The role of the antigen signal in LPS-induced B cell activation

by

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For my grandmother, Olga Mamchak

I kept my promise.

The experiments described in this thesis were carried out in the Division of Immunology and Cell Biology at the John Curtin School of Medical Research from March 1992 to August 1995, and at the Centenary Institute of Cancer Medicine and Cell Biology from May 1997 to December 1998. The work described in this thesis represents my own original work and has not been used to obtain any other degree. Unless otherwise stated, I have performed all the experiments described in this thesis.

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T cell independent type-1 B cell activation is characterised by the B cell response to LPS. This has been extensively studied by Coutinho and Möller who obtained bell shaped dose response curves for LPS-induced antigen specific antibody secreting cell (ASC) formation. Based on their experiments, Coutinho and Möller proposed the one signal model of B cell activation which suggested that surface immunoglobulin (sIg) passively binds to LPS (focusing it to the B cell surface) and that high doses of LPS induce B cell paralysis (Coutinho and Möller, 1975). The passive (non-signalling) role for sIg proposed by Coutinho and Möller is at odds with more recent experiments that demonstrate that sIg-mediated signals have an integral role in regulating B cell behaviour. A possible role for a sIg-mediated (antigen) signal is as a negative regulator of the LPS-induced B cell differentiation to ASCs. This thesis examines this possibility in an effort to reconcile Coutinho's and Möller's original experimental data with current knowledge about the antigen signal.

The experiments described in this thesis do not support Coutinho's and Möller's conclusion that high doses of LPS induce B cell paralysis. In the absence of high dose paralysis the down turn in LPS-induced polyclonal ASC formation could be accounted for by media effects or a toxic contaminant in Coutinho's and Möller's preparation of LPS, whereas the decline in antigen-specific ASC number could be due to a sIg-mediated signal. This prediction was tested by including trinitrophenol (TNP)-Ficoll in B cell cultures stimulated with LPS. TNP-Ficoll caused a dose-dependent decline in the number of LPS-induced anti-TNP ASCs. Thus, these results support the premise that sIg-mediated signals have a negative regulatory role in the production of LPS-induced ASCs.

In subsequent experiments, cyclosporine A (CsA) was used in an attempt to block the inhibitory effect of sIg-mediated signals on LPS-induced ASC formation. These experiments revealed that sIg could mediate two intracellular signals that differed in their sensitivity to CsA, threshold of activation, and effect on B cell behaviour. The CsA sensitive signal was required for anti-Ig induced proliferation, whereas the CsA insensitive signal inhibited proliferation and LPS-induced ASC formation. In addition, the results indicated that the two sIg-mediated signals were independently regulated. Further experiments using monoclonal anti-Ig antibodies supported this conclusion. Furthermore, these experiments suggested that the pattern of sIg-mediated signalling was determined by the physical form of the substitute antigen. Finally, the independence of the two sIg-mediated signals was confirmed through the use of B cells from CBA/N mice,

which do not proliferate in response mitogenic anti-Ig antibodies but are susceptible to the sIg-mediated inhibitory signal.

It was possible that the inhibitory signal induced by anti-Ig antibodies required the co-ligation of sIg and FcγRIIB. This possibility was examined through the use of a FcγRIIB blocking antibody and $F(ab')_2$ fragments of anti-Ig antibodies. The results demonstrated that co-ligation of sIg and FcγRIIB was not required for anti-Ig mediated inhibition of LPS-induced ASC formation. However, FcγRIIB-mediated signals did inhibit the CsA sensitive signal that was required for B cell proliferation. Thus, co-ligation of sIg and FcγRIIB appeared to selectively inhibited one component of the antigen signal. In a similar manner, interleukin (IL)-4 only affected one component of the antigen signal. Thus, IL-4 enhances the CsA sensitive signal without effecting the CsA insensitive signal.

An alternative explanation for the observed B cell behaviour was that it represented the response of different B cell populations, rather than being the result of different sIg-mediated signals. Experiments with electronically purified $sIgD^{hi}/CD23^{hi}$ and $sIgD^{lo}/CD23^{lo}$ B cell populations showed that both populations were susceptible to the two sIg-mediated signals. When stimulated with anti-Ig alone, or in combination with IL-4, the $sIgD^{hi}/CD23^{hi}$ population appeared to be more sensitive to the sIg-mediated positive signal for growth. However, in the presence of LPS, both B cell populations responded in a remarkably similar manner. Therefore, these results support to conclusion that ligation of sIg can induce more that one signal.

Combined, the experiments described in this thesis have led to the conclusion that sIg binding to its ligand can induce the activation of different independently regulated intracellular signalling pathways. The results suggest that differences in the physical form of the antigen will affect its binding to sIg, producing a unique ligand receptor interaction. This physical difference in the interaction between sIg and antigen appear to be translated into distinct patterns of intracellular signalling. In this way the antigen its self can direct the B cell response. For LPS-induced B cell activation, sIg-mediated signals shut down ASC formation while promoting B cell proliferation. Thus these results reveal a previously unrecognised role for the antigen signal in the regulation of TI type-1 B cell activation. In an immune response this may result in the proliferation of the small number of B cells that have a high degree of specificity for LPS while inducing antibody production from the large number of B cells with low specificity for LPS.

^{3}H-TdR	tritiated thymidine
AEC	3-amino-9-ethylcarbazole
AP	alkaline phosphatase
APC	antigen presenting cell
APL	altered peptide ligand
ASC	antibody secreting cell
ASF	antibody secreting foci
BCIP	5-bromo-4-chloro-3-indoylphosphate
BCM	B cell medium
BCR	B cell antigen receptor
BSA	bovine serum albumin
Btk	Bruton's tyrosine kinase
CD40L	CD40 ligand
C_H	constant heavy chain
cpm	counts per minute
CsA	cyclosporin A
CTM	cytotoxicity medium
DAG	diacyl glycerol
ddH2O	double distilled water
D_H	diversity region of the heavy chain Ig genes
DMF	dimethyl formamide
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNP	dinitrophenyl
EDTA	ethylenediaminetetraacetic acid
Elispot	enzyme linked immunoabsorbant spot assay
$F(ab')_2$	divalent antigen-binding fragment produced by pepsin digestion
F(ab)	fragment of antigen binding produced by papain digestion
FCS	fetal calf serum
FDC	follicular dendritic cell
FITC	fluorescein isothiocyanate
gαIgM	goat anti-mouse IgM
GAP	GTPase activating protein
HEL	hen egg lysozyme
HEL_{memb}	membrane bound HEL
HEPES	N-(2-hydroxyethyl)piperazine-N-2-ethanesulfonic acid

HES	hydroethyl starch
HGG	human gamma globulin
HI FCS	heat inactivated fetal calf serum
hi	high
hr	hour/s
HRP	horse radish peroxidase
IFN	interferon
Ig	immunoglobulin
IL	interleukin
$InsP_4$	inositol (1,3,4,5) phosphate
IP_3	inositol trisphosphate
ITAM	immunoreceptor tyrosine-based activation motif
ITIM	immunoreceptor tyrosine-based inhibitory motif
J_H / J_L	joining region for the heavy or light chain Ig genes
Kdo	2-keto-3-deoxyoctonic acid
lo	low
LPS	lipopolysaccharide
mAb(s)	monoclonal antibody(s)
MAP kinase	mitogen activated protein kinase
MHC	major histocompatibility complex
min	minute/s
MLC	mixed lymphocyte culture
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NBT	p-nitroblue tetrazolium chloride
NF-AT	nuclear factor for activated T cells
NK cell	natural killer cell
NNP	dinitrophenyl
OD	optical density
PALS	periarteriolar lymphocytic sheath
PBA	polyclonal B cell activator
PBS	phosphate buffered saline
PBS/T	PBS containing 0.05% Tween 20
PE	phycoerythrin
PI-3 kinase	phosphatidylinositol 3-kinase
PKC	protein kinase C
PLC	phospholipase C
PMA	phorbol 12-myristate 13-acetate
PTK	protein tyrosine kinase

RAG	recombinase activating gene
rαIgG	rabbit anti-mouse IgG
RBC	red blood cell
sαIg	sheep anti-mouse Ig
sαIg-AP	sheep anti-mouse Ig coupled to alkaline phosphatase
sαIg-HRP	sheep anti-mouse Ig coupled to horse radish peroxidase
SEM	standard error of the mean
SHIP	SH2 containing inositol phosphatase
SHP-1	SH2 containing phosphatase-1
sIg	surface immunoglobulin
sn	supernatant
TAPA	target of antiproliferative antibody
TCR	T cell antigen receptor
TD	T cell dependent
TGF	transforming growth factor
Th	helper T cell
TI	T cell independent
TNF	tumour necrosis factor
TNP	2, 4, 6-trinitrophenol
V_H / V_L	variable region for the heavy or light chain Ig genes
xid	X-linked immunodefiency

Chapter 1

General introduction

1.1 Introduction

Antigen recognition by lymphocytes is a necessary event in the generation of adaptive immune responses and follows the basic rules first proposed in Burnet's clonal selection theory (Burnet 1959). This theory suggested that lymphocytes would each possess a different antigen receptor, and that antigen recognition "selected" the appropriate responding cell. Burnet also suggested that antigen recognition would lead to clonal proliferation through which the population size of antigen specific cells could be increased and memory cells generated (Burnet 1959). In addition, Burnet suggested that clonal deletion of lymphocytes that recognised self-antigens would provide a mechanism of self-tolerance. These three principal components of clonal selection theory have subsequently been demonstrated experimentally (MacLennan and Gray 1986, Gray and Skarvall 1988, Gu *et al.,* 1991, McHeyzer-Williams *et al.,* 1993), confirming the broad details of the adaptive immune response envisaged by Burnet (Burnet 1959). The detailed behaviour of the adaptive immune system has been the subject of much recent research that has revealed an astonishing degree of cell interaction and molecular agility. Examination of the role of the antigen signal in these responses has revealed that antigen recognition can induce a diverse range of behaviours (Andersson *et al.,* 1974, Julius *et al.,* 1984, Nemazee and Bürki 1989b, Gu *et al.,* 1991, Peçanha *et al.,* 1991, Grandien *et al.,* 1993, Hartley *et al.,* 1993, Parry *et al.,* 1994b, Shokat and Goodnow 1995, Tarlinton *et al.,* 1997). The biochemical characterisation of the antigen signal has also exposed a complex pattern of intracellular signalling cascades which interact with each other and with signals from other receptors (Bijsterbosch and Klaus 1985, Harnett *et al.,* 1991, Carter and Fearon 1992, Snapper *et al.,* 1995a, Doody *et al.,* 1996, Sato *et al.,* 1996, Kiener *et al.,* 1997, Li *et al.,* 1997). However, despite substantial progress in the characterisation of the antigen signal, no cohesive model has arisen for how this signal may induce such variable responses. The role played by antigen recognition and subsequent signalling in the adaptive immune response is discussed further below, with particular emphasis on the B lymphocyte.

1.2. B lymphocytes view their environment and mediate effector functions through immunoglobulins

B lymphocytes survey their environment through surface immunoglobulin (sIg) which allows these cells to bind and internalise antigen. In addition to being expressed on the cell surface, immunoglobulins (Ig) can also be secreted into serum where they act as effector molecules or antibodies promoting the neutralisation and clearance of pathogens. The difference between cell surface and secreted forms of Ig is that the latter lacks the intermembrane and cytoplasmic regions that retain sIg within the cell membrane. A

typical Ig molecule consists of two pairs of heavy and light chain proteins (reviewed in Davies and Metzger 1983, Alzari *et al.,* 1988). The shorter light chain associates with the heavy chain to form a heterodimer that is held together by a disulphide bond (Figure 1.1). The two heterodimers combine to form an Ig molecule that is stabilised by further disulphide bonds formed between the heavy chains (Alzari *et al.,* 1988). Thus, Ig molecules have bilateral symmetry. The N-terminus of both the heavy and light chains contains a highly variable region, and it is the combination of these regions that form the antigen binding pocket (Davies *et al.,* 1983, Alzari *et al.,* 1988). Therefore, each antibody has two antigen binding sites, one at the N-terminus of each arm of the heterodimer (Figure 1.1). In contrast, the amino acid sequence of the C-terminus of the heavy chain is conserved (Figure 1.1) (Davies *et al.,* 1983, Alzari *et al.,* 1988). It is through this constant region that antibodies mediate effector functions by binding to cellular receptors or complement (Davies *et al.,* 1983). Enzymatic digestion has allowed for the preparation of different fragments of Ig. Papain cuts above the disulfide bonds that join the constant regions of the heavy chain generating a monovalent fragment of antigen binding known as the F(ab), and an Fc portion (Figure 1.1). In contrast, pepsin cuts below this disulphide bond producing a divalent antigen-binding fragment known as $F(ab')_2$ (Figure 1.1).

Immunoglobulins are encoded by multiple genes that can be rearranged

Within the murine genome, the genes that encode Ig are found in component parts and the final molecule is assembled from numerous alternatives (reviewed in Honjo 1983). The germline configuration of Ig heavy chain genes consists of between 200 to 1000 alternative variable (V_H) genes, twelve to fifteen diversity (D_H) genes, four joining (J_H) genes and eight constant (C_H) region genes (Honjo 1983). In contrast to the heavy chain, there are two light chains, kappa (κ) and lambda (λ). The κ light (L) chain is encoded by multiple (~250) V_L genes, five J_L genes and one C_L gene, whereas the chromosomal arrangement of the λ light chain genes consists of only two V_L genes, and four paired J_L and C_L genes (Honjo 1983). These genes are rearranged during B cell development to assemble and juxtapose a single V_H, D_H and J_H section together for the heavy chain and a V_L and J_L for the light chain (section 1.3) (reviewed in Alt *et al.,* 1992, Schatz *et al.,* 1992, Coffman *et al.,* 1993). Following Ig gene rearrangement, the VDJ sections of the heavy chains are transcribed in association with a C_H gene (Figure 1.2), and the recombined VJ light chain genes are expressed with a single a C_L region gene (Alt *et al.,* 1992, Schatz *et al.,* 1992, Coffman *et al.,* 1993). The numerous combinatorial choices in constructing the VDJ and the VJ sections provide a mechanism

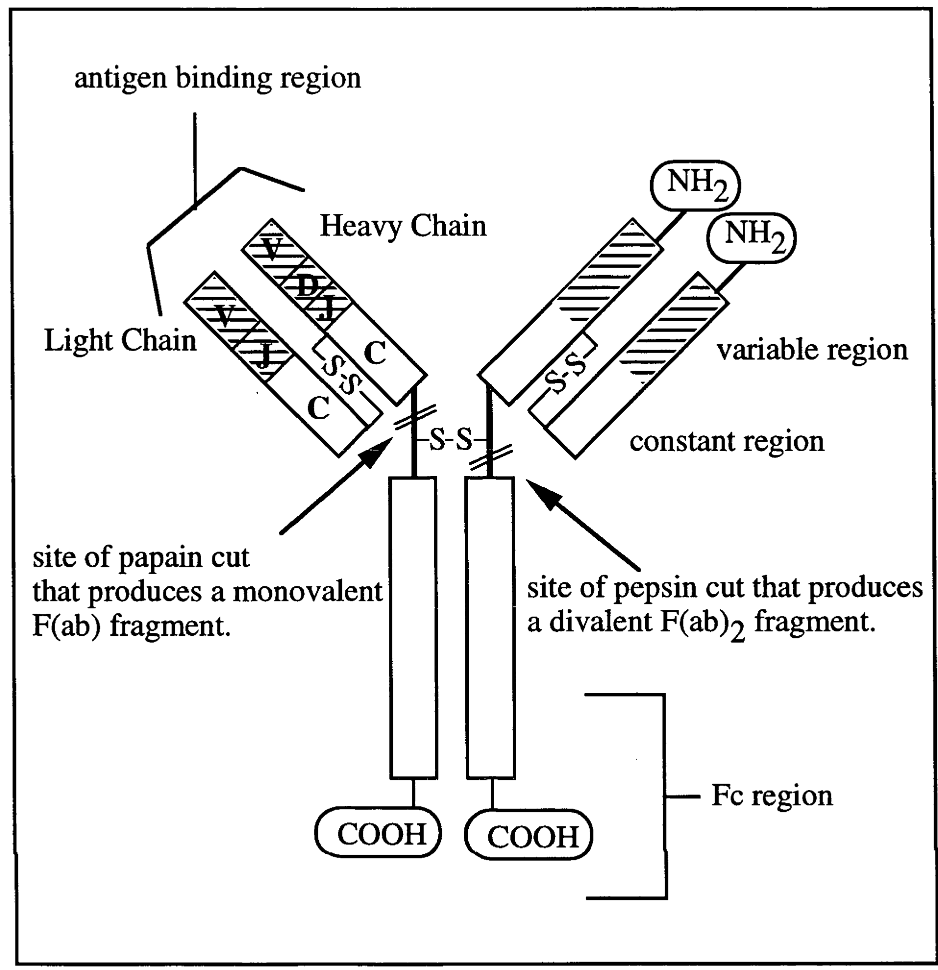

Figure 1.1 **The basic structure of antibody**

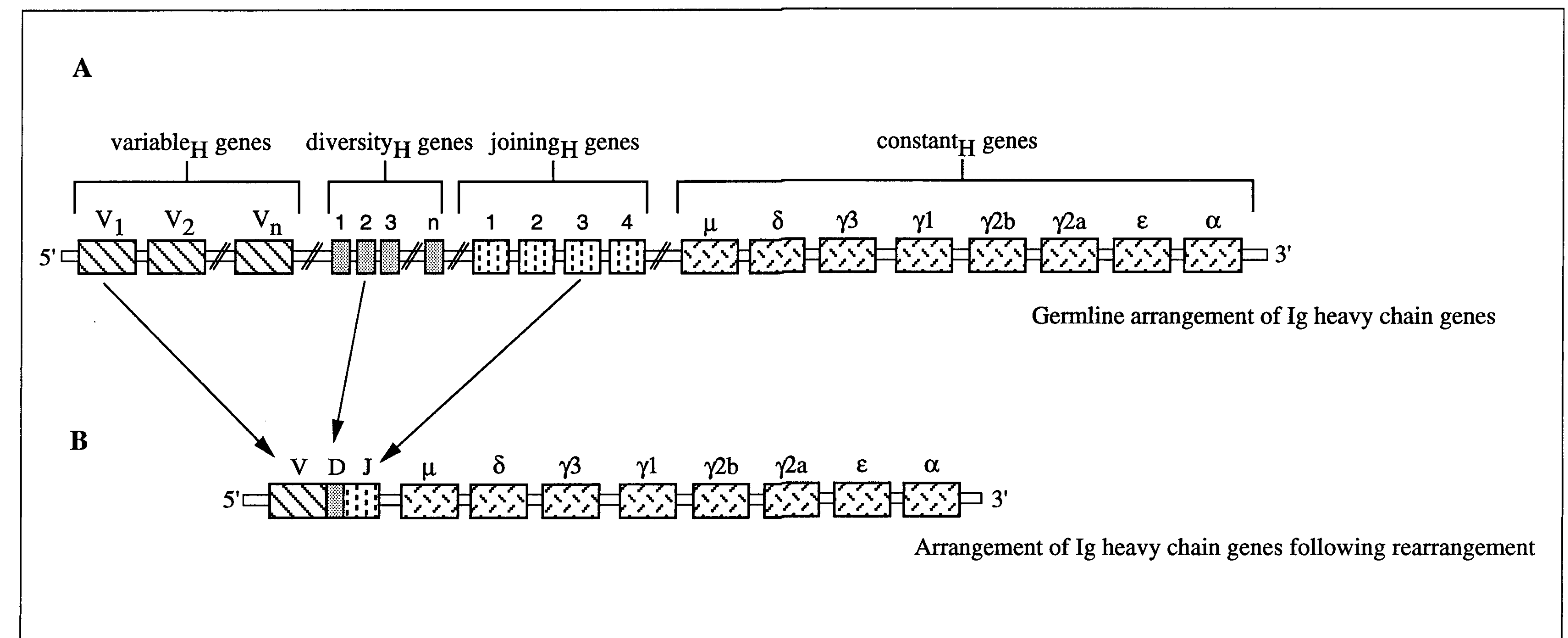

Figure 1.2 **The arrangement of the murine heavy chain Ig genes before and after gene rearrangement.**
As described in section 1.2, the Ig heavy chain genes that encode the variable region are found in component parts, with each component having a number of possible alternatives. The germline configuration of these genes (A) is rearranged during B cell development to bring one V, D, and J gene adjacent to each other to form a complete coding region for the varible heavy chain (B). Adapted from Alt *et al.,* (1992), Schatz *et al.,* (1992) and Coffman *et al.,* (1993).

by which antigen receptors with a diverse range of specificities can be generated. Despite the range of possible Ig gene combinations, each B cell expresses only one set of rearranged Ig genes with a single antigen specificity and light chain isotype. This phenomenon is known as allelic and isotype exclusion.

The constant region of the heavy chain determines the effector function of antibody
A complete Ig molecule can be assembled with a number of different alternative constant regions of the heavy chain of Ig, referred to as isotypes. The mouse possesses eight isotypes and all newly developed B cells express a receptor with two isotypes, initially IgM is expressed and as the B cell matures IgD is also expressed (Figure 1.4). The process of changing C_H genes is known as isotype switching. Murine B cells can switch to one of four different IgG isotypes or to IgA or IgE. Isotype switching plays an important role in the regulation of the immune response, as different antibody isotypes have specialised functions. Thus, IgG2a, IgG2b and IgG3 antibodies are able to fix and activate serum proteins (complement) that promote the destruction and clearance of extracellular pathogens. In contrast, IgE antibodies can bind to receptors on other cells of the immune system such as mast cells and, following cross-linking of the bound IgE, these cells degranulate releasing mediators of inflammation that can aid in the clearance of parasitic infections. Thus, the ability of B cells to switch isotypes allows for the production of antibodies that help target an appropriate immune response to a particular pathogen or antigenic challenge.

The C_H genes are organised within the murine genome in the following sequence: 5' $\mu - \delta - \gamma 3 - \gamma 1 - \gamma 2b - \gamma 2a - \varepsilon - \alpha$ 3' (Honjo 1983, Coffman *et al.,* 1993, Stavnezer 1996). The DNA between the C_H genes contains switch regions located at the 5' end of each gene with the exception of $C_H\delta$ (Coffman *et al.,* 1993, Stavnezer 1996). During isotype switching the DNA between switch regions is excised, the strands rejoined, and the DNA transcribed in its new order (Figure 1.3) (Coffman *et al.,* 1993, Stavnezer 1996). Thus, for a B cell to switch from IgM to IgG1, the genes for $\mu - \delta - \gamma 3$ are deleted and the genes for VDJ_H and $C\gamma 1$ heavy chain are transcribed together to form the Ig heavy chain (Coffman *et al.,* 1993, Stavnezer 1996). As switching from one isotype to another involves the deletion of the intervening DNA the B cell cannot switch back, therefore, isotype switching may only proceed in the 5' to 3' direction (Figure 1.3) (Coffman *et al.,* 1993). As the genes for the light chain and variable region of the heavy chain are not altered during switching, the specificity of the antibody remains the same (Coffman *et al.,* 1993, Stavnezer 1996). Thus, only the function of the antibody changes in response to isotype switching (Coffman *et al.,* 1993, Stavnezer 1996).

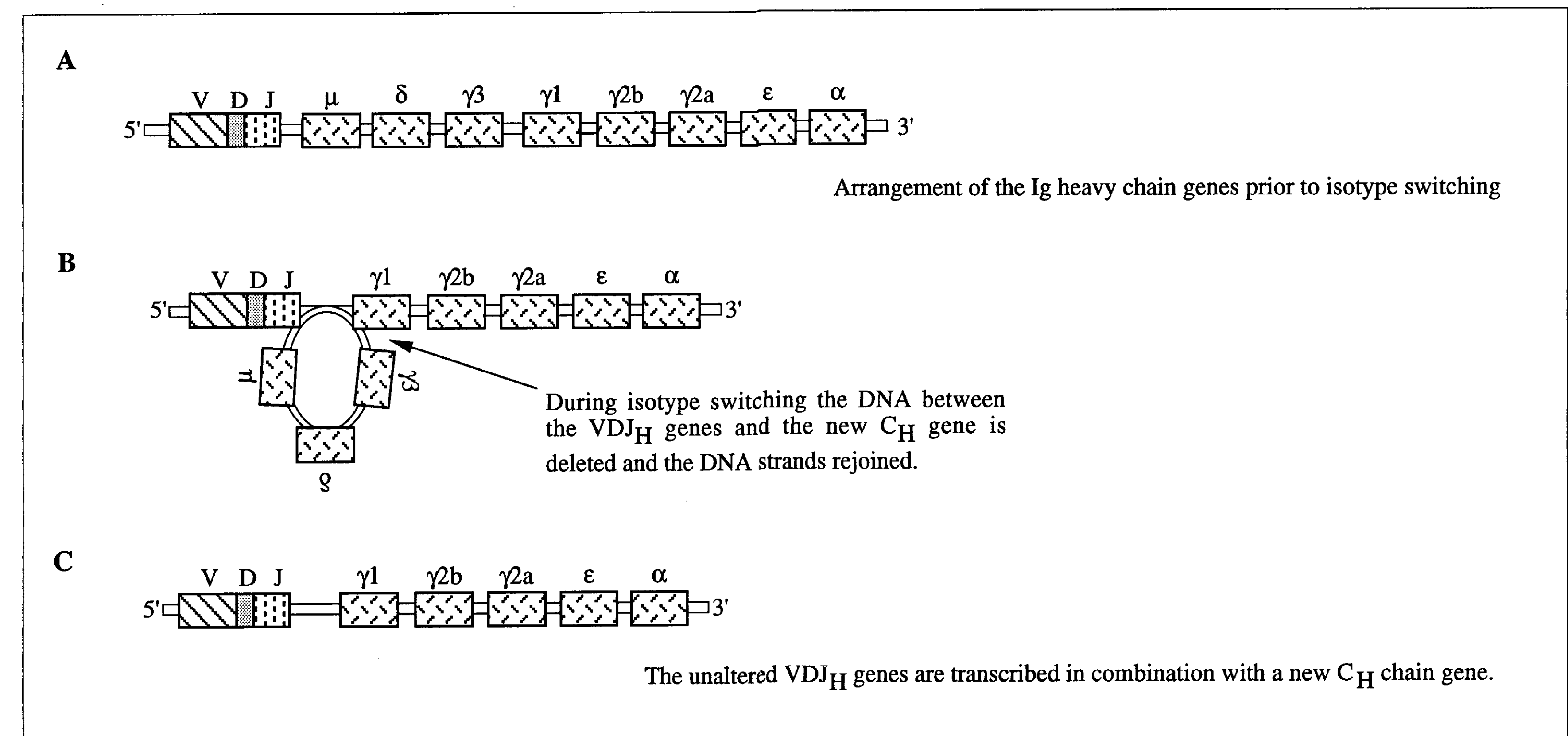

Figure 1.3 **Isotype switching.**
During B cell activation, the Ig heavy chain constant region that is transcribed may be changed without altering the specificity of the antibody. This occurs though deletional recombination, in which the germ line configuration of the constant heavy chain genes (A) is altered by the excision of the DNA between the VDJ_H genes and a new constant heavy chain gene (B). As a result of isotype switching the VDJ_H genes are place adjacent to the constant heavy chain gene which is to be transcribed (C). Adapted from Coffman *et al.*, (1993) and Stavnezer (1996)

1.3 The primary B cell repertoire is determined during B cell development

In the bone marrow pluripotent haematopoietic stem cells give rise to B cell precursors that may develop into one of two separate B cell lineages, B1a ($CD5^+$) or B2 ($CD5^-$), that differ in their behaviour and activation requirements (reviewed in Rolink and Melchers 1992, Kantor and Hertzenberg 1993, Tarlinton 1994). As the animal matures the number of $CD5^+$ B cell precursors in the bone marrow declines, whereas precursors of the B2 (often called conventional) B cell population continue to be produced for the life of the animal (Hayakawa *et al.,* 1985). The $CD5^+$ and conventional B cell populations may represent distinct B cell lineages and can be distinguished in the adult mouse by their expression of different cell surface antigens, their anatomical location and their capacity for self renewal. In the adult mouse $CD5^+$ B cells predominantly occupy the peritoneal and pleural cavities, appear to preferentially respond to carbohydrate antigens and are capable of self renewal (Hayakawa *et al.,* 1986, Herzenberg *et al.,* 1986, Förster *et al.,* 1991). In contrast, conventional B cells express CD23 and have higher levels of sIgD and lower levels of the major histocompatibility (MHC) class II antigen than B1a cells. These B cells are predominantly found in the spleen and lymph nodes and do not have the capacity for self-renewal but are replenished from the bone marrow throughout the life of the animal (Rolink *et al.,* 1992, Kantor *et al.,* 1993).

The four stages of conventional B cell development

The process of conventional B cell development can be divided into four stages that proceed from pro-B cells to pre-B cells to immature B cells and finally to mature B cells (Figure 1.4) (reviewed in Hardy 1990). Pluripotent haematopoietic stem cells generated in the bone marrow give rise to the B cell precursors known as pro-B cells (Hardy 1990). It is during the pro-B cell stage that Ig gene rearrangement commences with the fusion of the heavy chain D_H and J_H genes on both chromosomes (Figure 1.4) (Alt *et al.,* 1984). In pro-B cells where the DJ_H rearrangement is successful (translatable), the fusion of the D_H and J_H segments is followed by the rearrangement and fusion of the V_H gene to the DJ_H genes (Tonegawa 1983, Alt *et al.,* 1984). If the first rearrangement of the V_H genes is untranslatable, the process is repeated on the other chromosome (Tonegawa 1983, Alt *et al.,* 1984). Where both rearrangements are unsuccessful maturation is halted (Alt *et al.,* 1984). It has been proposed that pro-B cells that have untranslatable rearrangements on both chromosomes are deleted by apoptosis (reviewed in Osmond *et al.,* 1994). The successful joining of the V_H to the DJ_H segments marks the progression from the pro-B cell stage to the pre-B cell stage (Figure 1.4) (Hardy 1990).

	pro-B cell	**pre-B cell**	**immature B cell**	**mature B cell**
		$VDJC_H\mu$ ω and ι	IgM	IgM IgD
expression of surface Ig	nil	rearranged Ig heavy chain in combination with the surrogate light chains, ω and ι.	expression of sIgM consisting of rearranged heavy and light Ig chains.	expression of sIgM and sIgD.
Ig gene rearrangement	D_H to J_H DJ_H to V_H	VDJ_H V_L to J_L	VJ_L	completed
cell surface markers	B220, c-kit, CD44, CD40, CD19 and CD43.	B220, MHC class-II, CD44, CD40, CD19, Ig α and Igβ.	B220, MHC class-II, CD40, CD19, IgM, Ig α and Igβ.	B220, MHC class-II, CD40, CD19, IgM, IgD, Ig α and Igβ.

Figure 1.4 **B cell development.**

B cell development may be divided into four stages. This figure summarises some of the changes which occur during B cell development in terms of altered expression of cell surface markers and rearrangment of Ig genes. Adapted from: Hardy (1990); Melchers *et al.*, (1994) and Tarlinton (1994).

Pre-B cells express a functional antigen receptor

Following the productive rearrangement of the VDJ_H genes, these genes are transcribed in association with the Cμ heavy chain gene to form a functionally rearranged Ig heavy chain (Figure 1.4) (Alt *et al.,* 1992, Schatz *et al.,* 1992, Coffman *et al.,* 1993, Stavnezer 1996). In addition to producing the heavy chain, pre-B cells also make "surrogate" Ig light chain proteins ω and ι, that are encoded by the V_{preB} and λ5 genes, respectively (Tsubata and Reth 1990, Nishimoto *et al.,* 1991). The Ig heavy chain and surrogate light chains combine to produce an immature form of Ig that can be expressed on the pre-B cell surface (Tsubata *et al.,* 1990, Nishimoto *et al.,* 1991, Lassoued *et al.,* 1993). The expression of the immature antigen receptor on the pre-B cell surface requires it to be associated with Igα and Igß, two components of the mature B cell antigen receptor that are involved in signal transduction (section 1.6) (Hombach *et al.,* 1990, Lassoued *et al.,* 1993). The immature form of sIg expressed on pre-B cells has been shown to be a functional signalling molecule as ligation with anti-λ5 antibodies induces calcium mobilisation (Lassoued *et al.,* 1993). Although the role of these sIg-mediated signals in B cell development is not entirely clear, they may be involved in allelic exclusion at the Ig heavy chain locus (Loffert *et al.,* 1996). In addition, it has been proposed that signals generated through sIg binding a ligand, as yet unidentified, may promote pre-B cell survival (reviewed in Desiderio 1994, Melchers *et al.,* 1994, Pleiman *et al.,* 1994a). Thus, signals generated by the antigen receptor of pre-B cells may result in the positive selection of developing B cells based on the expression of a functional Ig heavy chain (Gu *et al.,* 1991, Tarlinton *et al.,* 1997).

