference in ELISA absorbance could be detected, but no effect on the amount of binding was seen.

The 7 anti-ids varied in their cross-reactivity patterns, but unlike the results obtained using polyclonal antisera, cross-reactions were not restricted to mabs of the same AChR binding specificity. The two anti-C7 anti-ids 6D2 and 8D8 did not cross-react with other ids, whereas the anti-D6 anti-id 3G6 reacted with C7. The cross-reactions of the four anti-G10 anti-ids were all different, indicating

that they each recognised different idiotopes. Cross-reactive ids were found between regions 4 and 5 (3G6 binding to D6 and C7); between regions 3 and 5 (5C5 binding to G10 and C7); and between regions 3, 4 and 5 (anti-ids 5F9, 10C1 or 10G3 binding to mabs C3, G10, D6 and C7).

As noted in chapter 4 the polyclonal anti-C3 and anti-G10 anti-ids did not distinguish between mabs C3 and G10. These two mabs were found to bind to the same region of AChR (see Table 3.2), and there was mutual cross-reaction between the polyclonal anti-ids and the ids both on the framework and inside the antigen binding site of each mab. However using monoclonal anti-ids distinctions could be made between the two mabs, revealing idiotopes on G10 not present on C3. Anti-id 5C5 did not bind at all to C3 and anti-id 10C1 only reacted at high concentrations. Anti-ids 5F9 and 10G3 both give extensive binding to C3, indicating a real difference between the idiotopes of G10 and C3.

Two of the anti-G10 anti-ids, 5C5 and 10C1 bound equally well to mabs C7 and G10. Similarly, the inhibition by soluble G10 and C7 was very much the same as well, suggesting that G10 and C7 certainly have a common idiotope recognised by 5C5 and 10C1. However, as 5C5 did not bind to C3, it might be that the idiotopes recognised by 5C5 and 10C1 are different, but this cannot be confirmed as competitive studies were not done between the two anti-ids. The other two anti-G10 anti-ids (5F9 and 10G3) also appeared to recognise a similar idiotope, as both cross-reacted strongly with C3 and less so with D6 and C7. Once again

competition between labelled and unlabelled anti-id may show the target idiotope(s) as being identical or otherwise.

It was difficult to draw quantitative conclusions about the relative abilities of soluble id to inhibit the binding of anti-id. Mab G10 inhibited the binding of each anti-G10 anti-id to varying degrees. Soluble C7 inhibited the binding of each of the anti-ids that cross-reacted with it, which was perhaps surprising since this mab was completely unable to inhibit the binding of 6D2 or 8D8 to C7. Anti-ids 6D2 and 8D8 both gave high OD readings even when used at high dilutions. It is possible that these anti-ids had a particularly high affinity for solid phase id, but unfortunately affinities could not be measured by this ELISA. Another consideration is that since IgM, is a pentamer, each anti-idiotype is repeated five times on each molecule. Therefore to inhibit all possible id binding sites one would require five times the amount of soluble id needed for a similar IgG anti-idiotype. Perhaps if the IgM molecules had been reduced to the monomeric constituent fragments it would have been easier to demonstrate inhibition by soluble id. IgM by natural design binds to regularly repeated epitopes. Idiotype immobilised on ELISA plates represents such epitopes to the IgM anti-ids and therefore is probably bound with high avidity.

Monoclonal anti-ids to anti-AChR antibodies have been raised in a number of studies of MG and EAMG. The anti-ids used by Lefvert (1988) to detect CRI in MG sera have

been discussed in Chapter 4. Agius et al. (1986) found that pre-treating rats with anti-id prior to a challenge with AChR suppressed the anti-AChR response and protected against AChR loss. Others have protected against passively transferred EAMG by a prior administration of polyclonal anti-id to anti-AChR mabs (Pachner et al. 1986).

Dwyer et al. (1986) have proposed an Idiotypic Connectivity on the basis of reactions between ids and anti-ids to different antigens, notably AChR and α-(1,3) dextran. They argued that the Idiotype Network can be perturbed by the id-anti-id interactions of disparate antigen systems resulting in the stimulation of autoantibodies. These autoantibodies are the anti-ids arising from the immune reaction to foreign antigens such as bacteria. They found that whereas control sera did not have any anti-dextran activity, 16% of the MG sera assayed did. One could postulate that the idiotypic consequences of an infection by certain bacteria could generate anti-AChR-like anti-ids which could lead to MG.

Monoclonal anti-idiotypic antibodies have been raised for a variety of reasons. Panels of anti-ids against a particular idiotype have been used to study the precise location of idiotopes relative to the antigen combining site. In this way Greenspan and Davie (1985) mapped the idiotopes of a murine anti-Streptococcal group A carbohydrate mab, and in a similar study the idiotopes of the anti-phosphorylcholine idiotype T15 have been mapped from close to the paratope down to the C_H1 region of the

molecule (Strickland et al. 1987). These two papers identified 4 and 6 distinct and overlapping idiotopes on their respective antibody molecules. Streicher et al. (1986) identified three clusters of idiotopes of an anti-myoglobin mab using a panel of monoclonal anti-ids one of which was inside the antigen-binding site; and Thanavala et al. (1988) raised monoclonal anti-ids to an anti-Hepatitis B surface antigen mab to define three different groups of idiotopes associated with that antibody.

5.4 Summary.

Fusions of the splenocytes of ten mice immunised with id and Freund's adjuvant did not produce any anti-idiotypic monoclonal antibodies. However monoclonal anti-idiotypic antibodies were raised against three anti-human AChR mabs, using the "single shot" intrasplenic technique. The two anti-ids against mab C7 bound exclusively to that id but their binding could not be inhibited by soluble id. The anti-D6 anti-id also cross-reacted with C7 but for technical reasons could not be investigated further. The four anti-G10 anti-ids all gave different patterns of cross-reaction, suggesting that they each recognised different idiotopes. Each was inhibited from binding to id in the ELISA by soluble id.

CHAPTER SIX

PURIFIED ANTI-IDIOTYPIC ANTIBODIES

6.1 Introduction.

It was necessary to purify the monoclonal anti-idiotypic antibodies in order to characterise them further. Purification was approached in two ways. Firstly affinity chromatography was tried using the anti-human AChR mab - Sepharose 4B columns prepared in Chapter 3. The feasibility of this approach was ascertained by purifying polyclonal anti-idiotypic sera raised and investigated in Chapter 4. The second approach was to partially purify and concentrate culture supernatants. To minimise the contamination of the anti-ids by exogenous proteins, the amount of FCS present in the growth media was reduced to 1%. Culture supernatants were then precipitated by ammonium sulphate and reconstituted in PBS.

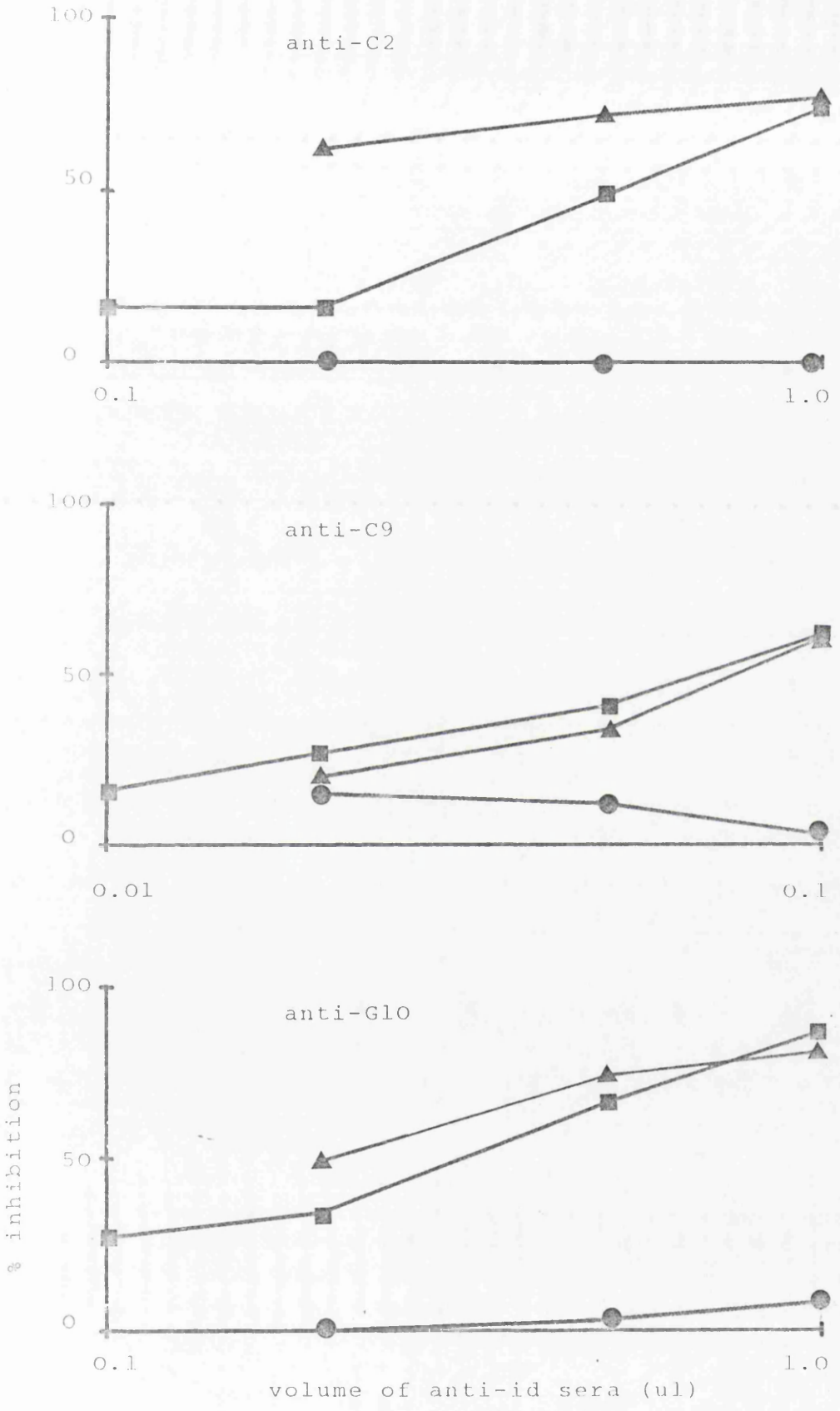
6.2 Results.

