The Avidity of Human IgG Subclasses

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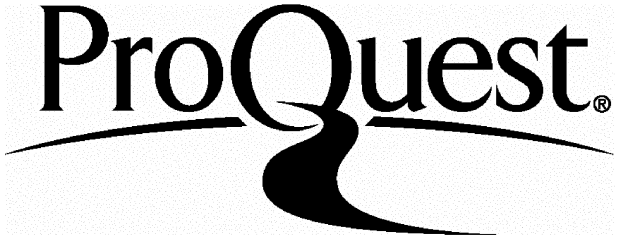

ABSTRACT

The four human IgG subclasses display greater than 90% sequence homology and their production is antigen restricted, with IgG1 and IgG3 being preferentially elicited against protein antigens and IgG2 against carbohydrate antigens. Although the titre of a particular subclass is clearly important in overcoming infection, it is becoming apparent that the functional affinity (avidity) of such a response is also crucial. These studies were therefore initiated to investigate aspects of antibody avidity of the four human IgG subclasses.

Utilising V region identical mouse-human IgG subclasses avidity differences were found by solid-phase avidity ELISA and biospecific interaction analysis (BIA). IgG4 was consistently of highest avidity and furthermore this reflected differences in dissociation rate constants. Following removal of the constant region such differences were abolished suggesting an involvement for the constant region in the control of human IgG subclass avidity.

Serum IgG subclass avidity specific for pneumococcal polysaccharide serotypes 3, 6, 19 and 23 were measured by solid-phase avidity ELISA following pneumococcal vaccination. A complex pattern of avidity emerged with serotype 3 specific antibodies generally being of higher avidity. Significant differences were obtained comparing serum antibodies from children below 2 years of age with children more than 2 years of age. Such differences may contribute to the higher incidence of bacterial infection observed in children less than 2 years of age.

Another aspect of IgG subclass avidity which has received little attention, is the control of affinity maturation. One possible area of regulation addressed in this study was the control of antibody affinity by cytokines. The effect of various

cytokines on antigen specific total IgG avidity utilising solid-phase avidity assays developed throughout this thesis. Preliminary evidence presented in this thesis suggest that the cytokines IL-6, IL-10, TNFα and IFNγ do not influence antibody avidity.

The development of solid phase avidity ELISAs and BIA opens up an exciting area of biology and future investigations should answer many of the remaining questions concerning human IgG subclass avidity.

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CONTENTS

CHAPTER 3: THE AVIDITY OF MOUSE-HUMAN CHIMERIC V REGION IDENTICAL MONOCLONAL IgG SUBCLASSES

CHAPTER 4: BINDING KINETICS OF MOUSE-HUMAN CHIMERIC V REGION IDENTICAL IgG SUBCLASSES

CHAPTER 5: THE AVIDITY OF IgG SUBCLASSES SPECIFIC FOR PNEUMOCOCCAL POLYSACCHARIDES

CHAPTER 6: THE EFFECT OF VARIOUS CYTOKINES ON SPECIFIC IN VITRO IgG AVIDITY

CHAPTER 7: GENERAL DISCUSSION

APPENDICES

List of Figures

List of tables

ABBREVIATIONS

Ab	antibody
Abs	absorbance
AET	S-2-aminoethylisothiouronium bromide
AFC	antibody forming cell
Ag	antigen
APC	antigen presenting cell
BCR	B cell receptor
BIA	biospecific interaction analysis
C	constant
CD	cluster of differentiation
CDR	complimentarity determining region
CTL	cytotoxic T lymphocyte
CWPS	cell wall polysaccharide
D	diversity
DNA	deoxyribonucleic acid
EBV	epstein barr virus
ELISA	enzyme linked immunosorbent assay
Fab	fragment antigen binding
Fc	fragment crystalline
FCS	foetal calf serum
FDC	follicular dendritic cell
FPLC	fast protein liquid chromatography
H	heavy chain
Hib	*Haemophilus influenzae* type b
HIV	human immunodeficiency virus
IFN	interferon
IGF	insulin growth factor
IgG	Immunoglobulin G
IL	interleukin
J	joining
K	equilibrium constant
k_a	association rate constant
k_d	dissociation rate constant
KLH	keyhole limpet hemocyanin
L	light chain
m∅	macrophage
mAb	monoclonal antibody
MHC	major histocompatibility complex
MPO	myeloperoxidase
NIP	4-hydroxy-3-iodo-5-nitrophenylacetyl
NK	natural killer
PBEF	pre B cell colony enhancing factor
PBS	phosphate buffered saline
PBSF	pre B cell growth stimulatory factor
PBS-Tw	phosphate buffered saline/0.5% Tween20

PBS-Tw-BSA	phosphate buffered saline/0.5% Tween20/1% BSA
PRP	polyribosylribitolphosphate
PS	polysaccharides
RNA	ribonucleic acid
RU	resonance unit
S	switch region
SCF	stem cell factor
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
sIg	surface immunoglobulin
SPR	surface plasmon resonance
φL	surrogate light chain
TCR	T cell receptor
T_{dep}	T dependent
TGFβ	transforming growth factor β
T_H	T helper lymphocyte
T_H1	T helper lymphocyte type 1
T_H2	T helper lymphocyte type 2
T_{ind}	T independent
TMC	tonsil mononuclear cell
TNF	tumour necrosis factor
T_S	T supressor lymphocyte
V	variable
VIP	vasoactive intestinal peptide
XLA	X-linked agammaglobulinemia

CHAPTER 1

General Introduction

INTRODUCTION

The Immune System

The immune system provides an individual with protection from infection by pathogens such as viruses, bacteria and parasites. The quality of the response depends upon the ability of the host to discriminate between self and non-self antigens and subsequently to elicit an appropriate immune response with which to eliminate potential pathogens. The immune system must, therefore, be carefully controlled since a breakdown in regulation may lead to disease.

Cells of the Immune System

Cellular (cell mediated), humoral (antibody mediated) and innate immunity work together to protect an individual from invasive pathogens. Immune responses are mediated primarily by lymphocytes and phagocytes, which arise from a mulipotent haematopoeitic stem cell(s).

Cellular immunity involves the cell mediated killing of pathogens by immune cells, such as neutrophils, macrophages (mϕ), natural killer (NK) cells, basophils, eosinophils and cytotoxic T lymphocytes (CTLs). Humoral immunity involves antibodies or immunoglobulins, produced by B lymphocytes (antibody producing plasma cells). The immunoglobulins bind to antigen and enhance phagocytosis of such antigen-antibody immune complexes. In addition, immunoglobulins work in association with complement (innate immunity) to promote complement mediated killing mechanisms.

T Lymphocytes

T lymphocytes originate in the bone marrow, develop and differentiate in the thymus and then travel through the circulation to primary and secondary lymphoid tissues. It is within such tissues that, by virtue of the T cell receptor (TCR), they recognise antigen expressed on an antigen presenting cell (APC), in association with MHC molecules. The TCR consists of an antigen binding portion formed by heterodimers of α and β or γ and δ polymorphic polypeptide chains. The TCR itself does not mediate cellular signalling pathways; these are elicited by an associated molecule, CD3, which consists of a complex of polypeptides. T lymphocytes are distinguished from other cells by the characteristic cell surface marker, CD3 man, whilst in the mouse, Thy1 is a characteristic marker.

T lymphocytes may be further divided into several subsets designated T helper (T_H), T cytotoxic (CTL) and T supressor (T_S) cells. CTLs are involved in cell mediated cytotoxicity and are characterised primarily by the expression of CD8. T_H cells provide help for B lymphocytes during an immune response via cell-cell contact and the production of soluble mediators, and they are characterised by the expression of CD4. T_S cells act by downregulating the immune response but, as yet no specific cell surface marker has been identified and their existence as a specific T cell subset remains controversial.

B Lymphocytes

B lymphocytes develop in the bone marrow and foetal liver. Mature B lymphocytes are responsible for immunoglobulin production which, when membrane bound, forms the characteristic B cell receptor (BCR). B lymphocytes also express MHC class II molecules and therefore may act as APCs, presenting antigen to CD4+ T lymphocytes. In addition, B lymphocytes have on their surface a variety of other markers, such as Fc receptors and CD40, which are essential for cell activation and proliferation.

The B cell receptor is specific for antigen and for the majority of antibody responses T cell help is also required. Protein antigens, which depend directly on T cell help in the form of cell-cell contact or cytokines, are referred to as T cell dependent (T_{dep}) antigens. Those antigens which do not require direct T cell help are designated T cell independent (T_{ind}) and are primarily carbohydrates. T_{ind} antigens are thought to stimulate B lymphocytes directly. Following interaction with antigen, providing that they receive the appropriate signals, B lymphocytes clonally expand and differentiate into antibody forming cells (AFC). Furthermore, long lived B lymphocytes, referred to as memory cells, may be produced and these retain the ability to produce specific antibody in response to subsequent antigen exposure.

Specific antibody is crucial in overcoming many infections. This is achieved by the antibody binding to antigen expressed on a pathogen and forming a bridge with the appropriate effector cell via Fc receptor binding. The type of effector response depends upon the isotype and subclass of the antibody produced.

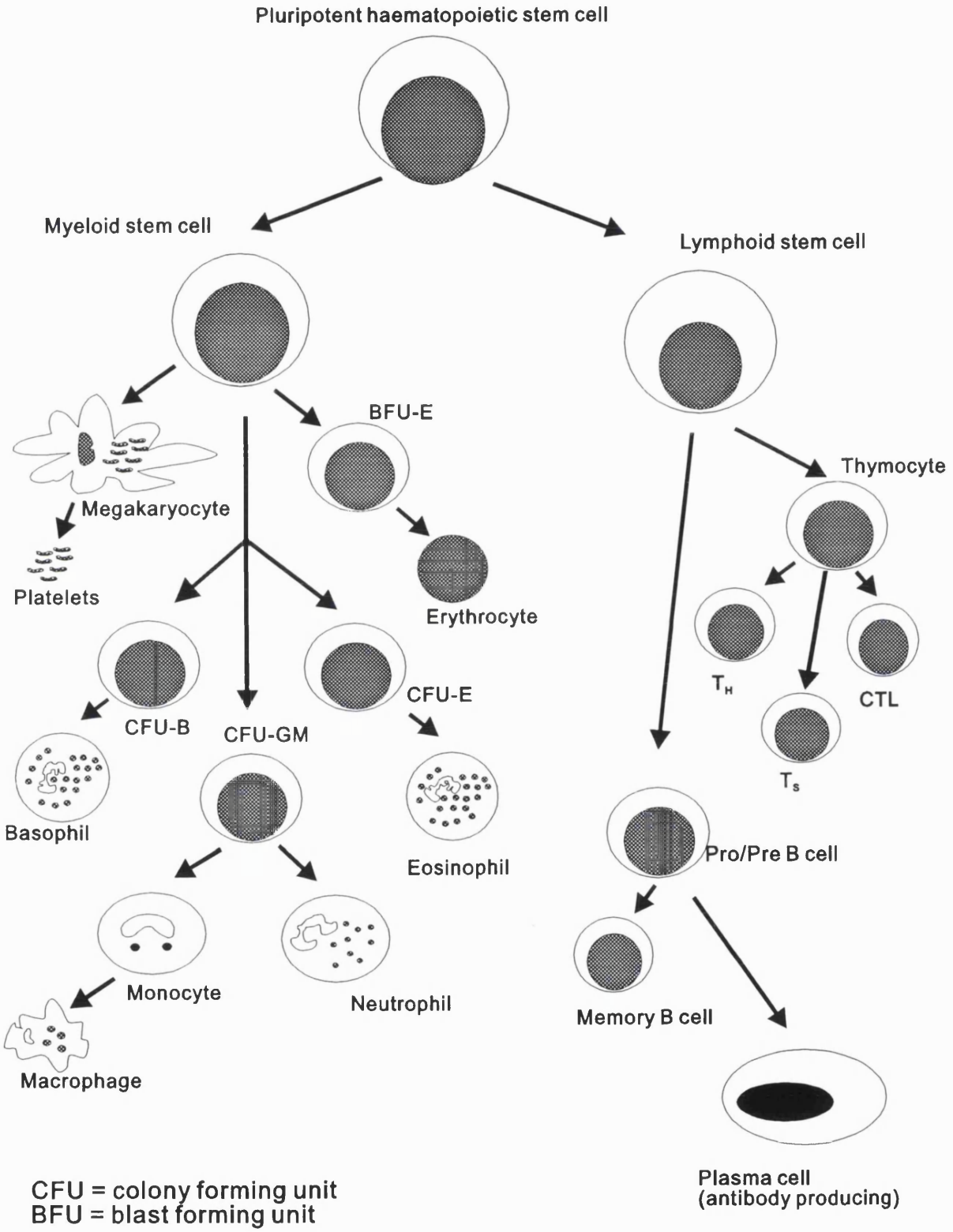


Figure 1.1 Cells Involved in The Immune System

B Cell Development

Lymphocyte development proceeds through various stages characterised by specific gene rearrangements and cell surface marker expression (Tonegawa, 1983; Alt et al. 1987), resulting in mature B and T cells expressing antigen specific immunoglobulin or TCR. It has been estimated that once the immune system is established there are approximately 5 x 10^{12} cells in the human B cell compartment, 10% of which are regenerating precursors and 90% resting mature B cells (Rolink and Melchers, 1993).

Different schemes exist for the nomenclature of B cell progenitors and precursors. Throughout this discussion the following nomenclature will be used: pro B cell, pre B-I cell, pre B-II cell, immature and mature B cell (Figure 1.2)

Pro B cells

Pro B cells are the earliest cells committed to the B cell lineage and may be defined as having immunoglobulin heavy (H) and light (L) chains in germline configuration, but have the ability to differentiate on stromal cells in the presence of IL-7 (Rolink and Melchers, 1993). The characterisation of early committed human lymphoid progenitors, however, remains elusive. The earliest human precursors committed to the B cell lineage identified so far express CD19, CD34, CD10, TdT, VpreB and do not express μH (reviewed by Melchers et al. 1995).

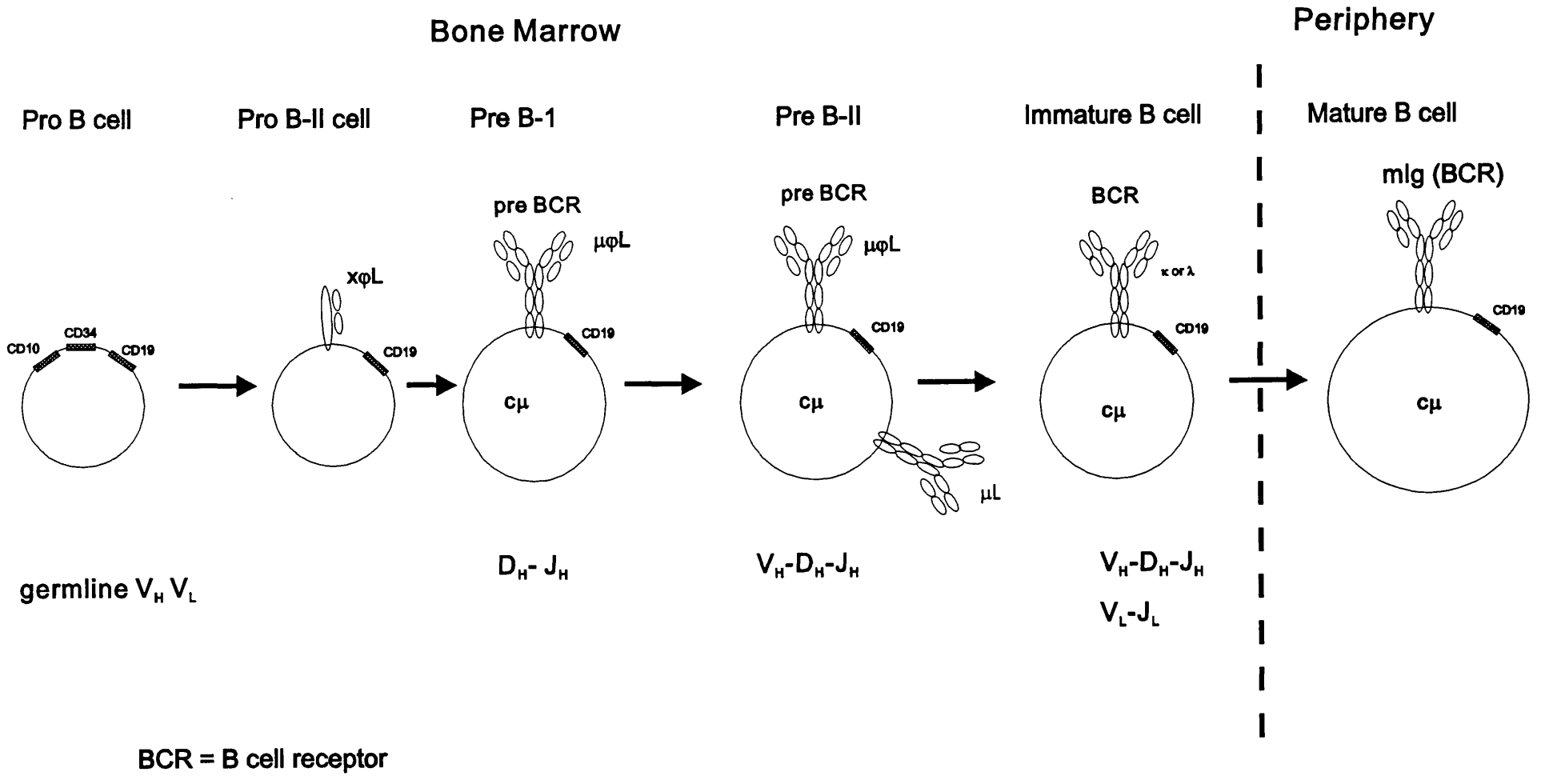


Figure 1.2 Human B cell development

Diagram showing gene rearrangements and immunoglobulin expressions during human B cell developmental stages, adapted from (Guelpa Fonlupt et al. 1994)

Pre B cells

B cell development is characterised by sequential immunoglobulin gene rearrangements, which begin in the heavy chain locus of pre B-I cells, in the D_H to J_H segments. In the mouse, pre B-I cells may be defined as having long term proliferative capacity on stromal cells in the presence of Il-7 and have undergone D_HJ_H rearrangements, whilst retaining germline L chain genes.

Bone marrow derived human B cell precursors grow in the presence of IL-7 similarly to mouse pro/pre B-I cells (Melchers et al. 1995). The cell-cell contact between human precursor B cells and stromal cells has been identified as CD49d/CD29 - CD106, and this interaction has furthermore been demonstrated to enhance IL-6 production which promotes B cell growth (Jarvis and LeBien, 1995). Such signals presumably enable the pro/pre B-I cells to proliferate and it is thought that loss of stromal contact stimulates pro/pre B-I cells to further differentiate.

In mice, large pre B-II cells have at least one of their IgH loci $V_HJ_HD_H$ rearranged. Expression of this μH chain cannot occur alone and is expressed in association with the surrogate L chain (φL), a heterodimer of the protein products of the *VpreB* and *λ5* genes, referred to as the pre B cell receptor (pre BCR). Mouse pro/pre B-1 cells express the φL chain in the cell cytoplasm or on the surface in association with the recently identified glycoproteins, gp130 and gp35-65 (Karasuyama et al. 1993; Melchers et al. 1994). The function for φL/gp130/gp35-65 is as yet unclear but may facilitate the rapid expression of the pre B cell receptor as soon as productive $V_HJ_HD_H$ rearrangements have occurred.

The pre B cell receptor appears to differentially signal to promote allelic exclusion (productive rearrangement of only one allele) and proliferation of pre B-II cells. At present, candidate ligands for the pre B cell receptor remain elusive (Melchers et al. 1995). Human B lineage cells express the φL chain throughout pro and pre B cell development but cell surface expression is confined to the pre B stages (Lassoued et al. 1993).

Immature B cells

Immature B cells are both $V_HD_HJ_H$ and V_LJ_L rearranged. L chain rearrangements are believed to occur in κ genes preferentially. Mouse immature bone marrow cells generated in vitro express κ:λ ratios of 10-20:1 (Ghia et al. 1995). Previously, it was believed that the rearrangement of L chain genes was arrested on autoreactive B cells. However, recent evidence following long term culture of bone marrow mouse B cells, has indicated that expression of L chains does not terminate L chain rearrangement. Sequential order exists in continued L chain rearrangements with κ giving way to λ expression but not vice versa and the cell can become surface immunoglobulin $(sIg)^-$ in both mice and humans (Ghia et al. 1995). Such secondary light chain rearrangements would be advantageous in rescuing sIg^+ cells when their sIg is autoreactive (Tiegs et al. 1993; Gay et al. 1993; Radic et al. 1993). This process is referred to as Ig receptor editing.

The role of cytokines

Various cytokines have been implicated in the control of B cell development. Growth and differentiation of pro and pre B cells is influenced by IL-7 (Namen et al, 1988), stem cell factor (SCF) (Billips et al, 1992), insulin growth factor (IGF-1) (Gibson et al, 1993), pre B cell growth stimulatory factor (PBSF) (Nagasawa et al, 1994) and pre B cell colony-enhancing factor (PBEF) (Samal et al, 1994). Factors that may inhibit B cell development have also been described and include IL-4 (Rennick et al, 1987), IL-1 (Dorshkind, 1988), TGFβ (Lee et al, 1989), IFNγ (Garvy and Riley, 1994), oestrogen (Medina et al, 1993) and IL-3 (Hirayama et al, 1994).

The role of tyrosine kinases

The cross linking of the pre B cell receptor via its putative ligand results in functional inactivation and promotes cell death. Signalling is not mediated directly by the pre B cell receptor but via associated molecules, Igα and Igβ, products of the *mb-1* and *b29* genes. Cross linking of the pre B cell receptor leads to the activation of a variety of tyrosine kinases including syk, lyn, fyn, fgr, blk and lck (Weschsler and Monroe, 1995). The identification of the gene responsible for X-linked agammaglobulinemia (XLA) as coding for the tyrosine kinase, btk, in humans underlies the importance of tyrosine kinases in regulating B cell development (Vetrie et al. 1993).

Generation of Diversity

The primary repertoire of immunoglobulin specificity is large and is estimated to exceed 1 million (Tonegawa, 1983). It is generated without exposure to exogenous antigen, by virtue of gene rearrangements, with additional diversity mediated by the imprecise joining of gene segments. The antigen binding portion of the immunoglobulin is formed by the recombination of V, (D) and J segments, V(D)J recombination is thus responsible for the generation of diversity.

V(D)J recombination is mediated by conserved heptamer and nonamer signal sequences separated by less conserved spacers. Efficient recombination requires one 12 base pair and one 23 base pair signal and results in precise heptamer to nonamer ligation of the two signal sequences (signal joint), together with imprecise joining of the flanking coding sequences (coding joint). This coding joint imprecision results from short additions of complementary or random nucleotides and/or small deletions. The mechanisms underlying V(D)J recombination have been extensively studied in mice, revealing it to be mediated by the protein products of the *rag-1* and *rag-2* genes. A recent study by McBlane et al. (1995) has demonstrated that RAG-1 and RAG-2 proteins are the only proteins required for the recognition and cutting at the V(D)J signal sequences. Additional proteins may however be involved in subsequent ligations. V(D)J recombination has also been shown to be associated with cell cycle status, occurring preferentially during two periods of quiescence or slow division, separated by a period of rapid proliferation. The exact reasons for this remain unresolved but have been suggested to be linked to levels of RAG-1/2 proteins (Lin and Desiderio, 1995).

Following recombination the V(D)J segment is expressed with a downstream Cμ gene segment to form a productive heavy chain. Light chain genes undergo similar recombination but lack the D segment and therefore diversity is greatest in the heavy chain gene. As mentioned earlier productive μH chains are expressed prior to μL chains at the pre B cell stage. The expression of productive L chains allows cytoplasmic IgM expression at the pre B-II cell stage. Membrane expression then occurs at the mature B cell stage.

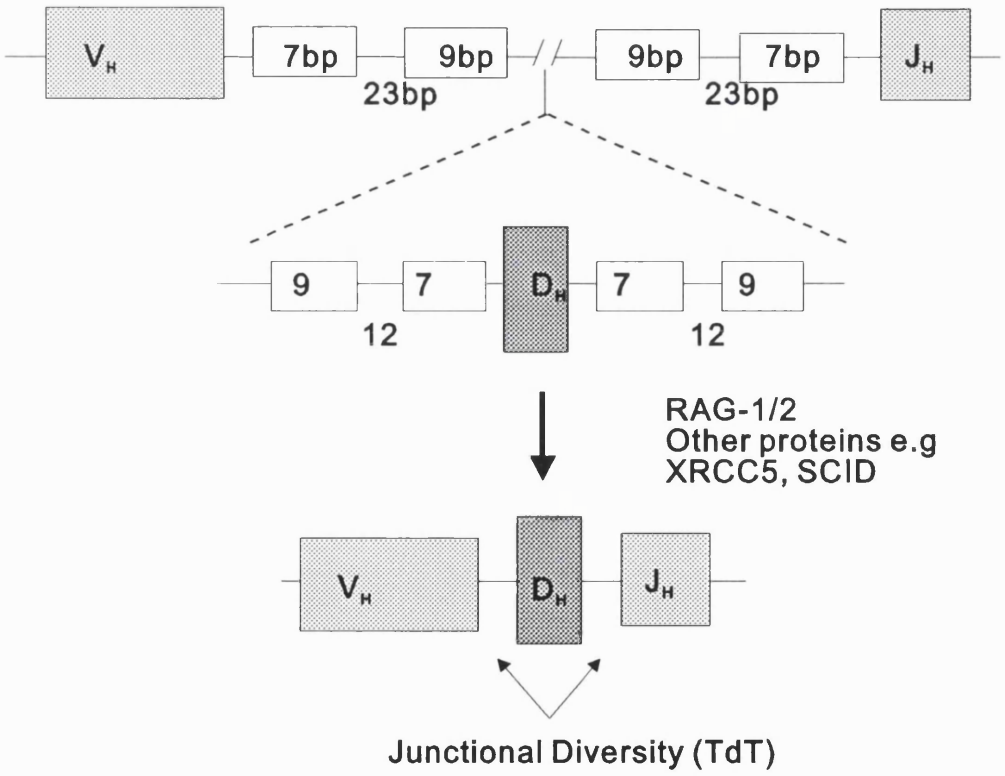


Figure 1.3 Proposed mechanisms involved in directing V(D)J recombination events

Immunoglobulin Isotype Switching

During an immune response a B cell may switch from expressing μH or δH chains to the production of another C_H: γ, ε or α, whilst still retaining the V region, thus ensuring that antigen specificity is maintained. Isotype (class) switching enables a B cell to produce an antibody with the appropriate effector functions required to eliminate a particular pathogen. Associated with the isotype switch is deletion of the DNA of the previous C_H chain and thus a B cell cannot switch back to expressing μH or δH chains.

Three models have been proposed to be responsible for the DNA deletion event (reviewed extensively by Harriman et al.1993).

(i) recombination between homologues

During mitosis four DNA strands are produced with only one allele being productive, these four strands could cross over at sites of homology upstream of the C_H gene giving rise to a daughter cell producing the V region joined to another C_H region. No evidence as yet has been presented to support this model, although it cannot be totally ruled out and probably occurs at low frequency.

(ii) unequal sister chromatid exchange

Sister chromatid exchange (SCE) involves the recombination of sister or duplicated chromosomes during metaphase. This model has been investigated in murine cells but such experiments have been unable to demonstrate an increase in SCE on chromosome 12.

(iii) looping out and deletion

Many lines of evidence accumulated in previous years have pointed to this model being responsible for isotype switching. In this model intervening DNA is looped out and excised as a 'switch circle' containing Cμ and the C_H switch region to which the cell has switched (Figure 1.4). Switch circles have been identified from mouse spleen cells stimulated using the alkaline lysis method (Matsuoka et al. 1990). So called "switch regions" located 5′ to the C_H genes except for Cδ facilitate homologous recombination and/ or focus the putative 'switch recombinase' enzyme (Figure 1.4).

Switch (S) Regions

Mouse S regions are tandemly repeated sequences 5′ of the C_H genes, including several distinct motifs. Sμ consists of (GAGCT)n(GGGGT) where n= 1-7, repeated approximately 150 times. Other S sequences contain multiple copies of these pentanucleotides as well as pentamers, ACCAG, GCAGC and TGAGC and the heptameric repeat (C/T)AGGTTG (Coffman et al. 1993). Sα and Sε tandemly repeated motifs are 40 and 80bp respectively and Cγ are 49 or 52bp. These S regions are particularly recombinogenic, with Sμ being 100 fold more recombinogenic than an average genomic site (Baar and Shulman, 1995). Human S regions also contain tandemly repeated sequences similar to the murine S regions, TGAGC and TGGGG. The recent sequencing of the Sγ1-4 regions of 2.3kb, 0.9kb, 1.5kb and 0.7kb respectively will hopefully permit elucidation of the mechanisms responsible for human IgG subclass switching (Mills et al. 1995).

The control of isotype switching by cytokines

Class switch recombination is preceded by the production of sterile (non-productive) germline transcripts from the C_H gene to be expressed (Stavenezer-Nordgren and Sirlin, 1986). Transcription of germline C_H RNA is initiated with I exons located 5′ to each S region, continues through the S region and terminates 3′ to the C_H gene, as shown in Figure 1.4 (Gauchat et al. 1990; Gerondakis, 1990; Rothman et al. 1990). The importance of I exons in class switch recombination is demonstrated in mice which lack Iγ1 and are unable to produce IgG1 (Thyphronitis et al. 1993). The preceding transcriptional activity of the C_H locus is believed to make the region accessible to factors that mediate class switch recombination, such as the putative switch recombinase. This hypothesis is referred to as the accessibility model (Stavenezer-Nordgren and Sirlin, 1986). Accessibility can be induced by creating (i) nucleosome free DNase I hypersensitivity sites for recombinase and RNA polymerase access (Berton and Vietta, 1990; Ford et al. 1992), or (ii) demethylation of DNA. The production of germline transcripts has been demonstrated to be under the control of cytokines, including IL-4, IL-13, IFNγ and TGFβ. Secondary signals provided by IL-5, IL-6, cognate T-B interactions mediated by CD40-CD40L and CD58-CD2 are then required to proceed to class switch recombination.

In the mouse, IL-4 has been shown to direct switching to IgE and IgG1 (Rothman et al, 1990; Esser et al, 1989), whereas in humans IL-4 directs switching to IgE and IgG4 (Ichiki et al, 1993). IL-13, a cytokine functionally related to IL-4, has also been demonstrated to direct switching to IgE in human foetal B cells (Punnonen and de Vries, 1994). In mice IFNγ promotes switching

to IgG2a (Snapper et al. 1988), whereas TGFβ promotes switching to IgA (Coffman et al. 1989). All these studies were performed *in vitro*, but recent *in vivo* analyses have supported the role of IL-4 in stimulating murine IgG1 and IgE (Nakanishi et al. 1995).

The mechanisms underlying cytokine driven class switching are unclear at present but presumably involve specific binding regions, such as the IL-4 response element identified 5′ to the ε switch site in mice (Ichiki et al. 1993). Interestingly, cholera toxin, a potent immunogen and adjuvant, has also been demonstrated to promote the formation of germline γ1 transcripts synergistically with IL-4 *in vitro* in murine LPS stimulated B cells. The mechanisms responsible were found to include increased intracellular cAMP, which augments germline transcript production (Lycke, 1993).

As mentioned earlier, human and mouse immunoglobulin isotype switching differ in their regulation. Studies of the cytokine regulation of human isotype switching have been facilitated in recent years by the availability of PCR primers for the switch sites, which have permitted the identification of germline transcripts. Much attention has focused on IL-4 mediated isotype switching and it has been found that IL-4 alone cannot direct human isotype switching. Among the additional signals identified are hydrocortisone (Jabara et al. 1993), CD58-CD2 (Diaz Sanchez et al. 1994) and CD40-CD40L interactions (Fujieda et al. 1995). The recent identification of vasoactive intestinal peptide (VIP) as a human switch factor for IgA (Kimata and Fujimoto, 1994) points towards the involvement of as yet unidentified cytokines and cytokine 'cocktails' in isotype switching.

With regard to the human IgG subclasses, studies by Fujieda et al. (1995) have demonstrated switching of human B cells to IgG1, IgG3 and IgG4 but not IgG2 by IL-4 and CD40 mAb. Kotowicz and Callard, (1993) have demonstrated increased production of IgG1, IgG2 and IgG3 by EBV stimulated human B cells using IL-4. Additionally, Kawano et al. (1995) have demonstrated increased production of human IgG subclasses using IL-6.

The mode of antigenic stimulus may affect the Ig isotype profile observed. Studies in mice have demonstrated that when stimulated with LPS, IL-4 promotes IgG1 and IgE selection, whereas IFNγ promotes IgG2a and TGFβ results in IgG2b and IgA production. When stimulated with $\alpha\delta$-dex IL-4 promotes only IgG1, IFNγ promotes IgG2a and IgG3 and TGFβ stimulates only IgA (Snapper and Mond, 1993). Furthermore, De Becker et al. (1994) have demonstrated in mice *in vivo* that antigen pulsed dendritic cells preferentially stimulated specific IgG2a and IgG1, whereas peritoneal macrophages favour the production of IgG1 and IgE. Thus the mode of B cell activation and type of APC in addition to the stimulating cytokines plays a role in determining the Ig isotype profile.

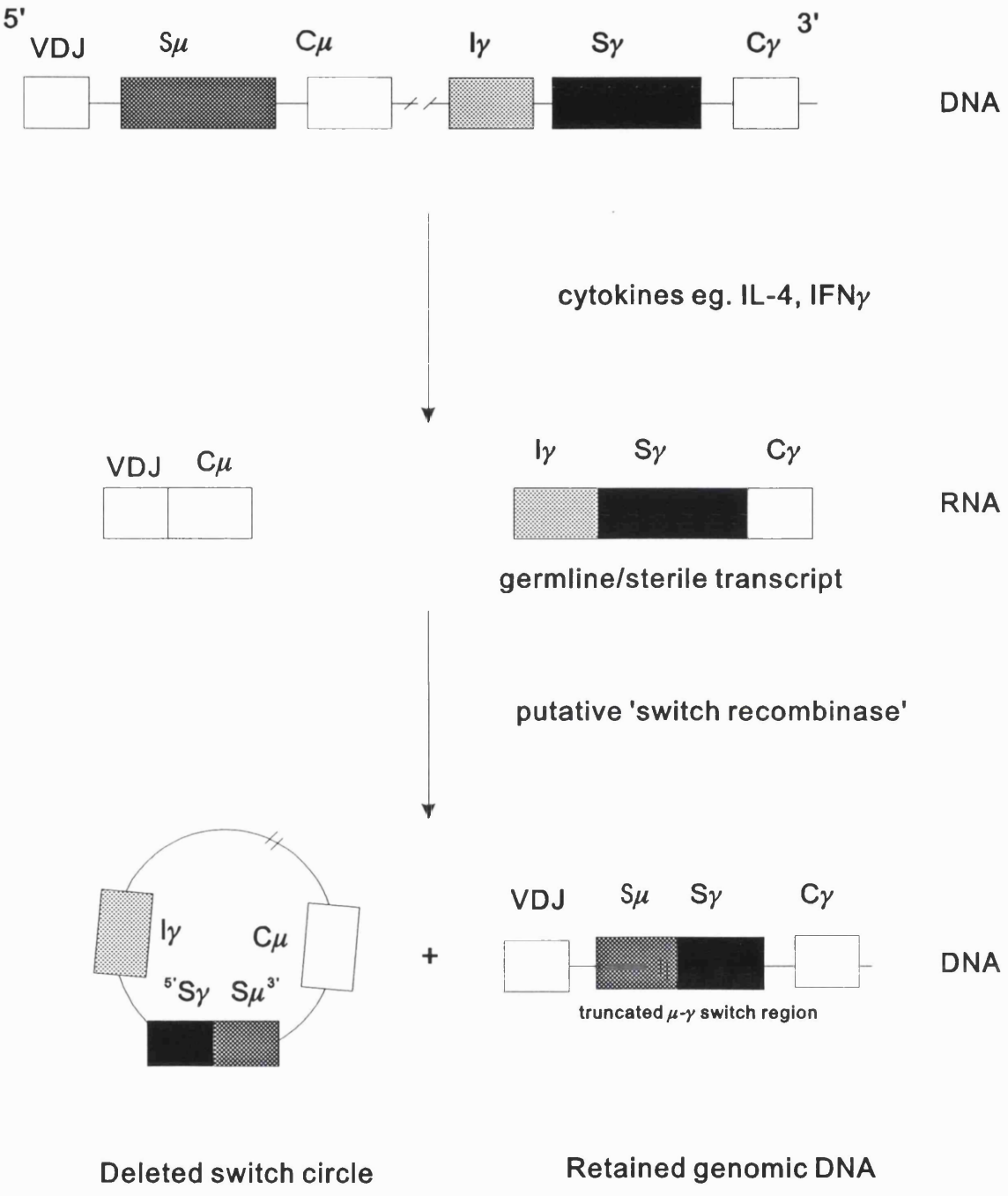


Figure 1.4 Mechanisms and Control of Immunoglobulin Isotype Switching

Affinity Maturation

During an immune response antibody affinity increases with time following initial exposure to antigen. This phenomenon is referred to as affinity maturation. Affinity maturation is due to somatic hypermutation of the variable region which physically occurs within structures called germinal centres in secondary lymphoid tissues.

Some mutations in the immunoglobulin V region give rise to higher affinity antibodies and B cells expressing lower affinity or self-reactive antibodies die by apoptosis. In contrast to Burnet's theory of positive and negative selection followed by clonal expansion (1957), it is now widely accepted that all B cells are programmed to die by apoptosis, unless rescued by a positive signal such as the CD40-CD40L interaction, in addition to sIg receptor cross-linking.

Following antigen stimulation mature B cells migrate to primary follicles of secondary lymphoid organs, including the spleen and tonsils, where they proliferate extensively to form germinal centres (Neuberger and Milstein, 1995). The germinal centre is divided into two main compartments, the dark and the light zone. B cells are activated in the T cell rich zones outside the follicles and on average only 3 B cell blasts colonise each follicle. Proliferating B cell blasts (centroblasts) are located in the dark zone giving rise to non-proliferating centrocytes in the follicular dendritic cell (FDC) rich light zone.

FDC are specialised APCs which take up and display antigen on their surface in the form of immune complexes. Centrocytes are believed to bind to antigen on FDCs and receive secondary survival signals from T cells. It is unclear whether germinal centres colonised with B cells activated by T_{ind} antigens exist

but such antigens may receive non-T cell help, and the true T_{ind} nature of the antigen needs to be clarified.

Human tonsillar B cell/FDC adhesion is mediated by CD54 and VCAM-1 on FDC binding to CD11a/CD18 and VLA-4 respectively on centrocytes (Koopman et al. 1991; Holder et al. 1992). The analysis of such interactions has been hampered by the poor identification of the true FDC and the small numbers of FDC which can be purified. The recent reports of FDC-like human cell lines FDC-1 (Clark et al. 1995) and HK (Kim et al. 1995) should help to clarify these reactions. Nevertheless, it is known that the anti-Ig cross linking signal is not enough to rescue B cells from apoptosis. Additional signals mediated by CD40-CD40L (Maclennan and Gray, 1986), B7.1:CD28/CTLA.4 (Han et al. 1995), CD20 engagement (Holder et al. 1995) and possibly CD19 and CD22 cross-linking are required (Chaouchi et al. 1995).

As yet the specific population of cells which undergo somatic hypermutation are undefined, although the triggering of somatic mutation occurs in human tonsil centroblasts during the transition from IgD^+, $CD38^-$, $CD23^+$ to IgD^-, $CD38^+$, $CD77^+$ (Pascual et al. 1994). Furthermore, a recent study by Liu et al. (1996) has shown that immunoglobulin class switching occurs after somatic mutation within the IgD^-, $CD38^+$, $CD77^-$ centrocytes.

Is somatic hypermutation random or directed ?

The pattern of somatic hypermutation of V genes has been extensively analysed in anti-hapten responses. Previously, somatic hypermutation was thought to be random, the existence of mutational hotspots being due to antigenic selection. Analysis of replacement versus silent mutations has demonstrated that somatic hypermutation is non-random (Berek and Milstein, 1988; Weiss et al. 1992). The location of such intrinsic hotspots have been identified by studying immunoglobulin passenger transgenes (transgenes that do not contribute to expressed antibody). In mouse and man a major intrinsic hotspot is in the second base of serine codons AGC/T, the existence of these unusual serine codons in the CDRs may favour local targeting to the complementarity determining regions responsible for antigen binding (Neuberger and Milstein, 1995). The recent sequencing of the whole human V_H locus (Cook and Tomlinson, 1995) and the analysis of transgenes should enable somatic mutation to be analysed in more detail. A recent study by Yelamos et al. (1995) has shown that replacement of a Vκ region with non-Ig human β globin sequences resulted in hypermutation of the human β globin gene, thus the V region itself is not required to direct hypermutation. The mechanisms responsible for somatic mutation remain enigmatic, but are believed to involve some form of error prone DNA repair process (Rajewsky, 1996). Various cis-acting elements have now been identified in vitro. The murine Igκ intron and 3′ enhancers appear to be critical (Betz et al. 1994; Yelamos et al. 1995). Transcription initiation may also be involved in targeted somatic mutation (Rajewsky, 1996).

Immunoglobulin: The Mediator of Humoral Immunity

Immunoglobulins comprise a four polypeptide chain unit of two identical light (L) chains and two identical heavy (H) chains which are cross linked by interchain disulphide bonds and stabilised by non-covalent interactions. Great progress has been made since the identification in the 1950's of five immunoglobulin classes (isotypes) in man characterised by specific heavy chains: μ, γ, δ, α and ε. In man immunoglobulin genes are located on chromosome 14 and in mice on chromosome 12 (Figure 1.5)

The L chains are folded into two globular domains while the heavy chains are folded into four or five domains depending on the immunoglobulin class/isotype. Some of the physio-chemical properties of human antibodies are illustrated in Table 1.1

Table 1.1 Physico-chemical properties of human immunoglobulin classes

	IgG	IgM	IgA	IgD	IgE
Heavy chain	γ1-4	μ	α1-2	δ	ε
Molecular weight (kDa)	146	950	160	175	190
Sedimentation constant	7S	19S	7S	7S	8S
No. of H chain domains	4	5	4	4	5
% carbohydrate	2-3	12	7-11	9-14	12
Half life (days)	23	5.8	5.1	2.8	2.5
Mean serum concentration (mg/ml)	13.5	1.5	3.5	0.03	5×10^{-5}

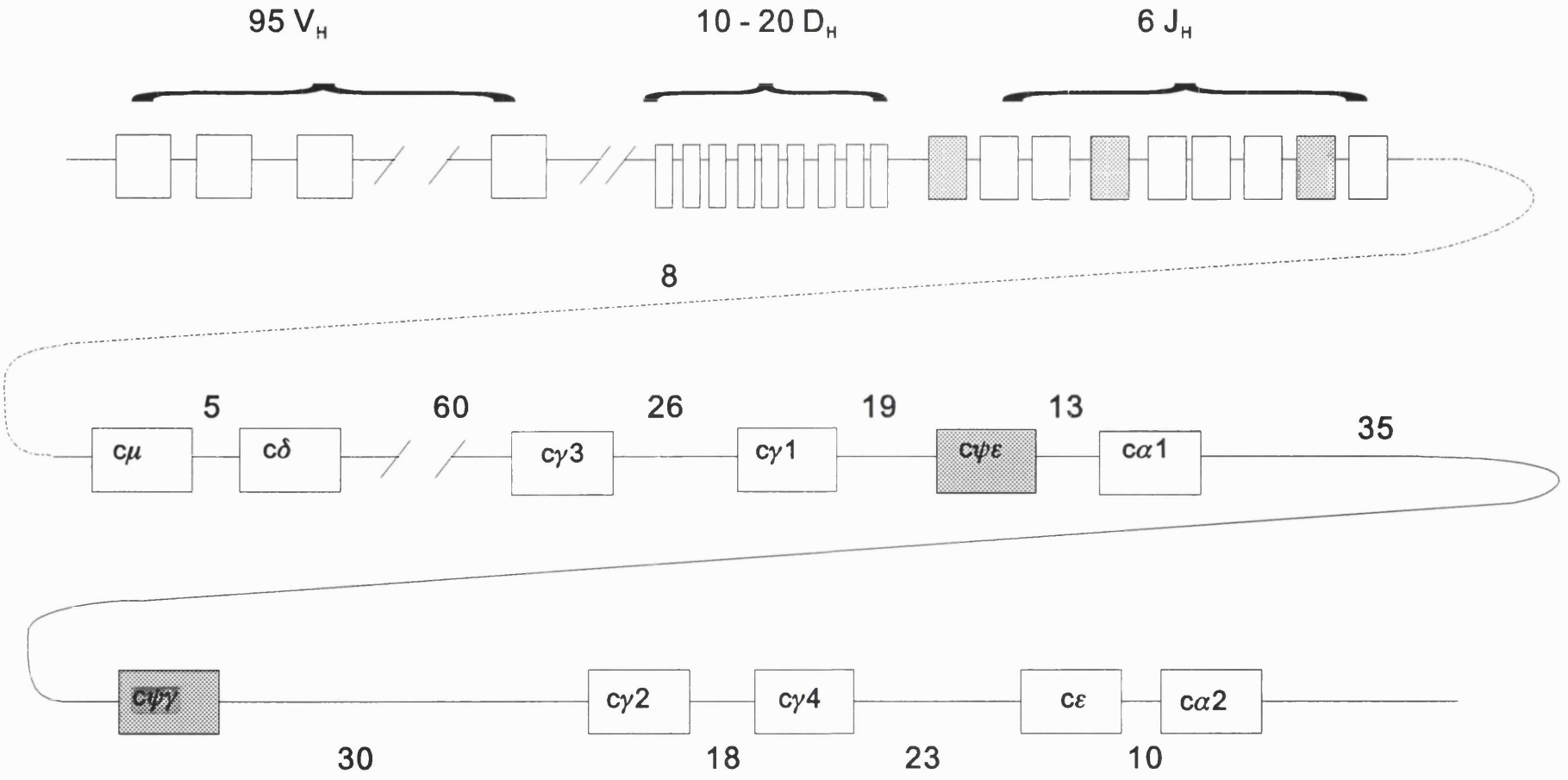


Figure 1.5 Gene organisation of the human immunoglobulin genome
Intron lengths in Kb, adapted from Male et al. (1996)

The N-terminal domains of both the heavy and light chains are highly variable (V domains), whilst the remaining domains are relatively constant (C domains), and are characteristic of the immunoglobulin class. The domains consist of a β barrel structure with seven (C domain) or nine (V domain) strands of β sheet stabilised by intrachain disulphide bonds. The variability of the V domain is clustered into hypervariable loops, sometimes referred to as complimentarity determining regions (CDRs). The hypervariable loops are brought together by the folding of the V domain and the heavy and light chains generate a surface of six hypervariable loops, which determine antibody specificity. This region has been called the paratope. The residues surrounding the hypervariable loops are termed framework residues and together with the rest of the V region constitute the fragment antigen binding (Fab) portion of the antibody molecule. The fragment crystalline (Fc) distal to the Fab region mediates the effector functions of antibodies, some of which are summarised in Table 1.2

Table 1.2 Biological properties of human immunoglobulin molecules

	IgM	IgG	IgA	IgD	IgE
Complement fixation	++	+	-	-	-
Platelet binding	-	+	-	-	-
Mast cell/basophil sensitisation	-	-	-	-	+
Transplacental transfer	-	+	-	-	-
Opsonisation capacity	-	+	+	-	-
Sensitisation for K cells	-	+	-	-	-
Binding to lymphocytes	T	T,B	T,B	-	T,B
Binding to mononuclear cells	+/-	+	-	-	-
Binding to neutrophils	+	+	+	-	-
Binding to protein A	+/-	+[a]	+/-[a]	-	-

[a] except for IgG3, not IgA1

Immunoglobulin G is the major isotype in normal human serum, accounting for 70-75% of total serum immunoglobulin. IgG is mounted early in the secondary response to antigenic challenge and its affinity is seen to increase during an immune response. Clinical deficiency of IgG is also associated with susceptibility to infection. IgG is therefore an important mediator of the humoral immune system and much research has focused upon elucidating the structure and function of IgG over the past three decades.

Immunoglobulin G: Structure and Function

IgG is a glycoprotein composed of two heavy and two light chains linked together via interchain disulphide bonds and non-covalent interactions (Porter, 1962). The light chains are solely associated with the Fab portion of the molecule and the heavy chains span both the Fab and Fc portions. A single disulphide bond connects the light and heavy chains whereas the number of inter heavy chain bonds differs from subclass to subclass. The IgG molecule has been shown by crystallography to be composed of domains, with each domain having a characteristic folding pattern called the immunoglobulin fold. The immunoglobulin fold consists of two surfaces of anti-parallel β sheet stabilised by a disulphide bridge. This is a characteristic structure shared by the members of the immunoglobulin supergene family, which include MHC and T cell receptor molecules.

The domains of IgG are designated V_H,V_L,C_L,C_H1,C_H2 and C_H3. Pairing between the V_H and V_L, C_L and C_H1 produces the Fab fragment, whereas pairing

between the C_H2 and C_H3 domains produces the Fc fragment (Figure 1.6). The domain pairing is stabilised by non-covalent interactions, except for the C_H2 domains, which have two N-linked branched carbohydrates interposed between them. The Fab fragment domain faces are also predominantly hydrophobic and therefore efficient domain pairing in the Fab fragment requires the removal of these faces from the aqueous environment.

The Fab arms are linked to the Fc region by virtue of a length of polypeptide, called the hinge. The hinge region is susceptible to proteolytic attack by enzymes such as papain and pepsin. Papain cleaves the IgG molecule N-terminal to the intrachain disulphide bridge, producing two Fab fragments, whereas peptic cleavage C-terminal to the disulphide bridges results in a divalent $F(ab')_2$ fragment and various Fc cleavage products including pFc'.

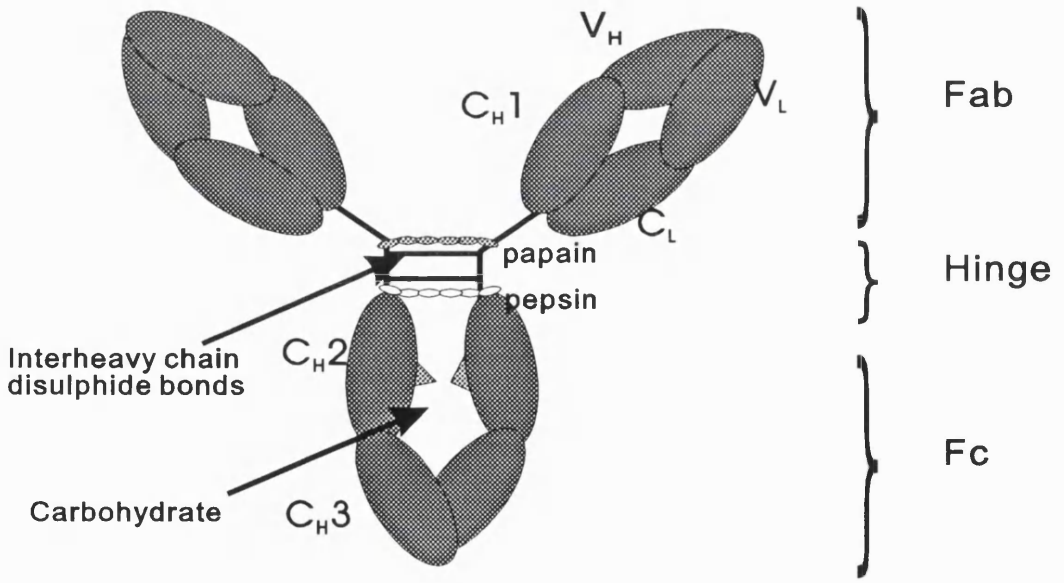


Figure 1.6 Domain Structure Of Immunoglobulin G

The IgG Subclasses

The four IgG subclasses were originally identified by the antigenic properties of their heavy chains (Dray, 1960; Terry and Fahey, 1964), which are determined by the amino acid sequence of the constant regions of the heavy chains. The four human IgG subclasses are designated IgG1, IgG2, IgG3 and IgG4. The mouse also has four subclasses called IgG1, IgG2a, IgG2b and IgG3. The different subclasses are defined by the C_H genes encoding the C_H1, hinge, C_H2 and C_H3 domains. The human constant domains are very similar with >90% sequence homology, suggesting that the four human IgG subclasses arose late in evolution by gene duplication after the emergence of the primates (Flanagan and Rabbitts, 1982). Further support for this conclusion comes from the observed differences in IgG subclass regulation in the mouse and humans, with IL-4 promoting switching towards IgG1 and IgE in mouse splenic B cells (Rothman et al. 1990; Esser et al. 1989) whereas IgG4 and IgE are promoted by IL-4 in humans (Ichiki et al, 1993). Whilst human IgG subclasses show more than 90% sequence homology between the C_H domains, sequence homology in the mouse is much less, with the C_H domains of IgG1 and IgG2a having only 60-70% homology. Heavy (γ) chain gene organisation in the mouse and human differ (Figure 1.7) and it is suggested that the human genes arose from a duplication of a region containing γ, ε and α genes, with the subsequent evolution of the IgG subclasses. This is supported by the antigenic and functional relationship between IgG1 and IgG3 and also between IgG2 and IgG4 (Callard and Turner, 1990).

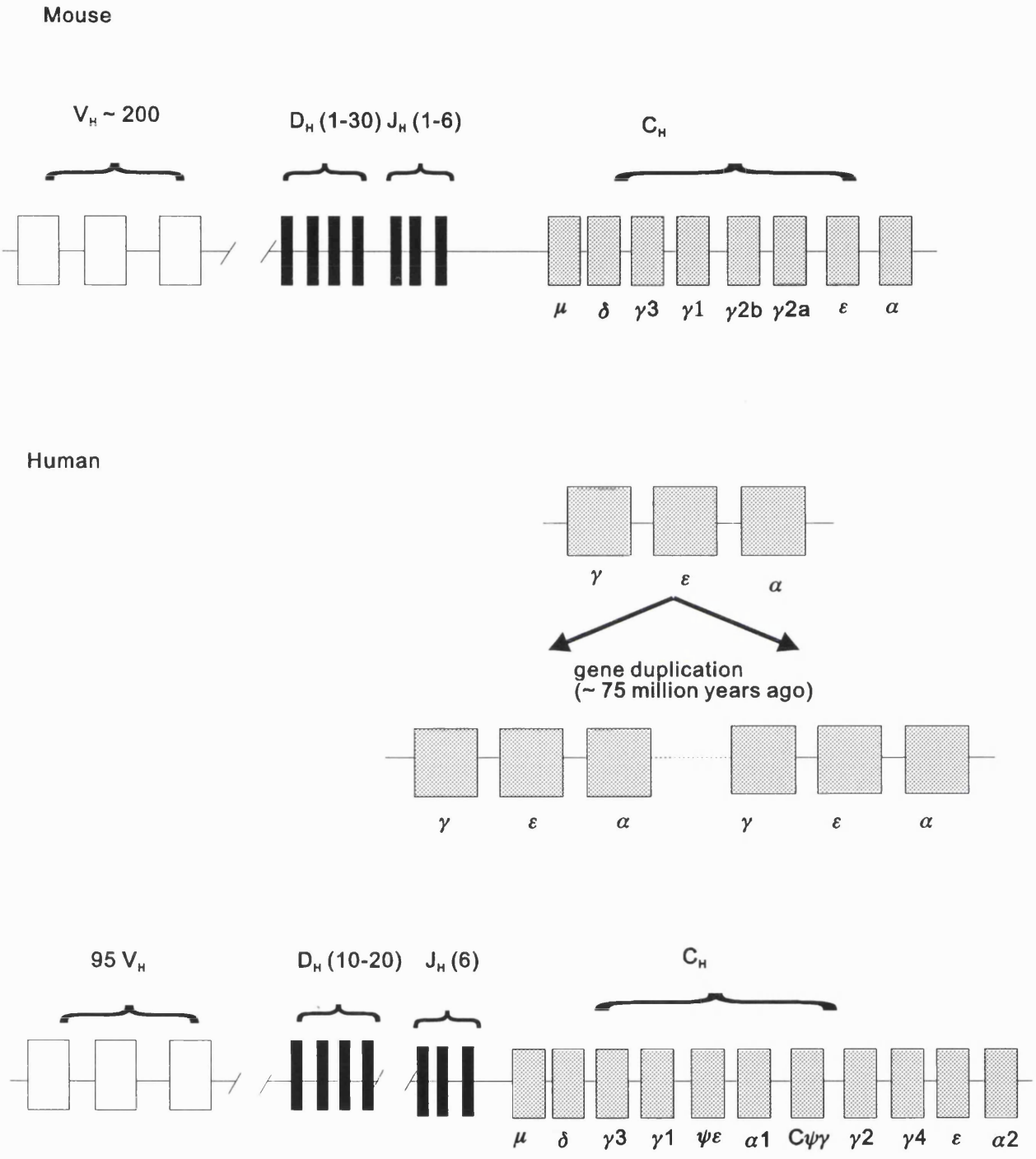


Figure 1.7 Gene Organisation of Human and Mouse IgG subclasses
Demonstrating possible gene duplication events responsible for the emergence of the human IgG subclasses

The region of greatest variability between the IgG subclasses resides in the hinge region. The hinge region comprises three parts, the upper hinge is defined as the peptide region from the N-terminal end of the C_H1 domain to the first Cys residue that forms a disulphide bond. The middle hinge stretches from the C-terminal end of the upper hinge to Ala231. The upper and middle hinge are encoded by separate exons and constitute the 'genetic' hinge. The lower hinge is then encoded by the C_H2 exon. The hinge regions of the subclasses are characterised by different numbers of amino acids, IgG1 has 15 amino acids, IgG2 and IgG4 have 12 amino acids and IgG3 has the longest most flexible hinge encoded by four exons yielding 62 amino acids. It is within the hinge region that flexibility is mediated (Figure 1.8). The variability of the hinge results in a relative flexibility between the subclasses of IgG3 > IgG1 > IgG4 > IgG2. This is also the rank order for expression of effector functions, such as complement fixation and interactions with Fcγ receptors.