Signals from the antigen receptor on pre-B cells regulate the expression of the Ig light chain genes

Light chain gene rearrangement commences after the formation of a functional Ig heavy chain and begins with the rearrangement of κ on one of the two chromosomes (Tonegawa 1983, Alt *et al.,* 1984). Signals from the antigen receptor on pre-B cells may play a role in promoting the rearrangement of the light chain Ig genes (Desiderio 1994, Melchers *et al.,* 1994, Pleiman *et al.,* 1994a). As described for the heavy chain genes, the V_L and J_L genes are rearranged prior to expression of the VJ_L with C_L region (Tonegawa 1983, Alt *et al.,* 1984). If there is no productive rearrangement of the κ genes on either chromosome, the process is repeated using the λ genes (Tonegawa 1983, Alt *et al.,* 1984). Once the light chain genes have been productively rearranged, they are transcribed and combine with the heavy chain to form a mature Ig molecule that is expressed on the surface of the developing B cell in association with Igα and Igβ (Figure 1.4) (Melchers *et al.,* 1994). The expression of sIgM marks the progression of the developing lymphocyte from the pre-B to the immature B cell stage (Hardy 1990). Once sIgM is expressed, transcription of the $V_{pre}B$ and λ5 genes is down-regulated

(Schatz *et al.,* 1992, Melchers *et al.,* 1994). Thus, sIg-mediated signals appear to regulate the transcription of the genes encoding the surrogate light chain (Desiderio 1994, Melchers *et al.,* 1994, Pleiman *et al.,* 1994a).

The antigen signal can inhibit the expression of proteins required for Ig gene rearrangement

Following expression of IgM on the surface of immature B cells the rearrangement of Ig genes is halted (Alt *et al.,* 1992, Schatz *et al.,* 1992, Coffman *et al.,* 1993). The cessation of Ig gene rearrangement is in part controlled by the down-regulation of the expression of the recombinase activation genes, RAG-1 and RAG-2 (Ma *et al.,* 1992). The RAG genes are expressed in lymphoid precursor cells and their expression is required for gene recombination (Schatz *et al.,* 1989, Oettinger *et al.,* 1990). Both RAG genes are expressed during Ig gene rearrangement, but levels progressively decline during the transition from an immature to a mature B cell (Melchers *et al.,* 1994). Mice which lack RAG-1 or RAG-2 are viable but have no mature T or B lymphocytes (Alt *et al.,* 1992). It is thought that sIg-mediated signals induce the down-regulation of RAG gene expression (Ma *et al.,* 1992). Support for the role of an antigen signal in regulating RAG gene expression can be obtained from *in vitro* experiments where the treatment of immature B cell lines with anti-IgM antibodies inhibits RAG gene expression (Ma *et al.,* 1992). In addition, a combination of phorbol 12-myristate 13-acetate (PMA) and calcium ionophores, which partially reproduces some of the signalling events mediated by sIg (section 1.6) (Klaus *et al.,* 1986), causes the down-regulation of RAG gene expression (Schatz *et al.,* 1992). Thus, there appears to be a feedback mechanism from sIg-mediated signals that regulates RAG gene expression (Schatz *et al.,* 1992).

1.4 The fate of the developing B cell is determined by its interaction with antigen

The number of immature B cells produced that express sIgM far exceeds the number that leave the bone marrow and develop into mature B cells (reviewed in Osmond 1986). This suggests that a large number of developing B cells are being deleted within the bone marrow. The B cells that are exported to the periphery are thought to have survived a process of negative selection (reviewed in Nossal 1994, Melchers *et al.,* 1995). There is also evidence to indicate that B cells undergo a positive selection at earlier stages of differentiation to determine which cells will progress onto the next phase of development (Desiderio 1994, Melchers *et al.,* 1994, Pleiman *et al.,* 1994a). The outcome of these sIg-mediated selective processes is discussed below.

1.4.1. Negative selection of self-reactive B cells - a choice between deletion and anergy

The final product of Ig gene rearrangement during development is a B cell that expresses a functional Ig with a single, randomly chosen, specificity. As Ig gene rearrangement is random it is inevitable that some B cells will be specific for self-antigens. These self-reactive B cells must be silenced in order to maintain self-tolerance and thereby prevent detrimental autoimmune responses (reviewed in Basten *et al.,* 1991, Nossal 1992). Burnet originally proposed that self-tolerance would occur as the result of cellular deletion following the interaction between the antigen receptor and a self-antigen (Burnet 1959). It has subsequently been demonstrated that this form of negative selection can occur during B cell development in the bone marrow, and is in part responsible for self-tolerance in the primary B cell repertoire (Nemazee and Bürki, 1989b, Nemazee and Bürki 1989a, Hartley *et al.,* 1991, Goodnow 1992). In addition, immature self-reactive B cells may also be silenced by the induction of a state of non-responsiveness (anergy) (Nossal and Pike 1980, Pike *et al.,* 1982, Nemazee and Bürki, 1989b, Hartley *et al.,* 1993). As illustrated in the following sections, the choice for the self-reactive B cell between clonal deletion or anergy appears to be dependent on the physical form of the antigen.

Anti-Ig antibodies provided the initial experimental evidence for clonal deletion

Lawton and Cooper demonstrated that mice treated from birth with anti-IgM antibodies failed to develop B cells (Lawton and Cooper 1974). Similar results were obtained in neonatal mice where treatment with anti-IgM antibodies resulted in the deletion of all developing B cells, excluding pro-B cells which do not express any form of sIg (Opstelten and Osmond 1985). These results indicated that ligation of sIg on developing B cells could cause the deletion of the cell. Additional *in vitro* experiments using WEHI-231 cells showed that anti-Ig antibodies could induce apoptosis in these cells (Hasbold and Klaus 1990). As the WEHI-231 cell line has been used as a model for immature B cells (Tisch *et al.,* 1988), these results have led to the suggestion that clonal deletion of self-reactive B cells occurs through the induction of apoptosis (Hasbold *et al.,* 1990).

B cells that recognise membrane bound self-antigens undergo clonal deletion

Additional support for clonal deletion has been obtained through a second experimental approach involving the use of transgenic mice that express Ig genes specific for self-antigens. Nemazee and Bürki developed a transgenic mouse model where B cells expressed sIgM specific for epitopes within the H-2^k allele of MHC class-I. In mice that expressed the H-2^d allele for MHC class-I, the anti-H-2^k transgenic B cells developed normally (Nemazee and Bürki, 1989b). In contrast, the progeny of a cross

between the transgenic H-2^d mouse and the H-2^k MHC mice lacked B cells, indicating that exposure to the self-antigen prevented the appearance of H-2^k specific B cells in the periphery (Nemazee and Bürki, 1989b). In additional experiments, bone marrow from the anti-H-2^k IgM transgenic mice was adoptively transferred into irradiated H-2^k recipients. Although B cell precursors that expressed low levels of anti-H-2^k IgM could be detected in the bone marrow, no anti-H-2^k B cells could be found in the periphery (Nemazee and Bürki, 1989a). From these experiments Nemazee and Bürki concluded that self-reactive anti-H-2^k B cells were deleted in the bone marrow following recognition of the self-antigen by sIg.

Similar results were obtained in a transgenic mouse model in which mice expressed two transgenes, where one encoded a novel self-antigen, hen egg lysozyme (HEL), and the other expressed anti-HEL Ig (Goodnow *et al.,* 1988). In the mice that express only anti-HEL Ig, B cells appear to develop normally and, following activation, secrete anti-HEL antibodies (Goodnow *et al.,* 1988). However, in double transgenic mice where HEL is expressed as a membrane protein (HEL_{memb}), self-reactive B cells are absent in the periphery (Hartley *et al.,* 1991, Goodnow 1992). In additional experiments using B cells from mice that express the transgenes for anti-HEL Ig, HEL_{memb} and bcl-2, transgenic B cells with an immature phenotype were found in the periphery (Hartley *et al.,* 1993). Constitutive expression of bcl-2 has been shown to inhibit the onset of apoptosis, and to prolong the life of lymphocytes (McDonnell *et al.,* 1989, Strasser *et al.,* 1990, Strasser *et al.,* 1991). Thus, these results indicate that the inhibition of apoptosis by bcl-2 allows immature self-reactive B cells to escape deletion in the bone marrow (Hartley *et al.,* 1993, Merino *et al.,* 1994). The experimental results from the two transgenic model systems clearly indicate that a sIg-mediated signal induced by the interaction with membrane bound self-antigens can cause apoptosis in developing self-reactive B cells resulting in clonal deletion.

Soluble self-antigens induce a hyporesponsive state in self-reactive B cells

Unlike clonal deletion, anergy was thought not to involve rapid deletion of self-reactive B cells, but instead the development of a non-responsive state (Nossal *et al.,* 1980, Pike *et al.,* 1982, Goodnow *et al.,* 1988, Hartley *et al.,* 1993). Clonal anergy was demonstrated in transgenic mice that expressed anti-HEL sIg and soluble HEL. In these double transgenic mice, HEL-specific B cells were released from the bone marrow and appeared in the periphery. These cells expressed normal levels of sIgD but only expressed a low level of anti-HEL sIgM (Goodnow *et al.,* 1989). Unlike normal B cells, the transgenic B cells did not secrete significant amounts of antibody when stimulated *in vivo* with HEL coupled to sheep red blood cells, or *in vitro* with lipopolysaccharide (LPS) (Goodnow *et al.,* 1989, Brink *et al.,* 1992). Originally it was

raising the question of the role of such anergic cells. However, it has since been demonstrated that the anergic HEL-specific B cells in the double transgenic mice have a short life span, surviving for only three to four days, in contrast to non-transgenic B cells which live four to five weeks (Hartley *et al.,* 1993, Fulcher and Basten 1994).

Anergic B cells can be activated and rescued from death

While self-reactive 'anergic' B cells in the double transgenic mice appear to be functionally silenced and are deleted a short time after being exported to the periphery, it is still possible to reverse the tolerising effect of self-antigens (Eris *et al.,* 1994). Eris *et al.,* (1994) demonstrated that anergic B cells would secrete antibody when cultured with activated T cells. Thus, anergy may represent a transitional state for the B cell in which signals from activated T cells could induce B cell activation rescuing it from death and resulting in antibody production, whereas in the absence of stimulatory T cell signals the B cell would be deleted. The reversal of anergy by activated T cells would be an unlikely event, however, as self-reactive B cells would have to receive T cell help within three to four days of leaving the bone marrow in an environment where self reactive T cells would also be tolerant (Fulcher *et al.,* 1994).

Thus, the interaction between sIg and self-antigens is responsible for the induction of tolerance in the primary B cell repertoire (Brink *et al.,* 1992, Cooke *et al.,* 1994, Monroe 1996). As illustrated by the HEL transgenic mice, different physical forms of the same antigen can induce distinct types of tolerance, or at least determine the time it takes a self reactive cell to die (Hodgkin and Basten 1995, Fulcher *et al.,* 1996). The different B cell response to membrane bound or soluble self-antigens suggests that their interaction with sIg results in distinct patterns of intracellular signalling. In the HEL transgenic system this may simply reflect the degree of sIg cross-linking induced by the self-antigen (reviewed in Goodnow 1992). If the strength of the sIg-mediated signal is simply proportional to the level of sIg cross-linking, then the membrane bound self-antigens - which presumably cause a higher density of sIg cross-linking - would deliver a stronger signals when compared to soluble self-antigens (Goodnow 1992). Thus, membrane bound self-antigens could induce a signal that exceeds the threshold for clonal deletion, whereas soluble self-antigens may induce a sub-threshold antigen signal allowing the self-reactive B cell to enter the periphery in search of additional activation signals, in the absence of which the anergic B cell dies (Hodgkin *et al.,* 1995).

1.4.2. Antigen driven positive selection

In addition to inducing tolerance the antigen signal is also involved in the positive selection of developing B cells (Gu *et al.,* 1991, Nadel *et al.,* 1993, Lam *et al.,* 1997,

Tarlinton *et al.,* 1997). The requirement for positive selection in B cell development was proposed as a result of analysis of the V_H chain genes represented in the pre-B cell and peripheral B cell population that revealed that the gene usage substantially differed between the two populations (Gu *et al.,* 1991). Gu *et al.,* (1991) suggested that this difference was the result of antigen dependent positive selection based on the expression of particular V_H chain genes. It is probable that positive selection during the pre-B cell stage occurs through the interaction of the immature sIg with an unidentified ligand or ligands (Gu *et al.,* 1991). Thus, in order to reach the periphery, developing B cells must survive a two stage process involving both positive and negative selection.

Thus far, this discussion has focused on the multiple roles that the antigen signal plays during B cell development. Clearly, the antigen signal regulates the primary repertoire by selecting cells that express a functional sIg by positive selection and by inducing self-tolerance. Therefore, the antigen signal can have opposing roles in B cell development by inducing cell survival or deletion. However, it is not obvious whether these apparently contradictory effects are due to variation in the antigen signal, or are the result of differences in the B cell based on maturation state or other signals it has received. Multiple and contradictory effects of sIg-mediated signals on B cell behaviour are also apparent in the varied roles that the antigen signal plays during B cell activation resulting in the formation of antibody secreting cells (ASCs).

1.5 B cell activation - what role does the antigen signal play?

B cell activation occurs as a result of antigen exposure to mature B cells. However, this response can be divided into two types based on the requirement for the involvement of T cells. Thus, B cells may be activated in either a T cell dependent (TD) or T cell independent manner (TI). The latter model can be sub-divided further into TI type-1 and TI type-2 categories. While there is no single definitive stimulatory role for the antigen signal in all forms of B cell activation, this signal plays an important regulatory role in the B cell response to both TD and TI antigens. This is discussed in the following sections.

1.5.1 An overview of TD B cell activation

In recent years TD B cell activation has been studied in considerable detail and can now be broken down into a sequence of steps (Figure 1.5). The initial step is the recognition of antigen by the B cell, which occurs through sIg binding to epitopes within the antigen. The subsequent antigen signal induces a number of changes in the B cell including *de novo* expression of cell surface receptors involved in T cell-B cell collaboration. Captured antigen is also internalised and, if it contains protein, is

processed and presented in association with class-II MHC on the B cell surface (Figure 1.5). Antigen presentation by B cells allows the B cell to interact with antigen-specific T cells initiating a dialogue that results in T cell activation and the provision of T cell help. This help is delivered through the interaction between novel cell surface molecules on the T cell and by the secretion of soluble cytokines, which together induce B cell activation and differentiation to ASCs (Figure 1.5). The influence of the antigen signal and the steps involved in TD B cell activation are described in greater detail below.

B cells process antigen and present it in association with class-II MHC

Antigen presentation by the B cell is a crucial step in successful B cell T cell collaboration, and is initiated when sIg binds to antigen and internalises it via receptor-mediated endocytosis (reviewed in Lanzavecchia 1990, Neefjes and Ploegh 1992, Neefjes and Momburg 1993). Although sIg binding to antigen can induce an intracellular signal, this signal is not required for the processing and presentation of antigen (Davidson *et al.,* 1990). Receptor mediated endocytosis directs the intact antigen into an acidic endosomal compartment from where the antigen is transferred into lysosomes (Lanzavecchia 1990, Neefjes *et al.,* 1992, Neefjes *et al.,* 1993). During the passage from the endosome to the lysosome, intact protein antigen is degraded into peptides that subsequently associate with class-II MHC molecules (Harding *et al.,* 1991). Once bound, the peptide stabilises the conformation of class-II MHC and the peptide / class-II MHC complex is exported to the B cell surface via the *trans*-golgi network (Lanzavecchia 1990, Neefjes *et al.,* 1992, Neefjes *et al.,* 1993).

Contact dependent signals between T and B cells amplify the initial antigen-dependent activating signals

Prior to the internalisation of antigen, the interaction between antigen and sIg generates transmembrane signals that can induce B cell migration in the spleen to the outer periarteriolar lymphoid sheath (PALS) where T cell-B cell collaboration is initiated by T cell recognition of antigen presented on the surface of the B cell (van den Eertwegh and Claassen 1992, Kelsoe and Zheng 1993). Antigen recognition by T cells occurs through the binding of the T cell antigen receptor (TCR) to the peptide / class-II MHC complex on the antigen presenting B cell (Figure 1.5, C) (Kronenberg *et al.,* 1986, Allison and Lanier 1987, Davis and Bjorkman 1988). The antigen signal also results in the expression of the B cell surface molecules, B7.1 and B7.2 (Hathcock *et al.,* 1994, Lenschow *et al.,* 1994). The interaction between B7.2 and its ligand, CD28, in combination with the interaction between the TCR and the peptide / class-II MHC complex, induces the *de novo* expression of CD40L on the T cell (de Boer *et al.,* 1993, Klaus *et al.,* 1994) and increases the expression of B7.2 on the B cell (Hathcock *et al.,* 1994, Stack *et al.,* 1994). The binding of CD40 to its ligand further up regulates the

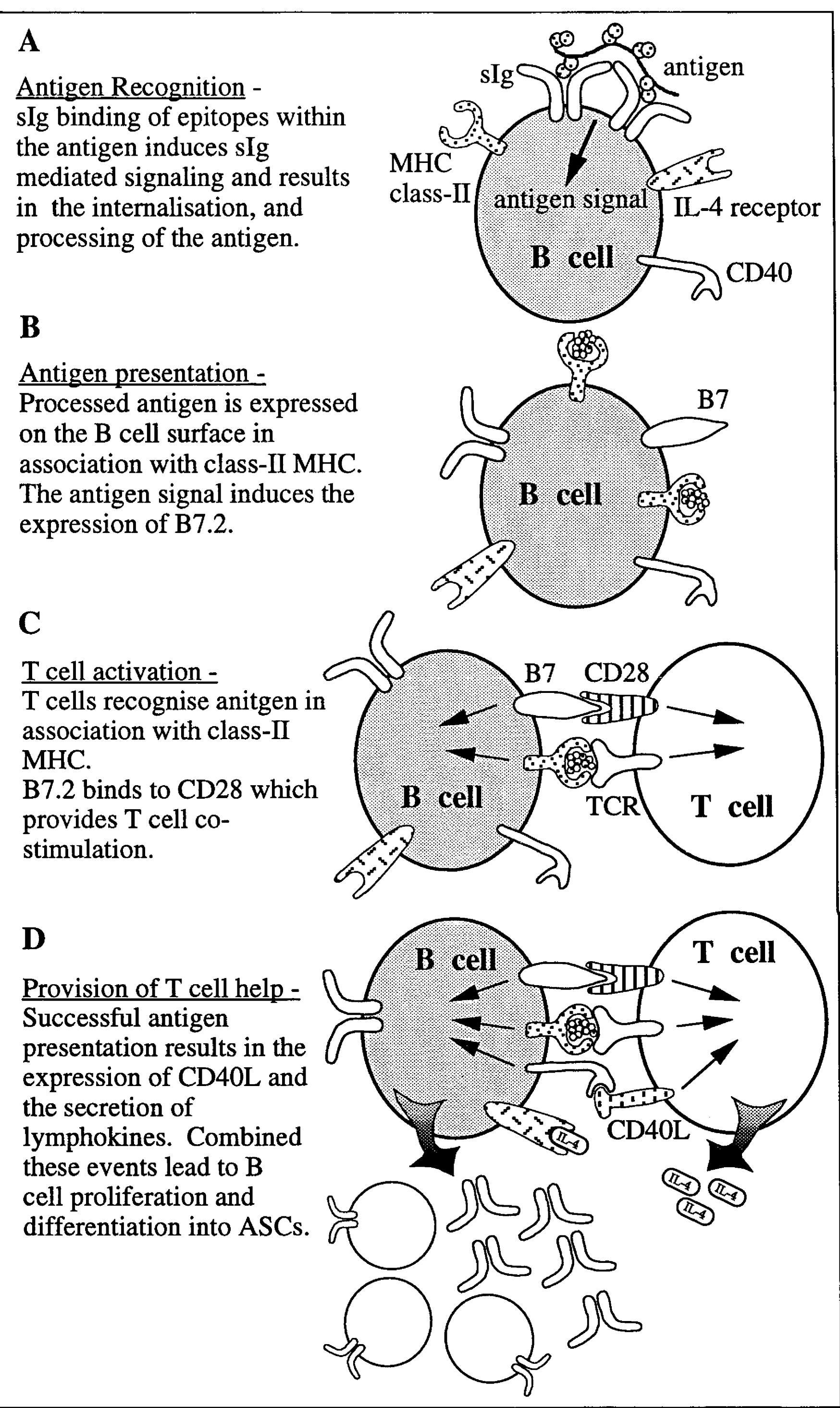

Figure 1.5 **Events in T cell B cell collaboration**

This Figure summarises some of the events that result in sucessful T cell B cell colaboration as described in section 1.5. Adapted from Hodgkin and Kehry (1992), Kehry and Hodgkin (1993), and Hodgkin and Basten (1995)

expression of B7.2, intensifying the level of costimulation to the T cell and thereby further raising the expression of CD40L (Ranheim and Kipps 1993, Hasbold *et al.,* 1994, Roy *et al.,* 1995). Thus, the interaction between the T and B cell surface antigens progressively amplifies the activating signals for both lymphocytes (Hodgkin *et al.,* 1995). The result of this interaction is in part dependent on the activation state of the T cell, as dictated by previous encounter with antigen (Lassila *et al.,* 1988, Ronchese and Hausmann 1993, Morris *et al.,* 1994). However, at a minimum the interaction between class-II MHC and the TCR, combined with B7.2 binding to CD28, stimulates T cell activation and the provision of T cell help delivered through CD40L and cytokines (Figure 1.5, C) (Noelle and Snow 1991, Hodgkin and Kehry 1992, Kehry and Hodgkin 1993, Hodgkin *et al.,* 1995). Thus, sIg-mediated signals can promote TD B cell activation by up-regulating the expression of B7.2, thereby promoting T cell activation and the provision of T cell help. Given this role for sIg-mediated signals it would not be unreasonable to conclude that the antigen signal played an obligatory role in this form of B cell activation, as originally proposed by Bretscher and Cohn (Bretscher and Cohn 1968, Bretscher and Cohn 1970). However, experimental evidence from *in vitro* systems where T cell activation was not dependent on antigen presentation by the B cell have demonstrated that the sIg-mediated signals themselves are not essential for TD B cell activation (Augustin and Coutinho 1980, Brian 1988, Hodgkin *et al.,* 1990) beyond the requirement to bring antigen specific cells together.

B cells can be activated by T cell contact and secreted cytokines in the absence of an antigen signal

The first indication that the antigen signal was not obligatory for B cell activation came from *in vitro* experiments with alloreactive T cell clones that could induce B cell differentiation to ASCs in the absence of an antigen signal (Augustin *et al.,* 1980). However, these conclusions were considered controversial. In separate experiments, sIg-mediated signals were found to be required for the activation of small naive B cells by T cell clones (Julius *et al.,* 1982) and for antigen specific B cell activation (Zubler and Kanagawa 1982). Subsequent work using T cell membranes provided support for the view that the sIg-mediated signals were not obligatory in TD B cell activation. These experiments allowed the minimum requirement for TD B cell activation to be identified. Thus, stimulation of B cells with membranes prepared from activated, but not resting, T cell clones induced B cell proliferation but not antibody production (Brian 1988, Hodgkin *et al.,* 1990). In contrast, the combination of IL-4 and IL-5 and activated T cell membranes enhanced the previously observed proliferation and induced B cell differentiation to ASCs (Hodgkin *et al.,* 1990). In each of these experiments the B cell response was polyclonal and occurred in the absence of antigen (Brian 1988, Hodgkin *et al.,* 1990). The essential T cell derived and contact-dependent ligand expressed on the

T cell surface following activation was subsequently identified as a ligand for CD40, a cell surface molecule constitutively expressed on B cells (Armitage *et al.,* 1992, Hollenbaugh *et al.,* 1992). Support for the role of CD40L in TD B cell activation was provided by Noelle *et al.,* who demonstrated that B cell proliferation and antibody production stimulated by activated T cell membranes and stimulatory cytokines could be inhibited by anti-CD40L antibodies (Noelle *et al.,* 1992a). In addition, stimulation of B cells with recombinant CD40L and IL-4 was shown to be sufficient to induce B cell proliferation and antibody production (Armitage *et al.,* 1992, Spriggs *et al.,* 1993). Thus, the interaction between CD40 and its ligand appears to represent an essential step in TD B cell activation (Figure 1.5, D). Further, these experiments demonstrate that B cell proliferation and polyclonal antibody production can be induced in the absence of a sIg-mediated signal.

T cell cytokines can amplify TD B cell activating signals

TD B cell activation to Ig secretion requires the presence of cytokines that are produced by the activated T cells (Figure 1.5, D) (Coffman *et al.,* 1988, Mosmann and Coffman 1989, Schmitz *et al.,* 1993). Cytokines have been divided into two groups based on their expression by two clones of T cells, referred to as T helper (T_h) type-1 and T_h type-2 (reviewed in Coffman *et al.,* 1988, Mosmann *et al.,* 1989). The T_h type-1 cytokines promote cell mediated immunity and include: IL-2, tumour necrosis factor-α (TNF-α), and interferon-γ (IFN-γ) (Coffman *et al.,* 1988, Mosmann *et al.,* 1989). In contrast, the T_h type-2 cytokines are stimulators of B cell responses and include: IL-4, IL-5, IL-6 and IL-10 (Coffman *et al.,* 1988, Mosmann *et al.,* 1989). In mice one of the most potent T_h type-2 cytokines for inducing TD antibody production is IL-4 (Coffman *et al.,* 1988). IL-4 can promote TD B cell activation by enhancing the B cell response to sIg and CD40 mediated signals (Howard *et al.,* 1982, Hodgkin *et al.,* 1991a, Banchereau *et al.,* 1993, Lane *et al.,* 1993, Maliszewski *et al.,* 1993), and by up-regulating the expression of B7.2, class-II MHC and CD40 (Vallé *et al.,* 1989, Hathcock *et al.,* 1994, Stack *et al.,* 1994). In this way the presence of IL-4 can amplify the activation signals between T and B cells.

Early TD antibody production in the foci

Antigen activated B cells migrate to the edge of the PALS where, following successful T cell-B cell collaboration, they proliferate and differentiate into ASCs (Liu *et al.,* 1991c, Kelsoe *et al.,* 1993). This response leads to the formation of an antibody secreting foci (ASF) at this site (Jacob *et al.,* 1991a). ASCs are formed within four to six days after exposure to TD antigens, and the early antibody produced is IgM. However, over time the B cells undergo isotype switching and secrete IgG (Jacob *et al.,* 1991a). Antibody production within the foci is maintained for approximately 10 days after the initial

exposure to antigen (Jacob *et al.,* 1991a) before the activated cells dissipate through apoptosis (Smith *et al.,* 1996) .

The antigen signal controls the selection of high affinity B cells

Soon after ASF appear, germinal centers begin to form in the follicular region of the splenic white pulp. Germinal centers are populated by B cells, T cells and a unique antigen presenting cell, the follicular dendritic cell (FDC) ((reviewed in Van Rooijen 1993, Tew *et al.,* 1997). FDCs express Fc and complement receptors which can retain antigen-antibody complexes for periods greater than a year (Nossal *et al.,* 1965, Tew *et al.,* 1997). It is within the germinal centers that high affinity ASCs are generated (reviewed in Liu *et al.,* 1992, Berek and Ziegner 1993, MacLennan 1994, Wagner and Neuberger 1996). Germinal centres arise from a small population of B cells (founder cells) that migrate to the primary lymphoid follicles and undergo rapid proliferation (Liu *et al.,* 1991c, Jacob and Kelsoe 1992). It has been suggested that these founders may have been previously activated in the foci (Jacob *et al.,* 1992). The proliferation of the founder gives rise to centroblasts, a rapidly dividing (6-7 hr division time) population of cells which do not express sIg (Liu *et al.,* 1991c, McHeyzer-Williams *et al.,* 1993). It is throught that during the division of the founder cells or centroblasts that point mutations are introduced into the variable region of the Ig genes (Berek *et al.,* 1991, Jacob *et al.,* 1991b). This process is known as somatic hypermutation and can lead to changes in the specificity or affinity of antibodies (MacLennan 1991, Wagner *et al.,* 1996).

Centroblasts give rise to centrocytes, a non-dividing population of cells that express the newly mutated sIg (Liu *et al.,* 1992, MacLennan 1994). As the mutations in the variable region of the Ig genes are random, it is possible that they may improve antigen binding. However, they may also change the specificity of Ig so that it now recognises self-antigens. Thus, as for the primary B cell repertoire, the specificity of the B cells that have undergone somatic hypermutation must be tested and self-reactive B cells again deleted (Nossal 1992, MacLennan 1994). Centrocytes that express high affinity antigen-specific antibody undergo positive selection that is thought to induce the up-regulation of the cell survival gene bcl-2, whereas cells that express sIg which has a reduced affinity for antigen, die presumably due to the absence of an antigen signal (Kelsoe 1996, Tarlinton and Smith 1997). In contrast, B cells that have developed autoreactivity undergo negative selection that is thought to induce apoptosis and maintain self tolerance (Han *et al.,* 1995, Pulendran *et al.,* 1995, Shokat *et al.,* 1995, Galibert *et al.,* 1996). The antigen signal generated as a result of sIg binding to antigen presented by FDCs in part determines the fate of B cells undergoing selection (Kelsoe 1996, Tarlinton *et al.,* 1997). However, while it is clear that the antigen signal regulates B cell selection in the germinal center, it is not obvious how ligation of sIg can both promote B

cell survival if induced by foreign antigen and induce cell death if mediated by self antigen. The resolution may depend on the site of antigen exposure. It is possible that when FDC present antigen to the B cell additional stimulatory receptors are engaged that will be absent from B cells seeing antigen in serum or on the surface of other cell types (Liu *et al.,* 1991d, Liu *et al.,* 1991e).

The germinal centre response also results in the formation of memory B cells (reviewed in Berek *et al.,* 1991, Nahm *et al.,* 1992, Gray 1993). The signals that regulate the developmental choice between B cell differentiation to ASCs or memory cells are still unclear but may involve an antigen signal (McHeyzer-Williams *et al.,* 1993).

These examples illustrate the different effects that sIg-mediated signals have on different aspects of the B cell response to TD antigens. Although the antigen signal is not required for TD B cell activation, it does promote B cell activation in response to these antigens. Thus, sIg-mediated signals could be considered to be positive regulators of T cell-B cell collaboration. In addition, sIg-mediated signals help to maintain B cell tolerance following somatic hypermutation. Therefore, the antigen signal can also have a negative effect on B cells activated by TD antigens. Clearly, sIg-mediated signals have inhibitory and stimulatory effects on B cell behaviour induced by TD antigens. A similarly complex role for the antigen signal is also found for the TI response as described in the following sections.

1.5.2. T cell independent B cell activation

TI antigens were initially identified because of their ability to induce an immune response in nude mice which lack T cells (reviewed in Kindred 1979). Subsequent experiments on the response of B cells from CBA/N mice which carry an x-linked immunodeficiency (*xid*) (Berning *et al.,* 1980, Thomas *et al.,* 1993) have subdivided TI antigens into two further types. TI type-1 antigens are able to induce antibody production in CBA/N B cells, whereas TI type-2 antigens are not (reviewed in Mosier *et al.,* 1977, Mond *et al.,* 1978, Scher 1982b). In addition to differing in their effect on CBA/N B cells, TI type-1 and TI type-2 antigens also differ in their requirement for sIg-mediated signals to induce B cell activation. TI type-2 antigens, which are characterised by large multivalent polysaccharides that contain repetitive epitopes, induce B cell activation through extensive cross-linking of sIg (Mosier and Subbarao 1982, Mond *et al.,* 1995). In contrast, TI type-1 antigens, exemplified by LPS, are thought to induce B cell activation in a manner that is independent of the antigen signal (Coutinho and Möller 1975).

LPS - A TI-type I antigen

LPS is a major component of the cell wall of gram-negative bacteria and it has a structure consisting of three sections: a polysaccharide region (the O-antigen); a core region; and lipid-A (Figure 1.6) (reviewed in Raetz 1990, Rietschel *et al.,* 1994). The O-antigen is the most distal component of LPS from the bacterial cell wall and is a hydrophilic polymer that consists of oligosaccharides made up of repeating glycosyl residues (Raetz 1990, Rietschel *et al.,* 1994). As gram negative bacteria synthesise O-antigens that contain different numbers of glycosyl residues, these antigens exhibit a high degree of heterogeneity (Raetz 1990, Rietschel *et al.,* 1994). The O-antigen is covalently linked to lipid-A by the core region (Figure 1.6) (Raetz 1990, Rietschel *et al.,* 1994). The core region is composed of hetero-oligosaccharides and can be divided into outer and inner regions (Raetz 1990, Rietschel *et al.,* 1994). It is the inner core region that binds to lipid-A, a phosphoglycolipid composed of fatty acids linked by ester and amine bonds to a glucosamine backbone (Figure 1.6) (Raetz 1990, Rietschel *et al.,* 1994). The composition of lipid-A is highly conserved and it is thought that lipid-A provides LPS with its capacity to activate B cells (Andersson *et al.,* 1973, Raetz 1990, Rietschel *et al.,* 1994).