6.2.1 Purification of polyclonal anti-ids.

Polyclonal anti-idiotypic sera raised to anti-AChR mabs C2, C9 and G10 were adsorbed onto homologous mab - Sepharose 4B beads. Bound anti-ids were eluted from the beads using 2M $NaClO_4$, dialysing the eluent against PBS. Figure 6. 1 shows that the anti-idiotypic activity (measured by the ability to inhibit mab binding to ^{125}I-BuTx-AChR) was completely removed from the sera by adsorption onto mab - Sepharose 4B beads. The anti-idiotypic antibodies were then regained by

Figure 6.1: Inhibition of homologous id binding to AChR by affinity purified polyclonal anti-idiotypes.

Varying dilutions of anti-id sera before (▲) and after (●) adsorption onto homologous mab-Sepharose 4B beads, and the eluted purified anti-id (■) were incubated with limiting amounts of anti-AChR mab before the addition of ^{125}I-BuTx-AChR.


anti-C2
100
50
0
0.1
1.0
anti-C9
100
50
0
0.01
0.1
anti-GlO
100
50
0
0.1
1.0
% inhibition
volume of anti-id sera (ul)

eluting from the beads.

6.2.2 Purification of monoclonal anti-ids.

a). Affinity chromatography.

Having established that polyclonal anti-idiotypic antibodies could be purified using mab - Sepharose 4B beads, the beads were used in an attempt to purify monoclonal anti-idiotypic antibodies from ascites fluid. The ELISA binding of the ascites fluid to id coated plates is shown in Figure 6.2. The pattern of binding was exactly that found using culture supernatants (see Fig. 5.2). However, because the non-specific binding of the ascites to uncoated ELISA plates was high, the results were somewhat varied.

Ascites fluid (0.25 - 0.5 ml) was circulated through 2.5 ml of mab - Sepharose beads packed into a column. Bound anti-idiotypic antibodies were eluted either in a citrate/ phosphate buffer (pH 3.5) or 2M $NaClO_4$. The peak fractions were pooled and dialysed immediately against PBS.

Purified anti-ids were titrated in an ELISA against homologous id (Figure 6.3). Except for anti-C7 (6D2) the binding of the purified anti-ids was very low.

b). Ammonium sulphate precipitation of culture supernatants.

Because the results of the affinity purified anti-ids were so poor, the anti-ids were partially purified and concentrated by ammonium sulphate precipitation of culture supernatant. The preparations were assayed by direct ELISA to ensure that the anti-idiotypic activity had been

Figure 6.2: ELISA binding of monoclonal anti-idiotypic ascites to idiotype.

Ascites fluid (diluted 1/5000 in PBST) was added to id (C2, C7, D6, G10 or T34) coated ELISA plates. Results are expressed as OD (450 nm) minus the background binding to uncoated plates.

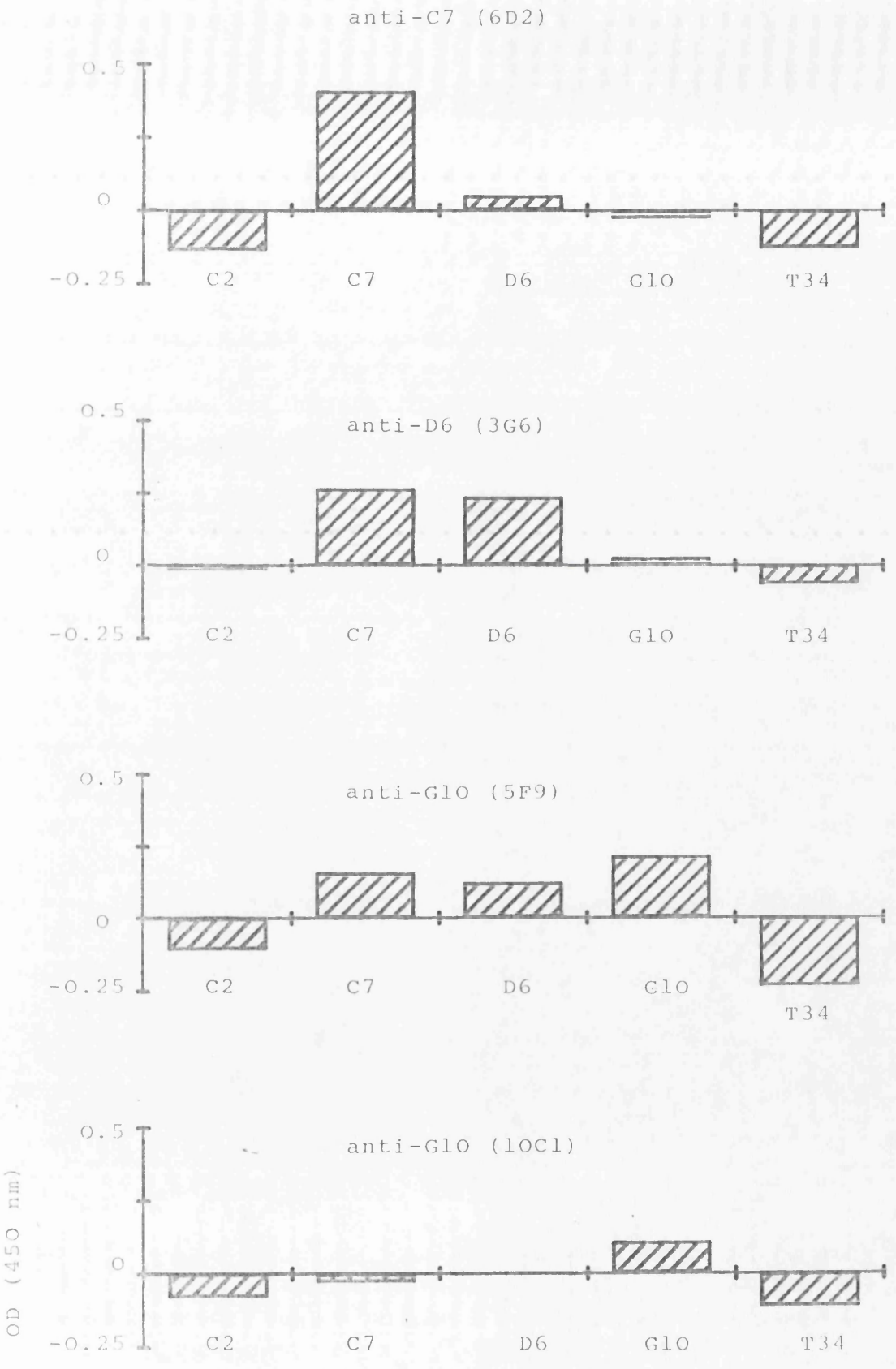
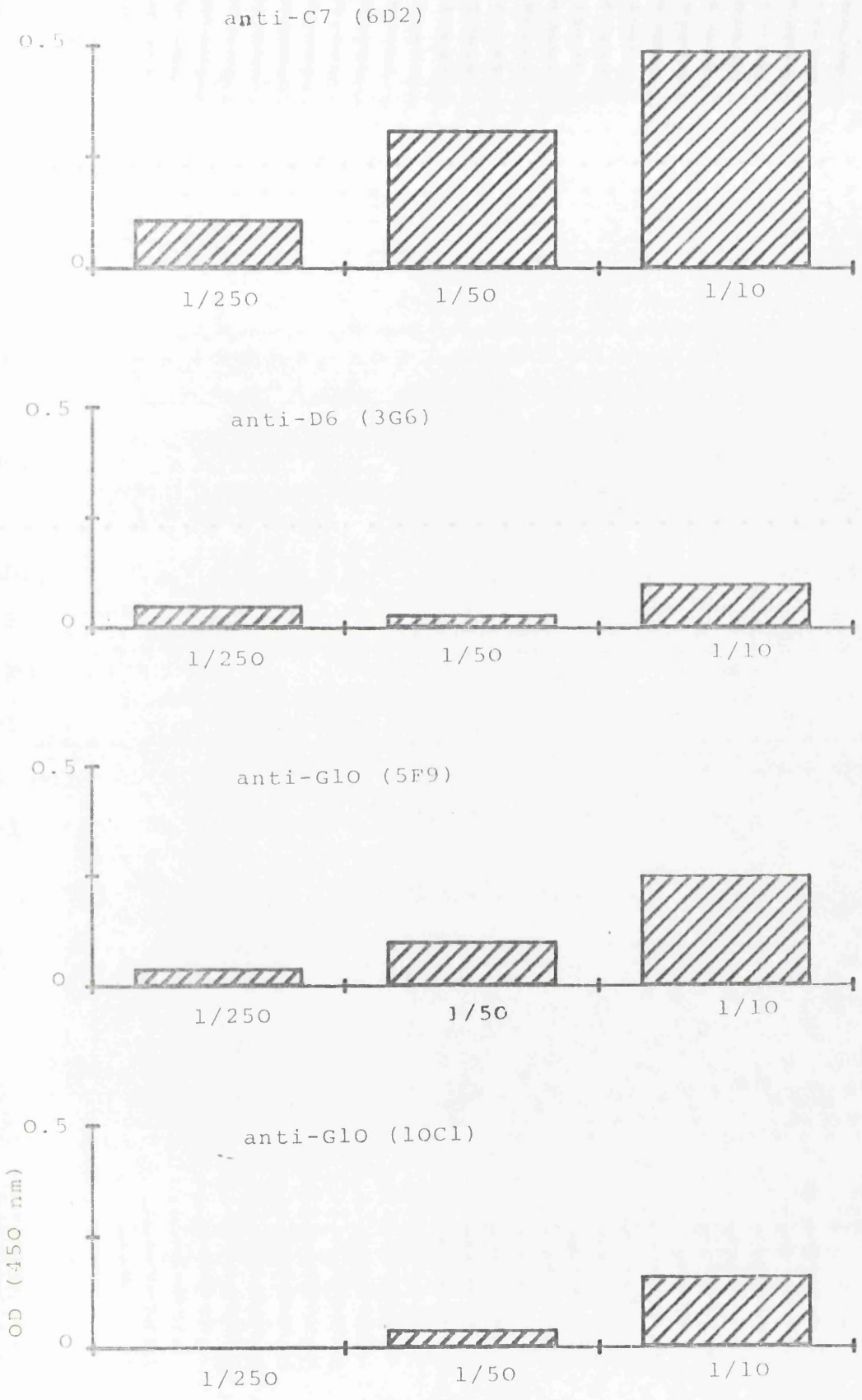

anti-C7 (6D2)
0.5
0
-0.25
C2
C7
D6
G10
T34
anti-D6 (3G6)
0.5
0
-0.25
C2
C7
D6
G10
T34
anti-G10 (5F9)
0.5
0
-0.25
C2
C7
D6
G10
T34
anti-G10 (10C1)
0.5
0
-0.25
C2
C7
D6
G10
T34
OD (450 nm)

Figure 6.3: ELISA titration of affinity purified monoclonal anti-idiotypes to homologous id.