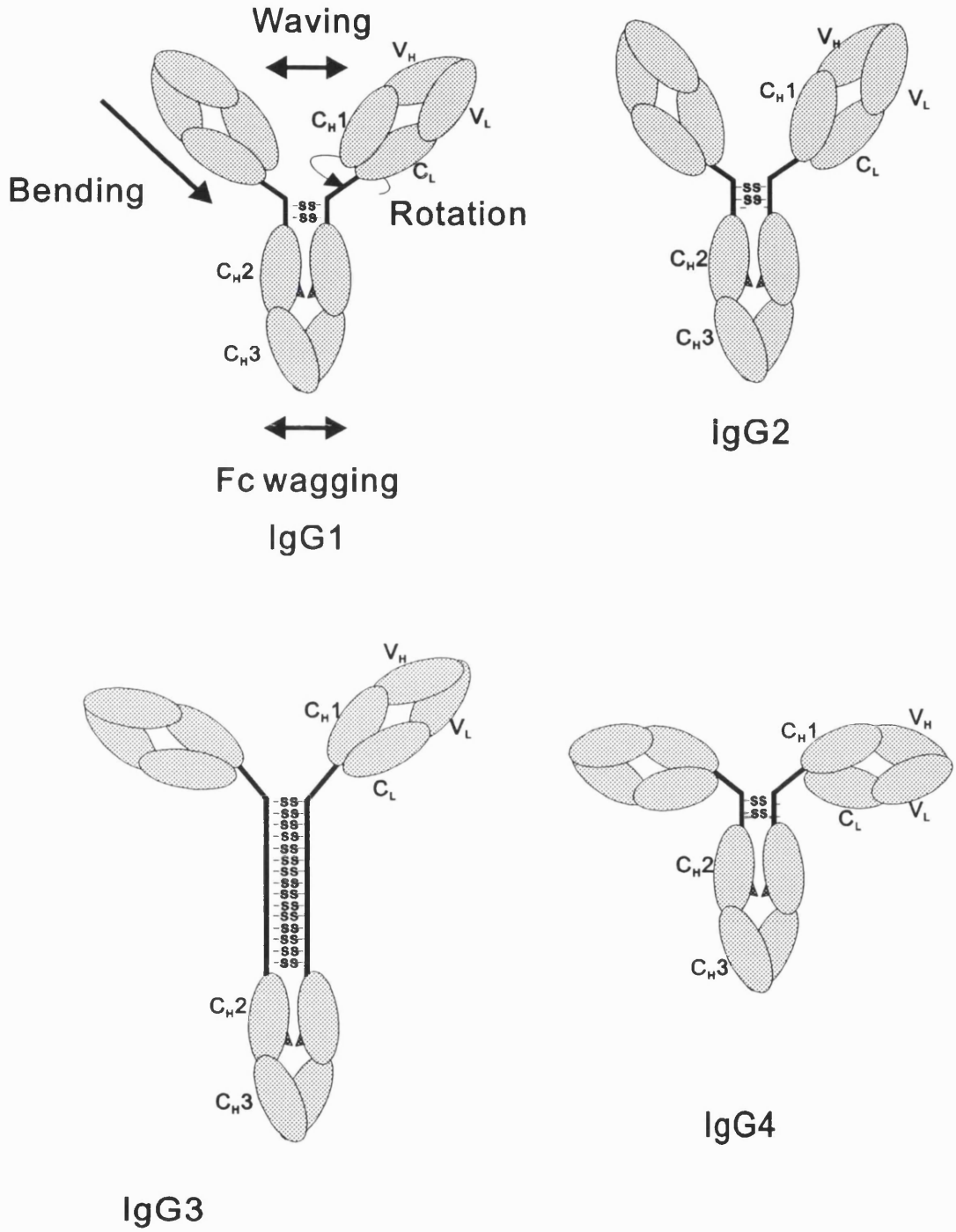


Figure 1.8 Domain Structure of the Human IgG Subclasses
Showing hinge length, number of hinge region disulphide bonds and areas of flexibility. Carbohydrate structure in the C_H2 domains are indicated by ▲

Gm Allotypes

Gm allotypes are antigenic determinants present on the constant regions of γ heavy chains which obey Mendelian laws of inheritance. Since their first description by Grubb (1956) many Gm allotypes have been characterised, shown in Table 1.3. Two systems of nomenclature exist for the classification of Gm allotypes, the World Health Organisation (WHO) recommends the numeric system, although both alphabetical and numerical systems are found in the literature

Table 1.3 Gm allotypes of human immunoglobulins adapted from Lefranc and Lefranc, (1990)

Subclass	Domain	Alphabetical	Numeric
IgG1	C_H3	G1m(a)	G1m(1)
	C_H3	G1m(x)	G1m(2)
	C_H1	G1m(f)	G1m(3)
	C_H1	G1m(z)	G1m(17)
IgG2	C_H2	G2m(n)	G2m(23)
IgG3	C_H3	G3m(b^0)	G3m(11)
	C_H2	G3m(b^1)	G3m(5)
	C_H3	G3m(b^3)	G3m(13)
	C_H2	G3m(b^4)	G3m(14)
	C_H3	G3m(b^5)	G3m(10)
	C_H3	G3m(c^3)	G3m(6)
	C_H3	G3m(c^5)	G3m(24)
	C_H2	G3m(g)	G3m(21)
	C_H2	G3m(u)	G3m(26)
	C_H3	G3m(v)	G3m(27)
	C_H3	G3m(s)	G3m(15)
	C_H3	G3m(t)	G3m(16)
	C_H3	G3m(g5)	G3m(28)

It is now well established that there is an association between Gm allotype and serum IgG subclass levels. Individuals who are homozygous for the G3m(b) allotype have higher IgG3 levels than G3m(g) homozygotes. Similarly, individuals homozygous for the G2m(n) allotype have higher IgG2 levels than individuals

typed as G2m(n)$^-$. A relationship between Gm allotype status and immune response to certain bacterial organisms, for example *Haemophilus influenzae*, *Streptococcus pneumoniae* and Meningococci, have been found. Polysaccharide specific antibody titre has been shown to be related to G2m(n) allotype (discussed in more detail in chapter 6). It is clear that Gm allotype exerts an effect on IgG subclass concentration, although the mechanisms underlying this relationship are as yet unresolved.

Structure-Function Relationships of the IgG Subclasses

It is within the Fc portion of the immunoglobulin molecule that effector functions are mediated, but there are important differences between the subclasses (Table 1.4)

Table 1.4 Biological and structural properties of IgG subclasses

	IgG1	IgG2	IgG3	IgG4
Heavy chain	γ1	γ2	γ3	γ4
Molecular weight of intact protein (kDa)	146	146	170	146
Hinge aa length	15	12	62	12
Light chain κ:λ	2.4	1.1	1.4	8
Inter-heavy chain disulphide bonds	2	4	11	2
Serum levels (mg/ml)	5-12	2-6	0.5-1	0.2-1
Half-life (days)	21-23	20-23	7	21
Complement fixation	++	+	++	-
Specific anti-CHO responses	++	+++	-	-/+
Specific anti-protein responses	++	-	++	-/+
Protein A binding	+	+	-	+
Protein G binding	+	+	+	+
FcγRI (CD64) interaction	+++	-/+	++++	++
FcγRII (CD32) interaction	+++	++	++++	++
FcγRIII (CD16) interaction	++++	+	++++	+

C1q binding is mediated by the motif Glu318-X-Lys320-X-Lys322. The context of the binding motif is of importance since the IgG subclasses differ in their ability to promote complement mediated cell lysis. Human IgG1 and IgG3 are very effective, IgG2 is effective only at high concentrations and IgG4 is not effective (Guddat et al. 1993; Bruggemann et al. 1987; Riechmann et al. 1988; Valim and Lachmann, 1991). Recent studies of hinge deleted IgG3 molecules has revealed that the long flexible hinge is not itself a prerequisite for C1q binding but that the inter H chain disulphide bond is essential for complement activation (Brekke et al. 1995).

Differences between the human IgG subclasses have been demonstrated for protein A binding, with IgG1, IgG2 and IgG4 binding whereas IgG3 does not. The critical residue for protein A binding is His435, which in the IgG3 of Caucasians is replaced by Arg. In oriental races IgG3 having the G3m(st) allotype is commonly found and this is associated with a histidine residue at position 435 and hence with protein A binding.

Another major effector system is the recognition of antibody coated target cells by cells expressing Fc receptors. Fc receptors mediate a variety of functions including phagocytosis (monocytes, macrophages, neutrophils) and antibody dependent cellular cytotoxicity (monocytes, macrophages, lymphocytes). Fcγ receptors fall into three categories FcγRIa,b (CD64, high affinity), FcγRIIa,b (CD32, intermediate affinity) and FcγRIIIa,b (CD16, low affinity) have been identified in mice and humans (Anderson and Looney, 1986) . FcγRI binds monomeric IgG whereas FcγRII and III only bind aggregated IgG in the form of immune complexes. The three FcγRs are probably derived from a common ancestral

gene and are members of the Ig gene superfamily (Fridman et al. 1992). FcγR binding has been shown to be mediated by the extreme N-terminal portion of the C_H2 domain. In particular, Leu235 has been demonstrated to be a critical residue in such binding (Woof et al. 1986; Canfield and Morrison, 1991).

The human IgG subclasses differ in their ability to bind Fc receptors. Human FcγRI is expressed on monocytes and macrophages and its expression on neutrophils may be induced by IFNγ in vitro (Perussia et al. 1983). The binding of the IgG subclasses differ with IgG3 exhibiting the highest affinity (Burton, 1985). IgG2 is thought to be incapable of binding to FcγRI (Walker et al. 1989), but may bind with very low affinity (Van Den Herik Oudijk et al. 1994). FcγRII is expressed on virtually all haematopoetic cells, whereas FcγRIII expression is restricted to macrophages, NK and mast cells (Weinshank et al. 1988; Daeron et al. 1990).

FcγR mediate a wide spectrum of activities, related to cell type. For example receptors expressed by macrophages mediate phagocytosis, endocytosis, antigen presentation and killing of IgG sensitised cells, a function also shared with NK cells (Fridman et al. 1992).

Human IgG Subclass Restriction

Human IgG subclass restriction was first described by Yount et al. (1968). The authors found a predominance of IgG2 antibodies specific for dextran, levan and teichoic acid and IgG1 specific for tetanus toxoid, diphtheria toxoid and blood group antigens in serum collected from adults immunised with a variety of antigenic preparations. These observations have since been confirmed and

extended by many investigators and it is now well recognised that antibody patterns are generally predictable, with IgG1, IgG3 and/or IgG4 being directed against T_{dep} protein antigens and IgG2 being produced in response to T_{ind} polysaccharide antigens.

Support for a predominantly IgG2 response directed against polysaccharide antigens was demonstrated following immunisation with *Haemophilus influenzae* vaccine (Shackelford et al. 1987; Makela et al. 1987; Hammarstrom et al. 1988). Furthermore, most antibody produced against polysaccharide antigens following infection with, organisms such as *Klebsiella pneumoniae* and *Niesseria mennigitidis*, is also of the IgG2 subclass (Hammarstrom et al. 1988; Seppela et al. 1984; Rautonen et al. 1986). In contrast the antibodies elicited following vaccination with a protein antigen, such as diphtheria or tetanus toxoid, are predominately found in the IgG1 and IgG3 subclasses (Makela et al. 1987). IgG subclass restriction is also observed in naturally occurring antibodies, for example IgG1 and IgG3 antibodies specific for varicella-zoster virus are found in healthy individuals as well as in those recovering from infection (Moyner and Michaelsen, 1988; Mathiesen et al. 1989). IgG subclass restriction is more complex than first thought. IgG1 antibodies are often observed concomitant with IgG2 responses specific for the polysaccharide capsule antigen, of *H.influenzae* (polyribosylribitolphosphate, PRP) (Shackelford et al. 1987), furthermore IgG2 antibodies specific for the protein antigen, hepatitis B core antigen, are elevated together with IgG1 following vaccination (Borzi et al. 1992).

It is also becoming clear that certain IgG subclasses are associated with disease states. A predominance of IgG1 and IgG3 directed against myeloperoxidase has been described in association with renal vasculitis (Esnault et al. 1991). Interestingly, infection with *Schistoma mansoni* is associated with antibodies of all four subclasses, but whereas IgG1 and IgG3 mediate killing of the parasite, IgG4 antibodies have been shown to block such killing mechanisms (Khalife et al. 1989). The route of antigen delivery has also been shown to affect the isotype profile with IgG1 and IgG3 predominating following natural hepatitis B infection (Persson et al. 1988), but IgG1 and IgG2 predominate following hepatitis B vaccination (Borzi et al. 1992). In contrast, IgG1 and IgG4 antibodies have been shown to dominate in hepatitis B-immunoglobulin complexes (Sallberg et al. 1991) and these authors propose that this is due to IgG3 complexes being effectively cleared from the circulation and therefore not being detectable in the serum.

The role of IgG4 is even more unclear than that of the other subclasses. It is known not to bind complement and binds poorly to FcγRs. IgG4 production has been demonstrated following secondary or tertiary immunisation and after chronic antigenic stimulation (Aalberse et al. 1983; Bird et al. 1990). IgG4 anti-egg antibodies have also been shown to be correlated with infection by *Schistoma mansoni* (Dunne et al. 1988). Initial studies speculated that IgG4 is of low affinity following secondary immunisation with the neo-antigen keyhole limpet hemocyanin (Devey et al. 1990) or hepatitis B (Persson et al. 1988) but other evidence suggests that high affinity IgG4 can be produced (Devey et al. 1990). Little work has been directed at understanding the role of IgG4 and as a result

the functional significance of the IgG4 subclass remains unclear. Taken together these studies suggest that human IgG subclass restriction exists but that the exact subclass elicited depends on the specific antigen, route of entry and dose.

The ontogeny of the human IgG subclasses

IgG subclasses also differ in their ontogeny. Children do not acquire adult IgG2 levels until 8 - 10 years of age, whereas adult levels of IgG1 and IgG3 are detected much earlier (Schur, 1979). This delay in IgG2 ontogeny results in young children being unable to mount an adequate response against polysaccharide antigens. This probably accounts for the frequent infections with encapsulated bacteria and poor response to polysaccharide vaccines observed in young infants. The responses that are elicited tend to be confined to the IgG1 subclass (Hammarstrom et al. 1988; Schatz and Barrett, 1987). The response mounted against protein antigens does not appear to be delayed in ontogeny and IgG1 antibodies specific for tetanus and diphtheria toxoid are detectable in children as in adults (Makela et al. 1987). This delay in ontogeny of the IgG2 subclass makes it possible to administer protein based vaccines such as tetanus and diphtheria to young children, whilst unconjugated polysaccharide vaccines are ineffective. The mechanisms underlying the delay in ontogeny of the IgG2 subclass remain unresolved.

Avidity of The Human IgG subclasses

Antibody binding affinity is defined as the strength of the interaction between a monovalent antibody and monovalent antigen; whereas the bivalent binding of an antibody to a complex antigen is referred to as functional affinity or avidity (Hornick, 1972). The affinity of an antibody is determined by the "fit" of the antigen in the binding groove of the Fab portion of the antibody formed by the heavy and light chain variable regions. The binding forces involved in the interactions between antigens and antibodies are of a non-covalent, purely physicochemical nature, the primary forces being Liftshitz-van der Waals, electrostatic and polar (hydrogen bonds) in nature (van Oss, 1994). The extent to which each of the primary bonds contribute varies between different interactions and thus determine antibody affinity. The affinity of an antibody plays a crucial role in determining its biological activity, with high affinity antibody being superior (Steward, 1981). In the assessment of the immune response to vaccines and natural infection it is important to characterise an antibody in terms of affinity as well as titre.

Whilst antibody affinity is predominantly determined by variable region structure, IgG subclasses directed against the same antigen have been shown to differ in affinity. Devey et al. (1985 and 1988), have demonstrated IgG subclass associated affinity differences for serum antibodies specific for tetanus toxoid from naturally infected, healthy controls or vaccinated individuals. The authors demonstrated a predominantly IgG1 and IgG4 response by specific ELISA, but when affinity was measured by the polyethylene glycol method, DEA elution or competitive binding ELISA the IgG1 antibody was of high affinity and IgG4

antibody was of low affinity. Furthermore the IgG4 response displayed restricted affinity heterogeneity. Persson and co-workers subsequently demonstrated affinity differences between serum IgG subclasses specific for hepatitis B surface antigen and pneumococcal polysaccharide derived from vaccinated adults. Employing affinity purification followed by globulin precipitation, IgG subclasses directed towards hepatitis B could be affinity ranked in the order IgG1 > IgG2 > IgG3 > IgG4, whereas the response to pneumococcal polysaccharide was restricted to IgG1 and IgG2, with IgG2 being of highest affinity (Persson et al. 1988). These findings were confirmed and extended by Wen et al. (1990), who also demonstrated IgG1 and IgG4 specific for hepatitis B surface antigen from naturally infected individuals. These authors found that individuals with chronic hepatitis had high levels of low affinity IgG1, whereas asymptomatic carriers had significantly lower levels and higher affinity. IgG subclass affinity differences associated with disease have subsequently been demonstrated for other systems such as high affinity anti-myeloperoxidase (MPO) IgG1 and IgG3 associated with renal vasculitis (Esnault et al. 1991). High affinity IgG1 and IgG3 have also been shown to be associated with recent streptococcal infections (Falconer et al. 1993) and high affinity IgG3 specific for the organism *M. catarrhalis* has also been described (Goldblatt et al. 1991).

Differential affinity maturation of the human IgG subclasses has been described for the neo-antigen keyhole limpet hemocyanin (KLH). Following primary immunisation IgG1, IgG2 and IgG3 antibodies were detected, with affinity maturation observed for IgG1. However, IgG4 antibodies were demonstrated only 1 year following primary immunisation, and they were of high affinity.

Coincidentally there appeared to be loss of high affinity IgG1 and the authors suggested a preferential switch with time from high affinity IgG1 to high affinity IgG4 (Devey et al. 1990).

The underlying mechanisms responsible for the differences in the affinity of IgG subclass antibodies specific for the same antigen are at present not understood, but may be due to differences in epitope specificity and consequently different variable (V) region gene usage or differences secondary to the constant region structure. The latter hypothesis is supported by recent evidence that murine antibody structures remote from the V region may influence affinity and function (Cooper et al. 1991; Bazin et al. 1992; Fulpius et al. 1993; Schreiber et al. 1993; Morelock et al. 1994; Cooper et al. 1994)

Aims of the work described in this Thesis

During the past few decades much progress has been made towards elucidating structural and functional properties of the human IgG subclasses. As outlined earlier the four human IgG subclasses display greater than 90% sequence homology and their production is antigen restricted, with IgG1 and IgG3 being preferentially elicited against protein antigens and IgG2 against carbohydrate antigens. However, many questions still remain unanswered. Although the titre of a particular subclass is clearly important in overcoming infection, it is becoming increasingly clear that the functional affinity (avidity) of such a response is also crucial. This study was therefore initiated to investigate aspects of antibody avidity of the four human IgG subclasses. The primary aim was to develop suitable assays for the measurement of serum antibody avidity and various ELISA based methods were explored for each system studied.

Although it is clear that avidity differences exist between human serum IgG subclasses specific for the same antigen, the exact mechanisms underlying such differences remain unclear. Recent studies in the murine system have highlighted the importance of the heavy chain constant region in influencing antibody avidity. Utilising two sets of V region identical monoclonal IgG subclasses, specific for the antigen TAG72 and the hapten NIP, the contribution to antibody avidity of structures distal to the V region domains were explored. Such avidity differences were further dissected employing the technique of biospecific interaction analysis. This approach extends the information gained from solid-phase avidity assays by providing data at the kinetic level.

The delay in ontogeny of the human IgG2 subclass has been suggested to play a role in the higher incidence of bacterial infections in infants. Despite its pivotal role, little work has been published investigating the avidity of such responses. Utilising pneumococcal antigen specific ELISAs, the titre and avidity of serum IgG subclasses specific for pneumococcal polysaccharides from children vaccinated with Pneumovax was investigated. This approach was employed in order to explore the relationship between age and IgG subclass avidity.

Another aspect of IgG subclass avidity which has received little attention, is the control of affinity maturation. One possible area of regulation addressed in this study was the control of antibody affinity by cytokines. The effect of various cytokines on total IgG avidity was investigated using the antigen specific human tonsil system and utilising solid-phase avidity assays.

CHAPTER 2

General Materials and Methods

GENERAL REAGENTS

Name, chemical formula, formula weight (where appropriate)	Supplier	Code
Acetic acid (CH_3CO_2H)	BDH	10001
Acrylamide (30% w/v), Bisacrylamide (0.8% w/v) (Protogel)	National diagnostics	EC890
Ammonium persulphate (APS), $(NH_4)_2S_2O_8$, FW 228.2	BDH	10032
Ammonium thiocyanate	Sigma	A0302
Bovine Serum Albumin	Sigma	A4503
Bromophenol blue	Sigma	B5525
Butan-1-ol	BDH	10061
Citric Acid, $C(OH)CO_2H(CH_2CO_2H)$, FW 210.1	BDH	10081
Coomassie brilliant blue R	Sigma	B0630
Cyanuric chloride crystals	Sigma	C5393
di-Sodium hydrogen orthophosphate	BDH	10249
Diethylamine	BDH	10341
DMEM	Sigma	D5796
Dimethyl sulphoxide (DMF)	Sigma	D5879
Ethyl alcohol	Hayman Ltd	1170
Foetal calf serum (FCS)	Sigma	F7524
Gentamycin	Roussel	N/A
Glycine, $C_2H_5NO_2$, FW 75.1	BDH	10119
Hepes (sodium salt)	Sigma	H0763
Horse serum (HS)	Life Technology	03-06055M
Hydrochloric acid	BDH	10125
Hydrogen peroxide (30% w/v), H_2O_2	Sigma	H1009
Influenza virus strains (NIB24, X97, X19)	NIBSC	N/A
L-glutamine	Sigma	G7513
Lymphocyte separation medium (Ficoll-Hypaque)	Flow	SDS6H
2-mercaptoethanol	Sigma	M6250
Bovine submaxillary mucin	Sigma	M3895
4-hydroxy-3-iodo-5-nitophenylacetic acid (NIP) -CAP-OSu	Genosys	IT-03-18140A
o-Phenylenediamine (OPD)	Sigma	P8287
p-nitrophenyl phosphate (pNPP)	Sigma	104105
Phosphate buffered saline (PBS) (tablets)	Unipath	BR14a
Percoll	Pharmacia	17-0891-01
Phenolphthalein	BDH	20088

Name, chemical formula, formula weight (where appropriate)	Supplier	Code
Poly-l-lysine	Sigma	P9155
RPMI 1640 with 25mM Hepes and L-glutamine	Gibco	041-02400M (liquid)
		079-03018P (powder)
RPMI 1640 with L-glutamine	Gibco	041-01875M
S-2-aminoethylisothiouronium bromide (AET)	Aldrich	A5460-1
20% sheep blood in alsevers	TCS ltd	N/A
Silver stain protein kit	Biorad	161-0443
Sodium azide, NaN_3, FW 65.0	BDH	10369
Sodium acetate	BDH	10236
di-sodium carbonate	BDH	10240
Sodium carbonate	BDH	10240
Sodium dihydrogenorthophosphate 1-hydrate,	BDH	10245R
$NaH_2PO_4.H_2O$, FW 156.0		
Sodium dodecyl sulphate (SDS)	Sigma	L4509
Sodium hydroxide pellets, NaOH, FW 40.0	BDH	10252
Sodium hydrogen carbonate, $NaHCO_3$, FW 84.0	BDH	10247
Streptavidin alkaline phosphatase conjugate	Amersham	RPN1234
Streptavidin peroxidase conjugate	Zymed	434323
Sulphuric acid, H_2SO_4	BDH	10276
TEMED, N,N,N',N' tetramethylethylenediamine, $C_6H_{16}N_2$	Sigma	T8133
Tris (hydroxymethyl)aminomethane, $C_4H_{10}NO_3$, FW 121.1	ICN	819638
Tween20 (polyoxyethylene(20)sorbitan monolaurate)	BDH	66368
Pneumoccocal polysaccharides - serotype 3	ATCC	169-X
- serotype 6		181-X
- serotype 19		205-X
- serotype 23		217-X

Antibodies	Supplier	Code
Mouse anti-human IgG biotinylated mAb, clone HP6045	Zymed	05-2440
Mouse anti-human IgG1 biotinylated mAb, clone HP6069	Zymed	05-3340
Mouse anti-human IgG2 biotinylated mAb, clone HP6002	Zymed	05-3540
Mouse anti-human IgG3 biotinylated mAb, clone HP6047	Zymed	05-3640
Mouse anti-human IgG4 biotinylated mAb, clone HP6025	Zymed	05-3840
Goat anti-human IgM biotinylated	Sigma	B-1265

All reagents were Analar grade or equivalent

* see Appendix 1 for full address of suppliers

BUFFERS AND SOLUTIONS

ELISA coating buffer

15mM sodium carbonate, (1.59g/litre), 35mM sodium hydrogen carbonate, (2.93g/litre) was prepared in 1litre double distilled water and pH adjusted to 9.6

Horse Radish Peroxidase Substrate Buffer

0.1M citric acid (21g/litre), 0.2M sodium hydrogen phosphate(28.5g/litre) was prepared in 1litre double distilled water. The substrate o-phenylenediamine (OPD) was dissolved in this buffer to give a 0.5mg/ml solution with 0.05% hydrogen peroxide.

Alkaline phosphatase substrate buffer

15mM sodium carbonate (1.59g/litre), 35mM sodium hydrogen carbonate (2.93g/litre), $MgCl_2$ (9.52mg/litre) prepared in double distilled water and pH adjusted to 9.6. The substrate p-nitrophenyl phosphate (pNPP) was dissolved in this buffer to give a 1mg/ml solution.

FPLC Buffers

Phosphate (600ml 0.1M Na_2HPO_4, 400ml 0.1M NaH_2PO_4)

0.1M glycine, pH adjusted to 2.7 with concentrated HCl

20% ethanol, wash buffer

All were prepared in double distilled water, filtered through a 0.2μm filter (Millipore) and degassed before use.

Holding medium

RPMI 1640 (powder, 104.3g/10 litres) was prepared in double distilled water and filter sterilised before use with hepes 25mM, gentamycin 50μg/ml, FCS 5% added.

SDS-PAGE running buffer

0.192M glycine, 0.025M Tris, 0.1% SDS was prepared in 10 litres of double distilled water, pH8.3

Protein sample buffer

4% w/v SDS, 20% w/v glycerol, 0.01% bromophenol blue in 0.01M Tris was prepared in double distilled water and pH adjusted to 6.8. For reducing conditions 4% 2-mercaptoethanol (v/v) added.

Gey's haemolytic Balanced Solution

Three solutions were prepared:

Solution A 650nM NH_4Cl, 25mM KCl, 5.6mM $Na_2HPO_4.12H_20$, 0.9mM KH_2PO_4, 28mM glucose, 0.0005% phenol red, 25g/l gelatine (Difco)

Solution B 21mM $KgCl_2.6H_2O$, 5.6mM $MgSO_4.7H_20$, 31mM $CaCl_2$

Solution C 267mM $NaHCO_3$

All solutions were prepared in double distilled water, sterilised by autoclaving and stored at 4°C. When required, 7 volumes of sterile double distilled water were mixed with 2 volumes of solution A (warmed to melt the gelatine) and 0.5 volumes each of solutions B and C.

Sources Of Sera

Pool of human serum: Clotted venous blood from sixty apparently healthy adults was collected at a single sitting, centrifuged and the serum pooled in equal amounts, aliquoted and stored at -70°C. This pooled serum is referred to as standard serum throughout. Serum for use in the influenza specific ELISA was prepared from the clotted venous blood of one healthy adult and stored aliquoted at -70°C.

Sources of sera used for individual studies are described elsewhere in the relevant chapters.

METHODS

General ELISA procedure

The antigen of choice diluted in coating buffer was adsorbed onto Immulon flat bottomed ELISA plates (Dynatech, Virginia, USA) (75μl/well). Unadsorbed antigen was then removed by washing once with PBS-0.05%Tween20 (PBS-Tw). The plates were either used immediately or stored dry after sealing under polythene and aluminium foil at 4°C for 1-3 months. Test samples were then added to relevant wells (75μl/well) and incubated for 2h at room temperature or 1h at 37°C in a humidified atmosphere. Unbound material was removed by washing four times with PBS-Tw. Bound material was detected by the addition of biotinylated antibodies diluted 1/1000 in PBS-Tw-1%BSA (PBS-Tw-BSA) (75μl/well) and incubated for 2h at room temperature or 1h at 37°C in a humidified atmosphere to prevent evaporation. Following washing with PBS-Tw a 1/5000 solution of streptavidin-peroxidase or 1/1000 solution of streptavidin alkaline phosphatase in PBS-Tw-BSA was added (75μl/well) and the plates incubated for a further 1h at room temperature or 37°C in a humidified atmosphere. Finally plates were again washed and chromogenic substrate solution (OPD or pNPP) added (75μl/well) and left for colour to develop at room temperature in the dark. The colour reaction was stopped by the addition of an equal volume of 2M sulphuric acid (OPD) or 3M NaOH (pNPP) and the absorbance of each well at 490nm (OPD) or 410nm (pNPP) was measured using an automatic ELISA plate reader (Titertek multiskan, Flow), linked to a computer employing Mikrofit Titertek software.

The conditions used for each antigen differed and are shown in Table 2.1

Table 2.1 Antigen Specific ELISA procedures: variations from general method.

Antigen	Coating Buffer	Adsorption Time	Other Variations
Mucin (10μg/ml)	Carbonate	16h 4°C	none
NIP-BSA (1μg/ml)	Carbonate	16h RT	Samples 2h RT All other incubations 1h 37°C
Pneumococcal polysaccharides (12.5μg/ml)	Carbonate	72h 4°C	none
Influenza virus (10μg/ml)	PBS	16h RT	Samples 16h RT, Detector 4h RT, Streptavidin-alkaline phosphatase 2h RT

Purification of mouse-human chimeric monoclonal IgG subclass antibodies

Tissue Culture

B72.3 hybridoma cell lines (IgG1 - Mouse Myeloma Cell (NSO), IgG2, IgG3, IgG4 - Chinese Hamster Ovary) were a gift from Dr. R. Owens, Celltech Ltd, Slough, Berks. Anti-NIP hybridoma cell lines, JW183 (IgG2) and JW184 (IgG4) were a gift from Dr. G. Williams, MRC, Cambridge, UK and THG-MP-2-19-3-8 (IgG3) was supplied by ECACC (Porton Down, UK). Unfortunately IgG1 anti-NIP producing cell lines were unavailable. For antibody purification, cells were allowed to grow to saturation in 0.5 litre DMEM (Gibco) supplemented with 10% foetal calf serum (FCS) (Sigma), 50μg/ml gentamycin (Roussel) and 2mM L-glutamine (Sigma). Cells were then pelleted by centrifugation at 250g for 5 minutes and the supernatant removed.

Purification of mouse-human chimeric IgG subclass antibodies

Culture supernatant was filtered through 0.45μm filter (Millipore) and 1litre concentrated to approximately 10ml using stirred concentrator cells (Amicon). The antibodies were purified by affinity chromatography on a protein-G Superose column (Pharmacia Biotech) using the FPLC system (Pharmacia Biotech). 0.1M glycine-HCL pH2.7 was used to elute bound antibody, which was immediately neutralised with solid Tris salt. Antibodies were then concentrated and dialysed against PBS-0.01% sodium azide using Centriprep-10 concentrators (Amicon). Purified proteins were analysed by reducing and non-reducing SDS-PAGE. Antibodies were then analysed by antigen specific ELISA for antigen binding

activity and to check for any subclass contamination. Antibody concentration was then evaluated by measuring absorbance at 280nm and assuming a value of $A^{1\%}_{1cm,280nm}$ of 13.6.

Coupling of type 3 pneumococcal polysaccharide and influenza virus strains to poly-L-lysine

Pneumococcal polysaccharide and influenza virus strains were coupled to poly-L-lysine using the method described by Gray (1979). A solution of polysaccharide at 1mg/ml in double distilled water or 100μg/ml of influenza virus was prepared. Antigen was made alkaline by the addition of 100μl of antigen solution to 500μl of 0.01M sodium hydroxide, 0.01% phenolphthalein which was mixed for about 10 seconds. This was then added to 0.5mg of cyanuric chloride crystals until the solution clarified. Finally, 100μl of poly-L-lysine at 1mg/ml was added and coupling was allowed to proceed for 2h at +4°C. Optimal coating conditions were found to be a 1/100 dilution of the antigen-poly-L-lysine solution diluted in coating buffer.

Coupling of NIP hapten to BSA

NIP hapten was coupled to BSA using the method described by Valim et al, 1991 (Lucisano Valim and Lachmann, 1991). Briefly 25mg of NIP were dissolved in 1ml dimethyformamide (DMF). A solution of 0.12g BSA in 1ml double distilled water/2.5ml 0.2M $NaHCO_3$, was then mixed with the NIP solution overnight at 4°C stirring continuously. Unconjugated NIP was removed by size

exclusion using a Sephadex G25 column (Pharmacia Biotech). Different epitope densities of NIP-BSA were then separated by ion exchange chromatography using a gradient of NaCl on a monoQ column (Pharmacia Biotech) linked to a FPLC system (Pharmacia Biotech). Molar ratios of NIP-BSA were then calculated in each fraction by absorbance at 430nm and 280nm respectively using molar extinction coefficients of 5 x 10^3 for NIP and 0.67 for BSA.

SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

10% or 7.5% polyacrylamide gels were prepared using the following stock solutions -

(i) Protogel (30% acrylamide)

(ii) 1M Tris-HCl pH8.8

(iii) 1M Tris-HCl pH6.8

(iv) 10% w/v SDS

(v) 1.5% ammonium persulphate (APS)

(vi) N,N,N',N' - tetramethylethylene diamine (TEMED)

1.5mm thick SDS-PAGE slab gels containing 10 or 7.5% acrylamide in 0.1% SDS Tris buffer pH8.8, 0.1% TEMED, 2.5% APS were cast in a Biorad "Protean II" apparatus and allowed to polymerise. Stacking gel containing 5% acrylamide in 0.1% SDS Tris buffer pH6.8, 0.5% TEMED, 5% APS were then cast and allowed to polymerise.

Sample preparation

Protein samples (20μl) were diluted 1:1 with sample buffer (4% w/v SDS, 20% w/v sucrose in 0.01M Tris pH 6.8, 0.01% bromophenol blue). Samples to be analysed under reducing conditions were diluted 1:1 in sample buffer containing 4% v/v 2-mercaptoethanol and boiled for 1minute. Molecular weight standards (Sigma) included on each gel contained the following proteins; myosin (205kDa); β-galactosidase (116kDa); phosphorylase (97.4kDa); bovine albumin (66kDa); egg albumin (45kDa) and carbonic anhydrase (29kDa).

Electrophoresis

The gels were run in a Biorad "Protean II" apparatus with a Tris/glycine buffer of 0.192M glycine, 0.025M Tris, 0.1% SDS pH 8.3. Gels were either run for approximately 4h at 200V or overnight at 45V. Following electrophoresis proteins were visualised by Coomassie brilliant blue R or silver staining.

Coomassie Staining

Gels were stained for approximately 1h in 0.025% Coomassie brilliant blue R (Sigma) in 50% methanol, 5% acetic acid and destained overnight in 10% methanol, 5% acetic acid.

Silver Staining

Gels were silver stained using the Biorad silver stain kit, according to the method of Merril et al. (1981). Gels were fixed for 60 minutes in 40% methanol/10% acetic acid (v/v) and 30 minutes twice in 10% ethanol/5% acetic acid (v/v). Oxidiser was then added for 5 minutes and the gel washed with double distilled water until all colour had been removed. Silver reagent was added for 30 minutes and the gel washed for 2 minutes with double distilled water. Developer solution was added and the reaction stopped with 5% acetic acid.

One dimensional electrophoresis of polysaccharides

Mobility of pneumococcal polysaccharides was analysed by one dimensional electrophoresis on cellulose acetate. Approximately 0.7μl of polysaccharide was loaded as a 3mm band on to a cellulose acetate sheet (Sartorious, Gottigen, W. Germany). Electrophoresis in 0.1M barium acetate pH 6.0 was performed for 4h at 7.5V. The acetate sheet was then stained with alcian blue solution for 30 minutes and destained in 5% acetic acid. Heparin standards were included on each sheet.

CHAPTER 3

The avidity of mouse-human chimeric V region identical monoclonal IgG subclasses as measured by solid-phase ELISA

INTRODUCTION

Antibody avidity (functional affinity) is expressed as the strength of the interaction between an antibody paratope, determined by variable region structure, binding to its complimentary antigen (Steward, 1981). Despite the importance of variable region structure, differences in avidity between naturally occurring IgG subclasses specific for the same antigen have been observed.

The avidity of naturally occurring IgG subclasses

Avidity differences between naturally occurring and vaccine induced IgG subclasses specific for the same antigen have been demonstrated by several investigators. Devey et al. (1985 and 1988) using DEA elution and competitive binding ELISAs found that, in individuals immunised with tetanus toxoid, IgG1 serum antibodies specific for tetanus toxoid have higher affinity than similar antibodies of the IgG4 isotype. Persson et al. (1988) using competitive binding ELISA demonstrated affinity differences between IgG subclasses directed against hepatitis B, which could be ranked in the order IgG1 > IgG2 > IgG3 > IgG4. Subclass associated affinity restriction has also been demonstrated for naturally occurring antibodies specific for *M. catarrhalis*, with IgG3 being of higher affinity than IgG1 (Goldblatt et al. 1991). Persson et al. (1988) have also shown that such subclass avidity differences are not restricted to protein antigens. Employing competitive binding ELISAs the authors found IgG2 antibodies specific for pneumococcal polysaccharide serotype 3 to be of higher affinity than IgG1.

IgG subclass avidity associated with disease states

Subclass associated affinity restriction has also been demonstrated in the disease state. Low affinity IgG1 reactive with hepatitis B core antigen has been shown to be associated with chronic liver disease whereas asymptomatic carriers have high affinity IgG1 (Wen et al. 1990). Circulating autoantibodies to myeloperoxidase found in patients with renal vasculitis are associated with a high titre of IgG1 antibodies, although using DEA elution, IgG3 antibodies specific for this antigen were reported to be of higher affinity than IgG1. Follow up studies in the same patient group demonstrated affinity maturation for IgG1 and IgG4 as IgG3 affinity decreased (Esnault et al. 1991). Antibody avidity differences have also been used to distinguish between primary exposure and reinfection. Primary rubella infection has been demonstrated to be associated with lower avidity IgG1 and IgG3 whereas reinfection gave rise to high affinity IgG1 and IgG3 (Thomas and Morgan Capner, 1988).

It would appear that serum IgG subclasses show affinity differences for the same antigen. The mechanisms underlying such differences are at present not understood, but may involve differences in epitope specificity and consequently different variable (V) region gene usage or differences in regions outside the V genes.

The influence on avidity of antibody structure remote from the V region

Evidence supporting the hypothesis that immunoglobulin domains distant from the V region may influence antibody binding has been presented in studies employing murine monoclonal antibodies. Fulpius et al. (1993) showed that an IgG1 switch variant of a murine monoclonal IgG3 parent lacked the expected rheumatoid factor activity, despite the fact that the mAbs possessed identical V regions. Similarly, Schreiber et al. (1993) demonstrated affinity differences between an IgG1 switch variant of a V region-identical murine monoclonal IgG3 parent specific for a *Pseudomonas* species, with IgG1 being of lowest affinity. Cooper et al. (1993 and 1994) have demonstrated differences in affinity between V region identical mouse monoclonal IgG1, IgG2b and IgG3 antibodies directed against N-acetyl-glucosamine (GlcNAc) of streptococcus group A carbohydrate with IgG3 being of the highest affinity.

It would appear that in the mouse, structures remote from the V region influences IgG subclass avidity. It is possible that structures outside the V region may also influence the avidity of human IgG subclasses and thus contribute to the avidity differences noted in polyclonal serum IgG subclasses specific for the same antigen. In order to investigate this possibility we have employed two matched sets of V region identical mouse-human chimeric IgG subclasses specific for two different antigens, namely the glycoprotein mucin and the hapten NIP.

B72.3 mAbs

mAb B72.3 (IgG1) was originally raised in mice following immunisation with a membrane enriched fraction of a human carcinoma (Colcher et al. 1981). B72.3 has been shown to react against a variety of human carcinomas such as those derived from the colon and ovary (Stramigoni et al. 1986). It is largely unreactive with normal human tissue, except for secretory endometrium (Thor et al. 1989).

B72.3 reacts with the antigen TAG72, identified as a mucin-like glycoprotein of high molecular weight (220-400kDa) purified from the LS-174T human colon cancer xenograft and is expressed in large amounts on bovine submaxillary mucin (Johnson et al, 1986). The epitope on TAG72 reactive with B72.3 has been reported as sialosyl-Tn(NeuAc2-6αGalNacα1-O-Ser/Thr) (Kjeldsen et al. 1988; Gold and Mattes, 1988). The TAG72 antigen is also expressed in foetal tissue and as such as been defined as an oncofoetal antigen (Thor et al, 1986). B72.3 mAb has been employed successfully for studying human carcinomas and for tumour imaging (reviewed by Adair, 1992).

Chimeric mAbs of B72.3 of all four IgG subclasses (γ1-γ4) have been successfully produced. These antibodies are secreted from the stable cell lines CHO, NSO and Sp2/0 as fully assembled tetramers and have been shown to retain their specificity for their antigen TAG72 (Adair, 1992).

Anti-NIP mAbs

A panel of cell lines secreting mouse-human chimeric mAbs directed against the hapten, NIP were initially constructed to investigate the effector functions of human antibodies, such as their ability to bind human C1q and mediate lysis of human erythrocytes in the presence of complement (Bruggemann et al. 1987). Since they were first described these antibodies have proven to be a valuable tool in the investigation of various functional aspects of the human antibody system (Garred et al. 1990; Valim and Lachmann, 1991; Michaelsen et al. 1991; Brekke et al. 1993; Allen et al. 1986).

Solid-phase avidity ELISAs

A wide variety of techniques have been described for measuring the affinity and kinetics of antigen-antibody interactions. Traditional methods such as equilibrium dialysis, spectrofluorometric methods and precipitation methods require antibody and antigen to be in solution, to be labelled, of high purity and available in relatively large amounts (reviewed extensively by Steward, 1986; Van Regenmortel and Azimadeh, 1994). These methods permit the determination of K values (equilibrium affinity constants) for a number of antigen-antibody interactions but are of limited use if polyclonal serum and large complex antigens are to be investigated.

The development of solid phase ELISA procedures has permitted the determination of antigen-antibody avidity of such impure preparations. Solid-phase avidity assays also bypass the need for labelling and are simple and convenient to perform in a routine laboratory. Two types of ELISA procedures

have been developed to measure antibody avidity; the competitive binding ELISA which employs free antigen to competitively inhibit antigen binding, and elution assays which employ a chaotropic agent such as thiocyanate, or a mild protein denaturant such as urea to disrupt antigen-antibody binding.

The Competitive Binding ELISA

Two types of competitive binding ELISAs have been described. In both procedures the binding of a constant concentration of antibody to antigen immobilised on an ELISA plate is inhibited by a range of concentrations of antigen free in solution. In the method described by Friguet et al. (1985) the antibody and antigen are incubated together for at least 12 hours to allow equilibrium to be reached before the concentration of free antibody is determined by using an antigen specific ELISA. K values can only be derived when this technique is applied to monoclonal antibodies and haptenic antigens. Where such conditions are not met, for example for polyclonal serum antibodies and multivalent antigens, avidity indices may be calculated as the amount of free antigen required to produce a 50% reduction in antibody binding.

In the method described by Rath et al. (1988) antibody and free antigen are added simultaneously to the ELISA plate. It is questionable whether accurate absolute affinity constants can be calculated employing this method since equilibrium is not approached. Nevertheless, relative avidity may be determined using avidity indices.

An adaptation of the competitive binding ELISA has been proposed by Azimzadeh and Van Regenmortel (1991), in which the amount of free antibody at

equilibrium is determined by separating free from bound antibody using ultracentrifugation and measuring free antibody in the supernatant. This method is restricted to situations where antibody and antigen can be separated by ultracentrifugation, and also suffers the disadvantage that the mAb must be labelled with peroxidase.

Elution ELISAs

Elution assays employ a mild protein denaturant such as guanidine hydrochloride (Inouye et al. 1984) or a chaotropic agent such as thiocyanate (Pullen et al. 1986) or diethylamine (Devey et al. 1985) at a variety of concentrations to disrupt antigen-antibody binding. The greater the amount of eluting agent required to reduce antibody binding by 50% the higher the relative avidity of the antibody. Such assays do not permit the calculation of an affinity constant but do allow the avidity of various antibodies specific for the same antigen to be ranked and have proven useful in a variety of experimental systems.

Applications of Solid-Phase Avidity ELISA procedures

The avidity assays described above have been employed to measure the avidity of a number of antigen-antibody interactions and some of these are listed in Table 3.1.

Table 3.1 Applications of solid-phase avidity ELISA procedures

Technique	Antigen	Reference
Competitive (Friguet et al, 1985)	β2 subunit E.coli tryptophan synthase	(Friguet et al. 1985)
	Factor V	(Annamalai et al. 1987)
	hirudin (inhibitor of thrombin)	(Schlaeppi et al. 1990)
	fibronectin	(Rostagno et al. 1991)
	group B streptoccoci, E. coli	(Wolff et al. 1992)
	lipopolysaccharide	(Bruderer et al. 1992)
	dsDNA	(Suenaga and Abdou, 1993)
	anti-nip IgG2a	(Jacobson et al. 1994)
	human IgG	(Kennedy et al. 1994)
	HIV V3 loop	(Seligman, 1994)
Competitive (Rath et al, 1988)	DNP-OVA	(Rath et al. 1988)
	P. falcipirum	(Ahlborg et al. 1993)
	myeloperoxidase (MPO)	(Ehrenstein et al. 1992)
	streptococcal antigens	(Wada et al. 1988)
DEA elution (Devey et al, 1985)	rubella	(Morgan-Capner and Thomas, 1988)
	MPO	(Esnault et al. 1991)
	M. catarrhalis, Fel d 1	(Goldblatt et al. 1993)
Thiocyanate elution (Pullen et al, 1986)	rubella	(Pullen et al. 1986)
	casein, β-lactoglobulin	(Jones et al. 1987)
	tetanus toxoid, diphtheria toxoid, cardiolipin	(Sutjita et al. 1988)
	phosphocholine	(Hall and Heckel, 1988)
	P. falcipirum circumsporozite (CS) protein	(Rickman et al. 1991)
	bovine heart cytochrome C oxidase	(Schneyder et al. 1991)
	pneumococcal polysaccharide serotype 3	(Gray and Shaw, 1993)
	M. catarrhalis, Fel d 1	(Goldblatt et al. 1993)
	poliovirus vaccines	(Mellander et al. 1993)
	poliovirus type 1	(Zaman et al. 1993)
	tetanus toxoid, E.coli	(Herias et al. 1993)
Urea elution	Rubella	(Rousseau and Hedman, 1988)
	T.gondii	(Hedman et al. 1989)
	Hepatitis C	(Wreghitt et al. 1990)
	T.gondii	(Holliman et al. 1994)

Aims of The Study

The possible influence of the human constant region on antibody avidity has, as yet, not been adequately investigated. Chimeric mouse-human mAbs consisting of identical mouse V regions combined with different human constant regions (γ1-γ4) provide an ideal tool with which to study this possibility. Two stable hybridoma cell lines producing B72.3 chimeric mAbs and anti-NIP chimeric mAbs were utilised to investigate IgG subclass associated antibody avidity employing solid-phase avidity ELISAs.

METHODOLOGY

Antibody Purification

Monoclonal antibodies were purified from culture supernatant by affinity chromatography on a Sephadex protein-G column using FPLC (as described in Chapter 2). The presence of heavy and light chains was then confirmed by 10% SDS-PAGE under reducing conditions. The antibodies had the expected molecular weight (including the expected earlier elution position of the IgG3 product) and the absence of aggregates was confirmed following size exclusion gel filtration employing a Superdex-200 column (Pharmacia) linked to an FPLC system. The subclass composition of each chimeric antibody preparation was then analysed by antigen specific ELISA, employing bovine submaxillary mucin (Sigma) as a capture antigen and mouse anti-human IgG subclass mAbs (Zymed) to detect specific subclasses, in order to exclude contamination. Finally, protein concentrations were determined by measuring absorbance at 280nm and assuming a value of $A^{1\%}_{1cm,280nm}$ of 13.6.

Conjugation of NIP to BSA

NIP was conjugated to BSA as described in Chapter 2. Briefly, unconjugated NIP was removed by gel filtration employing a Sephadex G25 column (NAP5, Pharmacia). Various NIP-BSA epitope densities were then separated by anion exchange employing a monoQ column using the FPLC system. 1ml of sample was loaded onto the column and a TRIS/HCl/NaCl pH7.6 gradient applied to elute bound material, 0.5ml fractions were collected and molar NIP to BSA ratios determined spectrophotometrically.

Solid-phase Avidity ELISAs

(i) Competitive binding ELISA

In this assay antibody avidity was measured by a modification of the antigen specific ELISA, including serial dilutions of free antigen to competitively inhibit antigen-antibody interactions (as described in Chapters 2 and 5). Results were then expressed as the log % reduction in absorbance in the presence of free antigen plotted against the concentration of free antigen. Avidity indices were then calculated as the amount of free antigen required to produce a 50% reduction in maximal absorbance.

(ii) Thiocyanate elution ELISA

The antibody avidity of the purified IgG subclasses was measured by a modification of the antigen specific ELISA, including an incubation step with various concentrations of ammonium thiocyanate to disrupt antigen-antibody binding (as described in Chapters 2 and 5). Results were then expressed as the log % reduction in absorbance in the presence of ammonium thiocyanate plotted against concentration of ammonium thiocyanate. Antibody avidity was displayed as an avidity index corresponding to the molar concentration of ammonium thiocyanate required to produce a 50% reduction in absorbance.

Calculations

The percentage change in absorbance for each serum at different molarities of ammonium thiocyanate or free antigen was obtained using the following equation:

$$100 \times \frac{\text{(mean Abs wells with PBS-Tw-BSA) - (mean Abs wells with } NH_4SCN \text{ or antigen)}}{\text{mean Abs wells PBS-Tw-BSA}}$$

The log % reduction in absorbance was plotted against molarity of ammonium thiocyanate. A line was then drawn on the graph at the log 50% (1.699). At the point where the line crossed the curves of individual sera, a perpendicular was dropped and the corresponding x value determined. This value was taken as the avidity index.

RESULTS

Purification of B72.3 and anti-NIP IgG subclass mAbs

10ml of concentrated culture supernatant were loaded onto a protein G column and bound antibody eluted with 0.1M glycine - HCl buffer pH 2.7. 1ml fractions were collected and neutralised immediately with 2M Tris Fractions corresponding to the protein peak were then pooled and the presence of heavy and light chains revealed by reducing 10% SDS-PAGE (Figure 3.1).

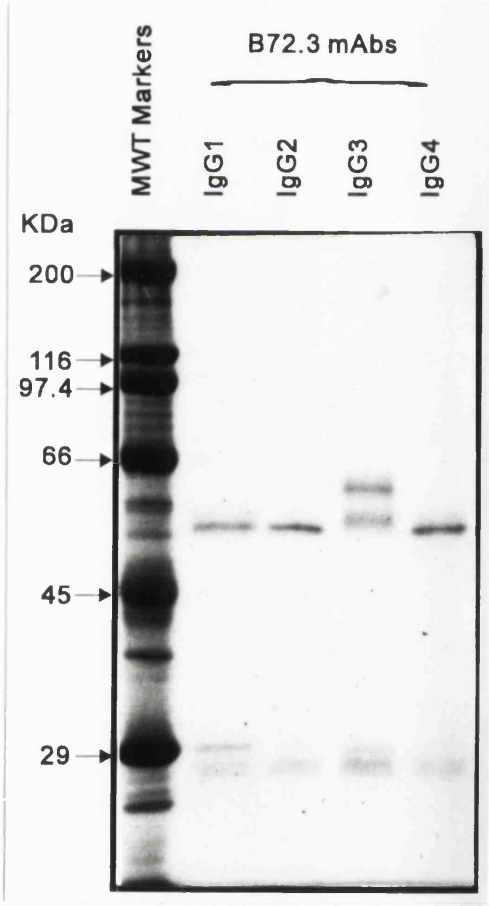


Figure 3.1 10% reducing SDS-PAGE of B72.3 mAbs
1ml fractions were collected from the protein G column. Samples were prepared under reducing conditions (as described in Chapter 2) and 40µl loaded onto the gel. Proteins were then visualised by Coomassie brilliant R staining. A major band can be seen at approximately 55kDa, representing heavy chains for B72.3 IgG1, IgG2, and IgG4 mAbs and a major band at approximately 25kDa, representing light chains. Two major bands were observed in the case of B72.3 IgG3 one approximately 55kDa and one approximately 60kDa.

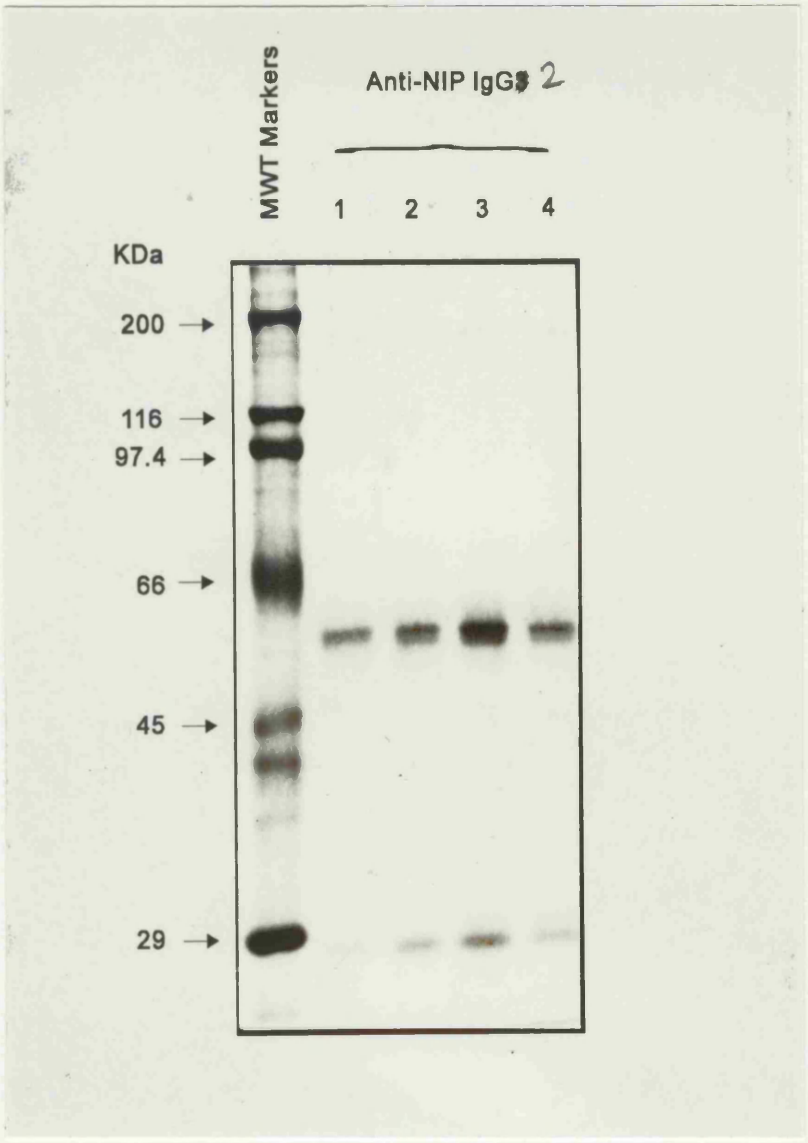


Figure 3.2a 10% reducing SDS-PAGE of anti-NIP mAbs

1ml fractions were collected from the protein G column. Samples were prepared under reducing conditions (as described in Chapter 2) and 40µl loaded onto the gel. Proteins were then visualised by Coomassie brilliant R staining.

Panel A represents anti-NIP IgG2 preparations from 4 separate column loadings. Major bands at the expected molecular weights for the heavy and light chains were observed at approximately 55 and 25kDa.

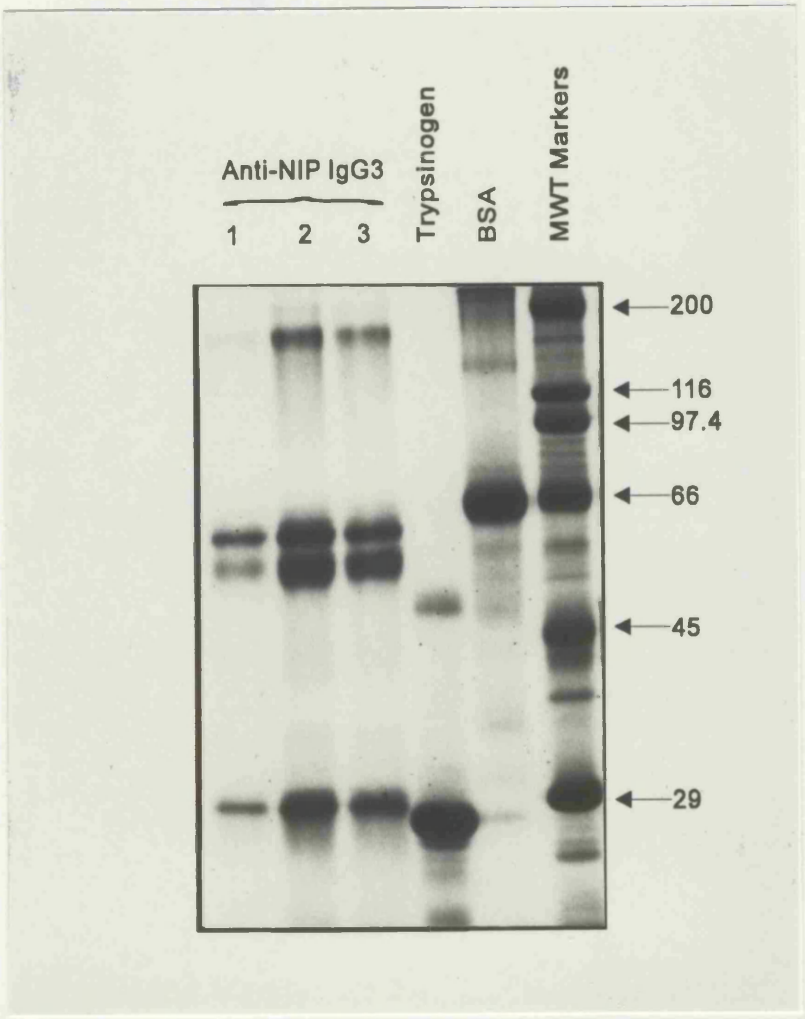


Figure 3.2b 10% reducing SDS-PAGE of anti-NIP mAbs
Panel B represents anti-NIP IgG3 preparations from 3 separate column loadings. Major bands were observed in all three preparations at approximately 60kDa, 55kDa presumably reflecting heavy chains and approximately 30kDa presumably reflecting light chains.