LPS-induced B cell responses

LPS is a potent stimulator of the immune system that can induce B cell proliferation and antigen-specific as well as polyclonal antibody production (Andersson *et al.,* 1972, Kearney and Lawton 1975). *In vivo* LPS-induced ASC formation may be antigen specific as demonstrated by the use of LPS-hapten conjugates (Lange *et al.,* 1983, Claassen *et al.,* 1986). Antigen specific antibody is primarily IgM, however, LPS can also induce the production of IgG2b and low levels of IgG3 (Claassen *et al.,* 1986). Therefore, LPS can induce isotype switching, and as with TD antigens this is regulated by cytokines. Thus, *in vitro* LPS and IFN-γ induce the production of IgG2a and inhibit the production of IgG3 (Snapper and Paul 1987, Snapper *et al.,* 1988b), whereas the combination of LPS and IL-4 promotes the production of IgG1 and IgE (Coffman *et al.,* 1986, Snapper *et al.,* 1988a). While B cells stimulated with LPS are capable of isotype switching, they do not undergo somatic hypermutation and affinity maturation (Mosier *et al.,* 1982) Therefore, the LPS-induced antibody response is generally of low affinity (Smith *et al.,* 1985). Finally, although LPS does not induce the formation of memory B cells there is evidence of a weak recall response. Rats immunised and subsequently challenged with trinitrophenol conjugated to LPS (TNP-LPS) produce a higher concentration of antibody with a different isotype profile than non-immunised animals (Zhang *et al.,* 1988).

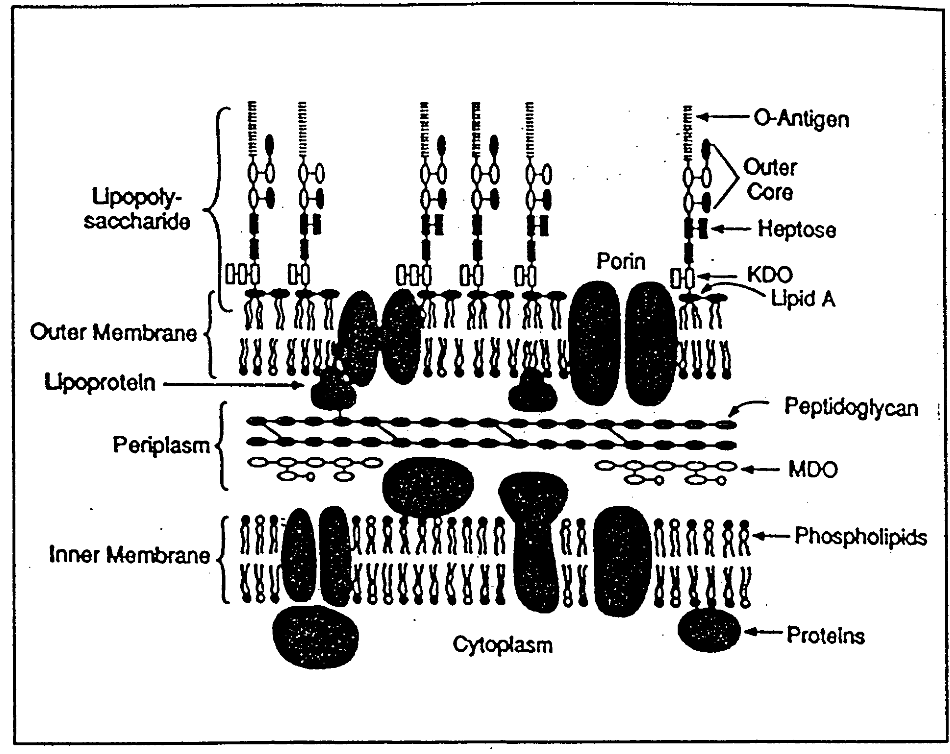

Figure 1.6 **Positioning of LPS on the bacterial cell wall.**

This figure illustrates the approximate orientation of LPS on the bacterial cell wall, where Lipid A is proximal, and the O-antigen is distal, to the outer membrane. Because of the exposed and highly variable nature of the O-antigen, most antibodies which are directed against LPS are specific for epitopes with this region. Taken from Ulevitch and Tobias (1995), with the permission of the *Annual Review of Immunology*. Volume 13 c1995, by Annual reviews Inc.

How does LPS-induce B cell activation?

While CD14 has been identified as a receptor for LPS on the surface of macrophages this antigen is not expressed on B cells and an alternative LPS receptor is yet to be found (Ziegler-Heitbrock and Ulevitch 1993, Ulevitch and Tobias 1995). Thus, the precise mechanism by which LPS-induces B cell activation is still to be defined. However, a long-standing explanation of how a non-specific mitogen such as LPS can give rise to specific antibody is due to Coutinho and Möller (reviewed in Coutinho *et al.,* 1975). They suggested that antigen- or hapten-specific sIg could passively focus hapten-LPS to the specific B cell and thereby promote the differentiation of antigen-specific B cells at hapten-LPS concentrations that were lower than required to induce a polyclonal response (Coutinho *et al.,* 1974a). Based on these conclusions, Coutinho and Möller proposed the one-signal model of B cell activation in which they suggested that all B cell activation, TD and TI, could be induced through the delivery of one antigen non-specific activating signal that did not involve any sIg-mediated signal (Coutinho *et al.,* 1975, Alarcon-Riquelme and Möller 1990, Möller *et al.,* 1991).

The influence of sIg-mediated signals on LPS-induced B cell activation

In contrast to Coutinho's and Möller's conclusions, separate experiments revealed that the antigen signal could have a negative influence on LPS-induced B cell activation. In these experiments the antigen signal was shown to inhibit LPS-induced ASC formation with little effect on LPS-induced proliferation (Andersson *et al.,* 1974, Kearney *et al.,* 1976, Andersson *et al.,* 1978, Kearney *et al.,* 1978, Grandien *et al.,* 1993). Although divalent anti-Ig reagents could inhibit LPS-induced ASC formation, monovalent F(ab) fragments were shown to have no effect (Andersson *et al.,* 1974, Andersson *et al.,* 1978). Thus, it appears that cross-linking of sIg with divalent anti-Ig reagents can induce a signal which inhibits LPS-induced B cell differentiation to ASCs.

TI type-2 B cell activation requires a sIg-mediated signal

TI type-2 B cell activation is typically induced by large multivalent polysaccharide antigens that contain repeated identical epitopes such as polymerised flagellin, haptenated-Ficoll, high molecular weight dextran and pneumococcal polysaccharide (Feldmann and Basten 1971, Coutinho and Möller 1973b, Coutinho *et al.,* 1974b, Mongini *et al.,* 1982, Mongini *et al.,* 1984). In addition, anti-μ dextran and anti-δ-dextran have been used *in vitro* to mimic TI type-2 B cell activation, allowing the characterisation of the B cell response to this class of antigens (Peçanha *et al.,* 1991, Peçanha *et al.,* 1993, Snapper *et al.,* 1995a, Snapper and Mond 1996). The multivalent nature of these antigens is essential to induce B cell activation through extensive cross-linking of sIg (Mond *et al.,* 1995). Thus, while polymerised flagellin can induce B cell activation, flagellin monomer does not (Feldmann *et al.,* 1971). A similarly dramatic

effect of polymerisation can be seen when the B cell response to soluble anti-μ or anti-δ is compared to that induced by those same antibodies chemically coupled to dextran (Peçanha *et al.,* 1991, Snapper *et al.,* 1995a) .

When used to stimulate B cells, TI type-2 antigens alone induce proliferation, whereas in combination with T_h type-2 cytokines they also induce antibody production (Peçanha *et al.,* 1991, Peçanha *et al.,* 1993). The antibody produced *in vivo* is predominantly IgM and IgG3 (Golding and Rittenberg 1982, Claassen *et al.,* 1986). As described for TD and TI type-1 antigens, the antibody isotype induced by TI type-2 antigens is regulated by cytokines as demonstrated *in vitro*. Thus, anti-δ-dextran in combination with IL-4 and IL-5 enhances IgM production and induces switching to IgG1 (Peçanha *et al.,* 1993). In contrast, B cells cultured with anti-Ig-dextran, IFN-γ and IL-5 undergo isotype switching to produce IgG3 and IgG2a (Snapper *et al.,* 1987, Snapper *et al.,* 1992). Further, anti-δ-dextran in combination with TGF-β and supernatants (sn) containing IL-4 and IL-5 have been shown to induce switching to IgA (Snapper *et al.,* 1991). These results clearly indicate the dependence of TI type-2 antigens on cytokines to induce antibody production. Despite this requirement, Th type-2 antigens can induce antibody production in the absence of T cells leading to the suggestion that natural killer cells may also provide the cytokines required for antibody production (Snapper *et al.,* 1994). In addition, as described for TI type-1 antigens, TI type-2 B cell activation does not induce somatic hypermutation and affinity maturation (Mosier *et al.,* 1982), or the generation of memory B cells (Zhang *et al.,* 1988).

A comparison of the role of the antigen signal in distinct types of B cell activation illustrates, again, the principle that this signal can have different and occasionally contradictory effects on B cells. Thus, sIg-mediated signals can promote TD B cell activation but are not themselves obligatory. In contrast, sIg may inhibit antibody production induced by TI type-1 antigens, but is not required for activation and proliferation. Finally, in TI type-2 B cell activation, sIg provides an essential activating signal. These diverse effects of the antigen signal on B cell activation could be due to variation in the sIg-mediated signal as a result of the interaction of sIg with different physical forms of antigen. Thus far, this discussion has focused on the diversity of B cell responses induced by the antigen signal. What then are the components of the sIg-mediated signal that could be differentially activated in response to different antigens? This question is addressed in the following sections.

1.6 The antigen (sIg-mediated) signal

The B cell antigen receptor consists of multiple subunits

The complete B cell antigen receptor (BCR) is made up of two components which have different functions: antigen recognition and signal transduction (reviewed in Cambier and Campbell 1990, Clark *et al.*, 1992, Reth 1992, Pleiman *et al.*, 1994a, Reth 1994). Antigen recognition occurs primarily through sIg, whereas signalling occurs through associated proteins (Cambier *et al.*, 1990, Clark *et al.*, 1992, Pleiman *et al.*, 1994a). The cytoplasmic region of sIgM and sIgD consists of only three amino acids. The other isotypes have somewhat longer cytoplasmic tails; sIgA has 14 amino acids, whereas sIgG and sIgE have 28 amino acids (Cambier *et al.*, 1990, Reth 1992, Pleiman *et al.*, 1994a). Thus, the cytoplasmic tails of sIgM and D were thought to be too short to be involved in signal transduction. This lead to the suggestion that sIg-mediated signalling must occur through sIg-associated proteins (Hombach *et al.*, 1988). These signalling proteins were later identified as Igα and Igβ (Hombach *et al.*, 1990, Kim *et al.*, 1993, Sanchez *et al.*, 1993).

Igα and Igβ are extensively glycosolated proteins composed of an Ig-like extracellular domain attached to long cytoplasmic tails of 61 and 48 amino acids, respectively (Hombach *et al.*, 1990, Kim *et al.*, 1993, Sanchez *et al.*, 1993). These two proteins form a heterodimer which can be co-expressed on the B cell surface with Ig. The association of IgM with the Igα/β heterodimer is essential for the successful transport and expression of IgM on the B cell surface (Hombach *et al.*, 1988, Hombach *et al.*, 1990, Campbell *et al.*, 1991). The cytoplasmic regions of both Igα and Igβ contain a conserved amino acid sequence called an immunoreceptor tyrosine-based activation motif (ITAM) (Cambier 1995). This motif is characterised by a conserved amino acid sequence: D/E-X_7-D/E-X_2-Y-X_2-L/I-X_7-Y-X_2-L/I in single amino acid letter code, where X is any amino acid (Cambier 1995). The tyrosine residues within the ITAM have been shown to be essential for signal transduction as substitution mutations of the tyrosine residues within the ITAM abolish the signalling function of the Igα/β heterodimer (Sanchez *et al.*, 1993). ITAMs are also found in the cytoplasmic tails of other molecules which are involved in signal transduction including components of the T cell antigen receptor (TCR) (CD3γ, δ, ε ζ, and η) and Fc receptors (γRII2a, εR1β and εR1γ) (Reth 1989, Clark *et al.*, 1992, Sanchez *et al.*, 1993).

Signal transduction through the BCR commences with the activation of protein tyrosine kinases

Signal transduction by the BCR involves the activation of a number of intracellular signalling pathways (reviewed in Cambier and Campbell 1992, Cambier *et al.*, 1994,

Gold and Defranco 1994, Harnett 1994, Bolen 1995, DeFranco 1997). Surface Ig-mediated signalling begins with the activation of protein tyrosine kinases (PTKs) that associate with the cytoplasmic tail of the Igα/β heterodimer. The kinases include: $p72^{syk}$, $p53/56^{lyn}$, $p55^{blk}$, $p59^{fyn}$ and $p56^{lck}$ (Clark *et al.*, 1992, Bolen 1995). One of the protein tyrosine kinases (PTK) activated after sIg cross-linking appears to be $p72^{syk}$ (Hutchcroft *et al.*, 1991, Weiss and Littman 1994). Once activated, $p72^{syk}$ may undergo autophosphorylation and phosphorylate other PTKs associated with the Igα/β heterodimer (Cambier *et al.*, 1994, Pleiman *et al.*, 1994a, Bolen 1995) The phosphorylation of the PTKs results in their activation, and they in turn phosphorylate tyrosine residues within the ITAM (Kimura *et al.*, 1996). The phosphorylation of the ITAM induces a conformational change in the cytoplasmic region of the Igα/β heterodimer which reveals additional binding sites for kinases known as SH2 domains (Johnson *et al.*, 1995, Takata and Kurosaki 1996). The conformational change of the Igα/β heterodimer brings about the re-orientation of the PTKs leading to heightened activation of these enzymes by cross- and auto-phosphorylation (Kurosaki *et al.*, 1995, Takata *et al.*, 1996).

Ligation of sIg mediates the activation of different signal transduction pathways

The PTKs associated with the Igα/β heterodimer bind and phosphorylate phospholipase C-γ (PLC-γ) (Carter *et al.*, 1991a), GTPase activating protein (GAP) (Kawauchi *et al.*, 1994) and phosphatidylinositol 3-kinase (PI-3 kinase) (Pleiman *et al.*, 1994b). After sIg cross-linking, each of these proteins are activated by phosphorylation (Figure 1.7) (Carter *et al.*, 1991a, Gold *et al.*, 1992, Cambier *et al.*, 1994). Once activated PLC-γ, GAP, and PI-3 kinase initiate three separate signal transduction pathways (Figure 1.7). The activation of PLC-γ results in the hydrolysis of inositol containing phospholipids within the cell membrane and the generation of the secondary messengers inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) (Berridge and Irvine 1989, Valentine *et al.*, 1992). The production of IP_3 mobilises calcium ions which in turn leads to the activation of the calcium dependent calmodulin kinase (Figure 1.7) (Valentine *et al.*, 1995). Calmodulin activates calcineurin which dephosphorylates the nuclear factor for activated T cells (NF-AT) promoting its migration from the cytoplasm into the nucleus (Figure 1.7) (Choi *et al.*, 1994, Venkataraman *et al.*, 1994). In contrast, DAG activates protein kinase C (PKC), (Nishizuka 1991) which can eventually lead to the activation of *junB*, a component of the transcription factor AP-1 (Figure 1.7) (Huo and Rothstein 1995).

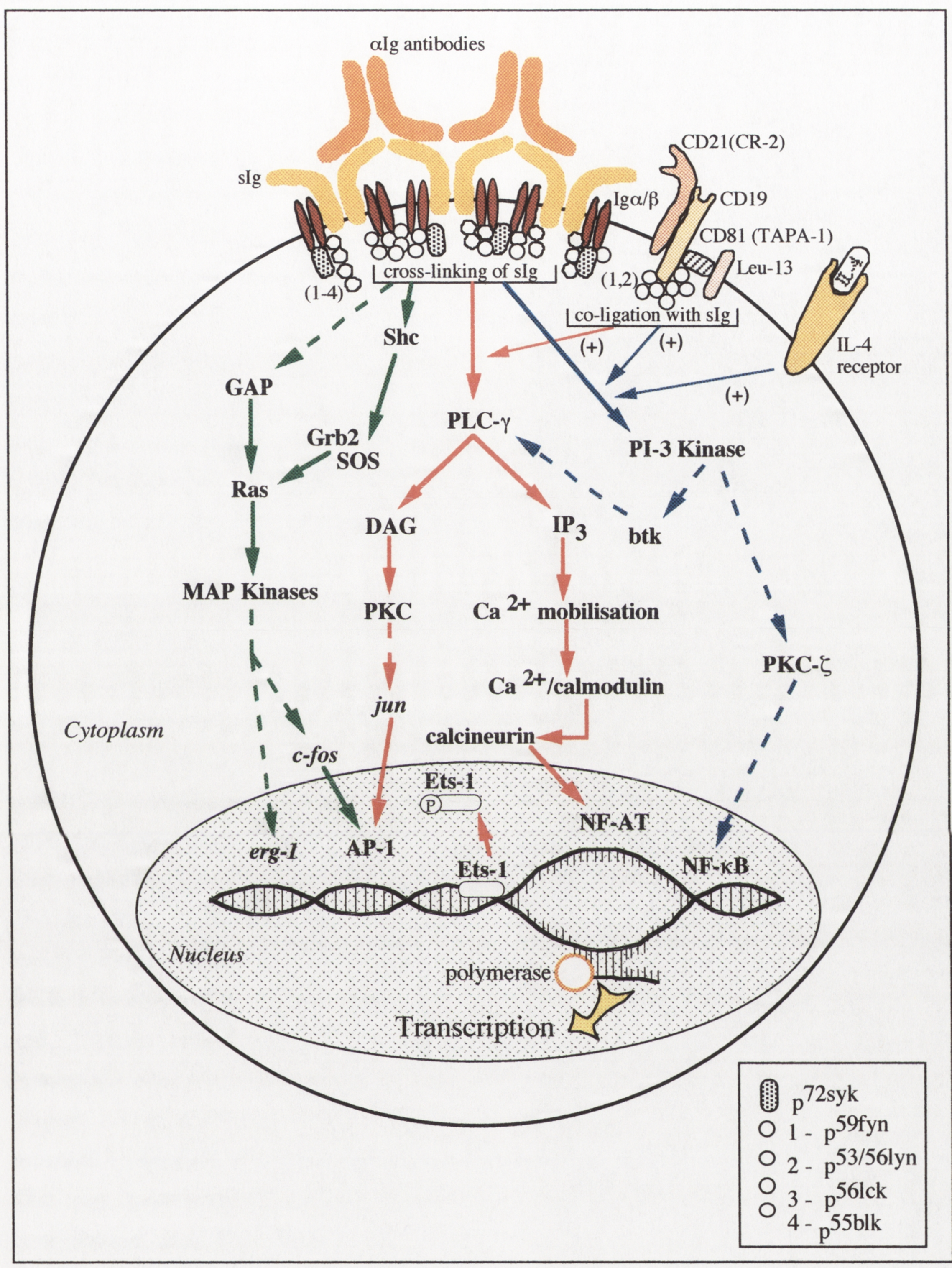

Figure 1.7. **A simplified view of sIg-mediated signalling.**

This figure illustrates some of the intracellular signalling pathways that are activated in response to cross-linking of sIg, and which ultimatly lead to the regulation of transcription. Also illustrated is how signalling from the IL-4 receptor and the CD21/CD19/CD81 complex can amplify the sIg-mediated signals. Addapted from DeFranco (1997), Gold and DeFranco (1994), Harnet (1994) and Cambier and Campbell (1992).

The downstream signalling events after the phosphorylation of GAP include the activation of p21^{ras} (Gold *et al.,* 1993, Harwood and Cambier 1993), which in turn leads to the activation of the mitogen activated protein (MAP) kinases (Harnett 1994). The MAP kinases phosphorylate transcription factors that include *c-jun* (Pulverer *et al.,* 1991), and can induce the expression of *egr-1* and *c-fos* (Figure 1.7) (DeFranco 1993, DeFranco 1997). Finally, activation of PI-3 kinase induces the downstream activation of phospholipase C-γ (Cambier *et al.,* 1994, Harnett 1994). In addition, PKC-ζ is also activated and, it is thought that PKC-ζ can interact with NF-κB and thereby promote transcription (Figure 1.7) (Liu *et al.,* 1991a). Thus, each of the three signal transduction pathways ultimately influences the activity of DNA binding proteins through which transcription is regulated. Combined, these pathways result in the transcription of *erg*-1, c-*myc*, *jun* and c-*fos*, all of which assist in the regulation of genes involved in B cell activation (Cambier *et al.,* 1992, Gold *et al.,* 1994).

although this pathway is not well understood

The antigen signal can be amplified by the signalling of co-receptors

The signalling induced by the BCR may be amplified through the involvement of co-receptors. One candidate is CD19 (reviewed in van Noesel *et al.,* 1993a, Tedder *et al.,* 1994, Fearon and Carter 1995). CD19 is expressed on the B cell surface in association with CD21 (complement receptor-2), CD81 (TAPA-1) and Leu-13 (Matsumoto *et al.,* 1991, Bradbury *et al.,* 1992, Matsumoto *et al.,* 1993). Combined, these molecules may form a unique signal transduction complex that can enhance BCR mediated signalling (Tedder *et al.,* 1994, Fearon *et al.,* 1995)). CD19 has been shown to associate with sIg and is phosphorylated by PTKs bound to the Igα/β heterodimer following ligation of the BCR (Chalupny *et al.,* 1993, van Noesel *et al.,* 1993b). In addition, co-ligation of sIg and CD19 has been shown to lower the threshold of B cell activation and to induce a synergistic increase in calcium mobilisation and the activation of PLC-γ and PI-3 kinase (Figure 1.7) (Carter *et al.,* 1991b, Carter *et al.,* 1992). The role of CD21 may be to promote co-ligation of CD19 and sIg by forming a bridge between the two complexes. This may occur when sIg binds to antigen that is part of an immune complex containing complement, and CD21 binds to the complement component (van Noesel *et al.,* 1993a, Fearon *et al.,* 1995). The co-ligation of sIg and CD21 has been shown to enhance B cell antibody production (Dempsey *et al.,* 1996). Finally, the role of CD81 and Leu-13 in this complex is less clear. However, ligation of Leu-13 has been reported to activate PTKs (Schick *et al.,* 1993) . Thus, intracellular signals induced by the CD21/CD19/CD81 complex could augment sIg-mediated signalling through the enhanced activation of PLC-γ and PI-3 kinase (Figure 1.7).

1.7 Aims of this thesis

When the effect of sIg-mediated signals in different forms of B cell activation is compared it is clear that the antigen signal does not have a single definitive role. While the role of the antigen signal in TD and TI type-2 B cell activation is relatively clear, its effect on LPS-induced B cell activation remains to be determined. According to Coutinho and Möller's one signal model of B cell activation sIg-mediated signals do not have a role in activating or regulating the B cell response (Coutinho *et al.,* 1974a, Coutinho *et al.,* 1975, Möller *et al.,* 1991). However, their conclusion is inconsistent with the effect of the antigen signal on TD and TI type-2 B cell activation, as well as the experimental evidence that sIg-mediated signals can inhibit LPS-induced ASC formation (Andersson *et al.,* 1974, Kearney *et al.,* 1976, Grandien *et al.,* 1993). Thus, the aim of this thesis is to reconcile these opposing views by re-examining the experimental foundation of the one signal model.

Chapter 2

The one signal theory of B cell activation revisited: A role for surface immunoglobulin in regulating T-independent antibody responses

2.1 Introduction[1]

The one signal theory of B cell activation, first proposed almost 20 years ago (Coutinho and Möller 1974, Coutinho and Möller 1975) argued against an active signalling role for surface immunoglobulin (sIg) in B cell activation. The theory still holds considerable interest today for a number of reasons. First, the model has been re-formulated to conform to new information of T-B collaboration and to re-state arguments against the evidence for a role of sIg mediated signals in activation (Möller. *et al.,* 1991). Second, one component of this theory - the proposal that one non-antigen specific signal is sufficient to activate B cells in the absence of antigen binding - is consistent with recent discoveries of the CD40-mediated B cell activation path by T cells (Noelle. *et al.,* 1992a, Hodgkin and Kehry 1993, Parker 1993). The model is, however, at odds with the overwhelming evidence for a sIg mediated signal (Cambier and Campbell 1990) and its role in T-dependent (TD) and some T-independent (TI) antibody responses (Peçanha. *et al.,* 1991, Cooke. *et al.,* 1994). The arguments and experimental basis of the one signal model then are worthy of re-examination in a modern context. In this paper we look at the experimental foundations of this theory and find that they suggest an alternate theory that, ironically, invokes a critical role for the transmission of a signal by sIg.

Experimental Foundations.

Three key empirical observations formed the foundation of the one signal theory of B cell activation (Figure 1). The first was that the dose response of B cells to polyclonal B cell activators (PBAs), such as lipopolysaccharide (LPS), generated a bell shaped dose response curve when total antibody secreting cells (ASCs) were measured in culture (Figure 2.1a) (Andersson. *et al.,* 1978). An antigen specific response, as detected by the number of anti-hapten ASCs, formed a subset of the total response and gave a similar bell shaped dose curve (Andersson, *et al.,* 1978). The second experiment used LPS that had been coupled to the hapten (4-hydroxy-3,5-dinitrophenyl)acetyl (NNP). The dose response curves for both the anti-hapten and total Ig secreting cell number were again bell shaped.

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1. This chapter has been published as a theoretical article in *Immunology and Cell Biology* (Mamchak and Hodgkin (1995) **73**:266-271), and is presented here in thesis format. It has been included as a chapter as it describes the theoretical foundations on which the experimental work described in this thesis is based.

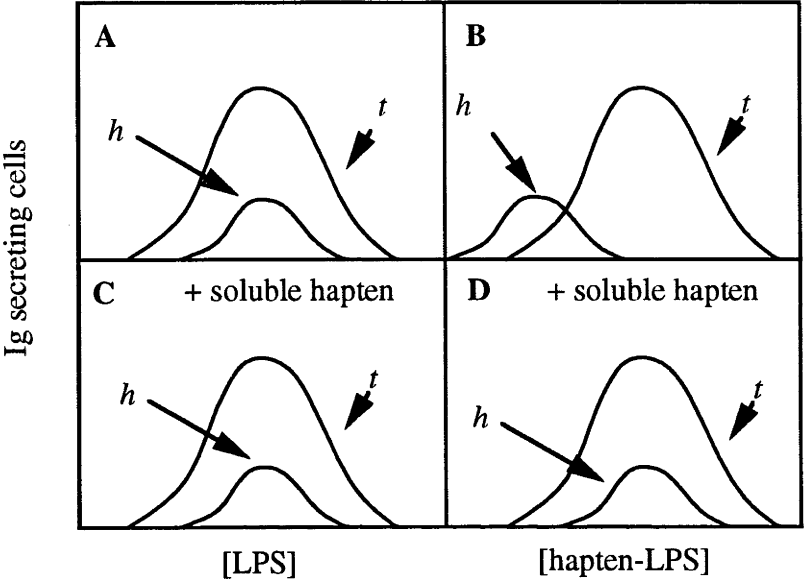

Figure 2.1. **The experimental foundation of the one signal theory of B cell activation.**

A. The dose response curve for LPS-induced total (t), or antigen specific (h), antibody secreting cell formation is bell shaped, with the specific antigen response forming a subset of the polyclonal response. B. By haptenating the LPS, the specific response displays a maximum at lower concentrations than the polyclonal response. C. Shows a similar experiment to that shown in (A) except that competing hapten is included in culture. The presence of the competitor does not affect the ability of LPS to induce the hapten specific response. D. Addition of free hapten to B cells stimulated with haptenated LPS inhibits the shift in dose curve seen in (B) and generates dose response curves that are similar to those seen in the presence of LPS alone, where the antigen specific response is a subset of the total antibody response (A).

However, peak production of antigen specific cells occurred at lower concentrations than the polyclonal response (Figure 2.1b) (Coutinho. *et al.,* 1974a). In the third experiment (Figure 2.1c and d) the NNP-specific fraction of the polyclonal response induced by LPS stimulation could not be blocked by soluble hapten (Figure 1c), whereas the leftward shift in antigen specific response following stimulation with NNP-LPS was inhibited (Figure 1d) (Coutinho, *et al.,* 1974a).

Coutinho and Möller's interpretation of these data (Coutinho and Möller 1975) is illustrated in Figure 2.2. To explain the bell shaped dose curves it was assumed that all B cells possessed similar receptors for PBAs that were independent of the sIg antigen receptor. It was also assumed that the response to any PBA would be dose dependent and that high levels of receptor binding induced a state of non-responsiveness (also referred to as paralysis). The occurrence of antigen-specific responses at low doses of haptenated LPS was proposed to occur because B cells with specificity for the hapten would use their sIg receptor to (passively) focus the LPS to their surface, thereby increasing interaction with the LPS receptor. Thus, hapten (or LPS)-specific B cells reached optimal and paralytic doses of LPS at lower concentrations than non-specific cells. To interpret the third experiment (Figure 2.1c and d) it was assumed that soluble hapten could block sIg-mediated focussing of LPS via hapten binding. Coutinho and Möller also noted that the inability of free hapten to inhibit the subset of anti-hapten B cells stimulated by LPS (Figure 1c) implied that antigen binding was not actually required for LPS to induce differentiation of antigen specific B cells (Coutinho and Möller 1975).

Although their experiments dealt exclusively with TI B cell stimulation, Coutinho and Möller extrapolated from their data to propose a model that included TD responses. This general model proposed that all B cell activation occurred through the focussing by sIg of one-non-antigen specific activating signal delivered either by a PBA or an antigen specific T cell (Coutinho and Möller 1975, Möller, *et al.,* 1991). Recently, Möller *et al.* strongly defended this model with a version of TD B cell activation where the only important role for sIg was that of processing antigen (Möller, *et al.,* 1991). Thus, by this view the specific B cell accumulates antigen derived peptides in association with MHC class II molecules on its surface, allowing recognition by MHC restricted helper T cells. Antigen recognition by collaborating T cells leads to the provision of a non-antigen specific set of B cell activating signals similar to those provided by the PBA (Coutinho and Möller 1974). Given that this theory evolved from studies on LPS, it is remarkable that this latter prediction was to some extent correct. Some T cell clones can activate B cells in vitro in this manner and, furthermore, once activated, the T helper cell delivers

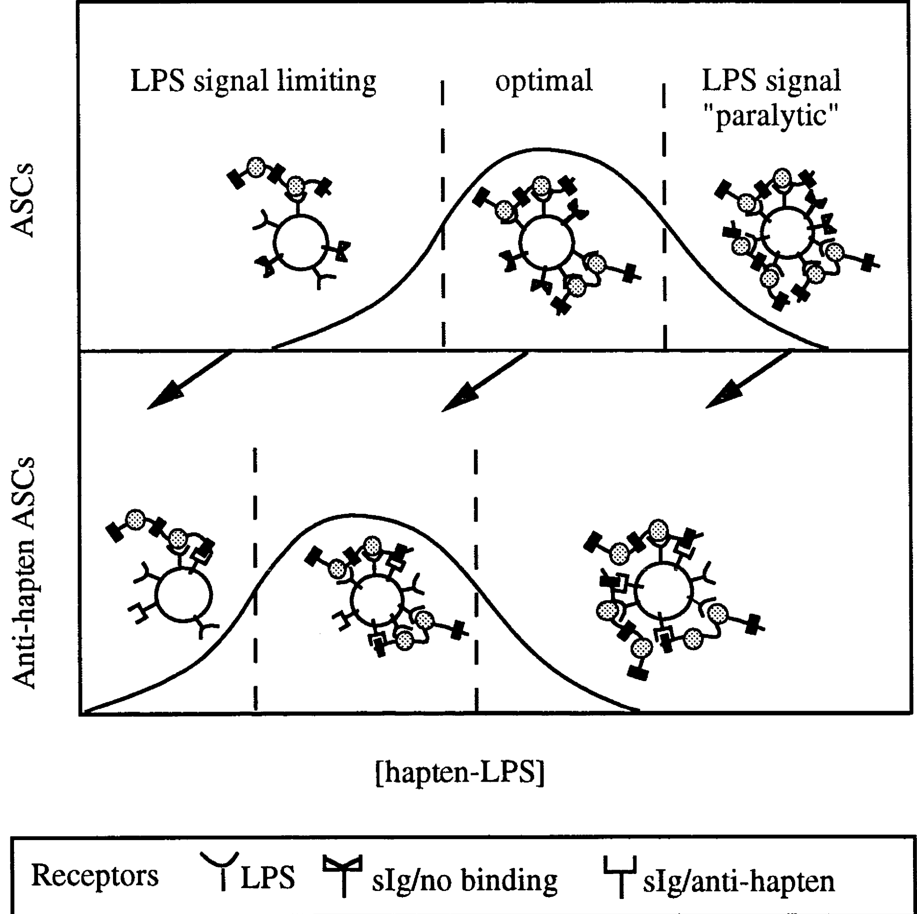

Figure 2.2 **The one signal model of B cell activation by LPS.**
The top panel shows the stimulation of non-hapten binding B cells by hapten-LPS. Interaction between LPS and its receptor on B cells induces a bell shaped dose curve, where at low concentrations receptor engagement is limiting, and at high occupation levels the LPS signal is paralytic. The lower panel illustrates how hapten recognition by sIg can alter the equilibrium of LPS binding by providing additional receptors for LPS capture. Importantly, however, binding hapten plays only a passive role in focussing LPS and doesn't alter the sensitivity of the B cell to LPS stimulation.

cell-cell contact and lymphokine-mediated signals that are sufficient to induce polyclonal B cell proliferation and antibody production (Hodgkin. *et al.,* 1990, Hodgkin and Kehry 1992). Thus, the essential signals to drive B cell differentiation to Ig secretion do not include antigen, although such signals may play other important roles (discussed further below).