Varying dilutions of monoclonal anti-idiotype, purified by mab-Sepharose 4B affinity chromatography were added to id-coated ELISA plates. Irrelevant binding to control IgG antibodies was always less than 0.02 OD units (not shown).


anti-C7 (6D2)
0.5
0
1/250
1/50
1/10
anti-D6 (3G6)
0.5
0
1/250
1/50
1/10
anti-G10 (5F9)
0.5
0
1/250
1/50
1/10
anti-G10 (10C1)
0.5
0
1/250
1/50
1/10
OD (450 nm)
dilution of anti-id

recovered (data not shown).

6.2.3 Assays of purified anti-idiotypic antibodies.

a). Inhibition RIA.

Purified anti-ids were tested for specific inhibition of mab binding to AChR. None of the anti-ids were able to inhibit either homologous id (Table 6.1), or heterologous id (Table 6.2) binding to AChR, indicating that the idiotopes recognised were not involved in AChR binding, and the anti-ids were not of the Ab2beta or Ab2gamma type.

b). Indirect RIA.

Figure 6.4 shows the indirect precipitation of mab-^{125}I-BuTx-AChR complexes by purified anti-ids. None of the anti-ids were able to precipitate mab-^{125}I-BuTx-AChR to any great degree, although there may have been some binding of anti-ids 6D2, 5C5, and 10G3.

Table 6.1: Lack of inhibition of homologous idiotype binding to AChR by purified monoclonal anti-idiotype.

		volume anti-id (ul)[a]				
id	anti-id	0	1	5	20	100
C7	6D2	5374	5591[b]	5288	5700	5569
C7	8D8	5374	5596	5590	5504	5629
D6	3G6	6122	6267	6338	6100	5974
G10	5C5	5340	5174	5011	5217	4959
G10	5F9	5340	4963	5240	5361	4989
G10	10C1	5340	4965	5300	5198	5290
G10	10G3	5340	5142	5376	5186	5200

[a] IgM concentration = 200 - 500 ug/ml.

[b] results expressed as cpm of ^{125}I-BuTx-AChR precipitated by idiotype in presence of anti-id.

Table 6.2: Lack of inhibition of heterologous id binding to AChR by purified monoclonal anti-idiotype.

anti-id	volume (ul)[a]	anti-AChR mab G10	C3	C7
-	-	4189[b]	4484	3178
5C5	2	4472[c]	4862	3254
"	5	4377	4581	3327
"	10	4628	4621	3289
"	25	4375	4528	3347
5F9	2	4591	5070	3514
"	5	4667	4989	3353
"	10	5205	4783	3314
"	25	4241	4799	3273
10C1	2	4572	5310	3562
"	5	5112	5620	3147
"	10	4610	4881	3688
"	25	4677	5436	3404
10G3	2	4938	5561	3346
"	5	4742	5049	3555
"	10	4748	5142	3647
"	25	4395	4939	3657

[a] IgM concentration = 200 - 500 ug/ml.

[b] cpm of ^{125}I-BuTx-AChR precipitated by mab alone.

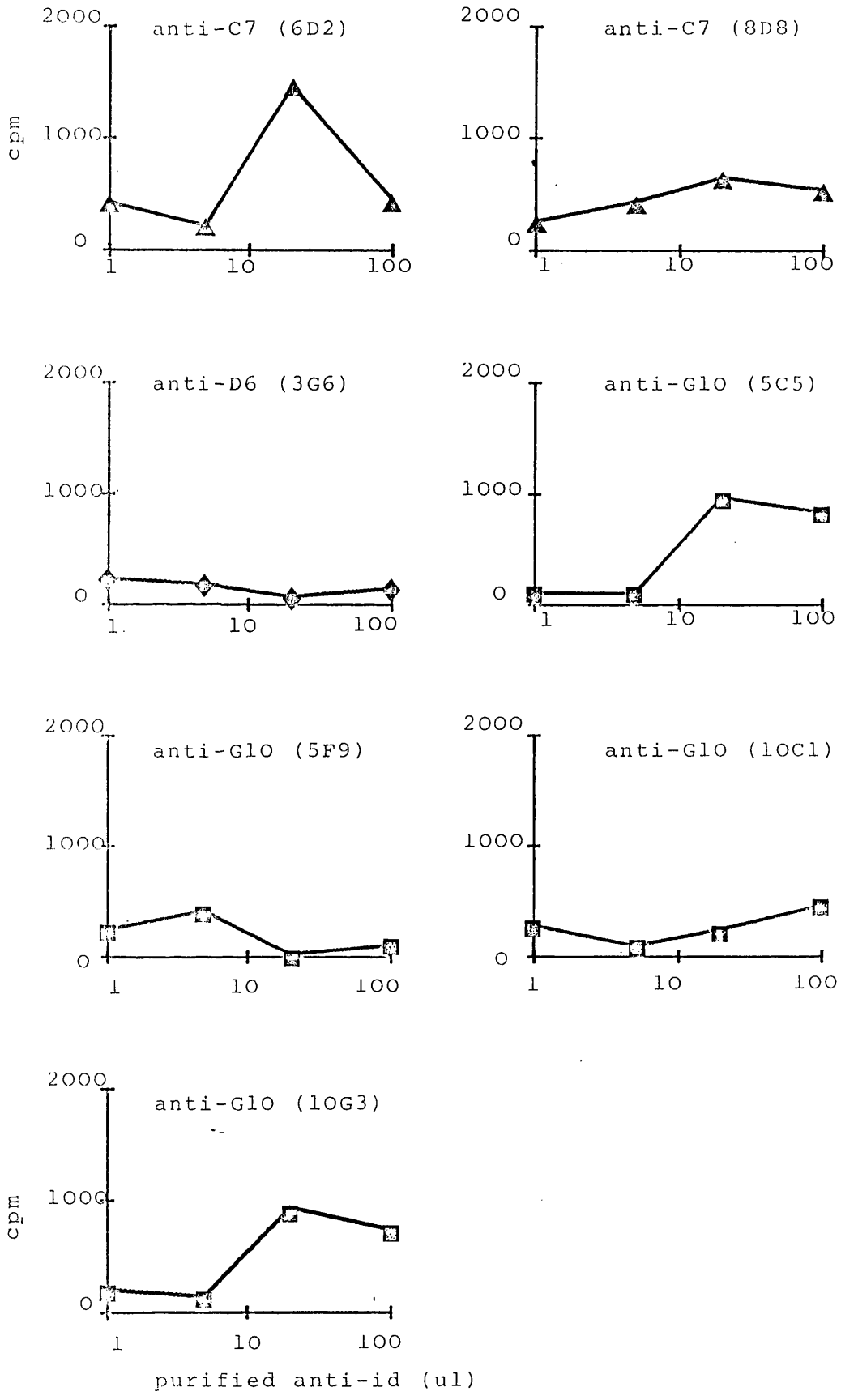
[c] cpm of ^{125}I-BuTx-AChR precipitated by mab in presence of anti-id.

Figure 6.4: Indirect binding of purified anti-idiotypes to id-^{125}I-BuTx-AChR by RIA.

^{125}I-BuTx-AChR was preincubated with limiting amounts of homologous id (▲ = id C7, ◆ = id D6, ■ = id G10) before the addition of increasing amounts of purified anti-id. ^{125}I-BuTx-AChR-id-anti-id complexes were precipitated with anti-mouse IgM.

ordinate = cpm id-^{125}I-BuTx-AChR precipitated.

abscissa = volume of anti-id (ul).


anti-C7 (6D2)
anti-C7 (8D8)
anti-D6 (3G6)
anti-G10 (5C5)
anti-G10 (5F9)
anti-G10 (10C1)
anti-G10 (10G3)
cpm
2000
1000
0
1
10
100
purified anti-id (ul)

c). Reverse ELISA.

As a preliminary study with a view to developing an assay for detecting shared idiotopes in MG sera, a reverse ELISA assay was devised. In this assay, the binding of soluble id to immobilised anti-id was measured. Varying dilutions of purified anti-id were coated onto ELISA plates and the amount of purified anti-id bound to the plastic evaluated, using HRP-anti-mouse IgM. Figure 6.5 shows that anti-ids 3G6, 5C5 and 10C1 bound well to the ELISA plates, whereas 6D2, 8D8, 3G6 and particularly 10G3 bound less well.

Although the anti-ids bound to the ELISA plates, when soluble id followed by HRP-anti-mouse IgG was added no significant binding (ie less than 0.2) was found (data not shown).

d) Capture ELISA.