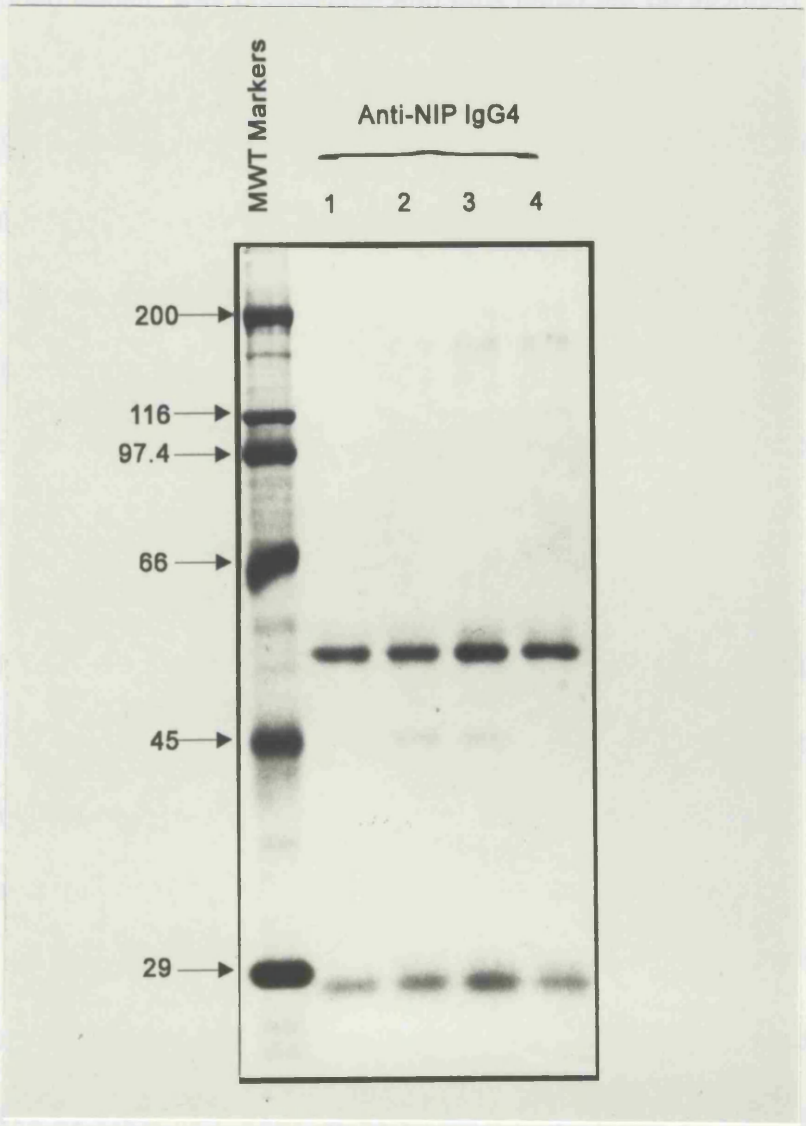


Figure 3.2c 10% reducing SDS-PAGE of anti-NIP mAbs
Panel C represents anti-NIP IgG4 preparations from 4 separate column loadings. Major bands at the expected molecular weights for the heavy and light chains were observed at approximately 55 and 25kDa. Minor bands were also observed in lanes 2 and 3 at approximately 45kDa.

When analysed by reducing 10% SDS-PAGE the presence of heavy and light chains were confirmed by the major banding patterns at approximately 55kDa and 25kDa observed for IgG1, IgG2 and IgG4 mAbs. Anti-NIP IgG1, as mentioned earlier, was unavailable and thus could not be included. Minor bands were observed for B72.3 IgG1 mAb corresponding to 116kDa, 97.4kDa , 66kDa and 29kDa. This banding pattern is the same as that observed for the molecular weight markers. These bands presumably reflect contamination of the lane with molecular weight markers and it is unlikely that these faint bands represent the presence of contaminating proteins. This was confirmed by further 10% SDS-PAGE analysis under reducing conditions (data not shown) and by analysis of the intact protein by non-reducing 7.5% SDS-PAGE (Figure 3.3)

Analysis of B72.3 IgG3 and anti-NIP IgG3 preparations by reducing 10% SDS-PAGE revealed the presence of a doublet at approximately 60-55kDa. The exact nature of these doublets is unclear but are unlikely to be due to contaminating proteins. These bands are possibly due to differing glycosylation patterns or alternatively, they may reflect incomplete reduction.

Preparations of mAbs were then pooled, diluted with PBS 0.1% azide and concentrated using centiprep-10 (Amicon) concentrators. Purified mAbs were analysed by 7.5% non-reducing SDS-PAGE (Figure 3.2) and their concentration calculated from their absorbance at 280nm.

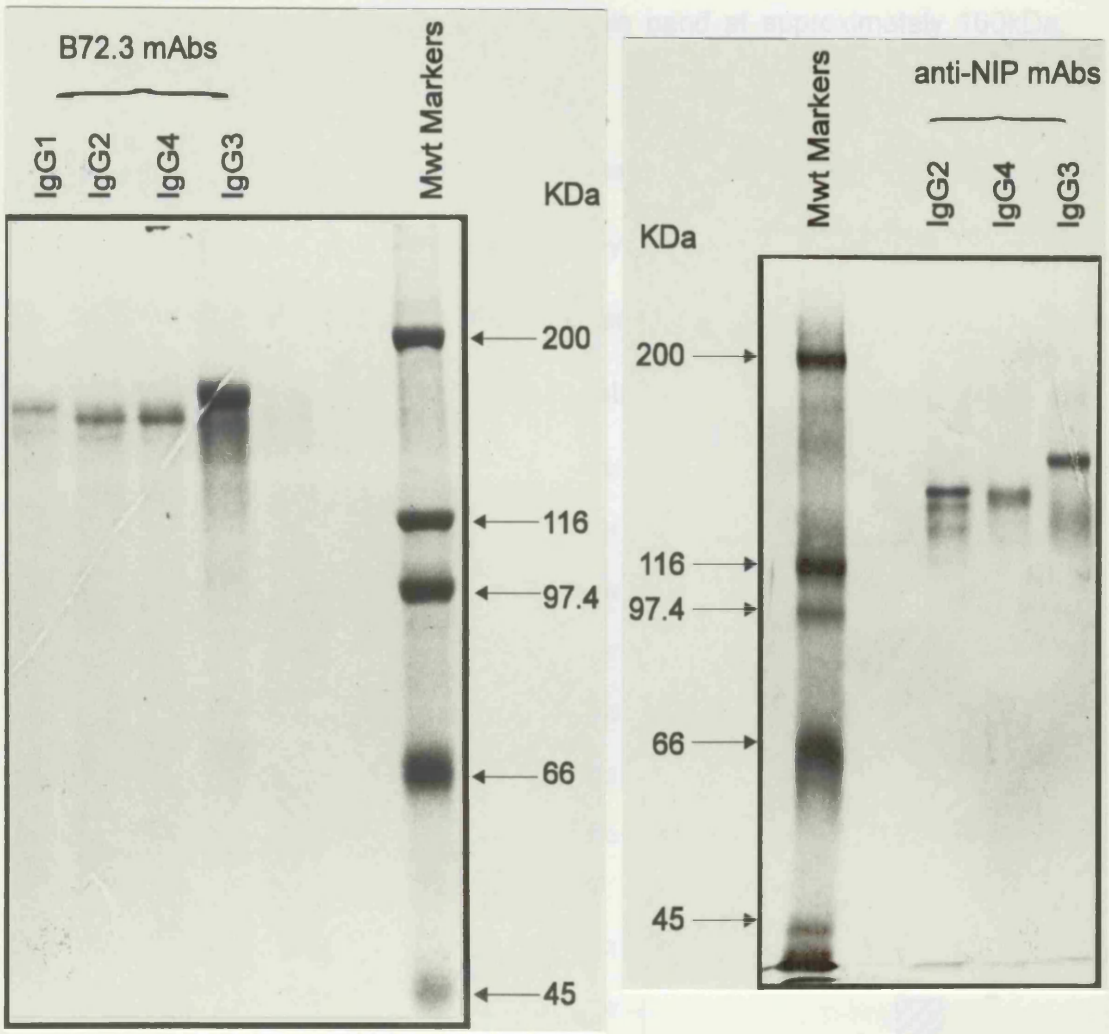


Figure 3.3 7.5% non-reducing SDS-PAGE of purified IgG subclasses
20μl of purified mAb was diluted 1:1 with sample buffer (as described in Chapter 2), loaded onto a 7.5% polyacrylamide gel and the gel run overnight at 50V. Proteins were then visualised by Coomassie brilliant blue staining. Panel A represents B72.3 mAbs and panel B represents anti-NIP mAbs. B72.3 mAbs IgG2 and IgG4 showed bands at approximately 150kDa, the IgG1 band was slightly heavier at approximately 160kDa and IgG3 gave a band at approximately 170kDa. A single band at approximately 150kDa can be seen for anti-NIP mAb IgG4, the lane containing anti-NIP mAb IgG2 showed the expected major band at 150kDa and two slightly smaller species. Anti-NIP mAb IgG3 gave a band at approximately 170kDa.

As expected analysis of purified B72.3 and anti-NIP mAbs by non-reducing SDS-PAGE demonstrated a single band having the expected molecular weights for IgG1, IgG2 and IgG4 of approximately 150kDa and IgG3 170kDa. In contrast B72.3 IgG1, was found to migrate as a single band at approximately 160kDa, possibly due to more extensive glycosylation. Furthermore, two minor additional bands were found in the anti-NIP IgG2 pattern. The exact nature of these is unclear, but may reflect degraded antibody fragments or perhaps differing glycosylation patterns. Antibodies were then analysed by antigen specific ELISA and found to be free of other contaminating subclasses (Figures 3.4 and 3.5).

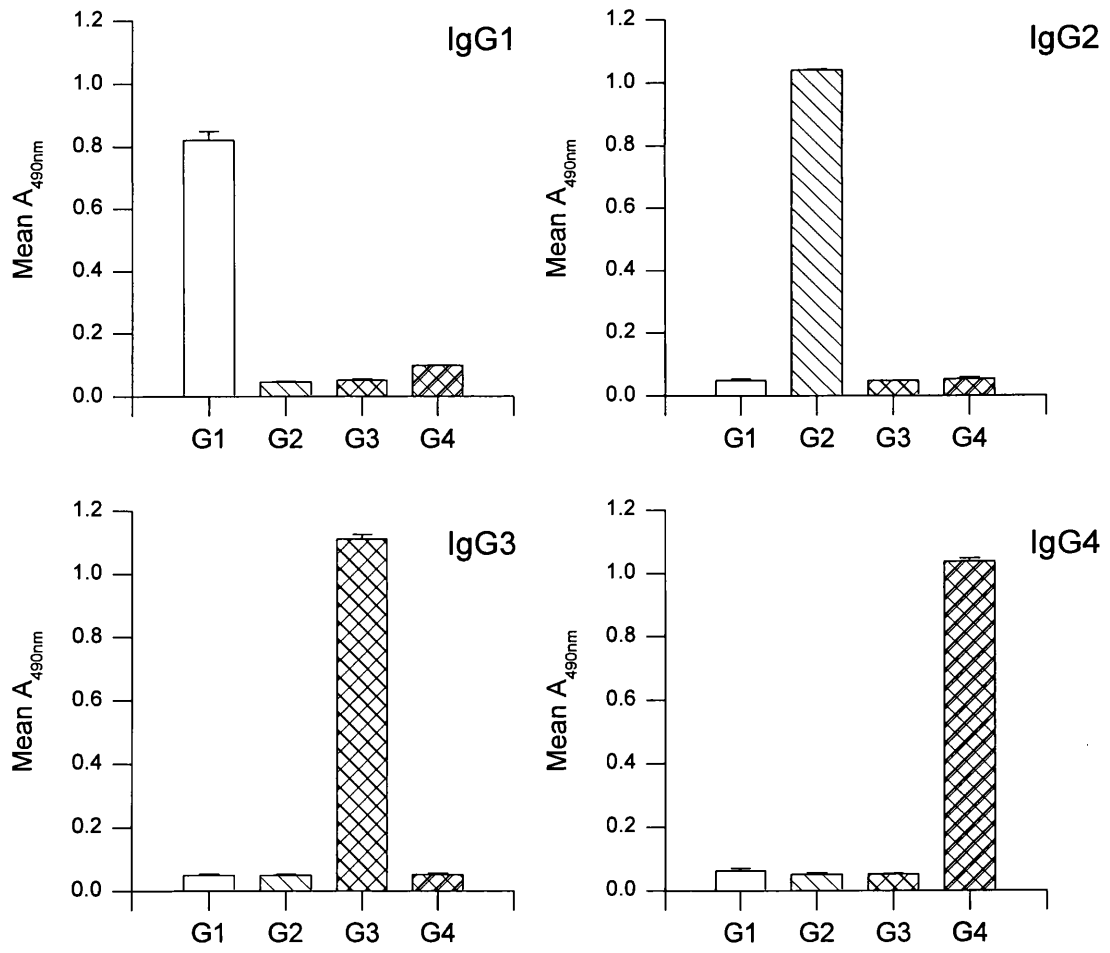


Figure 3.4 Mucin specific ELISA of purified B72.3 IgG subclasses
Each IgG subclass was bound to mucin adsorbed to Immulon2 plates at the same concentration and probed with antibodies specific for each subclass.

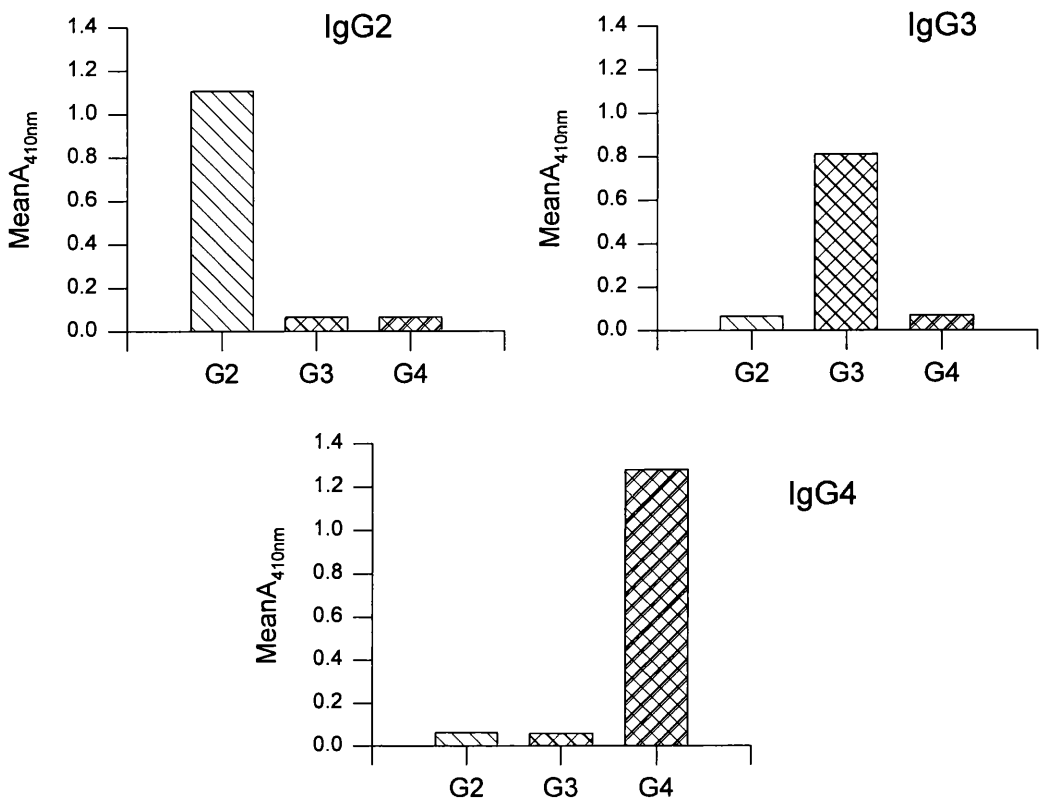


Figure 3.5 NIP specific ELISA of purified anti-NIP IgG subclasses
Each IgG subclass was bound to NIP_{12}-BSA adsorbed to Immulon2 plates at the same concentration and probed with antibodies specific for each subclass

Antigen Specific ELISAs

(a) Mucin specific ELISA for the measurement of B72.3 IgG subclasses

B72.3 mAbs recognise the TAG72 antigen present on bovine submaxillary mucin and therefore mucin was employed in an antigen specific ELISA for the analysis of B72.3 IgG subclass antibodies. Various conditions were investigated to optimise the ELISA sensitivity and these are described in detail in Chapter 2. Dose-response curves were then performed to determine the antibody concentration which would produce an absorbance of 1.0 for use in the avidity ELISAs (Figure 3.6).

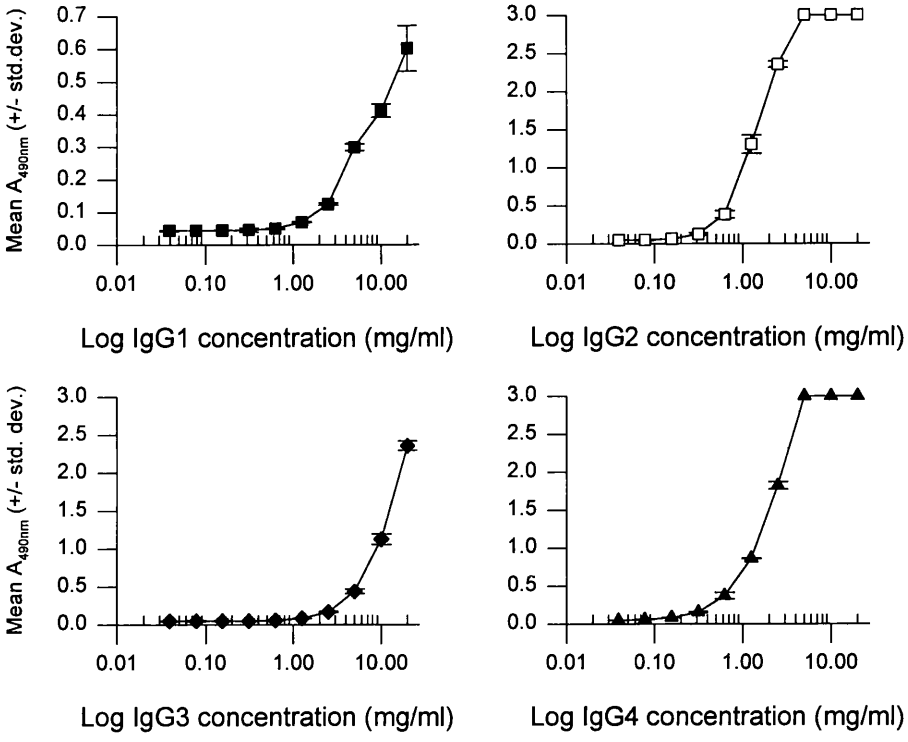


Figure 3.6 Dose-response curves obtained for B72.3 IgG1 (■), IgG2 (□), IgG3 (◆) and IgG4 (▲) binding to mucin
Extrapolating from the curves B72.3 mAbs gave an absorbance of approximately 1.0 at the following concentrations IgG1 - 20μg/ml, IgG2 - 1μg/ml , IgG3 - 10μg/ml and IgG4 - 2μg/ml.

(b) NIP-BSA specific ELISA for the measurement of anti-NIP IgG2, IgG3 and IgG4 subclasses

Anti-NIP IgG2, IgG3 and IgG4 mAbs are specific for the hapten, NIP. The hapten itself does not adsorb to polystyrene plates and therefore was conjugated to a carrier protein, BSA, to facilitate adsorption. Optimal conditions for the ELISA were investigated and are described in detail in Chapter 2. Dose-response curves were then constructed for each subclass binding to 3 different NIP-BSA epitope densities of NIP_{19}-BSA, NIP_{12}-BSA and NIP_8-BSA, to allow determination of the antibody concentration which gave an absorbance of approximately 1.0 for use in further avidity assays (Figure 3.7)

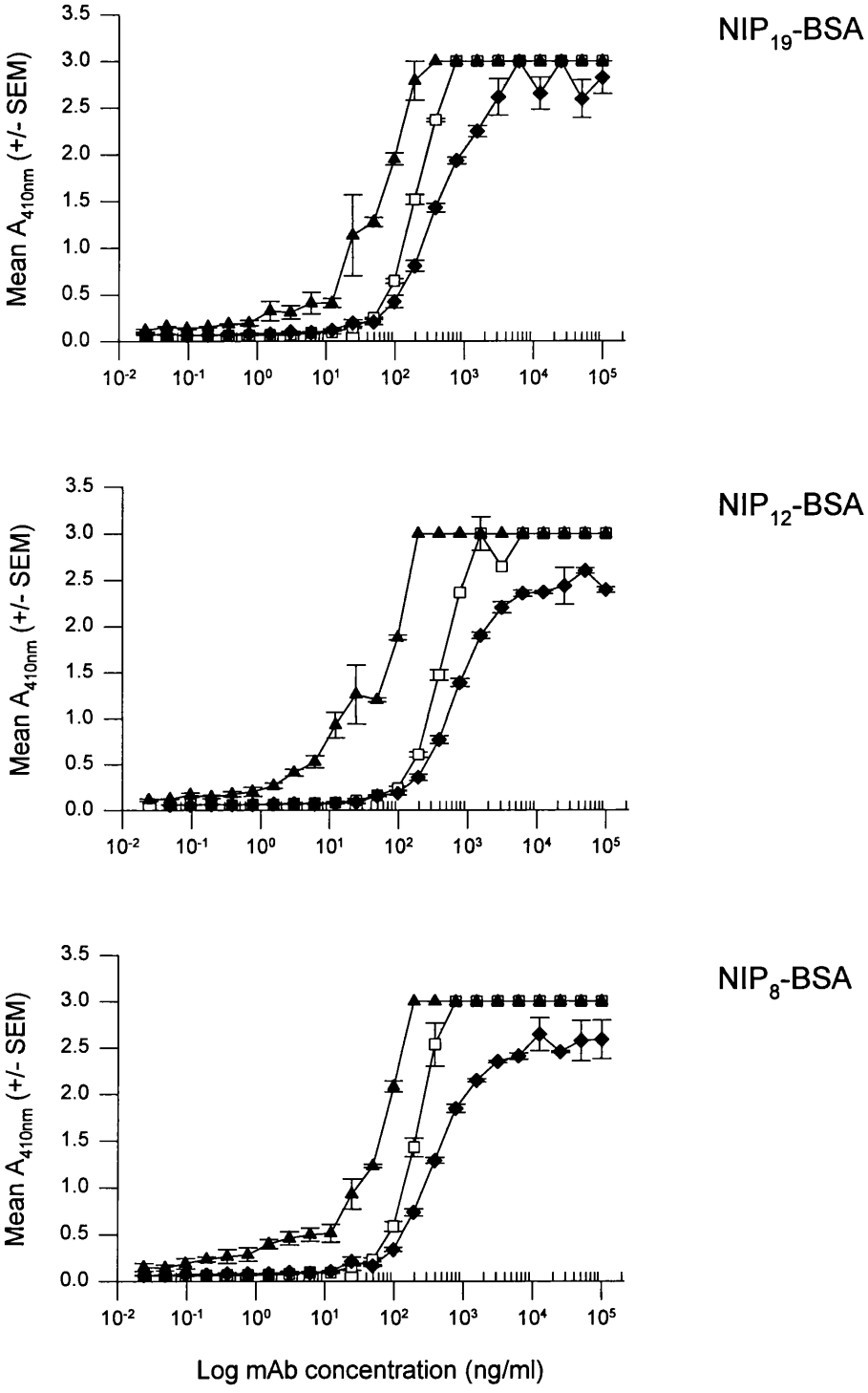


Figure 3.7 Dose-response curves obtained for anti-NIP IgG2 (□), IgG3 (◆) and IgG4 (▲) binding to NIP-BSA at epitope densities of NIP_{19}-BSA, NIP_{12}-BSA and NIP_8-BSA

Extrapolating from the curves anti-NIP mAbs gave an absorbance of approximately 1.0 at the following concentrations IgG2 - 100ng/ml , IgG3 - 400ng/ml and IgG4 - 500ng/ml.

Solid-Phase Avidity ELISAs

(i) Competitive binding ELISA: B72.3 mAbs

A modified mucin specific ELISA employing various concentrations of free mucin to compete for antibody binding was investigated for suitability in the measurement of B72.3 IgG subclass avidity. A detailed description of the method employed is described in Chapter 2.

Various concentrations of mucin were used and a range from 100μg/ml to 0.2μg/ml was found to be optimal. A concentration of mAb previously determined to give an absorbance of approximately 1.0 was incubated for 2h in the presence or absence of free mucin in solution. The degree of antibody binding to antigen was then investigated in a mucin specific ELISA. Results were expressed as the log of the percentage reduction in absorbance, with antibody binding when preincubated with PBS-Tw-BSA taken as 100% binding (Figure 3.8). Avidity indices were calculated as the amount of free antigen required to produce a 50% reduction in absorbance. The smaller the amount of free antigen required to produce a 50% reduction in binding the higher the corresponding antibody avidity. The ELISA was repeated five times on five different days and avidity indices for all four IgG subclasses calculated (Table 3.2).

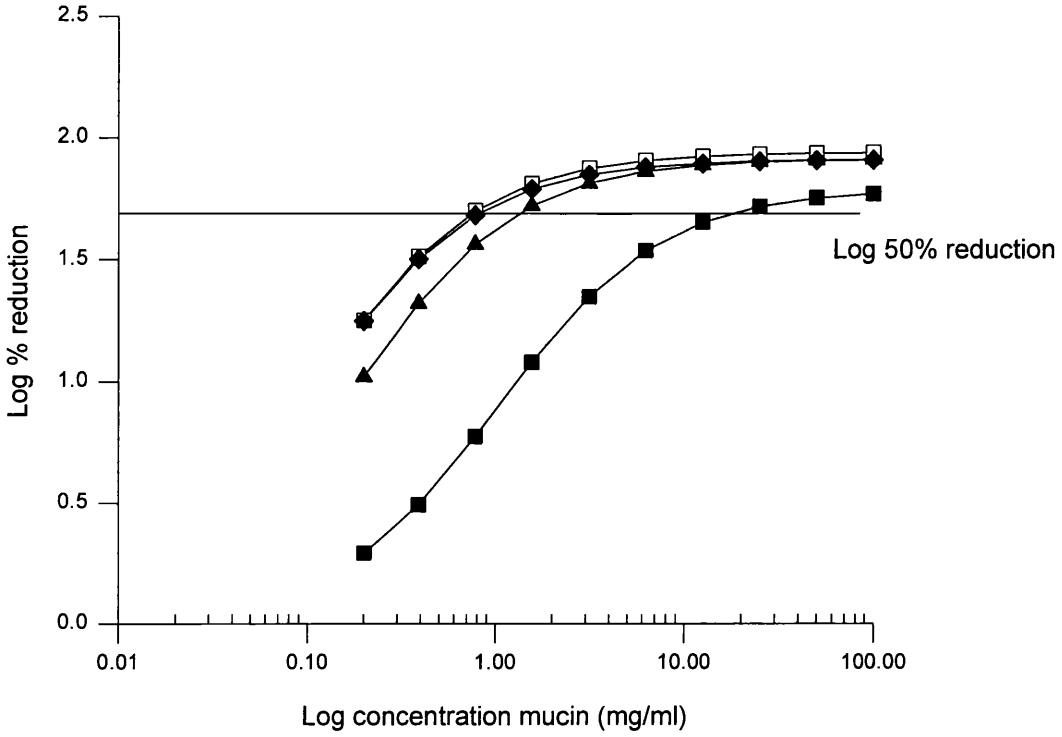


Figure 3.8 Competitive binding curves of B72.3 mAbs binding to mucin
Free mucin in solution was employed at a range of concentrations from 100μg/ml serially diluted to 0.2μg/ml to compete for binding of antibody to solid-phase antigen. All four IgG subclasses, IgG1 (■); IgG2 (□); IgG3 (◆) and IgG4 (▲) were investigated for antibody binding on the same ELISA plate.

Table 3.2 Avidity Indices derived for B72.3 mAbs measured by competitive binding ELISA

Experiment	IgG1	IgG2	IgG3	IgG4
1	2.29	0.90	1.15	1.00
2	ND*	0.75	0.75	1.35
3	6.25	0.68	0.38	0.73
4	1.23	1.92	1.75	2.54
5	1.90	2.38	4.56	0.63

* ND = not done

The interassay variations derived were as follows IgG1 = 77.6%, IgG2 = 58.3%, IgG3 = 97.1% and IgG4 = 61.9%. No conclusions could be reached regarding the avidity of B72.3 IgG subclasses employing the competitive binding ELISA due to the very high variability observed in the assay.

(ii) Competitive binding ELISA: anti-NIP mAbs

A modified NIP specific ELISA employing various free concentrations of NIP was explored for measuring avidity of the anti-NIP mAbs (as described in Chapter 2). NIP_{12}-BSA was employed from 1500ng/ml to 3ng/ml and the ELISA carried out as described for mucin (Figure 3.9).

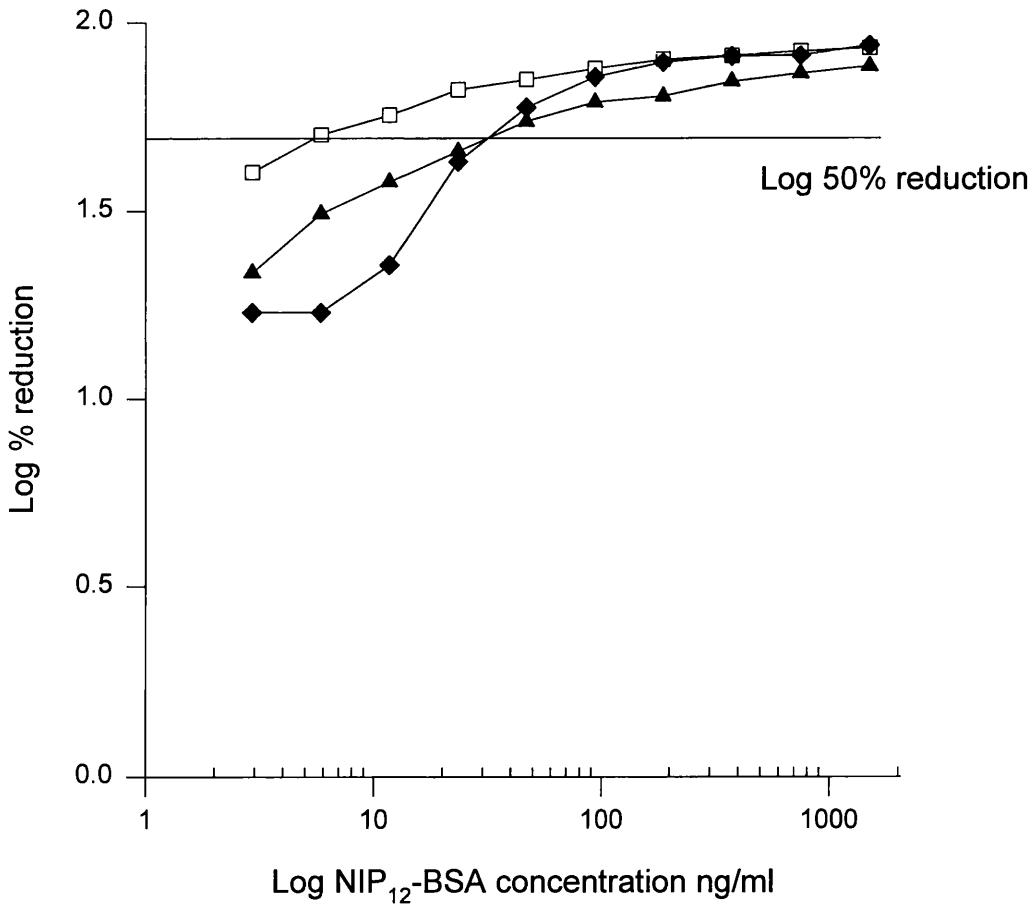


Figure 3.9 Competitive binding curves of anti-NIP mAbs binding to NIP_{12}-BSA

Free NIP_{12}-BSA in solution was employed over a concentration range from 1500ng/ml serially diluted to 3ng/ml to compete for binding of antibody to solid-phase antigen. All three IgG subclasses IgG2 (□), IgG3 (◆) and IgG4 (▲) were investigated for antibody binding on the same ELISA plate.

Avidity indices were then calculated as described previously for the B72.3 mAbs, as shown in Table 3.3

Table 3.3 Avidity indices derived for anti-NIP IgG2, IgG3 and IgG4 mAbs measured by competitive binding ELISA

Experiment	IgG2	IgG3	IgG4
1	13.8	ND#	222.0
2	9.2	ND	73.8
3	5.5	33.3	34.5
4	4.7	67.1	117.6

#ND = not done. Due to insufficient purified mAb being available.

The interassay variations for IgG2 and IgG4 were found to be:

IgG2 = 50.6%

IgG4 = 72.1%

These interassay variations were regarded as unacceptably high and the competitive binding ELISA was therefore not pursued further.

(iii) Thiocyanate elution ELISA

The antibody avidity of the B72.3 and anti-NIP mAbs was investigated employing the thiocyanate elution ELISA. Various concentrations of ammonium thiocyanate ranging from 0.1M to 10M were investigated to find optimal experimental conditions. The range 0.1M to 0.6M was found to be optimal for B72.3 mAbs, whereas 0.1M to 2M was optimal for anti-NIP mAbs.

It is theoretically possible that ammonium thiocyanate could disturb the binding of antigen to the polystyrene plate. This was investigated by preincubation of an antigen coated ELISA plate with 1M ammonium thiocyanate or PBS-Tw-BSA for 15 minutes before performing a standard curve of B72.3 mAbs or anti-NIP mAbs. Representative curves are shown in Figures 3.10 and 3.11.

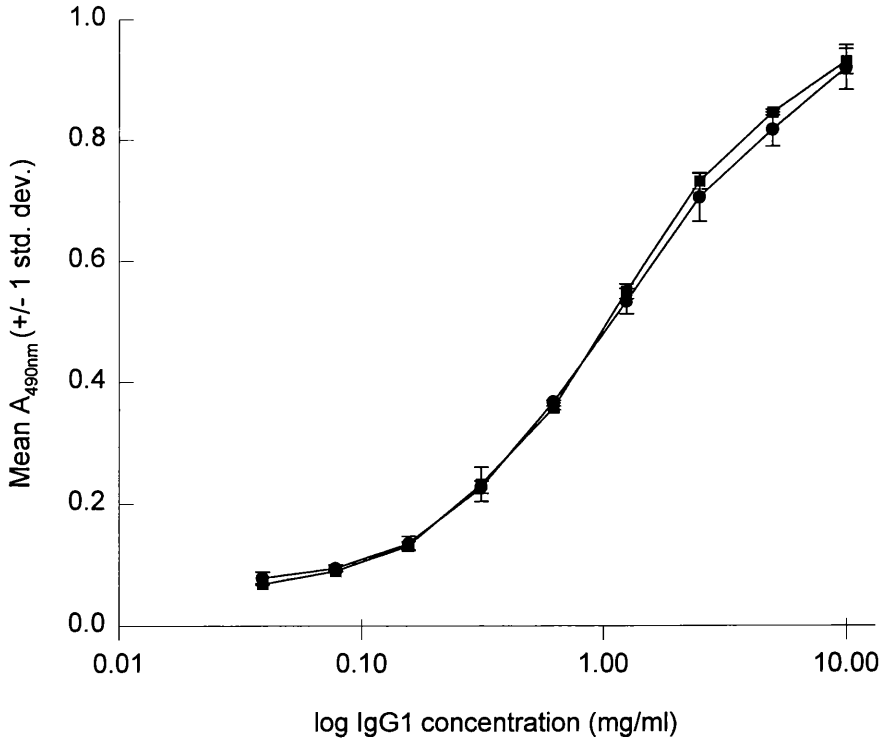


Figure 3.10 Dose-response curves for B72.3 mAbs
B72.3 IgG1 mAb binding to mucin adsorbed to Immulon 2 ELISA plate with (■) or without (●) preincubation with 1M ammonium thiocyanate prepared in PBS-Tw-BSA.

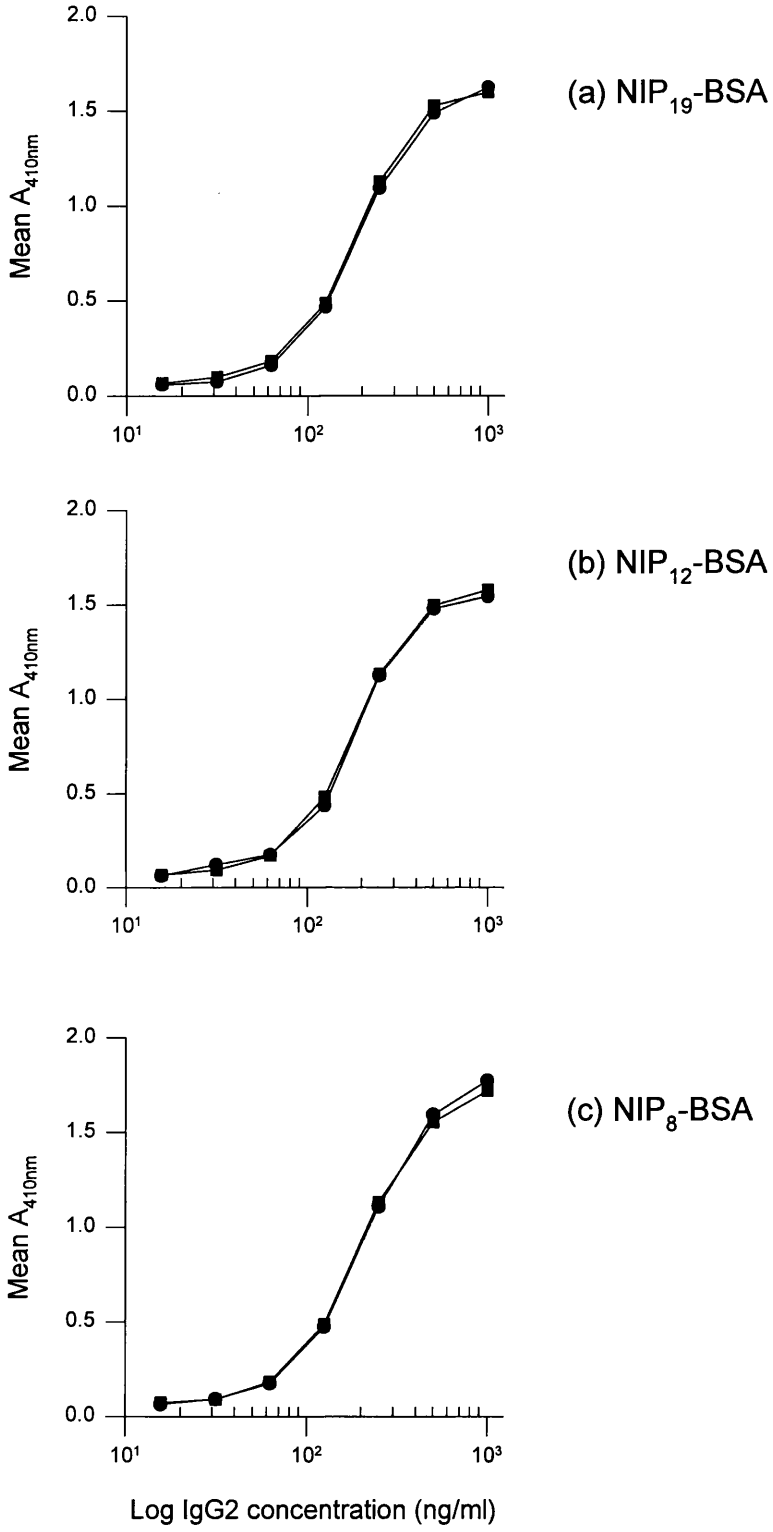


Figure 3.11 Dose-response curves for anti-NIP mAbs
Anti-NIP IgG2 mAb binding to NIP_{19}-BSA (a), NIP_{12}-BSA (b) and NIP_8-BSA (c) adsorbed to Immulon 2 ELISA plate with (■) or without (●) preincubation with 1M ammonium thiocyanate prepared in PBS-Tw-BSA

It can be seen from the dose-response curves obtained for B72.3 IgG1 mAb and anti-NIP IgG2 mAb that preincubation with 1M ammonium thiocyanate did not affect the binding of B72.3 mAbs or anti-NIP. It was concluded, therefore, that binding to the solid phase of mucin or NIP-BSA was not affected by treatment with ammonium thiocyanate.

The avidity of B72.3 IgG subclasses as measured by thiocyanate elution ELISA

The avidity of B72.3 IgG subclasses was investigated employing ammonium thiocyanate from 0.1M to 0.6M (Figure 3.12). Avidity indices were then calculated as the amount of ammonium thiocyanate required to produce a 50% reduction in absorbance (Table 3.4). The more ammonium thiocyanate required to produce 50% reduction in binding the higher the corresponding avidity of antibody. Hence a ranking of avidities of antibodies may be obtained.

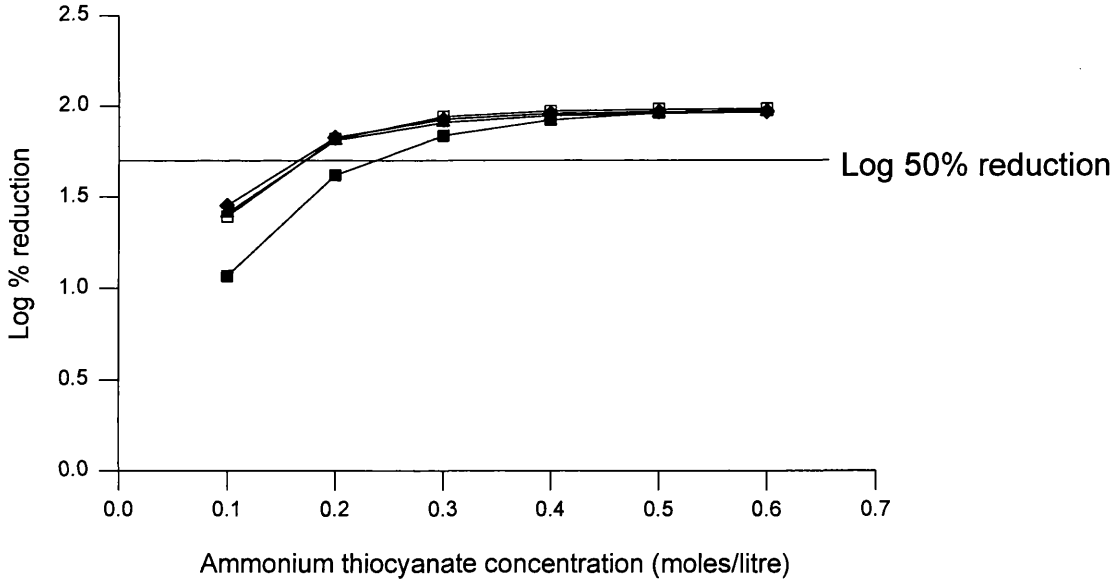


Figure 3.12 Ammonium thiocyanate elution ELISA using ammonium thiocyanate from 0.1M to 0.6M to disrupt antigen-antibody binding.
Results were expressed as the percentage reduction in absorbance at 490nm, taking antibody binding to mucin in the presence of PBS-Tw-BSA only as 100% binding. A representative curve is shown for B72.3 IgG1 (■), IgG2 (□), IgG3 (◆) and IgG4 (Δ) mAbs binding to mucin.

Table 3.4 Avidity Indices derived for B72.3 mAbs as measured by ammonium thiocyanate elution.

Experiment	IgG1	IgG2	IgG3	IgG4
1	0.26	ND	0.17	0.19
2	0.23	0.17	0.16	0.17
3	0.25	0.15	0.18	0.16
4	0.20	0.16	0.20	0.16
mean	0.24	0.16	0.18	0.17

The interassay variation was calculated and found to be within an acceptable range, as follows:

IgG1 = 11.1%, IgG2 = 6.25%, IgG3 = 9.6%, IgG4 = 8.3%.

Although B72.3 IgG1 appeared to have the highest avidity index, the variation in avidity indices between the subclasses was minimal. The thiocyanate elution ELISA was, therefore, unable to detect any differences in avidity between the B72.3 IgG subclasses.

The avidity of anti-NIP IgG2, IgG3 and IgG4 mAbs as measured by the thiocyanate elution ELISA

The avidity of anti-NIP IgG2, IgG3 and IgG4 mAbs were investigated using the thiocyanate elution ELISA. Figure 3.13 shows curves produced for anti-NIP mAbs in the presence of ammonium thiocyanate binding to various NIP-BSA complexes prepared at different epitope ratios.

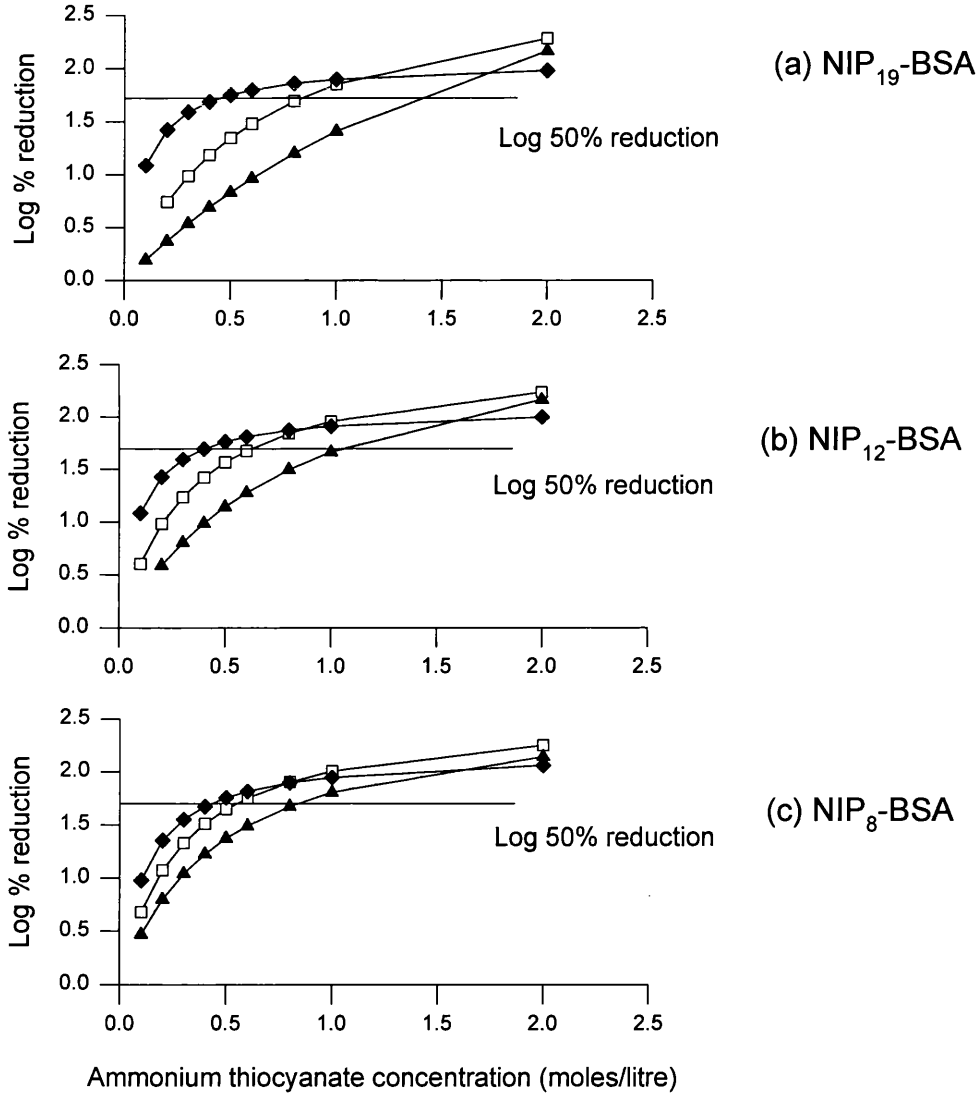


Figure 3.13 Ammonium thiocyanate elution ELISA using ammonium thiocyanate from 0.1M to 2M to disrupt antigen-antibody binding.
Results were expressed as the percentage reduction in absorbance at 490nm, taking antibody binding to NIP-BSA in the presence of PBS-Tw-BSA only as 100% binding. Representative curves are shown for IgG2 (□), IgG3 (◆) and IgG4 (▲) mAbs binding to NIP_{19}-BSA (a), NIP_{12}-BSA (b) and NIP_8-BSA (c).

Table 3.5 Avidity indices derived for anti-NIP IgG2, IgG3 and IgG4 mAbs measured by thiocyanate elution ELISA

Anti-NIP mAb	NIP_{19}-BSA	NIP_{12}-BSA	NIP_8-BSA
IgG2 - exp. 1	0.78	0.61	0.53
exp. 2	0.68	0.62	0.61
exp. 3	0.75	0.68	0.45
mean	0.74	0.64	0.53
IgG3 - exp. 1	0.39	0.38	0.39
exp. 2	0.46	0.43	0.49
exp. 3	0.31	0.31	0.37
mean	0.39	0.37	0.42
IgG4 - exp. 1	1.35	1.04	0.81
exp. 2	1.24	0.92	0.96
exp. 3	1.22	1.03	0.89
mean	1.27	1.00	0.87

Interassay variation was within acceptable range as follows:

NIP_{19}-BSA, IgG2 = 7%, IgG3 = 19%, IgG4 = 5.5%

NIP_{12}-BSA, IgG2 = 6%, IgG3 = 16%, IgG4 = 7%

NIP_8-BSA, IgG2 = 15%, IgG3 = 15%, IgG4 = 8.5%

Mean avidity indices were calculated and anti-NIP mAbs could be ranked in the order IgG4 > IgG2 > IgG3 for all three epitope densities investigated. In addition for all anti-NIP subclasses avidity could be ranked NIP_{19}-BSA > NIP_{12}-BSA > NIP_8-BSA. It must be noted that IgG3 had the lowest avidity index and differences between the epitope densities were minimal.

DISCUSSION

The development of solid-phase avidity ELISAs has made it possible to evaluate the avidity of antibodies without the need for lengthy purification, labelling procedures or complicated software packages. In this study the competitive binding ELISA and thiocyanate elution ELISA were employed to measure the avidity of two sets of V region identical mouse-human chimeric mAbs.

Choice of avidity ELISA

The competitive binding ELISA is the traditional solid-phase ELISA for measuring antibody avidity and has been validated in a number of antigenic systems, as shown earlier in Table 3.1. The competitive binding ELISA employed here was unable to measure the avidity of B72.3 mAbs, since high variability was observed between the calculated avidity indices obtained in separate assays. The exact reason for the high variability of the mucin specific competitive ELISA is unclear, but it possibly reflects the nature of the antigen once it is solubilised. Mucin is a highly polymorphic molecule consisting of repeating sheets of glycoprotein and such a highly repetitive molecule may fold up differently to display various epitopes. No evidence is available as yet but this would appear a likely explanation for the high variability observed here. In addition to the problems encountered with the B72.3 mAbs the avidity of the anti-NIP mAbs could also not be measured by competitive binding ELISA. The exact reasons for the observed high variability is unclear, but possibly arose from differing amounts of antigen being adsorbed to the ELISA plate. However, since antigen excess was

employed and the same antigen preparation was used on each occasion, this explanation appears unlikely.

In contrast to the difficulties experienced with the competitive binding ELISA, the thiocyanate elution ELISA proved to be highly reliable and avidity indices were calculated for B72.3 and anti-NIP mAbs specific for mucin and NIP-BSA respectively. Thiocyanate ions were shown not to disrupt antigen binding to the solid phase and interassay variation was acceptable.

Advantages of the thiocyanate elution ELISA

As discussed earlier a variety of solid-phase assays are currently available for measuring the avidity of antibody avidity. Results presented here suggest that the thiocyanate elution ELISA has many advantages over the more traditional competitive binding ELISA. The thiocyanate elution ELISA is reliable, requires no preincubation of samples and utilises small amounts of the sample. In contrast, the competitive binding ELISA was unreliable, required preincubation of the samples and also utilised larger amounts of samples. An alternative elution ELISA procedure is the DEA elution ELISA. Initial experiments, however were unable to measure the avidity of the B72.3 mAbs employing DEA to disrupt antigen-antibody binding (data not shown). Whilst, these experiments were being performed the chaotropic nature of DEA was investigated. It was found that DEA was not a true chaotrope and that it exerted its effect via pH (Goldblatt et al. 1993). The DEA elution ELISA was therefore not analysed further.

The avidity of B72.3 and anti-NIP IgG subclasses

Although, the thiocyanate elution ELISA was unable to determine avidity differences between B72.3 IgG1, IgG2, IgG3 and IgG4 mAbs, it was able to rank the avidity of the anti-NIP mAbs IgG2, IgG3 and IgG4 mAbs in the order IgG4 > IgG2 > IgG3 for all three epitope densities investigated.

Previous investigators have highlighted the importance of epitope density in the interpretation of anti-hapten responses. Valim and Lachmann, (1991) used anti-NIP IgG1, IgG2, IgG3 and IgG4 mAbs to demonstrate differences in binding to red blood cells coated with various NIP-BSA epitope densities. In addition, the extent to which each anti-NIP IgG subclass was able to mediate the complement cascade was also found to be dependent upon epitope density. In general the antibodies were able to bind and activate the complement cascade better at high epitope densities (Valim and Lachmann, 1991). The results presented here support this report with antibody avidity being highest at the highest epitope density investigated, (NIP_{19}-BSA) and lowest at the lowest epitope density, (NIP_8-BSA). Unfortunately, anti-NIP IgG1 was not available due to the hybridoma cell line having lost the relevant gene. It was not therefore possible to investigate all four IgG subclasses for anti-NIP avidity.

To date only one publication has addressed the role of the human constant region in relation to antibody avidity. In their paper Morelock and co-workers, used a competitive binding ELISA and were able to show avidity differences between V region identical IgG1, IgG2 and IgG4 antibodies specific for ICAM-1 and ranked the avidity of the antibodies IgG1>IgG4>IgG2 (Morelock et al. 1994). Although avidity differences were not detected for the B72.3 mAbs,

avidity differences were detected in this present investigation for anti-NIP mAbs with IgG4 being higher avidity than IgG2, supporting the findings of Morelock et al. (1994).

Using solid-phase avidity ELISAs, it appears that differences in avidity exist between human IgG subclasses specific for the same antigen. Since each set of mAbs possessed identical V regions, it is likely that the observed avidity differences for the anti-NIP mAbs are governed by structural features remote from the variable region

CHAPTER 4

Binding kinetics of mouse-human chimeric V region identical IgG subclasses

INTRODUCTION

An understanding of the interactions between biomolecules such as antigen-antibody complexes is crucial in furthering our knowledge of biological systems. A variety of methods are available with which to study antibody affinity, but very few permit the determination of kinetic properties. Traditional methods for measuring antibody affinity include equilibrium dialysis, fluorescence quenching, ammonium sulphate precipitation and solid-phase ELISAs (Van Regenmortel and Azimadeh, 1994). These methods do not in general allow the measurement of the kinetic properties of native molecules, since labelling of one or more of the reactants is usually required. In the case of solid-phase avidity assays accurate measurement of affinity constants are further complicated by the possibility of mass transport limitations (Karlsson et al. 1991). Furthermore, the majority of such techniques are static and since interactions between molecules are dynamic processes crucial information remains undetected with these methods.

Real-time biospecific interaction analysis (BIA) permits the determination of kinetic parameters (on and off rates) as well as equilibrium rate constants without the need for labelling. Using BIA, data is collected in real-time as the interaction occurs. Furthermore, interactions may be measured without extensive purification thereby avoiding structural changes that the labelling procedure may introduce.

Biosensor Technology

Biosensors were first described in 1962 by Clark and Lyons and the BIAcore™ biosensor manufactured by Pharmacia was the first biosensor instrument based on surface plasmon resonance (SPR). The BIAcore™ was originally utilised to measure antigen-antibody interactions but can now be exploited in other areas of biology such as DNA-protein interactions, receptor-ligand binding, signalling pathway analysis and epitope mapping (Malmqvist, 1993). BIAcore analysis of antibody affinity has been validated by a number of investigators. Pellequer and Van Regenmortel (1993), studying 3 mAbs to tobacco mosaic virus, found differences in affinity constants which were in good agreement with liquid-phase centrifugation and ELISA equilibrium titration methods. In order to study the ligand-analyte interaction in greater detail, BIA may also be utilised to analyse kinetic data. For example two mAbs specific for the same antigen were found to differ markedly in their rate constants, despite possessing identical affinity constants (Karlsson et al. 1991). The importance of measuring rate constants has recently been highlighted by the studies of VanCott et al. 1994, who showed that dissociation rate and not association was predictive of the neutralising capacity *in vitro* of mAbs reactive with peptides derived from the V3 loop of HIV-1 gp160.

Surface plasmon resonance (SPR)

The detection principle of the BIAcore™ is known as surface plasmon resonance (SPR). This is an optical phenomen observed at the interface between a metal film and liquid. Light of a critical wavelength is directed through a prism to a metal surface, part of the light - the evanescent wave - penetrates an order of one wavelength into the liquid at the metal surface. This causes energy to be absorbed by electrons (plasmons) at the metal surface, and this energy change is manifest as SPR. When SPR occurs, energy is lost to the electrons and the intensity of the reflected light drops accordingly. If all else within the system remains constant, the SPR signal is related to the liquid properties at the metal surface. In the BIAcore™ the SPR signal is detected by a photodetector array and expressed as resonance units (RU). 1000RU correlates with a change in reflected light of 0.1° (Fagerstam and Karlsson, 1995).

BIAcore™ Biosensor

The BIAcore™ instrument consists of a processing unit, a sensor chip, and a personal computer. Figure 4.1 shows the optical configuration of the BIAcore™.