2.2 The two signal theory

The most important competing theory of the time had a different history. Bretscher and Cohn's two signal model of B cell activation was a theoretical attempt to explain how a cell could distinguish self from non-self antigens (Bretscher and Cohn 1970). They argued that this could be achieved with two antigen recognition events, one supplied by B cell antigen recognition, and the second by associative recognition of another antigenic determinant by a helper mechanism (in modern terms a helper T cell). Both the two signal theory and the one signal model were consistent with this constraint, as even a passive role for sIg collecting antigen satisfies this requirement. The important difference is that Bretscher and Cohn argued that the first signal alone, without the second signal, must lead to tolerance. Hence a passive role for the B cell antigen receptor was not sufficient - the B cell had to perceive its contact with antigen to prime for the second signals or, in their absence, to tolerise (Bretscher and Cohn 1970).

With the benefit of hindsight, the ensuing debate was an interesting one. An experimental inductive model battling a theoretical deductive construction for numerical supremacy (one signal or two?). And, as is often the case, there was good evidence for both models, although not in the same systems. The chief point of difficulty was whether the first signal existed, and if so how was it triggered and what was its role?

2.3 The sIg mediated signal

In recent years the molecular mechanism of triggering the B cell surface Ig receptor has begun to be unravelled. In order to be expressed on the cell surface Ig must associate with the gene products from mb-1 and B-29, Ig α and Ig β respectively (Reth. *et al.,* 1991). These molecules form a heterodimer which functions as the signalling component of sIg (Pleiman. *et al.,* 1994a). Crosslinking of sIg by antigen brings the receptor complexes together to form a lattice. The formation of the receptor lattice can directly activate Ig α/β associated src-related kinases resulting in cross-phosphorylation and the activation of other kinases and their substrates (Pleiman, *et al.,* 1994a). Downstream of these initial activation events second messengers result in the movement of cytoplasmic

factors into the nucleus where they regulate gene transcription (Cambier and Campbell 1990).

The consequence of sIg signalling depends on both the maturation state of the B cell and receipt of other signals. An early role for the signal in deletion of immature, self reactive cells in the bone marrow is implied from recent studies (Nemazee and Bürki 1989b). Once the B cell has matured and entered the periphery, the sIg mediated signal can mediate various effects under different conditions. This can be illustrated with three examples corresponding to B cell activation by T cells and by two different TI activation protocols.

Role of antigen in TD responses

B cells exposed to antigen undergo a number of rapid changes that prepare them for interaction with T cells. These events include migration to T cell areas of lymphoid tissue (van Rooijen 1992), increased expression of adhesion and MHC molecules, and the de novo synthesis of ligands for CD28 (Cooke, *et al.,* 1994, Lenschow. *et al.,* 1994). In addition, antigen stimulated B cells are more sensitive to the helper signals provided by the T cell including ligation of CD40 on the B cell (Lane. *et al.,* 1993). Hence, it appears that antigen stimulation of the B cell will significantly promote interaction between the T and B cell. Furthermore, provision of the first signal (antigen), in the absence of T cell help, will apparently lead to a shorter B cell half life (Fulcher and Basten 1994), as predicted by the two signal theory (Bretscher and Cohn 1970). In addition to playing a role in early T-B interaction the antigen signal is implicated in the re-selection of high affinity B cells following somatic hypermutation in the germinal centre (Liu. *et al.,* 1989).

Role of antigen in TI responses

TI antigens have been divided into two categories: 1) TI-1 antigens (which include LPS) are all PBAs, and, 2) TI-2 antigens that are less mitogenic but are also multimeric polysaccharides (Scher 1982b). As has been already described, the TI-1 response does not require antigen recognition although sIg can focus the PBA and generate a specific response. Interestingly, treating LPS stimulated cells with anti-Ig reagents actually inhibits Ig secretion (this attribute will be discussed in greater detail below). B cell activation by TI-2 antigens are mimicked in vitro by anti-Ig and anti-Ig-dextran conjugates . In contrast to TI-1 activation, these agents seem to be critically dependent on the transmission of a sIg mediated signal rendering the cells sensitive to the differentiating effects of a range of cytokines (Snapper and Mond 1993).

2.4 Multiple roles for the B cell antigen signal

The obvious conclusion from the above discussion is that there is no universal role for the sIg-mediated signal. Therefore, the attempts of earlier workers to explain all of B cell behaviour within a single model necessarily underestimated the inherent complexity of the system. The multiple B cell differentiation paths and roles for sIg presumably evolved so that immunoglobulin production could be highly regulated and evoke classes of response appropriate to different categories of antigen. Of interest though, is the partial success of early logical arguments founded on either an experimental or theoretical base to predict B cell behaviour. The conclusions reached were valid for some modes of B cell activation and not others. For example, the two signal theory is approximately met by the current view of T-B collaboration but is inconsistent with activation by TI antigens or for T cell activation. The one non-specific mechanism is correct in predicting that the actual B cell activation events would occur without an obligatory role for antigen stimulation, however, under most situations in vivo, the enhancing effects of B cell antigen stimulation may be necessary.

2.5 An alternative theory of bell shaped curves for LPS activation

Anti-Ig and antigen stimulation during LPS exposure enhances B cell proliferation while inhibiting Ig secretion (Andersson. *et al.,* 1974, Kearney. *et al.,* 1976). The use of $F(ab')_2$ and F(ab) fragments of anti-Ig have shown that inhibition of PBA induced antibody production requires crosslinking of sIg and is not mediated by Fc receptors (Andersson, *et al.,* 1974, Grandien. *et al.,* 1993). These observations have not been satisfactorily placed within a theoretical framework. It seems reasonable to suggest that haptenated LPS, which is after all a multivalent antigen, could have the same effect as anti-Ig such that at a given concentration hapten specific B cells will receive a signal that inhibits Ig secretion and promotes proliferation. By this model, illustrated in Figure 2.3, the hapten (or LPS)-specific B cell population will focus low concentrations of LPS to the surface, as argued by Coutinho and Möller (Coutinho and Möller 1975), but increasing concentrations eventually result in sufficient binding of the receptor to transmit a sIg mediated signal that inhibits differentiation to Ig secretion and therefore generates the familiar bell shaped curve.

This alternative would not explain how similar curves are generated by the polyclonal response to LPS (Figure 1). Here, however, our data is at odds with Coutinho and Möller as we find that LPS-induced dose response curves can be complex, with the

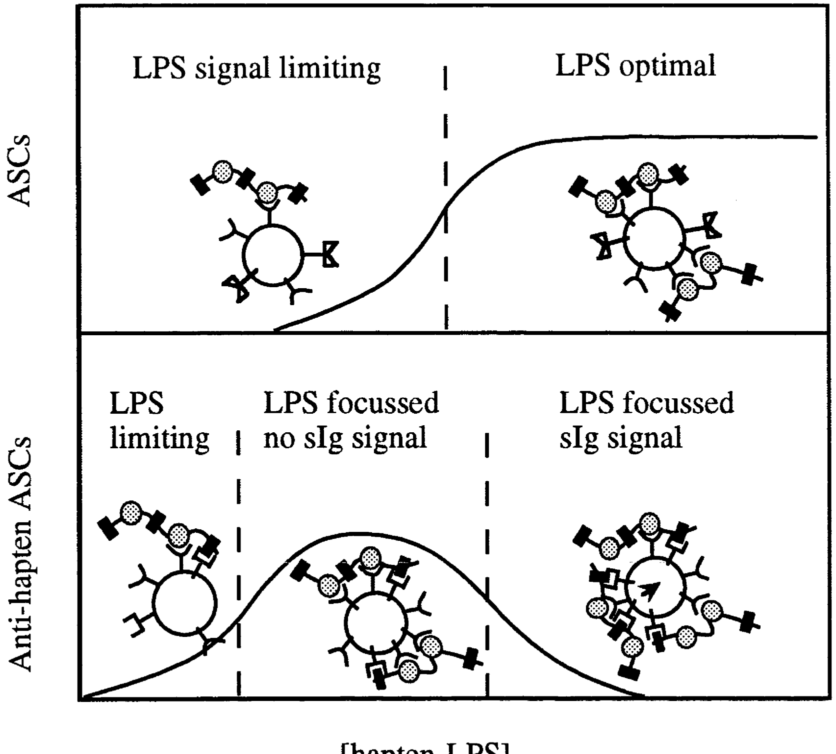

Figure 2.3. **An alternative explanation of LPS induced bell shaped dose response curves.**

This model argues that the true LPS dose curve follows a standard sigmoidal response (upper curve). For the anti-hapten response there will be a concentration region where hapten binding succeeds in focussing LPS to the surface without transmitting a signal. Higher concentrations result in sIg mediated signalling that promotes proliferation but prevents Ig secretion.

shape critically dependent on starting cell density and time of assay (Mamchak and Hodgkin, manuscript in preparation). If carefully performed, little inhibition is seen even at extremely high LPS concentrations (>500 mg/ml). Thus, we believe that the bell curve for total antibody production may be an artefact of the rapid growth of stimulated B cells and the complex kinetics of ASC formation at high doses of LPS. Upon re-examining the early literature it is apparent that the published bell shaped curves for anti-hapten responses are usually striking, showing inhibition to background levels at higher concentrations. In contrast the inhibition of the total Ig response at high LPS concentrations is less convincing with the peak response reduced by as little as 50% (Andersson. *et al.,* 1972, Coutinho, *et al.,* 1974a, Coutinho and Möller 1975).

2.6 Implications of sIg signalling inhibiting Ig production

The proposed negative signal transmitted by sIg during LPS stimulation would have the effect of ensuring that specific antibodies induced by LPS will be drawn from the low affinity range (Figure 2.4). Consistent with this prediction Smith et al (Smith. *et al.,* 1985) immunised C57BL/6 mice with TD and TI antigens coupled to the hapten (4-hydroxy-3-nitrophenyl)acetyl (NP). Anti-NP secreting hybridomas prepared from mice immunised with NP-LPS were of IgM subclass and of significantly lower affinity than those obtained from TI-2 or TD antigen priming. In fact, many of the LPS induced B cell clones were of such low affinity they were unable to be detected in a conventional plaque assay. Furthermore, the high affinity B cell response in the C57BL/6 mouse uses the l1 light chain and both the TI-2 and TD responses were dominated by l bearing B cells. In contrast the NP-LPS response consisted of low affinity k bearing B cells (Smith, *et al.,* 1985). This result suggests that the higher affinity l1 bearing precursor cells were not stimulated, consistent with an active suppression by antigen recognition.

Why would sIg actively maintain low affinity responses to TI-1 antigens? One possibility is that the mechanism serves as a form of memory cell generation. By proliferating rather than differentiating to Ig secretion, the higher affinity cells could form an expanded pool of specific cells to draw upon during subsequent antigen exposure. Alternatively, additional proliferation may be required for differentiation to non-IgM subclasses. Class switching will only be useful for higher affinity cells due to the significantly lower avidity of IgG compared to IgM. Other possibilities are that the high affinity B cells may differentiate into some other non-Ig secreting effector cell, or that high affinity antibody may be disadvantageous to an important effector function directed against LPS bearing microorganisms (ie complement activation).

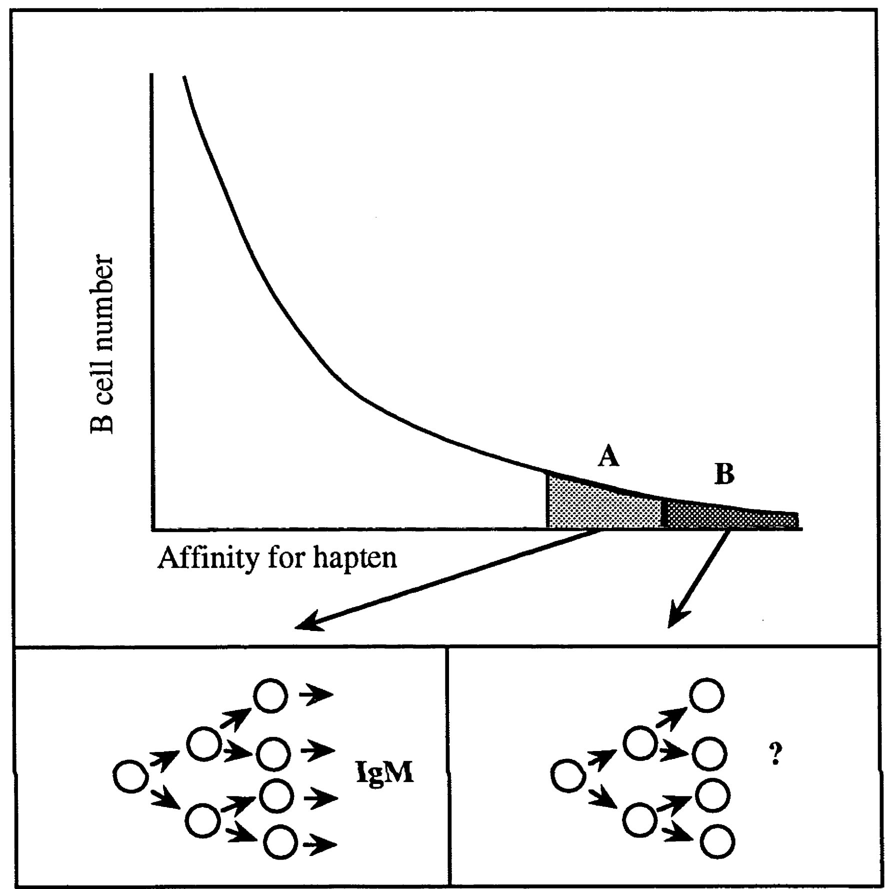

Figure 2.4. **Selection of low affinity cells by haptenated LPS.**
The curve in the upper panel shows a frequency distribution of B cell affinity for hapten. From the arguments outlined in the text it is proposed that only B cells that fall into a moderate affinity group (category A) will be stimulated by haptenated-LPS to secrete IgM. High affinity cells proliferate but won't secrete Ig. The function of this latter pathway of B cell response is unknown.

Evaluating the validity of these arguments and the importance of sIg in LPS responses will require further experiments. Of interest though is that the data that served as the foundation for a theory purporting to eliminate a role for antigen signalling in B cell activation, may ultimately help establish its importance.

Chapter 3

Materials and methods

3.1. Experimental animals

B cells were prepared from mice aged between 6 to 14 weeks. CBA/H mice were obtained from the animal facility at the John Curtin School of Medical Research (JCSMR, Canberra, Australia), or purchased from the Animal Resources Centre (Perth, Australia) and housed at the Centenary Institute of Cancer Medicine and Cell Biology (Sydney, Australia). Male CBA/N mice were obtained from the Walter and Eliza Hall Institute (WEHI, Melbourne, Australia). Prior to use CBA/N mice were housed within the animal facility at the Centenary Institute. All animals housed at the Centenary Institute were kept in microisolator cages under specific pathogen free conditions.

3.2. B cell preparation

Reagents

Red blood cell (RBC) lysis buffer: 10 mM potassium bicarbonate, 0.15 M ammonium chloride, 0.1 mM ethylenediaminetetraacetic acid (EDTA) and 5% fetal calf serum (FCS), pH 7.3.

RPMI/1640: A powder preparation of RPMI/1640 with L-glutamine was purchased from Trace (Sydney, Australia. Multicell product No 60 001PF) and made up in irrigation water obtained from Baxter Health Care (Old Toongabbie, Australia). The medium was supplemented further with, 1 g/L sodium bicarbonate, 4.6 g/L N-(2-hydroxyethyl) piperazine-N-2-ethanesulfonic acid (Hepes), 100 µg/ml streptomycin sulfate and 60 µg/ml benzylpenicillin. The supplemented media is referred to as RPMI (Hepes).

Cytotoxicity medium (CTM): RPMI (Hepes) was further supplemented with 10 mM Hepes and 0.3% bovine serum albumin (BSA), pH 7.4. BSA was obtained from Sigma (St Louis, MO. product No. A-9543) and contained ≤ 0.1 ng/mg endotoxin.

B cell medium (BCM): At JCSMR BCM was prepared by supplementing RPMI (Hepes) with 10% heat inactivated (45 minutes at 56°C) FCS (HI FCS; Commonwealth Serum Laboratories, Melbourne) which had been previously screened for low intrinsic B cell mitogenicity, $5x10^{-5}$ M mercaptoethanol, 10 mM Hepes pH 7.4, 0.1 mM non-essential amino acids (Sigma, St Louis, MO), 1 mM sodium pyruvate (Gibco BRL, Grand Island, New York), 2 mM glutamine (Sigma, St Louis, MO), 60 µg/ml benzyl penicillin and 100 µg/ml streptomycin. At the Centenary Institute BCM was made using commercially prepared RPMI/1640 that contained 2 mM L-glutamine (Gibco BRL,

Grand Island, NY). This media was used to prepare BCM as described above with the exception that the 2 mM glutamine was not added.

All solutions were sterilised by filtration through 0.2 μm membranes (Millipore, Bedford, MA) and stored at 4°C.

T cell depleting antibodies: A cocktail of the rat anti-mouse CD4, RL172 anti-CD8, 31M, and anti-Thy1, 30H-12 (Ledbetter and Herzenberg 1979) were used for T cell depletion. Hybridomas for the anti-CD8 and anti-CD4 antibodies were a gift from Dr H R MacDonald (Lausanne, Switzerland). These antibodies were used as hybridoma supernatants (sn) for T cell depletion.

The hybridomas were grown in mixed lymphocyte culture (MLC) medium. MLC medium was prepared with 100 g of DMEM (Gibco, Grand Island, NY. product No. 430-1600) dissolved in 10 L of double distilled water, supplemented with 40 g D-glucose, 60 mg folic acid, 360 mg L-asparagine, 1.160 g L-arginine HCl and 20 g sodium bicarbonate. The solution was sterilised by filtration through 0.2 μm membranes and stored at 4°C. Prior to use MLC media was supplemented with, 2 mM glutamine, 1 mM sodium pyruvate, 10 mM HEPES, 50 ml HI FCS, 5 x 10^{-5}M 2-ME, 60 μg/ml penicillin and 100 μg/ml streptomycin. The hybridoma sn were titrated using a colourimetric assay to determine the optimal dilution for T cell depletion.

Complement: Rabbit or guinea pig complement, which had been screened for low toxicity, was used to deplete antibody treated T cells. Each batch was tested to determine the optimal specific killing dilution. Rabbit complement was obtained lyophilized from Cederlane Laboratories (Hornby, Ontario). Lyophilized guinea pig complement was obtained from the Institute of Medical and Veterinary Science (Adelaide, Australia) and used at a final concentration of 2 mg/ml. The complement was dissolved and diluted in cold CTM immediately prior to use. The complement solution was filter sterilised by passage through a 0.2 μm membrane, and kept on ice until use.

Percoll: Percoll was purchased from Pharmacia (Uppsala, Sweden), and a 100% stock solution was prepared by diluting 9 parts percoll with 1 part 10 x concentrated sterile phosphate buffered saline (PBS). The 100% stock solution has a density of 1.123 g/ml at 20°C and was diluted to 75%, 65% and 50% percoll solutions with sterile 1 x PBS (having densities of 1.0945 g/ml, 1.0819 g/ml, and 1.062 g/ml at 20°C, respectively). All percoll solutions were kept sterile and stored at 4°C.

<u>Staining antibodies:</u> The anti-B220 FITC conjugate was a gift from Dr P. Lalor (WEHI, Melbourne), whereas the anti-B220-PE was purchased from PharMingen (San Diego, CA). Both antibodies were titrated on unseparated B cell populations and the staining analysed on the FACScan to determine the optimal concentration for staining.

Methods

<u>Colourimetric assay for titrating antibody concentration</u>

To monitor the efficacy of T cell depleting antibodies a colourimetric assay was developed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT). Thymic lymphocytes (thymocytes) were used as the target cells. These cells were prepared by resecting thymi from mice and preparing single cell suspensions by pressing gently through stainless steel sieves. These cells were washed in CTM before use. The MTT assay was performed by preparing two-fold serial dilutions of antibody containing hybridoma sn across a 96 well round bottom microtitre plate in a final volume of 50 µl CTM (Nunc, Denmark). A total of 10^6 thymocytes in CTM were then added to each well and the cells kept on ice for 30 minutes. The cells were then washed by centrifugation at 500 x g for 2 mins, and resuspension in 100 µl CTM. These cells were spun again and the sn removed. The pelleted thymocytes were resuspended in 100 µl of complement solution (see below), diluted in CTM and the cells incubated at 37°C for 30 mins. Cells were then washed again and resuspended in 100 µl CTM. To each well 10 µl of MTT solution was added and the plate incubated at 37°C for one hour. During the incubation an insoluble formazan precipitate is formed that is resuspended in 100 µl of isopropanol containing 0.04 M HCl. The optical density (OD) of each well was then read at a wavelength of 570 nm against a reference of 650 nm using a ThermoMAX® colourimetric plate reader (Molecular Devices, Palo Alto, CA).

<u>Preparation of B cells</u>

Mice were killed by CO_2 asphyxiation, the spleens removed and placed in RPMI (Hepes) containing 10% FCS (RPMI/10% FCS). A single cell suspension was prepared by dispersing the spleens through a wire mesh. The spleen cells were washed by centrifugation at 500 x g for 5 mins and resuspended in RPMI/10% FCS. The cells were pelleted again and resuspended in 5 ml RBC lysis buffer / spleen. The cells were then washed again in RPMI/10% FCS and viable cell number counted by trypan blue exclusion using an improved Neubauer Haemocytometer (Germany). To remove adherent cells, the cells were then resuspended at 1 x 10^7 viable cells / ml in RPMI (Hepes) containing 2% FCS and 10 ml aliquots placed in plastic petri dishes (Falcon 1005, Becton and Dickinson, Palo Alto, CA). These petri dishes were incubated in a humidified atmosphere, at 37°C and 5% CO_2 for one hour, to allow adherent cells to attach to the plastic. The non-adherent cells were recovered by rinsing the base of the

petri dishes with 10 mls of warm RPMI/10% FCS/plate. Recovered cells were then washed twice in cold CTM, and the viable cell number determined by trypan blue exclusion.

To deplete T cells, splenic cells were resuspended at 10^7 viable cells / ml in cold CTM, and a cocktail of anti-T cell antibodies ($^1/100$ dilution of 30H-12, $^1/5$ dilution of 31M and $^1/30$ dilution of RL172) was added and left on ice. After 30 minutes the cells were washed twice, the cells counted and resuspended in CTM at 10^7 viable cells/ml. Complement was then added and the cells incubated in a water bath at 37°C for 45 minutes. Following the incubation, the cells were washed and resuspended in BCM. The viable cell number was again determined by trypan blue exclusion. Unless otherwise stated the B cell population produced following this method was used in the experiments described in this thesis.

Flow cytometry

The purity of the unseparated B cell preparation was determined by flow cytometric analysis using a Becton and Dickinson FACScan (Palo Alto, CA). An aliquot of approximately 10^6 cells were transferred into plastic 5 ml tubes (Falcon 2054, Becton and Dickinson, CA) and washed in sterile PBS containing 5% FCS (PBS/FCS). Cells were stained with 20 µl of phycoerythrin (PE) labelled anti-B220 antibody (RA3-6B2)(Coffman 1982) or a fluorescein isothiocyanate (FITC) anti-B220 antibody conjugate diluted in PBS / 5% FCS. The cells were incubated on ice for 30 minutes, after which time they were underlaid by pasteur pipette with 300 µl of HI FCS and washed in 1 ml of cold PBS/5% FCS. The B cells were resuspended in 500 µl of PBS/FCS and kept on ice until analysis. Routine analysis of the unseparated B cell preparation revealed that it contained greater than 85% $B220^+$ cells. Analysis of the data was conducted using CellQuest software (Becton and Dickinson) version 1 or 2.

Preparation of small and large B cells

When required, small and large B cells were prepared by separation on a percoll density gradient. A discontinuous percoll gradient was prepared by layering 2.5 mls of 75%, 65% and 50% percoll in pre-cooled 15 ml centrifuge tubes (Falcon, Becton and Dickinson, Palo Alto, CA). Cells were then added to the percoll gradient in 2.5 mls of RPMI/10% FCS at a cell density of 2 x 10^7 cells/ml. Gradients were then centrifuged at 1835 x g for 25 minutes at 4°C, with the brake off. Large B cells were obtained from the interface between the 65% and 50% layers, whereas small dense B cells were obtained from the interface between the 65 and 75% layers. After recovery cells were washed in RPMI/10% FCS by centrifugation at 1322 x g at 4°C for five minutes. Cells

were washed a further three times in BCM at 1101 x g at 4°C for five minutes. The viable cell number was determined by trypan blue exclusion.

3.3. Methods for monitoring B cell responses

Reagents

Antibodies : Goat anti-mouse IgM (gαIgM) antibody was purchased from Cappel (West Chester, PA, catalogue No. 0611-0201). Rat anti-mouse kappa antibody, 187.1 (Gilligan *et al.,* 1988), rat anti-mouse IgM antibody, 331.12 (Kincade *et al.,* 1981) and mouse anti-mouse IgD^a allotype antibody, AMS15.1 (Stall and Loken 1984) , and rat anti-β galactosidase antibodies, GL113 and GL117 were prepared and purified by Ms S. Hwa Chin (JCSMR, Canberra). Purified rat anti-mouse IgM antibody, BET-2 (Kung *et al.,* 1981), mouse anti-mouse IgD antibody, AMS9.1 (Stall *et al.,* 1984) and rat anti-mouse FcγRIIB, 2.4G2, were gifts from Dr P. Lalor (WEHI, Melbourne). Purified rat anti-mouse IgD, 1.19 (Julius *et al.,* 1984), and rat anti-mouse IgM, B7.6 (Brines and Klaus 1992), were prepared and purified by Dr J. Hasbold (Centenary Institute, Sydney). Rabbit anti-mouse IgG antibody and its $F(ab')_2$ fragment were purchased from Serotec (Oxford, UK. catalogue Nos. STAR37 and STAR8B, respectively).

The GL113 and GL117 were used as rat IgG1 and IgG2a isotype controls, respectively.

Sheep anti-mouse Ig antibody (sαIg) and sheep anti-mouse Ig antibody coupled to horse radish peroxidase (sαIg-HRP) were obtained from Silenus (Melbourne, Victoria - catalogue No. BE23A and PD12F, respectively). Sheep anti-mouse total Ig antibody coupled to alkaline phosphatase (sαIg-AP) was purchased from Silenus (Melbourne, Victoria - catalogue No. DAP) and used at a final concentration of 1 μg/ml. Prior to use sαIg was stored at -20°C, whereas sαIg-HRP and sαIg-AP were stored at 4°C.

The hybridoma cells for 187.1, 331.12 and AMS15.1 were a gift from Dr P. Lalor (WEHI, Melbourne), whereas the hybridomas for 1.19 and B7.6 were provided by Dr G. Klaus (National Institute for Medical Research, Mill Hill, UK). The hybridomas for GL113 and GL117 antibody were provided by Dr J. Abrams (DNAX, Palo Alto, CA).

Cyclosporin A (CsA): CsA was a gift from the Sandoz Pharmaceuticals Corporation (Switzerland). A 1 mg/ml stock solution of CsA stock was prepared in dimethyl sulfoxide (DMSO) and stored at -20°C.

LPS: LPS isolated by phenol extraction from *Salmonella typhosa* was obtained from Sigma (St Louis, MO.). LPS was dissolved in BCM, the solution sterilised by filtration

through 0.2 μm membranes and stored at 4°C. This mode of sterilisation was compared to boiling and UV irradiation and was found to yield LPS preparations with equivalent potency (personal communication, Dr Hodgkin. Centenary Institute, Sydney).

IL-4: Recombinant murine IL-4 expressed in *Escherichia coli* and purified by affinity chromatography using the anti-IL-4 antibody, 11B11 (Ohara and Paul 1985), was a generous gift from Dr R. Kastelein (DNAX, Palo Alto).

Ficoll and TNP-Ficoll: Ficoll-400 was obtained from Pharmacia (Uppsalla, Sweden). A 20 mg/ml stock solution of Ficoll-400 was prepared in BCM and sterilised by filtration through 0.2 μm membranes. Ficoll haptenated with TNP (TNP-Ficoll) was a gift from Dr P. Lalor (WEHI, Melbourne).Ficoll solutions were stored at 4°C.

PMA: PMA was purchased from Sigma (St Louis, MO). A 10 μg/ml stock solution was prepared in double distilled water and sterilised by filtration through 0.2 μm membranes. The stock solution was stored at -20°C until required.

All solutions were diluted to the desired concentration in BCM immediately prior to use.

^{3}H-Thymidine: ^{3}H-methyl 1,2, thymidine at 1 mCi/ml was obtained from Amersham, (Uppsala, Sweden - catalogue No. TRK565) or ICN Pharmaceuticals Inc (Costa Mesa, CA. - catalogue No. 2404205). A stock solution, prepared by making a $^1/20$ dilution of the ^{3}H-thymidine concentrate in BCM, was stored at 4°C.

Carbonate Buffer: 0.02 M Na_2CO_3 and 0.076 M $NaHCO_3$, pH 9.1.

Acetate buffer: 0.05 M sodium acetate, pH 5.0.

PBS Tween-20 (PBS/T): PBS containing 0.05% Tween 20 (Sigma, St Louis, MO.)
PBS/T-BSA: PBS/T containing 1%.

The carbonate, and acetate buffers and PBS/T-BSA were sterilised by passage through 0.2 μm membranes and kept at 4°C, whereas PBS/T was stored at room temperature.

Horseradish peroxidase (HRP) substrate: 25 mg of 3-amino-9-ethylcarbazole (AEC) was dissolved in 2 mls of dimethyl formamide (DMF). The AEC solution was then added to 10 mls of 0.05 M acetate buffer, and filtered through a 0.2 μm membrane to remove the precipitate. This solution was diluted with 85 mls of acetate buffer, to which

40 μl of 30% H_2O_2 had been added. The HRP substrate was kept in the dark at 4°C and used within one week.

Alkaline Phosphatase (AP) substrate: 15 mg of 5-bromo-4-chloro-3-indoylphosphate toluidine (BCIP) and 30 mg of p-nitroblue tetrazolium chloride (NBT) were dissolved in 1 ml of DMF. This solution was immediately diluted in a 100 mls of buffer containing 0.1 M $NaHCO_3$ and 1 mM $MgCl_2$, pH 9.8. The AP substrate could be kept at 4°C in the dark for up to a week.

TNP-BSA: TNP was conjugated to BSA resulting in a conjugation ratio of 16 TNP : 1 BSA (TNP_{16}-BSA). This reagent was prepared and provided by Dr P. Lalor (WEHI, Melbourne). The TNP-BSA conjugate was used as a coat for ELISpot assays to determine the number of anti-TNP ASCs. This solution was stored in the dark at 4°C.

ELISpot plates: In all ELISpot assays Millipore microtiter 96-well filtration plates (Germany, catalogue No. STHA09610) were used.