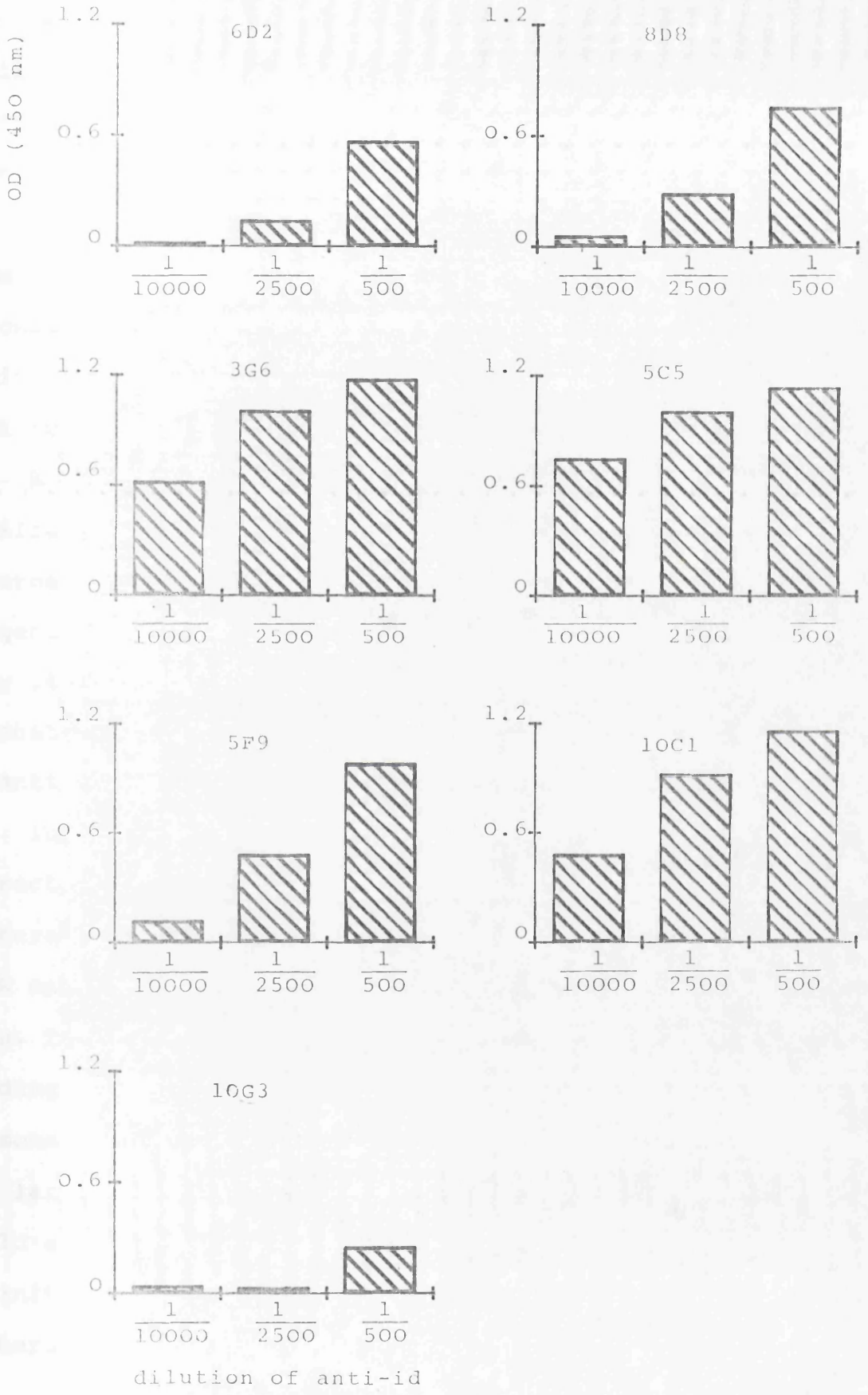
To increase the sensitivity of the reverse ELISA, the wells were coated with anti-mouse IgM antibodies to try to capture more anti-id. However the results obtained were no better then those of plates coated directly with anti-id (data not shown).

Figure 6.5: Binding of purified monoclonal anti-idiotypes to ELISA plates.

ELISA wells were coated with increasing amounts of anti-id, and the binding determined using HRP-anti-IgM.

ordinate = absorbance reading at 450 nm.

abscissa = dilution of anti-id coated to ELISA plates.


OD (450 nm)
1.2
0.6
0
6D2
8D8
3G6
5C5
5F9
10C1
10G3
1/10000
1/2500
1/500
dilution of anti-id

6.3 Discussion.

Two methods were used to purify anti-idiotypic antibodies. Polyclonal anti-idiotypic sera to three different mabs were purified by chromatography using mab - Sepharose 4B beads. However, this technique, when applied to the purification of monoclonal anti-idiotypic antibodies from ascites fluid yielded very poor results. This lack of success could not be attributed to poor anti-idiotypic activity of the ascites as each sample was shown to bind well to id in the ELISA.

However, monoclonal anti-idiotypes were partially purified by ammonium sulphate precipitation of culture supernatants. To avoid unnecessary contamination by exogenous proteins the cells were grown in media containing only 1% FCS. The cells grew well in this media, and ammonium sulphate precipitation of the supernatants gave good amounts of anti-idiotypic antibodies.

These purified monoclonal anti-ids were then characterised further. None of the monoclonal anti-ids appeared to be directed against the paratope of the anti-AChR mabs as they were unable to inhibit the binding to AChR. The reciprocal experiment ie inhibition of anti-id binding to id by AChR could not be done for technical reasons. Denervated human muscle is not rich enough in AChR for large quantities to be purified; 100 g of tissue would yield at most 10 ug of AChR following purification by affinity chromatography using cobra toxin-conjugated to Sepharose 4B. Such amounts of AChR would not be sufficient

to enable one to do a full inhibition experiment for one anti-id.

The results of the binding of anti-id to pre-formed id-AChR complexes were inconclusive. As discussed in Chapter 4, the binding of id to AChR may induce conformational changes in framework idiotopes making them unrecognisable to anti-id. Such conformational changes have been postulated for antigen-antibody modulation of epitopes (Berzofsky 1985) and for id-anti-id modulation of idiotopes (Campbell et al. 1986). Indeed, of the 47 polyclonal anti-ids assayed only 4 bound to $F(ab')_2$-AChR complexes, a lower number than one might have expected. Poor binding of anti-id to id-AChR complexes should not be viewed as conclusive evidence for a lack of binding to framework idiotopes.

The failure of the reverse ELISA to detect soluble id binding to immobilised anti-id was suprising, since it had been shown that id was capable of reacting with anti-id in solution. However the IgM anti-ids may have bound to the ELISA wells in a crab-like fashion via the variable region arms, thus occluding the idiotope combining sites. The capture assay should have rectified such a problem by anchoring the IgM molecules through the mu chains thereby exposing the idiotope combining sites. Unfortunately this modification did not improve the situation.

6.4 Summary

Polyclonal anti-idiotypic antibodies were affinity purified using mab - Sepharose 4B beads. However, as poor

yields were obtained by affinity chromatography of ascites fluid, monoclonal anti-idiotypic antibodies were purified by ammonium sulphate precipitation of culture supernatant. None of the monoclonal anti-idiotypes were found to inhibit mab binding to AChR indicating that they were probably directed to idiotopes outside the paratope. The purified monoclonal anti-idiotypes bound well to ELISA plates, but the binding of idiotype to immobilised anti-idiotype was not detected.

CHAPTER SEVEN

CONCLUDING REMARKS

The Idiotype Network is of great interest to those wishing to understand the factors involved in initiating and sustaining an autoimmune disease such as myasthenia gravis. It seems clear that cross-reactive idiotypes are a feature of non-organ specific diseases like systemic lupus erythematosus and rheumatoid arthritis, but in an organ-specific disease such as myasthenia gravis the existence of CRIs is still not universally accepted. To this end I have examined the idiotypes expressed by a panel of ten anti-human AChR mabs using both polyclonal and monoclonal anti-idiotypic antibodies.

Initially the mabs were characterised by the inhibition of mab binding to AChR by $F(ab')_2$ fragments of each mab. This confirmed the grouping of the mabs into five AChR binding regions by P. Whiting (1986b).

The polyclonal anti-idiotypic sera used in this study were raised in syngeneic mice by immunising with idiotype and Freund's adjuvant. However, from the fusions of the splenocytes of ten mice immunised with idiotype and Freunds adjuvant not one monoclonal anti-idiotypic antibody was detected. Using idiotype emulsified with Freund's adjuvant may not be the most suitable method of immunisation for the generation of monoclonal anti-idiotypes, even though all the mice used had good levels of anti-idiotypic antibodies in the sera prior to fusion. However, using a direct

intrasplenic injection followed by fusion 3 or 4 days later, hybridomas secreting monoclonal anti-idiotypic antibodies were prepared against three anti-human AChR mabs.

The polyclonal anti-ids were mostly directed against paratope-associated idiotopes. These anti-ids were defined as Ab2gamma using the nomenclature of Jerne et al. (1982), and not the more frequently used Ab2beta (internal image) designation. Rajewsky and Takemori (1983) commented that even for an idiotope invariably associated with antibodies of given hapten specificity and recognised by a hapten inhibitable anti-id, it did not necessarily follow that the idiotope coincided with the hapten binding site. The monoclonal anti-ids were not directed against AChR binding site idiotopes as they did inhibit the binding of id to AChR, and were therefore termed Ab2alpha. Ab2alpha anti-ids are directed against framework determinants of antibody molecules.

The polyclonal anti-ids detected a certain degree of cross-reactivity between the idiotypes, but sharing was restricted to mabs binding to the same region of AChR. Such an observation is consistent with an idiotope being associated with one particular epitope. The cross-reactive idiotopes detected on the anti-AChR mabs using the monoclonal anti-ids were not restricted to mabs of the same binding region.

It would be interesting to have sequence data on the V region genes encoding each of the anti-human AChR mabs to help resolve the cross-reactive idiotypic patterns observed

between the mabs, especially those of C3 and G10. Using polyclonal anti-idiotypic sera the idiotypes of mabs C3 and G10 were almost indistinguishable, both inside and outside the antigen binding site. However, one of the four monoclonal anti-ids to G10 detected an idiotope present on G10 but not on C3. The sequences of the CDRs of C3 and G10 may show which CDRs are identical or nearly identical, and which CDRs account for the idiotypic differences between the two mabs. Likewise, sequence data may account for the difference in idiotypy between mabs C7 and G3, the two mabs that define binding region 5 of human AChR. Similarly the differences in the group I mabs where C2 and F8 were idiotypically distinct from C9 and B8 may also be explained in terms of the V region sequences.

Cross-reactive inhibition of MG sera by the polyclonal anti-ids was not detected, perhaps for two reasons. Firstly, individual levels of idiotype in MG sera may be so low that even though the polyclonal anti-ids do cross-react with human antibodies it is impossible to detect it by an inhibition assay. Secondly, these particular murine mabs may not share idiotypes with the human anti-AChR antibodies.