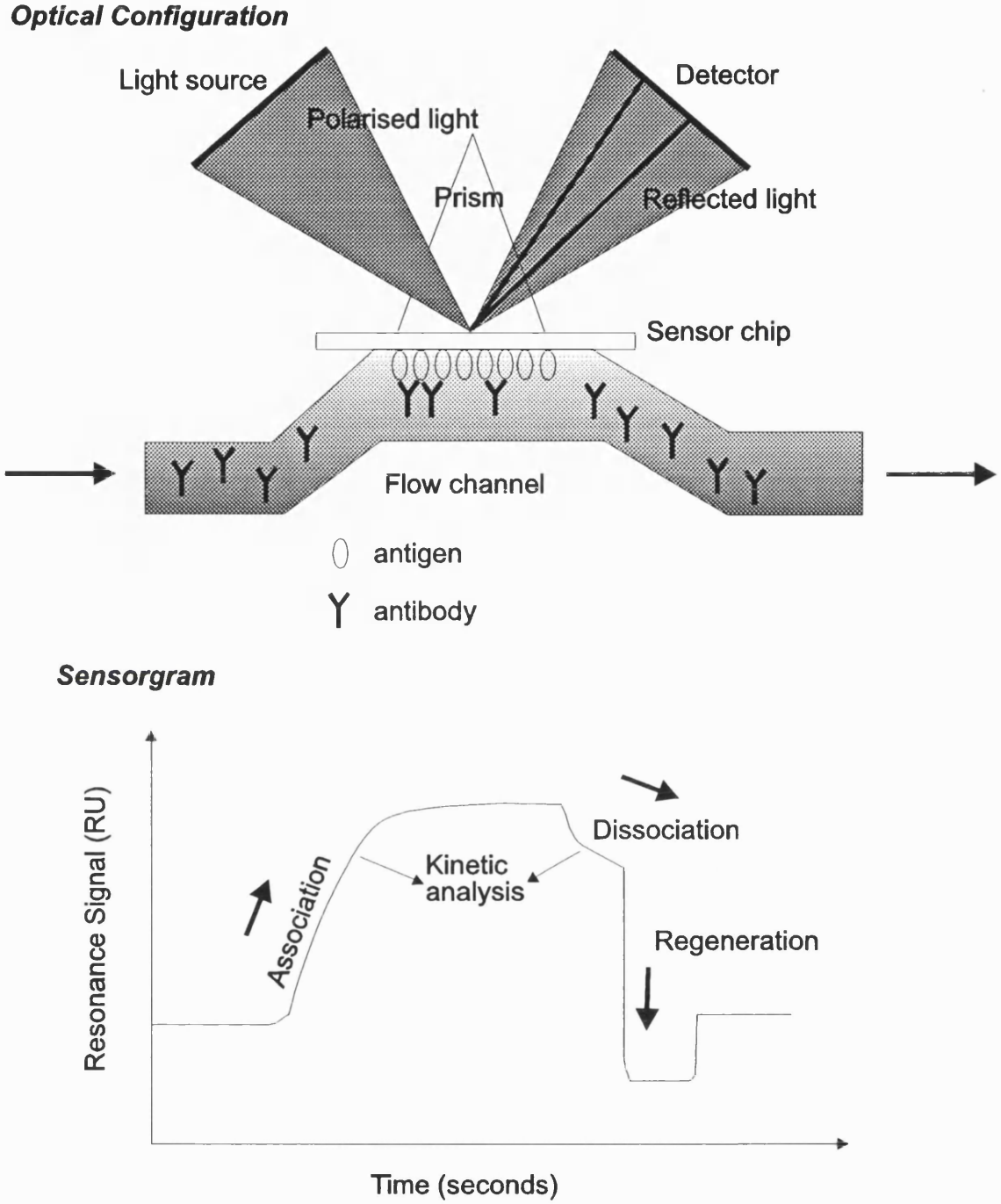


Figure 4.1 Optical configuration of the BIAcore™ Biosensor
The sensor chip consists of a thin gold film (50nm) attached to a glass surface. The gold film is covered with a carboxylated dextran matrix (100nm) to which the ligand of interest may be covalently coupled. The use of carboxylated dextran allows ligand to be bound for example by exploiting amine groups following activation of the matrix with carbodiimide-N-hydroxysuccinimide. Analyte can then be passed over the dextran matrix by virtue of a sample flow loop and binding to ligand measured in real time, which is plotted as resonance units against time and expressed as a sensorgram. Following each experimental cycle the sensor chip can then be regenerated by a pulse with a suitable buffer and reused.

Kinetic and affinity analysis

By monitoring the rate in change of resonance units over time in a sensorgram the kinetics and affinity of analyte binding can be calculated. The association phase of the sensorgram (Figure 4.1) is a function of the kinetic properties of the reaction as long as mass transport effects are not limiting. Mass transport effects can be determined by virtue of flow rate, flow cell properties and the diffusion coefficient of the analyte (Malmqvist, 1993). The dissociation rate of the interaction may then be analysed by measuring the dissociation of analyte in buffer flow after the sample flow has passed. However, it is important that the analyte is free of aggregates which may cause disturbances in the evaluation (Griffiths et al. 1993). The association equilibrium constant can then be determined as the ratio of the association and dissociation rate constants, or alternatively from a series of experiments during which equilibrium is reached (Karlsson et al. 1991).

Various models for kinetic analysis of the interaction studies are included in the analytical software supplied with the BIAcore™, although for most cases employing purified homogeneous preparations a simple one-site model will most probably be sufficient (Appendix 2). The principles of kinetic analysis used with the BIAcore™ are reviewed in detail elsewhere (Fagerstam and Karlsson, 1995).

Aims of the Study

Previous studies presented in Chapter 3 had demonstrated avidity differences between V region identical mAbs specific for the hapten, NIP. However, solid-phase avidity ELISAs proved unable to detect any avidity differences between B72.3 mAbs reactive with mucin. Despite possessing similar avidity indices, it was possible that the B72.3 mAbs differed with respect to kinetics. In order to investigate avidity differences of the anti-NIP mAbs further and to dissect out any avidity differences between the B72.3 mAbs, the kinetics of binding of these mAbs were analysed using BIA. The role of the human immunoglobulin constant region on antibody avidity was also investigated by measuring the binding kinetics of $F(ab')_2$ fragments obtained from B72.3 mAbs.

METHODOLOGY

Preparation of $F(ab')_2$ Fragments

B72.3 and anti-NIP mAbs were purified from culture supernatant, as described fully in Chapter 3.

$F(ab')_2$ fragments were obtained from B72.3 IgG1, IgG2, IgG3 and IgG4 mAbs. Antibodies were dialysed against 0.2M NaAc pH4.5 overnight and pepsin (Sigma) added at previously determined optimal enzyme:substrate ratios; 1:100 (10h) for B72.3 IgG1, 1:100 (2h) for B72.3 IgG3 and 1:500-1000 (1h) for B72.3 IgG2 and IgG4. Digestion was carried out at 37^0C and the reaction stopped by neutralisation with 2M Tris. $F(ab')_2$ fragments were then separated by size exclusion gel filtration using a Superdex-200 (Pharmacia) column operated by FPLC and $F(ab')_2$ concentrations analysed by absorbance at 280nm and assuming a value of $A^{1\%}_{1cm,280nm}$ of 15.

Immobilisation of Antigen to the Sensor Chip

(a) *Mucin*

Bovine submaxillary mucin (Sigma) was immobilised on the dextran layer of a sensor chip (Pharmacia Biosensor) in 10mM citrate buffer pH3.8. Firstly, the dextran layer was activated for 7 minutes at a flow rate of 5µl/min using an amine coupling kit (Pharmacia Biosensor) containing EDC/NHS which puts active N-hydroxysuccinamide esters onto the dextran which will then bind primary amines and other nucleophilic groups. Non-activated sites were blocked with 1M ethanolamine-HCl pH8.5. Approximately 930 RU of mucin were immobilised by

passing 35μl of 200μg/ml mucin in citrate buffer at a flow rate of 5μl/min. Various concentrations of mAb were then passed over the immobilised matrix for kinetic analysis. The matrix was regenerated with 10M NH_4SCN after each cycle, with no significant loss of RU.

(b) *NIP-BSA*

NIP conjugated to BSA at the ratio NIP_{12}-BSA was immobilised to a sensor chip as described above except that the buffer used for immobilisation was 10mM sodium acetate pH2.6. Approximately 530 RU were immobilised onto the sensor chip by passing 35μl of NIP_{12}-BSA at 200ng/ml in acetate buffer at a flow rate of 5μl/min. The matrix was regenerated similarly to the mucin chip with 10μl of 10M NH_4SCN at a flow rate of 5μl/min after each cycle, with no significant loss of RU.

Kinetic Calculations

Rate constants k_a and k_d were calculated using BIAcore™ Biaevaluation software analysis, assuming the relationship Ab + Ag $\leftrightarrow$ AbAg (see Appendix 2).

RESULTS

Preparation of B72.3 chimeric IgG subclass F(ab')$_2$ fragments

Pepsin digestion was employed to cleave the IgG subclasses to produce F(ab')$_2$ fragments. Firstly, a time course was followed to determine the optimal conditions with which to obtain the maximal amount of F(ab')$_2$ fragments, according to the method of Turner et al. (1970a,b). All four IgG subclasses were dialysed overnight against 0.2M NaAc pH4.5 and pepsin added to give enzyme substrate ratios of 1:100, 1:500 or 1:1000. The reaction was performed at 37°C and stopped at various time points by neutralisation with 2M tris. The samples were then analysed by non-reducing 10% SDS-PAGE (Figures 4.2 and 4.3).

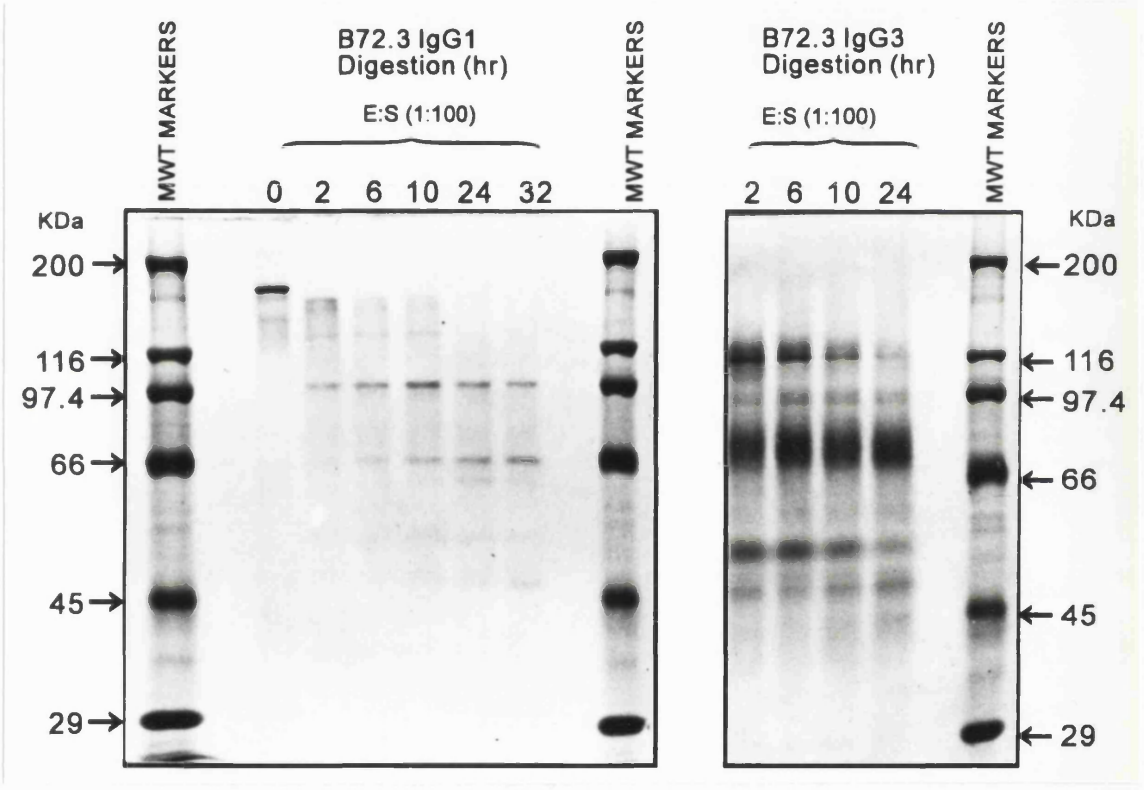


Figure 4.2 10% non-reducing SDS-PAGE of pepsin digested B72.3 IgG1 and IgG3

Approximately 0.5mg of IgG1 and IgG3 were dialysed against 0.2M sodium acetate pH4.5 overnight at 4°C. Pepsin was added to give an enzyme:substrate ratio of 1:100 and the reaction allowed to proceed at 37°C and stopped by neutralisation with 2M Tris at the time points indicated, 20μl of each sample were then diluted 1/2 with protein sample buffer and analysed by SDS-PAGE. Proteins were then visualised using silver staining.

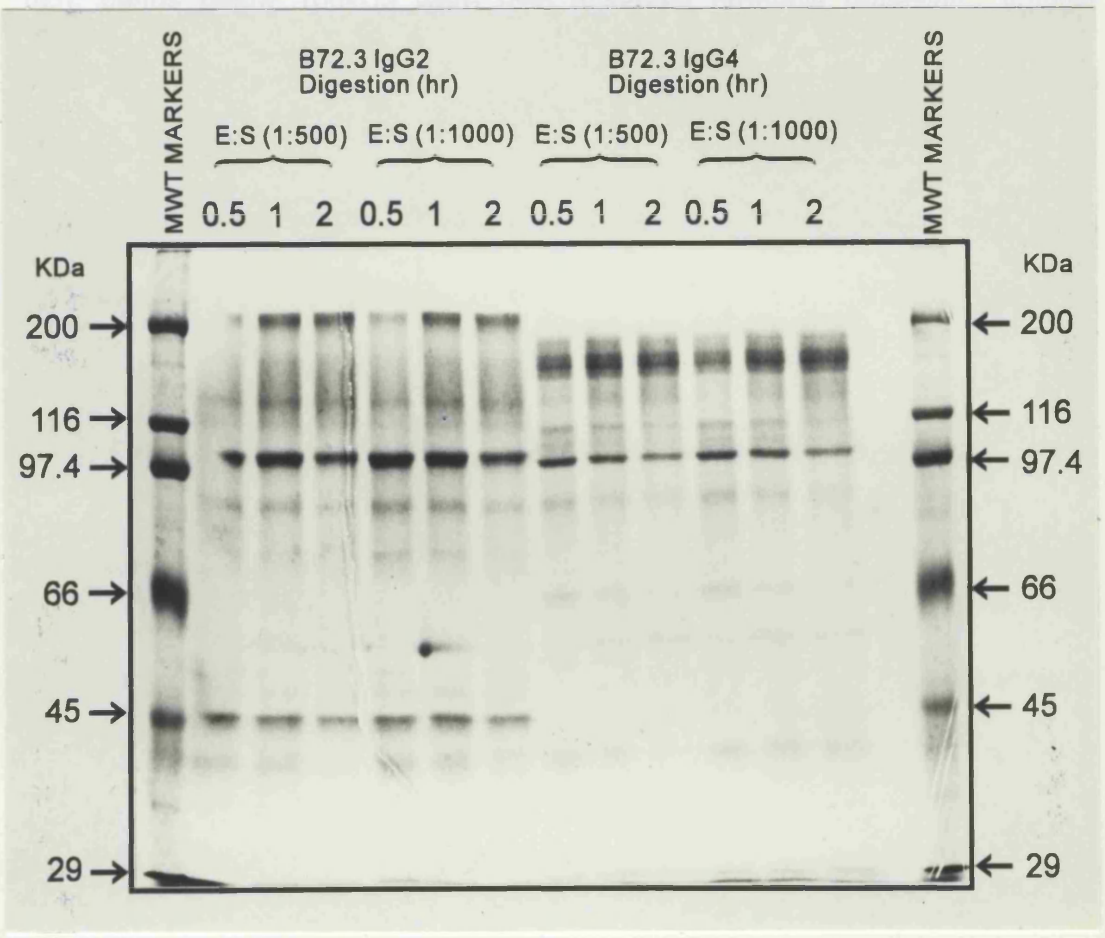


Figure 4.3 10% non-reducing SDS-PAGE of pepsin digested B72.3 IgG2 and IgG4

Approximately 0.5mg of IgG2 and IgG4 were dialysed against 0.2M sodium acetate pH4.5 overnight at 4°C. Pepsin was added to give an enzyme:substrate ratio of 1:500 or 1:1000 and the mixture incubated at 37°C. The reaction was stopped by neutralisation with 2M Tris at the time points indicated. 20μl of each sample were then diluted 1/2 with protein sample buffer and analysed by SDS-PAGE. Proteins were then visualised using silver staining.

Digesting IgG1 at an enzyme: substrate ratio of 1:100 with pepsin gave a band at approximately 100kDa at each of the time points investigated. However, incompletely digested antibody was observed at 2, 4 and 6h, as shown by the appearance of bands between 150 and 100kDa (Figure 4.2). At 4, 6, 10, 24 and 32h, bands below 100kDa were also observed probably reflecting smaller fragments of antibody. The strongest band, at 100kDa, representing $F(ab')_2$ fragments, was seen at 10h (Figure 4.2).

In the case of the IgG2 chimeric protein no $F(ab')_2$ fragments were observed using an enzyme:substrate ratio of 1:100 for 10h. All of the observed digestion products were below 100kDa, suggesting that the conditions were too harsh (data not shown). Accordingly the enzyme:substrate ratio was reduced to 1:500 and 1:1000 and the time of digestion reduced. A band at 100kDa was observed at all time points (Figure 4.3). Little or no undigested antibody was observed, although bands were observed in all lanes corresponding to fragments of less than 100kDa. The strongest band at 100kDa was observed in a preparation obtained at an enzyme:substrate ratio of 1:1000 for 0.5h.

The optimal conditions for IgG3 digestion were found to be an enzyme:substrate ratio of 1:100 for 2h at 37°C since a band at approximately 120kDa was observed at 2, 6 and 10h indicating the presence of IgG3 $F(ab')_2$ fragments, with the strongest band being observed at 2h. No undigested antibody was observed but smaller digestion fragments were observed at all time points investigated and there was no evidence of the 120kDa band at 24h (Figure 4.2)

As with IgG2 an enzyme:substrate ratio of 1:100 was found to be too harsh for the digestion of IgG4 (data not shown) and therefore the enzyme:substrate ratio was changed to 1:500 and 1:1000 and the time of digestion reduced. Bands at approximately 100kDa representing $F(ab')_2$ fragments were observed at all time points investigated as were undigested and smaller fragments. The strongest band at 100kDa was seen at both enzyme:substrate ratios at 0.5h. Optimal conditions for pepsin digestion of B72.3 mAbs are shown in Table 4.1

Table 4.1 Optimal conditions for the pepsin digestion of B72.3 mAbs

mAb	Enzyme:Substrate	Time (hours)
IgG1	1:100	10
IgG2	1:1000	0.5
IgG3	1:100	2
IgG4	1:1000	0.5

Employing these conditions 1-5mg of antibody were digested and $F(ab')_2$ fragments purified by gel filtration using a 300 x 10mm Superdex-200 column (Pharmacia), which has a mean particle size of 34μm, bead size 24-44μm, with a separation range of 10 - 600 kDa, using the FPLC system. The void volume of the column was investigated employing blue dextran (40,000 kDa) and found to be 9ml and a calibration curve constructed using proteins of known molecular weight (Figure 4.4). Extrapolating from the calibration curve proteins of 100kDa should be eluted at Ve/Vo = 1.59.

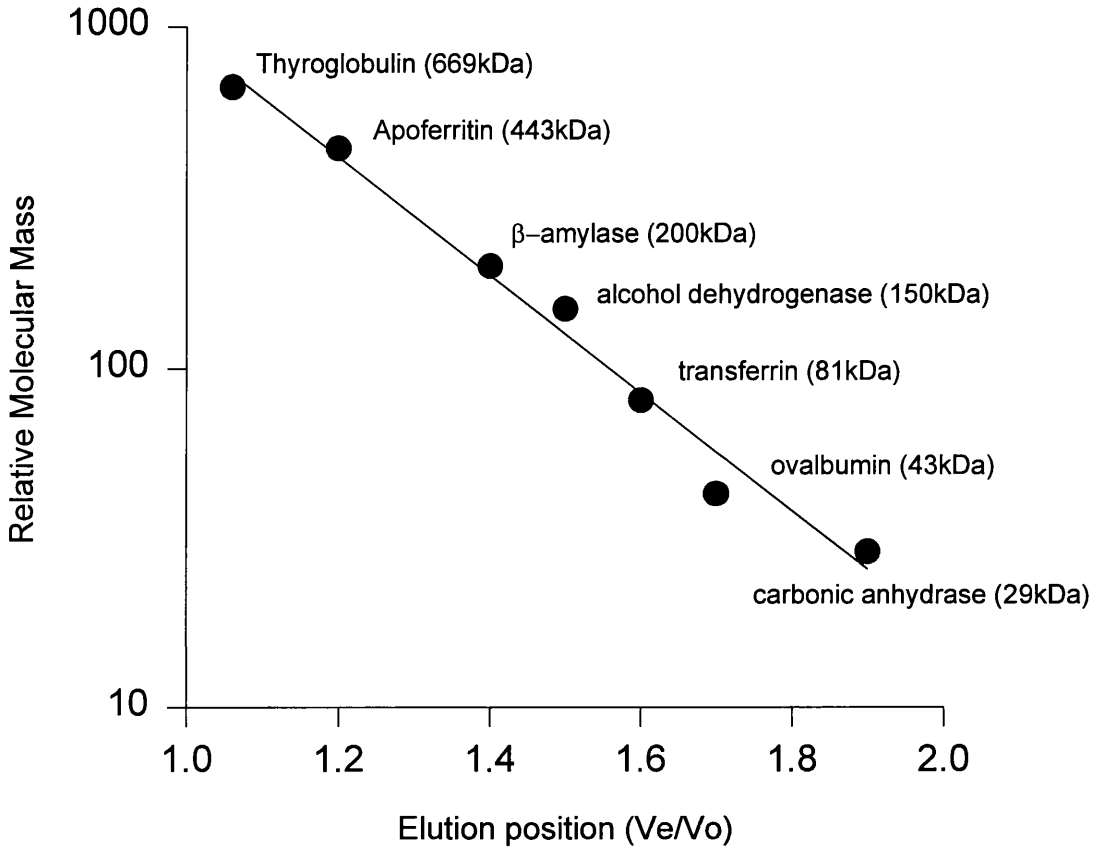


Figure 4.4 Superdex-200 column calibration
The elution volumes of a range of standards (Ve) relative to the void volume (Vo) of the column were plotted against relative molecular mass. The equation of the regression line shown is: log y = 4.7 -1.7x and the regression coefficient was 0.983.

Each pepsin digested antibody was separated by gel filtration on the Superdex-200 column using the FPLC system (Figures 4.5, 4.6, 4.7 and 4.8) and the fractions corresponding to approximately 100kDa at Ve/Vo = 1.59 analysed by SDS-PAGE (Figure 4.9, 4.10 and 4.11).

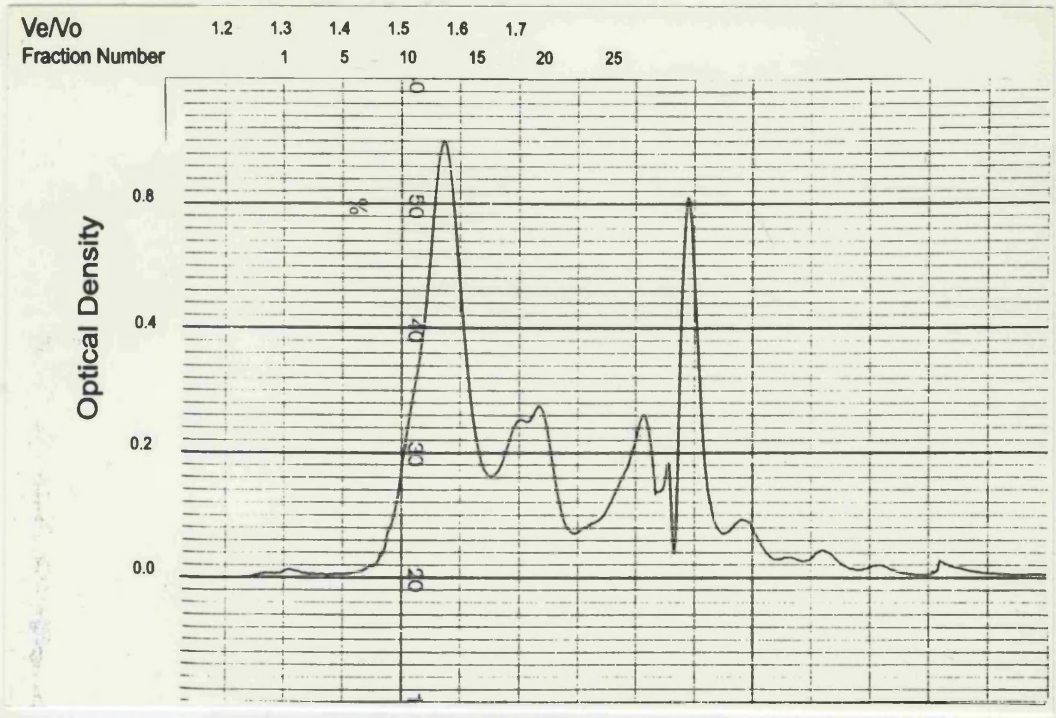


Figure 4.5 Elution profile of B72.3 IgG1 mAb digested with pepsin
200µl of sample were loaded onto the Superdex-200 column, proteins were eluted using PBS and 0.5ml fractions collected. Fractions corresponding to a Ve/Vo value of 1.5-1.6 were then analysed by SDS-PAGE (10% non-reducing) for the presence of 100kDa proteins representing $F(ab')_2$ fragments.

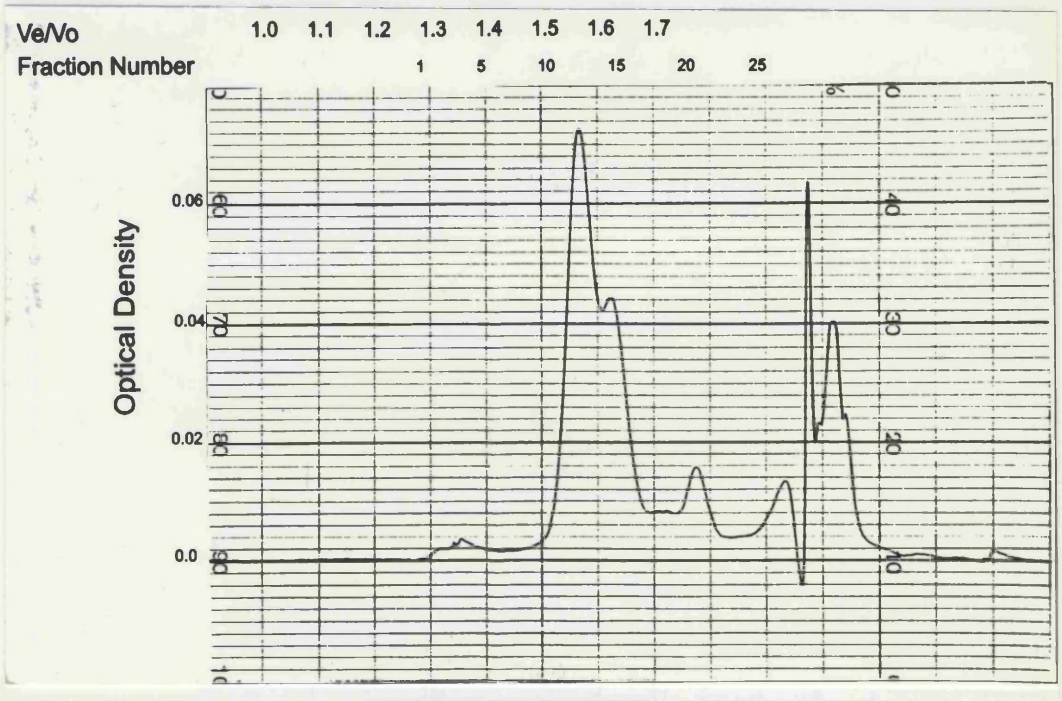


Figure 4.6 Elution profile of B72.3 IgG2 mAb digested with pepsin
200µl of sample were loaded onto the Superdex-200 column, proteins were eluted using PBS and 0.5ml fractions collected. Fractions corresponding to a Ve/Vo value of 1.5-1.6 were then analysed by SDS-PAGE (10% non-reducing) for the presence of 100kDa proteins representing $F(ab')_2$ fragments.

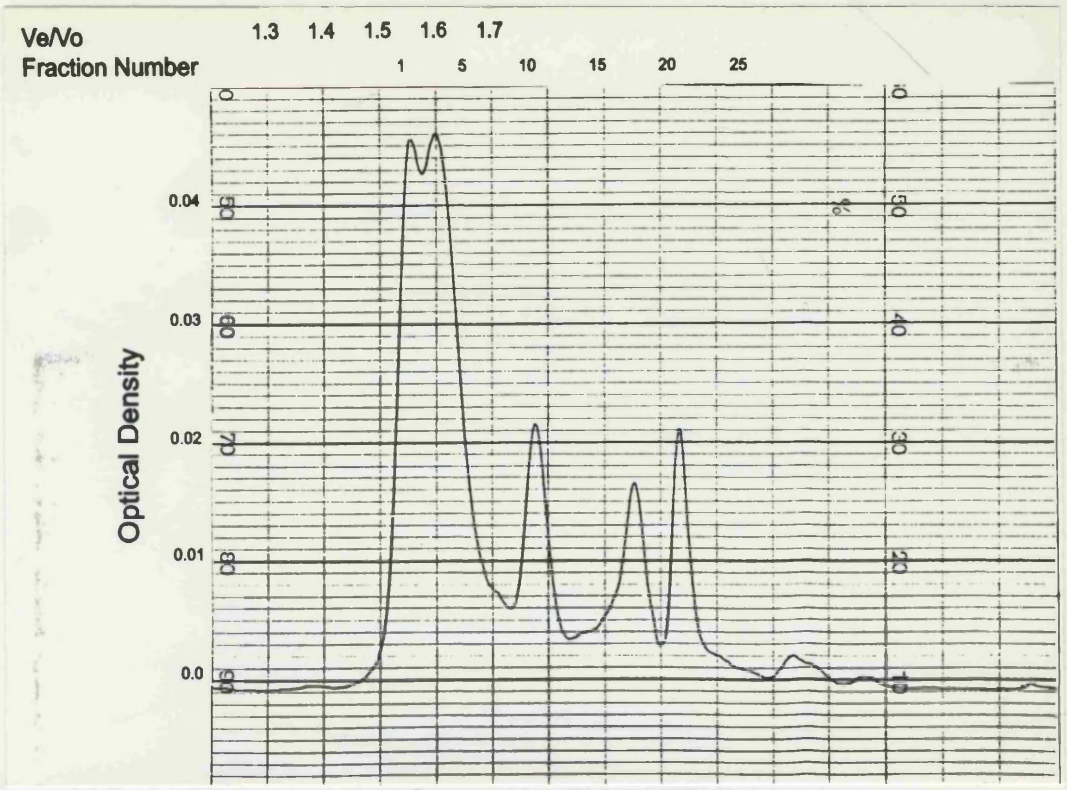


Figure 4.7 Elution profile of B72.3 IgG3 mAb digested with pepsin

200µl of sample were loaded onto the Superdex-200 column, proteins were eluted using PBS and 0.5ml fractions collected. Fractions corresponding to a Ve/Vo value of 1.5-1.6 were then analysed by SDS-PAGE (10% non-reducing) for the presence of 100kDa proteins representing $F(ab')_2$ fragments.

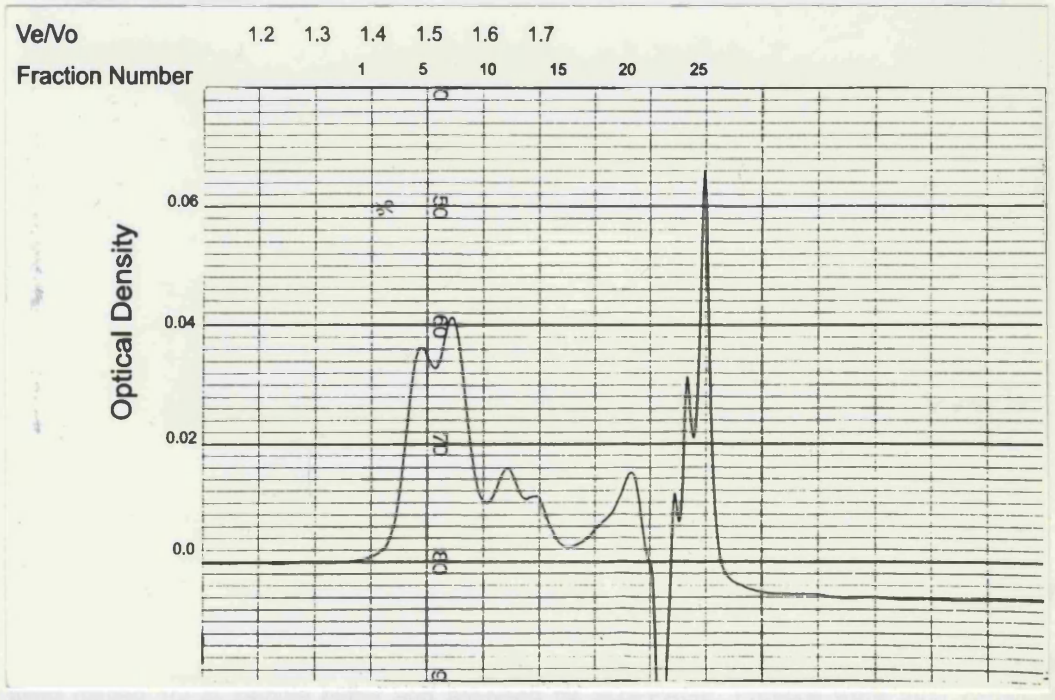


Figure 4.8 Elution profile of B72.3 IgG4 mAb digested with pepsin

200µl of sample were loaded onto the Superdex-200 column, proteins were eluted using PBS and 0.5ml fractions collected. Fractions corresponding to a Ve/Vo value of 1.5-1.6 were was then analysed by SDS-PAGE (10% non-reducing) for the presence of 100kDa proteins representing $F(ab')_2$ fragments.

Fractions corresponding to a Ve/Vo value of 1.5-1.6 were then analysed by non-reducing 10% SDS-PAGE, in order to determine the fraction containing $F(ab')_2$ fragments (Figures 4.9 - 4.11).

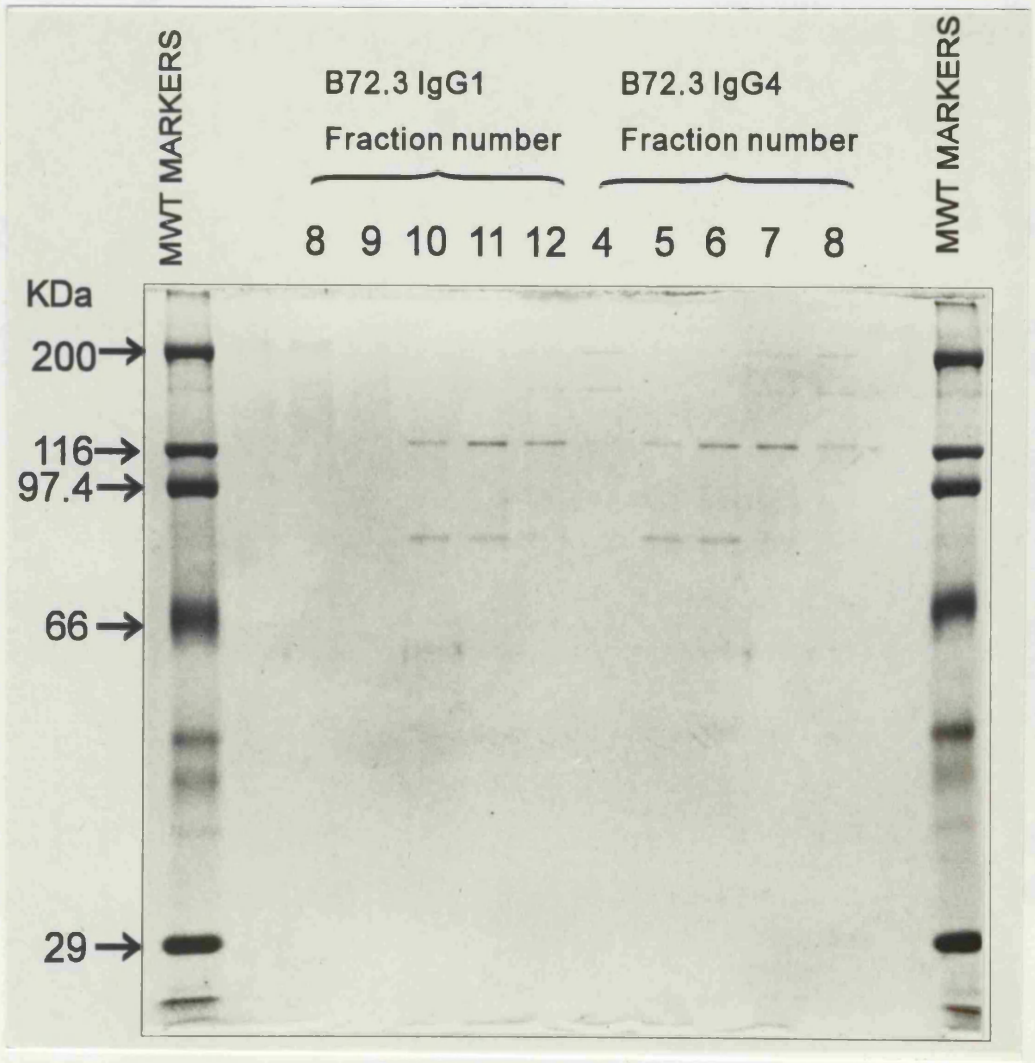


Figure 4.9 10% non-reducing SDS-PAGE of B72.3 IgG1 and IgG4 pepsin digestion fractions eluted from Superdex-200

20µl samples from fractions corresponding to the expected elution position of proteins of 100kDa were diluted 1/2 in sample buffer and analysed by SDS-PAGE. Proteins were then visualised using silver staining. Major bands at 100kDa were observed in fractions 10 - 12 for B72.3 IgG1 and fractions 5 - 8 for B72.3 IgG4. It can be seen that fraction 12 for B72.3 IgG1 was free of contaminating species and therefore was assumed to contain the IgG1 $F(ab')_2$ fragments. Fraction 7 for B72.3 IgG4 was free of contaminating species and thus this fraction was assumed to contain the IgG4 $F(ab')_2$ fragments. The protein concentration was then determined spectrophotometrically

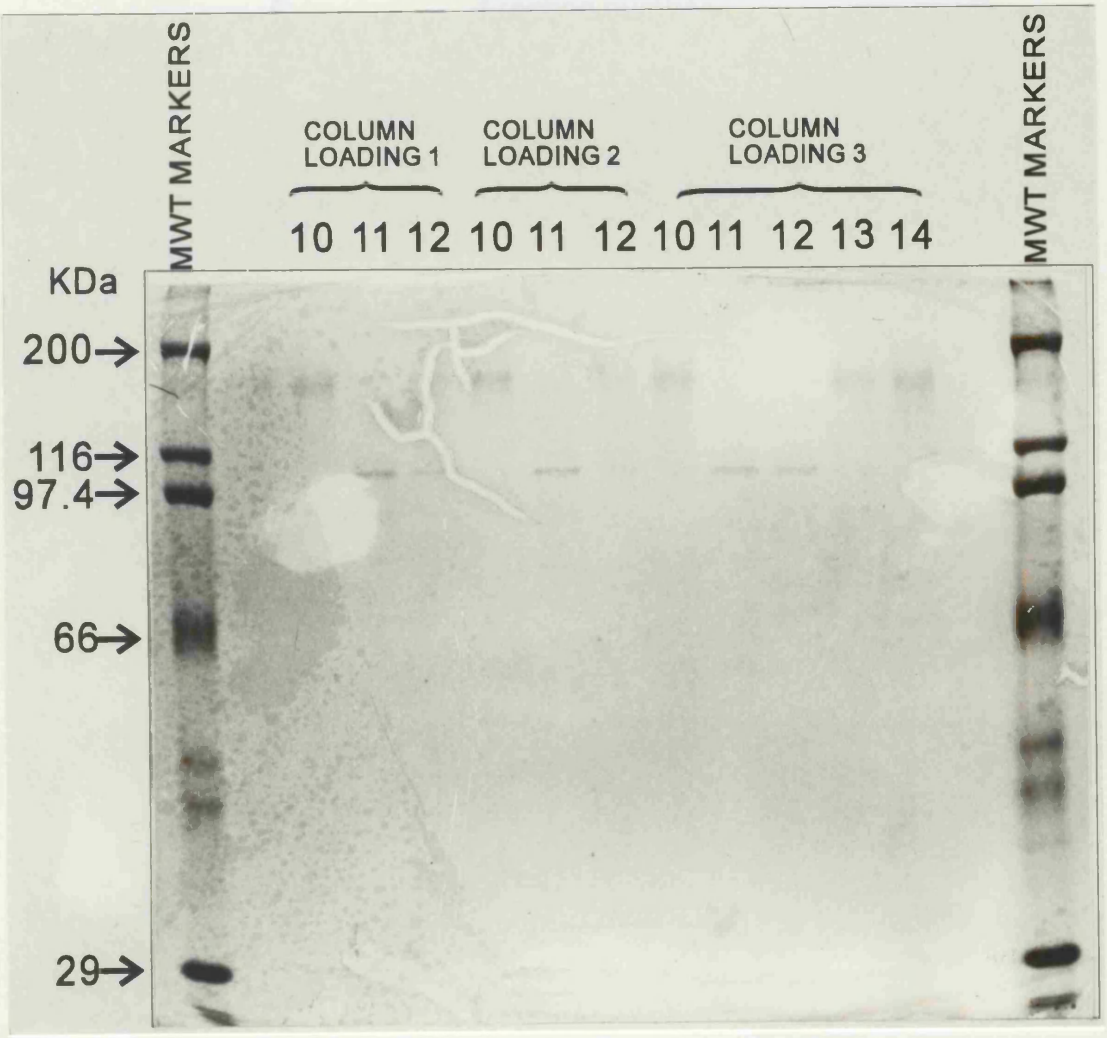


Figure 4.10 10% non-reducing SDS-PAGE of B72.3 IgG2 pepsin digestion fractions eluted from Superdex-200

20µl samples from fractions corresponding to the expected elution position of proteins of 100kDa were diluted 1/2 in sample buffer and analysed by SDS-PAGE. 3 separate column runs were performed for B72.3 IgG2 due to the sample volume size. Proteins were then visualised using silver staining. Major bands at 100kDa were observed in fraction 11 for column loadings 1 and 2 and in fractions 11 and 12 for column loading 3. These fractions were free of contaminating species and assumed to contain the IgG2 $F(ab')_2$ fragments. The samples were then pooled and the protein concentration determined spectrophotometrically.

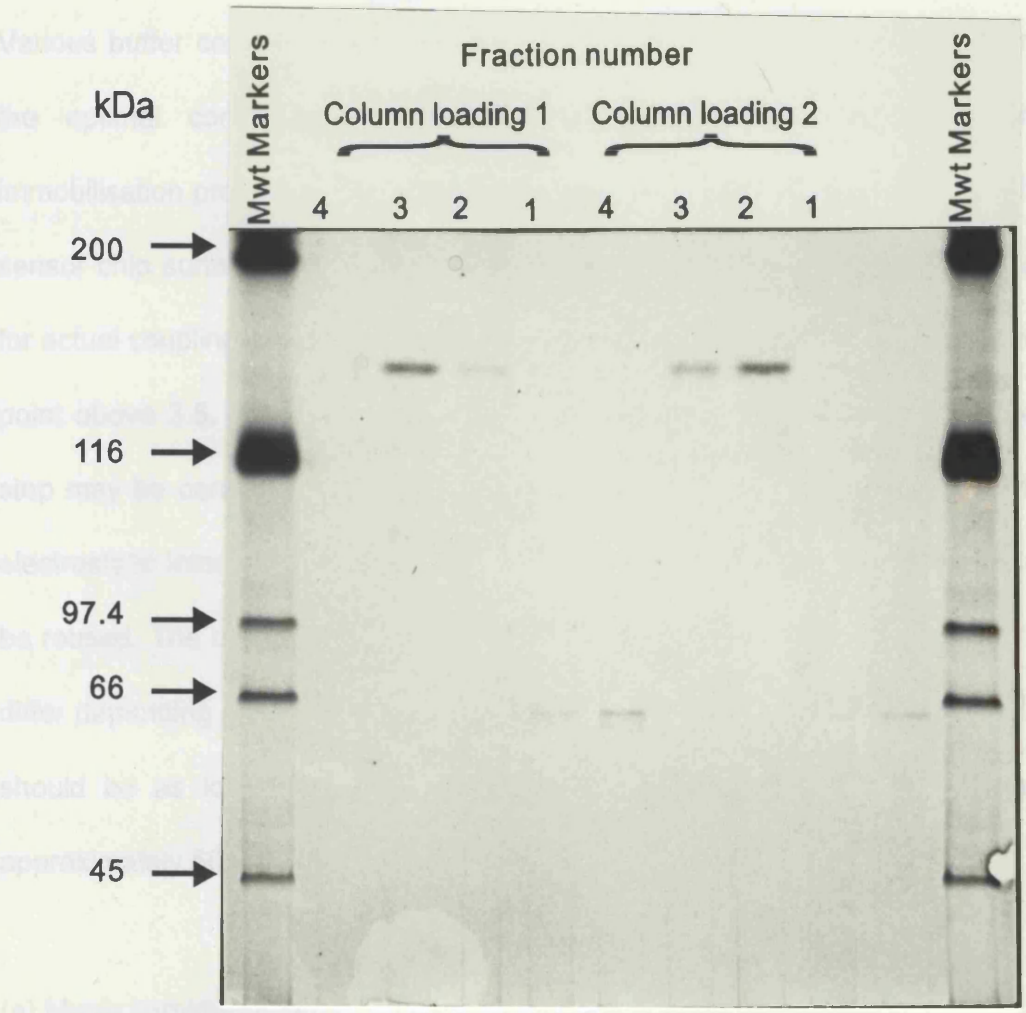


Figure 4.11 7.5% non-reducing SDS-PAGE of B72.3 IgG3 pepsin digestion fractions eluted from Superdex-200

20µl samples from fractions corresponding to the expected elution position of proteins of 120kDa were diluted 1/2 in sample buffer and analysed by SDS-PAGE. 2 separate column runs were performed for B72.3 IgG3 due to the sample volume size. Proteins were then visualised using silver staining. Major bands at 120kDa were observed in fraction 3 for column loading 1 and in fractions 2 and 3 for column loading 2. These fractions were free of contaminating species and assumed to contain the IgG2 $F(ab')_2$ fragments. The samples were then pooled and the protein concentration determined spectrophotometrically.

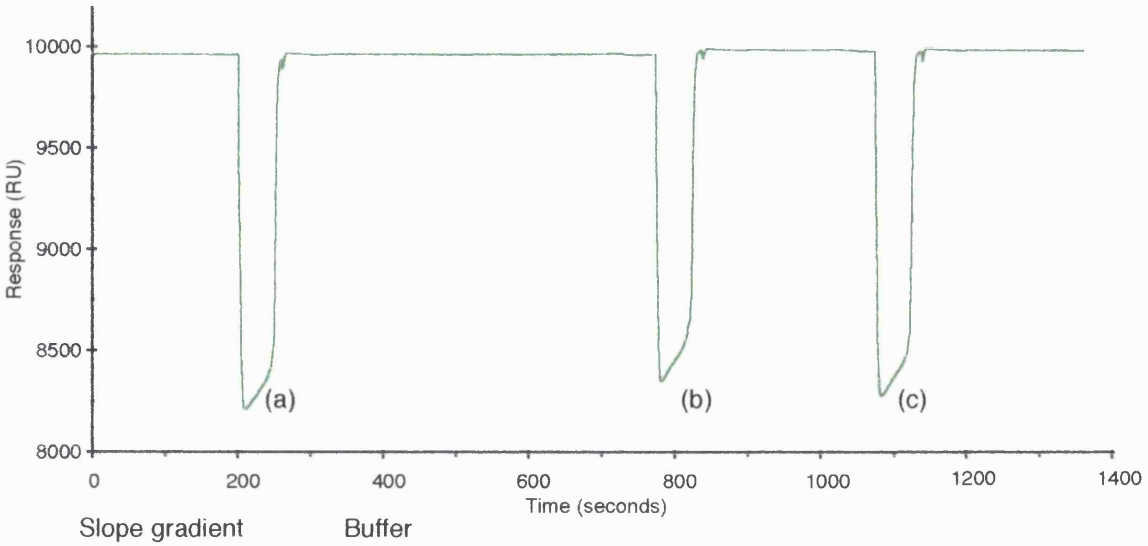
BIAcore™ Analysis

Immobilisation of mucin and NIP-BSA to the sensor chip

Various buffer conditions and activation times were analysed in order to define the optimal conditions for immobilisation of the two antigens. In the immobilisation procedure the negative charge of the carboxylated dextran on the sensor chip surface is exploited to concentrate the sample close to the surface for actual coupling. For this to be achieved the sample should have an isoelectric point above 3.5. If the pI of the sample is unknown a simple preconcentration step may be carried out using various buffers of different pH to investigate the electrostatic interaction. Preconcentration is reversible and the sensor chip may be reused. The optimal resonance units (RUs) immobilised onto the sensor chip differ depending upon the nature of the application. For kinetic analysis the RUs should be as low as possible to minimise mass transport effects. Typically approximately 500 RU should be sufficient.

(a) Mucin immobilisation

No significant preconcentration of mucin was achieved using 10mM sodium acetate pH4.5. Preconcentration was, however, successful with 10mM citrate buffer pH3.8 (Figure 4.12). Optimal immobilisation conditions were found to be activation of the sensor chip with the amine coupling kit (NHS/EDC) for 7 minutes followed by blocking of unactivated sites with 1M for ethanolamine for 2 minutes, and immobilisation of mucin at 200μg/ml for 7 minutes, all at a flow rate of 5μl/min (Figure 4.13). Approximately 930RU of mucin were immobilised and this sensor chip employed for subsequent kinetic studies.



	Slope gradient	Buffer
(a)	5.14135	10mM citrate pH3
(b)	5.78281	10mM citrate pH3.8
(c)	5.80866	10mM citrate pH4.5

Figure 4.12 Preconcentration of mucin to a sensor chip.
Non-covalent binding of mucin to a sensor chip dextran matrix, was followed by passing mucin at 200μg/ml in 10mM citrate buffer, at various pH values, in a continuous flow at 5μl/min over the sensor chip. The gradient of the slope represents the amount of antigen bound to the sensor chip. The higher the slope gradient the greater the amount of bound antigen. The observed dip in RU is due to buffer change.

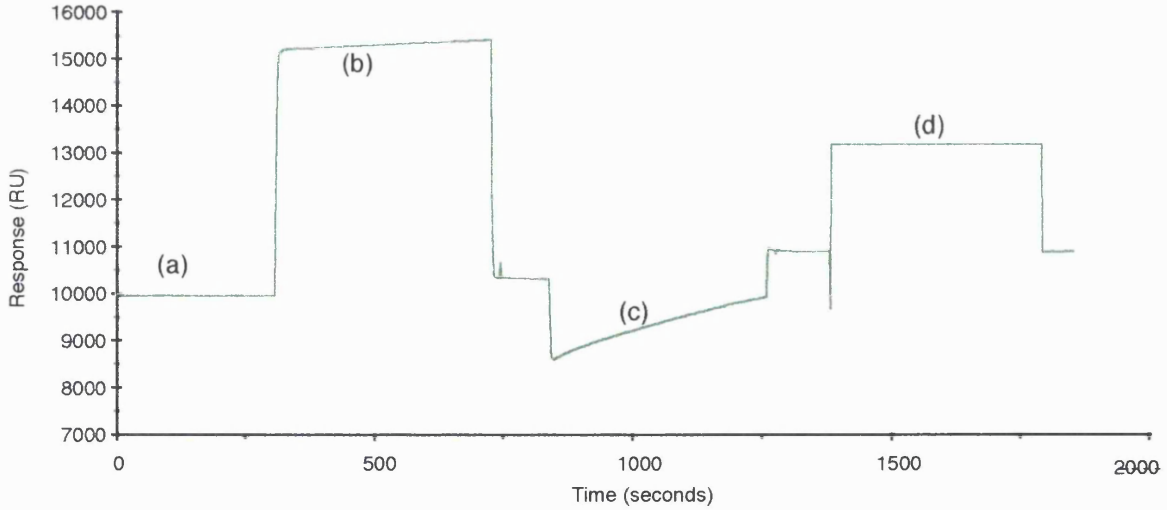


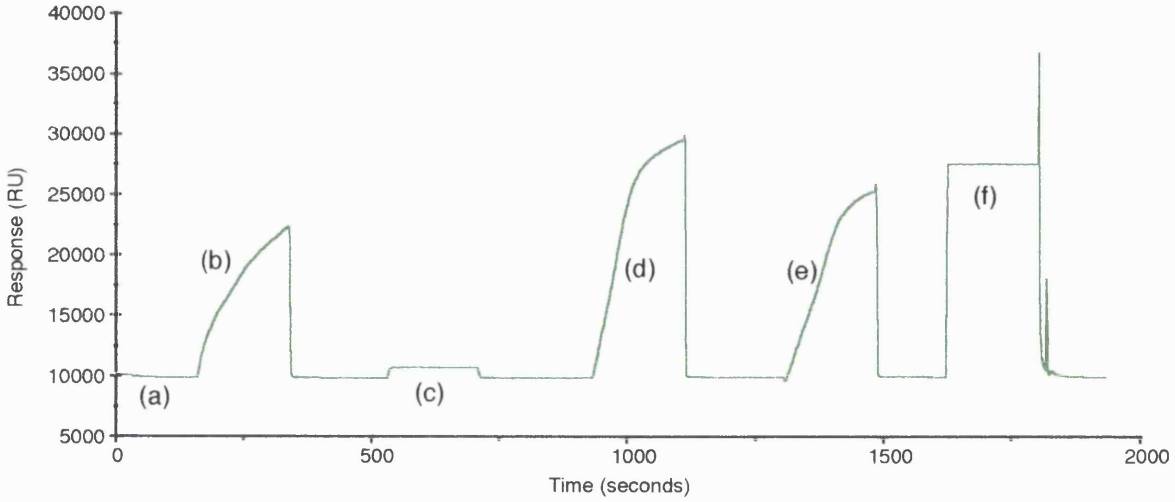
Figure 4.13 Immobilisation of mucin to a sensor chip
(a) baseline (b) activation of sensor chip with NHS/EDC (c) binding of mucin at 200μg/ml in 10mM citrate pH3.8 (d) blocking of unbound sites with 1M ethanolamine. The amount of mucin immobilised was 927 RU.

(b) NIP-BSA

No significant preconcentration of NIP_{12}-BSA occurred using 10mM sodium acetate at pH4.5. However, preconcentration was improved by lowering the pH of the acetate buffer and pH2.6 was found to give the greatest preconcentration effect (Figure 4.14). Immobilisation of NIP_{12}-BSA was then carried out using the method described above but employing 10mM sodium acetate pH2.6 and NIP_{12}-BSA was at 200ng/ml with acetate buffer. Approximately 530RU of NIP_{12}-BSA were immobilised and this sensor chip was employed for further kinetic studies (Figure 4.15).

Optimal Binding Conditions and Specificity

Before employing the sensor chip in binding studies an evaluation was made of the regeneration conditions and the specificity of the sensor chip were studied. Various regeneration buffers were investigated, 100mM NaOH pH 11.3 was found to give reasonable regeneration, but following repeated cycles with B72.3 IgG2 at 100μg/ml, it was found that a build up of resonance units had occurred. It was concluded that 100mM NaOH pH11.3 was not an ideal regeneration solution following repeated use. 10M NH_4SCN was then investigated since this is known to disrupt antigen-antibody interactions without destroying specificity. Following repeated use no significant loss of RU was observed and regeneration was good. It was decided that 10M NH_4SCN was to be used as the regeneration buffer in experimental cycles.



	Slope gradient	Buffer pH
(a)	baseline	
(b)	21.47245	500ng/ml pH2_6
(c)	0.48834	500ng/ml pH4.5
(d)	4.40986	200ng/ml pH2.5
(e)	6.44810	100ng/ml pH2.6
(f)	Regeneration with 10M ammonium thiocyanate	

Figure 4.14 Preconcentration of NIP_{12}-BSA to a sensor chip

Non-covalent binding of NIP_{12}-BSA to a sensor chip dextran matrix was followed by passing NIP_{12}-BSA diluted in 10mM sodium acetate buffer at various pH values, in continuos flow at 5µl/ min for 3 minutes over the sensor chip. The gradient of the slope produced represents the amount of antigen bound to the sensor chip. The larger the gradient the more antigen is bound. The sensor chip was then regenerated with a 3 minute pulse of 10M ammonium thiocyanate

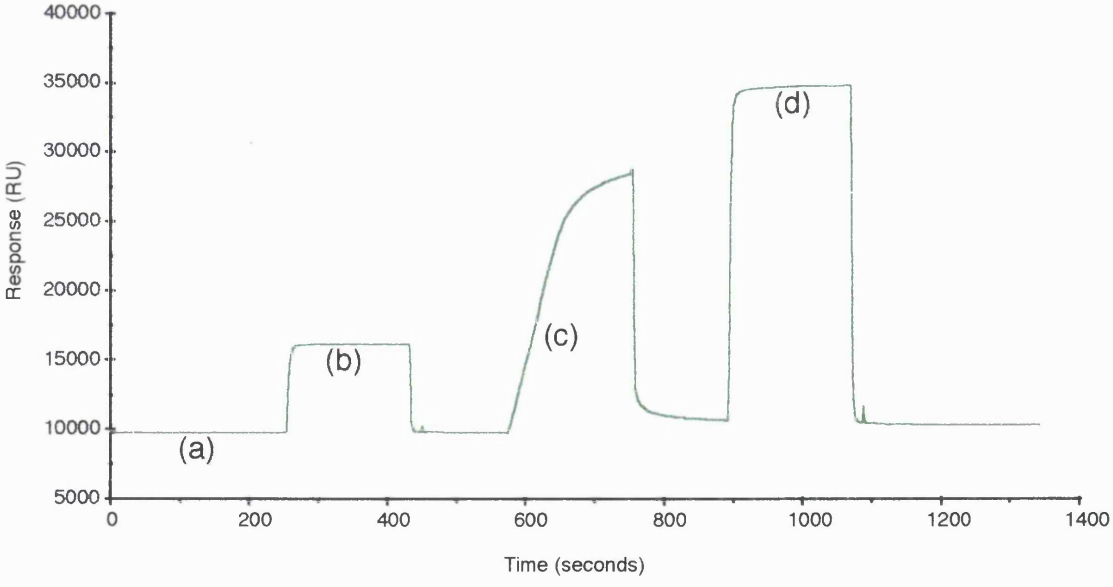


Figure 4.15 Immobilisation of NIP_{12}-BSA to a sensor chip

(a) baseline (b) activation of the sensor chip with NHS/EDC (c) Binding of NIP_{12}-BSA at 100ng/ml in 10mM acetate buffer pH 2.6 (d) blocking of any unbound sites with 1M ethanolamine. 531 RU of NIP12:1BSA were successfully immobilised to the sensor chip.

(a) Mucin

The specificity of the mucin sensor chip was then investigated. It was found that B72.3 IgG2, IgG3 and IgG4 all bound to the mucin sensor chip when used at 25μg/ml, but showed no reactivity to a blank flowcell containing dextran matrix alone (Figure 4.16). The observed increase in RU was due to changes in the buffer utilised. Furthermore, irrelevant isotype matched mouse-human IgG2, IgG3 and IgG4 chimeric antibodies specific for the hapten NIP did not bind to the mucin sensor chip (Figure 4.16). It appeared, therefore that the B72.3 mAbs were specific for the mucin bound to the dextran matrix.