Methods

Measuring B cell proliferation

B cell proliferation was determined by ^{3}H-thymidine incorporation over a 4 hr pulse. In a typical experiment, B cells were diluted to the desired starting cell density in BCM, and placed in 96-well flat-bottom plates (Nunc, Denmark) to a final volume of 200 μl/well. The B cells were incubated in a humidified atmosphere at 37°C and 5% CO_2. After the desired incubation period, 20 μl of ^{3}H-thymidine in BCM, containing 1 μCi, was added to each well. The plates were then incubated for a further 4 hours at 37°C. After the second incubation, plates were harvested by a 96-well cell harvester (Pharmacia Wallac 1295-004 Betaplate™ - Turku, Finland) which transferred the cells of each well to glass fibre filter mats (Pharmacia Wallac, Turku, Finland). These filter mats were then dried in an oven and, once dry, the filters were placed in bags and scintillant added. The bags were then sealed and the level of ^{3}H-thymidine incorporation per well determined in a Betaplate™ liquid scintillation counter (Pharmacia Wallac 1205, Turku, Finland).

Measuring antibody production using an ELISpot assay

The number of antibody secreting cells was determined by a modified ELISpot assay based on the assay, originally described by Sedgwick and Holt (1983) (Sedgwick and Holt 1983). B cells were cultured in BCM, under various experimental conditions, at a starting cell density of 5 x 10^4 cells/well in 96-well flat-bottom microtitre plates and a final volume of 200 μl/well. The B cells were incubated in a humidified atmosphere at 37°C and 5% CO_2. After the desired incubation period cells were harvested and

transferred into fresh 96-well round-bottom plates and washed twice in BCM (Nunc, Denmark) by centrifugation . The cells were then counted and diluted to the desired density in BCM, and transferred into ELISpot plates. These ELISpot plates were coated with 100 µl/well sαIg or TNP-BSA in carbonate buffer and incubated overnight at 4°C. The following day the coat solution was flicked out and the plates blocked with 200 µl/well of PBS containing 2% FCS for an hour at 37°C. Wells were then washed three times in 200 µl PBS. Once transferred, to ELISpot plates the cells were placed in a CO_2 incubator at 37°C, and left undisturbed for 4 hr. After incubation the fluid in the well was flicked out of the wells, and the plates washed three times with PBS, and three times with PBS/T. The plates were blotted dry and then processed for spot formation by adding 100 µl of sαIg-HRP or sαIg-AP in PBS/T-BSA to each well, and the plates left overnight at 4°C. The following day plates were washed four times with PBS. After washing 100 µl/well of the appropriate HRP or AP substrate was added to each well. The plates were then monitored for the formation of spots. The reaction was stopped by washing the plates thoroughly in tap water. Plates were allowed to dry on the bench and the number of spots per well counted using a low power microscope.

Chapter 4

An alternate view of B cell responses to LPS: The absence of high dose paralysis

4.1. Introduction

Coutinho and Möller's concept of a non-specific B cell activating signal (1975d) has been borne out by experiments which revealed that in the absence of an antigen signal, activated T cell membranes, or the interaction between CD40 and its ligand, was sufficient to stimulate B cell proliferation and, in combination with T_h type-2 lymphokines, antibody secretion (Brian 1988, Hodgkin *et al.,* 1991b, Kehry *et al.,* 1992, Noelle *et al.,* 1992a, Noelle *et al.,* 1992b, Armitage *et al.,* 1993, Spriggs *et al.,* 1993). However, the proposed non-signalling role for sIg has not withstood the test of time. It is now clear that sIg can mediate transmembrane and intracellular signals (reviewed in Cambier *et al.,* 1990, Cambier *et al.,* 1992, Reth 1992, Cambier *et al.,* 1994, DeFranco 1997) and that these signals play subtle and variable roles in the regulation of B cell behaviour that are dependent on the type of B cell activator and the differentiation state of the cell (Andersson *et al.,* 1974, Kishimoto *et al.,* 1975, Brunswick *et al.,* 1988, Hasbold *et al.,* 1990, Peçanha *et al.,* 1991, Brines *et al.,* 1992, Grandien *et al.,* 1993, Parry *et al.,* 1994a, Snapper *et al.,* 1995a). In chapter 2 a hypothesis is described which attempts to reconcile the one signal model with sIg-mediated signalling. This hypothesis suggests that the antigen signal may have played a role in the generation of bell-shaped dose response curves for LPS-induced antigen-specific ASC formation that formed the basis of the original one signal theory (Mamchak and Hodgkin 1995). This chapter experimentally re-examines two important conclusions of Coutinho and Möller (1975d); the inhibitory nature of high doses of LPS, and the lack of any role for sIg-mediated signalling in the anti-hapten response.

4.2. Results

4.2.1. Dose response curves for LPS-induced ASC formation do not provide evidence of high dose paralysis

To examine the parameters under which stimulation with LPS generated bell-shaped dose response curves, B cells were cultured in media containing LPS under varying culture conditions. The variables explored were: dose, time in culture, starting cell population, ^{3}H-TdR incorporation, and the generation of ASCs. The initial experiment was performed using a B cell population prepared from spleen cells that had been depleted of RBCs, adherent cells and T cells. After 3 days in culture, B cell differentiation into ASCs was apparent at very low concentrations (10 ng/ml) of LPS, and the number of ASC increased in a dose-dependent manner up to 30 μg/ml (Figure 4.1A). However, at higher concentrations of LPS a small decline in the number of ASCs was observed (Figure 4.1A). The dose response curve obtained on day 4 resembled that from the previous day, with the exception that the maximal response occurred at a lower dose (1 μg/ml) of LPS (Figure 4.1B). As before, there was a modest reduction in the number of ASCs at high (10-1000 μg/ml) concentrations of LPS (Figure 4.1B). On day 5, the dose-response curve differed from that seen on the previous two days (Figure 4.1C). While the maximal response occurred at 1 μg/ml, there was no decline in ASC number at high doses of LPS (Figure 4.1C). An additional feature of LPS-induced ASC formation was that the total number of ASCs increased over time. Thus, the maximal response on day 5 represented a 33% increase over that obtained on day 3 (Figure 4.1A-C).

4.2.2. The dose response curve for LPS-induced proliferation is dependent on the time of assay

In conjunction with measuring LPS-induced ASC formation, the pattern of proliferation was also determined (Figure 4.1D-F). After 3 days the dose response curve for LPS-induced proliferation appeared to be conventional, where the level of ^{3}H-TdR incorporation corresponded to dose (Figure 4.1D). The following day the apparently simple relationship between LPS dose and ^{3}H-TdR incorporation had deteriorated (Figure 4.1E). In contrast, to the results obtained on day 3, the day 4 dose response curve had a biphasic pattern, with peaks of ^{3}H-TdR incorporation occurring at 20 ng/ml and 1 mg/ml (Figure 4.1E). This biphasic pattern of response was also evident on day 5 (Figure 4.1F). Further, unlike the increase in LPS-induced ASC number between days 3 to 5, the overall level of ^{3}H-TdR incorporation decreased with time (Figure 4.1D-F).

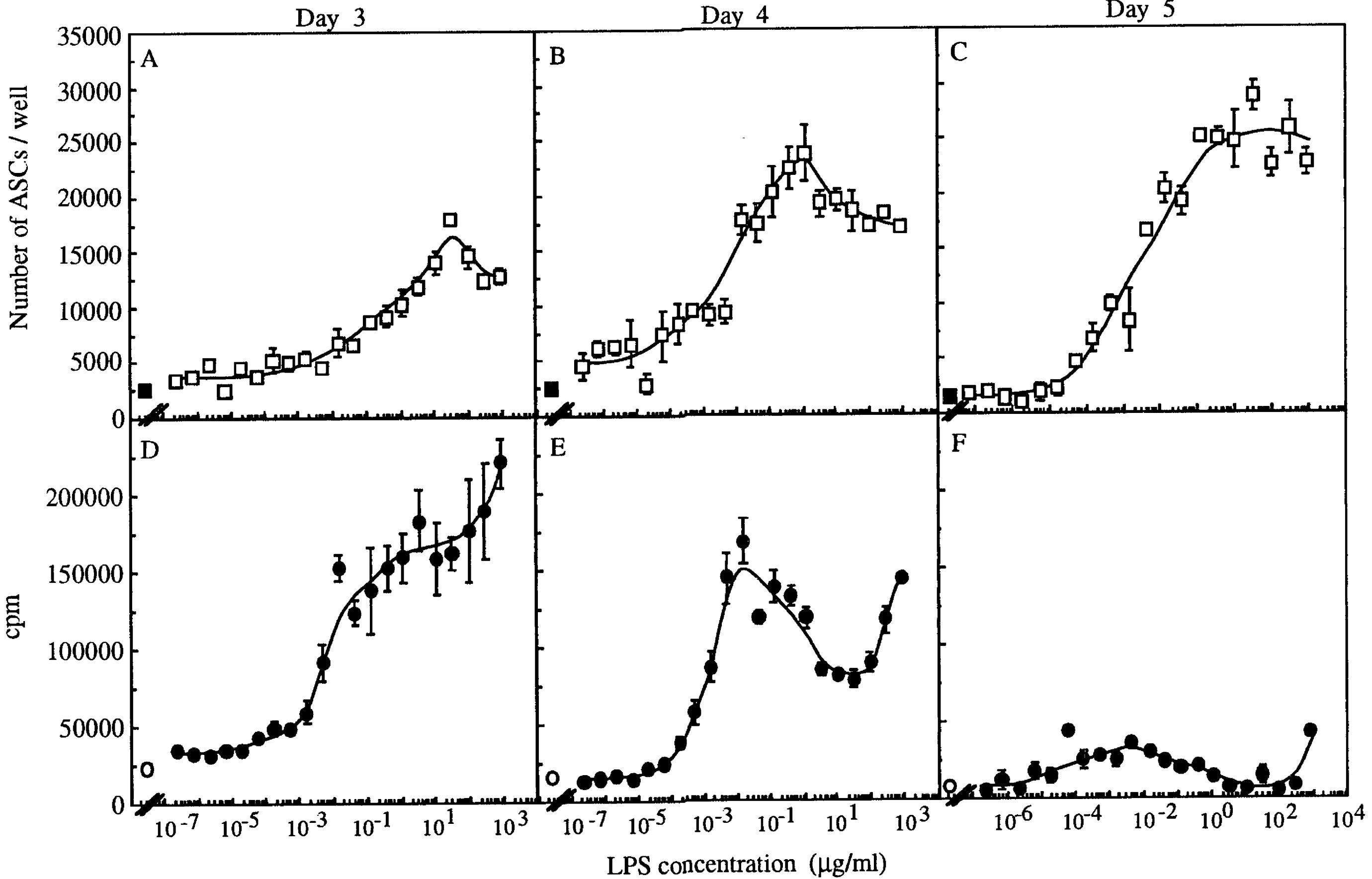

Figure 4.1. **LPS-induces complex dose response curves for proliferation and ASC formation which vary with time.**

B cells were prepared as described in section 3.2.2 and cultured in BCM (5 x 10^4 cells/ 200 μl well) containing varying concentrations of LPS. Panels A-C illustrate the results obtained for ASC formation as measured by Elispot assay (□), whereas panels D-F show the pattern of proliferation as determined by a ^{3}H-TdR incorporation assay (●). Both the Elispot and ^{3}H-TdR incorporation assays were performed as described in chapter 3. The data illustrated represent the mean of triplicate cultures ± 1 x standard error (SEM). Background proliferation (○) and ASC formation (■) are indicated.

The results illustrated in Figure 4.1 do not support Coutinho and Möller's (1975d) tenet of high dose paralysis by LPS. Instead they suggest that B cell differentiation may be delayed at high doses of LPS but will occur late in the time course. However, it is possible that the discrepancy between these results and those described by Coutinho and Möller (1974a/b, 1975a) were the product of using different starting cell populations. To eliminate this possibility the experiment described in Figure 4.1 was repeated using RBC depleted spleen cells in an attempt to better approximate the culture conditions originally used by Coutinho and Möller (Coutinho *et al.,* 1973, Coutinho *et al.,* 1974a).

4.2.3. The dose response curves for LPS stimulation of RBC depleted spleen cells do not show evidence of high dose paralysis

In cultures containing RBC depleted spleen cell, LPS-induced ASC, were detected after 48 hours in culture, however, the down turn in ASC number at high doses of LPS illustrated in Figure 4.1, panels A and B, was not evident (Figure 4.2B, inset). Instead there was a dose-dependent increase in the number of ASCs with the maximal response forming a plateau that extended over a two log increase in LPS concentration (Figure 4.2B). The dose response curve obtained on the following day was similar to that obtained on day 2, with the exception that the overall number of ASCs had increased by approximately ten fold (Figure 4.2C). Finally, on day 4 while the number of ASCs was almost identical to that seen on the preceding day at LPS doses below 100 μg/ml, at higher concentrations the number of ASCs had increased by two fold over that observed on day 3 (Figure 4.2C and D).

4.2.4. The dose response curves for LPS-induced ^{3}H-TdR incorporation indicate that proliferation precedes differentiation to ASCs

As before, the level of proliferation of RBC deleted spleen cells was also determined (Figure 4.2E-H). In contrast to LPS-induced ASC formation, proliferation was above the background at all LPS concentrations after only 24 hrs (Figure 4.2E). On day 2, the level of ^{3}H-TdR incorporation had increased, generating a dose response curve where proliferation was proportional to dose below 6μg/ml and at higher concentrations the level of ^{3}H-TdR incorporation formed a plateau (Figure 4.2F). On the following day the overall level of proliferation had declined (Figure 4.2G), and by day 4 it had almost returned to background with the exception of cultures containing 1 mg/ml LPS. In these cultures the level of ^{3}H-TdR incorporation was still approximately twice that obtained at lower doses (Figure 4.2H).

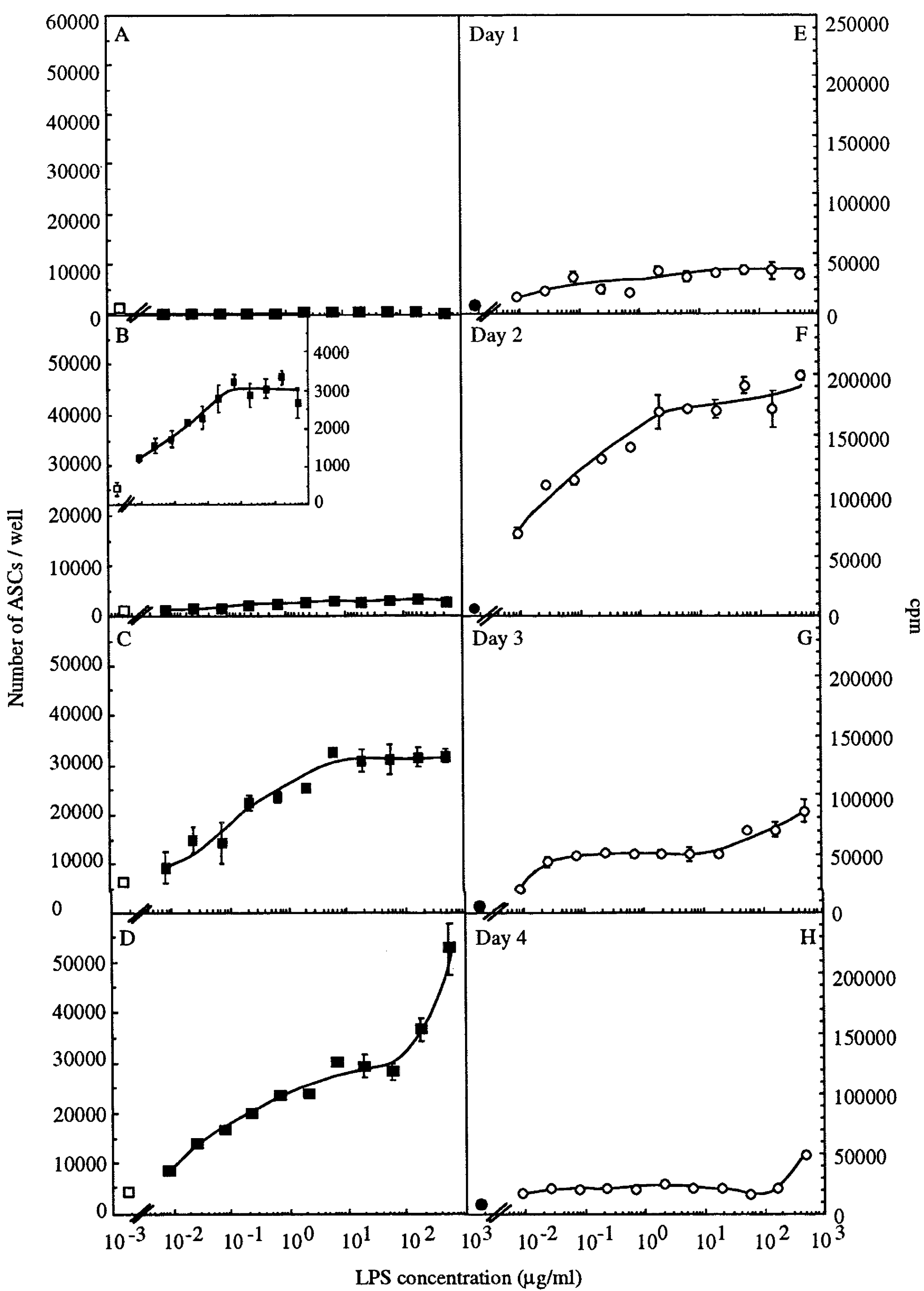

Figure 4.2. **High doses of LPS do not induce B cell paralysis.**

Spleen cells were obtained from CBA/H mice and depleted of RBCs, as described in section 3.2. These cells were then cultured in BCM (10^5 cells / 200μl well) containing varying concentrations of LPS as described in Figure 4.2. Panels A-D illustrate the results obtained for ASC formation as measured by Elispot assay (■), whereas panels E-G show the pattern of proliferation as determined by ^{3}H-TdR incorporation assay (O). For both ASC formation and ^{3}H-TdR incorporation the results are plotted using the same scale to allow direct comparison of the data obtained on different days. Both the Elispot and ^{3}H-TdR incorporation assays were performed as described in chapter 3. Inset in panel B shows the detail of the LPS-induced ASC response on day 2. The data illustrated represent the mean of triplicate cultures ± 1 x SEM. Background proliferation (●) and ASC formation (□) are indicated.

Although there are a number of differences between the dose response curves for ASC formation illustrated in Figures 4.1 and 4.2 neither set of data provides evidence of obligatory high dose paralysis as described by Coutinho and Möller (1975d). Instead these data suggest that B cell differentiation into ASCs was commensurate with the level of stimulation by LPS. However, at very high doses of LPS there appears be a slight delay in this response in cultures containing the more purified B cell population (Figure 4.1A and B). This delay in B cell differentiation was not apparent in the dose response curves for B cell proliferation (Figures 4.1 and 4.2). Finally, a comparison of the results obtained for proliferation and ASC formation indicate that LPS-induced B cell proliferation precedes differentiation, and that ASCs persist in culture without dividing.

4.2.5. Dose response curves for LPS-induced proliferation are influenced by the starting cell density and the time of assay

An obvious difference between the two experiments, illustrated in Figures 4.1 and 4.2, was the shape of the dose response curves obtained for proliferation. As both experiments were conducted under similar culture conditions it is possible that the variation between the two experiments was due to differences in the cell population and / or starting cell densities between the two experiments. A simple experiment to determine if differences in the two cell populations were responsible for the variation in pattern proliferation LPS-induced proliferation of RBC depleted spleen cells and B cells using the same starting cell density (Figure 4.3). The results obtained from this experiment demonstrated that the two cell populations generated virtually identical dose response curves and displayed the same pattern of behaviour as illustrated in Figure 4.1D-F. Therefore, it would appear that the differences between the dose response curves for proliferation illustrated in Figures 4.1 and 4.2 were a reflection of the starting cell densities used in the two experiments. The influence of cell density on LPS-induced proliferation was previously observed by Andersson *et al.,* (1978) and Melchers *et al.,* (1975b). Given the similarity in the dose response curves of RBC depleted spleen cells and the more purified B cell population it was decided to use the second population in subsequent experiments in order to reduce the influence of other cell types in the B cell response to LPS.

To further examine the role of starting cell density in generating the complex dose response curves for LPS-induced proliferation B cells were cultured at different starting cell densities and ^{3}H-TdR incorporation measured over a number of days. As illustrated in Figure 4.4, the shape of the dose response curve was affected by both the starting cell density and the time of harvest. One of the most notable general features was that early in culture (days 1 and 2) the level of ^{3}H-TdR incorporation at each starting cell density

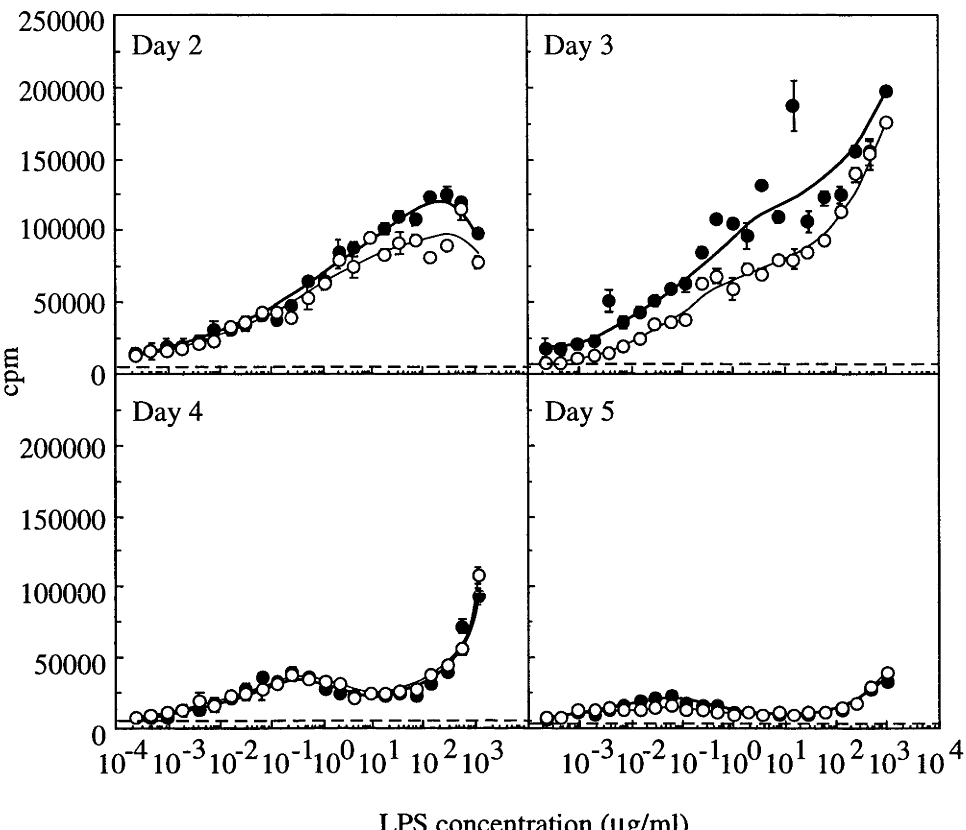

Figure 4.3. **RBC depleted spleen cells and B cells produce identical dose response curves for LPS-induced proliferation.**

This figure compares the dose response curves obtained for LPS-induced proliferation of RBC depleted spleen cells (●) and B cells (○), prepared as described in section 3.3.2. In this experiment cells were cultured in BCM (5 x 10^4 cells / 200 μl well) containing varying concentrations of LPS, and the level of proliferation measured by ^{3}H-TdR incorporation assay, as described in chapter 3. The data illustrated represents the mean of triplicate cultures ± 1 x SEM. Background proliferation (— —) is indicated.

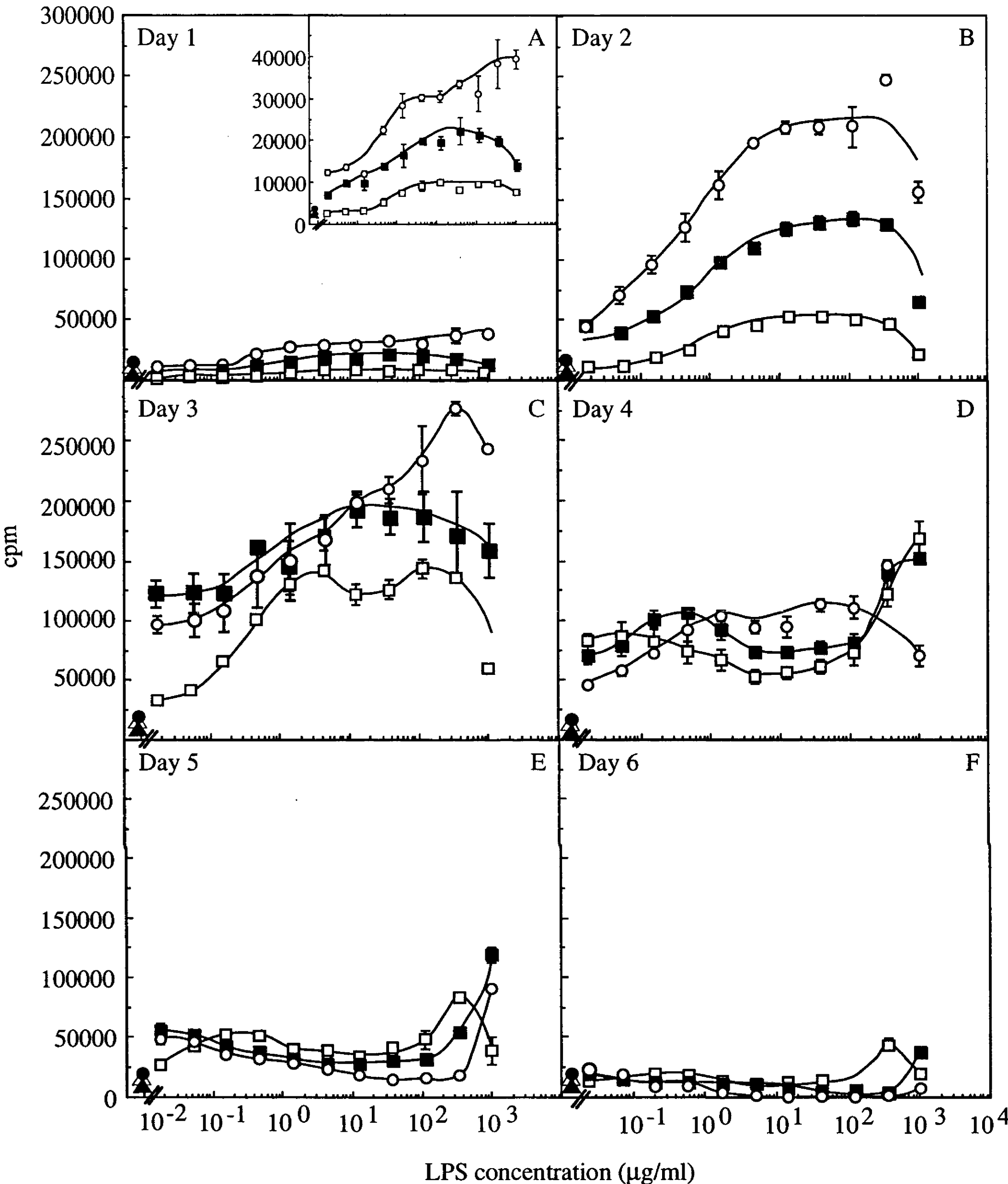

Figure 4.4. **The dose response curve for LPS-induced B cell proliferation is dependent on the starting cell density and time of assay.**

B cells were prepared as described in section 3.2 and cultured at different starting cell densities (10^5 (O); 5 x 10^4 (■); 2.5 x 10^4 (□) / 200 µl well) in BCM containing varying concentrations of LPS. At the time point indicated, proliferation was measured by ^{3}H-TdR incorporation as described in chapter 3. To allow direct comparison between days, the data were plotted on the same scale. The inset in panel A shows the detail of B cell proliferation on day 1. The data illustrated represent the mean of triplicate cultures ± 1 x SEM.

Background proliferation: 10^5 (●); 5 x 10^4 (△); 2.5 x 10^4 (▲) are indicated.

was conventional, producing a dose-dependent increase in proliferation up to 10 μg/ml and forming a plateau at higher concentrations (Figure 4.4A and B). However, in cultures containing the highest dose of LPS (1 mg/ml) ^{3}H-TdR incorporation was slightly reduced (Figure 4.4B). Taken in isolation this feature might be interpreted as high dose inhibition by LPS, however, on subsequent days the level of ^{3}H-TdR incorporation at 1 mg/ml was quite high, revealing a small lag in the response similar to that observed for ASC formation (Figure 4.1A-C) rather than complete inhibition (Figure 4.4D and E). The other feature of note was that by days 3 and 4 the overall level of ^{3}H-TdR incorporation had peaked, and the proportional relationship between starting cell number and proliferation had deteriorated (Figure 4.4C and D). Thus, on day 4 the overall level of ^{3}H-TdR incorporation had declined, and the highest level of proliferation was obtained in cultures containing low to moderate starting cell densities (Figure 4.4D). The effect of these kinetics combined with the delayed response at the very high concentrations was to create a series of complex wave like curves that could be either bell-shaped, biphasic or essentially linear (Figure 4.4).

4.2.6. LPS-induced B cell proliferation depletes the media of essential nutrients, which causes a decline in ^{3}H-TdR incorporation

Although the LPS dose response curves appear complex, a relatively simple explanation of the kinetic changes observed for ^{3}H-TdR incorporation in Figures 4.1 to 4.4, was that LPS stimulated B cells will grow to a limit set by the culture conditions. Hence, lower LPS concentrations, or reduced starting cell number would allow this limit to be reached later in culture, generating the wave-like features of the response curves (Figure 4.4C-E). An alternative explanation was the generation of suppressor cells and / or cellular interactions which elicit different outcomes depending on the initial culture conditions. These two possibilities can be easily be distinguished by replenishing the media in which B cells are growing. The first hypothesis predicts that replenishing the medium would prolong B cell proliferation, whereas the second suggests that cell interactions are responsible and that media replacement will have no effect. To determine which of the two alternatives was correct, LPS-induced proliferation was measured in matched B cell cultures where the media was replenished with fresh media containing the starting concentration of LPS at 12 hourly intervals in one set of cultures but not in the other (Figure 4.5). Both the control (no replenishment) and the experimental culture generated almost identical dose response curves for the first 72 hrs (Figure 4.5), indicating that the media was not limiting proliferation during these early time points. However, after 72 hrs, the level of ^{3}H-TdR incorporation in the control cultures declined to the background level over a period of 48 hrs (Figure 4.5). In contrast, in cultures which had received fresh media, the level of proliferation remained well above the

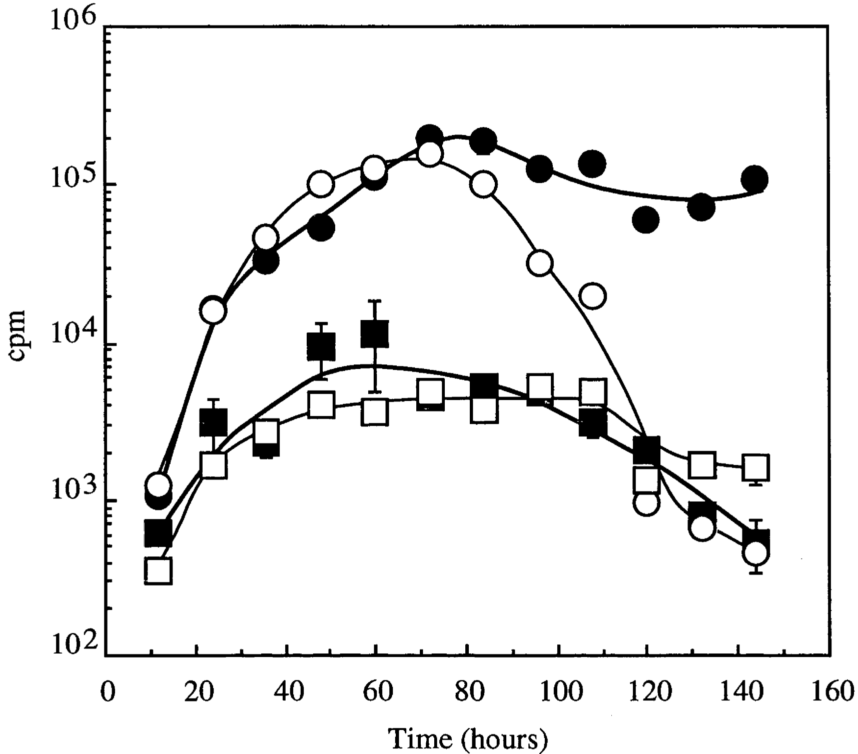

Figure 4.5. **Regular media replenishment prolongs LPS-induced B cell proliferation.**

B cells were prepared as described in 3.2 and cultured in BCM (5 x 10^4 cells / 200 μl well) containing LPS (50 μg/ml). At 12 hourly intervals half the media was removed and replaced with fresh BCM containing 50μg/ml LPS (●). Replicate B cell cultures were prepared but remained undisturbed for the duration of the assay (O). The response of control B cell cultures prepared and incubated in the absence of LPS and subjected to the same experimental regime are shown (media replenished - ■ , undisturbed - □). At the time points indicated B cell proliferation was measured by ^{3}H-TdR incorporation as described in chapter 3. The data illustrated represent the mean of triplicate cultures ± 1 x SEM.

background for the duration of the assay (Figure 4.5). These results are consistent with the premise that depletion of the media in high density cultures leads to an inability to maintain proliferation beyond day 3.