Although I was unable to develop a successful assay for the detection of CRI in MG sera, the monoclonal anti-idiotypic antibodies raised in this study may prove a useful tool for investigating immune regulation in response to AChR. Molecular biology is now providing the means of producing antigens such as human AChR hitherto difficult to isolate in sufficient quantities. cDNA of the α-subunit of

human AChR has been cloned in this laboratory and plentiful amounts of expressed protein are now readily available. An animal model of MG established using recombinant mammalian AChR rather than AChR purified from the electric organs of electric eels would lend itself to idiotypic manipulation using the monoclonal anti-idiotypic antibodies. It would be interesting to see if there are any functional differences between anti-idiotypes to paratope and framework idiotopes.

It is interesting that the polyclonal and monoclonal anti-ids reveal different types of shared ids. It is feasible that the immunisation regimes select for different types of anti-ids, although Capra and Bona (1988) report that there are few rules governing their production. It is possible that the monoclonal anti-ids are directed against "regulatory" idiotopes (ReI), as described by Paul and Bona (1982). Bona et al. (1984) defined a ReI by the following criteria:

a) they function as autoimmunogens

b) they are shared by various members of an idiotype network pathway which may include antibodies with different specificities

c) they are recognised by regulatory T cells that control the expansion of clones bearing the ReIs.

An example of the latter point is the dominant CRI seen in the secondary response to hen egg lysozyme, selected by idiotope-specific T cells (Metzger et al. 1981). Bona et al. also suggested that only ReI are immunogenic in a syngeneic and autologous system, and that ReIs are antigen non-

inhibitable, i.e. they lie outside the paratope. Bottomly (1984) has also argued that a ReI would need to be widely shared on antibodies of differing specificities in order to be effective regulators within an Idiotype Network. In other words, a ReI would be a recurrent or CRI. She proposed that ReIs would differ from other ids in that they would be present in very high levels as part of `natural antibodies`. A corollary of this hypothesis is that anti-ids that do not react with naturally occurring antibodies would not detect non-regulatory idiotopes. She envisaged ReIs interacting with idiotope-specific T helper cells (Thid), and her studies showed that id found in normal serum plays an important role in the maturation and/or function of these cells. Indeed during ontogeny T and B cell repertoires could be co-selected in order to create a functional idiotypic network.

One could simple interpret a recurrent idiotope as being a marker of a germline V gene, and a private idiotope as the product of somatic mutation arising during the maturation of an immune response. The recombination of V-D-J gene segments of the heavy chain forms CDR3, which is less susceptible to somatic mutation than either CDR1 or CDR2. CDR3 would therefore be an ideal candidate for a ReI as it would be carried by antibodies of different antigenic specificities, and encoded by germline genes. Such ReIs would not be selected out by somatic mutation.

When assessing the significance of CRI and ReI there are many factors to consider apart from overall dominance of

the antibody pool. Zanetti (1985) does not agree with the proposal of Bottomly (1984) that ReIs must be dominant idiotopes. In their studies of the idiotypes of the anti-thyroglobulin response Zanetti et al. (1984) found that an id present in small amounts had a considerable capacity to regulate the autoantibody production. By analogy an antibody presenting only a small fraction of the total antibody pool may be highly pathogenic.

In contrast, Ebling et al. (1988) have reported that three cross-reactive idiotopes account for 85% of the total immunoglobulin repertoire in the sera of of the nephritic NZB/NZW F1 mice, a strain that spontaneously develop SLE. They proposed that two of these ids were ReIs, and that they were also pathogenic as both were found on glomerular Ig deposits. They hypothesise that pathogenic autoantibodies carrying these ReI are subject to substained up-regulation by autoreactive ReI-recognising T helper cells. Such a hypothesis of `idiotypic spreading` of aberrently functioning ReI would be consistant with the interactions proposed by Bottomly (1984).

The Idiotype Connectivity proposed by Dwyer et al. (1986) of families of 'super organiser' idiotypes may also fit in with a regulatory Idiotype Network. These workers demonstrated an extensive idiotypic connectivity between antibodies that make up the response to AChR and α-1,3-dextran, a determinant present on certain bacteria. They reported that idiotypic connections formed an elaborate network linking these two disparate antigen systems, and the

two responses were interconnected by key anti-idiotypes. They suggested that their findings may provide a model for the initiation of autoimmunity, which may develop as a consequence of perturbations of the normal operation of regulatory idiotypic networks.

There have been numerous studies of anti-idiotypic stimulation of the immune system in the absence of antigen. It has been shown that manipulation by anti-ids to non-antigen site idiotopes (Ab2alpha) as well as internal image (AB2beta) anti-ids can regulate the expression of antigen binding antibodies to, for example Hepatitis B surface antigen (Schick et al. 1987), Streptococcal group A carbohydrate (Monafo et al. 1987) and phosphorylcholine (Huang et al. 1988). These experiments demonstrate a second area in which researchers have sought to manipulate the immune system via the Idiotype Network, namely alternative vaccines.

Recent advances in molecular biology have led to new strategies for producing alternative vaccines. The determination of precise amino acid sequences has meant that either synthetic peptides or recombinant proteins can be used to replace conventional vaccines (reviewed by Steward and Howard 1987). An problem of using synthetic peptides or bacterially expressed recombinant proteins is their lack of glycosylation, an important factor when trying to elicit immunity to bacterial pathogens etc. A second consideration is the difficulties in establishing protective immunity in

infants against infections for which natural immunity does not develop until late in ontogeny. These two points have been addressed by several researchers, for example, McNamara et al. (1984) who found that immunisation with anti-idiotype protected mice from infection by Streptococcal pneumoniae; and Stein and Soderstrom (1984) who showed that neonatal administration of anti-idiotype primed for protection against Escherichia coli infection.

Anti-idiotypes have also been used to protect against parasitic infection such as Schistosoma mansoni (Gryzch et al. 1985) and Trypanosoma rhodesiense (Sacks et al. 1982). However, an anti-idiotype induced response to Trypanosoma cruzi did not confer active protection against the parasite (Sacks et al. 1985).

Also under investigation are potential anti-idiotype vaccines for viral infections. Using anti-idiotypic mabs to anti-poliovirus type II idiotypes, Uytdehaag and Osterhaus (1985) raised Ab3 antibodies that also bound to the polio virus. However this Ab3 response was not sufficient to protect against the virus as all mice primed with anti-idiotype succumbed to a challenge . An anti-idiotype vaccine providing immunity against hepatitis B virus (HBV) has been developed by Kennedy et al. (1986), who found that chimpanzees, when immunised with rabbit anti-idiotypes to anti-HBV idiotypes were protected against HBV infection. This latter example of an alternative vaccine may have been superceded by the routine use of a recombinant hepatitis B vaccine.

Idiotypic and anti-idiotypic antibodies are potentially powerful agents for anti-cancer therapy. Anti-idiotypes have been raised against the idiotypic determinants of the surface Ig of some lymphoid tumours. Injection of these anti-idiotypes into the patients resulted in some success in controlling the tumours (Meeker et al. 1985a; Stevenson et al. 1986). Problems associated with such treatment include swamping of anti-idiotype by excess serum idiotype, toxicity from idiotype-anti-idiotype complexes, and endocytosis of anti-idiotype bound to surface idiotype by the tumour. Although these problems may be overcome by various means, another difficulty is that tumours may escape treatment by mutation of the expressed idiotype, either spontaneously (Raffled et al. 1985), or anti-idiotypically induced (Meeker et al. 1985b). This is compounded by the need to "tailor-make" anti-idiotypes for each patient (Thielemans et al. 1984).

Experiments have shown that tumours may also be controlled using idiotypic antibodies to the tumour antigens themselves. It is thought that the idiotypes generate the formation of anti-idiotypes which then regulate the idiotype network in such a way that tolerance to the tumour is broken. The spread of the cancer is then controlled by natural antibodies triggered by internal image anti-idiotypes rather than by tumour antigen (Koprowski et al. 1984). Further results have shown that experimentally raised Ab3 antibodies were capable of in vitro lysis of tumour cells (Herlyn et al. 1987).

So far as autoimmunity goes the problem still remains of how to explain the break in self tolerance in terms of the Idiotype Network. Of course there are many other factors to consider in autoimmunity. Bottazzo et al. (1983) have proposed a scheme in which aberrant MHC class II expression can cause autoimmune endocrine diseases in individuals genetically predisposed by certain HLA haplotypes. As mentioned in Chapter 1 perturbations in the Idiotype Network could be lead to autoimmune disease (see Fig. 1.8). Idiotypes or anti-idiotypes to a foreign infection could coincidentally be the idiotypes to an autoantigen; or regulatory T cells are activated by cross-reactive idiotopes present on antibodies to both foreign and self antigen.

Perhaps the ultimate goal is the specific suppression of autoimmunity by idiotypic manipulation. Male (1986) has proposed ways in which autoimmune diseases could conceivably be manipulated idiotypically. At the B cell level the idea of direct blockade of autoantibody by anti-idiotype may not be such a profitable one, as problems associated with the formation and deposition of immune complexes have been envisaged. Another potential problem of idiotypic modulation of B cells is that, like the B cell neoplasms, the idiotype may mutate. This may not be a drawback in autoimmunity if by mutating the antibody looses its autoreactivity. However, suppression of one particular clone of idiotypes may allow a silent clone to take its place and continue the autoimmune response. In the study of Ebling et al. (1988) cited above, they sought to suppress the onset of nephritis in NZB/NZW F1

mice using anti-ids to a ReI. They found that although the appearance of the ReIs was delayed in those mice treated with anti-id, the ReIs eventually escaped from control and fatal nephritis ensued. The need to identify recurrent idiotypes that are also present on the pathogenic autoantibodies is yet another consideration, if one is hopeful of providing a widely available anti-idiotypic therapy.

For these reasons interest has turned to the T cell compartment. The autoimmune T cell response may well be more oligoclonal than the B cell response, and as the TCR is not thought to somatically mutate, idiotypic suppression of the TCR may be a more fruitful alternative of id-specific manipulation. Indeed, it has been shown in EAE that vaccination with attenuated or suboptimal doses of active autoimmune T cell clones can provide long term protection against the induction of EAE (Lider et al. 1988).