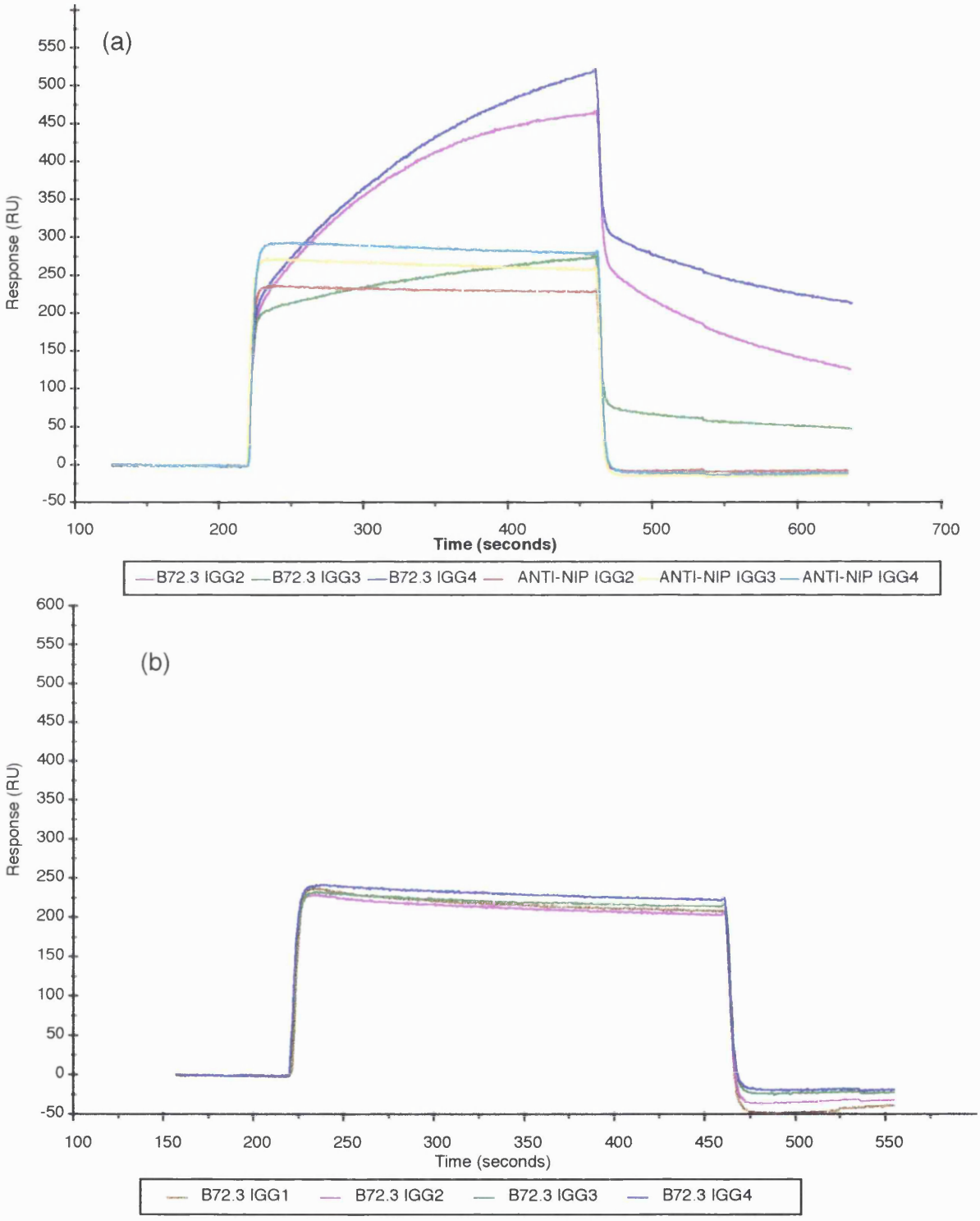


Figure 4.16 Sensorgrams illustrating the specificity of the mucin sensor chip

(a) B72.3 and anti-NIP mAbs at 25μg/ml were passed over the mucin sensor chip and binding followed for 6 minutes. Sample flow was then replaced with HBS buffer and the dissociation phase observed (b) B72.3 mAbs at 25μg/ml were passed over a blank flow cell using similar conditions to those employed in (a).

(b) NIP-BSA

The specificity of the NIP-BSA sensor chip was investigated as described above. When used at 25μg/ml the anti-NIP mAbs bound to the NIP-BSA sensor chip but did not bind to a blank flow cell (dextran matrix alone). In contrast, isotype matched mouse-human B72.3 mAbs specific for mucin did not bind to the NIP-BSA sensor chip (Figure 4.17). It was therefore concluded that the anti-NIP mAbs were able to bind specifically to the NIP-BSA antigen.

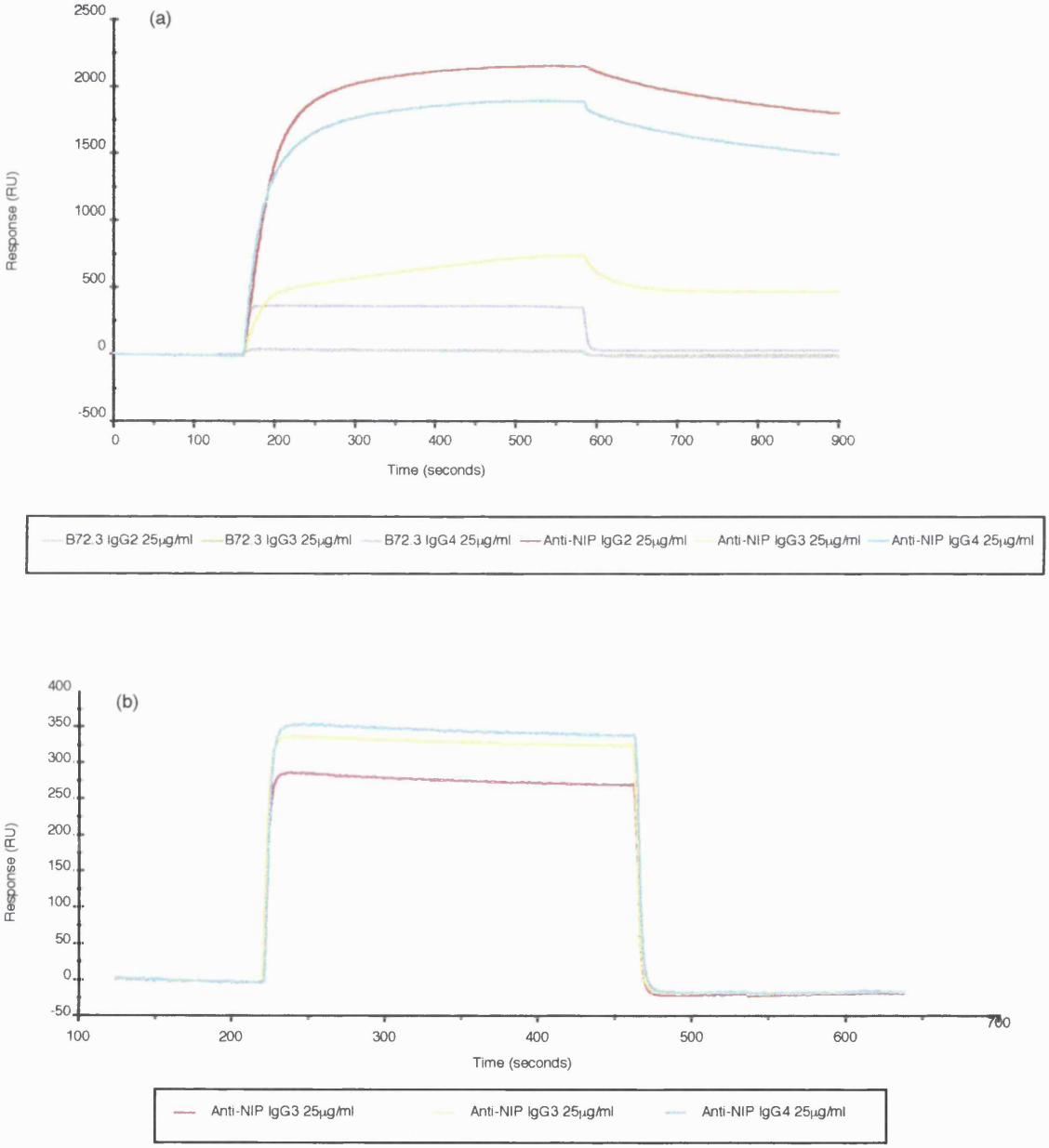


Figure 4.17 Sensorgrams illustrating the specificity of the NIP_{12}-BSA sensor chip

(a) B72.3 and anti-NIP IgG2, IgG3 and IgG4 mAbs at 25μg/ml were passed over the NIP-BSA chip and binding was observed for 8 minutes. The antibody sample flow was then stopped and buffer alone passed across the sensor chip, allowing dissociation to be observed. (b) anti-NIP mAbs at 25μg/ml were passed over a blank flow cell similarly to above.

Real-time biospecific interaction analysis (BIA) of chimeric IgG subclasses

(a) B72.3 IgG subclasses

Mucin binding sensorgrams were obtained for all four B72.3 IgG subclasses and for $F(ab')_2$ fragments of IgG1, IgG2 and IgG4 over a range of concentrations. The low yield of $F(ab')_2$ fragments from IgG3 precluded kinetic analysis. Each IgG subclass had different binding characteristics (Figures 4.18, 4.19 and 4.20). The maximal resonance units achieved for each subclass during the time course of the injection phase differed with IgG4 having the highest maximal response. Furthermore the dissociation curves of the four antibodies showed subtle differences. IgG1 and IgG2 both demonstrated rapid initial dissociation phases whereas the dissociation of both IgG3 and IgG4 appeared to be slower. These differences were observed at each concentration of antibody investigated.

In contrast to the intact parent antibodies, the binding curves for the $F(ab')_2$ fragments of IgG1, IgG2 and IgG4 were strikingly similar to each other, showing rapid association followed by rapid dissociation (Figure 4.21). In the case of IgG2 and IgG4 there was a difference between the patterns obtained using the intact antibodies and those obtained with the $F(ab')_2$ fragments.

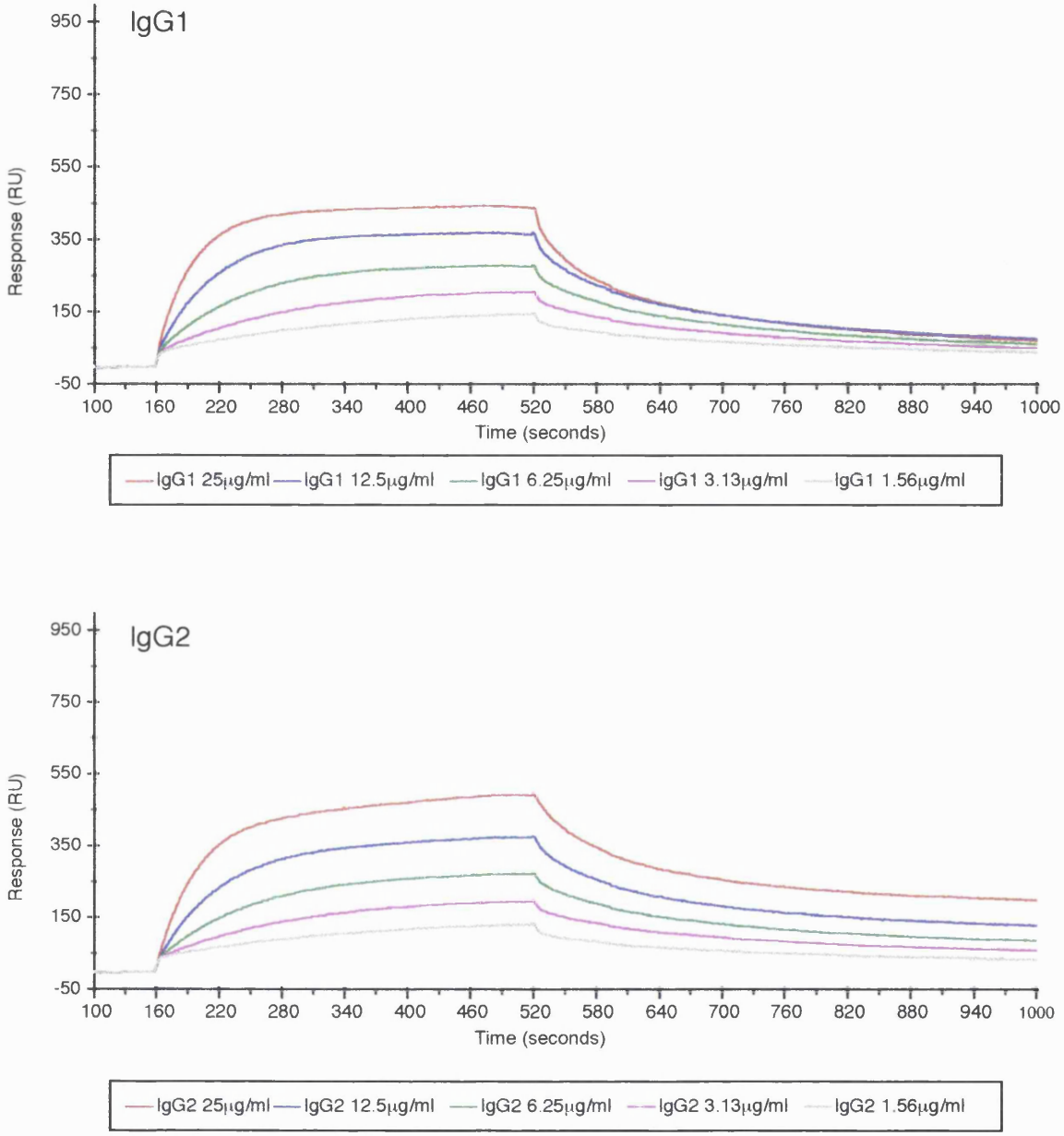


Figure 4.18 Overlay of mucin binding sensorgrams produced for IgG1 and IgG2 B72.3 mAbs

Antibody binding was monitored for 5 minutes using a continous flow of antibody at various concentrations and a flow rate of 5μl/min. Dissociation of binding was then followed for approximately 8 minutes using a continous flow of buffer.

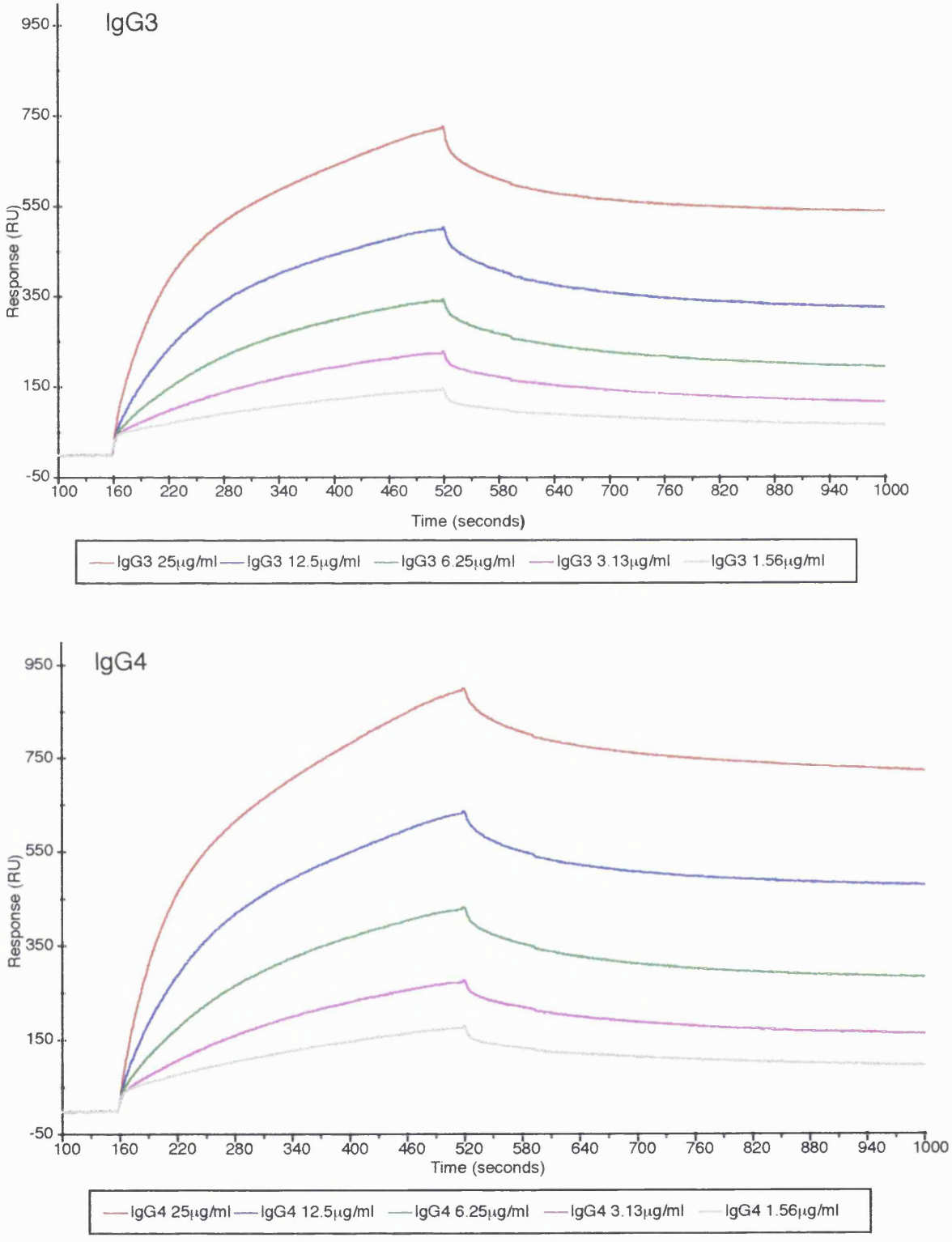


Figure 4.19 Overlay of mucin binding sensorgrams produced for B72.3 mAbs IgG3 and IgG4

Antibody binding and dissociation were followed as described in Figure 4.18

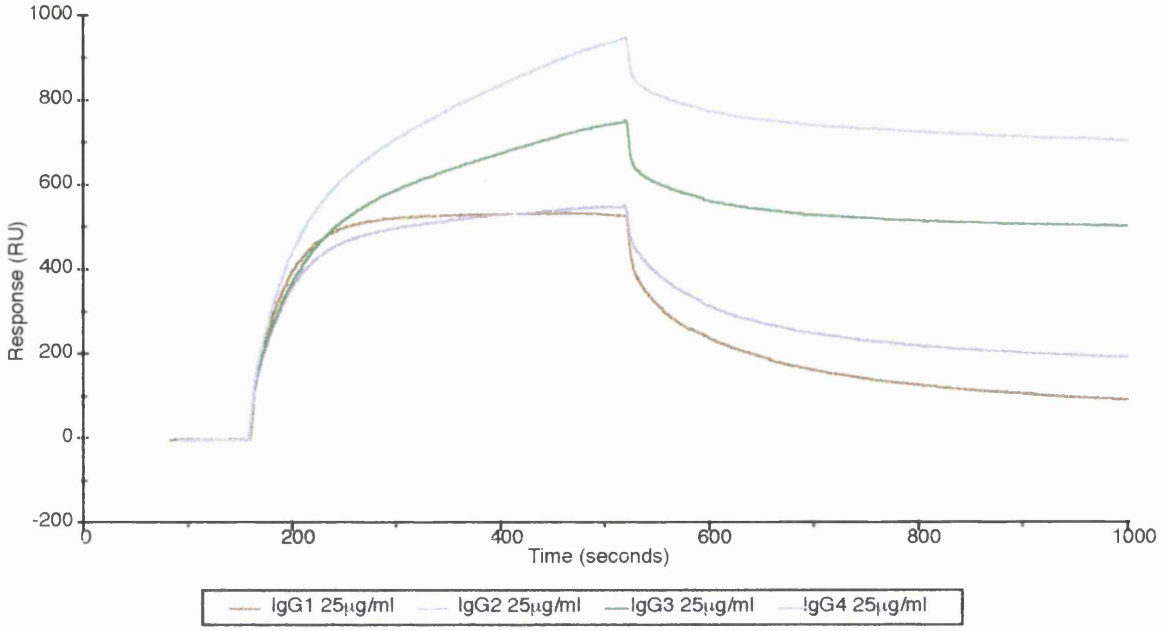


Figure 4.20 Overlay of mucin binding sensorgrams obtained for B72.3 IgG subclasses

Binding of B72.3 mAbs at 25µg/ml was followed by passing antibody over the mucin sensor chip at a flow rate of 5µl/min for 6 minutes. Dissociation was then observed by replacing the antibody flow with continuous buffer flow

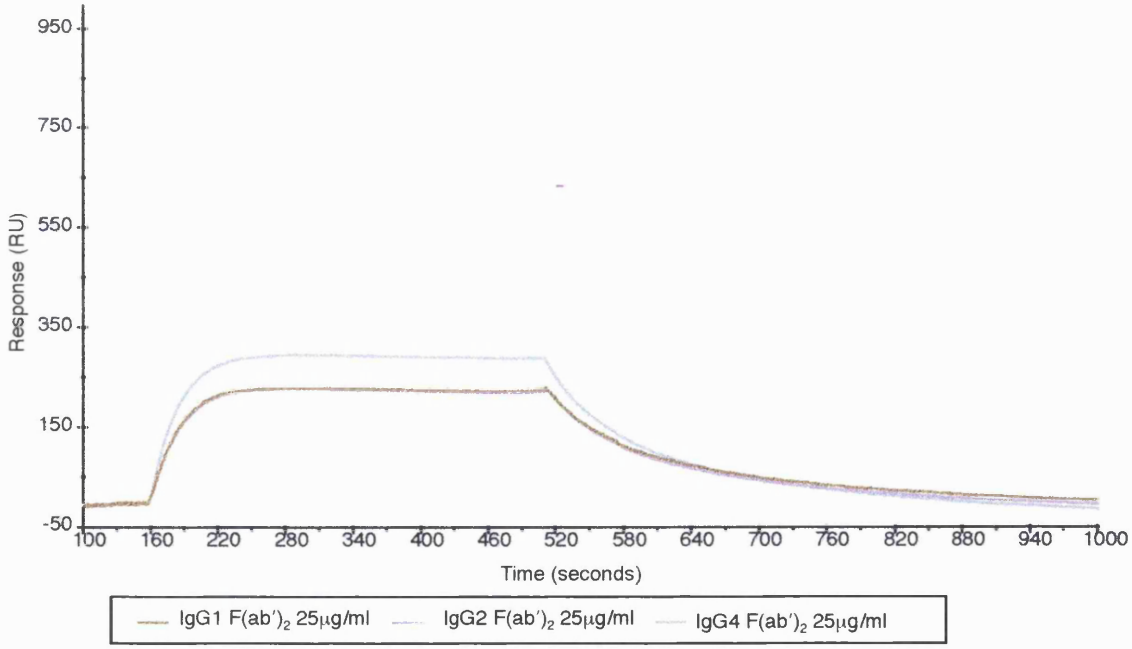


Figure 4.21 Overlay of mucin binding sensorgrams obtained for B72.3 peptic $F(ab')_2$ fragments

B72.3 IgG1, IgG2 and IgG4 peptic $F(ab')_2$ fragments at 25µg/ml were passed over the mucin specific sensor chip at a flow rate of 5µl/min. Binding was observed for 8 minutes. Dissociation was then followed by replacing the antibody with a continuous flow of buffer.

(b) Anti-NIP IgG subclasses

NIP:BSA binding sensorgrams were obtained for anti-NIP IgG2, IgG3 and IgG4 mAbs over a range of concentrations (Figures 4.22, 4.23 and 4.24). Different binding characteristics were observed for all three subclasses. The dissociation curves of the four antibodies exhibited subtle differences with IgG3 being the fastest. Interestingly, the binding curves obtained for IgG2 and IgG4 were very similar.

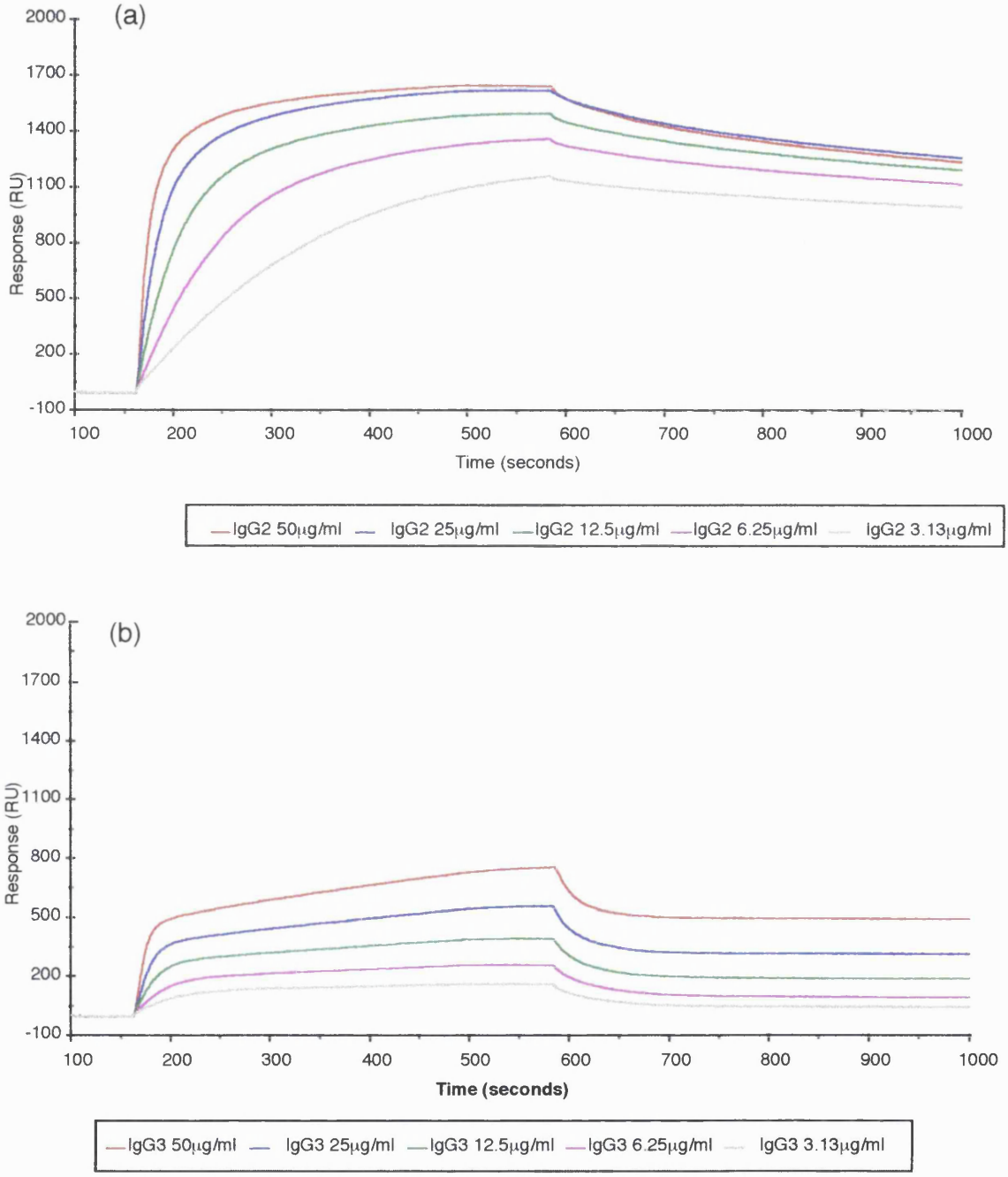


Figure 4.22 Overlay of NIP binding sensorgrams produced for IgG2 and IgG3 anti-NIP mAbs

Antibody binding was monitored for 7 minutes using a continuous flow of antibody at 5ul/min. Antibody sample was then replaced with buffer alone and dissociation of binding was then followed for approximately 7 minutes.

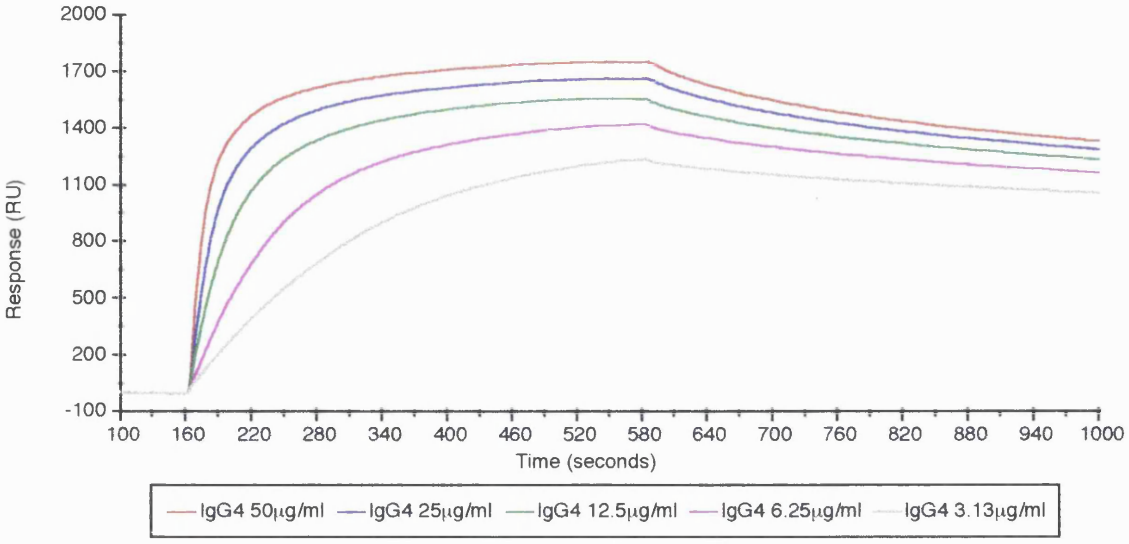


Figure 4.23 Overlay of NIP binding sensorgrams produced for IgG4 anti-NIP mAb
Antibody binding was monitored for 7 minutes using a continuos flow of antibody at 5µl/min. Dissociation of binding was then followed by replacing the antibody sample with a continous flow of buffer.

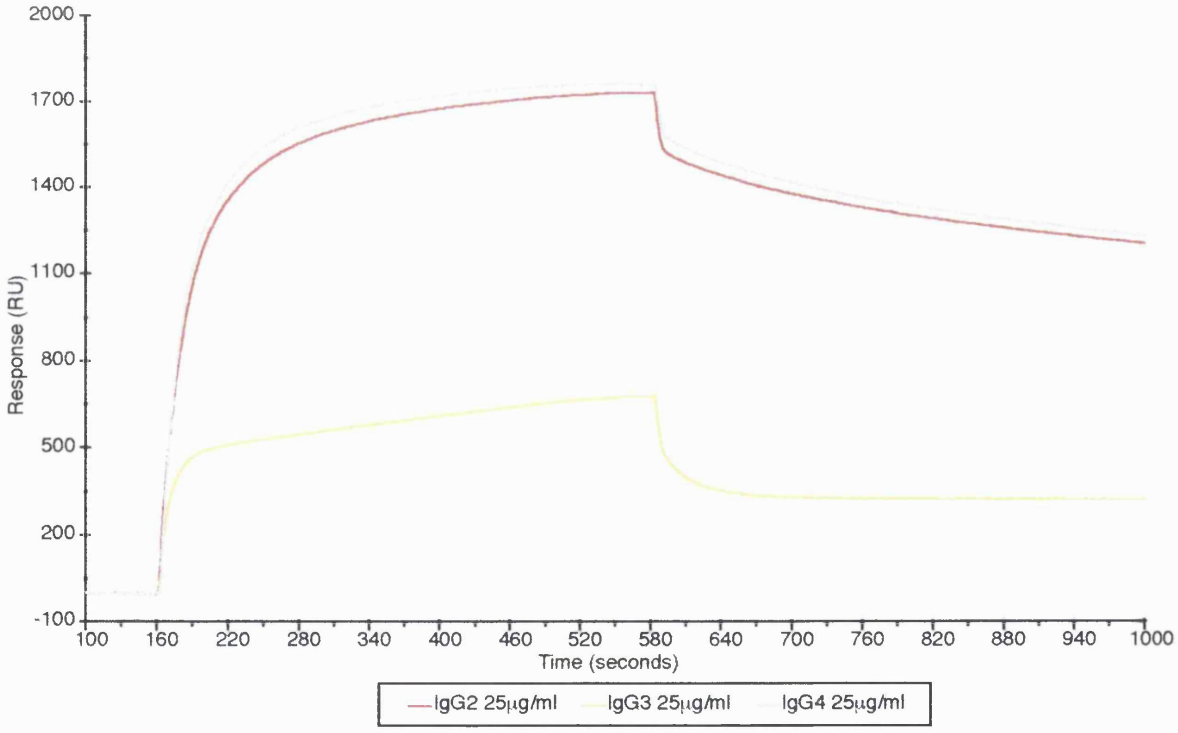


Figure 4.24 Overlay of NIP_{12}-BSA binding sensorgrams
Binding of anti-NIP IgG2, IgG3 and IgG4 mAbs at 25µg/ml was followed by passing the mAbs over the NIP_{12} -BSA sensor chip at a flow rate of 5µl/min for 8 minutes. Dissociation was then observed by flowing sample buffer over the sensor chip.

Apparent Rate Constants

(a) B72.3 IgG subclasses and $F(ab')_2$ fragments

Association and dissociation rate constants were calculated for each IgG subclass and $F(ab')_2$ fragment studied. A range of five different concentrations of each antibody were analysed. Apparent association rate constants were found to be similar for both intact antibodies and $F(ab')_2$ fragments (Table 4.2). However the apparent dissociation rate constants were found to differ between the IgG subclasses with IgG1 having the fastest dissociation rate and IgG4 the slowest dissociation rate. The $F(ab')_2$ fragments were found to have dissociation rates similar to each other and all were faster than the respective intact antibodies. Calculating affinity constants (K_A) it was found that the mAbs could be ranked in the order IgG4 > IgG3 > IgG2 > IgG1

Table 4.2. Apparent rate constants of intact chimeric B72.3 IgG subclass proteins and peptic $F(ab')_2$ fragments studied[a].

IgG subclass	[mAb] nM	$K_aM^{-1}s^{-1}$ ($x10^5$)	$k_d s^{-1}$ ($x10^{-3}$)	$K_A M^{-1}$ ($x10^8$)
IgG1	170 - 10	1.92 (+/- 0.08)	3.21 (+/- 0.13)	0.6
IgG2	170 - 10	1.98 (+/-0.13)	2.56 (+/- 0.03)	0.8
IgG3	150 - 20	1.41 (+/- 0.10)	1.16 (+/- 0.06)	0.9
IgG4	170 - 10	1.68 (+/- 0.18)	0.69 (+/- 0.06)	2.4
IgG1 $F(ab')_2$	250 - 15	1.62 (+/- 0.03)	4.01 (+/- 0.15)	0.4
IgG2 $F(ab')_2$	250 - 15	2.09 (+/- 0.08)	3.93 (+/- 0.12)	0.5
IgG4 $F(ab')_2$	250 - 15	1.93 (+/- 0.13)	3.71 (+/- 0.10)	0.5

a) Rate constants were calculated as the mean +/- SEM of a range of concentrations of antibodies. The same portion of the binding curve was chosen for analysis at each antibody concentration. Results are expressed as the mean value obtained from five different antibody concentrations, molar values were determined assuming a molecular weight of 146kDa for IgG1, IgG2 and IgG4, 170kDa for IgG3 and 100kDa for $F(ab')_2$ fragments.

(b) Anti-NIP IgG subclasses

Association and dissociation rate constants were calculated for each of the anti-NIP subclasses studied over a range of five different concentrations. Both apparent association rate constants and apparent dissociation rate constants differed (Table 4.3). Apparent association rate constants could be ranked in the order IgG3 > IgG4 > IgG2 and dissociation rate constants in the order IgG3 > IgG2 > IgG4. On the basis of the calculated affinity constants (K_A), the avidity of the mAbs could be ranked in the order IgG4 > IgG2 > IgG3.

Table 4.3. Apparent rate constants[a] of intact chimeric anti-NIP IgG subclass proteins studied.

IgG subclass	[mAb] nM	$k_a M^{-1}s^{-1}$ ($x10^5$)	$k_d s^{-1}$ ($x10^{-3}$)	$K_A M^{-1}$ ($x10^8$)
IgG2	340 - 10	2.02 (+/- 0.18)	0.96 (+/- 0.06)	2.1
IgG3	290 - 18	4.52 (+/- 0.22)	10.00 (+/- 0.4)	0.45
IgG4	340 - 10	2.35 (+/- 0.28)	0.85 (+/- 0.05)	2.8

(a) Rate constants were calculated as the mean +/- SEM of a range of concentrations of antibodies. The same portion of the binding curve was chosen for analysis at each antibody concentration. Results are expressed as the mean value obtained from five different antibody concentrations, molar values were determined assuming a molecular weight of 146kDa for IgG2 and IgG4 and 170kDa for IgG3.

DISCUSSION

V region identical humanised antibodies of the four IgG subclasses have been used to demonstrate that such antibodies exhibit differences in their apparent dissociation rate constants despite possessing identical V regions. Whilst standard techniques of affinity measurement permit the derivation of equilibrium constants, the BIAcore™ provides detailed analysis of the association and dissociation phases of the antigen-antibody interaction. The major differences in the kinetics of the antibodies studied here resided in their respective dissociation rates. Such differences appear to be due to the influence of the constant region on antibody binding, since they were abolished following the removal of the constant region by pepsin digestion.

Employing V region identical mouse-human chimeric IgG subclasses specific for mucin similar association rate constants were observed for each IgG subclass. In contrast the dissociation rate constants (k_d) showed marked differences and could be ranked in the order IgG1 > IgG2 > IgG3 > IgG4. Using the calculated equilibrium affinity constants (K_A) it was found that antibody avidity could be ranked in the order IgG4 > IgG3 ≥ IgG2 > IgG1. In contrast, $F(ab')_2$ fragments derived by pepsin digestion of the parent IgG1, IgG2 and IgG4 mAbs did not show any differences in rate or equilibrium constants but exhibited rate and equilibrium constants lower than their respective parent mAbs. Utilising a different set of V region identical mouse-human chimeric mAbs specific for the hapten NIP, similar differences were observed with IgG4 having the highest avidity and IgG3 the lowest avidity. Unfortunately data were not available for IgG1 since the hybridoma cell line did not express antibody.

Morelock and co-workers (1994), using the less sensitive technique of competitive binding ELISA, were able to show functional affinity differences between V region-identical IgG1, IgG2 and IgG4 antibodies specific for ICAM-1. In their study the functional affinity of the antibodies was ranked IgG1>IgG4>IgG2 suggesting that the functional affinity differences could be explained by flexibility in the hinge regions. The greater flexibility of the IgG1 hinge region may permit bivalent binding, in contrast to that of IgG2, the isotype thought to have the least flexible hinge region (Dangl et al. 1988; Tan et al. 1990; Schnieder et al. 1988; Oi et al. 1984). In this study, IgG4 was found to be of highest avidity using two separate sets of mAbs directed against two different antigens. Since IgG4 is known to have a relatively inflexible hinge, it is unlikely that hinge flexibility is the sole explanation for the differences noted above.

Differences in the functional affinity of IgG subclasses have been investigated more extensively in the murine system. Fulpius et al. (1993) showed that an IgG1 switch variant of an IgG3 parent lacked the expected rheumatoid factor activity despite having an identical V region. Similarly, Schrieber et al. (1993) demonstrated functional affinity differences between an IgG1 switch variant of a V region-identical IgG3 parent specific for a *Pseudomonas* species, with IgG1 having the lowest avidity. Cooper et al. (1994) have demonstrated differences in binding kinetics determined by BIA between mouse IgG1, IgG2b and IgG3 directed against N-acetyl-glucosamine (GlcNAc) of streptococcus group A carbohydrate with IgG3 having the highest affinity. These authors suggested that the higher functional affinity of mouse IgG3 specific for GlcNAc was due to molecular co-operativity of IgG3, whereby IgG3 antibodies bound in

close proximity to antigen undergo non-covalent Fc-Fc interactions stabilising the complex. This might explain the consistent finding of higher IgG3 functional affinity in the various murine systems studied although it is unlikely to explain differences in the human IgG subclasses since they do not appear to display co-operative binding (Greenspan and Cooper, 1992). Furthermore, the human functional equivalent of murine IgG3 is believed to be IgG2 which is thought to have the least hinge flexibility (Oi et al. 1984; Dangl et al. 1988; Schnieder et al. 1988; Tan et al. 1990) while human IgG3, in contrast to all the murine IgG subclasses has a long flexible hinge. Such differences highlight the restricted structural and functional homology between the mouse and human IgG subclasses and underlines the problems in extrapolating data from the murine to the human IgG system (Callard and Turner, 1990). Most of the IgG subclasses are believed to have arisen since speciation occurred in the mammals and hence groups such as rodents, ungulates and primates have widely different patterns of subclass proteins.

The functional significance of the differences in the apparent dissociation rate constants demonstrated remains unclear. However, the ability to separately analyse association and dissociation kinetics by the BIAcore™ may prove crucial in our understanding of certain biological phenomena. Foote and Milstein (1991) have shown that the association constant of antibodies specific for the hapten 2-phenyl-5-oxazalone may be critical for B cell selection and that concurrent with affinity maturation there is also kinetic maturation. With regard to antibody function, the neutralising capacity of a panel of antibodies reactive with the V3

loop of HIV has recently been shown to correlate directly with dissociation rate (VanCott et al. 1994).

Summary

It appears likely that the differences observed here in the binding kinetics of chimeric mouse-human IgG subclasses may be due to structural differences in the human constant regions although the exact mechanisms underlying such differences are at present unclear. The consistent finding of high avidity IgG4 mAbs is of interest, since it has been noted that high avidity IgG4 is produced following secondary and tertiary immunisation with the neo-antigen keyhole limpet hemocyanin (Devey et al. 1990). The exact mechanisms giving rise to such high avidity IgG4 are unclear but hinge flexibility seems unlikely to be the sole explanation. It remains to be established whether such avidity differences between the different human IgG subclasses exist in naturally occurring antibodies. The observation that engineered V region identical mAbs display avidity differences may be of importance when designing therapeutic antibodies.

CHAPTER 5

The avidity of IgG subclasses specific for pneumococcal polysaccharides

INTRODUCTION

Previous chapters have described experiments demonstrating a difference in avidity between IgG subclasses specific for the same antigen. Removal of the Fc portion of the B72.3 mAbs by peptic digestion abolished these differences, suggesting an influence on avidity of the constant region. These studies, however employed engineered mouse-human IgG subclasses. Chimeric antibodies may differ functionally when compared to naturally occurring antibodies for a variety of reasons including the potential for differences in glycosylation patterns. This chapter therefore describes the exploration of IgG subclass avidity differences in polyclonal serum IgG subclasses specific for clinically relevant bacterial antigens, namely the capsular polysaccharides of *S.pneumoniae*.

Streptococcus pneumoniae

Despite the widespread use of antibiotic therapy, infection with encapsulated bacterial pathogens, such as *Streptococcus pneumoniae* and *Haemophilus influenzae* type b, results in high morbidity and mortality. These bacteria cause a variety of clinical infections including meningitis, pneumonia, pericarditis and osteomyelitis. *S. pneumoniae* is the most common bacterial causes of otitis media in children. The virulence of *S. pneumoniae* is attributed to the polysaccharide capsule which is resistant to complement mediated killing and phagocytosis. This capsule is also the target for protective antibodies and is incorporated into the currently available pneumococcal vaccine. There are 90 different pneumococcal polysaccharide serotypes of which 23 are included within

the current unconjugated polysaccharide vaccine. Nevertheless the protection afforded by the current vaccine is short-lived and the efficacy of the vaccine has not been proven in high risk groups, including infants, immunocompromised patients and the elderly. The increasing prevalence of antibiotic resistant pneumococcal strains has focused attention upon the development of a pneumococcal vaccine that will be effective in high risk groups.

Immune Responses to Pneumococcal Polysaccharides

The success of *S. pneumoniae* as a pathogen is partly due to its resistance to complement mediated lysis. Immunity to *S. pneumoniae* and to encapsulated bacteria in general is dependent upon the existence of specific antibody. The major IgG subclass raised against pneumococcal polysaccharides in adults is IgG2, and opsonisation is strongly correlated with IgG2 levels (Viathrsson et al. 1994; Lortan et al. 1993). It is unclear exactly how IgG2 mediates this increase in opsonisation, since IgG2 is traditionally thought to be poor at activating complement. However, studies by Burton and Woof (1992) have demonstrated complement activation by IgG2 at high epitope densities.

As discussed in Chapter 1, children under 2 years of age are unable to mount an adequate IgG2 response to carbohydrate antigens. As a result anti-capsular polysaccharide antibody responses are poor below 2 years of age and IgG2 dominant adult type responses are not achieved until 8 - 10 years (Kumararatne and Jefferis, 1990).

Pneumococcal polysaccharide antigens are classified as T independent type 2 antigens. A precise definition of T cell independence is lacking, but in general, these antigens deliver prolonged and persistent signalling to B cells without the need for MHC class II restricted T cell help. This is not sufficient to ensure B cell responsiveness and cytokines such as IL-3, GMCSF and IFNγ are also required (Mond et al, 1995). The source of these cytokines is believed to be from non-T cells, such as natural killer cells (NK) and T cells themselves. Anti-polysaccharide antibodies are not thought to undergo somatic hypermutation, and whether germinal centres colonised by T independent antigen specific B cells even exist remains controversial (Mond et al. 1995; Maclennan, 1994).

Protective immunity to *S. pneumoniae* is believed to be reflected in the level of serotype specific anti-capsular polysaccharide antibodies, with low levels increasing the risk of invasive disease (Ambrosino et al. 1987; Austrian, 1989). Studies of protective levels of specific antibodies have been hampered in the past by the unavailability of a reference serum and variations between laboratories in assay conditions. Many published studies have employed the whole vaccine, containing 23 serotypes in the case of Pneumovax, as the antigen in solid-phase assays. This is unsuitable for assessing whether an individual has protective antibody levels, since a strong response to one serotype, for example the highly immunogenic serotype 3, may hide an overall poor response (Hazelwood et al. 1992 and 1993). Feldman et al. (1994) have demonstrated that the method of adsorbing the antigen to the solid phase may also affect the antibody level measured. These authors showed that the titre of serum antibodies specific to serotype 3 differed, depending on whether a poly-L-

lysine conjugation method or tyramine conjugation method was used to adsorb the polysaccharide to the solid-phase. Another factor influencing specific antibody titres is the existence of antibodies directed against the cell wall polysaccharide which are largely non-protective, giving a falsely high titre (Goldblatt et al. 1992; Viathrsson et al. 1994). This may be overcome by adsorbing out anti-CWPS antibodies prior to measuring serotype specific titres.

Clearly, a need has arisen for a consensus assay protocol for the measurement of specific anti-pneumococcal polysaccharide antibody levels if we are to assess adequately the efficacy of pneumococcal vaccines and collaborative studies with this goal are currently underway.

Factors influencing the anti-pneumococcal polysaccharide response

(i) Gm Allotypes

Human allotypes have been described for the $\alpha 2$, $\gamma 1$, $\gamma 2$, $\gamma 3$ and ε heavy chains and for the κ light chain. It is well established that serum IgG subclass levels correlate directly with Gm allotype status, for example G2m(n)$^+$ homozygotes have higher serum IgG2 levels than G2m(n)$^-$ homozygotes. Certain Gm and Km allotypes are also associated with characteristic immune responses to bacterial antigens. Much of the work presented so far exploring correlations between allotype status and anti-polysaccharide antibody response, have utilised *H.influenzae* type b (Shackelford et al. 1985; Granoff et al. 1986; Granoff et al. 1988; Lenoir et al. 1988; Sarvas et al. 1990). Such, allotype associations are not restricted to the Hib response. An association between pneumococcal serotype specific IgG2 antibodies and G2m(n) status has been observed (Sarvas et al.

1989). In addition, *Moraxella catarrhalis* outer membrane protein IgG3 levels are significantly lower in individuals homozygous for the G3m(n) allotype (Goldblatt et al. 1994).

The clinical relevance of allotype associations is unclear and conflicting evidence has been presented with regard to the risk of invasive disease. Takala et al. (1991) found no increased risk of Hib disease associated with the G2m(n)-/- genotype, whereas Granoff et al. (1986) have suggested an association between the Km(1) allotype and invasive Hib disease.

(ii) *Pneumococcal Antibody Avidity*

Since antibody avidity is mainly determined by its V region, the V region utilised may influence the quality of the antibody produced. The majority of studies exploring the V region gene usage in anti-polysaccharide responses have been carried on anti-*H. influenzae* type b antibodies. The human anti-Hib antibody repertoire is of limited structural diversity being restricted to the IgG1 and IgG2 subclasses (Shackelford et al. 1987; Makela et al. 1987), with κ light chain predominating (Ambrosino et al. 1990; Granoff et al. 1993), and a limited number of V region genes being utilised. Heavy chain V genes are usually of the V_H3 subgroup (Scott et al. 1989; Adderson et al. 1991; Silverman and Lucas, 1991). Serological and sequencing studies of the human anti-Hib V_L repertoire revealed domination by VκII-A2 expression, with little or no somatic mutation (Scott et al. 1991). The functional significance of this restricted V region gene expression remains unclear. However, a study by Granoff et al. (1993) has demonstrated a correlation between VκII-A2 expression and high specific antibody avidity from

individuals vaccinated with the Hib polysaccharide conjugated to the outer membrane protein complex of *Niesseria meningitidis* (Hib PS-OMPC) or Hib polysaccharide conjugated to tetanus toxoid (Hib PS-T) vaccines, compared with VλIII expressing antibodies. In contrast, it was found that individuals vaccinated with the Hib polysaccharide oligomers coupled to CRM_{197}, a mutant diphtheria toxin, (Hib PS-Oligo CRM) produced high avidity antibodies irrespective of their V region gene expression. Furthermore, when the anti-Hib IgG1 antibodies were separated on the basis of their idiotype expression, designated, Hib-Id1, Hib-Id2 or Hib-Id0 it was found that Hib-Id1 expressing antibodies elicited by Hib PS-Oligo CRM had higher avidity that Hib-Id1 expressing antibodies elicited by Hib-OMPC. A direct correlation was demonstrated between the molecular form of the vaccine, V_L usage and antibody avidity (Lucas and Granoff, 1995). Although the underlying mechanisms are unclear it would appear that the molecular form of the antigen dictates both V region usage and antibody quality.

It is clear that infants who are at high risk from infection do not respond well to the pneumococcal polysaccharide based vaccine as measured by antibody titre (Goldblatt et al. 1992; Hazelwood et al. 1993), although, it is increasingly apparent that antibody titre alone is not the sole factor influencing protection by antibody. The avidity of the antibody response is also of importance, with high avidity specific antibody being superior (Steward, 1981; Einhorn et al. 1986; Granoff et al. 1993; Lucas and Granoff, 1995). A high titre of relatively low avidity antibody may confer limited protection from infection, whereas a lower titre of high avidity antibody may be important in overcoming infection. Little work to date has been published exploring the antibody avidity of

anti-pneumococcal polysaccharide responses (Konradsen et al. 1994; Konradsen, 1995). Investigations of various Hib conjugate vaccine antibody response have highlighted the importance of carrier protein choice in influencing antibody quality. The assessment of antibody quality reflected in antibody avidity will therefore be crucial when evaluating pneumococcal polysaccharide conjugate vaccines.

Pneumococcal Polysaccharide Vaccines

Due to the poor antibody response of children to pure polysaccharides, polysaccharide-protein conjugate vaccines have been developed in an attempt to improve the response by converting a T cell independent polysaccharide antigen into a T cell dependent antigen. This strategy has proved highly successful in improving the immunogenicity of the Hib vaccine in young infants and reducing the incidence of invasive *Haemophilus influenzae* type b infection. Hib conjugate vaccines are now included in the routine infant immunisation schedule in a number of countries world-wide and have all but eradicated invasive Hib infection in vaccinated populations. The development of pneumococcal polysaccharide conjugate vaccines will be more difficult due to the large number of serotypes involved. Preliminary evidence is encouraging and vaccination studies in the chinchilla model of otitis media, employing pneumococcal polysaccharide serotypes 6B, 14, 19F and 23F conjugated to *Niesseria meningitidis* outer membrane protein (OMPC) have demonstrated enhanced immunogenicity and protection from infection (Giebink et al. 1993 and 1996). Clinical studies in healthy adults have demonstrated enhanced immunogenicity of pneumococcal

polysaccharide serotype 12F conjugated to diphtheria toxoid (Fattom et al. 1990) and serotypes 6B, 14, 18C, 19F and 23F linked to non-toxic mutant diphtheria toxin (CRM_{197}) (Ahmed et al. 1996). Furthermore, trials in high risk groups such as patients with Hodgkin's disease (Chan et al. 1996) and children below 2 years old (Steinhoff et al. 1994; Leach et al. 1996) have also found enhanced immunogenicity of protein conjugated pneumococcal serotypes. Very recent data however demonstrates some of the potential complications of pneumococcal conjugate vaccines, with a failure to show enhanced immunogenicity of some of the serotypes included the 5 valent CRM_{197} conjugate in HIV infected adults (Ahmed et al. 1996) and children (King et al. 1996).

The choice of protein conjugate will be important when evaluating a pneumococcal polysaccharide conjugate vaccine. Extensive studies with the Hib conjugate vaccines have revealed that the nature of the conjugate influences immunogenicity. There are three Hib conjugate vaccine formulations currently available, Hib PS outer membrane protein complex (Hib PS-OMPC) vaccine which consists of high molecular weight Hib PS covalently coupled to *Niesseria meningitidis* outer membrane protein, Hib PS oligomers conjugated to a non-toxic mutant diphtheria toxin (Hib PS-Oligo CRM) and Hib-PS conjugated to tetanus toxoid (Hib PS-T). These conjugates vary in their immunogenicity in infants, for example Hib PS-OMPC has been shown to stimulate antibody production in 2 month old infants following a single dose, whereas Hib PS-Oligo CRM does not (Einhorn et al. 1986).

Aims of the Study

Due to the qualitative differences observed in antibodies elicited following vaccination with HIB conjugate vaccines, it will be important to assess anti-pneumococcal antibody avidity when evaluating the different pneumococcal vaccine formulations. This study was designed to analyse the avidity of anti-pneumococcal antibody produced following vaccination with the currently available pure polysaccharide pneumococcal vaccine. The development of methods to measure anti-pneumococcal serum antibody avidity will thus enable antibody quality to be evaluated following immunisation with pneumococcal conjugate vaccines.

Initially I set out to determine whether the pneumococcal serotype specific solid-phase assays could be adapted for the measurement of serum pneumococcal specific IgG subclass avidity. Once this was established the assay could be used to (i) explore whether there are avidity differences in the antibodies raised against different serotypes and (ii) to investigate a possible relationship between age and pneumococcal specific antibody avidity.

METHODOLOGY

Serum antibodies reactive with pneumococcal polysaccharide serotypes 3, 6B, 19F and 23F were measured by specific ELISA as described in detail in chapter 2. The avidity of IgM, IgG and IgG subclass antibodies were then analysed by modifying the antigen specific ELISAs as follows:

Competitive Binding ELISA

Sera diluted in PBS-Tw-BSA with or without free antigen (12 or 2h RT)
↓
Sera incubated in precoated ELISA plates (2h RT)
↓
Biotinylated anti-human IgG subclass antibodies (2h at RT)
↓
Streptavidin-peroxidase conjugate (1h at RT)
↓
0.5mg/ml OPD in PO_4/citrate/0.05% H_2O_2 (15-30min RT)
↓
Colour reaction stopped with 2M H_2SO_4
↓
A_{490nm}

Thiocyanate Elution

Sera diluted in PBS-Tw-BSA to give an A_{490nm} ~ 1.0 (2h RT)
↓
Ammonium thiocyanate added to the plate 0-4M for 15min at RT
↓
Biotinylated anti-human IgG subclass antibodies (2h at RT)
↓
Streptavidin-peroxidase conjugate (1h at RT)
↓
0.5mg/ml OPD in PO_4/citrate/0.05% H_2O_2 (15-30min RT)
↓
Colour reaction stopped with 2M H_2SO_4
↓
A_{490nm}

Assay volumes were 75μl/well throughout and the plates were washed four times with PBS-Tw between each step.

The avidity ELISAs were developed using standard serum to measure the avidity of antibodies specific for pneumococcal polysaccharides serotypes 3, 6B, 19F and 23F. These serotypes are included in the commercially available 23 valent Pneumovax vaccine and are clinically relevant serotypes in paediatric practice.

RESULTS

Antibodies to pneumococcal polysaccharides serotypes 3, 6B, 19F and 23F from pooled human serum (standard serum*).*

Antibodies from standard serum were investigated by antigen specific ELISA as described in chapter 2. Dose-response curves were obtained for IgM, IgG total and IgG subclasses (Figure 5.1). IgM and IgG total antibodies were detected in the standard serum and as expected for a polysaccharide antigen the major IgG subclass was IgG2. The dilution of serum which gave an A_{490nm} of approximately 1.0 was calculated from these curves for use in further avidity ELISAs. A dilution of 1/160 for serotype 3, 1/640 for serotype 6B, 1/300 for serotype 19F and 1/100 serotype 23F gave an A_{490nm} of 1.0 for polyclonal IgG2 responses.

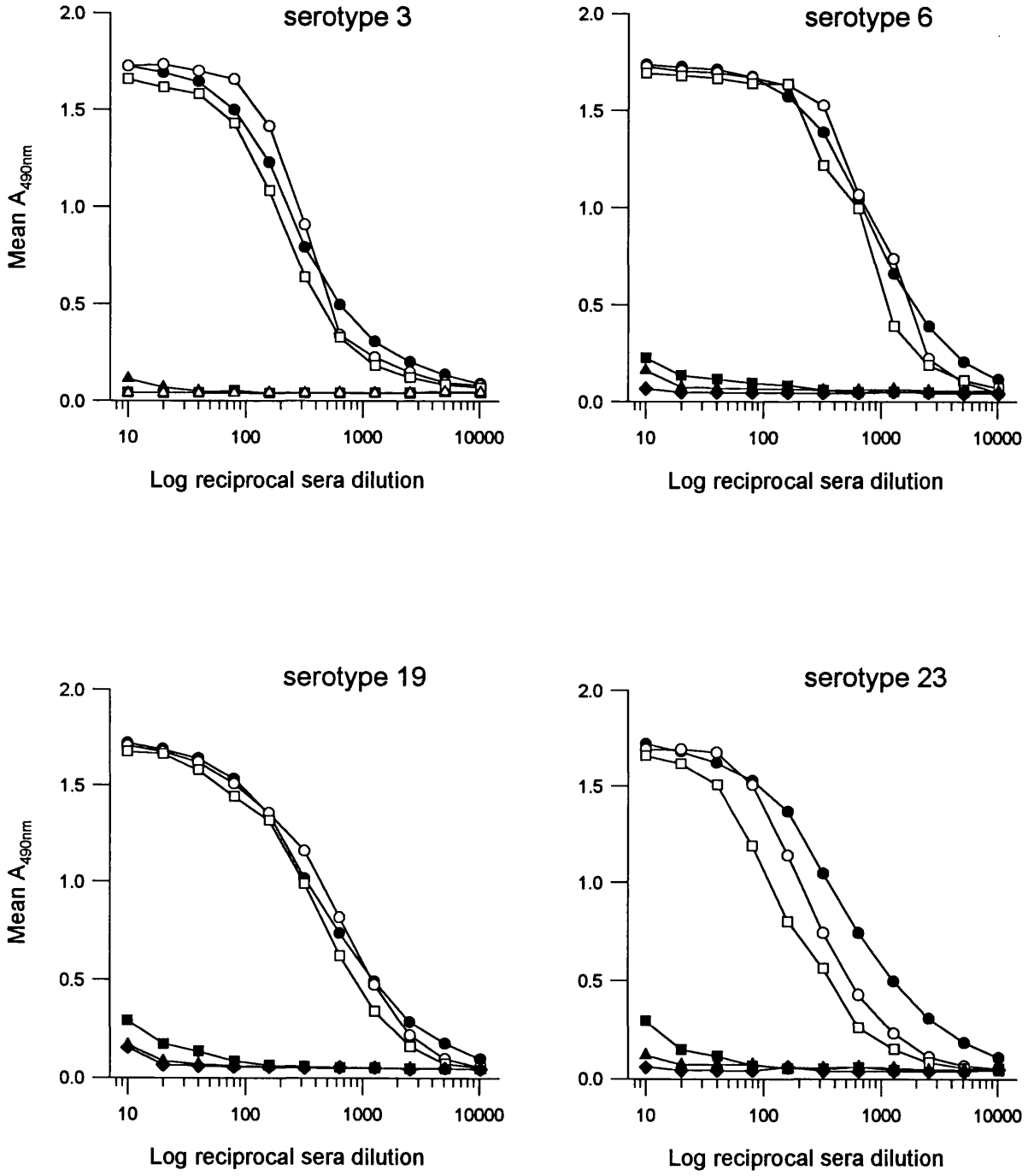


Figure 5.1 Dose-response curves for standard serum
Standard serum was serially diluted in PBS-Tw-BSA and binding of IgM (●), IgG total (O), IgG1 (■), IgG2 (□), IgG3 (◆) and IgG4 (▲) antibodies to pneumococcal polysaccharide serotypes 3, 6B, 19F and 23F were measured by specific ELISA.