The experiment illustrated in Figure 4.5 indicated that replenishment of cultures with media containing LPS could prolong B cell proliferation. Before drawing a conclusion however, it was important to show that the prolonged ^{3}H-TdR uptake in cultures in which the media had been replenished was not due to the provision of fresh LPS. The argument could be made that the LPS was depleted in the control cultures, and therefore at late times the two culture groups were not receiving the same level of stimulation. To distinguish between these two possibilities, B cells were cultured in media which contained LPS and the supernatants from these cultures harvested and stored after 2 to 4 days. Fresh B cells were prepared and cultured in new media, or in the previously harvested supernatants to which LPS had been added. As illustrated in Figure 4.6 each of the cultures generated the same pattern of proliferation over time, where ^{3}H-TdR incorporation reached a peak on day 2. Importantly, the level of ^{3}H-TdR uptake was inversely proportional to the length of time cells had originally been grown in the supernatant (Figure 4.6). As the level of LPS would be the same, or greater, in the cultured supernatant than in fresh media these results discount the possibility that consumption of LPS was responsible for the down turn in proliferation. Instead they indicate that the depletion of media components caused the rapid reduction in ^{3}H-TdR uptake after 72 hours (Figure 4.6).

4.2.7. The role of the antigen signal in altering LPS-induced antigen-specific antibody production

The observation that LPS-induced paralysis was not obligatory contradicted the conclusion reached by Coutinho and Möller (Coutinho *et al.*, 1975), and raised a question about the B cell response to LPS. If LPS does not induce B cell paralysis at high concentrations, what was the cause of the bell-shaped dose response curves observed by Coutinho and Möller (1975d) for ASC formation? The results illustrated in Figures 4.5 and 4.6 suggest that depletion of the media results in a decline in LPS-induced B cell proliferation. Although the down turn in LPS-induced proliferation did not correspond with a decline in ASC numbers under the culture conditions described in this chapter (Figures 4.1 and 4.2), it is possible that under the culture conditions employed by Coutinho and Möller media depletion may have played a role in the generation of their bell-shaped dose response curves. Alternatively their preparation of LPS could have contained toxic contaminants which at high doses of LPS were sufficient to inhibit B cell differentiation. However, neither media depletion nor toxic

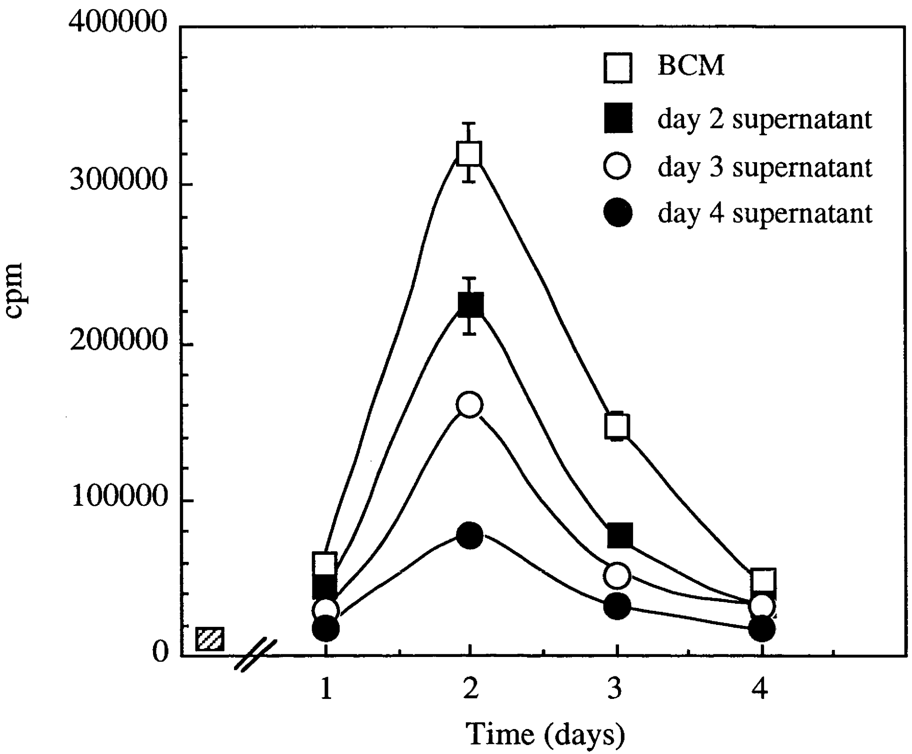

Figure 4.6. **Depletion of the media results in a reduction in LPS-induced B cell proliferation.**

B cells were prepared as described in section 3.2 and cultured (5 x 10^5 cells/ml) in BCM containing LPS (50 μg/ml). The supernatants from these cultures were harvested on days 2, 3 and 4 and stored. Fresh B cells were prepared and cultured (5 x 10^4 cells / 200μl well) in either BCM (□) or the previously harvested supernatants (day 2 - ■ , day 3 - ○ , and day 4 - ●) in the presence of LPS (50 μg/ml). The level of proliferation in these cultures was measured over a number of days by ^{3}H-TdR incorporation as described in chapter 3. The data illustrated represent the mean of triplicate cultures ± 1 x SEM. The background cpm for cells only is indicated (▨).

contaminants would account for the down turn in the number of LPS-induced antigen-specific ASCs observed by Coutinho and Möller (Coutinho *et al.,* 1974a) at much lower overall LPS concentrations. As the number of cells involved in an antigen-specific response would be small, their rapid proliferation would not deplete the culture media sufficiently to cause the decline in antigen-specific ASCs. In addition, as the down turn in antigen-specific ASC numbers occurred at low doses of hapten-LPS it is unlikely that contaminants in Coutinho and Möller's LPS preparation would have caused the inhibition of this response. An alternative explanation for the antigen-specific response has been proposed which suggests that the a sIg-mediated (antigen) signal could inhibit LPS-induced antigen-specific ASC formation (Mamchak *et al.,* 1995). This hypothesis predicts that the cross-linking of sIg by a hapten coupled to a non-mitogenic carrier would induce a signal which could inhibit LPS-induced antigen-specific ASC formation in a manner similar to that postulated in chapter 2 for haptenated-LPS. In contrast, Coutinho and Möller's one signal model suggests that such a reagent would not influence the anti-hapten response (Coutinho *et al.,* 1975, Möller *et al.,* 1975). To test the alternative hypothesis, B cells were cultured with LPS in the presence of TNP-Ficoll to provide a sIg-mediated signal, and the number of anti-TNP ASCs measured.

4.2.8. Ficoll does not alter LPS-induced B cell proliferation or ASC formation

Prior to using TNP-Ficoll to determine its effect on LPS-induced anti-TNP ASC formation, it was necessary to determine if Ficoll alone had any effect on B cell behaviour. As illustrated in Figure 4.7 Ficoll did not induce proliferation above the background and had only a slight inhibitory effect on LPS-induced proliferation, whereas TNP-Ficoll had virtually no effect on LPS-induced B cell proliferation (Figure 4.7). These results indicated that Ficoll was non-mitogenic and did not significantly alter LPS-induced proliferation. Next the effect of Ficoll on TNP-specific ASC formation was determined. A proportion of LPS stimulated B cells secrete antibodies that bind to TNP-BSA coated ELISpot plates (Figure 4.8). Increasing the coat concentration of TNP-BSA allows for more positive cells to be detected, presumably as the affinity cut off of detection is lowered. The addition of concentrations of Ficoll up to 5μg/ml did not affect the generation of TNP-BSA specific ASCs in culture (Figure 4.8).

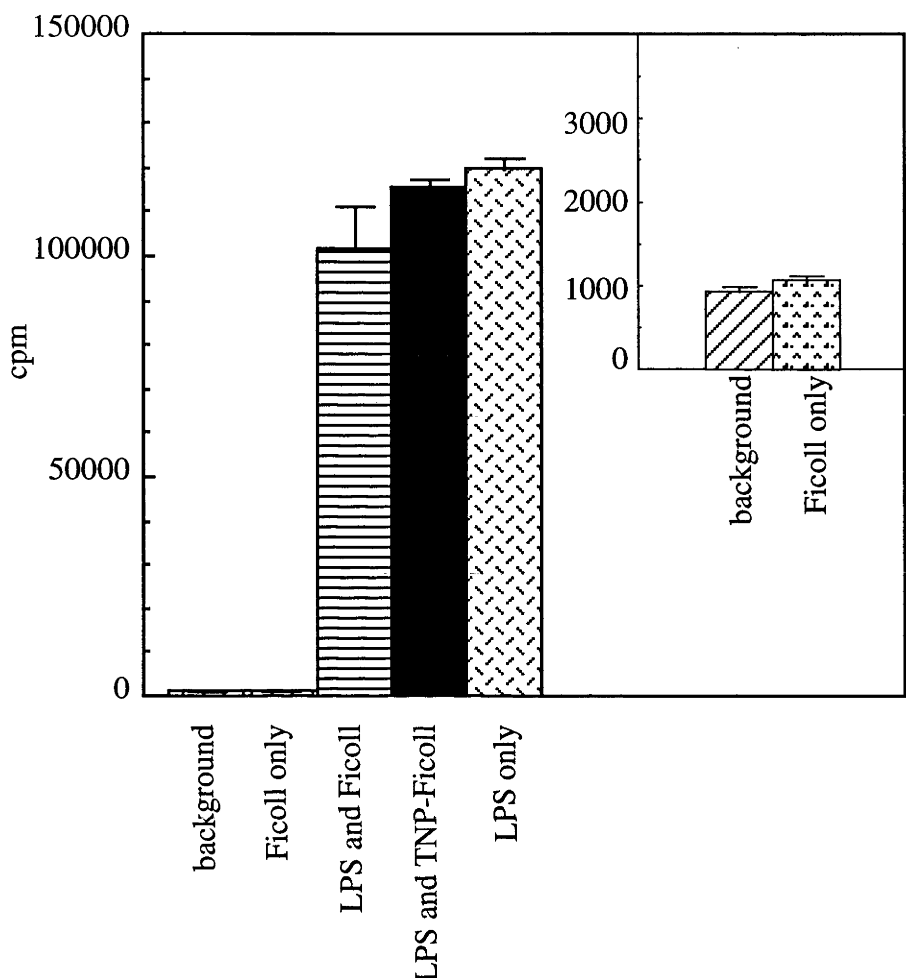

Figure 4.7. **The effect of Ficoll on B cell proliferation.**
B cells were prepared as described in section 3.2 and cultured in BCM (5×10^4 cells / 200 μl well) containing Ficoll (5μg/ml) alone, or in combination with LPS (50μg/ml). Similar cultures were also prepared which contained TNP-Ficoll (5μg/ml) and LPS. After 3 days B cell proliferation was measured by ^{3}H-TdR incorporation as described in chapter 3. The inset shows the detail of the background proliferation and that induced by Ficoll alone. The data illustrated represent the mean of triplicate cultures ± 1 x SEM.

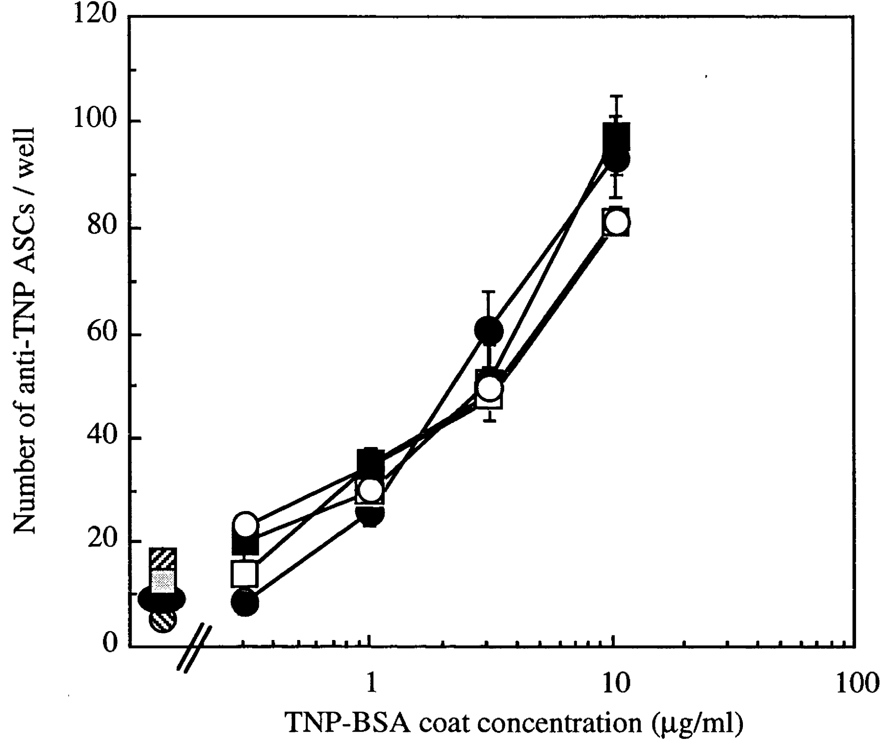

Figure 4.8. **Ficoll does not alter LPS-induced antigen-specific ASC formation.**

B cells were prepared as described in section 3.2 and cultured in BCM (5 x 10^4 cells / 200 μl well) containing 50 μg/ml LPS alone (●), or in combination with three different concentrations of Ficoll (5 - □ , 0.5 - ○ , and 0.05 μg/ml - ■). After 3 days the cells were transferred to Elispot plates which had previously been coated with varying concentrations of TNP-BSA. The use of different TNP-BSA concentrations to coat Elispot plates made it possible to determine the effect of Ficoll on the LPS-induced formation of anti-TNP ASCs of different affinities. The number of anti-TNP ASCs was then determined by Elispot assay as described in chapter 3. Background: cells only (⬬); Ficoll only: 5 μg/ml (▨); 0.5 μg/ml (▩); and 0.05 μg/ml (◍) are indicated. The data illustrated represent the mean of triplicate cultures ± 1 x SEM, with the anti-BSA response subtracted.

4.2.9. TNP-Ficoll inhibits the formation of LPS-induced antigen-specific ASCs

Having shown that Ficoll was a suitable non-mitogenic non-inhibitory matrix for haptenation, the effect of TNP-Ficoll on the LPS-induced anti-TNP response was determined (Figure 4.9). As before, the presence of Ficoll in B cell cultures containing LPS did not inhibit the formation of anti-TNP ASCs (Figure 4.9). In contrast, the addition of TNP-Ficoll to LPS-stimulated B cell cultures resulted in a dose-dependent decline in the number of antigen-specific ASCs (Figure 4.9). At a TNP-BSA coat concentration of 1 μg/ml the detectable number of anti-TNP ASCs was much lower than observed at higher coat concentrations which may reflect the small number of high affinity anti-TNP ASCs induced by LPS (Figure 4.9). However, the inhibition of anti-TNP ASC formation by TNP-Ficoll was still evident even at low TNP-BSA concentrations (Figure 4.9C). These results support the hypothesis that a sIg-mediated signal can induce a decline in the number of LPS-induced hapten-specific ASCs.

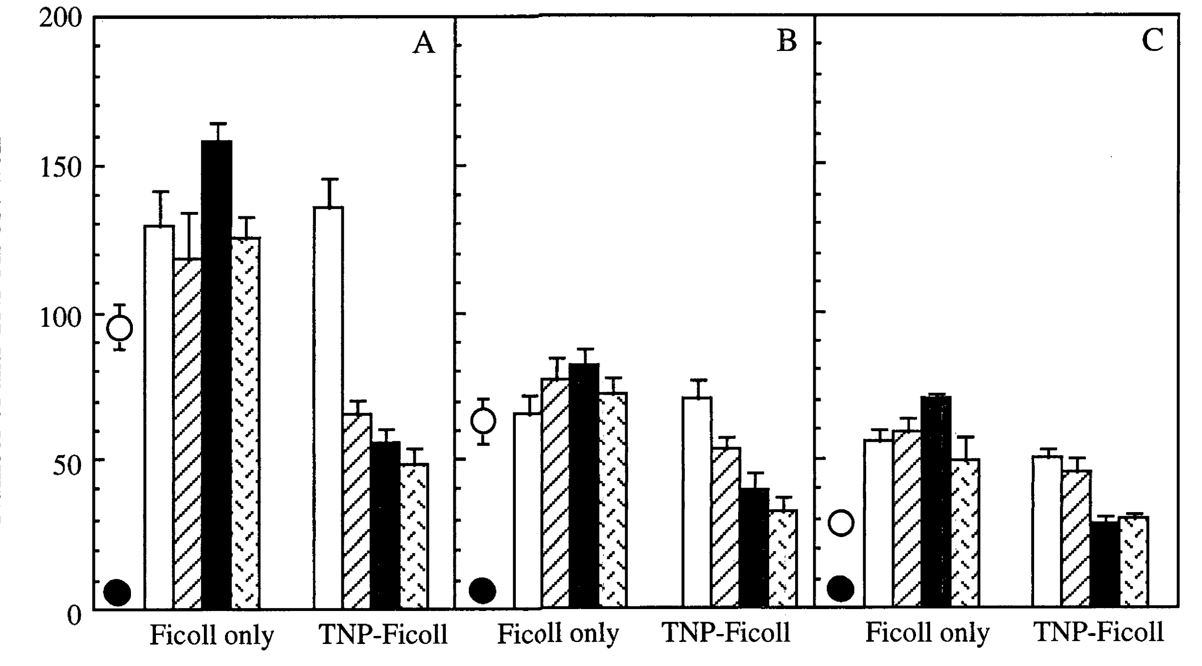

Figure 4.9. **TNP-Ficoll inhibits LPS-induced anti-TNP ASC formation in a dose dependent manner.**

B cells were prepared as described in section 3.2 and cultured in BCM (5×10^4 cells / 200 µl well) containing 50 µg/ml LPS alone (○); or in combination with different concentrations of Ficoll or TNP-Ficoll (0.005 - □ , 0.05 - ▨ 0.5 - ■ , and 5 µg/ml - ⬚). After 3 days the number of anti-TNP ASCs was determined by Elispot assay as described in chapter 3 using Elispot plates which had been previously coated with different concentrations of TNP-BSA (panel A -10 µg/ml; panel B - 3 µg/ml; and panel C - 1 µg/ml). The data illustrated represent the mean of triplicate cultures ± 1 x SEM with the anti-BSA response subtracted. ASC fomation from cultures containing cells only (●) is indicated.

4.3. Discussion

The experiments described in this chapter were initially designed to reproduce the bell shaped dose response curves described by Coutinho and Möller (1994b) for LPS-induced ASC formation. However, they revealed that high doses LPS did not induce obligatory paralysis, although they could, under certain circumstances, cause a delay in B cell differentiation (Figure 4.1). Given the absence of LPS-induced paralysis the down turn in ASC number described by Coutinho and Möller (1994b) must have been due to some other cause. As described in chapter 2, the decline in the number of LPS-induced antigen-specific ASCs may have been due to the antigen signal, and the results illustrated in Figure 4.9 support this hypothesis. In addition, the results described in this chapter reveal that LPS can induce complex patterns of B cell proliferation (Figures 4.1 and 4.4).

4.3.1. The complex pattern of LPS-induced proliferation conforms to a relatively simple model

Despite the apparent complexity of the dose response curves for LPS-induced B cell proliferation it was possible to generate similar curves through a relatively simple model. The wavelike features of the experimentally derived dose response curves can be explained as a combination of two independent patterns of proliferation. Figure 4.10A, illustrates the expected changes in dose response curves with time of a cell with unlimited growth potential responding in conventional fashion to a titrated stimulus. At early time points the response will yield a typical sigmoidal curve with a plateau region of optimal stimulation (Figure 4.10A). With time the proliferating cells will deplete the culture media, and the rate of growth will slow and begin to fall (Figure 4.10A). Cells containing less stimulus will deplete the media more slowly, and will for a time contain a greater number of dividing cells (Figure 4.10A). The utilisation of media generates a wave of bell curves, the peak of which occurs at lower concentrations with increasing time (Figure 4.10A). Reducing the starting cell number would have the effect of lengthening the time it takes to reach the point of media depletion. As illustrated in Figures 4.5 and 4.6, the reduction in the rate of growth was due to depletion of the media and not to an intrinsic property of the B cells. The capacity of B cells to maintain prolonged growth if subcultured has previously been observed (Melchers *et al.,* 1975, Zauderer and Askonas 1976). As a first approximation of LPS-induced proliferation, the model illustrated in Figure 4.10A matches the observed B cell behaviour (Figures 4.1, 4.2 and 4.4). However, the experimental dose response curves exhibit an additional complexity. At very high concentrations of LPS (≥1 mg/ml) the B cell response appears to be delayed (Figure 4.4). By altering the model of B cell proliferation in Figure 4.10A by substituting the response at the highest LPS

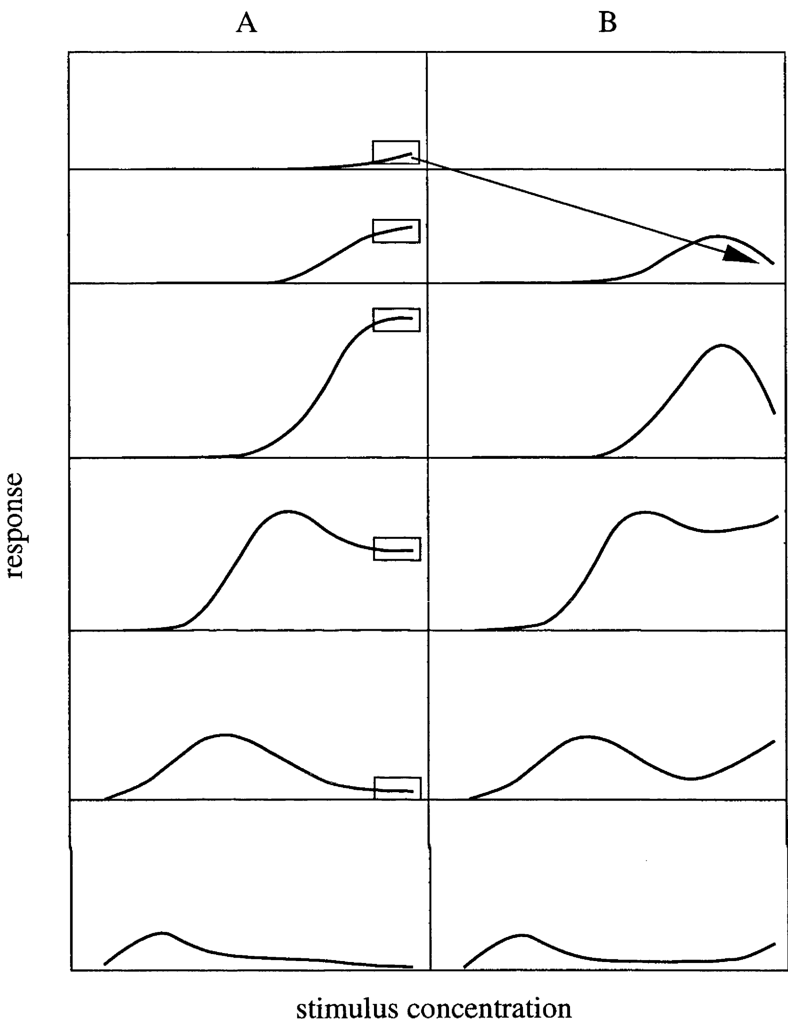

Figure 4.10. **A model of B cell proliferation.**

This figure depicts the expected dose response curves for two models of B cell growth. Panel A illustrates the predicted dose response curves over time for the proliferation of B cells with unlimited growth potential, where the cells are responding to the titrated stimulus in a conventional manner. Panel B shows the predicted dose response curves over time for the proliferation of B cells with unlimited growth potential, where high doses of the stimulus result in a delay in the response. Thus, the dose response curve for high concentrations of the stimulus (boxed section) in panel A has been used to replace the dose response curve for high concentrations of the stimulus of the following time interval in panel B.

concentration (boxed section of curve) with that of the previous time interval, the delayed response can be incorporated into the model (Figure 4.10B). Through this method, complex dose curves were constructed (Figure 4.10B), which contained the same wave-like pattern of proliferation as observed experimentally (Figures 4.1 and 4.3). Thus, the combined effect of growth to a culture-imposed limit and a delayed response at very high concentrations of stimuli yields bell-shaped curves for proliferation of two different types. One appears early in culture and is caused by the delayed response at high concentrations of LPS (Figure 4.10B). The other appears later and seems to be an artefact of high density culture (Figure 4.10A).

4.3.2. Speculation on the cause of bell-shaped dose response curves for LPS-induced polyclonal ASC formation

Although the results obtained for LPS-induced B cell proliferation may be explained by a combination of media depletion and a delayed response at high doses of LPS, the bell-shaped curves described for polyclonal ASC formation (Andersson *et al.,* 1972) are more difficult to account for. The results described in this chapter indicate that B cells proliferate prior to differentiating to ASCs (Figures 4.1 and 4.2). It is possible that in Coutinho and Möller's experiments, which were conducted at very high starting cell density (10^7 cells/ml) the rapid proliferation of so many cells at high doses of LPS depleted the media before the B cells could undergo sufficient rounds of division to differentiate. However, this proposition was not supported by results from an experiment which used the same cell density as Coutinho and Möller, in which the proliferative response waned rapidly, but the number of ASCs increased with time and did not show any evidence of high dose paralysis (data not shown). Alternatively, the down turn in the total ASC numbers could have been the result of toxic contaminants within Coutinho and Möller's preparation of LPS. If the toxic contaminants were present at low concentrations they might only exert their inhibitory effect at high concentrations of LPS, thereby mimicking high dose paralysis. Finally the results illustrated in Figure 4.1, reveal that under appropriate culture conditions high doses of LPS can induce a small decline in the number of ASCs, which generates dose response curves that are reminiscent of the bell-shaped curves described by Coutinho and Möller (1994b). Taken in isolation such dose response curves may have led to the conclusion that high doses of LPS-induce B cell paralysis.

4.3.3. Previous work on LPS-induced B cell activation which is inconsistent with the principle of high dose paralysis

Close examination of some original papers on the B cell response to LPS also revealed variations in the shape of the dose response curve for ASC formation with time. In one experiment the dose response curve for LPS-induced anti-DNP ASC formation did not

show any evidence of high dose paralysis after 24 hours in culture. Instead the dose response curve was conventional, with the number of ASCs being proportional to dose (Coutinho *et al.,* 1973). In this experiment the anti-DNP response was representative of the total antibody response, as LPS was not conjugated to the hapten and therefore could not be focussed to the B cell surface by anti-DNP sIg. In contrast to the results obtained on the previous day, the dose response curve for day 2 showed evidence of a down turn in ASC number at high doses (100 µg/ml) of LPS (Coutinho *et al.,* 1973). In addition to the change in the shape of the dose response curve over time, the number of ASCs had increased at all LPS concentrations between the days 1 and 2 (Coutinho *et al.,* 1973). Taken in isolation, the day 2 dose response curve could be interpreted as evidence of high dose paralysis. However, when the results from both days are considered it is clear that the shape of the dose response curve was determined by the time of assay. Thus, although it was possible to generate bell-shaped dose response curves for LPS-induced ASC formation, the shape of the curve appeared to be due to the culture conditions and time of assay, rather than obligatory high dose paralysis. The results described in Figure 4.1 suggest that the effect of high concentrations of LPS is to delay B cell differentiation, which could be misinterpreted as LPS-induced paralysis if the data for a single day were taken in isolation, as Coutinho and Möller may have done.

In addition, Coutinho and Möller's published data was not always consistent on the degree, or time of onset of paralysis (Andersson *et al.,* 1972, Coutinho *et al.,* 1974a, Coutinho *et al.,* 1975), and some of the B cell responses they observed were inconsistent with the behaviour predicted by a model which invokes high dose paralysis by LPS. For example, Coutinho and Möller examined the effect of a combination of sub-optimal concentrations of DNP-LPS and LPS on the anti-DNP ASC response (Coutinho *et al.,* 1975). According to Möller and Coutinho's one signal model, a high dose of LPS in combination with DNP-LPS should induce a more severe inhibition of the optimal anti-DNP response than a low dose of LPS in combination with DNP-LPS. However, a detailed examination of the data reveals that the converse was true (Coutinho *et al.,* 1975). Although these results do not conform to the behaviour that would be predicted if high doses of LPS induced B cell paralysis, they are consistent with the hypothesis that sIg-mediated signals inhibit LPS-induced antigen-specific ASC formation. Further, the results from more recent experiments in which B cells were coated with anti-TNP antibodies (which were not capable of inducing an intracellular signal) and stimulated with TNP-LPS produced evidence of sIg-mediated focussing but not of the LPS-induced paralysis (Alarcon-Riquelme *et al.,* 1990) which would have been expected to occur if the tenet of high dose paralysis were correct.

4.3.4. The antigen signal can inhibit LPS-induced differentiation of antigen-specific B cells

So far, the discussion has focussed on the polyclonal response induced by LPS and the view that the down turn in the number of ASCs at high doses of LPS was not due to paralysis, but rather was the result of an artefact of the culture conditions. Although this hypothesis may account for the bell-shaped curves for the total ASC number it cannot explain the bell curves obtained for antigen-specific responses. An alternative explanation for the dose response curves for LPS-induced antigen-specific ASC formation was proposed in which sIg-mediated signals inhibit LPS-induced B cell differentiation (Mamchak *et al.,* 1995). This hypothesis was supported by the experimental results obtained with TNP-Ficoll (Figure 4.10). As the presence of Ficoll alone did not inhibit LPS-induced ASC formation, the inhibitory effects of TNP-Ficoll (Figure 4.9) were probably due to the interaction between TNP and anti-TNP-sIg. Further support for the inhibitory role of sIg-mediated signals can be obtained from the experiments in which B cells were stimulated with a combination of anti-Ig antibodies and LPS. In these experiments anti-Ig antibodies, or their $F(ab)_2$ fragments, inhibited LPS-induced ASC formation in a dose-dependent manner (Andersson *et al.,* 1974, Kearney *et al.,* 1976, Andersson *et al.,* 1978, Kearney *et al.,* 1978, Sidman and Unanue 1978). The conclusion that sIg-mediated signals inhibit LPS-induced ASC formation is inconsistent with Coutinho and Möller's one signal model. However, it does present an explanation for their antigen-specific dose response curves which does not invoke LPS-induced paralysis and is consistent with a regulatory role for the antigen signal in limiting the affinity of T-independent antibody responses (Mamchak *et al.,* 1995). The potential for sIg-mediated signals to regulate the B cell response to LPS is examined further in chapter 5.

Chapter 5

Surface Ig-mediates two signals with different thresholds of initiation and sensitivity to cyclosporin A

5.1. Introduction

In chapter 2 a model was proposed that argued the ability of anti-Ig reagents to inhibit LPS-induced ASC formation (Andersson *et al.,* 1974, Kearney *et al.,* 1976, Andersson *et al.,* 1978, Kearney *et al.,* 1978), was the result of sIg-mediated signals which actively regulated the affinity of antibody produced following TI type-1 B cell activation (Mamchak *et al.,* 1995). One way to distinguish experimentally between this model and that described by Coutinho and Möller (1975), which invokes high dose paralysis by LPS, was to induce an antigen signal in B cells stimulated with LPS in the presence an inhibitor of sIg-mediated signalling. A possible candidate was CsA, as this drug has been shown *in vitro* to inhibit sIg-mediated signals, but not LPS-induced B cell responses (Dongworth and Klaus 1982, O'Garra *et al.,* 1986, Klaus 1988). If CsA inhibits sIg- and not LPS-induced signalling then it could be used to determine whether antigen stimulation is responsible for the bell shaped dose response curves which have been described for LPS-induced ASC formation (Andersson *et al.,* 1972, Coutinho *et al.,* 1973, Coutinho *et al.,* 1975) as predicted by Mamchak and Hodgkin (1995). To examine whether CsA could be used in this way, it was important to first show that this drug would prevent the ability of anti-Ig reagents to inhibit LPS-induced ASC formation as originally shown by Andersson *et al.,* (1974). This chapter describes the results of experiments designed to test this prediction.