In the future, idiotypic-specific TCR targetted immunotherapy may be feasible for an autoimmune disease such as myasthenia gravis.

REFERENCES

ABDOU N. I., Wall H., Lindsley H. B., Halsey J. F. & Suzuki T. (1981) J. Clin. Invest. **67:** 1297-1304

AGIUS M. A. & Richman D. P. (1986) J. Immunol. **137:** 2195-2198

AGIUS M. A., Geannopoulos C. J., Fairclough R. H. & Richman D. P. (1988) J. Immunol.**140:** 62-68

ALLISON A. C. (1971) Lancet **ii:** 1401-1403

ALT F. W., Blackwell T. K. & Yancopoulos G. D. (1987) Science **238:** 1079-1087

AOKI T., Drachman D. B., Asher D. M., Gibbs C. J., Bahmanyar S. & Wolinski J. S. (1985) Neurology **35:** 185-192

BARKAS T., Mauron A., Roth B., Alliod C., Tzartos S. J. & Ballivet M. (1987) Science **235:** 77-80

BARKAS T., Gabriel J. M., Mauron A., Hughes G. J., Roth B., Alliod C., Tzartos S. J. & Ballivet M. (1988) J. Biol. Chem. **263:** 5916-5920

BARNETT A. H., Eff C., Leslie R. D. G. & Pyke D. A. (1981) Diabetologia **20:** 87-93

BATCHELOR J. R., Welsh K. I., Tinoco R. M., Dokery C. T., Hughes G. R. V., Bernstein R., Ryan P., Naish P. F., Aber G. M., Bing R. F. & Russel G. I. (1980) Lancet **i:** 1107-1109

BATCHELOR J. R. & Welsh K. I. (1982) in `Clinical Aspects of Immunology` (4th edn, Lachmann P. J. & Peters D. K. eds., Blackwell Press, Oxford) pp 283-306

BEHLKE M. A., Spinella D. G., Chou H. S., Sha W., Hartl D. L. & Loh D. Y. (1985) Science **229:** 566-570

BERZOFSKY J. A. (1985) Science **229:** 932-940

BERMAN P. W. & Patrick J. (1980) J. Exp. Med. **151:** 204-223

BILLINGHAM R. E., Brent L. & Medawar P. B. (1956) Nature **172:** 603-606

BJORKMAN P. J., Saper M. A., Samroui B., Bennett W. S., Strominger J. L. & Wiley D. C. (1987) Nature **329:** 506-511

BONA C. A., Victor-Kobrin C., Manheimer A. J., Bellon B. & Rubenstein L. J. (1984) Immunol. Rev. **79:** 25-44

BOTTAZZO G. F., Pujol-Borrel R., Hanafusa T. & Feldmann M. (1983) Lancet **ii:** 1115-1119

BOTTOMLY K. (1984) Immunol. Rev. **79:** 45-61

BRUCK C., Drebin J. A., Glineur C. & Portetelle D. (1986) Meths. Enzymol. **121:** 587-596

BUCKNELL R. C., Dixon A. St. J., Glick E. N., Woodland J. & Zutshi D. W. (1975) Br. Med. J. **1:** 600-602

BURNET F. M. (1959) `The Clonal Selection Theory of Acquired Immunity`, Vanderbilt University Press, Nashville, USA

CAMPBELL M., Bieber M., Levy R. & Teng N. N. H. (1986) J. Immunol. **136:** 2983-2988

CAPRA J. D. & Bona C. (1988) Immunol. Today **9:** 98-100

CHANG C. E. & Lee C. Y. (1962) Ann. Int. Pharmodyn. Ther. **144:** 241-257

CHOTHIA C. & Lesk A. M. (1987) J. Molec. Biol. **196:** 901-917

CLEVELAND W. L., Wassermann N. H., Sarangarajan R., Penn A. S. & Erlanger B. F. (1983) Nature **305:** 56-57

COHEN I. R. & Cooke A. (1986) Immunol. Today **7:** 363-364

COMPSTON D. A. S., Vincent A., Newsom-Davis J. & Batchelor J. R. (1980) Brain **103:** 579-601

CONTI-TRONCONI B. M. & Raftery M. A. (1982) Ann. Rev. Biochem. **51:** 491-530

CROWLEY J. J., Goldfrein R. D., Schrohenloher R. E., Spiegelberg H. L., Silverman G. J., Mageed R. A., Jefferis R., Koopman W. J., Carson D. A. & Fong S. (1988) J. Immunol. **140:** 3411-3418

DAVIS M. M. & Bjorkman P. J. (1988) Nature **334:** 395-402

DAWKINS R. L., Christiansen F. T., Kay P. H., Garlepp M., McCluskey J., Hollingsworth P. N. & Zilko P. J. (1983) Immunol. Rev. **70:** 5-22

DELVES P. J. & Roitt I. M. (1984) Clin. Exp. Immunol. **57:** 33-40

DeSHAMBO R. M. & Krolick K. A. (1986) J. Immunol. **137:** 3135-3139

DEVAUX C. A., Phillips M. L. & Delovitch T. L. (1984) J. Immunol. **133:** 2595-2602

DIAMOND B. & Scharff M. D. (1984) Proc. Natl. Acad. Sci. (USA) **81:** 5841-5844

DRESSER D. W. (1962) Immunology **5:** 161-168

DWYER D. S., Bradley R. J., Urquhart C. K. & Kearney J. F. (1983) Nature **301:** 611-614

DWYER D. S., Vakil M. & Kearney J. F. (1986) J. Exp. Med. **164:** 1310-1318

DWYER D. S. in Ann. N. Y. Acad. Sci. (1987) **505:** p 154

EBLING F. M., Ando D. G., Panosian-Sahakian N., Kalunian K. C. & Hahn B. H. (1988) J. Autoimmunity **1:** 47-61

EL-ROIEY A., Sela O., Isenberg D. A., Feldman R., Colaco B. C., Kennedy R. C. & Shoenfeld Y. (1987) Clin. Exp. Immunol.

67: 505-515

ENGEL A. G. & Arahata K. (1987) Ann. N. Y. Acad. Sci. **505:** 326-332

FAMBROUGH D. M., Drachman D. B. & Satyamurti S. (1973) Science **182:** 293-295

FINER-MOORE J. & Stroud R. M. (1984) Proc. Natl. Acad. Sci. (USA) **81:** 155-159

GARLEPP M. J., Dawkins R. L. & Christiansen F. T. (1983) Br. Med. J. **286:** 338-340

GEORGE S. & Schenck J. R. (1983) Anal. Biochem. **130:** 416-419

GOMEZ C. M. & Richman D. P. (1985) J. Immunol. **135:** 234-241

GOODNOW C. C., Crosbie J., Adelstein S., Lavoie T. B., Smith-Gill S. J., Brink R. A., Pritchard-Briscoe H., Wotherspoon J. S., Loblay R. H., Raphael K., Trent R. J. & Basten A. (1988) Nature **334:** 676-682

GORZYNSKI T. J., Krco C. J. & David C. S. (1985) J. Immunogenetics **12:** 267-279

GREENSPAN N. S. & Davie J. M. (1985) J. Immunol. **134:** 1065-1072

GRUBECK-LOEBENSTEIN B., Londei M., Greenall C., Pirich K., Kassal H., Waldhausl W. & Feldmann M. (1988) J. Clin. Invest. **81:** 1608-1614

GRZYCH J. M., Capron M., Lambert P. H., Dissous C., Turres S. & Capron A. (1985) Nature **316:** 74-76

HANAFUSA T., Pujol-Borrell R., Chiovato L., Russell R. C. G., Doniach D. & Bottazzo G. F. (1983) Lancet **ii:** 1111-1115

HARCOURT G. & Jermy A. (1987) Immunol. Today **8:** 319-322

HARCOURT G. C., Sommer N., Rothbard J., Willcox H. N. A. &

Newsom-Davis J. (1988) J. Clin. Invest. **82:** 1295-1300

HARRIS D. E., Cairns L., Rosen F. S. & Borel Y. (1982) J. Exp. Med. **156:** 567-584

HEININGER K., Hendricks M., Toyka K. & Kalb H. (1983) Muscle & Nerve **6:** 386-387

HEIDENREICH F., Vincent A., Roberts A. & Newsom-Davis J. (1988) Autoimmunity **1:** 285-297

HEMMI S., Fenner H., Binder C., Binz H., Winterhalter K. & Wigzell H. (1985a) Scand. J. Immunol. **21:** 565-574

HEMMI S., Fenner H., Binz H., Winterhalter K. & Wigzell H. (1985b) Scand. J. Immunol. **21:** 575-584

HERLYN D., Ross A. H., Iliopoulos D. & Koprowski H. (1987) Eur. J. Immunol. **17:** 1649-1652

HOHLFELD R., Toyka K. V., Heininger K., Grosse-Wilde H. & Kalies I. (1984) Nature **310:** 244-246

HORSFALL A. C. & Isenberg D. A. (1988) J. Autoimmunity **1:** 7-30

HUANG J-Y., Ward R. E. & Kohler H. (1988) Immunology **63:** 1-8

HUMPHREY J. H. & White R. G. (1970) Immunology for Medical Students (3rd. edition) Blackwell Scientific Publications, p 606

ISENBERG D. A., Shoenfeld Y., Madaio M. P., Rauch J., Reichlin M., Stollar B. D. & Schwartz R. S. (1984) Lancet **ii:** 417-422

ISENBERG D. A. & Shoenfeld Y (1988) Autoimmunity **1:** 67-75

IVERSON G. M & Lindenmann J. (1972) Eur. J. Immunol. **2:** 195-197

JERNE N. K. (1974) Ann. Immunol. (Paris) **125C:** 373-389

JERNE N. K., Roland J. & Cazenave P. A. (1982) EMBO J. **1:** 243-247

KAPPLER J. W., Roehm N. & Marrack P. (1987) Cell **49:** 273-280

KAPPLER J. W., Staerz U, White J. & Marrack P. C. (1988) Nature **332:** 35-40

KAO I. & Drachman D. B. (1977) Science **196:** 527-529

KEAY S., Rasmussen L. & Merigan T. C. (1988) J. Immunol. **140:** 944-948

KEESEY J., Lindstrom J. M., Cokely H. & Herrman C. (1977) N. Engl. J. Med. **296:** 55