Competitive Binding ELISA

The avidity of IgM, IgG total and IgG2 antibodies derived from standard serum specific for pneumococcal polysaccharide serotypes 3, 6B, 19F and 23F were measured by competitive binding ELISAs employing free polysaccharide to inhibit binding. The more free polysaccharide required to inhibit binding by 50% the lower the avidity of the antibody. Whilst absolute affinity constant (K) values cannot be calculated, a ranking of relative avidity of the antibodies may be obtained.

(a) Using the method modified from Friguet et al. (1985), where free antigen and antibody are incubated for 12h prior to analysis, with free polysaccharide from 10μg/ml to 0.02μg/ml to competitively inhibit binding, IgM, IgG total and IgG2 binding curves were obtained for responses specific for serotypes 3, 6B, 19F and 23F (Figure 5.2). 50% avidity indices could be calculated for serotypes 6B, 19F and 23F. Avidity indices could not be obtained for serotype 3 specific antibodies, due to the inability of free polysaccharide to inhibit serotype 3 specific antibodies by 50%.

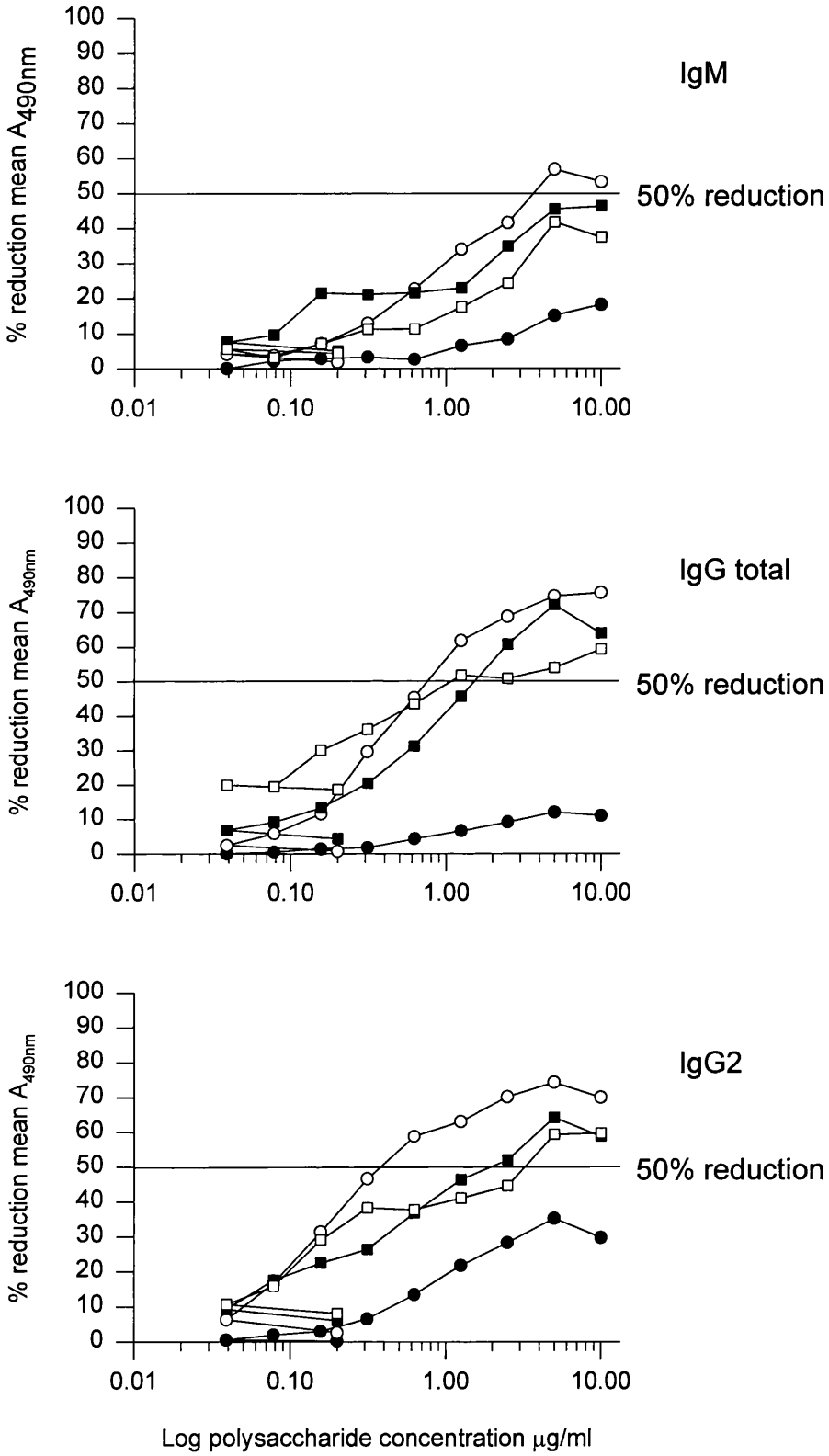


Figure 5.2 Competitive binding curves
Standard serum was diluted with PBS-Tw-BSA and incubated for 12h at RT with free polysaccharide from 10μg/ml to 0.02μg/ml. IgM, IgG total and IgG2 antibodies specific for serotypes 3 (●), 6B (○), 19F (■) and 23F (□) were then analysed by antigen specific ELISA.

It was possible that insufficient polysaccharide was employed to inhibit binding of serotype 3 specific antibodies and therefore competitive binding was analysed employing polysaccharide from 500μg/ml to 1μg/ml. It was found that polysaccharide at 500μg/ml was unable to inhibit antibody binding up to 50% (Figure 5.3).

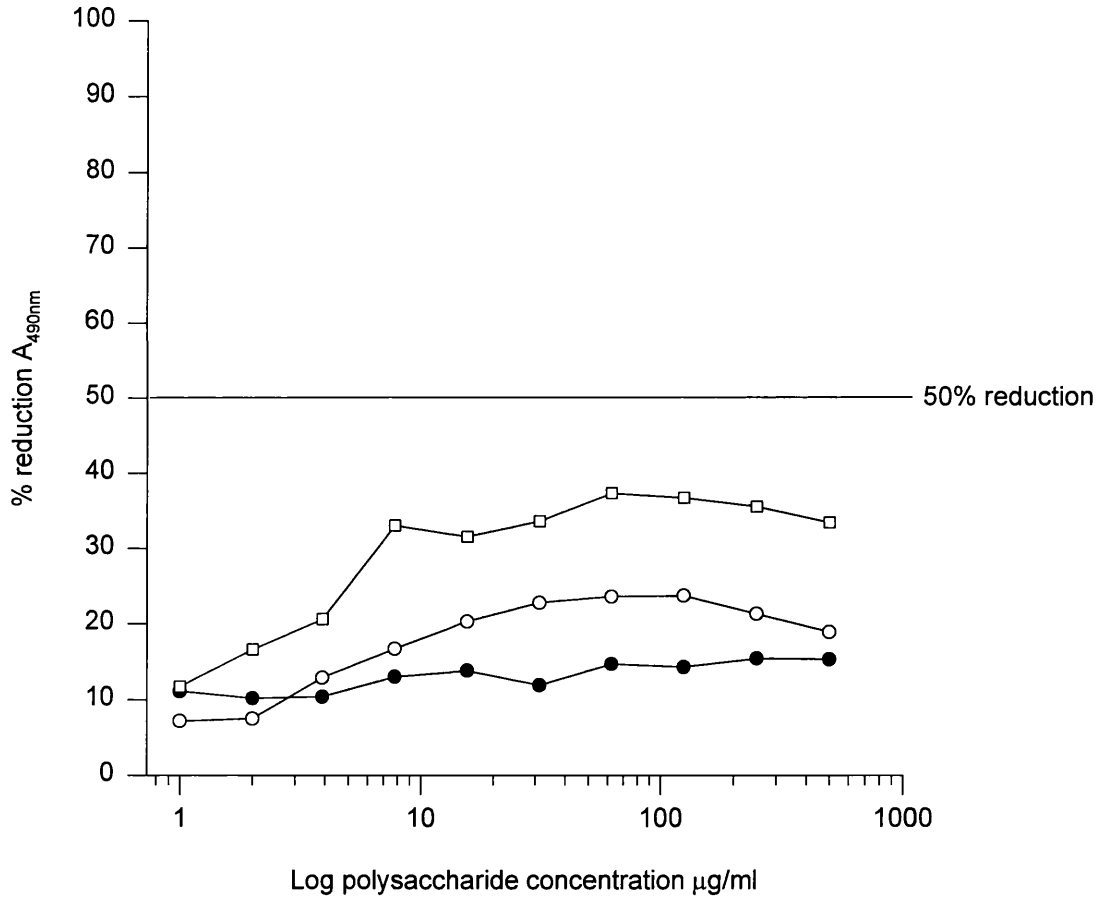


Figure 5.3 Polysaccharide serotype 3 competitive binding curves
Standard serum was diluted with PBS-Tw-BSA and incubated for 12h at RT with free polysaccharide from 500μg/ml to 1μg/ml. IgM (●), IgG total (O) and IgG2 (□), antibodies specific for serotype 3 were then analysed by antigen specific ELISA.

(b) Using the method modified from Rath et al. (1988), where the free antigen and antibody are incubated together for 2h in the presensitised ELISA plate, competitive binding curves were obtained for IgM, IgG total and IgG2 antibodies specific for polysaccharide serotypes 3, 6B, 19F and 23F employing free polysaccharide from 100μg/ml to 0.2μg/ml (Figure 5.4). Competitive binding curves were obtained for serotypes 3, 6B, 19F and 23F but antibodies to serotype 3 were not significantly inhibited at any concentration of polysaccharide investigated.

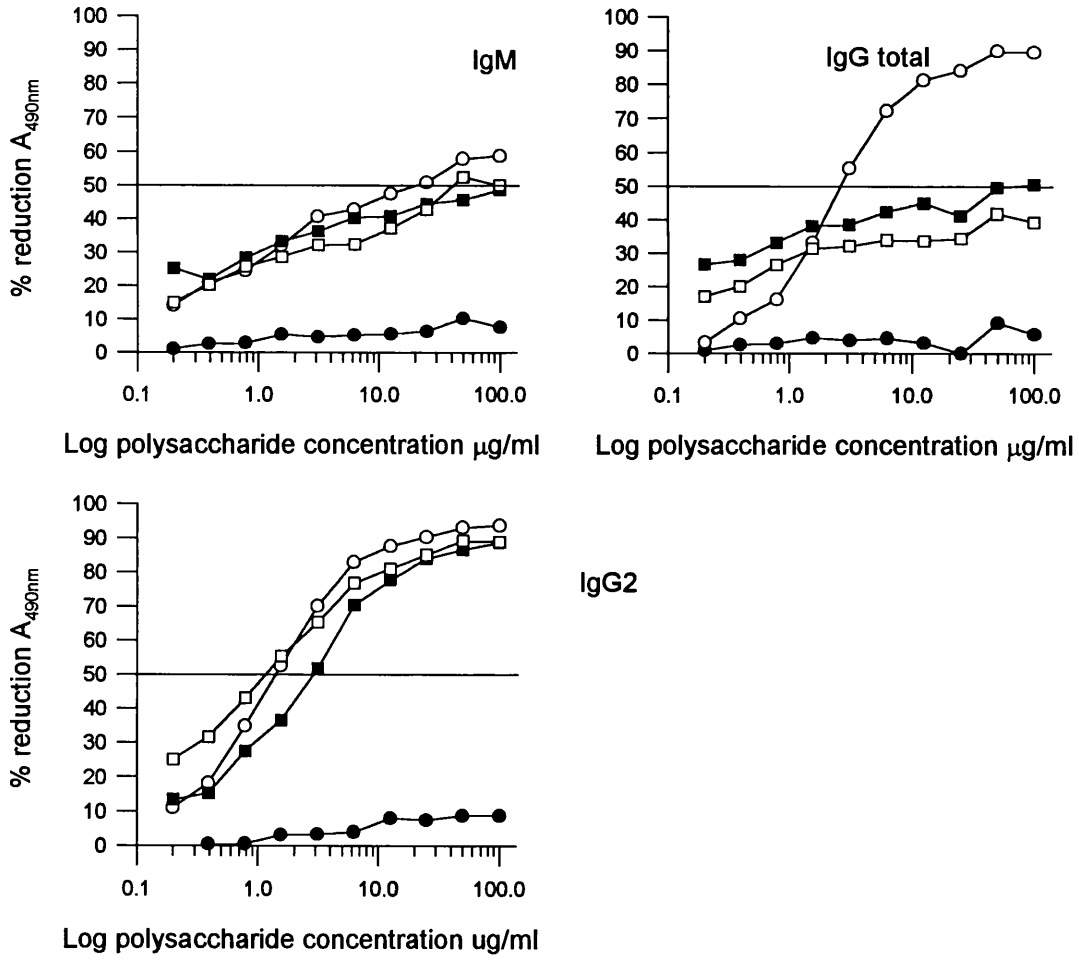


Figure 5.4 Competitive binding curves
Standard serum was diluted with PBS-Tw-BSA and incubated simultaneously for 2h at RT with free polysaccharide from 100μg/ml to 0.2μg/ml in the precoated ELISA plate. IgM, IgG total and IgG2 antibodies specific for serotypes 3 (●), 6B (O), 19F (■) and 23F (□) were then analysed by antigen specific ELISA.

Antibodies specific for polysaccharide serotype 3 could not be inhibited by free polysaccharide in the competitive binding ELISAs investigated. It has been reported that antibody responses specific for the cell wall polysaccharide (CWPS) of the pneumococcal capsule may interfere with binding and give a falsely high absorbance value in serotype specific ELISAs (Goldblatt et al. 1992). Hence it was possible that antibody responses to polysaccharide serotype 3 were not inhibited by free polysaccharide due to the presence of anti-CWPS antibodies. To address this possibility a competitive binding ELISA was performed comparing standard serum containing anti-CWPS antibodies (unadsorbed) and standard serum without anti-CWPS antibodies (adsorbed). Competitive binding curves were obtained for IgM, IgG total and IgG2 antibodies employing the method modified from Rath et al. (1988) (Figure 5.5). Antibodies specific for polysaccharide serotype 3 could not be inhibited by free polysaccharide regardless of the presence or absence of anti-CWPS antibodies. The presence of anti-CWPS antibodies was not responsible for the inability of free polysaccharide to inhibit serotype 3 specific antibody.

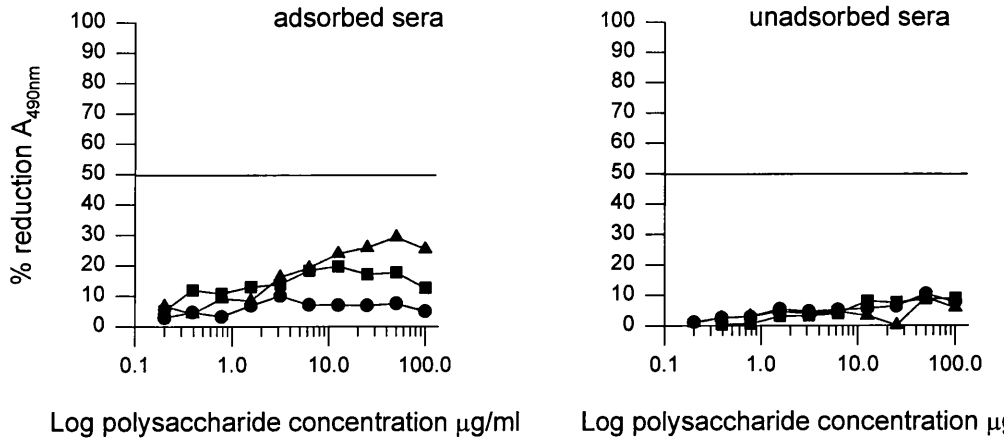


Figure 5.5 Competitive binding curves
Standard serum was diluted with PBS-Tw-BSA and incubated for 2h at RT with free polysaccharide from 100μg/ml to 0.2μg/ml in the precoated ELISA plate. IgM (●), IgG total (▲) and IgG2 (■), antibodies specific for serotypes 3 from adsorbed and unadsorbed sera were then analysed by antigen specific ELISA.

Relative antibody avidity as measured by competitive binding ELISA

It was not possible to measure the avidity of serotype 3 specific antibodies employing the conditions described. Relative antibody avidity specific for serotypes 6B, 19F and 23F were explored employing the method modified from Rath et al. (1988), as good competitive binding curves were obtained and this ELISA was rapid and convenient to perform. Avidity indices were calculated as the amount of free polysaccharide required to produce a 50% reduction in binding for IgM, IgG total and IgG2 antibodies specific for polysaccharide serotypes 6B, 19F and 23F (Table 5.1). The higher the avidity index, that is the more free polysaccharide required to inhibit binding, the lower the corresponding antibody avidity.

Table 5.1 Relative antibody avidity indices[a]

Antibody	Serotype 6B	Serotype 19F	Serotype 23F
IgM - exp. 1	ND[b]	>100	>100
- exp. 2	10.5	>100	>100
- exp. 3	>100	>100	>100
IgG - exp. 1	ND	2.7	6.0
- exp. 2	2.95	2.6	1.1
- exp. 3	4.0	4.6	1.8
IgG2 - exp. 1	ND	2.7	3.9
- exp. 2	1.5	1.0	6.7
- exp. 3	1.8	3.6	2.0

[a] avidity indices were calculated as the amount of free antigen required to give a 50% reduction in absorbance at 490nm. Avidity indices could not be calculated for antibody responses to serotype 3.

[b] not done

Interassay variation was calculated for serotypes 19F and 23F as follows:

Serotype 19F IgG = 50%, IgG2 = 43.3%

Serotype 23F IgG = 88%, IgG2 = 78.8%.

50% avidity indices could not be calculated for IgM antibodies. Since 50% inhibition was not achieved by 100μg/ml of free polysaccharide, this suggests that IgM antibodies are of low avidity. Antibody avidity for responses specific for pneumococcal polysaccharide serotype 3 could not be measured employing the competitive binding ELISAs. The exact reasons for this are unclear since previous investigators were able to measure the avidity of human (Persson et al. 1988) or murine (Van Dam et al. 1989) serum antibodies specific for pneumococcal serotype 3. It is possible that the serotype 3 specific IgG was of low avidity, however high avidity serotype 3 specific antibody was detected employing the thiocyanate elution ELISA, described later. Poor solubility of the polysaccharide may account for the inability of serotype 3 to inhibit antibody binding. Furthermore interassay variation for all assays was unacceptably high, which may reflect the variability of epitopes displayed when the polysaccharide is in solution, or difficulty in controlling the amount of polysaccharide in solution due to poor polysaccharide solubility. The competitive binding ELISA was therefore not pursued any further.

Thiocyanate Elution ELISA

Another solid-phase avidity ELISA was explored which employed the chaotropic ion, ammonium thiocyanate, to disrupt antigen-antibody binding. To rule out the possibility that ammonium thiocyanate was disrupting solid-phase antigen binding, standard curves were constructed following incubation of the precoated ELISA plate with PBS-Tw-BSA or ammonium thiocyanate. Figure 5.6 shows the standard curves produced for serum IgG2 antibodies binding to each pneumococcal serotype. It was found that ammonium thiocyanate did not affect binding of serotypes 6B, 19F and 23F to the ELISA plate but that it did reduce serotype 3 binding.

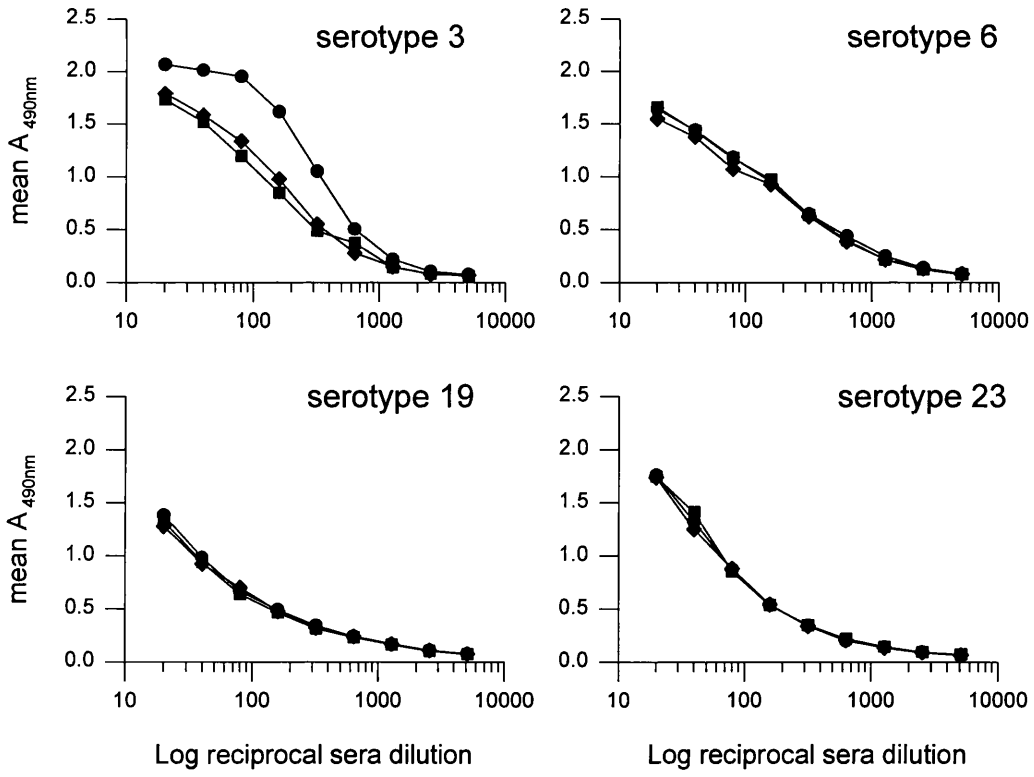


Figure 5.6 Dose-response curves of pneumococcal specific IgG2 antibodies
Binding curves were constructed from standard serum IgG2 binding to pneumococcal polysaccharide serotypes 3, 6B, 19F and 23F in the presence of PBS-Tw-BSA (●), 10M NH4SCN (■) or 5M NH4SCN (◆).

To ensure that the ammonium thiocyanate was not chemically altering the polysaccharides, the mobility of the polysaccharides was investigated using one-dimensional electrophoresis as described in detail in Chapter 2. As seen in Figures 5.7, 5.8 and 5.9, mobility of serotypes 6B, 19F and 23F was not affected by treatment with ammonium thiocyanate. Serotype 3 did not migrate in the cellulose, suggesting that it did not have a sufficient negative charge. No change in mobility was noted following treatment with ammonium thiocyanate. (Figure 5.10).

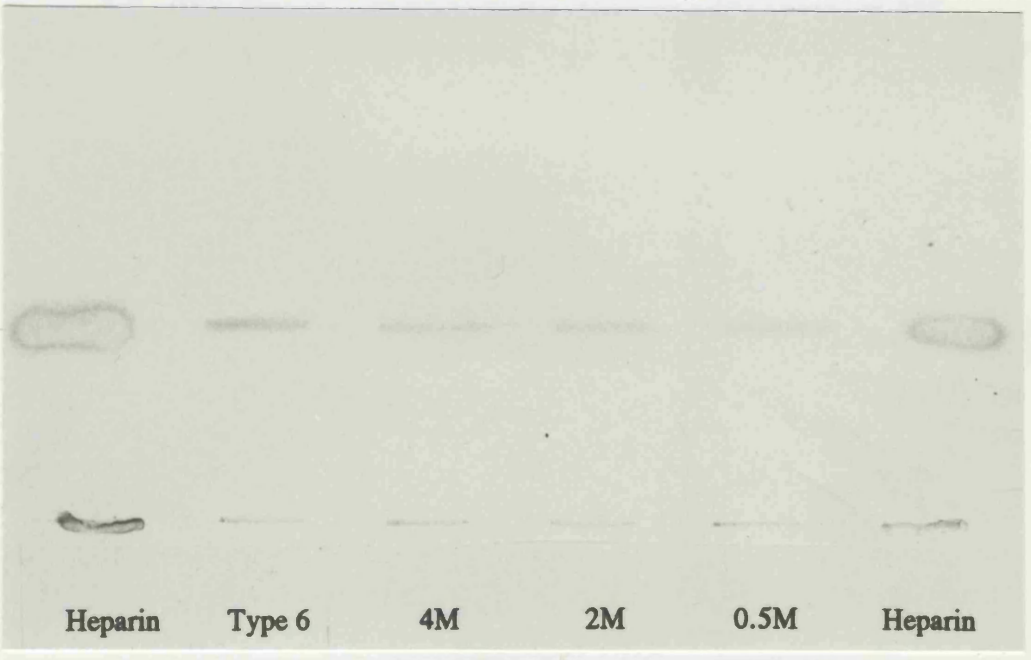


Figure 5.7 Mobility of pneumococcal polysaccharide serotype 6B
Polysaccharide mobility was measured by one dimensional electrophoresis with or without various concentrations of NH_4SCN.

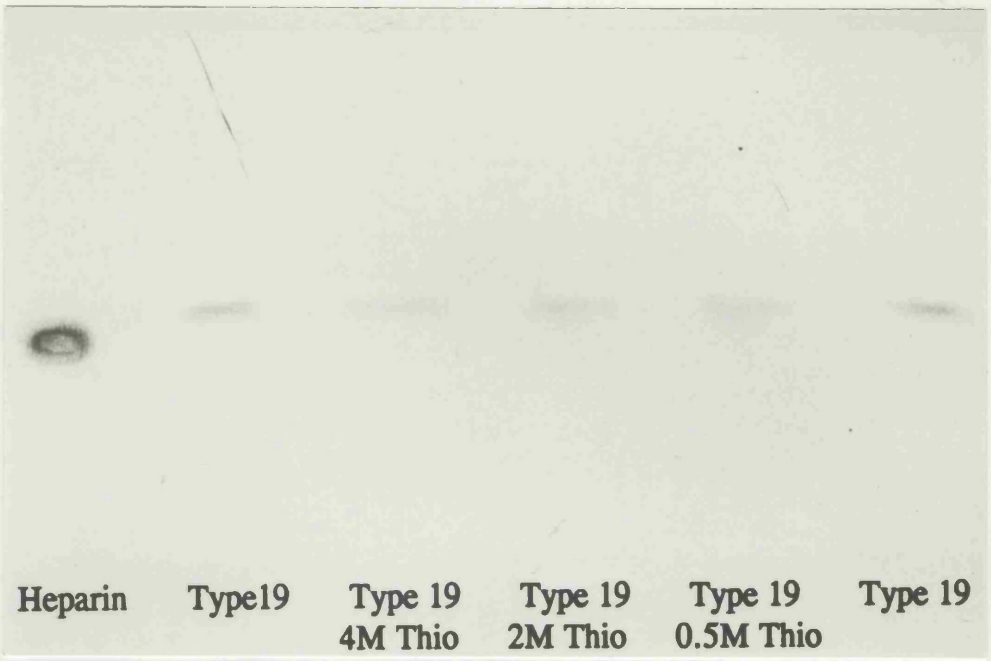


Figure 5.8 Mobility of pneumococcal polysaccharide serotype 19F
Polysaccharide mobility was measured by one dimensional electrophoresis with or without various concentrations of NH_4SCN.

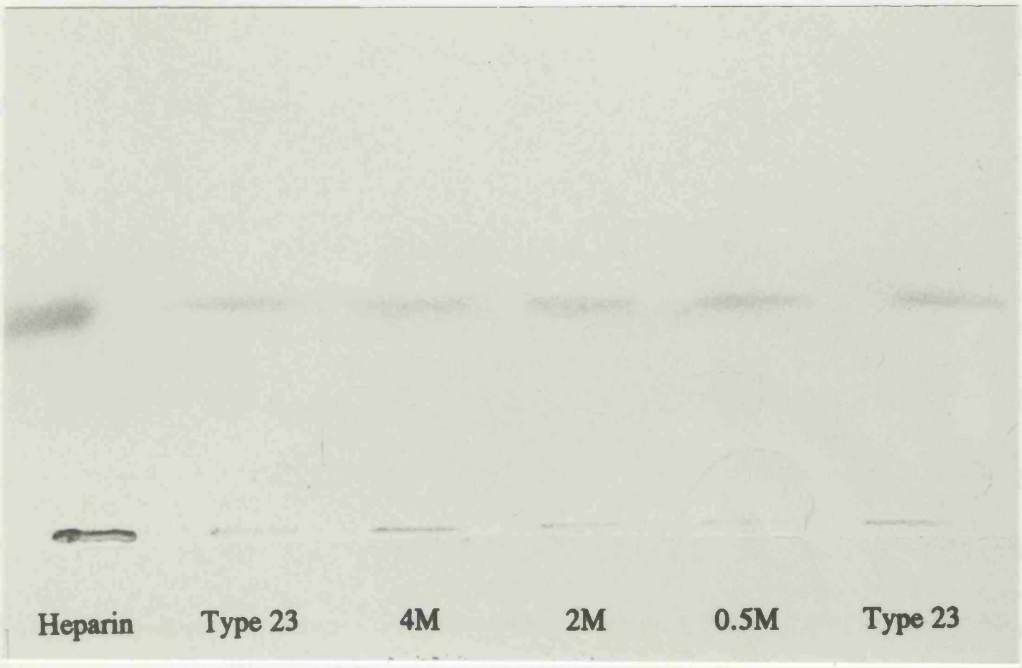


Figure 5.9 Mobility of pneumococcal polysaccharide serotype 23F
Polysaccharide mobility was measured by one dimensional electrophoresis with or without various concentrations of NH_4SCN.

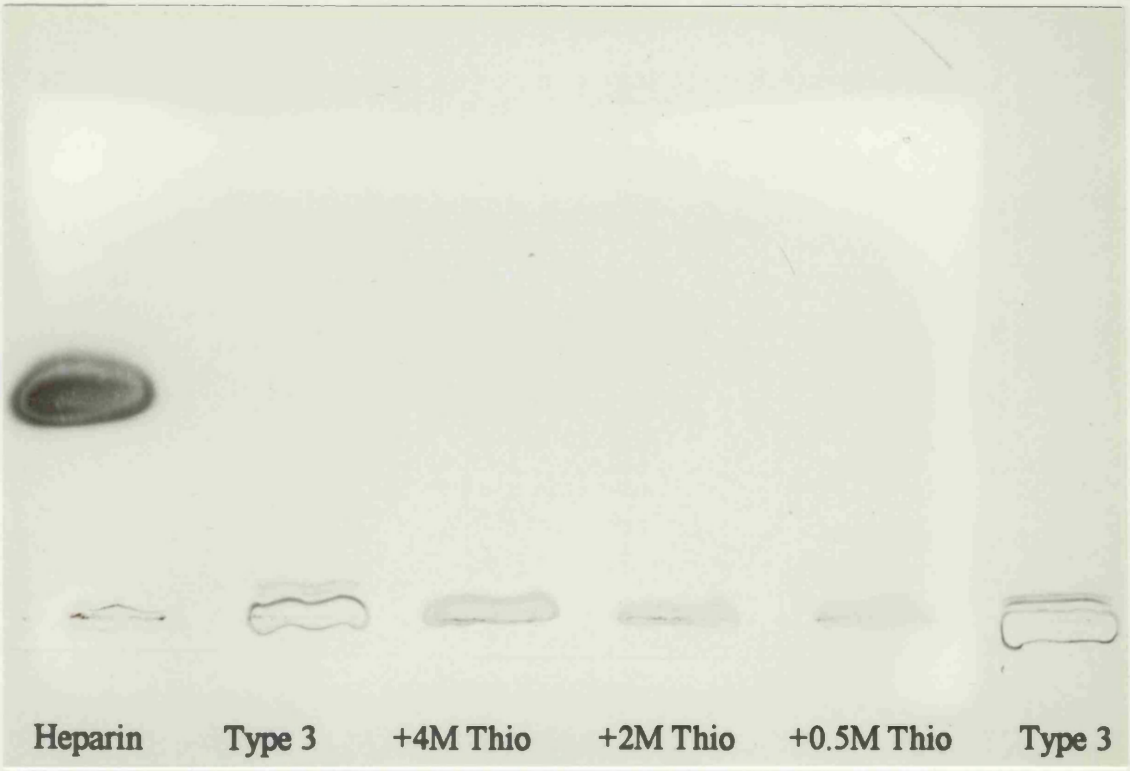


Figure 5.10 Mobility of pneumococcal polysaccharide serotype 3
Polysaccharide mobility was measured by one dimensional electrophoresis with or without various concentrations of NH_4SCN.

The observed disruption in binding of antibodies to serotype 3 following ammonium thiocyanate incubation was therefore not due to a modification in charge of the bound antigen but was presumably due to a disruption in adsorption. In order to overcome this problem serotype 3 was conjugated to poly-L-lysine to increase stability of the binding to the ELISA plate as described by Gray, (1979). Experiments were carried out to determine optimal conditions for serotype 3-lysine specific ELISA and a coating concentration of 1μg/ml was found to be optimal. Standard curves of IgG2 binding were then constructed following preincubation of coated plates with PBS-Tw-BSA or ammonium thiocyanate as described earlier. It was found that preincubation of the serotype3-lysine plate with ammonium thiocyanate did not affect the standard

curve obtained (Figure 5.11). Serotype 3-lysine was therefore employed in further assays to measure the avidity of serotype 3 specific serum antibodies.

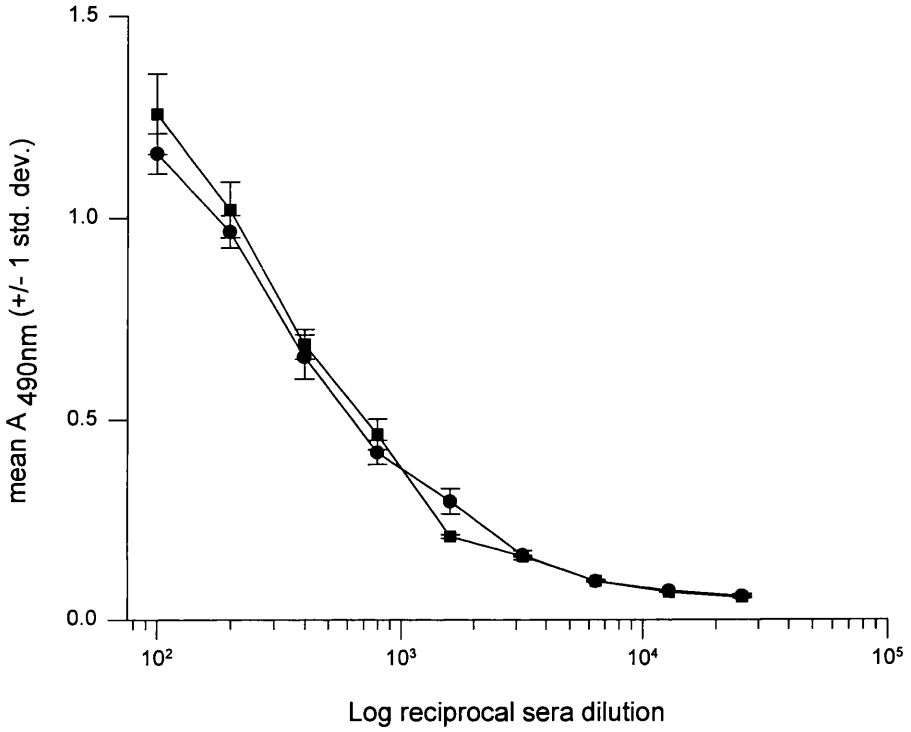


Figure 5.11 Dose-response curves of standard serum IgG2 antibodies
Binding to standard serum IgG2 antibodies to pneumococcal polysaccharide serotype 3 conjugated to poly-l-lysine adsorbed to an Immulon 2 polystyrene plate, was measured with (■) or without (●) a 15min preincubation with 10M NH_4SCN.

Experiments were initially carried out to determine the optimal range of concentration of ammonium thiocyanate for use in subsequent experiments. Ammonium thiocyanate was added as an additional 15min incubation step from 0M to 10M and the percentage reduction in absorbance at 490nm calculated at each concentration of thiocyanate. Binding curves were then constructed by

plotting the log % reduction in absorbance against the molar concentration of ammonium thiocyanate. It was found that a good binding curve was obtained with ammonium thiocyanate from 0.5M to 4M (Figure 5.12).

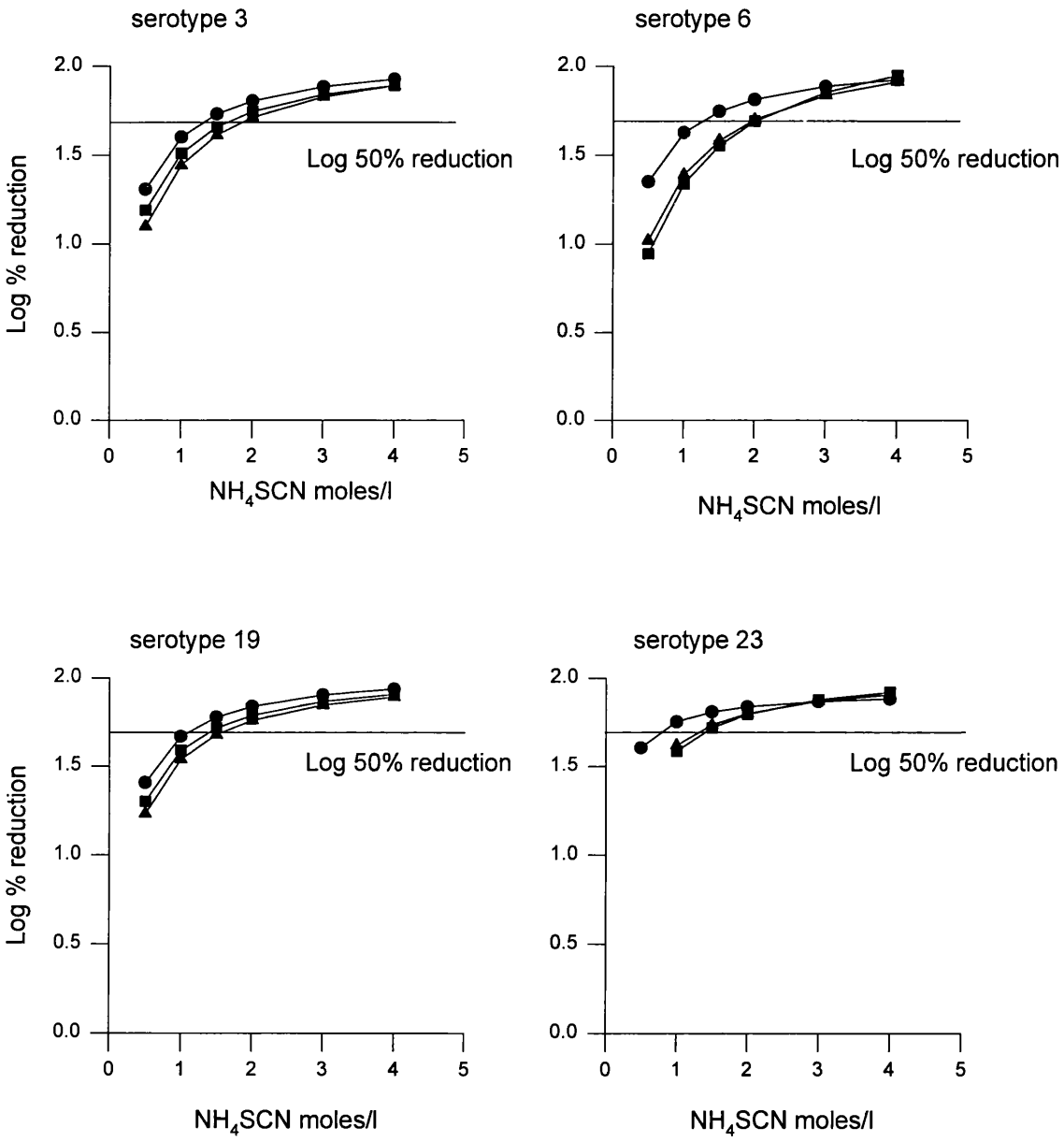


Figure 5.12 Thiocyanate elution binding curves
Binding curves were produced for IgM (●), IgG (▲) and IgG2 (■) antibodies specific for pneumococcal polysaccharide serotypes 3, 6B, 19F and 23F in the presence of ammonium thiocyanate.

In order to measure the avidity of standard serum antibodies specific for pneumococcal polysaccharide serotypes 3, 6B, 19F and 23F ammonium thiocyanate at 4M, 2M, 1.5M, 1M and 0.5M was used to disrupt antigen-antibody binding. Avidity indices were calculated as the amount of ammonium thiocyanate required to produce a 50% reduction in absorbance at 490nm (mean of four experiments).

Table 5.2 Mean avidity indices for serum antibodies specific for pneumococcal polysaccharide serotypes measured by thiocyanate elution ELISA

Serotype	IgM	IgG	IgG2
3	1.2	1.6	1.6
6B	1.2	1.6	1.8
19F	1.3	1.4	1.3
23F	1.3	1.6	1.3

Interassay variations:

Serotype 3 IgM = 6.5%, IgG = 3.5%, IgG2 = 12.7%

Serotype 6B IgM = 12.2%, IgG = 10.9%, IgG2 =9%

Serotype 19F IgM = 14.9%, IgG =10.7%, IgG2 =7.8%

Serotype 23F IgM = 4%, IgG = 20%, IgG2 =18.9%

Interassay variation was within acceptable levels and, as expected, serum IgM antibodies were of lower avidity than IgG antibodies. Differences in avidity indices were, however, small between serotypes and no conclusion could be reached regarding the avidity of standard pooled serum antibodies specific for different serotypes.

Responses of Children Immunised with Pneumovax to Pneumococcal Polysaccharides

Patient selection: Normal healthy children are not routinely vaccinated with Pneumovax. Children with sickle cell disease are, however, susceptible to pneumococcal infection and it is recommended that they are vaccinated with pneumococcal vaccine. 35 patients with sickle cell disease attending the sickle cell clinic of The Queen Elizabeth Hospital For Sick Children, Hackney, London, were vaccinated with the 23 valent Pneumovax vaccine. Serum samples were prepared from venous blood taken pre vaccination and 1 month post vaccination and stored frozen at -70°C.

Antibody Response: IgG total and IgG1, IgG2 and IgG3 subclasses were measured by antigen specific ELISA for serotypes 3, 6B, 19F and 23F. Each serum sample was diluted in PBS-Tw-BSA and absorbance at 490nm read. At the time of analysis a pneumococcal standard serum was unavailable and therefore absolute titres were not analysed. Response to polysaccharide was thus expressed as the fold increase in absorbance following vaccination. Serum were taken as positive if a two fold or more increase was observed (Goldblatt et al. 1992). 26 out of 35 sera responded to one or more of the serotypes described above and these sera were analysed further in the avidity assays.

The Avidity of Serum IgG and IgG Subclass Antibodies Specific for Pneumococcal Polysaccharides Measured by Thiocyanate Elution ELISA

The avidity of IgG total, IgG1, IgG2 and IgG3 antibodies were analysed by thiocyanate elution ELISA in positive serum samples. Avidity indices were then calculated as the amount of ammonium thiocyanate required to give 50% reduction in absorbance.

IgG total avidity of serum antibodies

Measurement of pneumococcal serotype specific serum IgG revealed that 24/35 (69%) patients responded to serotype 3, 6/35 (17%) patients responded to serotype 6B, 2/35 (6%) patients responded to serotype 19F and 20/35 (57%) patients responded to serotype 23F. The avidity of serum IgG total antibodies of positive samples was then measured employing the thiocyanate elution ELISA.

As described in Chapter 1 pneumococcal capsular polysaccharides are characterised as T cell independent antigens and therefore are not expected to exhibit affinity maturation. Although, the IgG response to the pneumococcal polysaccharides increased more than two fold, the avidity of IgG total serum antibodies did not increase 1 month following vaccination with the Pneumovax vaccine. The results presented here were as expected for a polysaccharide antigen.

Only 2 patients responded to serotype 19 and therefore was not included for analysis. Comparing serum IgG avidity between the serotypes it was found that pre vaccination serum IgG specific for serotype 6B was of higher avidity than

IgG specific for serotype 3, or 23. Post-vaccination, however, serum IgG specific for serotype 3 was of higher avidity than serotype 6 or 23 (Figure 5.13). Following immunisation with Pneumovax it can be seen that serum IgG specific for serotypes 6 and 23 is of slightly lower avidity than pre-vaccination.

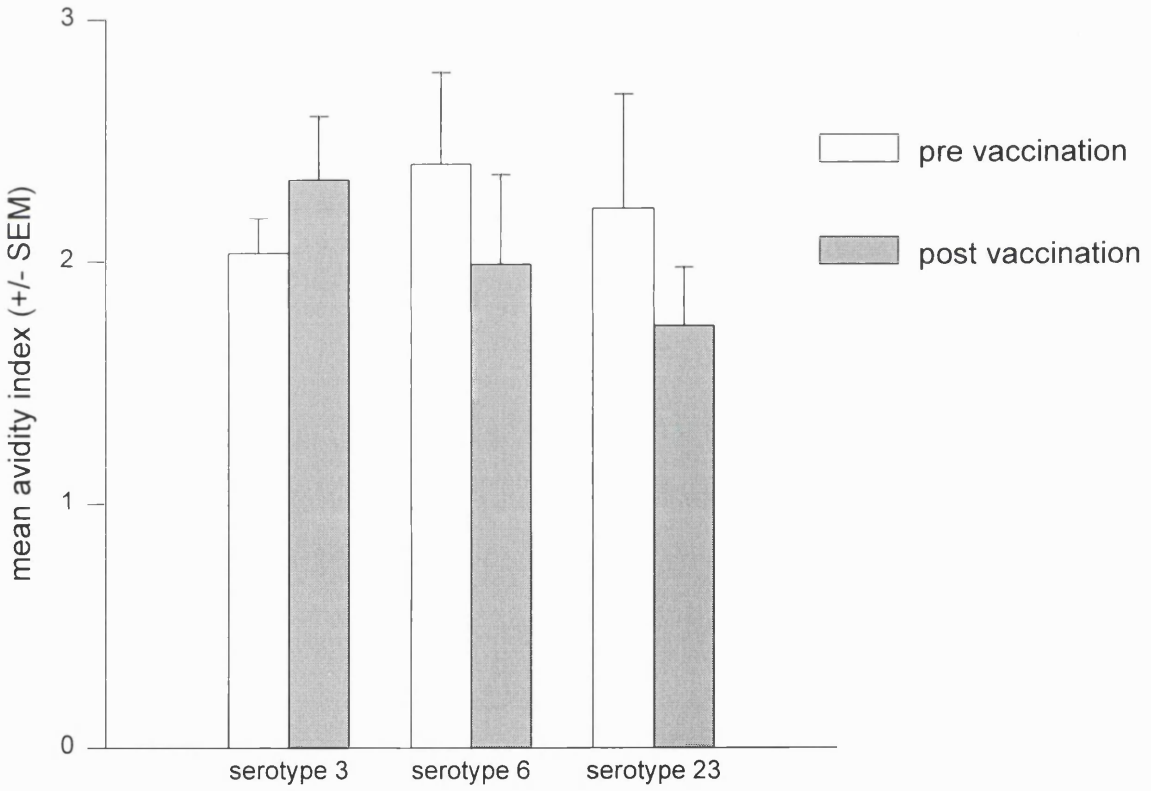


Figure 5.13 Mean avidity indices produced for serum IgG specific for pneumococcal polysaccharides serotypes 3, 6B, 19F and 23F

The avidity of serum IgG total was measured by the thiocyanate elution ELISA employing ammonium thiocyanate from 0.5M to 4M. Avidity indices were then calculated as the amount of ammonium thiocyanate required to produce a 50% reduction in absorbance. Mean avidity indices (+/- the SEM) were calculated. Only two patients responded to serotype 19 and therefore this data was not analysed.

IgG subclasses: titre and avidity

Pneumococcal serotype specific IgG1 and IgG2 subclass responses were also measured in this patient group (Table 5.4) Of the 35 patients vaccinated 10 were aged 2 years or less and 8 of these responded to one or more pneumococcal serotypes.

In the two years and under age group IgG1 was detectable in 6/10 (60%) patients specific for serotype 3 and 4/10 (40%) for serotype 23F. No serotype 6B or 19F specific IgG1 was detectable. IgG2 was detectable for 5/10 (50%) specific for serotype 3, 1/10 (10%) to serotype 6B and 2/10 (20%) to serotype 23F. None of the 2 years and under age group responded with an IgG2 response to serotype 19F.

Table 5.3 Numbers of patients responding to Pneumovax with serotype specific IgG1 and IgG2 antibodies

Serotype	IgG1	IgG2
3	15/35 (46%)	19/35 (54%)
6B	0	5/35 (14%)
19F	0	2/35 (10.5%)
23F	7/35 (20%)	6/35 (17%)

Avidity indices for serum IgG1 and IgG2 antibodies were measured as described previously for IgG total antibody. Mean avidity indices were calculated for pre and post vaccination serum IgG1 and IgG2 specific for pneumococcal serotypes 3 and 23 (Figure 5.14). The small numbers of patients that produced specific IgG1

and IgG2 for serotypes 6B and 19F, precluded analysis. Comparing serotype 3 and 23F specific IgG1 and IgG2, differences in avidity for serotype 3 pre vaccination were observed, with IgG1 being of higher avidity than IgG2. Also, serotype 23F specific IgG1 was of higher avidity that IgG2 antibodies post vaccination.

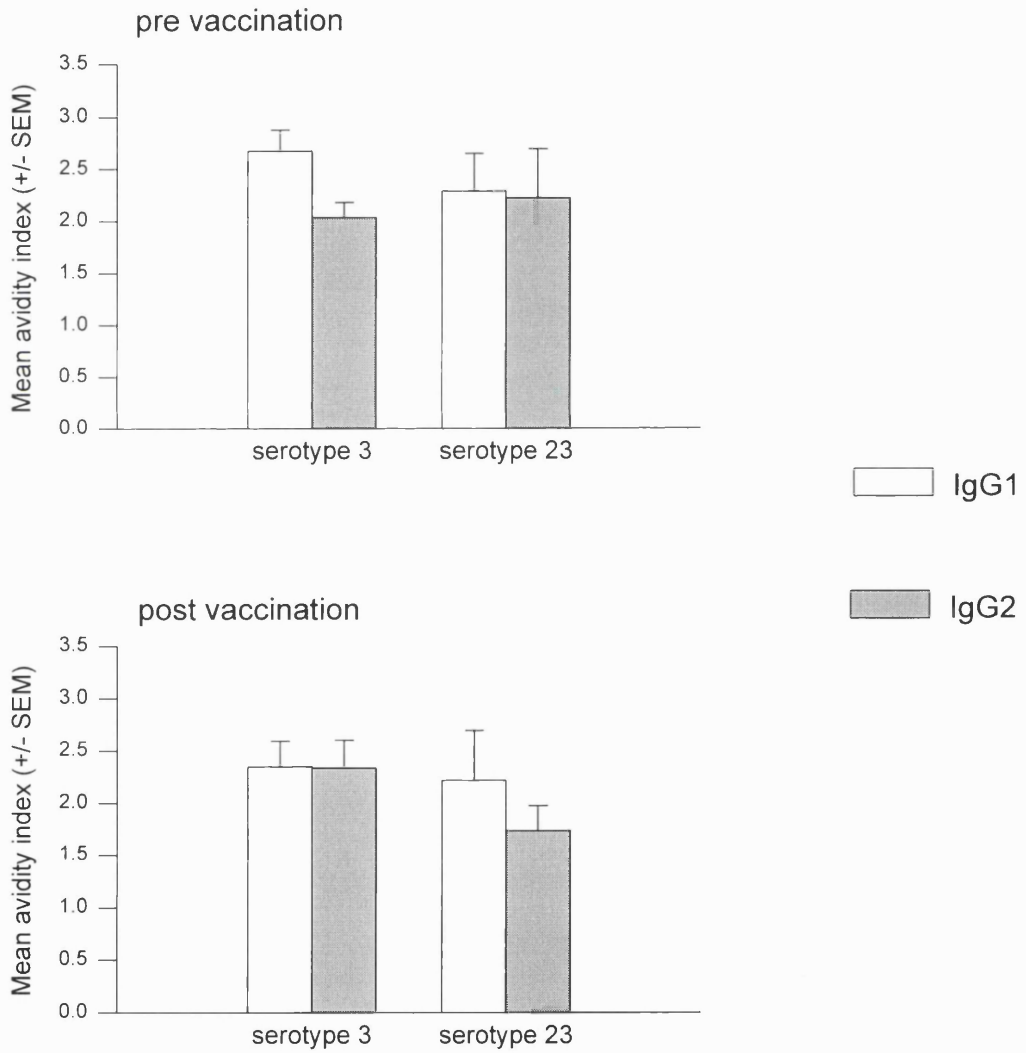


Figure 5.14 Mean avidity indices of serum IgG1 and IgG2 antibodies specific for pneumococcal polysaccharide serotypes 3, 6B, 19F and 23F
Avidity indices were calculated as described previously for Figure 5.13 for pre and post vaccination serum samples.

Serum antibody avidity and age

Age related IgG subclass restriction has been observed for polysaccharide antigens, with infants less than two years of age being unable to mount an IgG2 response to polysaccharides. This inability to mount an adequate anti-polysaccharide response has been linked to the higher incidence of bacterial infections in young children. However, in the patient group investigated here, 50% of patients aged 2 years or less produced an IgG2 response specific for one or more serotype and 60% an IgG1 response. In order to explore the relationship between age and antibody avidity, the patients were grouped into two age groups, less than or greater than two years and mean antibody avidity compared. Since patient numbers were small mean avidity indices could only be calculated for serotypes 3 (IgG total, IgG1 and IgG2) and 23F (IgG total and IgG1) where $n>2$ for each age group (Figures 5.15 and 5.16).

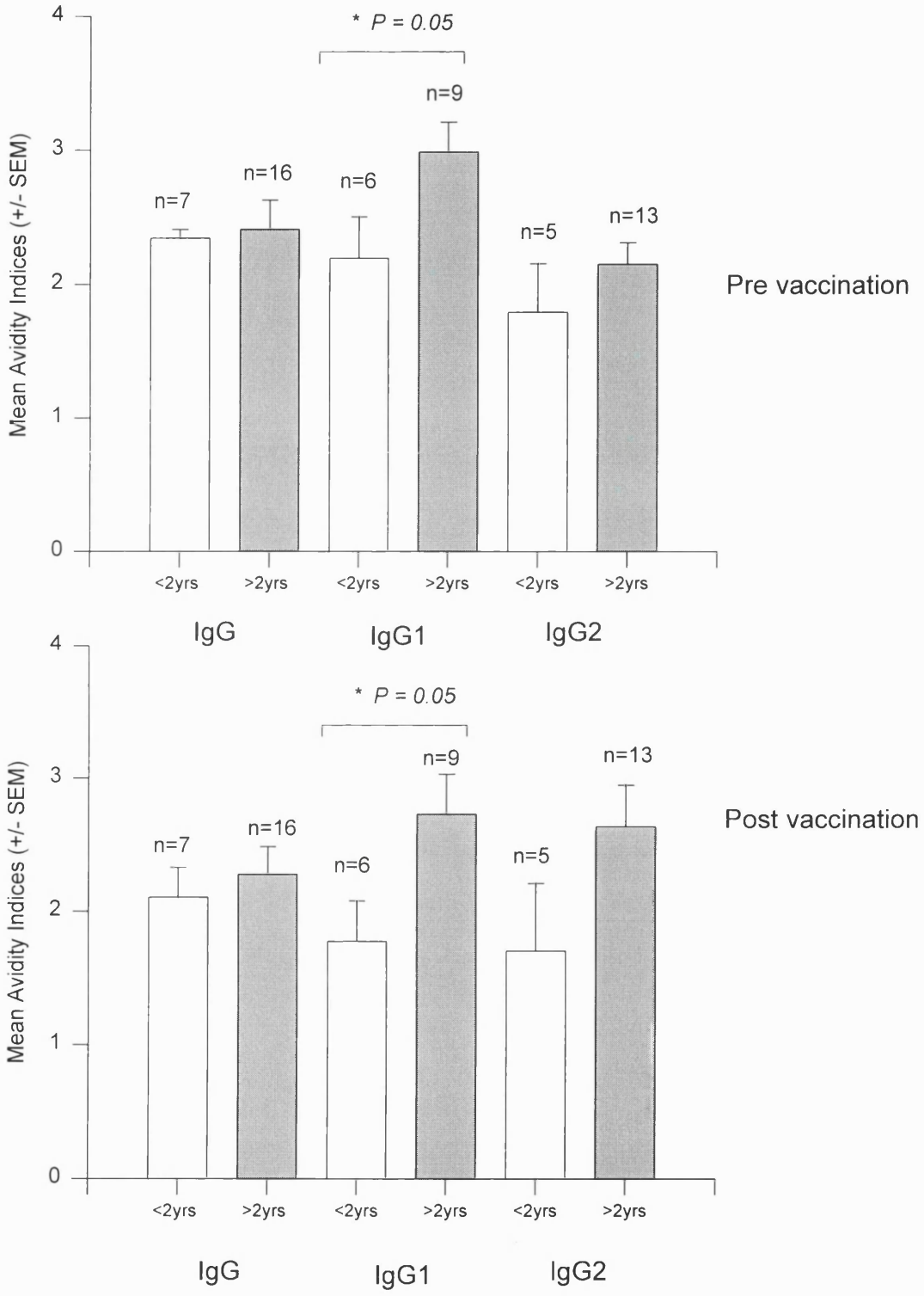


Figure 5.15 Mean avidity indices for serum IgG antibodies specific for pneumococcal polysaccharide serotype 3

Avidity indices were calculated by thiocyanate elution ELISA for pre and post vaccination serum IgG total, IgG1 and IgG2 subclasses, as described previously (Figure 5.13). The patients were then grouped into two age groups, <2years and >2years of age and mean avidity index calculated for each age group. Significance tests were unpaired Student t-tests.