5.2. Results

5.2.1. Goat anti-mouse IgM (gαIgM) induces different effects on B cell proliferation which vary according to antibody dose

To explore the role of the antigen signal in TI type-1 activation, B cells were stimulated with a combination of LPS and gαIgM. The anti-Ig antibody was used to trigger a signal from sIg, and LPS was used as a TI type-1 B cell activator. CsA, which has been shown to inhibit sIg-, but not LPS- induced responses (Dongworth *et al.,* 1982, O'Garra *et al.,* 1986, Klaus 1988) was used to block sIg-mediated signals in these cultures. Incubation of B cells with high doses (10-20 μg/ml) of gαIgM resulted in proliferation above the background (Figure 5.1). However, an unexpected feature of the dose response curve was the inhibition of proliferation at low to moderate concentrations (1 ng -1 μg/ml) of gαIgM (Figure 5.1). The inclusion of 40 ng/ml CsA in these cultures inhibited proliferation (Figure 5.1). This dose of CsA completely inhibited gαIgM-induced ^{3}H-TdR incorporation, while having only a small inhibitory effect on LPS-induced proliferation (Figures 5.1 and 5.2). As higher concentrations of CsA resulted in the inhibition of the LPS-induced ^{3}H-TdR incorporation, whereas lower doses did not completely inhibit the B cell response to gαIgM (data not shown), a concentration of 40 ng/ml of CsA was used in all subsequent experiments. Also noteworthy was that addition of CsA to unstimulated B cell cultures resulted in a significant (~70%) inhibition of the background proliferation (Figures 5.1 and 5.2). These results demonstrate that gαIgM was able to induce a measurable sIg-mediated signal which was sensitive to the action of CsA. Having determined the B cell response to gαIgM and CsA, it was possible to test the hypothesis that sIg-mediated signals are involved in the regulation of LPS-induced antibody production.

5.2.2. Anti-Ig mediated inhibition of LPS-induced ASC formation is CsA insensitive

In B cell cultures containing gαIgM and LPS, the presence of the anti-Ig antibody strongly inhibited ASC formation (Figure 5.3), as previously described (Andersson *et al.,* 1974, Kearney *et al.,* 1976, Andersson *et al.,* 1978, Kearney *et al.,* 1978). Given the inhibitory effect of CsA on gαIgM-induced proliferation it was anticipated that CsA would block the anti-Ig mediated decline in ASC number. However, as illustrated in Figure 5.3, CsA had almost no effect on the gαIgM-mediated inhibition of LPS-induced formation of ASCs. In fact the addition of CsA to cultures containing gαIgM and LPS caused a slight further reduction in the overall number of ASCs (Figure 5.3). These results show that not all B cell behaviour in response to sIg-mediated signalling was CsA sensitive, and that the plan to use this drug to address the role of the antigen signal in regulating the affinity of TI type-1 antibody responses could not be pursued.

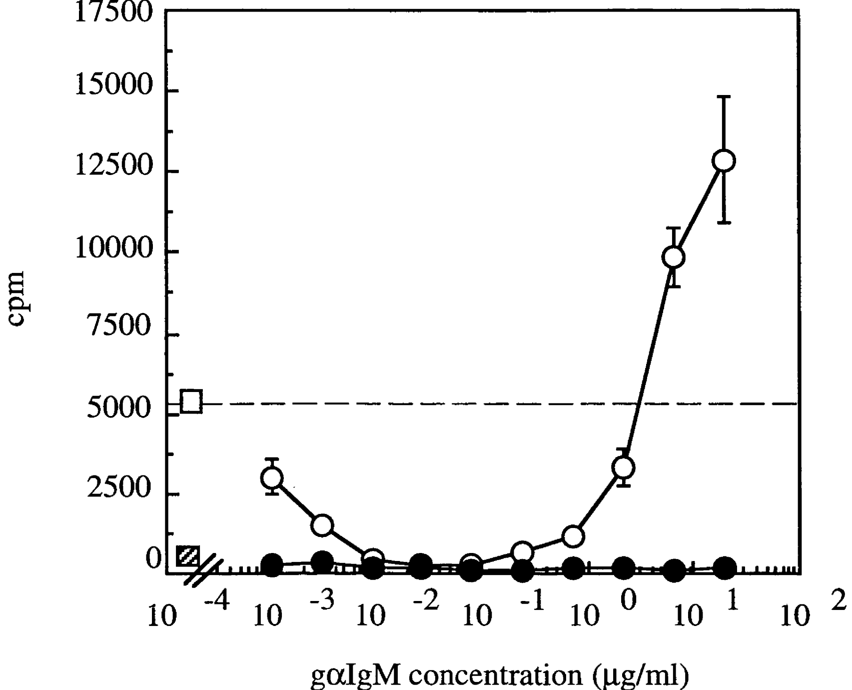

Figure 5.1. **GαIgM-induced B cell proliferation is inhibited by CsA.** B cells were cultured in BCM (5 x 10^4 cells / 200 µl well), containing varying concentrations of gαIgM (O). Replicate cultures were also prepared which contained gαIgM and 40 ng/ml CsA (●). Cells were incubated in a humidified atmosphere at 37°C and 5% CO^2. After 3 days B cell proliferation was measured by ^{3}H-TdR incorporation as described in chapter 3. Background proliferation: cells only (□) and CsA only (▨). The data illustrated represents the mean of triplicate cultures, expressed with 1 x SEM.

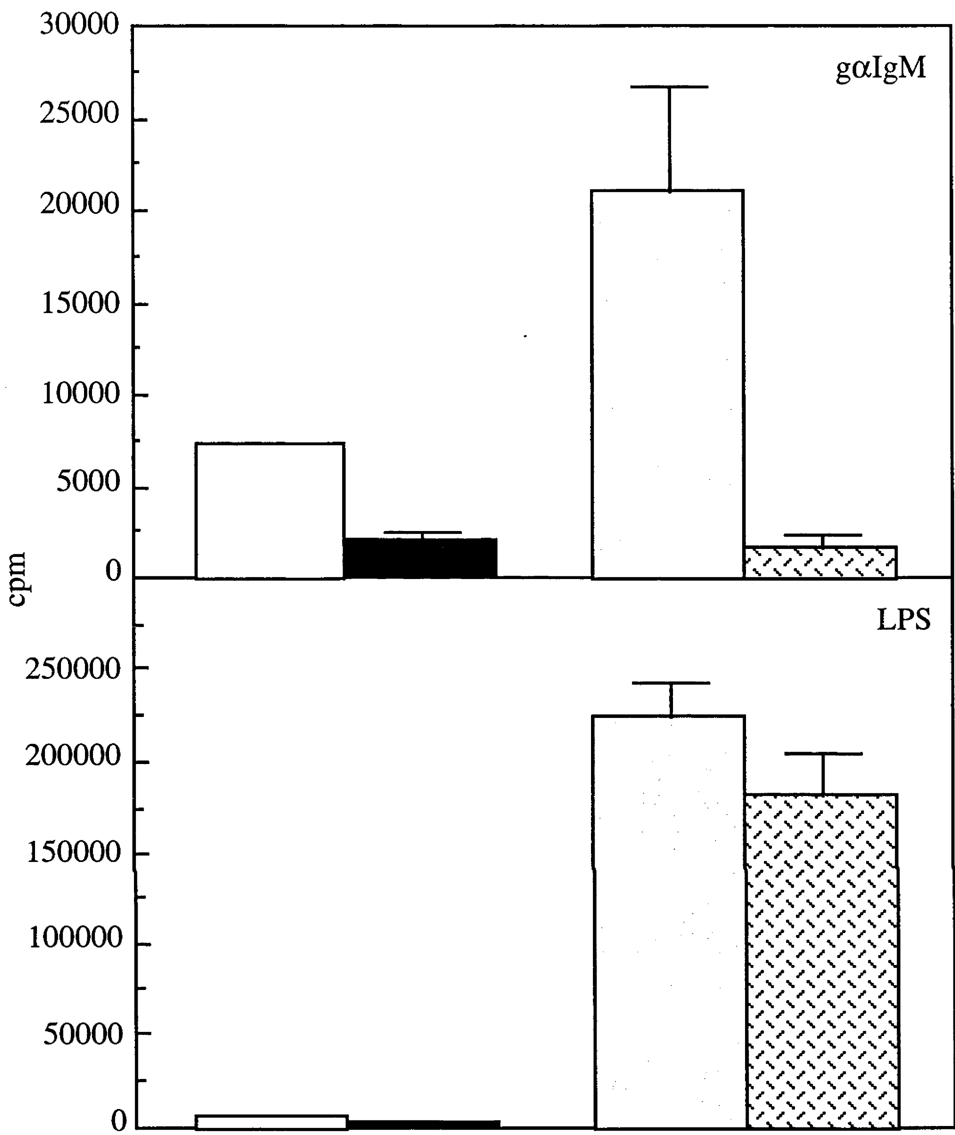

Figure 5.2 **CsA inhibits gαIgM- and not LPS-induced B cell proliferation.**

B cells were cultured as described in *Figure 5.1*, in the presence of 10 μg/ml gαIgM or 50 μg/ml LPS (☐). Replicate cultures were prepared which contained CsA (40 ng/ml) in combination with LPS, or gαIgM (☐). After 3 days B cell proliferation was measured by ^{3}H-TdR incorporation as described in chapter 3. Background proliferation: cells only (☐) and CsA only (■). The data illustrated represents the mean of triplicate cultures, expressed with 1 x SEM.

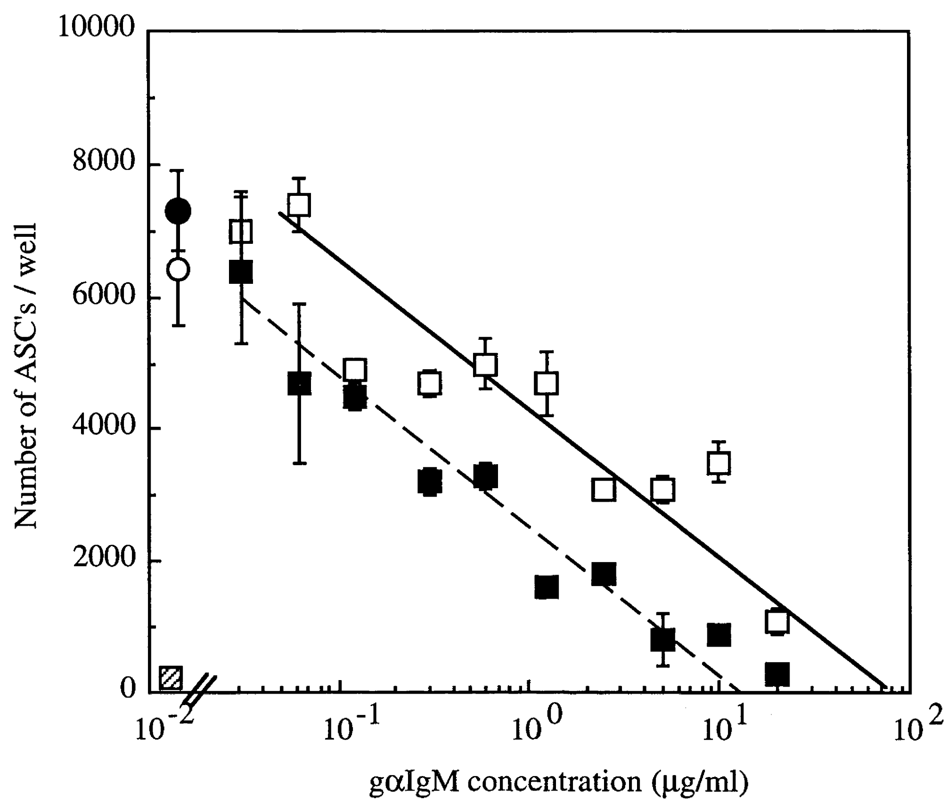

Figure 5.3 **GαIgM inhibition of LPS-induced ASC formation is CsA insensitive.**

The total numbers of ASCs formed after B cells were cultured as described in *Figure 5.1* in the presence of varying concentrations of gαIgM and 50 μg/ml LPS (□), or gαIgM, LPS and 40 ng/ml CsA (■), was determined by Elispot assay. After 3 days in culture the B cells were transferred to Elispot plates coated with sheep anti-mouse Ig and the Elispot assay performed as described in chpater 3. Background antibody production: LPS only (O), LPS and CsA (●), and cells only (▨). The data illustrated represents the mean of triplicate culture, expressed with 1 x SEM.

5.2.3. GαIgM alters LPS-induced proliferation in two opposing ways which differ in their sensitivity to CsA

In addition to monitoring the generation of ASCs, the proliferation of B cells stimulated with LPS and gαIgM was also measured in the presence and absence of CsA (Figure 5.4). In these cultures gαIgM altered LPS-induced proliferation in two apparently contradictory ways. As illustrated in Figure 5.4, low doses (< 1 μg/ml) of gαIgM inhibited LPS-induced ^{3}H-TdR incorporation, whereas higher antibody concentrations induced a dose-dependent increase in proliferation. The addition of CsA to these cultures did not affect the low dose inhibition of proliferation by gαIgM, but did prevent the increase in ^{3}H-TdR incorporation at high doses of antibody (Figure 5.4). Furthermore, in the presence of CsA inhibition of LPS-induced proliferation by gαIgM followed the same dose response curve as obtained for the reduction in ASC number (Figure 5.5). It was also of interest to note that the low concentrations of gαIgM which inhibited LPS-induced B cell proliferation, were similar to those which significantly reduced the level of spontaneous ^{3}H-TdR incorporation (Figure 5.1). Thus, gαIgM appeared to induce two independent effects on B cells which were dependent on dose. Low concentrations of gαIgM inhibited spontaneous or LPS-induced proliferation and ASC formation, whereas high antibody concentrations promoted proliferation while inhibiting LPS-induced B cell differentiation to ASCs.

5.2.4. LPS-induced proliferation is inhibited by PMA

The results obtained with gαIgM and CsA indicate that the two sIg-mediated signals differ in their sensitivity to CsA, with the inhibitory signal being CsA insensitive, whereas the positive signal for growth was CsA sensitive (Figures 5.1 and 5.5). While the ability of CsA to inhibit B cell proliferation induced by cross-linking of sIg was as expected (O'Garra *et al.,* 1986, Klaus 1988), the presence of an addition CsA insensitive signal which inhibited proliferation and ASC formation was a surprise (Figure 5.5). The inability of CsA to block the sIg-mediated inhibition of LPS-induced B cell activation provided a clue as to the identity of the intracellular signalling components involved in the negative signal. One potential candidate was PKC, which is activated as part of the sIg-mediated intracellular signalling pathways (Figure 1.4) (Cambier *et al.,* 1990, Cambier *et al.,* 1992, Cambier *et al.,* 1994, Harnett 1994), and which induces a number of B cell responses that are insensitive to CsA, such as the up-regulation of MHC class-II (Klaus and Hawrylowicz 1984, Klaus 1988, Testi *et al.,* 1989). To test the hypothesis that PKC could be involved in the sIg-mediated inhibitory signal, B cells were cultured with the phorbol ester, PMA, which is know to activate PKC

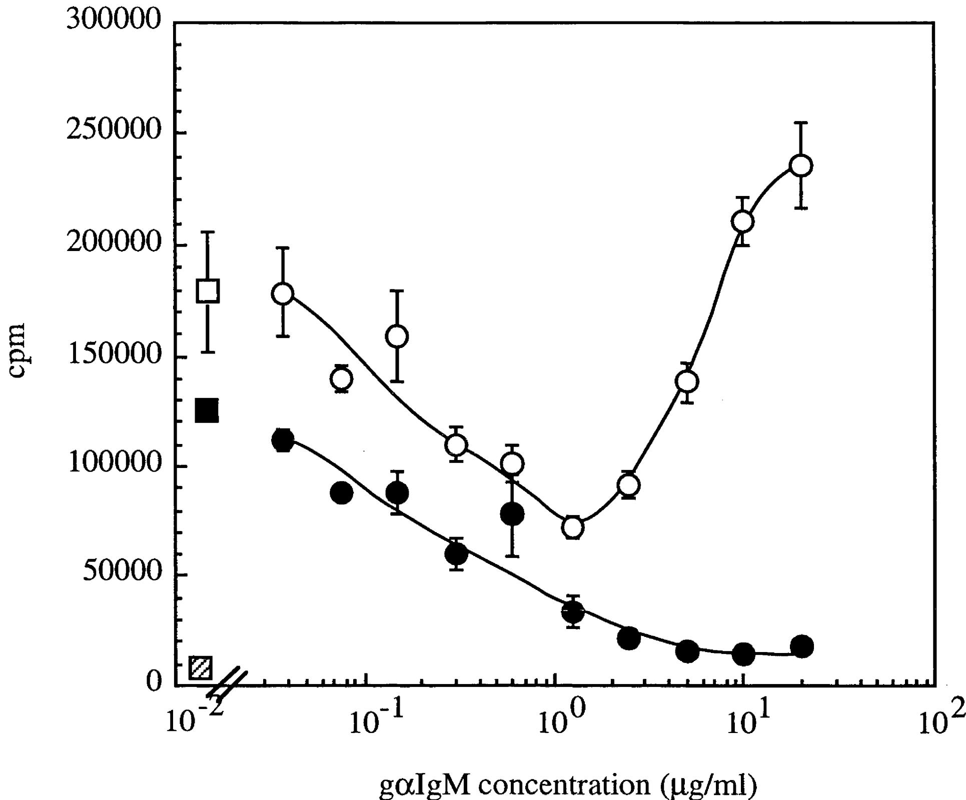

Figure 5.4. **Co-culture of gαIgM and LPS reveals different effects of sIg mediated signals which differ in their sensitivity to CsA .**

B cells were cultured as described in *Figure 5.1*, in the presence of LPS (50 μg/ml) and varying concentrations of gαIgM (○). Replicate cultures were also prepared which contained gαIgM, LPS and 40 ng/ml CsA (●). After 3 days B cell proliferation was measured by ^{3}H-TdR incorporation as described in chapter 3. Background proliferation: LPS only (□), LPS and CsA (■), and cells only (▨). The data illustrated represents the mean of triplicate cultures, expresed with 1 x SEM.

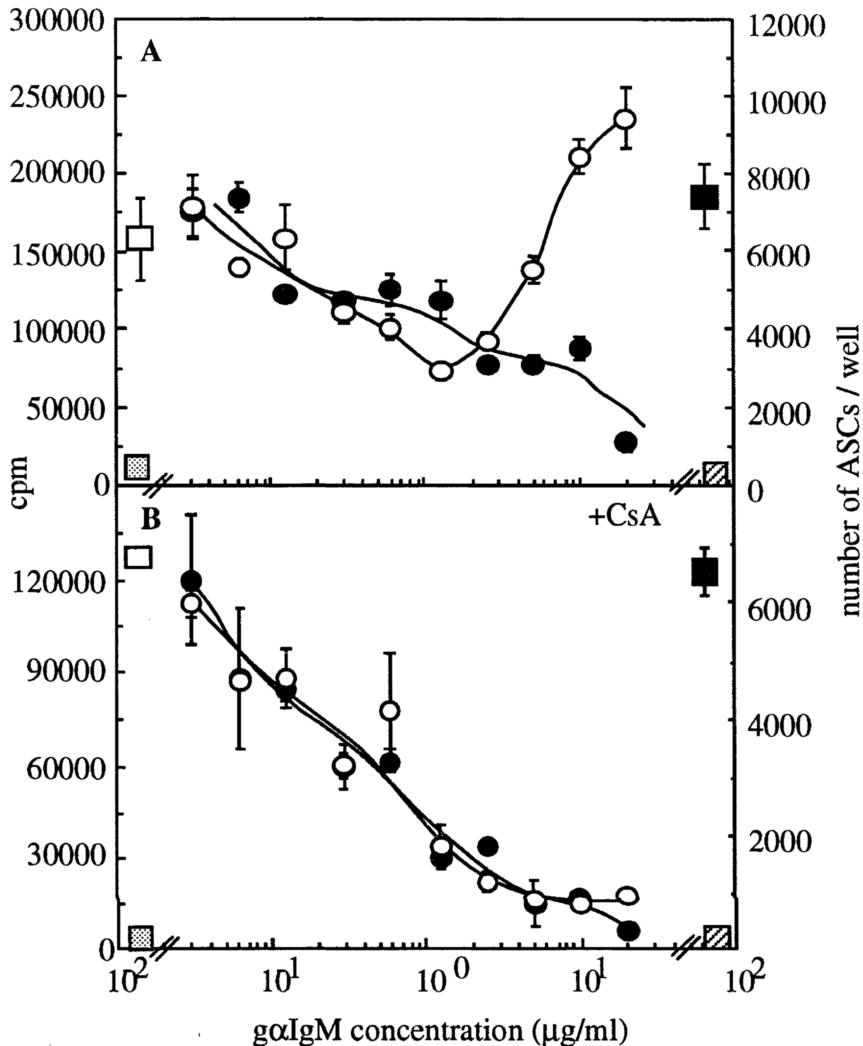

Figure 5.5. **Inhibition of LPS-induced proliferation and ASC formation by gαIgM generates similar dose response curves in the presence of CsA .** This *Figure* overlays the data illustrated *Figures 5.3 and 5.4* to demonstrate the relationship between ASC number and proliferation. In the absence of CsA (panel A) the dose response curves for ASC formation (●) and ^{3}H-TdR incorporation (O) diverge at gαIgM concentrations which exceed 3 μg/ml. However in the presence of CsA (panel B) a similar dose response curve is generated for both ASC number and proliferation. Background proliferation: LPS with and without CsA (□) and cells only (▩). Background ASC number: LPS with and without CsA (■) and cells only (▨). As before the data illustrated represents the mean of triplicate cultures, expressed with 1 x SEM.

(Berridge and Irvine 1984, Rejendra and Hokin 1990). Stimulation of B cells with PMA alone resulted in a decline in the background proliferation (Figure 5.6). A similar result was obtained when B cells were cultured with a combination of PMA and LPS. In these cultures PMA inhibited LPS-induced proliferation (Figure 5.6), as previously described (Högbom *et al.,* 1987, Gupta *et al.,* 1988).

5.2.5. Different preparations of gαIgM differ in their signalling capacity

During the course of these experiments two different preparations of gαIgM were tested, and it was found that the dose response curves for B cell proliferation differed between the two preparations (Figure 5.7). At low concentrations of gαIgM the dose response curves for proliferation induced by the different batches of antibody virtually overlayed each other (Figure 5.7). However while the inhibitory effect of the two batches of antibody was very similar, their ability to induce proliferation differed. Thus, the concentration at which the different preparations of gαIgM were able to induce proliferation above the background differed by approximately 10 fold, with batch 1 being more stimulatory than batch 2 (Figure 5.7). These results indicate that the relative strength of the positive signal differed between preparations of gαIgM. More importantly the results suggest that the ability of gαIgM to induce the inhibitory signal was independent of the induction of the positive signal, indicating that the two sIg-mediated signals are independently regulated.

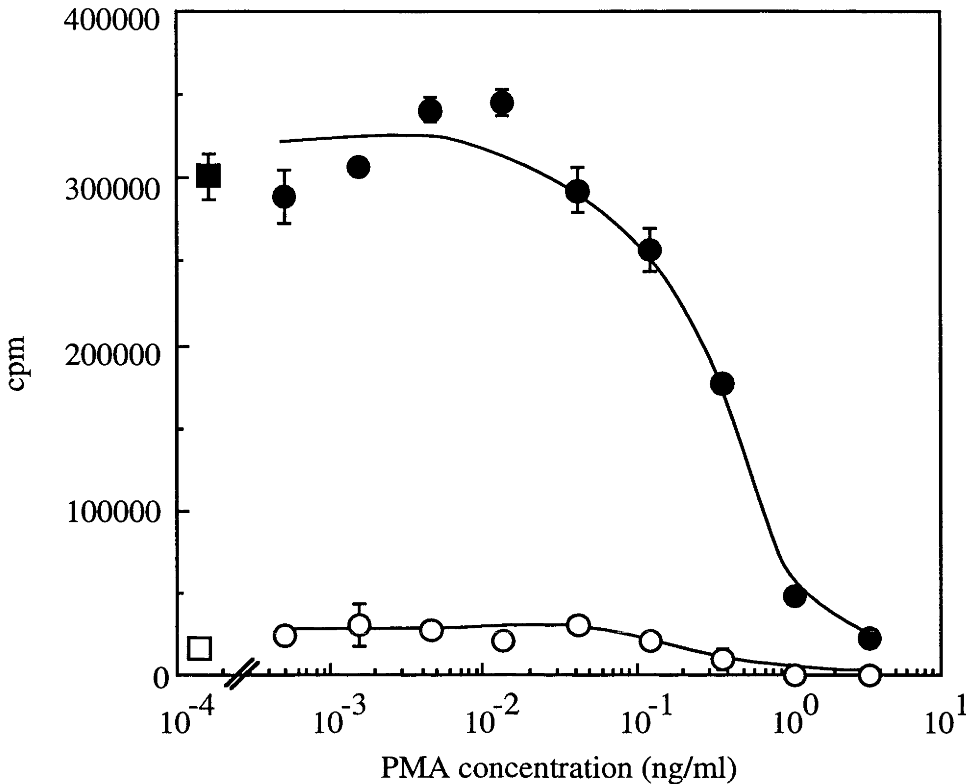

Figure 5.6. **PMA inhibits LPS-induced B cell proliferation.**

B cells were cultured in BCM (5 x 10^4/ 200 µl well), in the presence of varying concentrations of PMA (○). Replicate B cell cultures were also prepared which contained LPS (50 µg/ml) and PMA (●). After 3 days B cell proliferation was measured by ^{3}H-TdR as described in chapter 3. Background proliferation: cells only (□) and LPS only (■). The data illustrated represents the mean of triplicate cultures, expressed with 1 x SEM.

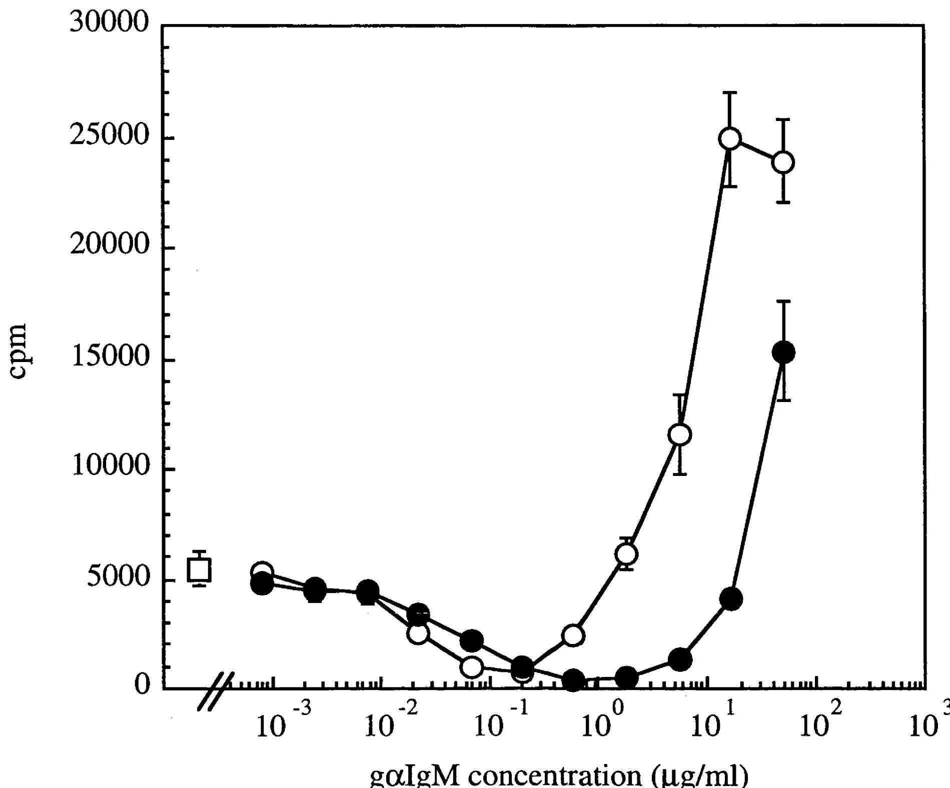

Figure 5.7. **The response to different batches of gαIgM suggests that the inhibitory and stimulatory signals are independent of each other.** B cells were cultured in BCM (5 x 10^4 cells / 200 μl well) in the presence of varying concentrations of different batches of gαIgM - batch 1 (O) and batch 2 (●). After 3 days B cell proliferation was measured by ^{3}H-TdR incorporation as described in chapter 3. Background proliferation (□). The data illustrated represents the mean of triplicate cultures, expressed with 1 x SEM.

5.3. Discussion

5.3.1. Surface-Ig mediated signals can inhibit LPS-induced ASC formation while promoting B cell proliferation

It has been accepted that TI type-1 antigens, such as LPS, are intrinsically stimulatory for B cells (Andersson *et al.,* 1972, Andersson *et al.,* 1973, Coutinho and Möller 1973a, Coutinho *et al.,* 1973b, Coutinho *et al.,* 1975, Möller *et al.,* 1975) and that during the immune response to these antigens, specificity is achieved through sIg binding to epitopes within the TI type-1 antigen which focus it to the B cell surface resulting in the activation of antigen specific cells (Figure 4.1) (Coutinho and Möller 1974, Coutinho *et al.,* 1975, Coutinho *et al.,* 1975, Möller *et al.,* 1975). In the alternative model proposed by Mamchak and Hodgkin (1995) it was suggested that B cells which receive a sIg-mediated signal at the same time as the TI type-1 activation signal will be prevented from making antibody (Figures 2.3 and 5.8), resulting in a decline in LPS-induced ASC number and generating a bell shaped dose response curve, as observed by Coutinho and Möller (Coutinho *et al.,* 1975). If correct, this model could account for the results obtained by Coutinho and Möller (1975d) whilst incorporating a signalling role for sIg (Mamchak *et al.,* 1995). The experiments described here were initially designed to test this proposal, and the results are consistent with the alternative explanation for the bell shaped dose response curves for LPS-induced ASC formation (Mamchak *et al.,* 1995). However they indicate that a modification of the original model is required to account for the unexpected effects of sIg-mediated signals on LPS-induced proliferation. As before, the antigen signal can inhibit LPS-induced ASC formation, but in B cells which strongly bind antigen (and which presumably have high affinity for antigen) this signal appears to also induce the cells divide more quickly while not secreting antibody (Figure 5.8). This modification would still result in a bell shaped curve for LPS-induced antibody production, as observed by Coutinho and Möller (1975). If correct antigen specific B cells would be induced to proliferate and not secrete antibody, however, it is not obvious as to why this mechanism may have evolved. It is possible that the antigen specific B cells expand to generate a memory cell population available for subsequent antigen exposure. Alternatively, the progeny of these cells might be activated to secrete antibody once the epitope density is reduced, either due to the presence of soluble antibody sequestering antigen or by antigen clearance. The appearance of highly antigen specific antibodies late in the response would expedite the complete eradication of the antigen.