KELSOE G., Reth M. & Rajewsky K. (1981) Eur. J. Immunol. **11:** 418-423

KENNEDY R. C., Eichberg J. W., Lanford R. E. & Dreesman G. R. (1986) Science **232:** 220-223

KIEBER-EMMONS T. & Kohler H. (1986) Immunol. Rev. **90:** 29-48

KILLEN J. A. & Lindstrom J. M. (1984) J. Immunol. **133:** 2549-2553

KILLEN J. A., Hochschwender S. M. & Lindstrom J. M. (1985) J. Neuroimmunol. **9:** 229-241

KIRCHNER T., Hoppe F., Muller-Hermelink H. K., Schalke B. & Tzartos S. (1987) Lancet **i:** 218

KLAVINSKIS L. S., Willcox N., Oxford J. & Newsom-Davis J. (1985) Neurology **35:** 1381-1384

KOHLER G. & Milstein C. (1975) Nature **256:** 495-497

KOHNO Y., Berkower I., Minna J. & Berkofsky J. A. (1982) J. Immunol. **128:** 1742-1748

KOPROWSKI H., Herlyn D., Lubeck M., DeFreitas E. & Secir H. F. (1984) Proc. Natl. Acad. Sci. (USA) **81:** 216-219

KUBALEK E., Ralston S., Lindstrom J. M. & Unwin N. (1987) J. Cell Biol. **105:** 9-18

KUNKEL H. G., Mannik M. & Williams R. C.(1963) Science **140:** 1218-1219

LANG B., Vincent A. & Newsom-Davis J. (1982) J. Immunol. Methods. **51:** 371-381

LANG B., Newsom-Davis J., Prior C. & Wray D. (1984) J. Physiol. **357:** 28P

LANG B., Roberts A. J., Vincent A. & Newsom-Davis J. (1985) Clin. Exp. Immunol. **60:** 637-644

LEFVERT A. K. (1981) Scand. J. Immunol. **13:** 493-497

LEFVERT A. K. (1988) J. Autoimmunity **1:** 63-72

LEFVERT A. K., Pirkanan R. & Svanborg E. (1985) J. Neuroimmunol. **9:** 41-53

LEFVERT A. K., Sunden H. & Holm G. (1986) Scand. J. Immunol. **23:** 655-662

LENNON V. A., Lindstrom J. M. & Seybold M. E. (1975) J. Exp. Med. **141:** 1365-1375

LENNON V. A. & Lambert E. H. (1980) Nature **285:** 238-240

LENNON V. A. & Lambert E. H. (1981) Ann. N.Y. Acad. Sci. **377:** 77-96

LIDER O., Reshef T., Bernaud E., Ben-Nun A. & Cohen I. R. (1988) Science **239:** 181-183

MacDONALD H. R., Schneider R., Lees R. K., Howe R. C., Acha-Orbea H., Festenstein H, Zinkernagel R. M. & Hengartner H. (1988) Nature **332:** 40-45

MALE D. K. (1986) Clin. Exp. Immunol. **65:** 1-9

MALE D., Pryce G., Quartey-Papafio R. & Roitt I. (1983) Eur.

J. Immunol. **13:** 942-947

MALE D., Pryce G. & Roitt I. (1985) Molecular Immunol. **22:** 255-263

MARRACK P. & Kappler J. (1987) Science **238:** 1073-1079

MATSUYAMA T., Fukumori J. & Tanaka H. (1983) Clin. Exp. Immunol. **51:** 381-386

McNAMARA M. K., Ward R. E. & Kohler H. (1984) Science **233:** 1325-1326

MEEKER T. C., Lowder J., Maloney D. G., Miller R. A., Thielemans K., Warnke R. & Levy R. (1985a) Blood **65:** 1349-1363

MEEKER T. C., Lowder J., Cleary M. L., Stewart S., Warnke R., Sklar J. & Levy R. (1985b) N. Engl. J. Med. **312:** 1654-1665

METZGER D. W., Furman A., Miller A. & Sercarz E. E. (1981) J. Exp. Med. **154:** 701-712

MOELLER G. (1988) Scand. J. Immunol. **27:** 247-250

MONAFO W. J., Greenspan N. S., Cebra-Thomas J. A. & Davie J. M. (1987) J. Immunol. **139:** 2702-2707

MORGAN A., Isenberg D. A., Naparstek Y., Rauch J., Duggan D., Khiroya R., Staines N. A. & Schattner A. (1985) Immunology **56:** 393-399

MOURAD W., Pelletier G. & Hebert J. (1988) Immunology **63:** 397-401

NEWSOM-DAVIS J., Pinching A. J., Vincent A. & Wilson S. G. (1978) Neurology **28:** 266-272

NIELSEN F. C. Rodgaard A., Djurup R., Somnier F & Gammeltoft S. (1985) J. Immunol. Methods **83:** 249-258

NODA M., Furutami Y., Takahashi H., Toyosato M., Tanabe T., Shimizu S., Kikyotani S., Kayano T., Hirose T., Mayama S. & Numa S. (1983) Nature **305:** 818-823

NOSSAL G. J. V. (1983) A. Rev. Immunol. **1:** 33-62

NUSSENBLATT R. B. & Silverstein A. M. (1985) in "The Autoimmune Diseases" (eds. N. R. Rose & I. R. Mackay, Academic Press) pp371-398

OUDIN J. & Michel M. (1963) C. R. Hebd. Seanc. Acad. Sci. (Paris) **257:** 805-808

OWEN R. D. (1945) Science **102:** 400-401

PACHNER A. R., Sourojon M. & Fuchs S. (1986) J. Neuroimmunol. **12:** 205-214

PATRICK J. & Lindstrom J. M. (1973) Science **180:** 871-872

PAUL W. E. & Bona C. (1982) Immunol. Today **3:** 230-234

PHILLIPS M. L., Harris J. F. & Delovitch T. L. (1984) J. Immunol. **133:** 2587-2594

PINCHING A. J. Peters D. K. & Newsom-Davis (1976) Lancet **ii:** 1373-1376

PLOTZ P. H. (1983) Lancet **ii:** 824-826

RAFFLED M., Neckers L., Longo D. L. & Cossman J. (1985) N. Engl. J. Med. **312:** 1653-1658

RAJEWSKY K. & Takemori T. (1983) A. Rev. Immunol. **1:** 569-607

ROBERTS A., Perera S., Lang B., Vincent A. & Newsom-Davis J. (1985) Nature **317:** 737-739

SACKS D. L., Esser K. M. & Sher A. (1982) J. Exp. Med. **155:** 1108-1119

SACKS D. L., Kirchoff L. V., Hieny S. & Sher A. (1985) J. Immunol. **135:** 4155-4163

SCADDING G. K., Vincent A., Newsom-Davis J. & Henry K. (1981) Neurology **31:** 935-943

SCHECHTER Y., Maron R., Elias D. & Cohen I. R. (1982) Science **216:** 542-545

SCHICK M. R., Dreesman G. R. & Kennedy R. C. (1987) J. Immunol. **138:** 3419-3425

SCHLUEP M., Willcox N., Vincent A., Dhoot G. K. & Newsom-Davis J. (1987) Ann. Neurol. **22:** 212-222

SCHWARTZ M., Novick D., Givol D. & Fuchs S. (1978) Nature **273:** 543-545

SHIBAHARA S., Kubo T., Perski H. J., Takahashi H., Noda M. & Numa S. (1985) Eur. J. Biochem. **146:** 15-22

SHOENFELD Y., Isenberg D. A., Rauch J., Madaio M. P., Stollar B. D. & Schwartz R. S. (1983) J. Exp. Med. **158:** 718-730

SIMPSON J. A. (1960) Scot. Med. J. **5:** 419-436

SOMMER N., Harcourt G., Willcox N., Nagvekar N & Newsom-Davis J. (1988) J. Neurology (Suppl.) **235:** 62

SOUROUJON M., Barchan D. & Fuchs S. (1985) Immunol. Lett. **9:** 331-336

SOUROUJON M., Pachner A. R. & Fuchs S. (1986) Neurology **36:** 622-625

SPITZ M., Spitz L., Thorpe R. & Eugui (1984) J. Immunol. Methods. **70:** 39-43

STEFFANSON K., Dieperink M. E., Richman D. P., Gomez C. M. & Marton L. S. (1985) N. Engl. J. Med. **312:** 221-225

STEIN K. E. & Soderstrom T (1984) J. Exp. Med. **160:** 1001-1011

STEVENSON F, K., Wrightham M., Glennie M. J., Jones D. B., Cattan A. R., Feizi T., Hamblin T. J. & Stevenson G. T. (1986) Blood **68:** 430-436

STEWARD M. W. & Howard C. R. (1987) Immunol. Today **8:** 51-58

STREICHER H. Z., Cuttitta F., Buckenmeyer G. K., Kawamura H., Minna J. & Berzofsky J. A. (1986) J. Immunol. **136:** 1007-1014

STRICKLAND F., Gleason J. T. & Cerny J. (1987) Molecular Immunol. **24:** 631-635

TAKEMORI T., Tesch H., Reth M. & Rajewsky K. (1982) Eur. J. Immunol. **12:** 1040-1046

TEH H. S., Kiesielow P., Scott B., Kishi H., Uematsu Y., Bluthmann H. & von Boehmer H. (1988) Nature **335:** 229-233

THANAVALA Y. M., Bond A., Tedder R., Hay F. C. & Roitt I. M. (1985) Immunology **55:** 197-204

THANAVALA Y. M., Moore M. J., Tedder R., Hay F. C. & Roitt I. M. (1988) Immunology **63:** 575-577

THIELEMANS K., Maloney D. G., Meeker T., Fujimoto J., Doss C., Warnke R. A., Bindl J., Gralow J., Miller R. A. & Levy R. (1984) J. Immunol. **133:** 495-501

TODD J. A., Bell J. I. & McDevitt H. O. (1987) Nature **329:** 599-604

TONEGAWA S (1983) Nature **302:** 575-581

TOYKA K. V., Drachman D. B., Pestronk A. & Kao I. (1975) Science **190:** 397-399

TZARTOS S. J. & Lindstrom J. M. (1980) Proc. Natl. Acad. Sci. (USA) **77:** 755-759

TZARTOS S. J., Seybold M. E. & Lindstrom J. M. (1982) Proc.