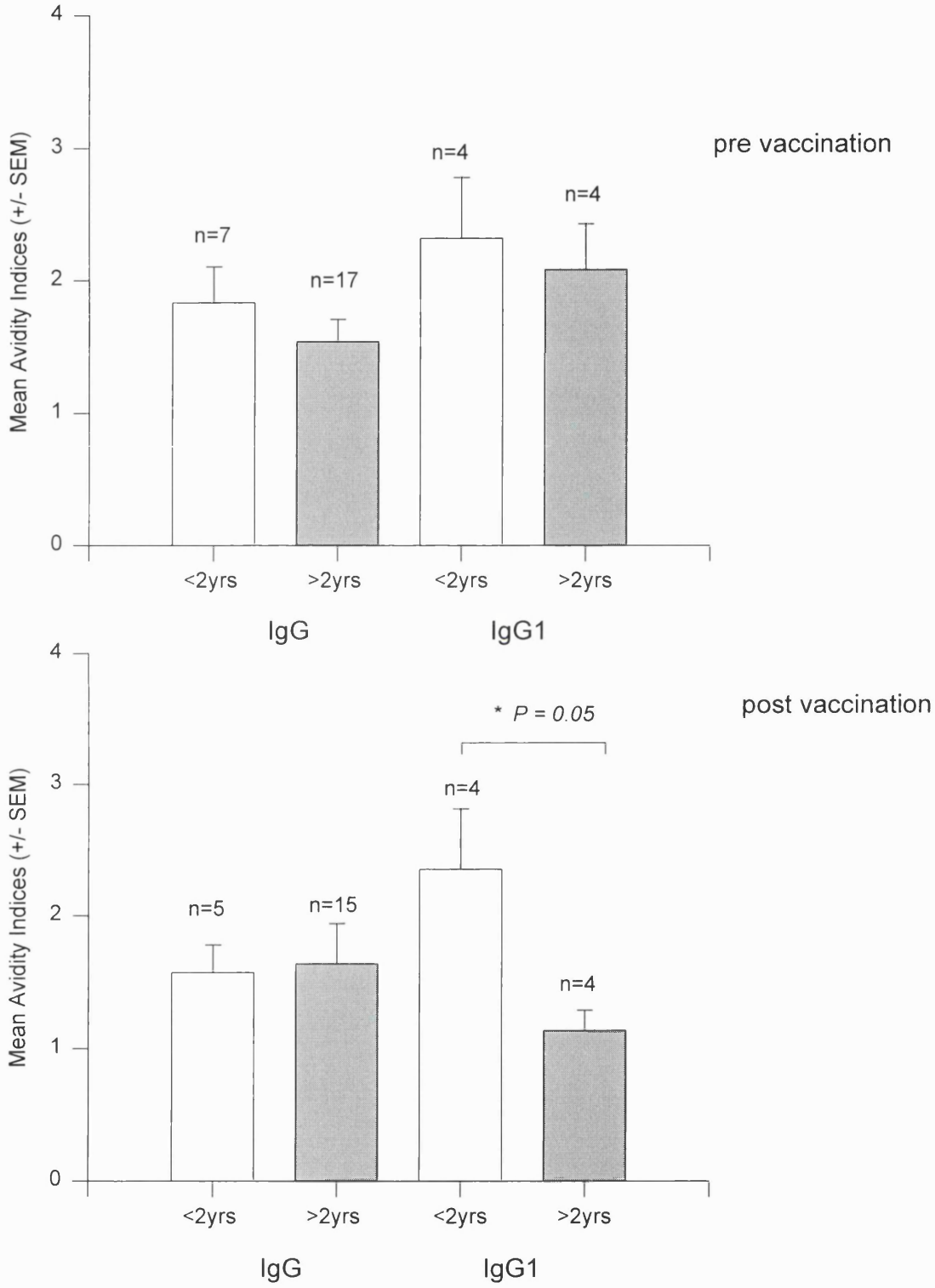


Figure 5.16 Mean avidity indices produced for serum IgG antibodies specific for pneumococcal polysaccharide serotype 23F

Avidity indices were calculated by thiocyanate elution ELISA for pre and post vaccination serum IgG total and IgG1 subclasses, as described previously (Figure 5.13). The patients were then grouped into two age groups, <2years and >2years of age and mean avidity index calculated for each age group. The small number of patients which produced serotype 23F specific IgG2 precluded inclusion for analysis. Significance tests were unpaired Student t-tests.

It can be seen from Figure 5.15 that avidity indices appear to be lower for infants less than two years of age in both pre and post vaccination categories for total IgG, IgG1 and IgG2 antibodies directed against serotype 3. However, this difference was not statistically significant for total IgG and IgG2 but did reach statistical significance for the IgG1 response ($p=0.05$, unpaired Student's t-test).

In contrast, IgG1 antibodies specific for serotype 23F produced in children less than 2 years old were of higher avidity than children > 2 years old. Only two children less than two years old produced IgG2 antibodies specific for serotype 23F and this data could not be analysed.

DISCUSSION

The development of solid-phase avidity ELISAs has made it possible to evaluate the avidity of polyclonal serum antigen specific antibodies. Whilst it is not possible to derive a K value these assays permit the derivation of avidity indices which allow the avidity of antibodies specific for the same antigen to be ranked. In this present investigation two avidity ELISAs were developed and evaluated for the measurement of the avidity of serum antibodies specific for pneumococcal polysaccharide serotypes 3, 6B, 19F and 23F.

Competitive binding ELISAs

Competitive binding ELISAs are the most widely used solid-phase avidity ELISAs. Employing the methods of Friguet et al. (1985) and Rath et al. (1988) the relative antibody avidity of serum IgM, IgG or IgG2 antibodies specific for serotype 3 could not be measured. Previously, Persson et al. (1988) were able to measure the avidity of human serum pneumococcal serotype 3 IgG1 and IgG2 antibodies by a competitive binding ELISA using free antigen from 0.195μg/ml to 50μg/ml. In a subsequent study Van Dam et al. (1989) were able to inhibit the binding of murine serum antibodies to solid-phase pneumococcal serotype 3 by free polysaccharide or oligosaccharide. The inability of free polysaccharide to inhibit antibody binding of human pooled serum antibodies, suggests that serotype 3 specific antibodies are of low avidity. Serotype 3 specific antibody avidity indices similar to the other serotypes were detected employing the thiocyanate elution ELISA. It is possible that solid-phase antigen displays different epitopes to those displayed in solution and therefore antibody binding to

the solid-phase cannot be inhibited by liquid phase antigen. The exact reasons for the observed inability of free polysaccharide to inhibit specific serotype 3 antibody binding however remain unclear and require further investigation. It was possible to measure relative avidity of serotype 6B, 19F and 23F serum antibodies by competitive ELISA. However, further investigation revealed high interassay variability, which precluded the use of this assay. The reasons for such high variability are unclear but may in part be due the impurity of the polysaccharide preparations, and day to day variation in the solubility of the polysaccharides.

Elution ELISAs

Relative serum antibody avidity was also investigated by employing elution ELISA which uses the chaotropic ion, thiocyanate, to disrupt antigen-antibody binding. Thiocyanate ions, are known to disrupt the weak bonds stabilising the antigen-antibody complex, without affecting specificity (Dandliker et al. 1967). The extent to which thiocyanate ions disrupt antigen-antibody interactions will therefore be directly related to the strength of antibody avidity. Interassay variability of the thiocyanate elution was acceptably low and the avidity of serum IgG and IgG subclass antibodies specific for all four pneumococcal polysaccharide serotypes could be analysed.

This investigation highlights the importance in evaluating various solid-phase ELISAs for each antigen-antibody system to be investigated. The nature of the antigen may determine which assay is the best for each particular system. In the current investigation the thiocyanate elution ELISA was found to be the

most reproducible and therefore was chosen to explore the avidity of serum antibodies specific for pneumococcal polysaccharides further. It must however be noted that for the measurement of serotype 3 specific antibody avidity, the polysaccharide was conjugated to poly-L-lysine. It is possible that conjugation to poly-L-lysine, although a widely accepted method for adsorbing polysaccharides to polystyrene, may alter the polysaccharide confirmation such that different epitopes are displayed relative to the native molecule. Since embarking upon this study, antibody titre and avidity has been demonstrated to be affected by the adsorption method employed. Feldman et al. (1994) found that streptococcal type III conjugated to poly-L-lysine coats with a ten fold lower efficiency than a tyramine conjugate and therefore the poly-L-lysine based method was biased towards detection of only higher avidity antibodies. This may explain why the competitive ELISA appeared to suggest that serotype 3 specific antibodies were of low avidity, while the elution ELISA, using the poly-L-lysine conjugation method, pointed towards high avidity serotype 3 antibody compared to serotypes 6B, 19 and 23F.

The avidity of pneumococcal capsular polysaccharide (pneumococcal capsular polysaccharide) specific serum antibodies

In this study the avidity of serum antibodies specific for pneumococcal serotypes 3, 6B, 19F and 23F were assessed employing the thiocyanate elution ELISA in 26 out of 35 patients vaccinated with the 23 valent unconjugated pneumococcal vaccine. Of the four serotypes investigated, serotypes 23F and 3 were the most immunogenic, followed by serotype 6B, with serotype 19F showing the lowest number of responders. Pneumococcal serotypes 6B, 14, 19F and 23F are known to be relatively poor immunogens (Barrett et al. 1984; Scott et al. 1989) whereas serotype 3 is highly immunogenic in children. This is reflected in the less immunogenic serotypes accounting for the majority of paediatric pneumococcal diseases (Henrichsen, 1979). The results presented here also demonstrated high immunogenicity for serotype 3 and low immunogenicity for serotypes 6B and 19F. In contrast, to previous reports however, serotype 23F showed high immunogenicity in the group of patients investigated here. Nevertheless it should be noted that the patient group under investigation was highly selected and it is possible that children with sickle cell disease may respond differently than healthy children to Pneumovax. A recent study from our laboratory has demonstrated that this group of patients responded normally to Hib conjugate vaccine immunisation (Goldblatt et al. 1996).

Pneumococcal polysaccharide antigens are classified as T cell independent type 2 antigens, which are antigens that stimulate antibody production in the absence of MHC class II restricted T cell help (reviewed in detail by Mond et al. 1995). T cell independent antigens are not found in germinal

centres where affinity maturation occurs and therefore are not expected to manifest affinity maturation (Maclennan, 1994). As expected for a polysaccharide antigen, despite the increase in antibody response 1 month post vaccination in the patients studied, no statistically significant difference in avidity was observed between pre and post vaccination serum IgG total, IgG1 or IgG2 antibodies.

Investigating the avidity of serotype 3 and 23F specific IgG1 and IgG2 antibodies it was found that pre vaccination serotype 3 specific IgG1 was of higher avidity than IgG2 antibodies. In contrast, post vaccination serotype 3 specific IgG1 and IgG2 antibodies did not display any avidity differences. The reasons underlying the observed avidity differences pre vaccination are unclear. Persson et al, (1988), using the competitive binding ELISA, found serotype 3 specific IgG1 to be of lower avidity than IgG2 in adult sera and in agreement with the results presented here they found IgG2 to be of lower avidity than IgG1 in infants less than 2 years of age. Naturally acquired antibodies are raised against the pneumococcal polysaccharides which are present in the respiratory tract, whereas following vaccination the antibodies elicited are specific for pneumococcal antigens delivered intramuscularly. It is conceivable that the route of antigenic delivery may affect antibody avidity. Nevertheless, it should be noted that avidity differences between serotype 23F specific IgG1 and IgG2 were not found for this group of patients. As mentioned previously, poly-L-lysine conjugation has also been demonstrated to introduce a bias towards the detection of higher avidity antibodies by thiocyanate elution and this possibility deserves further investigation. Furthermore, the patient numbers in this study were relatively small, and larger patient groups are required to resolve this issue.

The relationship between age and antibody avidity

Since the anti-pneumococcal capsular polysaccharide antibody response has been shown to be delayed in ontogeny (Hazelwood et al. 1993), the relationship between age and antibody avidity was explored. Previous investigators have shown that infants less than 2 years old are more likely to produce an IgG1 response to pneumococcal capsular polysaccharide antigens (Hammarstrom et al. 1988; Schatz and Barrett, 1987) but the protective capacity of this response is unclear. Although 80% of infants aged 2 years or less responded well to Pneumovax, a complex pattern of avidity emerged, with the IgG1 antibody response to serotype 3 being of lower avidity, whereas IgG1 specific for serotype 23F was of higher avidity. This finding may account for the low protective capacity of Pneumovax in this age group. Due to the small numbers of patients available for study it is unclear whether this finding is specific for children with sickle cell disease or is a general phenomena in children less than two years of age. It is clear however that polysaccharide specific antibody avidity does differ with respect to age. The adaptation of the solid-phase avidity ELISA for the measurement of pneumococcal polysaccharide specific antibody avidity will permit the investigation of antibody responses to the newly available pneumococcal polysaccharide conjugate vaccines in greater detail.

Summary

This investigation highlights the importance of evaluating various solid-phase assays when measuring the avidity of serum antibodies. The nature of the antigen under investigation may determine which assay is best for each particular application. The results obtained here for pneumococcal capsular polysaccharide antigens suggested that the thiocyanate elution ELISA was the most reproducible and therefore this was chosen to explore the avidity of serum antibodies specific for pneumococcal capsular polysaccharide antigens.

The finding that infants less than 2 years of age in addition to their reported low titre response to Pneumovax, demonstrate avidity differences for pneumococcal polysaccharide antigens, suggests that it may be important to determine avidity in association with antibody titre when assessing the immunogenicity of a vaccine. Studies are at present ongoing within the laboratory with larger patient groups to ascertain whether this is a general feature of pneumococcal capsular polysaccharide specific antibodies, or whether it is specific for sickle cell patients. In addition, the protective capacity of such low avidity responses needs clarification, as do the mechanisms responsible for the delay in ontogeny of anti-pneumococcal capsular polysaccharide antibody quality.

CHAPTER 6

The effect of various cytokines on specific in vitro IgG avidity

INTRODUCTION

Following recognition of an antigen a B cell is primed to develop into a plasma cell producing specific antibody. During the course of the primary immune response, B cells can switch the immunoglobulin isotype that they produce and also somatically hypermutate to form high avidity antibody. The mechanisms underlying the process of somatic hypermutation remain unresolved but it is known that hypermutation is specific for the immunoglobulin V regions and that accessory molecules are required to rescue from apoptosis those B cells that have somatically mutated their V regions.

The use of adjuvants to enhance the immune response

Vaccination has been very successful in combating many infectious diseases, but the immunogenicity of some vaccine formulations are poor in certain population groups. Attention has therefore focused upon improving vaccine immunogenicity through the use of conjugates and adjuvants. The mechanisms by which adjuvants enhance vaccine immunogenicity remains controversial, although a depot effect, local recruitment of immune cells, complement activation and APC targeting have all been implicated. Cytokines are also known to profoundly influence the immune response. In mice IL-2 and IFNγ secreted by $T_H1$cells promote cellular immunity and IL-4, IL-5, IL-6, IL-9 and IL-10 secreted by T_H2 cells promotes humoral immunity. Recent studies have therefore focused upon the use of cytokines in enhancing the immune response elicited following vaccination.

The influence of cytokines as adjuvants upon the immune response

Aluminium hydroxide is the only adjuvant at present licensed for use in humans. Due to the problems of toxicity associated with many adjuvants, such as local granuloma formation, the use of cytokines to enhance vaccine immunogenicity has become increasingly attractive. IL-12 has been investigated in various murine models as a vaccine adjuvant. Schijns et al. (1995) demonstrated that IL-12 injected at the time of vaccination increased immunity against pseudorabies virus infection of mice. Furthermore, this was shown to coincide with an increase in the titre of virus specific IgG2a. Similarly, Miller et al. (1995), studying a murine model of listeria infection, showed that heat killed *Listeria monocytogenes* became immunogenic when injected with IL-12. Moreover, Germann et al. (1995) found that IL-12 was a potent adjuvant for enhancing humoral immunity to keyhole limpet hemocyanin (KLH) by inducing IgG2a, IgG2b and IgG3 production. It is, however, unclear how IL-12 exerts its immune enhancing effects. Schijns et al. (1995) have suggested that IL-12 adjuvant activity is largely mediated by IFNγ, due the absence of IL-12 mediated virus specific IgG2a production in IFNγ receptor deficient mice or following anti-IFNγ treatment.

The effect of cytokines upon antibody affinity

Although, the isotype of an antibody is clearly an important parameter in determining protective capacity, protection against invasive disease does not always correlate with antibody titre. As highlighted throughout this thesis the avidity of an antibody is an important parameter of biological activity. It is possible, therefore, that in addition to antibody isotype and T cell responses,

cytokines may potentiate antibody avidity. Holland et al. (1990) demonstrated that IFNγ, tuftsin and IL-1α enhanced antibody titre to bacillus Calmette-Guérin (BCG) in mice, but only IFNγ enhanced antibody affinity. Similarly, Rizzo et al. (1992) employing an adoptive transfer system in mice found that cytokines were able to influence antibody avidity. In their system keyhole limpet hemocyanin (KLH) specific CD4+ TH2 clones (IL-4 and IL-5 producing) or TH1 (IL-2 and IFNγ producing) induced B cell memory and affinity maturation.

All the studies described so far have been in animal models and it is not known whether human cytokines exhibit such modulatory effects upon the immune system occur in humans. Murine studies are not directly analogous to the situation in humans, particularly with regard to cytokines and IgG subclasses (Callard and Turner, 1990). It appears that in mice cytokines such as IFNγ enhance antibody affinity. The role of other cytokines such as IL-12 in potentiating antibody affinity warrants further investigation, especially if such cytokines are to be utilised as vaccine adjuvants. The exact mechanisms underlying the role of cytokines in the modulation of antibody affinity remain to be resolved. However, recent studies of human germinal centre B cells point towards a role for CD40/CD40L interactions (reviewed by Liu et al. 1992), CD20 engagement (Holder et al. 1995), CD23 and IL-1α (Liu et al. 1992) in the rescue of B cells from apoptosis, believed to be important in selecting B cells expressing high affinity antibody.

Somatic Hypermutation

Analysis of V region gene mutations in antibodies specific for phenyloxazolone secreted by hybridomas showed that somatic hypermutation was targeted to the V region at a high rate (Griffiths et al. 1984). The pattern of somatic hypermutation is non-random and replacement mutations are overepresented in the CDRs, the region responsible for direct binding to antigen (reviewed by Rajewsky, 1996). Although the mechanism responsible for somatic mutation is unknown it is believed to involve some type of error prone DNA repair and recent studies suggest the involvement of cis-acting elements (Betz et al. 1994; Yelamos et al. 1995). DNA transcription initiation may also play a role (Peters and Storb, 1996). The availability of methods for purifying germinal centre B cells into subpopulations at various stages of affinity maturation (Pascual et al. 1994) and progress in understanding DNA repair mechanisms, will permit the elucidation of the molecular basis of somatic hypermutation in the very near future.

Affinity maturation and memory cell generation occurs in secondary lymphoid tissues called germinal centres. Somatic hypermutation is believed to occur at the centroblast stage in the dark zone, whereas immunoglobulin isotype switching occurs before hypermutation (Liu et al. 1996). Since immunoglobulin isotype switching is under the control of various cytokines (discussed in detail in Chapter 1), it is possible that the process of affinity maturation may also be influenced by soluble mediators such as cytokines and accessory molecules.

Cytokines

Cytokines are pleiotropic protein molecules which act in a paracrine or autocrine manner mediating short range signals between cells via specific cell surface receptors. They are of central importance in the regulation of the immune response, in particular lymphocyte activation and immunoglobulin isotype switching. A comprehensive review of all cytokines is outside the scope of this introduction. The cytokines used in the studies described here were interleukin-6, interleukin-10, tumour necrosis factor-α and interferon-γ and the major features of these cytokines are described below.

Interleukin-6 (IL-6)

The IL-6 gene has been cloned from humans, rats and mice and the protein shows 42% homology between humans and mice. The human IL-6 receptor has also been cloned and is expressed on monocytes, hepatocytes and lymphocytes. Several biological properties have been ascribed to IL-6. It stimulates B cells to differentiate and produce immunoglobulin (Hirano et al. 1986) and causes upregulation of acute phase proteins (Mackiewicz et al. 1991). IL-6 has been suggested to play a protective role in inflammation by stimulating host defence mechanisms (acute phase proteins) and clearance of pathogens (immunoglobulin production) (reviewed by Remick, 1994).

Interleukin-10 (IL-10)

IL-10 and its corresponding receptor have both been cloned in humans and mice. IL-10 is produced by T_H2 cells, γδ T cells, B cell lymphomas, macrophages, mast cells and keratinocytes (reviewed by Fidel, 1994). IL-10 suppresses proliferation of T_H1 cells, thus inhibiting the production of TH1 cytokines (Fiorentino et al. 1989 and 1991). IL-10 has also been shown to inhibit costimulatory molecules on antigen presenting cells (Fiorentino et al. 1991; Ding and Shevach, 1992), enhance the growth of mast cells (Thompson-Snipes et al. 1991) and upregulate MHC class II expression on B cells (Go et al. 1990).

Tumour necrosis factor-α (TNFα)

The TNF gene has been cloned in humans and mice, as well as in many other species. TNF exists in two forms TNFα and TNFβ (lymphotoxin) which share approximately 30% amino acid homology. TNFα was used in this study. TNFα is a 17kDa non-glycosylated protein of 157 amino acids in humans which is secreted as a propeptide with an extra 76 amino acids. TNFα exists as a trimer of identical subunits and is found in soluble or membrane bound form on macrophages. The gene for the TNFα receptor has been cloned and exists in two forms, 75kDa and 55kDa (Loetscher et al. 1990; Lewis et al. 1991). The receptors may be shed from the cell surface and soluble TNF receptor is able to bind to and inactivate TNFα. TNFα has many biological activities, some of which are shared with IL-1. TNFα induces the synthesis of a variety of cytokines including IL-6, IL-8, IL-1, IFNγ and GM-CSF, which is believed to be crucial for the role of TNFα as a mediator of inflammation (reviewed by Tracey, 1994). A

role for TNFα has been implicated in the pathogenesis of many diseases including cachexia, septic shock syndrome, cerebral malaria, meningitis and rheumatoid arthritis (reviewed by Tracey, 1994). In the case of B cells TNFα exhibits proliferation enhancing effects, although it is thought to play a rather ancillary role for B cell responses. Since, TNFα is secreted by B cells it may act to enhance B cell responses by inducing other cytokines such as IL-6.

Interferon-γ (IFNγ)

IFNγ was first described as a substance having antiviral activity. The gene for IFNγ has been cloned in humans and mice and the primary cells that secrete it are T_H1 cells, CD8 T cells (Vilcek et al. 1985; Schreiber and Celada, 1985; Mosmann and Coffman, 1989), γδ T cells (Bluestone et al. 1991; Morita et al. 1991) and NK cells (Handa et al. 1983; Bancroft et al. 1987). IFNγ's main role is within delayed type hypersensitivity responses. It upregulates MHC class I and II expression on antigen presenting cells thus increasing their ability to present antigen at sites of injury (reviewed by Fidel, 1994). IFNγ acts on B cells by inhibiting class II expression (Mond et al. 1986) and inhibits B cell responses to IL-4 as reviewed by Defrance and Banchereau (1990). Conversely, IFNγ promotes antibody formation by augmenting MHC class II expression on accessory cells which stimulates the interaction of these cells with T cells.

Aims of the Study

Previous studies in the mouse have investigated the potential of cytokines to act as adjuvants in modulating the immune response and have demonstrated that IL-12 and IFNγ potentiate the immune response to a variety of antigens. Furthermore, IFNγ enhances antibody avidity. Accessory molecules are also known to be involved in the rescue of human germinal centre B cells from apoptosis. It is possible, therefore, that cytokines may influence human antibody avidity and this study was thus initiated in an attempt to study the effect of cytokines on human antibody affinity.

Initially solid-phase avidity ELISAs were explored for the measurement of influenza specific IgG in standard serum. Following the establishment of influenza specific avidity ELISAs, these were explored for the investigation of antigen specific IgG produced from tonsillar mononuclear cells (TMC) and E^- B cells. Utilising human TMCs and E^- B cells stimulated with the influenza virus, the possible effect of the cytokines IL-6, IL-10, IFNγ and TNFα upon antigen specific IgG production and avidity was investigated by solid-phase ELISA. This system was chosen to evaluate the role of cytokines in the modulation of antibody avidity as it is a well established system within the laboratory and has been extensively investigated previously (Callard, 1979). These cytokines were chosen because of their availability within the laboratory and also because they have previously been shown to have differing activities on B cells. IL-6 is known to enhance immunoglobulin production, IL-10 upregulates B cell MHC class II expression, TNFα enhances IL-6 production and IFNγ has been demonstrated to enhance antibody avidity in mice. (*The studies described in this chapter are of a preliminary nature and could not be developed further due to time constraints)*

METHODOLOGY

Isolation of Human Tonsillar B cells

Excised human tonsils were obtained fresh from The Royal National Ear, Nose and Throat NHS Trust Hospital, London. Tonsils were washed briefly with 70% IMS to kill surface micro-organisms and the IMS subsequently removed by brief washing with holding medium. Tonsillar cells were obtained by teasing into holding medium. Tonsil mononuclear cells (TMCs) were then separated by gentle layering of 15ml of tonsillar cells onto 9ml of Ficoll gradient followed by centrifugation at 1000g for 20 minutes. TMCs were removed from the interface layer and washed twice in holding medium. Cell viability was then analysed using trypan blue exclusion. T cells were removed by E rosetting with AET treated SRBCs. AET/SRBC (2.5ml of 10% suspension) were added to 5 x 10^7 TMCs in 10ml holding medium supplemented with 10% FCS. The cells were then pelleted at 250g for 20 minutes at 4°C and incubated for 1h on ice. The E rosettes were resuspended by gently rocking to avoid disturbing the rosettes and 15ml of this suspension layered onto 9ml of Percoll (specific gravity 1.080) and centrifuged at 1000g for 20 minutes. The interface layer containing the E^- B cells was removed and cell viability analysed using trypan blue exclusion. E^- B cells were stained for B cell, T cell and monocyte content using the FACScan (Becton Dickinson). E^- B cells were routinely <0.5% $CD3^+$ (T cell), <0.5% $CD14^+$ (monocyte/macrophage) and > 97% $CD19^+/CD20^+$ (B cell). E^+ (T cells) were recovered by carefully removing the remaining Percoll, resuspending the pellet in Gey's haemolytic balanced solution and centrifuging at 250g for 5min. The supernatant containing

lysed red cells was poured off and the pellet containing the E^+ (T cells) resuspended in holding medium.

Antibody production by human TMC and B cells stimulated with influenza virus

TMCs at a concentration of 4 x 10^6/ml or E^- B cells at 0.5-1 x 10^6/ml were cultured in 1ml of RPMI 1640 medium supplemented with 10% horse serum (HS) (Sigma), 50μg/ml gentamycin (Gibco) and 2mM L-glutamine (Gibco), in capped 12 x 75mm tubes (Falcon Beckton-Dickinson, New Jersey) with or without influenza virus, at 0.2μg/ml for TMCs and 2μg/ml for E^- B cells. E^- B cells were also incubated with 1 x 10^6/ml E^+ T cells or IL-2 at 5U/ml to provide T cell help (Callard, et al, 1986). Various cytokines were added to the experimental cultures. All the cultures were then incubated at 37°C, 5% CO_2, humidified atmosphere for 6 days. All cultures were performed at least in triplicate. Cultures were then washed with holding medium to remove HS and resuspended in 0.5ml RPMI 1640 supplemented with 10% FCS (Sigma), 50μg/ml gentamycin (Roussel) and 2mM L-glutamine (Sigma) and incubated overnight at 37°C, 5% CO_2, humidified atmosphere. Cells were then pelleted by centrifugation at 100g for 5 minutes and culture supernatant removed and frozen at -20°C.

Antigen Specific ELISA

Influenza specific ELISA procedures were performed as described in detail in Chapter 2. Briefly, influenza virus at 10μg/ml diluted in PBS was adsorbed to Immulon 4 (Dynatech) plates and unsaturated sites blocked with PBS-Tw-BSA. Standard serum was then serially diluted in PBS-Tw-BSA across the plate and samples added to the presensitised plate diluted 1/2 with PBS-Tw-BSA. Binding was allowed to occur for 16h at RT and unbound antibody removed by extensive washing with PBS-Tw. The plate was incubated for 4h at RT with the relevant detector antibodies diluted 1/1000 in PBS-Tw-BSA, followed by extensive washing with PBS-Tw. Streptavidin-alkaline phosphatase diluted 1/1000, or streptavidin-peroxidase diluted 1/5000 in PBS-Tw-BSA, was then added and the plate incubated for 2h at RT. A chromogenic substrate (pNPP or OPD) was then applied and the colour reaction allowed to develop for approximately 30 minutes. Absorbance at 410nm or 490nm was then read using an automatic plate reader.

Recombinant Human Cytokines

Cytokine	Source	Specific Activity
IL-2	Amersham International	2×10^6 U/mg
IL-6	Immunex	not known
IL-10	Genzyme	not known
TNFα	Kind gift from N.Klein (ICH)	4×10^7 U/mg
IFNγ	Genzyme	2×10^7 U/mg

IL-2 was used at a concentration of 5U/ml, IL-6 at 20U/ml, IL-10 at 100ng/ml, TNFα at 5ng/ml and IFNγ at 100U/ml, which are the concentrations routinely used in the laboratory.

Influenza Virus

Influenza virus was a kind gift from Dr. J. Wood (NIBSC, South Mimms, UK) as a sucrose gradient purified preparation. The strains used were X97 (H3N2), NIB24 (H5N2) and X19 (H1N1). The original suspension was stored at a concentration of 20mg/ml at 4°C with 0.01% azide. A working stock dilution was then prepared containing 100μg/ml of virus in holding medium supplemented with 50μg/ml gentamycin without FCS.

RESULTS

The IgG subclass antibody profile of standard serum to influenza virus

The natural influenza specific IgG subclass antibodies in the standard serum were evaluated by antigen specific ELISA (Figure 6.1). Using three different viral strains, the major IgG subclass detected was IgG1, as expected for a protein antigen.

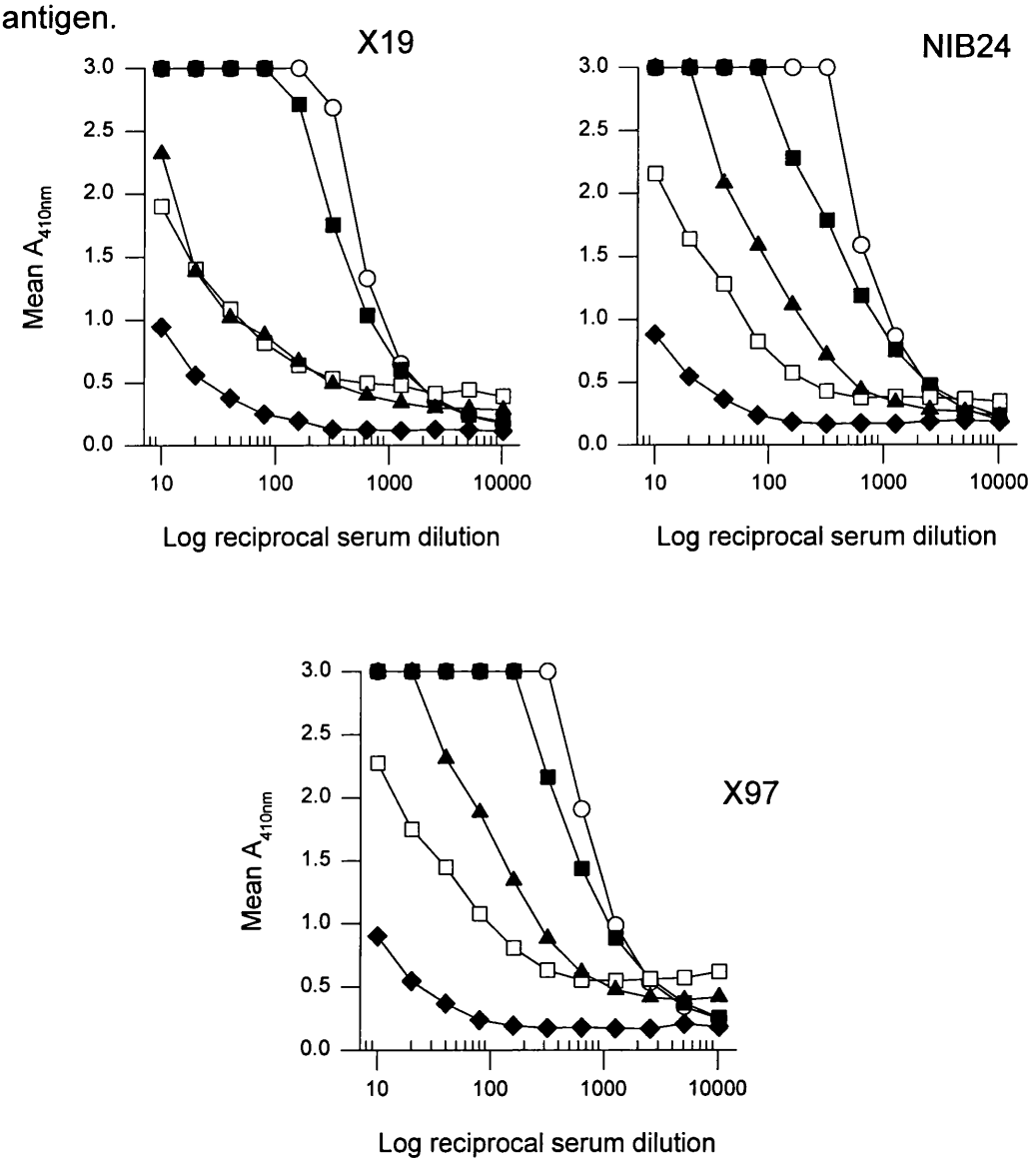


Figure 6.1 Influenza specific IgG subclass response from standard serum
The IgG total (O), IgG1 (■), IgG2 (□), IgG3 (◆) and IgG4 (▲) response to individual influenza virus strains, X19; NIB24 and X97, from the standard serum was evaluated by antigen specific ELISA.

The detection of influenza specific total IgG antibodies in tonsillar cell cultures

Standard curves were constructed employing a standard individual serum, serially diluted in PBS-Tw-BSA from 1/50 to 1/51200, using Mikrotek software. Results were expressed as units/ml of antibody, with a serum dilution of 1/50 corresponding to 1000units/ml. A representative curve is shown in Figure 6.2.

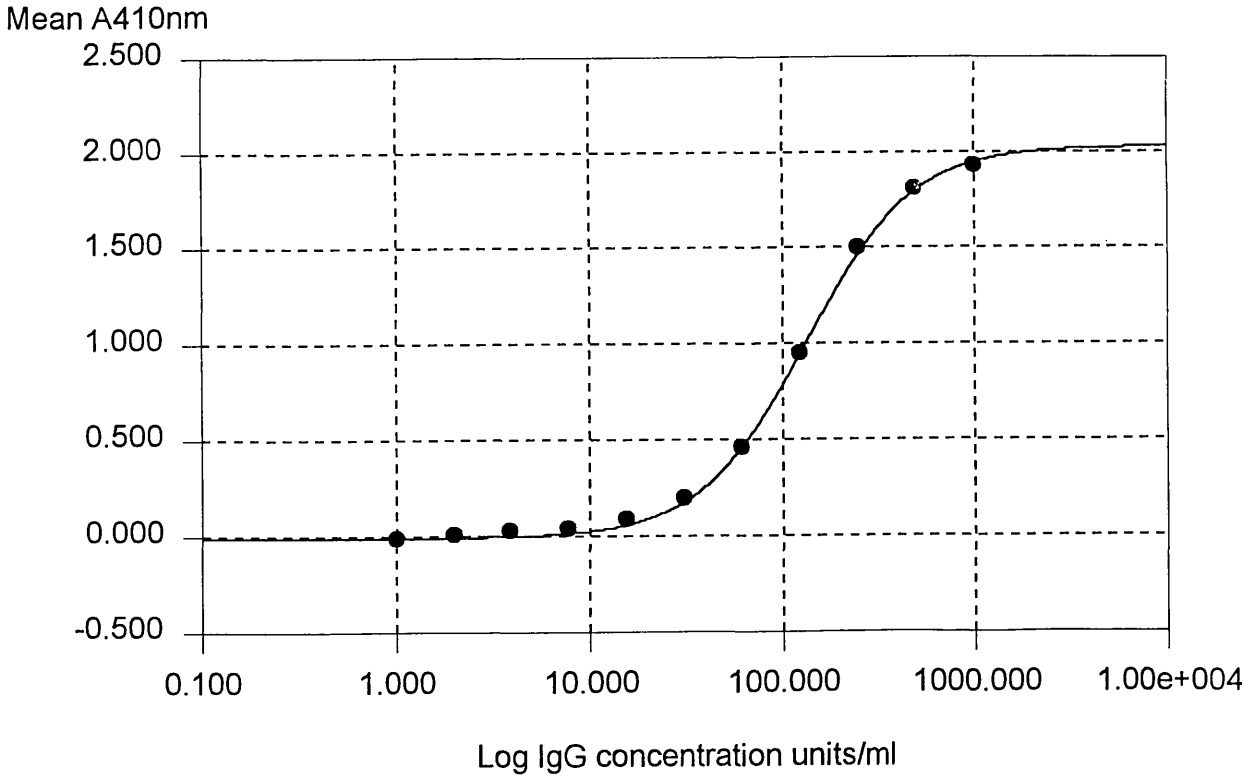


Figure 6.2 IgG total standard curve
Influenza specific IgG present in experimental samples were evaluated by extrapolating from the standard curve obtained employing an individual serum. A specific standard serum IgG level was unavailable, and therefore a dilution of 1/50 was given an arbitrary value of 1000units/ml. The algorithm "four-parameter" was applied for calculating concentrations from the standard curves, using the Mikrotek software package (Dynatech).

The effect of cytokines on influenza specific IgG production

E^- B cells were stimulated with influenza virus, as described earlier, to produce influenza specific IgG. Cytokines, TNFα (5ng/ml), IFNγ (100units/ml), IL-6 (20units/ml) and IL-10 (100ng/ml), were added on day 0 and specific IgG production measured on day 7 using the influenza specific ELISA. Figure 6.3 shows IgG production for three experiments performed on cells separated from three different tonsils.

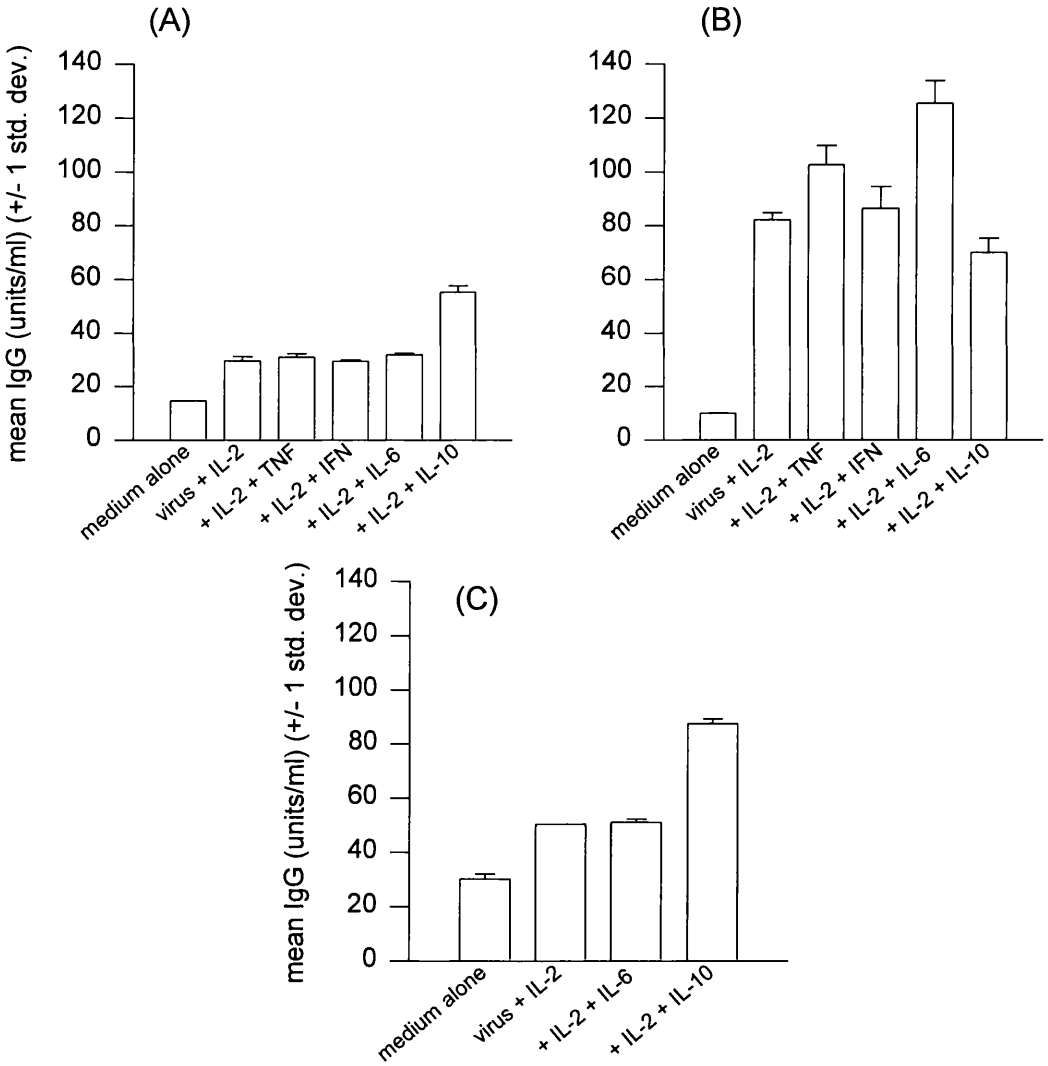


Figure 6.3 The effect of cytokines, TNFα, IFNγ, IL-6 and IL-10 upon influenza specific IgG production by E^- B cells

E^- B cells were stimulated with influenza virus and IL-2 with or without cytokines. Influenza specific IgG was measured following culture for 7 days, as described previously in Chapter 2, by antigen specific ELISA.

Stimulation with influenza virus (strain X19) and IL-2, employed as a T cell replacing factor, was found to increase production of influenza specific IgG from E^- B cells(Figure 6.3). Results presented in Figure 6.3 are for three different tonsil preparations. It was found that the cytokines TNFα, IFNγ, IL-6 and IL-10 differed in their ability to affect influenza stimulated IgG production of different tonsil preparations. TNFα, IFNγ and IL-6 did not alter IgG production from tonsil A, whereas IL-10 increased antibody production. Results obtained from tonsil C were similar to tonsil A with IL-6 not altering IgG production and IL-10 increasing IgG production. Unfortunately, an insufficient number of cells were recovered from tonsil C and therefore could not be investigated for TNFα and IFNγ effects. Similarly, IFNγ did not alter IgG production from tonsil B. In contrast, TNFα and IL-6 appeared to increase IgG production, whereas IL-10 did not. The exact reasons for the observed variability in the observed response between E^- B cell preparations are unclear but may be due to the preparations containing differing numbers of resting and activated B cells. Inter-tonsil variability is to be expected, due to differences in responsiveness to the influenza virus. Quantitative differences in B cell subpopulations are also found between tonsils which may also influence the response.

The development of influenza specific avidity ELISA procedures

(i) Thiocyanate elution ELISA

The avidity of standard serum IgG total antibody was measured employing the thiocyanate elution ELISA, as described extensively in Chapters 2 and 3. It is theoretically possible that ammonium thiocyanate could disturb the binding of influenza virus to the polystyrene plate. This was investigating by constructing dose-response curves following pre-incubation of the influenza coated ELISA plate with PBS-Tw-BSA or various concentrations of ammonium thiocyanate (Figure 6.4). Pre-incubation with ammonium thiocyanate from 2M to 5M appeared to disturb the binding of influenza virus to the polystyrene plate.

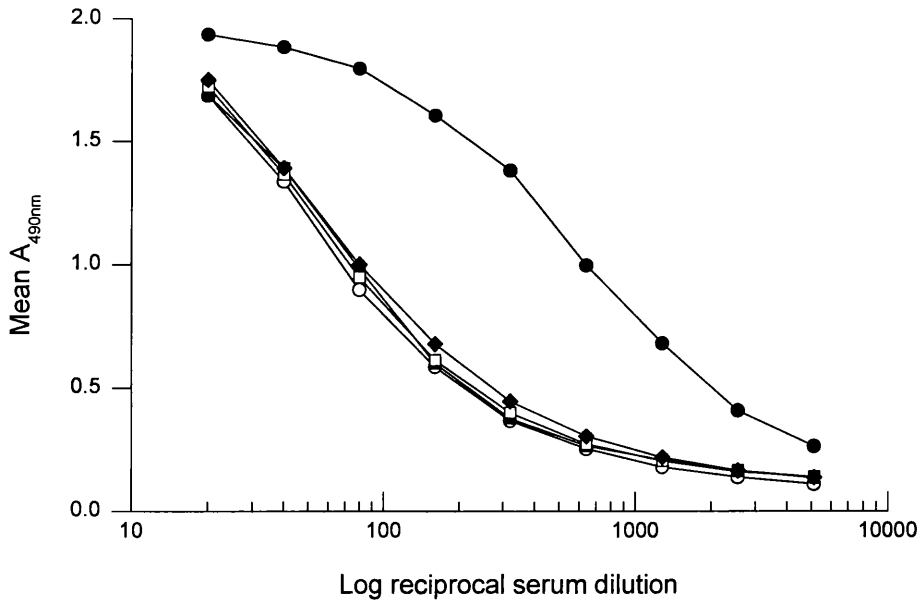


Figure 6.4 Dose-response curves obtained for standard serum IgG
Standard serum IgG binding to influenza virus adsorbed to Immulon 4 plates, following preincubation for 15 minutes with PBS-Tw-BSA (●), 5M NH_4SCN (O), 4M NH_4SCN (■), 3M NH_4SCN (□) or 2M NH_4SCN (◆).

In order to improve the binding of influenza virus to the polystyrene plate, the virus was conjugated to poly-L-lysine (as described in Chapter 2). Immulon 4 (Dynatech) plates were then coated with the influenza-lysine conjugate for 16h at RT and the antigen specific ELISA procedure completed, as described in Chapter 2. As shown in Figure 6.5, preincubation with ammonium thiocyanate did not appear to disrupt the binding to polystyrene of influenza conjugated to poly-L-lysine. Influenza virus conjugated to poly-L-lysine was therefore employed for the measurement of antibody avidity.

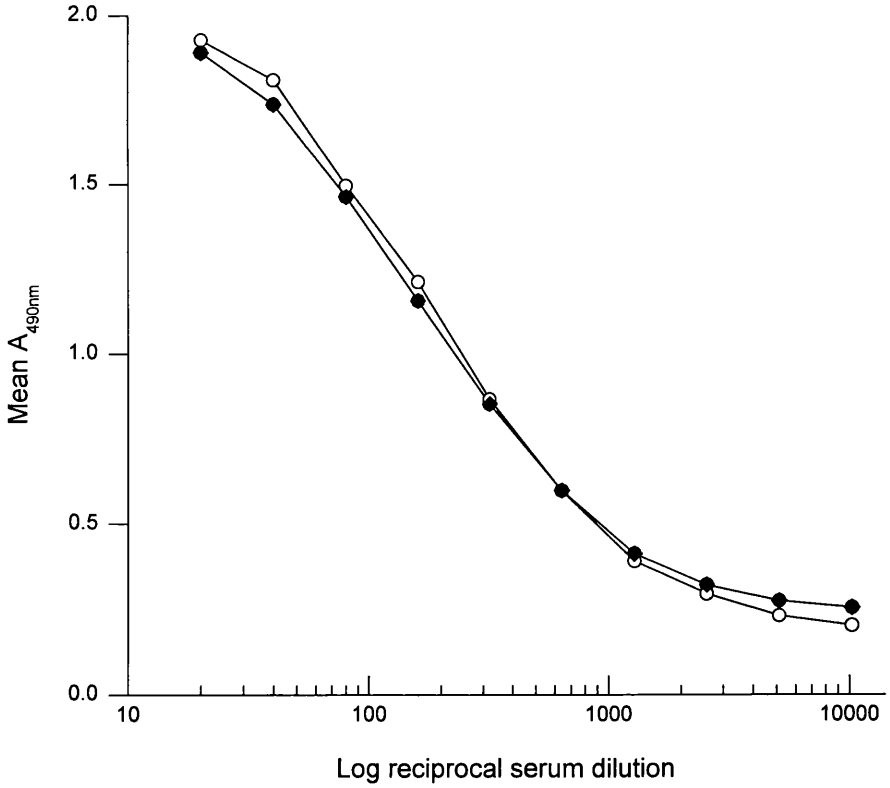


Figure 6.5 Dose-response curves obtained for influenza - lysine specific standard serum IgG

Standard serum IgG binding to influenza virus adsorbed to Immulon 4 plates, was detected following preincubation for 15 minutes with PBS-Tw-BSA (●), 5M NH_4SCN (O) or 2M NH_4SCN (◆).

The avidity of standard serum IgM and IgG antibodies was investigated using ammonium thiocyanate from 0.5M to 6M to disrupt the antibody-antigen interaction (Figure 6.6). Avidity indices were then calculated as the amount of ammonium thiocyanate required to produce a 50% reduction in absorbance (Table 6.1). The more ammonium thiocyanate required to produce a 50% reduction in absorbance the higher the antibody avidity.

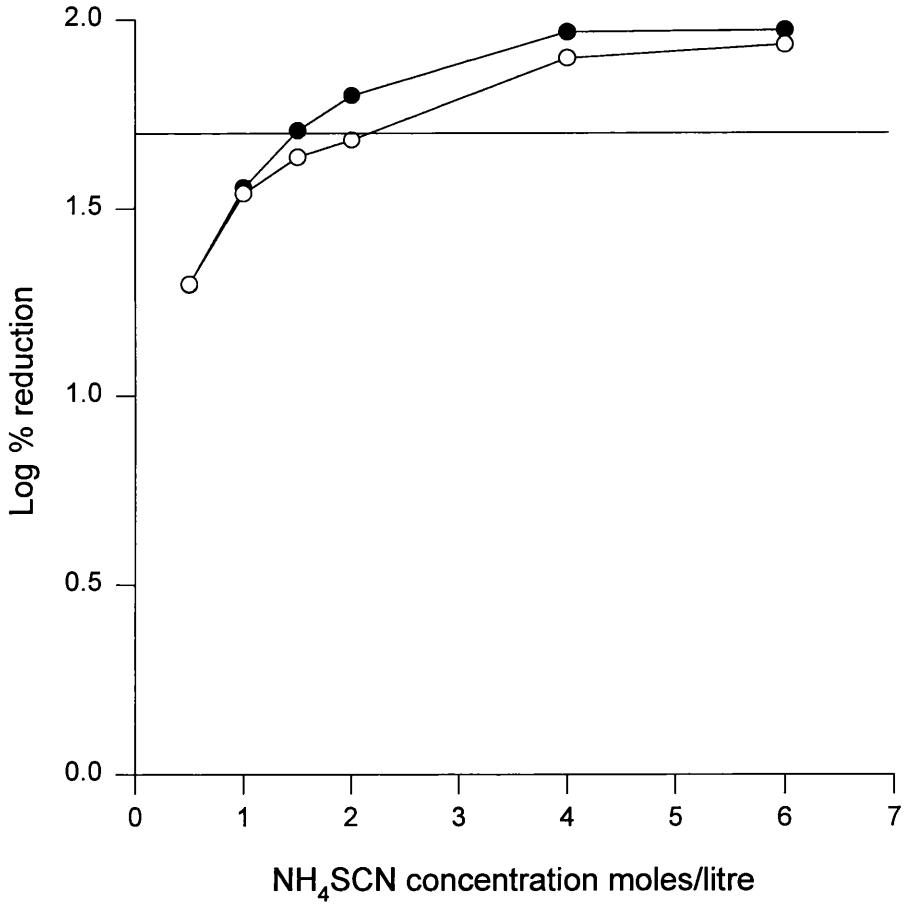


Figure 6.6 Ammonium thiocyanate elution ELISA using ammonium thiocyanate from 0.5M to 6M to disrupt serum derived antibody-antigen binding

Results were expressed as the percentage reduction in absorbance at 490nm produced in the presence of ammonium thiocyanate. The absorbance at 490nm produced in the presence of PBS-Tw-BSA was taken as 100% binding. A representative curve is shown for standard serum IgM (●) and IgG (O) antibodies binding to influenza-lysine.

Table 6.1 Avidity Indices obtained for standard serum IgM and IgG antibodies binding to influenza-lysine.

Experiment	IgM	IgG
1	1.45	2.20
2	1.80	2.40
3	1.60	2.30
4	1.80	2.20

Avidity indices were calculated as the molarity of ammonium thiocyanate required to inhibit antibody binding by 50%. Each experiment was performed on different days employing the same assay conditions.

The variation in avidity indices between assays was within the acceptable range: IgM = 10.2%, IgG = 4.2%.

Furthermore, as expected, IgM antibodies were of lower avidity than IgG antibodies. The thiocyanate elution ELISA utilising influenza virus conjugated to poly-L-lysine was thus further employed to measure antibody avidity from TMC culture supernatant.

The avidity of influenza specific IgG produced from TMCs

The avidity of influenza specific TMC produced IgG was measured by thiocyanate elution using ammonium thiocyanate at concentrations ranging from 0.5M to 6M, as described previously, to disturb antibody-antigen binding. However, it was found that antibody binding was not decreased employing this molarity of ammonium thiocyanate. An increased molarity of ammonium thiocyanate from 8M to 20M was therefore employed. Figure 6.7 shows that antibody-antigen binding could still not be disturbed even by increasing the molarity of ammonium thiocyanate, suggesting that the influenza specific IgG was of very high avidity.

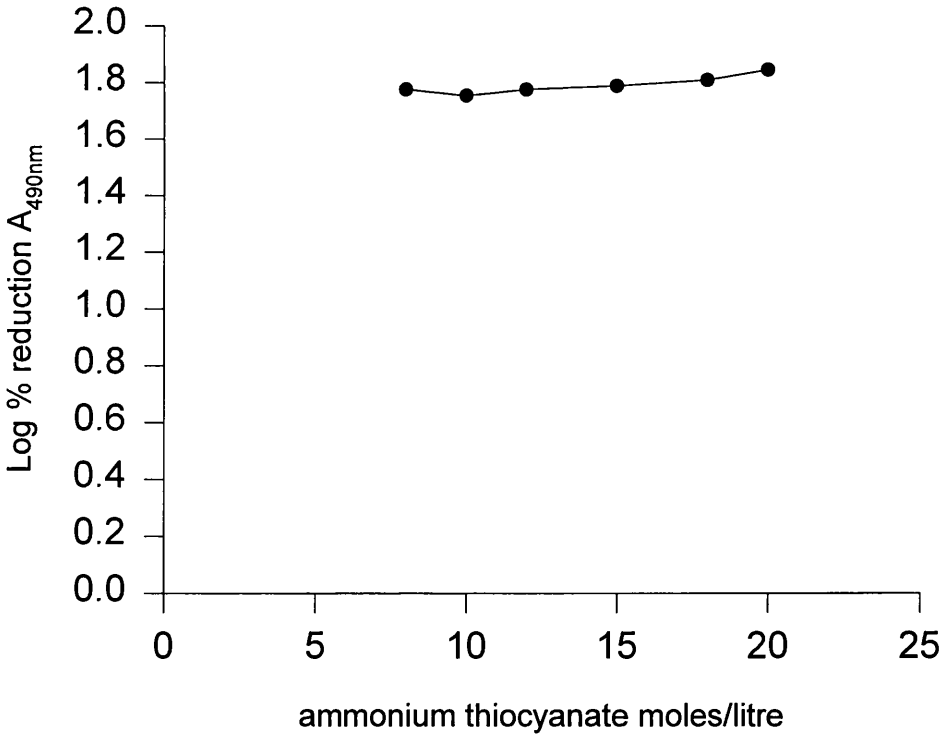


Figure 6.7 The avidity of influenza specific IgG produced from TMCs, measured by thiocyanate elution ELISA

TMCs were stimulated with influenza virus and IgG avidity was measured by thiocyanate elution ELISA, as described previously in Figure 6.6.

(ii) Competitive binding ELISA

Since, the avidity of influenza specific IgG produced by TMCs could not be measured by the thiocyanate elution ELISA, the competitive binding ELISA was explored employing the method of Rath et al. (1988) as described previously in Chapter 5. Free influenza from 100μg/ml was used to competitively inhibit antibody-antigen binding (Figure 6.8). The assay was investigated employing supernatants from two different TMC preparations. It was found that IgG detected from TMCs purified from tonsil D was of higher avidity than tonsil E by two competitive binding ELISAs performed on separate days.

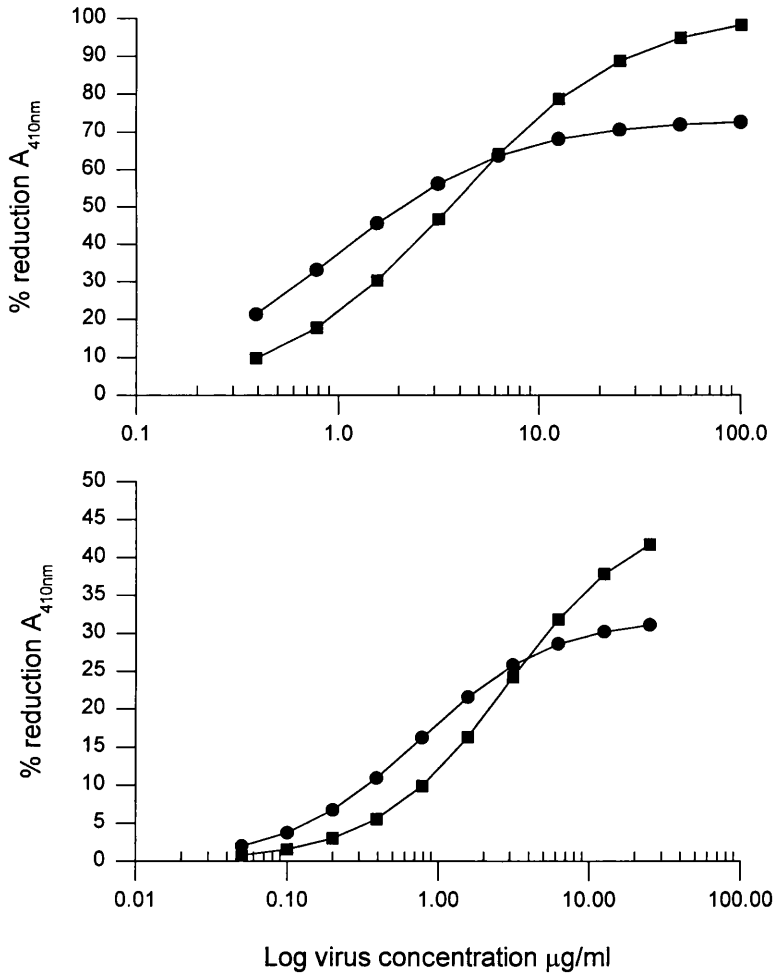


Figure 6.8 Competitive binding curves
TMCs were stimulated to produce IgG by influenza virus and the supernatant harvested on day 6. The avidity of the influenza specific IgG was measured by competitive binding ELISA employing influenza virus from 100μg/ml (a) or 25μg/ml (b) for TMC D (●) and TMC E (■).