Natl. Acad. Sci. (USA) **79:** 188-192

TZARTOS S. J., Sophianos D. & Efthimiadis A. (1985) J. Immunol. **134:** 2343-2349

TZARTOS S. J., Hochschwender S., Vasquez P. & Lindstrom J. M. (1987) J. Neuroimmunol. **15:** 185-194

TZARTOS S. J., Kokla A., Walgrave S. L. & Conti-Tronconi B. M. (1988) Proc. Natl. Acad. Sci. (USA) **85:** 2899-2903

UNANUE E. R. (1984) A. Rev Immunol. **2:** 395-

UYTDEHAAG F. G. C. M. & Osterhaus A. D. M. E. (1985) J. Immunol. **134:** 1225-1229

VINCENT A. (1988) J. Autoimmunity **1:** 131-142

VINCENT A. & Newsom-Davis J. (1982) Clin. Exp. Immunol. **49:** 257-265

VINCENT A. & Bilkhu M. (1982) J. Immunol. Methods **51:** 359-369

VOGEL Z., Sytkowski A. J. & Nirenberg M. W. (1972) Proc. Natl. Acad. Sci. (USA) **69:** 3180-3184

WEIGLE W. O. (1971) Clin. Exp. Immunol. **9:** 437-447

WHITING P. (1984) PhD Thesis, University of London.

WHITING P. J., Vincent A. & Newsom-Davis J. (1985) Eur. J. Biochem. **150:** 533-539

WHITING P. J., Vincent A. & Newsom-Davis (1986a) Neurology **36:** 612-617

WHITING P. J., Vincent A., Schluep M. & Newsom-Davis J. (1986b) J. Neuroimmunol. **11:** 223-235

WIKLER M. , Franssen J. D., Collignon C., Leo O., Mariame B., Van de Walle P., De Groote D. & Urbain J. (1979) J. Exp. Med. **150:** 184-195

WILLCOX N. & Vincent A. (1988) in "The B lymphocyte in human disease". (eds A. G. Bird & J. E. Calvert, Oxford University Press, Oxford) pp 469-506

WILLCOX H. N. A., Newsom-Davis J. & Calder L. (1983) Clin. Exp. Immunol. **54:** 378-386

WILSON S., Vincent A. & Newsom-Davis J. (1983) J. Neurol. Neurosurg. Pschiatry **46:** 377-382

WOOD H. (1988) PhD. Thesis, University of London.

WU T. T. & Kabat E. A. (1970) J. Exp. Med. **132:** 211-250

ZAMVIL S., Nelson P., Trotter J., Mitchell D., Knobler R., Fritz R. & Steinmann L. (1985) **317:** 355-358

ZANETTI M. (1985) Immunol. Today **6:** 299-302

ZANETTI M., DeBaets M. & Rogers J. (1983) J. Immunol. **131:** 2452-2457

ZANETTI M., Rogers J. & Katz D. H. (1984) J. Immunol. **133:** 240-243

ZUBERI R. I., Katz D. H. & Zanetti M. (1988) J. Autoimmunity **1:** 31-46

APPENDIX ONE: CALCULATION OF DATA USED IN FIGURE 4.2

anti-human AChR mab	[A] cpm ^{125}I-BuTx-AChR precipitated	[B] average cpm	[C] corrected cpm
C2 $(F(ab')_2)$	4325 4665	4495	4085
C2 (whole mab)	5243 5036	5139	4729
C9 $(F(ab')_2)$	4727 4604	4666	4256
C9 (whole mab)	5036 4756	4896	4486
F8 $(F(ab')_2)$	4712 4600	4656	4246
F8 (whole mab)	6250 6144	6197	5787
B3 $(F(ab')_2)$	4500 4416	4458	4048
B3 (whole mab)	5892 6012	5952	5542
C3 $(F(ab')_2)$	4517 4342	4430	4020
C3 (whole mab)	5453 5532	5493	5083
C7 $(F(ab')_2)$	3738 3384	3561	3157
C7 (whole mab)	5140 5651	5396	4986
normal mouse serum	363 456	410	
positive anti-human AChR serum	8867 9353	9100	8700

[A] cpm ^{125}I-BuTx-AChR precipitated by limiting amounts of each mab $(F(ab')_2$ or whole mab) in duplicate

[B] average cpm of above counts

[C] corrected cpm = average cpm - cpm precipitated by normal mouse serum alone

total cpm of ^{125}I-BuTx-AChR precipitable in assay is given by the positive anti-human AChR serum

APPENDIX ONE CONTINUED

idiotype ($F(ab')_2$ or mab)	anti-id	volume (µl)	E cpm	F corrected cpm	G % inhibition
C2 $F(ab')_2$	anti-C2	0.010	4149	3739	8
"	"	0.025	4197	3787	7
"	"	0.100	2738	2328	43
C2 mab	"	0.010	4941	4531	4
"	"	0.025	4404	3994	16
"	"	0.100	2669	2259	52
C9 $F(ab')_2$	anti-C9	0.0010	4155	5745	12
"	"	0.0025	2589	2179	49
"	"	0.0100	2302	1892	55
C9 mab	"	0.0010	4896	4486	0
"	"	0.0025	3897	3487	22
"	"	0.0100	1499	1089	76
F8 $F(ab')_2$	anti-F8	0.05	4491	4081	4
"	"	0.20	3727	3317	22
"	"	1.00	1856	1446	66
F8 mab	"	0.05	5076	4666	19
"	"	0.20	5393	4983	14
"	"	1.00	1846	1436	75
B3 $F(ab')_2$	anti-B3	0.010	4547	4137	0
"	"	0.025	4563	4153	0
"	"	0.100	3461	3051	25
B3 mab	"	0.010	5633	5223	6
"	"	0.025	3590	3180	43
"	"	0.100	2075	1665	70
C3 $F(ab')_2$	anti-C3	0.05	4620	4210	0
"	"	0.20	3710	3300	18
"	"	1.00	450	40	99
C3 mab	"	0.05	5390	4980	2
"	"	0.20	2395	1985	61
"	"	1.00	425	15	100
C7 $F(ab')_2$	anti-C7	0.01	3020	2610	17
"	"	0.02	2412	2002	36
"	"	0.05	2263	1853	41
"	"	0.10	1577	1167	63
C7 mab	"	0.01	4896	4486	10
"	"	0.02	3971	3561	29
"	"	0.05	3438	3028	39
"	"	0.10	2730	2320	54

idiotype, in limiting amounts (see overleaf) was preincubated with increasing amounts of anti-id before the addition of ^{125}I-BuTx-AChR.

E_{cpm} = ^{125}I-BuTx-AChR precipitated by idiotype preincubated with anti-id.

$F_{\text{corrected cpm}}$ = cpm - cpm precipitated by normal mouse serum alone (410cpm)

$$G_{\%\text{ inhibition}} = \frac{\text{value of column C - value of column F}}{\text{value of column C}} \times 100$$

for each idiotype - anti-id interaction

APPENDIX TWO: CALCULATION OF DATA USED IN FIGURE 4.3

anti-human AChR mab	A cpm ^{125}I-BuTx-AChR precipitated	B average cpm	C corrected cpm
C2	4707 4734	4721	4271
C9	5036 4756	4896	4446
F8	2751 2636	2694	2244
B3	4952 4741	4847	4397
C3	4405 4452	4429	3979
C7	5529 5773	5651	5201
normal mouse serum (1µl)	520 394	450	
positive anti-human AChR serum	9880 10031	9956	9506

A cpm of ^{125}I-BuTx-AChR precipitated by limiting amounts of each mab (in duplicate)

B average of duplicate values

C corrected cpm = average cpm - cpm precipitated by 1µl normal mouse serum alone

APPENDIX TWO CONTINUED

D idiotype	anti-id	volume of anti-id (µl)	E cpm	F corrected cpm	G % inhibition
C2 +	anti-C2	0.010	4506	4056	5
C2 +	"	0.025	3726	3276	23
C2 +	"	0.100	1859	1409	67
C2 −	"	0.010	4254	3804	11
C2 −	"	0.025	4353	3903	9
C2 −	"	0.100	2252	1802	58
C9 +	anti-C9	0.0010	4453	4003	10
C9 +	"	0.0025	4017	3567	20
C9 +	"	0.0100	1628	1178	74
C9 −	"	0.0010	4896	4446	0
C9 −	"	0.0025	3897	3447	22
C9 −	"	0.0100	1499	1049	76
F8 +	anti-F8	0.010	2967	2517	0
F8 +	"	0.025	2693	2243	0
F8 +	"	0.100	2100	1650	26
F8 −	"	0.010	2702	2252	0
F8 −	"	0.025	2826	2376	0
F8 −	"	0.100	2171	1721	23
B3 +	anti-B3	0.010	4324	3874	12
B3 +	"	0.025	2853	2403	45
B3 +	"	0.100	1316	866	80
B3 −	"	0.010	4169	3719	15
B3 −	"	0.025	2862	2412	45
B3 −	"	0.100	1244	794	82
C3 +	anti-C3	0.010	4973	4523	0
C3 +	"	0.025	4184	3734	6
C3 +	"	0.100	3971	3521	12
C3 −	"	0.010	4584	4134	0
C3 −	"	0.025	5006	4556	0
C3 −	"	0.100	3683	3233	19
C7 +	anti-C7	0.010	5006	4556	12
C7 +	"	0.025	4550	4100	21
C7 +	"	0.100	2594	2144	59
C7 −	"	0.010	5138	4688	10
C7 −	"	0.025	4295	3845	26
C7 −	"	0.100	2379	1929	63

D denotes idiotype preincubated (+) or not preincubated (−) with 1µl normal mouse serum before the addition of increasing amounts of anti-id

E cpm = ^{125}I-BuTx-AChR precipitated by idiotype in the presence of anti-id

F corrected cpm = cpm − cpm precipitated by 1µl normal mouse serum alone (450)

G % inhibition = $\frac{\text{value of column C} - \text{value of column F}}{\text{value of column C}} \times 100$

for each idiotype − anti-id interaction