The effect of cytokines TNFα, IFNγ, IL-6 and IL-10 upon influenza specific IgG avidity

The effect of cytokines TNFα, IFNγ, IL-6 and IL-10 upon IgG production and avidity secreted from TMCs or E^- B cells stimulated with influenza virus, was investigated by antigen specific and competitive binding ELISA.

Each tonsil was separated into TMC and E^- B cell preparations and the experiments performed in parallel. Following a 6 (TMC) or 7 (E^- B cells) day culture with or without added cytokine, the supernatants were harvested and IgG production measured by antigen specific ELISA. Figure 6.9 shows the total IgG produced from the two different tonsils employed.

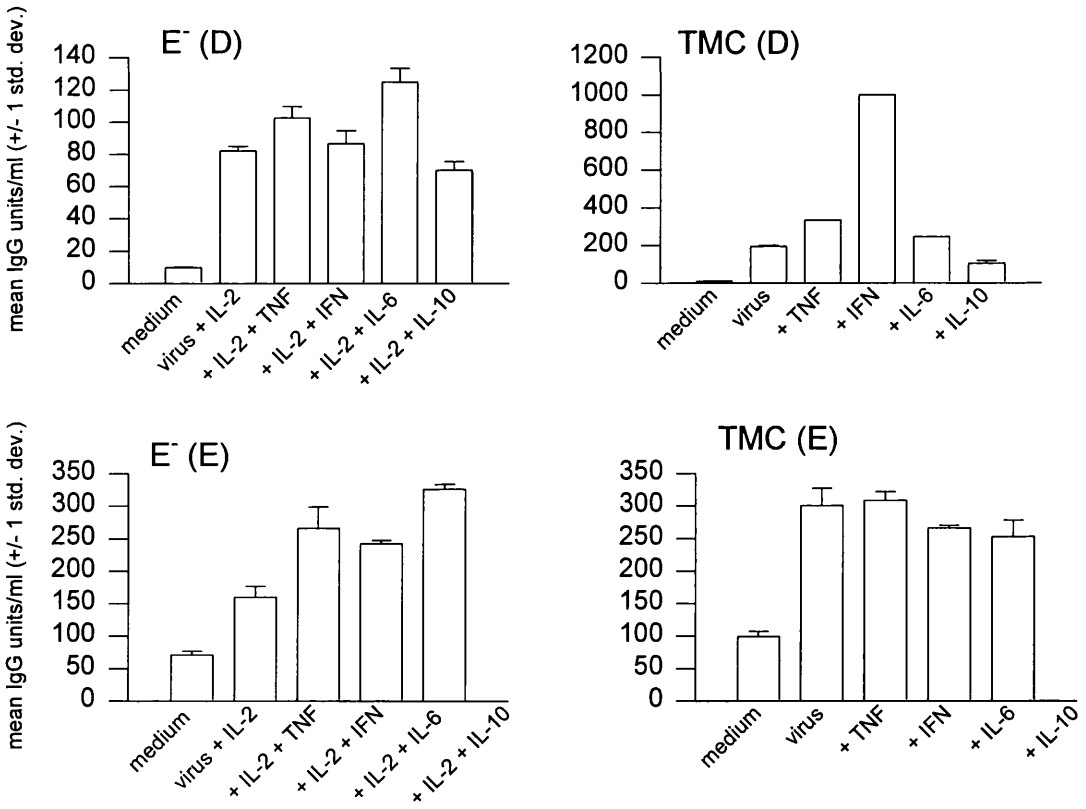


Figure 6.9 Influenza specific IgG produced from two different tonsils
TMC or E^- B cells were stimulated to produce IgG antibody with influenza virus in the presence or absence of the additional cytokines. Cell supernatants were then harvested on day 6 (TMC) or day 7 (E^- B cell) and specific IgG levels measured by antigen specific ELISA.

Influenza specific IgG avidity was evaluated employing influenza virus at concentrations ranging from 100μg/ml to 0.4μg/ml to competitively inhibit antibody binding. Avidity indices were then calculated as the amount of free virus required to inhibit antibody binding by 50% (Figures 6.10 and 6.11).

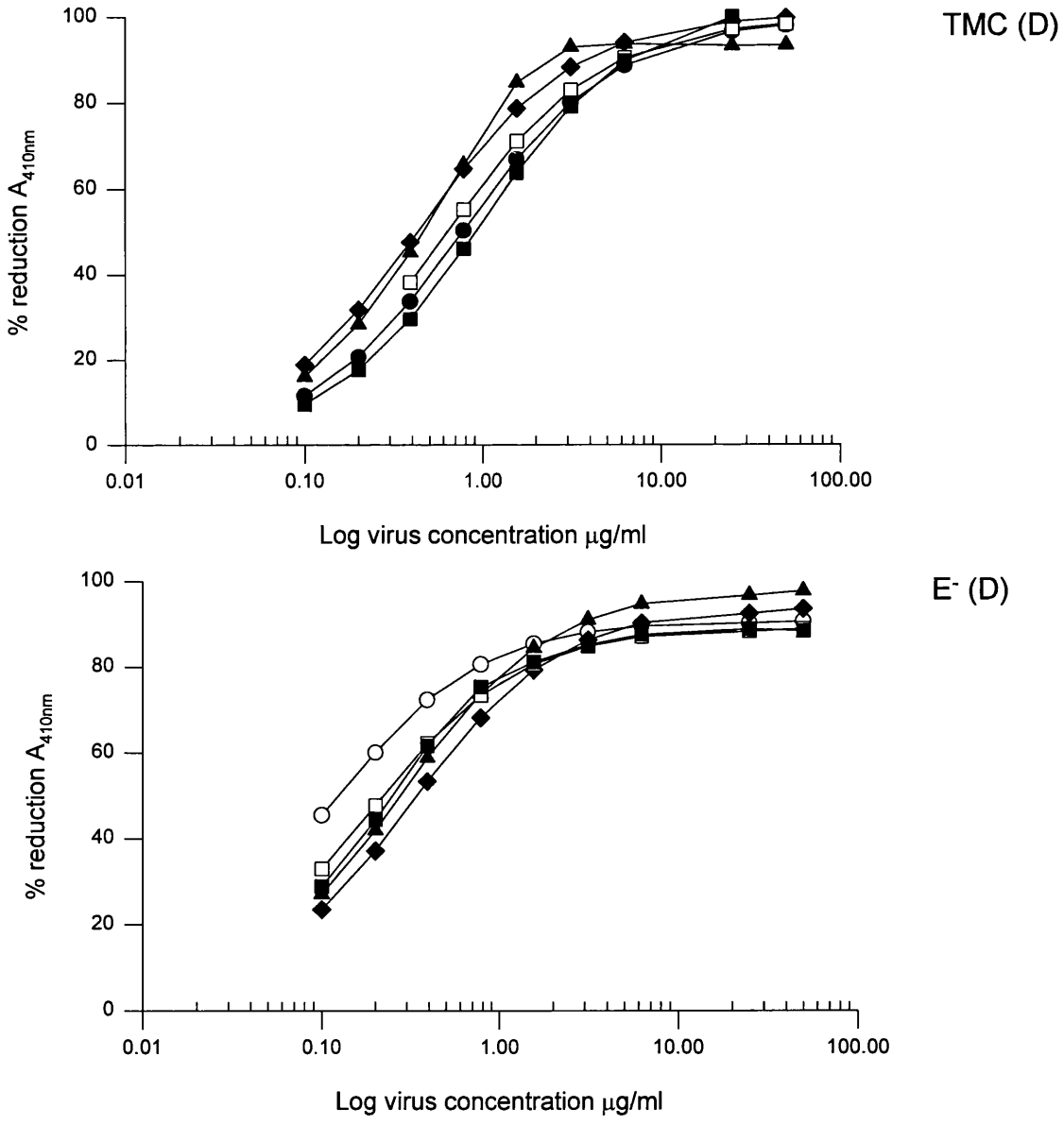


Figure 6.10 Competitive binding curves
Human tonsillar TMC or E⁻s were stimulated to produce influenza specific IgG in the presence of IL-6 (■), IL-10 (♦), TNFα (♦), IFNγ (◆) or virus alone (●).IgG avidity was then analysed by competitive binding ELISA, as described previously in chapter 2.

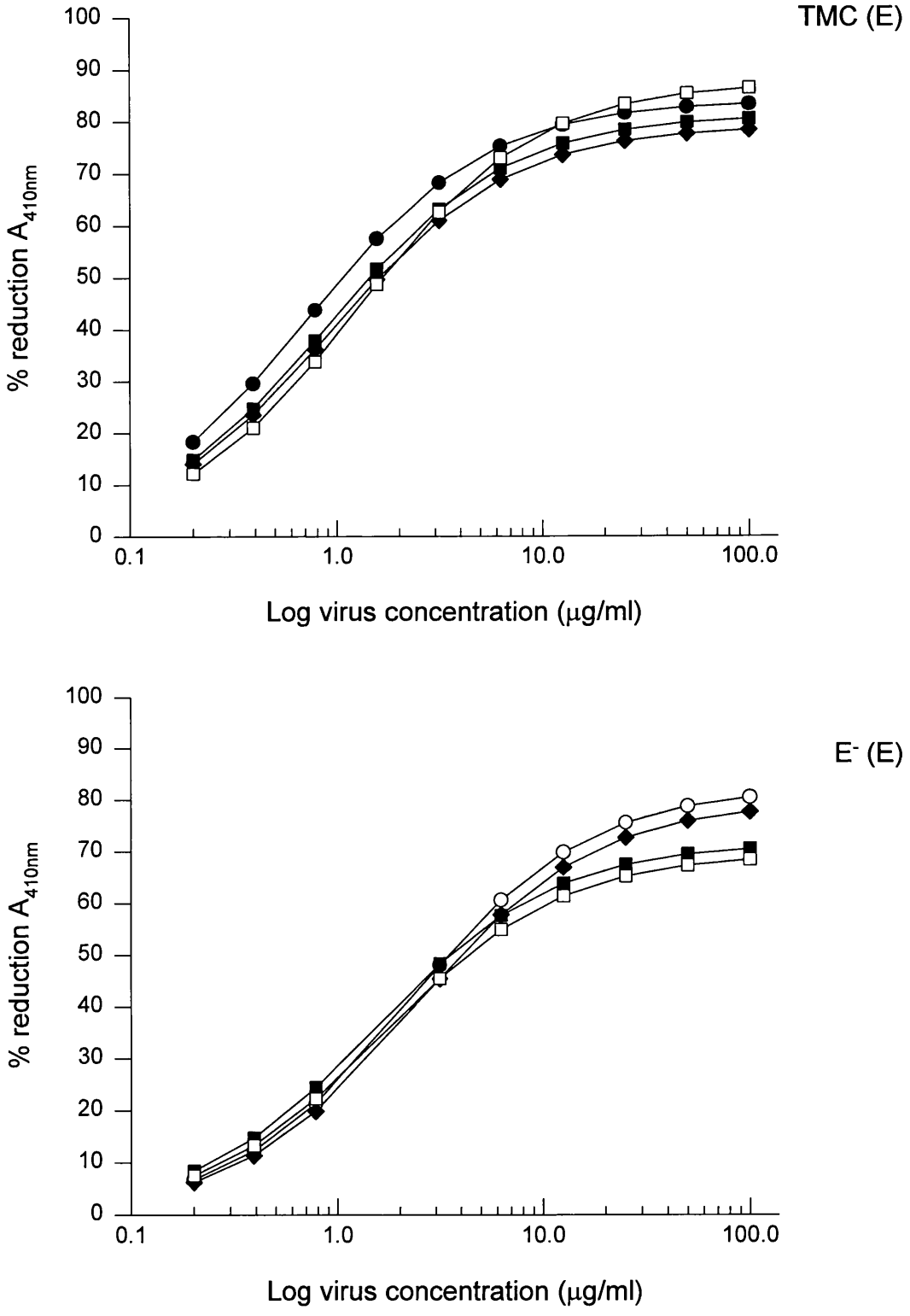


Figure 6.11 Competitive binding curves
Human tonsillar TMC or E⁻s were stimulated to produce influenza specific IgG in the presence of IL-6 (■), IL-10 (▲), TNFα (▼), IFNγ (◆) or virus alone (●).IgG avidity was then analysed by competitive binding ELISA, as described previously in chapter 2.

Avidity indices were then calculated as the amount of free virus required to produce 50% reduction in binding (Tables 6.2 and 6.3). The larger the avidity index the smaller the corresponding antibody avidity.

Table 6.2 Avidity indices produced for influenza specific IgG obtained from TMC preparations incubated with influenza virus, with or without various cytokines.

Tonsil	Medium alone	+ IL-6	+ IL-10	+ TNFα	+ IFNγ
D	0.77	0.44*	0.48	0.96	0.66
E	1.2	1.6	ND#	1.5	1.7

* avidity indices were derived from the competitive binding curves shown in Figures 6.10 and 6.11, as the amount of influenza virus required to inhibit antibody binding by 50%.
# ND = not done. Insufficient cells were recovered from tonsil E which precluded the analysis of IL-10.

Table 6.3 Avidity indices produced for influenza specific IgG obtained from tonsil E - B cell preparations incubated with influenza virus, with or without various cytokines.

Tonsil	Medium alone	+ IL-6	+ IL-10	+ TNFα	+ IFNγ
D	0.13	0.35*	0.28	0.26	0.23
E	3.6	4.2	ND#	3.6	4.6

* avidity indices were derived from the competitive binding curves shown in Figures 6.10 and 6.11, as the amount of influenza virus required to inhibit antibody binding by 50%.
# ND = not done. Insufficient cells were recovered from tonsil E which precluded the analysis of IL-10.

From the avidity indices obtained it can be seen that the addition of cytokines IL-6, IL-10, TNFα or IFNγ appeared to decrease influenza specific IgG avidity. Except for tonsil D where TNFα appeared to increase influenza specific IgG avidity obtained from TMCs.

DISCUSSION

During an immune response to protein antigens, in addition to isotype switching, the affinity of specific antibody produced from a B cell may increase, a process referred to as somatic hypermutation. Somatic hypermutation is known to occur in germinal centres at the centroblast stage within the dark zone. The interaction of surface immunoglobulin with antigen is not sufficient to promote cell survival, and it is postulated that accessory molecule interactions may be involved in the selection of germinal centre B cells which have undergone somatic hypermutation and immunoglobulin class switching (reviewed by Maclennan, 1994). Immunoglobulin class switching is known to be influenced by cytokines such as IL-4 and TGFβ. Little work, however, has been published exploring the role of cytokines in the modulation of antibody affinity. In mice, antibody affinity has been shown to be enhanced by the cytokine, IFNγ (Holland et al. 1990) and by T_H1 or T_H2 cell subsets in adoptive transfer experiments (Rizzo et al. 1992). At the time of writing no work has been published exploring the potential influence of cytokines on human antibody affinity.

The development of solid-phase avidity ELISA procedures

In the past the analysis of polyclonal antibody avidity, such as serum and culture derived antibodies was hampered by the lack of a suitable assay. However, rapid progress has been made in recent years in the development of solid-phase assays which enable such avidity measurements and various solid-phase assays are now available. As previously stressed not all of these assays are suitable for

the particular antibody-antigen interaction under investigation and care must be taken to select an appropriate method.

Data presented in previous chapters have suggested that the thiocyanate elution ELISA was the most reproducible ELISA procedure for avidity determinations. The thiocyanate elution ELISA was thus investigated for the measurement of influenza specific IgG but unfortunately, proved to be unsuitable for detecting the avidity of TMC produced IgG avidity.

The avidity indices determined for IgG specific for pneumococcal polysaccharide antigens was approximately 1.6, and for H. influenzae PRP a value of 0.3 has been determined within our laboratory (data not shown). In contrast the avidity indices determined for influenza specific IgG was 2.5. suggesting that serum derived influenza specific antibody avidity was relatively high. The response of human tonsillar cells to influenza virus is expected to be a memory B cell response. The influenza specific IgG produced will have been subject to somatic mutation and selection for high avidity antibodies. The stimulation of tonsil cells with influenza virus might therefore be expected to expand this population of B cells. The results obtained here with the thiocyanate elution ELISA appear to confirm the high avidity measured in the influenza specific ELISA. However, poly-L-lysine conjugated influenza virus was employed in the thiocyanate elution ELISA. Poly-L-lysine conjugation is known to enhance antigen binding to the polystyrene plate thus increasing epitope density (Feldman et al. 1994). The detection of high avidity antibodies might therefore be due in part to the use of poly-L-lysine. The measurement of TMC produced influenza specific IgG using the competitive binding ELISA also suggested that the

antibody was of high avidity. Although the possibility cannot be ruled out, it is unlikely that poly-L-lysine conjugation was responsible for the observed high avidity IgG.

The effect of cytokines, IL-6, IL-10, TNFα and IFNγ, on influenza specific IgG produced from tonsillar cells

The influence of cytokines on the avidity of influenza specific IgG was investigated on two different tonsil preparations and conflicting results were obtained. In general, IL-6, IL-10, TNFα and IFNγ appeared to decrease the avidity of influenza specific IgG from tonsil E as measured by competitive binding ELISA, whereas IL-6, IL-10 and IFNγ appeared to increase the avidity of influenza specific IgG produced from TMCs separated from tonsil D. No firm conclusions regarding the influence of IL-6, IL-10, TNFα or IFNγ on antibody avidity can be reached from just two experiments. Difficulties with responsiveness to influenza virus between tonsils precluded the analysis of further preparations. Further experiments are required to determine the role, if any, of cytokines in modulating antibody avidity.

Since relatively unseparated populations of tonsillar cells were analysed here, it is possible that variations in B cell subpopulations may explain the inconsistent results obtained. Further purification of germinal centre B cell subpopulations may resolve this issue. The observed decrease in antibody avidity upon addition of exogenous cytokine could be due to the cytokines promoting cell death and the investigation of apoptosis and necrosis during the culture period would therefore be desirable in future experiments. Since, whole

virus was employed as the antigen for the investigation of influenza specific IgG avidity, the response measured would have been heterogeneous. It is possible therefore that a small amount of very high avidity antibody specific for one epitope would be preferentially detected. It is known that haemagglutinin (HA) is the major surface glycoprotein of influenza virus and important antigenic sites have been identified (Kalyan et al. 1994). The use of specific peptides corresponding to these antigenic sites may permit a less heterogeneous population of antibodies to be investigated.

Summary

An influence of the cytokines Il-6, IL-10, TNFα and IFNγ on influenza specific IgG avidity could not be unequivocally demonstrated. The potential for cytokines to influence antibody avidity possibly by promoting somatic mutation, rescuing somatically mutated B cells from apoptosis and by the expansion of memory B cell clones cannot, however, be discounted. The data presented in this chapter is preliminary and the caveats mentioned previously must be recognised. The solid-phase ELISA for the measurement of influenza specific IgG avidity has potential for future investigations of cytokine modulation of antibody avidity.

CHAPTER 7

General Discussion

The immunoglobulin isotype elicited in response to antigenic challenge is crucial in overcoming infection. Following an initial encounter with antigen IgM is the major isotype produced. During the immune response isotype switching and affinity maturation occurs and high affinity IgG is mounted early in the secondary response. It is well documented that the specific antibody titre raised is an important indicator of protection following natural infection or vaccination. A high titre of specific antibody, however, does not necessarily correlate with protection from infection and the quality of the antibody response, reflected in the avidity of antibody may also be important. Investigations described in this thesis have explored (i) the role of structure remote from the V region in influencing antibody avidity (ii) the biological significance of avidity differences within IgG subclasses and (iii) factors influencing specific antibody avidity.

Analysis of Antibody Avidity

Traditional methods for measuring antibody avidity have relied upon the availability of pure antibody and antigen preparations. Furthermore, the requirement in most such methods for a labelling step may alter protein integrity. In the past, therefore, it has not been possible to determine the avidity of serum antibody responses, and this has resulted in a lack of information regarding the relative biological importance of antibody avidity.

More recently, ELISA based methods have been explored for the measurement of serum antibody avidity. Many investigators have determined equilibrium constants (K) employing a competitive binding ELISA, although the validity of such measurements remain controversial. Since antigen and antibody

are no longer in solution and proteins are known to undergo denaturation when adsorbed to plastic, it is questionable whether the K value reflects the binding of the antibody under investigation to its natural epitope. The valency of the bound antibody is also difficult to determine in the competitive binding ELISA and the K value may thus be underestimated if all the IgG is not bivalently bound. Moreover, differences in K values determined for the same antigen-antibody reaction are observed when comparing solution and solid-phase assays, in general solid-phase assays give higher K values than solution phase assays. Alternative methods which have been described for measuring antibody avidity in polyclonal serum responses do not rely upon the determination of a K value. The measurement of avidity is expressed as an avidity index, which describes the amount of free antigen or elution reagent required to inhibit antibody binding by 50%.

The competitive binding ELISA has attracted the most attention and was originally validated by comparing the avidity of a panel of anti-dinitrophenol (DNP) mAbs obtained by equilibrium dialysis and a competitive binding ELISA (Rath et al. 1988). This assay has subsequently been used to measure serum antibody avidity specific for a number of antigens including V3 loop peptides of HIV (Seligman, 1994), tetanus toxoid (Devey et al. 1988) and streptococcal antigens (Wada et al. 1988; Falconer et al. 1993).

In the studies described in this thesis solid-phase avidity ELISAs were developed for the measurement of both monoclonal and polyclonal serum antibody avidity. The competitive binding ELISA is thought of as the 'gold-standard' with which to compare other solid-phase avidity ELISAs. Results

obtained here demonstrated high variability using the competitive binding ELISA for the detection of antibodies specific for mucin, pneumococcal polysaccharides and the hapten, NIP, conjugated to BSA. Furthermore, the competitive binding ELISA was unable to measure antibody avidity specific for pneumococcal serotype 3. The underlying reasons responsible for this remain unresolved, but several explanations are possible. The nature of the antigen in solution may determine the extent to which the free antigen is able to inhibit binding. The epitopes displayed in solution may be sufficiently different from those on the solid-phase antigen thus preventing solution phase antigen from competing for antibody bound to the solid-phase antigen. It is possible that the antibodies binding to pneumococcal serotype 3 were of very high avidity and would therefore require a higher concentration of free antigen to compete for binding. Difficulty in achieving solubility of the polysaccharide antigen may also contribute to interassay variation.

Further experiments are clearly required in order to understand these difficulties. Since mucin is a large complex glycoprotein, the use of pure TAG72, the antigen reactive with the B72.3 set of mAbs, may minimise the variability of the mucin specific ELISA. Improving the purity and solubility of the pneumococcal polysaccharide preparation and perhaps the use of oligosaccharides as inhibitors such as those employed by Van Dam et al. (1989) could be used in the future to standardise the pneumococcal competitive binding ELISA.

An alternative solid-phase ELISA used in this thesis was the elution ELISA, in which antibody-antigen binding is disrupted by a chaotropic agent

included in an additional incubation step. Various chaotropes have been explored including DEA, thiocyanate and urea. Experiments performed in our laboratory demonstrated that DEA exerts its disruptive effect via pH and therefore can no longer be regarded as a true chaotrope (Goldblatt et al. 1993). In contrast, thiocyanate is well established as a true chaotrope (Dandliker et al. 1967). Attention was therefore directed towards the thiocyanate elution ELISA.

Conflicting evidence has been presented throughout the literature in respect of the reliability of the thiocyanate elution ELISA. Macdonald et al. (1988) comparing six monoclonal antibodies specific for the hapten DNP demonstrated good correlation between avidity measured by traditional equilibrium dialysis and by the thiocyanate elution ELISA. In contrast, Hall and Heckel, (1988) found a poor correlation between the thiocyanate elution ELISA and equilibrium dialysis. The thiocyanate elution ELISA has, however, been successfully used to measure the avidity of serum derived antibody specific for a wide variety of antigens such as rubella (Pullen et al. 1986), poliovirus (Mellander et al. 1993), *M. catarrhalis* (Goldblatt et al. 1993) and tetanus toxoid (Herias et al. 1993).

Results presented throughout this thesis have highlighted the importance of the type of method employed to measure antibody avidity. In my hands the thiocyanate elution ELISA proved to be the most reliable solid-phase assay. In particular a good correlation was demonstrated between the avidity of anti-hapten (NIP) mAbs measured by the thiocyanate elution ELISA and equilibrium constants determined by biospecific interaction analysis (BIA).

Antibody Binding Kinetics

Research has now begun to focus upon the investigation of ligand-analyte interactions in further detail. Employing state of the art technology it has become possible to observe ligand-analyte binding in real-time and to use this information to analyse kinetic data. The biological importance of kinetic analysis was first observed by Foote and Milstein, (1991). These authors studied the binding constants of antibodies specific for 2-phenyl-5-oxazolone and demonstrated an increase in the association rate constants during antigen driven B-cell selection. It was suggested that concomitant with affinity maturation a process of kinetic maturation might also occur. VanCott et al. (1994) subsequently found that although of similar affinity, the neutralising capacity of antibodies specific for the V3 loop of HIV correlated with the dissociation rate constant and not the association rate constant. The advent of the BIAcore™, described fully in Chapter 4, has allowed detailed kinetic analysis of many biological interactions in the laboratory. Although, initially used to study antibody-antigen binding, applications for this technology have rapidly expanded in the past few years. Ward et al. (1992;1995) have investigated the IL-6 and IL-6 receptor interaction and mapped important residues involved in receptor binding. Johanson et al. (1995) also investigating cytokine-cytokine receptor interactions have demonstrated using the BIAcore™ that conformational changes occur upon binding of IL-5 to its receptor. The field of cell signalling has also exploited BIAcore™ technology and many investigators have analysed ligand binding to Src homology domains. Cell adhesion molecule interactions have also been analysed using the BIAcore™ (van der Merwe et al. 1994), as have MHC-peptide

(Khilko et al. 1995) and zinc finger DNA binding proteins (Yang et al. 1995). The use of the BIAcore™ to measure serum derived antibody avidity has been described recently (Bakker et al. 1995), and it should now be possible to analyse antibody avidity in various situations, for example following vaccination. Applications of BIAcore™ technology are expanding rapidly and full automation in the future will permit, the rapid screening of novel compounds in drug design and the screening of phage libraries for high avidity mAbs for use in therapy.

IgG Subclass Avidity Differences

Prior to embarking upon these studies avidity differences between naturally occurring or vaccine induced IgG subclasses specific for the same antigen had been noted by several investigators. It was postulated that differential V region usage may be responsible for the observed avidity differences. Experiments performed with murine V region identical monoclonal antibodies demonstrated a role for the constant region in influencing antibody avidity. It was possible therefore that structure remote from the variable region might also influence human antibody avidity. Investigations described here employing solid-phase avidity ELISA indicated avidity difference between IgG subclasses specific for the hapten, NIP. In contrast, V region identical IgG subclasses specific for the TAG72 antigen of mucin appeared to possess similar avidity. Kinetic analysis of the V region identical mAbs specific for TAG72, showed that the avidity differences between the IgG subclasses, reflected differences in the dissociation rate constants. It does appear therefore that structures remote from the V region can influence antibody avidity.

The underlying mechanisms responsible for the observed avidity differences are presently unclear. It has been suggested that the degree of segmental flexibility is related to the antibody avidity. The ability of the Fab arms to rotate freely is believed to increase the chance of the antibody being able to bind bivalently to its antigen and thus increase its observed avidity. Segmental flexibility is mediated via the hinge region of the antibody, the longer the hinge the greater the flexibility (Dangl et al, 1988). Segmental flexibility of IgG subclasses has been ranked as IgG2b > IgG2a > IgG3 > IgG1 in mice and IgG3 > IgG1 > IgG4 > IgG2 in humans. If the previous hypothesis is correct murine IgG2a and human IgG3 would be expected to be of the highest avidity. Results obtained for studies in mice employing V region identical IgG subclasses have, however, demonstrated that despite possessing identical V regions mouse IgG3 was of higher avidity (Fulpius et al. 1993; Schreiber et al. 1993; Cooper et al. 1994). Cooper et al. (1994) postulated that the consistent finding of high avidity murine IgG3 was due to IgG3 exhibiting molecular co-operativity. This involves non-covalent Fc-Fc interactions creating antibodies of higher valency than non-cooperative antibodies, but no evidence has been presented to support this theory.

In the studies described in this thesis using two different sets of mAbs the IgG4 subclass was found to have the highest avidity. Since human IgG4 has a relatively inflexible hinge, segmental flexibility cannot explain the differences in avidity observed here between IgG subclasses. The area of greatest sequence variability within the IgG subclasses resides within the hinge region, and it is to be expected that variations in avidity between the IgG subclasses would be

mediated via the hinge region. Although removal of the constant region via peptic digestion clearly demonstrated that the constant region influenced antibody avidity, it would be of interest to examine the potential role of the hinge region in greater detail. Horgan et al. (1993) found that deletion of the IgG1 hinge did not affect peptide binding whereas hinge deleted IgG4 showed increased binding as measured by solution and solid-phase binding assays. This suggests that the hinge region may influence the binding of IgG4 to antigen. Site-directed mutagenesis could also be employed to explore the relative importance of specific amino acids, such as those involved in glycosylation.

Clearly, further investigation is required to elucidate the structural features within the human constant region responsible for affecting antibody avidity and binding kinetics. Although evidence presented here appears to suggest that segmental flexibility mediated by the hinge region is not involved, detailed structural analysis of these mAbs is required in order to determine whether these chimeric mAbs exhibit the expected hinge flexibility patterns. Since it would appear from the results presented here that hinge flexibility does not confer high antibody avidity it would be of interest to examine the degree of glycosylation expressed by these mAbs. Since glycosylation influences effector function and antibody stability, glycosylation may also contribute to antibody avidity.

The role of antibody avidity in protection

Engineered antibodies may differ structurally from naturally occurring molecules. Glycosylation patterns are particularly important, since the two C_H2 domains do not exhibit any protein-protein contacts and interdomain pairing is thus mediated by sugar residues. If the protein is not glycosylated the conformation of the domains is altered and biological function impaired. For example, aglycosylated antibodies have been demonstrated to lack C1q binding. With regard to the two sets of chimeric mAbs employed in this study, the anti-NIP mAbs have been extensively studied and shown to form the expected tetrameric structure and to have glycosylation patterns comparable to their human counterparts (Bruggemann et al. 1987). Chimeric B72.3 IgG subclasses are also secreted as fully assembled tetramers. Chimeric B72.3 IgG4 has been demonstrated to express normal glycosylation patterns, but IgG1 - 3 mAbs have not been analysed (Lund et al. 1993).

The observation that V region identical chimeric IgG subclasses display avidity differences will be of importance when choosing the isotype of antibody for therapeutic use. The biological significance of such IgG subclass differences remains to be fully investigated and was the rationale for studying the avidity of serum derived antibody specific for the clinically relevant bacteria *S. pneumoniae.*

It is well documented that infants under the age of two do not mount an adequate immune response against polysaccharide antigens. A delay in the ontogeny of the IgG2 subclass has been postulated to be responsible for the high incidence of invasive disease in this group of individuals, which imposes

limitations on the usefulness of pure polysaccharide vaccines. Infants under the age of two are, however, able to mount a polysaccharide specific IgG1 response but whereas IgG2 levels have been correlated with protection, the protective capacity of the IgG1 response is not known.

Comparing IgG1 and IgG2 serum antibody responses mounted in children before and after vaccination with the pure polysaccharide pneumococcal vaccine, infants below the age of two were found to have IgG1 specific for serotype 3 of lower avidity than older children, as determined by the thiocyanate elution ELISA. In contrast, IgG1 specific for serotype 23 was of higher avidity than IgG2. It should be noted, however, that pneumococcal serotype 3 required linkage to poly-L-lysine to permit stable adsorption and although, widely employed, conjugation of antigens may alter antigenic integrity. Pneumococcal serotype 23 was employed in its native form and the results obtained contrasted to those obtained for serotype 3. Further investigations employing different conjugation procedures or perhaps alternative avidity assays are required to resolve this issue.

The protective capacity of an antibody is related to its titre, effector function and avidity. Results presented here suggest that it is possible that the low protective capacity of pure polysaccharide vaccines in infants is not only related to their inability to produce polysaccharide specific IgG2 but also due to differences in antibody avidity elicited specific for different serotypes. Polysaccharide specific antibodies are not thought to display affinity maturation and therefore it is unlikely that the difference in avidity observed between the two subclasses is a result of affinity maturation concomitant with class switching from

IgG1 to IgG2. It is possible that differential V gene usage or the Gm allotype expressed may be responsible for the observed avidity differences. Previous studies suggested positive correlations between Gm allotype status, antibody titre and protection from invasive disease. Only one study so far has investigated the relationship of Gm allotype with antibody avidity and no correlation was found by ELISA (Konradsen et al, 1994). Since we have found that the antibody structure remote from the V region influences antibody avidity it is possible that Gm allotype may influence antibody avidity. Gm allotyping of the serum samples and correlation with known IgG subclass avidity of these samples would resolve this issue. Differential V region usage of the anti-polysaccharide responses may also be responsible for the observed avidity differences and studies are ongoing in our laboratory to address this question.

There is a trend towards the development of vaccines based upon pneumococcal polysaccharide vaccines conjugated to proteins in order to overcome the poor immunogenicity of carbohydrate antigens in children less than two years old. Studies with *H. influenzae* protein conjugates have demonstrated antibody avidity differences between different conjugates (Lucas and Granoff, 1995). It will therefore be important to evaluate the avidity of the antibody response elicited by these vaccines and examine whether these responses correlate with protection.

The Control of Somatic Hypermutation

Antibody binding is mediated by the V region, particularly within the hypervariable regions referred to as the complimentarity determining regions (CDRs). Immunoglobulin V region sequences are modified from the germ line via a process called somatic hypermutation after the recognition of antigen, which in conjunction with selection results in an increase in antibody affinity. The factors controlling affinity maturation and the molecular mechanisms underlying somatic hypermutation remain uncharacterised. Insights into these processes may permit modulation of the immune response, particularly following vaccination, and may be utilised to improve the avidity of antibodies produced for use in therapy.

Previous studies have identified a role for the transcriptional machinery in somatic hypermutation. A model has been proposed whereby single or double stranded DNA nicks occur followed by sequence alteration and DNA repair introducing mutations. No studies have as yet identified biochemical activities associated with targeted somatic hypermutation. Concomitant with selection by antigen trapped by follicular dendritic cells, additional signals, such as the CD40/CD40L are required to promote cell survival rather than apoptosis. Little evidence has been presented to date investigating the role that cytokines may play in affinity maturation in humans. Studies in mice however have shown that IFNγ increases the avidity of antibody specific for BCG. Preliminary results presented here studying IL-6, IL-10, TNFα and IFNγ, failed to demonstrate any effect upon the avidity of influenza specific IgG produced by human tonsillar B cells, as measured by competitive binding ELISA. Nevertheless a role for these cytokines in modulating antibody affinity cannot be dismissed. It was possible

that the mixed germinal centre B cell population used in these studies was responsible for the inability of the cytokines employed to modulate antibody avidity. Recent studies have demonstrated that somatic hypermutation is triggered at the centroblast stage (Pascual et al. 1994). The tonsillar B cells employed in the present investigation would have contained naive unmutated and memory mutated B cells, as well as the centroblasts undergoing somatic hypermutation. Clearly, a heterogeneous population of antibodies with differing avidity would thus be present. Furthermore, since most individuals have been exposed to the influenza virus, this could result in an expansion of the memory population of B cells, with skewing of the antibody towards high avidity. It would follow from this that the results presented here suggest no role for IL-6, IL-10, TNFα or IFNγ in modulating avidity of antibodies secreted by memory B cells. Dissection of the germinal centre B cells into various subpopulations should permit the investigation of the role of accessory molecules including cytokines in more detail. In conjunction with the analysis of antigen specific antibody avidity by solid-phase assays, binding kinetic studies employing the BIAcore™ and molecular analysis of specific V regions will allow the investigation of antibody avidity in greater detail.

Concluding Remarks and Future Perspectives

Whilst it is clear that the isotype and antibody titre elicited during an immune response is crucial in overcoming infection, evidence is now accumulating that antibody avidity must also be taken into account. The analysis of antibody avidity in greater detail employing state of the art technology should help to illuminate the immune responses elicited following many natural infections and vaccination. Furthermore an understanding of the structural features and factors underlying antibody avidity will aid in the production of therapeutic antibodies and the design of immunogenic vaccines.

Previous studies have been frustrated by the limitations imposed by past technology. The availability of biospecific interaction analysis within the laboratory opens up an exciting area of scientific research. The analysis in detail of ligand-analyte interactions in many areas of biology such as signalling, vaccine development, drug design and receptor biochemistry, will hopefully lead towards novel discoveries.

Unanswered questions still remain regarding the mechanisms underlying the avidity differences noted between the IgG subclasses. The application of molecular techniques, in particular V gene usage of the IgG subclasses, together with a detailed analysis of the mutations accumulated within the V regions should shed some light upon the features underlying the processes of somatic hypermutation. Detailed structural analysis of the human IgG subclasses will lead to a better understanding of the structural constraints influencing antibody avidity. A concerted effort utilising the latest technologies will hopefully give us a better picture of how the immune response works as a whole and the role

antibody avidity has to play within this context. Such findings should enable us to modulate the immune response in an effort to improve individual protection from infectious disease, to enhance the usefulness of therapeutic antibodies and improve vaccine effectiveness.

Appendix 1

Names and Addresses of Suppliers

UK unless otherwise stated

American Type Tissue Culture Collection
12301 Parklawn Drive
Rockville
Maryland
20582 USA

Amersham International
Amersham Place
Little Chalfont
Buckinghamshire

Amicon Ltd
Upper Mill
Stonehouse
Gloucestershire

BDH (Merck Ltd)
Merck House
Poole
Dorset

Bio-Rad Laboratories
Maylands Avenue
Hemel Hempstead
Herts

Dynatech Laboratories
Daux Road
Billingshurst
Sussex

Genosys Biotechnologies Inc.
162A Cambridge Science Park
Milton Road
Cambridge

Genzyme
50 Gibson Drive
Kings Hill
West Malling
Kent

Hayman Ltd
70 Eastways Industrial Park
Witham
Essex

ICN (Flow) Biomedicals Ltd
Unit 18
Thame Park Business Centre
Thame Oxon

Millipore
The Boulevard
Blackmore Lane
Watford
Herts

National Diagnostics
Unit 4
Fleet Business Park
Itlingslane
Hessle Hull

NIBSC
South Mimms
Potters Bar
Herts

Pharmacia Biotech/Biosensor
23 Grosvenor Road
St. Albans
Herts

Roussel of Ireland Ltd
Dublin 2
Republic of Ireland

Sigma Ltd
Fancy Road
Poole
Dorset

Zymed Laboratories
458 Carlton Court
So San Francisco
CA 9480

Appendix 2

BIAcore™ Kinetic Analysis

Various models for kinetic analysis of the interaction studies are included in the analytical software supplied with the BIAcore™, although for most cases a simple one-site model will most probably be sufficient when employing purified homogeneous preparations. The principles of kinetic analysis used with the BIAcore™ are reviewed in detail elsewhere (Fagerstam and Karlsson, 1995).

Briefly, using first order kinetics, the rate equation of analyte A reacting with ligand B to form AB depends on the free concentrations of A and B and the stability of the AB complex and may be written as:

$$d[AB]/dt = k_a[A][B] - k_d[AB] \quad (1)$$

where k_a is the association rate constant ($M^{-1}s^{-1}$) and k_d the dissociation rate constant (s^{-1}).

In the BIAcore the ligand B is immobilised onto the sensor chip and therefore its concentration remains constant. The concentration of bound analyte is therefore equal to [AB]. In the BIAcore [AB] is proportional to the resonance units, R_A, and total free ligand may be calculated as $R_{max} - R_A$. Since analyte is passed over the sensor chip continuously its concentration may be regarded as static and equation (1) may therefore be rewritten as:

$$dR_A/dt = k_aC (R_{max} - R_A) - k_dR_A \quad (2)$$

where C is the concentration of analyte

Rearranging this equation at equilibrium, where $dR_A/dt = 0$ and introducing the affinity constant $K = k_a/k_d$ (M^{-1}) the equation becomes

$$R_A/C = KR_{max} - KR_A \qquad (3)$$

the affinity constant may then be calculated form a plot of R_A/C vs R_A.

Similarly rearranging the basic kinetic equation as follows:

$$dR_A/dt = k_aCR_{max} - (k_aC + k_d)R_A \qquad (4)$$

rate constants may be evaluated from a plot of dR_A/dt vs R_A

Kinetic analysis was achieved using BIAevaluation software supplied with the BIAcore™ . Non-linear fitting was applied assuming a model of antigen-antibody binding of Ab + Ag ↔ AbAg. The same portion of the binding curve was used for each concentration of antibody overlaid onto one sensorgram and avoiding the very early phase of the curve which may be complicated by mass transport effects.

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Immunology 1996 **88** 169–173

Human constant regions influence the antibody binding characteristics of mouse–human chimeric IgG subclasses

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SUMMARY

Although antibody affinity is primarily determined by immunoglobulin variable region structure human IgG antibodies of the four subclasses specific for the same antigen have been shown to differ in their affinity. To explore the influence of the immunoglobulin constant region on functional antibody affinity, a set of V region identical mouse–human chimeric IgG subclasses specific for TAG72 (tumour-associated glycoprotein) were studied. Biomolecular interaction analysis (BIA) was used to determine the binding kinetics of whole IgG subclasses and $F(ab')_2$ fragments. Despite identical V regions, binding kinetics differed for the four subclasses. The apparent dissociation rate constants of the intact immunoglobulins ranked IgG4 < IgG3 < IgG2 < IgG1. In contrast, analysis of the binding characteriztics of the $F(ab')_2$ fragments derived from IgG1, IgG2 and IgG4 revealed identical binding kinetics. The structure of the constant regions of the humanized IgG subclass antibodies clearly influenced functional antibody affinity, as has been described for the murine IgG subclasses. The exact mechanism for this phenomenon remains obscure but such differences should be taken into account when designing or choosing antibodies for therapeutic use.

INTRODUCTION

Antibody binding affinity is defined as the strength of the interaction between a monovalent antibody and monovalent antigen whereas the bivalent binding of an antibody to a complex antigen is referred to as functional affinity or avidity.[1] The affinity of an antibody is determined by the 'fit' of the antigen in the binding groove of the Fab portion of the antibody formed by the heavy- and light-chain variable regions. Following encounter with an antigen *in vivo* an increase in antibody affinity over time is observed and this is known as affinity maturation.[2] Affinity maturation has been shown to be due to a combination of clonal selection and somatic hypermutation,[3] the latter resulting in a high rate of mutation in the complementarity determining regions (CDR) of the variable region genes. The functional affinity of an antibody plays a crucial role in determining biological activity such as the ability to clear antigen, with high affinity antibody being superior.[4] In the assessment of the immune response to vaccines and natural infection it is important therefore to characterize an antibody in terms of functional affinity as well as titre.

Whilst antibody affinity is predominantly determined by variable region structure, human antibodies of different IgG subclasses which bind the same antigen have been shown to differ in functional affinity. This was first described for tetanus toxoid[5,6] but has also been shown for a variety of other antigens including hepatitis B surface antigen,[7] *Moraxella catarrhalis*[8] and the neo-antigen keyhole limpet haemocyanin.[9]

The underlying mechanisms for differences in the functional affinity of IgG subclasses specific for the same antigen are at present not understood, but may be due to differences in epitope specificity and consequently different variable (V) region gene usage or differences secondary to the constant region structure. The latter hypothesis is supported by recent evidence that murine antibody structure remote from the V region may influence affinity and function.[10–15]

To explore the role of the human constant region in influencing functional antibody affinity we have utilized a set chimeric antibodies having different human IgG subclass constant regions linked to identical murine V regions directed against a mucin-like glycoprotein (TAG72, tumour-associated glycoprotein) purified from the human colonic cancer xenograft, LS174T.[16] This molecule is found in large amounts on bovine submaxillary mucin which has been utilized as the target antigen in these studies. Avidity and binding kinetics of the mouse–human chimeric antibodies were investigated by biomolecular interaction analysis (BIA) using a BIAcore™ instrument (Biosensor AB, Uppsala, Sweden). This technology utilizes surface plasmon resonance to detect binding events at

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the surface of a sensor chip composed of a dextran matrix to which binding partners of interest may be immobilized, allowing binding to be observed in real time and rate constants to be calculated.[17,18]

MATERIALS AND METHODS

B72.3 monoclonal antibodies

B72.3 chimeric cell lines (IgG1, Mouse Myeloma Cell; IgG2, IgG3, IgG4, Chinese Hamster Ovary) were obtained from Celltech Therapeutics Ltd (Slough, UK). For antibody purification cells were allowed to grow to saturation in 0·5 l Dulbecco's modified Eagle's minimal essential medium (DMEM) (Gibco, Paisley, UK) supplemented with 10% fetal calf serum (FCS; Globepharm, Esher, UK), 50 U/ml of penicillin, 50 μg/ml streptomycin (Gibco) and 1% non-essential amino acids (Gibco). Culture medium was then centrifuged, the supernatant filtered through a 0·45 μm filter and 1 l concentrated to approximately 10 ml using stirred concentrator cells (Amicon, Stonehouse, UK). The antibodies were purified by affinity chromatography on a Sephadex–Protein G column using fast protein liquid chromatography (FPLC; Pharmacia LKB Biotechnology, Uppsala, Sweden). A buffer of 0·1 M glycine–HCl pH 2·7 was used to elute the bound antibody and the pH of this eluate was immediately neutralized with solid Tris. Antibodies were then concentrated and dialysed against phosphate-buffered saline (PBS) pH 7·2 containing 0·01% sodium azide using Centriprep-10 concentrators (Amicon). The presence of both heavy and light chains was confirmed using reducing 10% sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS–PAGE) under reducing conditions and the absence of aggregates confirmed by non-reducing SDS–PAGE. The antibodies had the expected molecular weight (including the expected earlier elution position of the IgG3 product) following size exclusion gel filtration employing a Superdex-200 column (Pharmacia) linked to an FPLC system. The subclass composition of each chimeric antibody preparation was then analysed by antigen-specific enzyme-linked immunosorbent assay (ELISA), employing bovine submaxillary mucin (Sigma) as a capture antigen and mouse anti-human IgG subclass monoclonal antibodies (mAb) (Zymed, San Francisco, CA) to detect specific subclasses, in order to exclude contamination. Finally, protein concentrations were determined by measuring absorbance at 280 nm and assuming a value of $A^{1\%}_{1cm,280}$ of 13·6.

Preparation of F(ab')$_2$ fragments

F(ab')$_2$ fragments were obtained from the IgG1, IgG2, IgG3 and IgG4 subclasses by pepsin digestion. Antibodies were dialysed against 0·2 M sodium acetate buffer pH 4·5 overnight and pepsin (Sigma) was added at the following previously determined optimal enzyme : substrate ratios; IgG1, 1 : 100 for 10 hr; IgG2, 1 : 500–1000 for 1 hr; IgG3, 1 : 100 for 2 hr; and IgG4, 1 : 500–1000 for 1 hr. All digestions were carried out at 37° and the reaction was stopped by neutralization with 2 M Tris. F(ab')$_2$ fragments were then separated using a Superdex-200 (Pharmacia) column linked to an FPLC system and appropriate fractions were used without further concentration. The protein concentration of each preparation was determined by absorbance at 280 nm assuming a value of $A^{1\%}_{1cm,280}$ of 15·0.

Measurement of binding kinetics by biospecific interaction analysis

Antibody binding kinetics were measured using the BIAcore™ system (Biosensor AB) which permits real-time biospecific interaction analysis (BIA) by allowing analyte to pass over a sensor chip, to which antigen is covalently bound. Binding is detected by a change in refractive index expressed as resonance units (RU) depicted as a sensorgram. Analysis of the sensorgram permits the determination of rate constants.

Immobilization and kinetic measurements

Bovine submaxillary mucin (Sigma) was immobilized onto the dextran matrix of a CM5 sensor chip (Biosensor AB) in 10 mM citrate buffer pH 3·8. The dextran layer was activated for 7 min at a flow rate of 5 μl/min using an amine coupling kit (Biosensor AB) containing *N*-ethyl-*N'*-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) and *N*-hydroxysuccinamide (NHS). Approximately 900 resonance units (RU) of mucin were immobilized to the sensor chip by passing through the flow cell 35 μl of 200 μg/ml mucin in citrate buffer at a flow rate of 5 μl/min. Remaining active sites were then blocked with 1 M ethanolamine–HCl pH 8·5. Various concentrations of mAb were then passed over the immobilized mucin for kinetic analysis. After each measurement, bound antibody was washed out with 10 M NH_4SCN with no significant loss of immobilized mucin.

Kinetic calculations

Apparent rate constants k_a and k_d were calculated using BIAevaluation software from Biosensor AB. The interaction was treated as monovalent of the type antibody (Ab) + antigen (Ag) ↔ AbAg to simplify the mathematical treatment of the data, even though a portion of the AbAg complex is likely to have become an Ab2Ag complex. Detailed descriptions of the mathematical analysis have been reviewed extensively elsewhere.[19,20]

RESULTS

Kinetic measurements using real-time biospecific interaction analysis

The purity of the chimeric B72.3 mAb was checked by SDS–PAGE, specific ELISA and size exclusion gel filtration and found to be free of contaminating proteins, free of aggregates and to consist entirely of the relevant subclass (data not shown). Mucin-binding sensorgrams were obtained for all four B72.3 IgG subclasses and F(ab')$_2$ fragments of IgG1, IgG2 and IgG4 over a range of concentrations. The low yield of F(ab')$_2$ fragments from IgG3 precluded kinetic analysis. Each IgG subclass had different binding characteristics (Fig. 1). Maximal resonance units achieved for each subclass during the time–course of the injection differed with IgG4 having the highest maximal response. While antibodies at the same concentration might be expected to have similar maximal resonance units, the interaction curves observed here displayed a degree of heterogeneity. Furthermore, dissociation curves for the four antibodies showed subtle differences. IgG1 and IgG2 both demonstrated rapid initial dissociation phases while the dissociation curves for IgG3 and IgG4 appeared to be slower. These differences were observed at each concentration of antibody investigated.

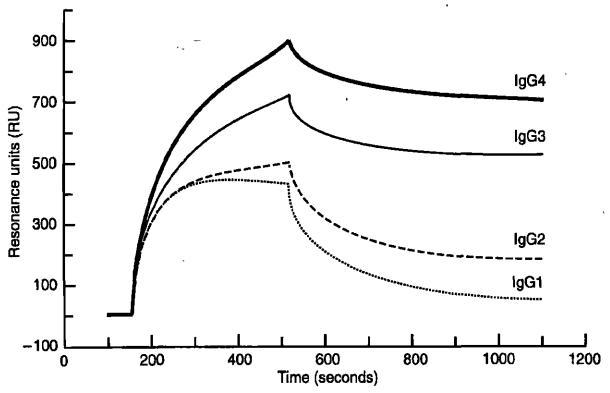

Figure 1. Sensorgrams for all four human IgG subclasses binding to mucin covalently attached to a sensor chip. Each preparation was analysed at a concentration of 170 nM.

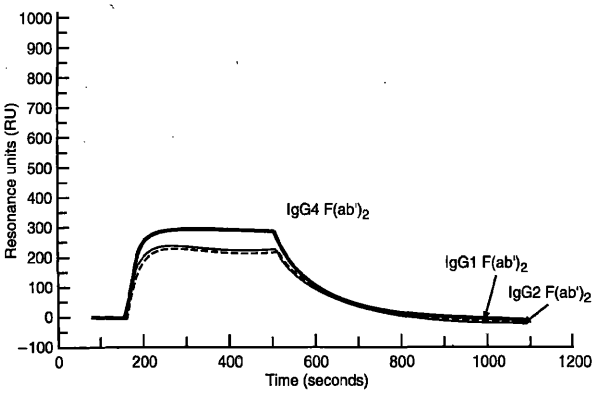

Figure 2. Sensorgrams obtained for $F(ab')_2$ fragments of human IgG1, IgG2 and IgG4 binding to mucin. All preparations were examined at a cocentration of 250 nM. The sensorgrams for the IgG1 and IgG2 fragments are virtually superimposable.

In contrast to the intact parent antibodies, binding curves for the $F(ab')_2$ fragments of IgG1, IgG2 and IgG4 were strikingly similar to each other, showing rapid association followed by rapid dissociation (Fig. 2). For IgG2 and IgG4 there was a significant difference in the pattern obtained using the intact antibodies compared with the patterns obtained with the $F(ab')_2$ fragments.

Apparent rate constants

Association and dissociation rate constants were calculated for each IgG subclass and $F(ab')_2$ fragment studied. A range of five different concentrations of each antibody were analysed. Apparent association rate constants were found to be similar for both intact antibodies and $F(ab')_2$ fragments (Table 1). However, apparent dissociation rate constants were found to differ between the IgG subclasses with IgG1 having the fastest dissociation rate and IgG4 the slowest dissociation rate. The $F(ab')_2$ fragments were found to have dissociation rates similar to each other and all were faster than the respective intact antibodies.

DISCUSSION

We have utilized V region identical humanized antibodies of the four IgG subclasses to show that despite possessing identical V regions such antibodies exhibit differences in apparent dissociation rate constants. Whilst standard techniques of affinity measurement permit the derivation of equilibrium constants, BIA provides detailed analysis of the association and dissociation phases of the antibody–antigen interaction. The antibodies studied here showed a degree of heterogeneity in their association phase, although the major differences in the antibody kinetics was in their respective dissociation rates. Such differences appear to be due to the influence of the constant region on antibody binding, as shown by their abolition following the removal of the constant region by pepsin digestion.

Morelock *et al.*, using the less sensitive technique of competitive binding ELISA, were able to show functional affinity differences between mouse–human chimeric, V region-identical IgG1, IgG2 and IgG4 antibodies specific for intracellular

Table 1. Apparent rate constants of intact chimeric IgG subclass proteins and peptic $F(ab')_2$ fragments studied*

IgG subclass	Antibody range (nM)	k_a M^{-1} $second^{-1}$ ($\times 10^5$)	k_d $second^{-1}$ ($\times 10^{-3}$)
IgG1	170–10	1·92 (±0·08)	3·21 (±0·13)
IgG2	170–10	1·98 (±0·13)	2·56 (±0·03)
IgG3	150–20	1·41 (±0·10)	1·16 (±0·06)
IgG4	170–10	1·68 (±0·18)	0·69 (±0·06)
IgG1 $F(ab')_2$	250–15	1·62 (±0·03)	4·01 (±0·15)
IgG2 $F(ab')_2$	250–15	2·09 (±0·08)	3·93 (±0·12)
IgG4 $F(ab')_2$	250–15	1·93 (±0·13)	3·71 (±0·10)

*Rate constants were calculated as the mean ± standard error of a range of concentrations of antibodies. The same portion of the binding curve was chosen for analysis for each antibody concentration. Results are expressed as the mean value obtained from five different antibody concentrations, molar values were determined assuming a molecular weight of 146 000 MW for IgG1, IgG2 and IgG4, 170 000 MW for IgG3 and 100 000 MW for $F(ab')_2$ fragments

adhesion molecule-1.[14] In their study the functional affinity of the antibodies was ranked IgG1 > IgG4 > IgG2 suggesting that the functional affinity differences could be explained by the respective subclass hinge flexibilities. The greater flexibility of the IgG1 hinge region may permit bivalent binding, in contrast to that of IgG2, the isotype thought to have the least flexible hinge region.[21–24] In our study, however, IgG1 and IgG2 had similar binding kinetics suggesting that hinge flexibility is unlikely to be the sole explanation for the differences noted above.

Differences in the functional affinity of IgG subclasses have been investigated more extensively in the murine system. Fulpius *et al.* showed that an IgG1 switch variant of an IgG3 parent lacked the expected rheumatoid factor activity despite having identical V regions.[12] Similarly, Schreiber *et al.* demonstrated functional affinity differences between an IgG1 switch variant of a V region-identical IgG3 parent specific for a *Pseudomonas* species, with IgG1 being of lowest avidity.[13] Cooper *et al.* have demonstrated differences in binding kinetics determined by BIA between mouse IgG1, IgG2b and IgG3 directed against *N*-acetyl-glucosamine (GlcNAc) of streptococcus group A carbohydrate with IgG3 being of the highest affinity.[15] The authors have suggested that the higher functional affinity of mouse IgG3 specific for GlcNAc is due to molecular co-operativity of IgG3, whereby IgG3 antibodies bound in close proximity to antigen undergo non-covalent Fc–Fc interactions stabilizing the complex. This might explain the consistent finding of higher IgG3 functional affinity in the murine literature although it is unlikely to explain differences in human IgG subclasses as they do not appear to display co-operative binding.[25] Furthermore, the human functional equivalent of murine IgG3 is IgG2 which is thought to have the least hinge flexibility[21–24] while human IgG3, in contrast to all the murine IgG subclasses, has a long flexible hinge. Such differences highlight the restricted structural and functional homology between the mouse and human IgG subclasses and underlines the danger in extrapolating data from the murine to the human IgG system.[26]

The functional significance of the differences in the apparent dissociation rate constants demonstrated here are as yet, unclear. However, the ability to analyse separately association and dissociation kinetics by BIA may prove crucial in the understanding of certain biological phenomena. Foote and Milstein have shown that the association constant of antibodies specific for the hapten 2-phenyl-5-oxazalone may be critical for B-cell selection and that concurrent with affinity maturation there is also kinetic maturation.[27] With regard to antibody function, the neutralizing capacity of a panel of antibodies reactive with the V3 loop of human immunodeficiency virus has recently been shown to correlate directly with dissociation rate.[28]

It appears likely that the differences observed here in the binding kinetics of chimeric mouse–human IgG subclasses may be due to structural differences in the human constant regions although the exact mechanism underlying such differences is at present unclear. In addition it remains to be established whether such differences exist between the different subclasses in a naturally occurring antibody response. Engineered antibodies may differ in their functional affinity and hence in their function, depending upon their subclass. Such consideration may be of importance when considering the choice of antibodies for therapeutic use.

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