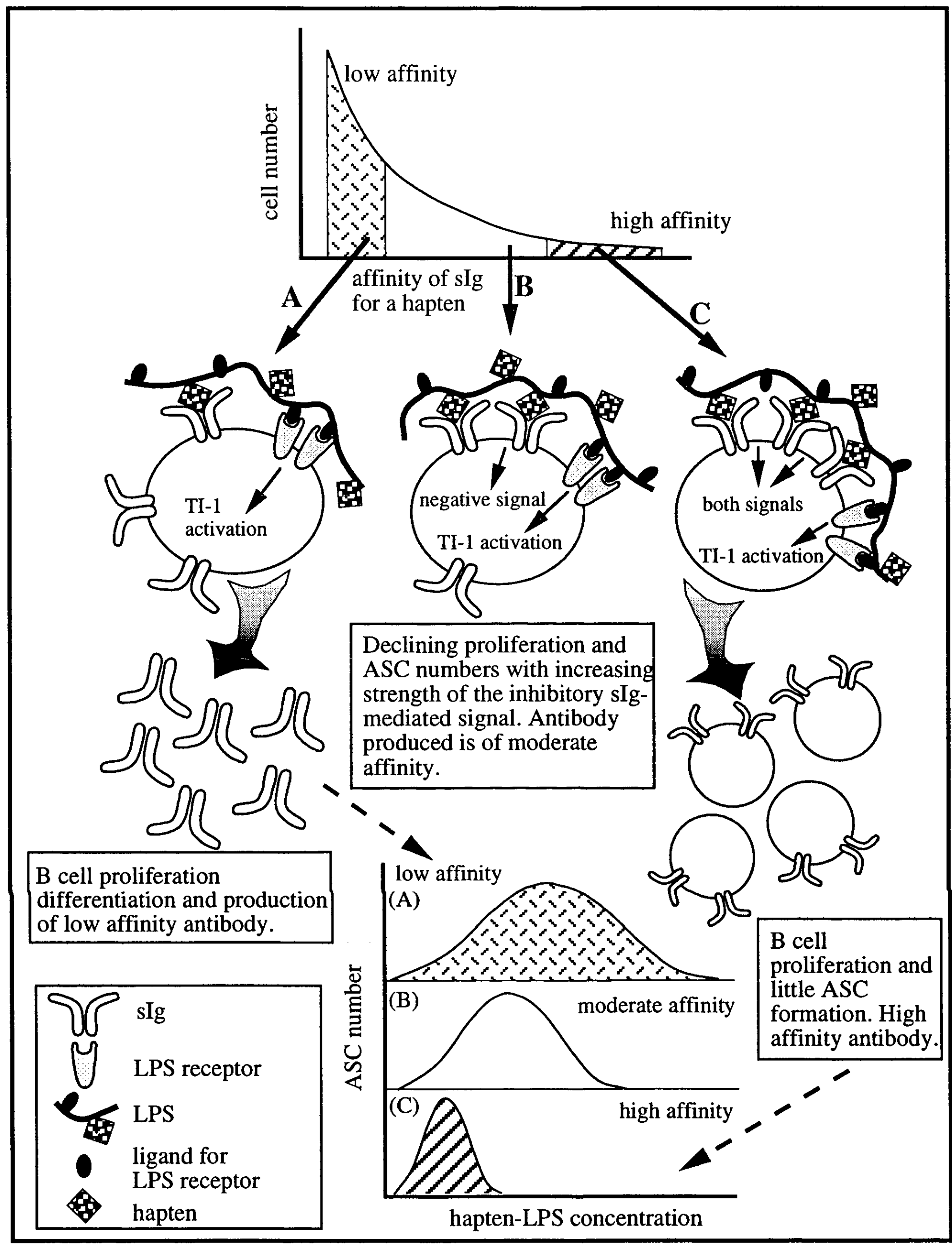

Figure 5.8. **A model in which sIg-mediated signals regulates the affinity of LPS-induced ASCs.**

A) B cells which express sIg which has a low affinity for hapten will focus LPS to the cell surface and be induced to divide and differentiate to ASCs. These cells represent the majority of LPS-induced ASC and secrete low affinity antibodies. **B)** Surface Ig which binds the hapten with a moderate affinity results in the generation of the negative sIg-mediated signal. In this instance the number of B cells which undergo LPS-induced proliferate and differentiation to ASC declines as the strength of the negative sIg-mediated signal increases. **C)** Surface Ig which binds to the hapten with high affinity generate both a positive and negative sIg-mediated signal. These B cells will proliferate but produce little or no antibody.

While the experiments described in this chapter demonstrated that cross-linking of sIg by gαIgM could inhibit LPS-induced ASC formation and at the same time promote proliferation (Figure 5.5), the results also reveal an additional inhibitory effect of gαIgM. At low concentration, or in the presence of CsA, gαIgM-induced a signal capable of inhibiting spontaneous and LPS-induced B cell proliferation (Figures 5.1 and 5.5). The inhibitory effect of gαIgM on proliferation appears to be controlled by the same sIg-mediated signal that inhibits LPS-induced B cell differentiation into ASCs (Figure 5.5). Although the inhibition of proliferation may be countered by the sIg-mediated positive signal for growth, the positive signal does not affect the inhibition of ASC formation (Figures 5.5, and 5.8). Finally the positive CsA sensitive signal seems to be related to the ability of the anti-Ig reagent to promote B cell proliferation independently of LPS, a property previously determined to be a correlate of TI type-2 antigenic stimulation (Peçanha *et al.,* 1991, Mond *et al.,* 1995). The different effects of the two sIg-mediated signals are summarised in Figure 5.9.

5.3.2. Speculation on the intracellular signalling components involved in the positive and negative sIg-mediated signals

The use of CsA and PMA in these experiments has provided some indication of the secondary messengers which may be involved in the different sIg-mediated signals (Figure 5.10). CsA inhibits a number of signalling events which occur as a result of calcium mobilisation and include the activation, and migration from the cytoplasm to the nucleus of transcription factors, such as NF-AT (Emmel *et al.,* 1989, Bierer *et al.,* 1991, Jain *et al.,* 1993, Venkataraman *et al.,* 1994) and NF-κB (Emmel *et al.,* 1989). The inhibitory action of CsA appears to be the result of the inhibition of the Ca^{2+}/calmodulin dependent phosphatase, calcineurin (Liu *et al.,* 1991b, Clipstone and Crabtree 1992, Fruman *et al.,* 1992, O'Keefe *et al.,* 1992). Once inside the cell CsA specifically binds to a group of compounds called the cyclophilins, forming a ligand receptor complex (Handschumacher *et al.,* 1984). The CsA-cyclophilin complex binds to, and inhibits the action of calcineurin (Liu *et al.,* 1991b, Clipstone *et al.,* 1992, Fruman *et al.,* 1992, O'Keefe *et al.,* 1992). Inhibition of calcineurin by CsA can prevent the dephosphorylation, and subsequent migration into the nucleus of NF-AT (Emmel *et al.,* 1989, Granelli-Piperno *et al.,* 1990, Bierer *et al.,* 1991, Jain *et al.,* 1993, Venkataraman *et al.,* 1994). In this way the presence of CsA could block the activation and migration of NF-AT, and other transcription factors, which promote the expression of a number of genes (Jain *et al.,* 1993), and thereby prevent sIg-induced B cell proliferation.

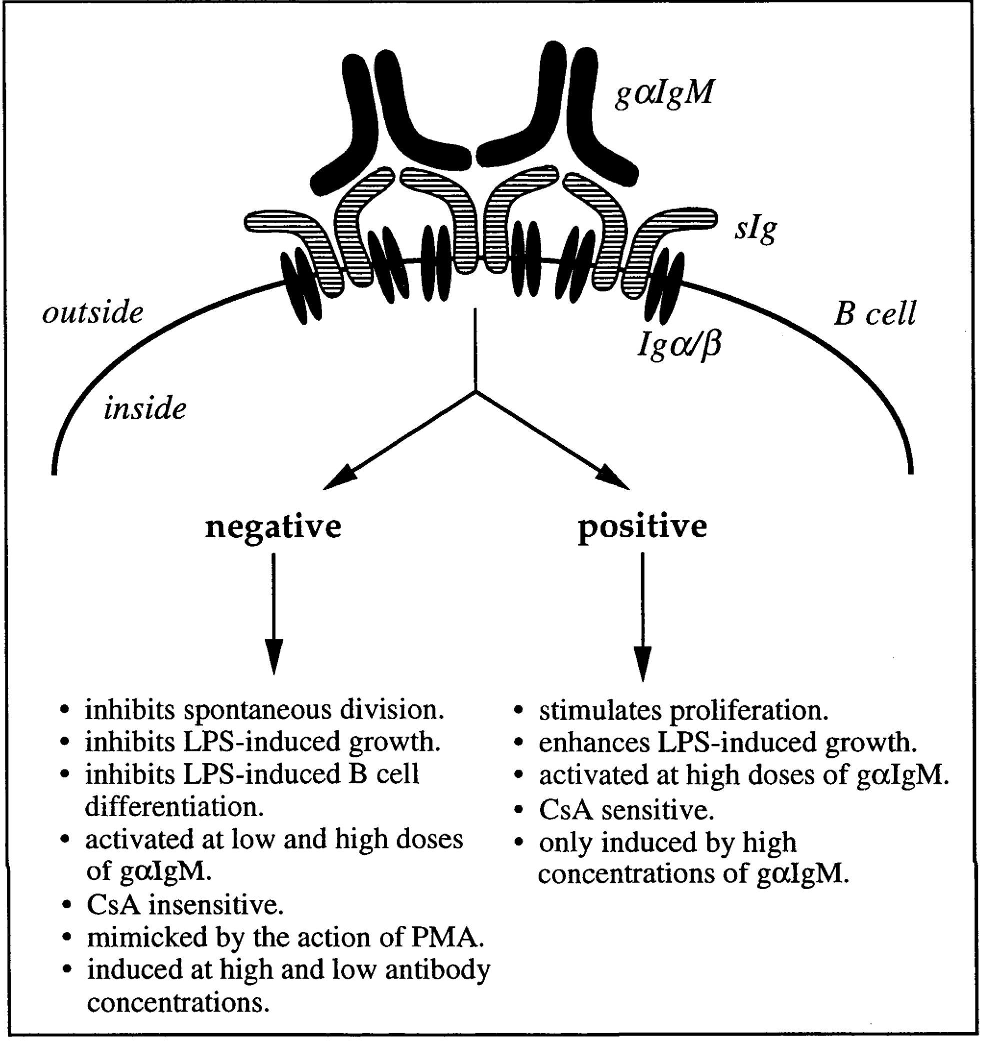

Figure 5.9. **A summary of the B cell response to the two sIg-mediated signals .**

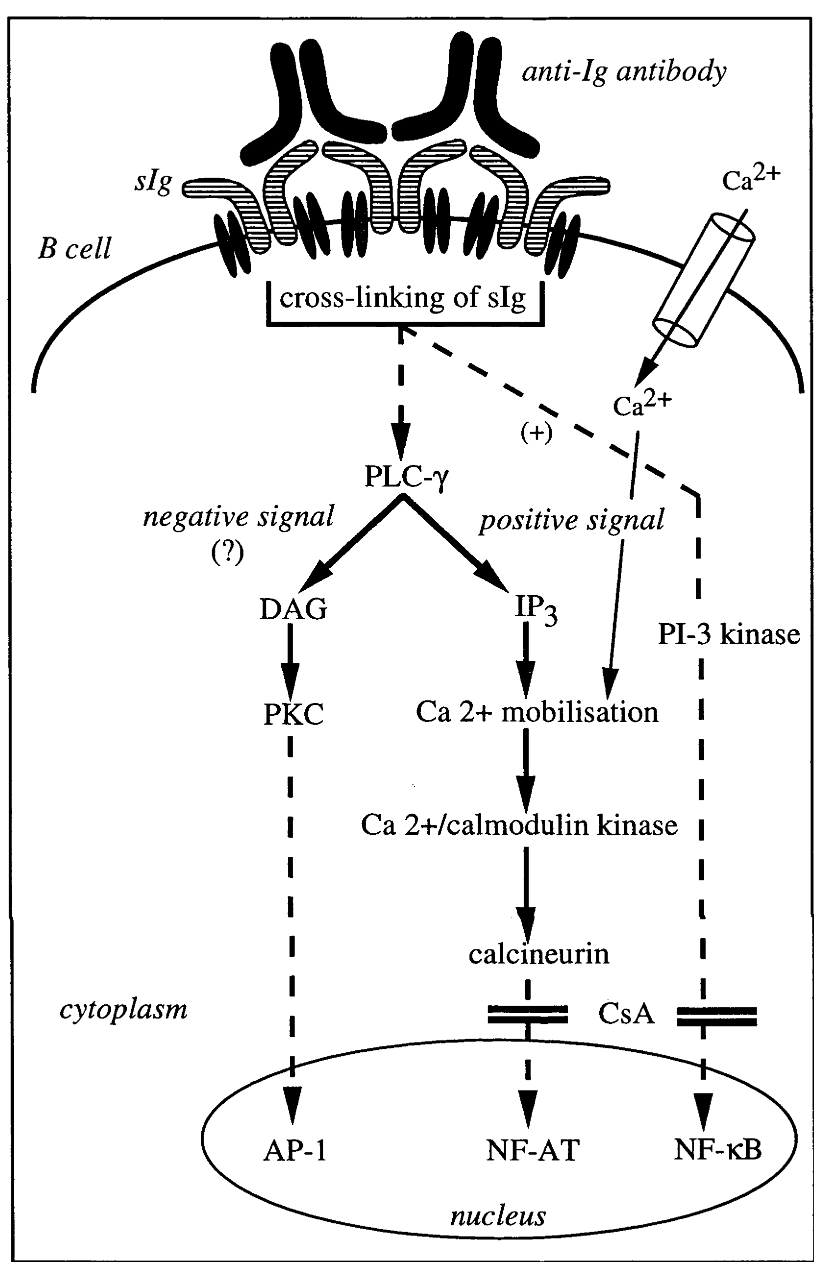

Figure 5.10. **Components of sIg-mediated signalling pathways which may constitute the positive and negative signals.**

The model put forward in this chapter suggests that cross-linking of sIg initiates two signals. The positive signal is inhibited by CsA, and may involve the activation of calcineurin. In contrast the negative signal is CsA insensitive, and may involve the activation of PKC. It is the activation, and interaction, of both pathways which is proposed to determine B cell behaviour.

The failure of CsA to prevent gαIgM-mediated inhibitory effects, and the inhibition of LPS-induced proliferation (Figure 5.6) (Högbom *et al.,* 1987, Gupta *et al.,* 1988) and ASC formation by phorbol esters (Isakson and Simpson 1984, Högbom *et al.,* 1987) suggests that the negative signal may involve the activation of PKC (Figure 5.10). However, the regulation of B cell responses by sIg-mediated signals may not be quite this simple, as PKC is also a component of the intracellular signalling which results in the activation of B cells by both LPS (Gupta *et al.,* 1988, Klaus 1988, Shapira *et al.,* 1994) or anti-Ig reagents (Klaus 1988, Cambier *et al.,* 1990, Cambier *et al.,* 1992, Cambier *et al.,* 1994, Harnett 1994) (Figures 1.4 and 5.10). In addition, the combination of phorbol esters and calcium ionophores has been shown to induce B cell proliferation (Klaus *et al.,* 1986). Therefore, the B cell response may not be simply due to the activation of PKC, or the mobilisation of calcium, but could depend on the strength of, and interaction between, these and other signals. Although this hypothesis is speculative and requires further experimentation to determine the relationship between these two pathways and B cell behaviour, it does suggest that sIg-mediated signals provide more information to the B cell about the nature of the bound antigen than has previously been anticipated.

5.3.3. Factors which could affect the type (positive or negative) of sIg-mediated signalling

Irrespective of the intracellular signalling pathways involved, the data clearly implies that there are two paths. What then are the differences which result in the activation of each? While the cause of the divergent behaviour induced by gαIgM is unknown, the results indicate that the B cell response is affected by the concentration (Figures 5.1 and 5.5) and batch of gαIgM (Figure 5.7). As gαIgM is a polyclonal antibody different batches could vary in the frequency of anti-Ig antibodies which have a particular specificity and affinity for certain epitopes. The variation between batches of gαIgM could result in differences in the pattern of sIg-mediated signalling and subsequent B cell behaviour. Finally, the inhibitory effects of gαIgM are unlikely to be due to co-ligation of sIg and the FcγRIIB which results in Fc-mediated inhibition (Sidman and Unanue 1976, Phillips and Parker 1983) as the Fc region of goat antibodies are reported not to bind to FcγRIIB (Sieckmann *et al.,* 1978a, Sieckmann *et al.,* 1978b, Sidman and Unanue 1979). Therefore, gαIgM-mediated inhibition of proliferation and ASC formation would appear to be caused by sIg-mediated rather than Fc-mediated signals. The influence of the specificity and affinity of anti-Ig reagents, and the role of Fc-mediated signals on sIg-induced B cell behaviour is explored in the following chapter.

The results described in this chapter suggest a model in which cross-linking of sIg with gαIgM results in the induction of at least two independently regulated signals which have different functional consequences for the B cell. Clearly, one of the affects of sIg-mediated signalling was to inhibit LPS-induced ASC formation. This result supports the model proposed in chapter 2 in which sIg-mediated signals are responsible for the bell shaped dose response curves observed by Coutinho and Möller (1975) for LPS-induced ASC formation. Further, these results are consistent with the view that sIg-mediated signals regulate the affinity of LPS-induced ASCs (Mamchak *et al.,* 1995). Finally the data suggests that the pattern of sIg-mediated signals is dependent on the specific physical interaction between sIg and gαIgM. The effect of varying the physical form of the surrogate antigen and B cell behaviour is explored in the following chapter through the use of monoclonal antibodies.

Chapter 6

Monoclonal antibodies provide further evidence for the independent regulation of two sIg-mediated signals

6.1. Introduction

The antigen signal has been shown to have a number of different and apparently contradictory effects on B cell behaviour. One experimental approach which has been employed to examine the role of this signal has been to use anti-Ig antibodies as surrogate antigens. Anti-Ig stimulation of B cells has revealed that the resulting sIg-mediated signal can induce a number of effects which include: increased cell size (DeFranco *et al.,* 1982b, Klaus *et al.,* 1985b), proliferation (Sieckmann *et al.,* 1978a, Parker *et al.,* 1979, Sidman *et al.,* 1979, Klaus *et al.,* 1985a, Brunswick *et al.,* 1988), expression of cell surface markers (Mond *et al.,* 1981, Lenschow *et al.,* 1994), apoptosis (Hasbold *et al.,* 1990, Parry *et al.,* 1994a, Parry *et al.,* 1994b) and, in combination with cytokines, antibody secretion (Kishimoto *et al.,* 1975, Peçanha *et al.,* 1991). These experiments have provided an insight into the role of the antigen signal in a number of different areas of B cell biology supporting the conclusion that anti-Ig reagents provide an effective way of examining the B cell response to antigen. However the use of anti-Ig antibodies to study the B cell response to antigen has often drawn criticism because of the different and sometimes contradictory responses these antibodies induced, leading to the proposition that B cell behaviour induced by anti-Ig antibodies was not representative of the B cell response to natural antigens (Möller 1978).

A hypothesis which could explain the variation in the B cell response to anti-Ig reagents is suggested by the results described in the previous chapter. According to this hypothesis the antigen signal is not immutable, but may instead be composed of a number of signals which can vary in strength and are independently triggered by B cell receptor engagement. Given this possibility, the question arises as to what triggers the different components of the antigen signal. It is probable that variations in the antigen signal are the result of physical differences in the specific interaction between sIg and antigen. If correct this model would allow the re-interpretation of disparate results obtained with anti-Ig antibodies published in the literature, and provide a better understanding of how the B cell responds to antigen. Thus, this hypothesis was explored here through the use of different anti-Ig antibodies.

6.2. Results

6.2.1. Variation in inhibitory and enhancing capacity of seven monoclonal antibodies

Initially seven mAbs were tested for their ability to induce B cell proliferation in the presence and absence of IL-4, and their responses compared to that of gαIgM (Figures 6.1 and 6.2). Properties of the seven mAbs used in this experiment are summarised in Table 6.1.

Each of the mAbs had a different effect on B cell proliferation (Figures 6.1 and 6.2). Although proliferation in response to gαIgM occurred in the absence of IL-4, the addition of this cytokine enhanced the level of ^{3}H-TdR incorporation (Figure 6.1A), as previously described (Howard *et al.,* 1982, Hodgkin *et al.,* 1991a). The inclusion of IL-4 to B cell cultures stimulated with gαIgM also altered the shape of the dose response curve, as the low dose inhibition induced by gαIgM alone was no longer apparent (Figure 6.1A). Three of the mAbs, AMS-9.1, 1.19 and B7.6, were also able to induce proliferation in the absence of IL-4, and as observed for gαIgM (Figures 6.1 and 6.2). The presence of IL-4 in these cultures enhanced the level of ^{3}H-TdR incorporation induced by AMS-9.1, 1.19 or B7.6 (Figures 6.1 and 6.2), as previously reported (Julius *et al.,* 1984, Goroff *et al.,* 1986, Brines and Klaus 1991, Udhayakumar *et al.,* 1991). In addition while the anti-κ antibody, 187.1, alone did not induce proliferation, the combination of 187.1 and IL-4 stimulated an increase in ^{3}H-TdR incorporation like that observed for gαIgM under similar culture conditions (Figure 6.1A and B).

In contrast to the mitogenic mAbs, BET-2 and 331.1 did not induce proliferation, irrespective of the presence of IL-4 (Figure 6.1C and D). Both BET-2 and 331.1 inhibited spontaneous proliferation in the absence of IL-4, in a manner similar to that observed for low doses (< 1 μg/ml) of gαIgM (Figure 6.1A, C and D). However in the presence of IL-4, B cell proliferation in response to a high dose (20 μg/ml) BET-2 was slightly enhanced over that observed in the control (IL-4 only) cultures (Figure 6.1C), whereas the addition of IL-4 to cultures containing 331.1 did not alter the inhibitory effect of this mAb (Figure 6.1D). Thus, both 331.1 and BET-2 appeared to induce an inhibitory signal while causing little, if any, detectable positive response. A weak inhibition of spontaneous proliferation was also observed with low doses (< 5 μg/ml) of AMS-9.1 in the absence of IL-4 (Figure 6.1E, inset). Finally unlike the other mAbs used in this experiment, AMS-15.1 did not produce any measurable effect on B cell proliferation, even in the presence of IL-4

Table 6.1: Monoclonal anti-Ig antibodies

antibody	specificity	isotype	raised in	reference
BET -2	anti-mouse μ	IgG1	rat	(Kung *et al.*, 1981, Udhayakumar *et al.*, 1991)
331.12	anti-mouse μ	IgG2b	rat	(Kincade *et al.*, 1981, Udhayakumar *et al.*, 1991)
B7.6	anti-mouse μ	IgG1	rat	(Julius *et al.*, 1984, Brines *et al.*, 1992).
187.1	anti-mouse κ	IgG1	rat	(Gilligan *et al.*, 1988)
AMS-9.1	anti-mouse δ^a	IgG2a	mouse expressing δ^b	(Stall *et al.*, 1984)
AMS-15.1	anti-mouse δ^a	IgG2a	mouse expressing δ^b	(Stall *et al.*, 1984)
1.19	anti-mouse δ	IgG2a	rat	(Brines *et al.*, 1992)

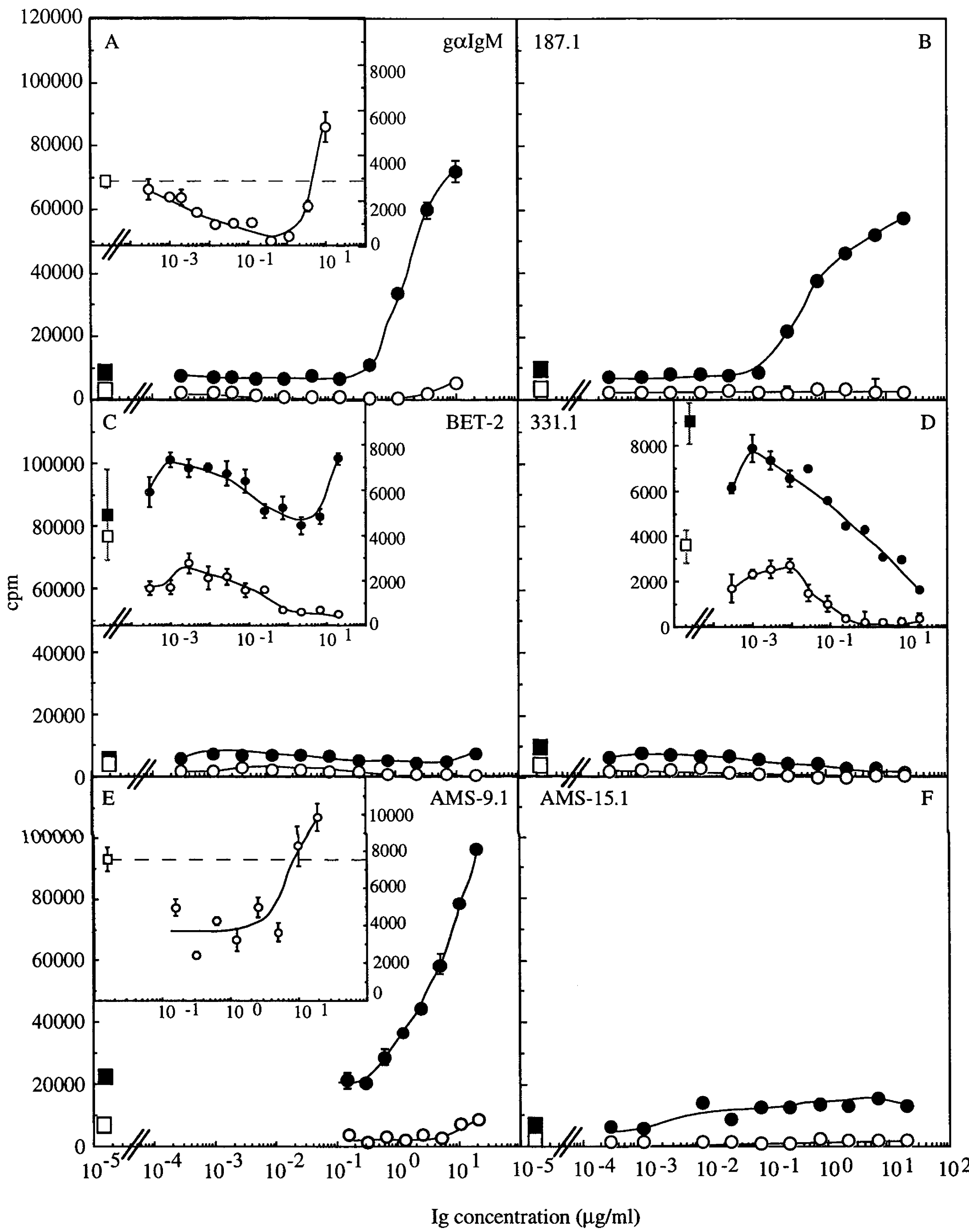

Figure 6.1. **Different anti-Ig antibodies display varying effects on B cell proliferation.**
B cells were cultured in BCM containing varying concentrations of anti-Ig antibodies at a starting cell density of 5 x 10^4 cells / 200 μl well (O). Replicate cultures were also prepared which contained anti-Ig antibodies in combination with 100 U/ml of IL-4 (●). After 3 days B cell proliferation was measured by ^{3}H-TdR incorporation as described in chapter 3. Background proliferation: cells only (□), and IL-4 only (■) as indicated. For each anti-Ig dose cultures were prepared in triplicate and the data illustrated represents the mean of the response ± 1 x SE. **A** - gαIgM, inset shows detail of the response to gαIgM only; **B** - 187.1; **C** - BET-2, inset shows detail of the response to BET-2 with and without IL-4; **D** - 331.1, inset shows detail of the response to 331.1 with and without IL-4; **E** - AMS-9.1, inset shows detail of the response to AMS-9.1 only; **F** - AMS-15.1.

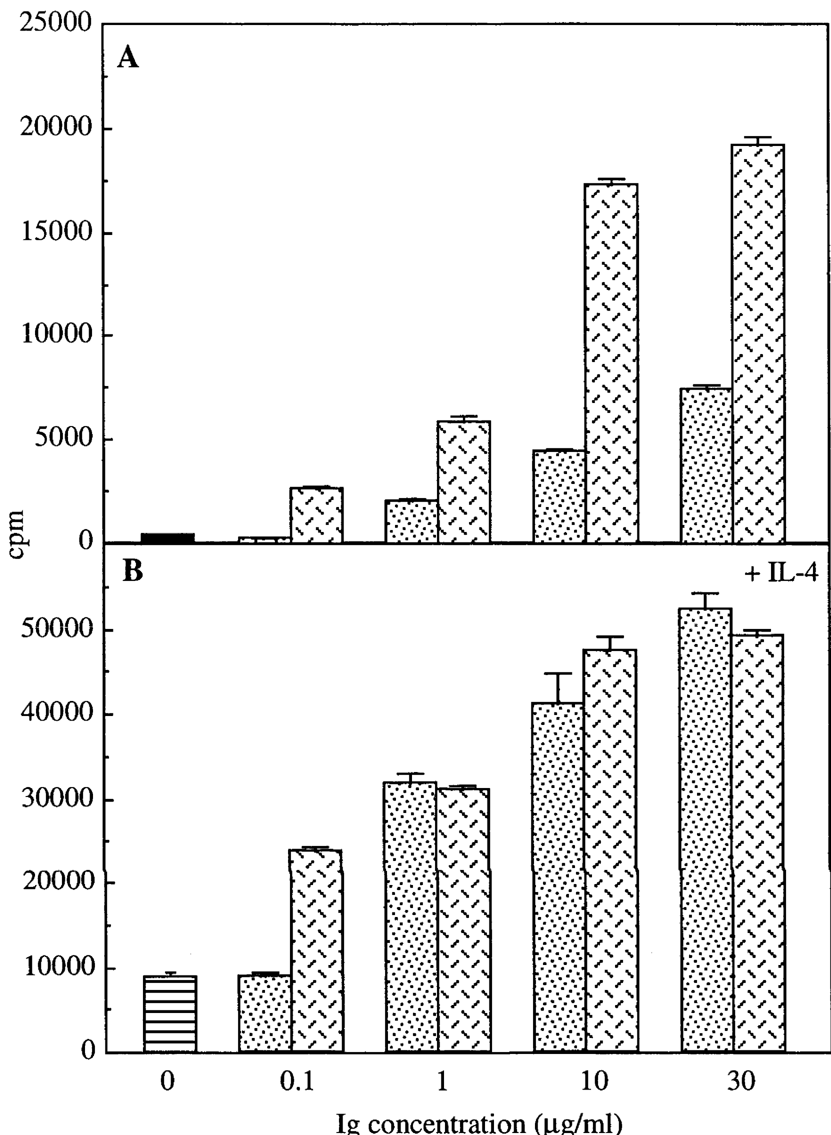

Figure 6.2. **Two anti-Ig mAbs which are mitogenic in the absence of IL-4.** Small percoll purified B cells were prepared as described in chapter 3 from CBA x C57/B6 mice and cultured in BCM (2 x 10^5 cells / 200 µl well) in the presence of varying concentrations of the anti-IgD mAb, 1.19 ( ), or the anti-IgM mAb, B7.6 ( ). Replicate wells were prepared which contained the either 1.19 or B7.6 in combination with 10 U/ml of IL-4. After 3 days B cell proliferation was determined by ^{3}H-thymidine incorporation, as described in chapter 3. Background proliferation; cells only ( ), and IL-4 only ( ) is indicated. The data illustrated represents the mean of triplicate cultures ± 1 x SE. This experiment was performed by Dr J. Hasbold (Centenary Institute, Sydney) who generously gave permission for the data to appear in this thesis.

Figure 6.1F). Isotype matched control mAbs had no influence on the behaviour of the B cells, irrespective of the presence of IL-4 (data not shown).

Together the results illustrated in Figures 6.1 and 6.2 indicate that cross-linking of sIg induces intracellular signals which result in B cell proliferation which can be enhanced by IL-4. In addition they reveal that some anti-Ig reagents (331.1) can inhibit spontaneous proliferation in a manner that is not sensitive IL-4 (Figure 6.1D). Having established the pattern of B cell responses to anti-Ig antibodies alone, and in combination with IL-4, the effect of these mAbs on LPS-induced proliferation was examined.

6.2.2. Different anti-Ig antibodies have distinct effects on LPS-induced B cell proliferation

Figure 6.3 illustrates the effect of different anti-Ig antibodies on LPS-induced proliferation, and as described for the B cell response to mAbs and IL-4 the B cell behaviour differed between the various antibodies. As before the response to mAbs was compared to that induced by gαIgM, as described in chapter 5 (Figures 5.4 and 6.3A). The dose response curve generated by titrating 187.1 in the presence of LPS was reminiscent of that obtained with gαIgM under similar culture conditions, with the exception that moderate to high doses (> 1 μg/ml) of 187.1 did not enhance LPS-induced ^{3}H-TdR incorporation (Figure 6.3A and B). While low doses (< 1 μg/ml) of 187.1 inhibited LPS-induced proliferation, higher antibody concentrations generated a plateau of proliferation which extended over a twenty fold increase in antibody dose (Figure 6.3B). The addition of CsA to these cultures resulted in a more severe inhibition of LPS-induced proliferation. However in the presence of CsA the previously observed plateau in proliferation was still apparent but occurred at a substantially reduced level of ^{3}H-TdR incorporation (Figure 6.3B). Both BET-2 and 331.1 inhibited LPS-induced ^{3}H-TdR incorporation in a dose-dependent manner, generating response curves which were almost identical (Figure 6.3C and D, respectively). The addition of CsA to these cultures did not alter the shape of the dose response curve induced by either antibody, however, the overall level of proliferation was slightly lower (Figure 6.3C and D). The complete inhibition of LPS-induced proliferation by these anti-Ig reagents suggest that BET-2 and 331.1 are full agonists of the negative signal. Thus, while BET-2 and 331.1 induced a similar inhibitory effect to that observed at low doses of gαIgM, these mAbs did not demonstrate any positive influence on LPS-induced proliferation even at high concentration (Figure 6.3).

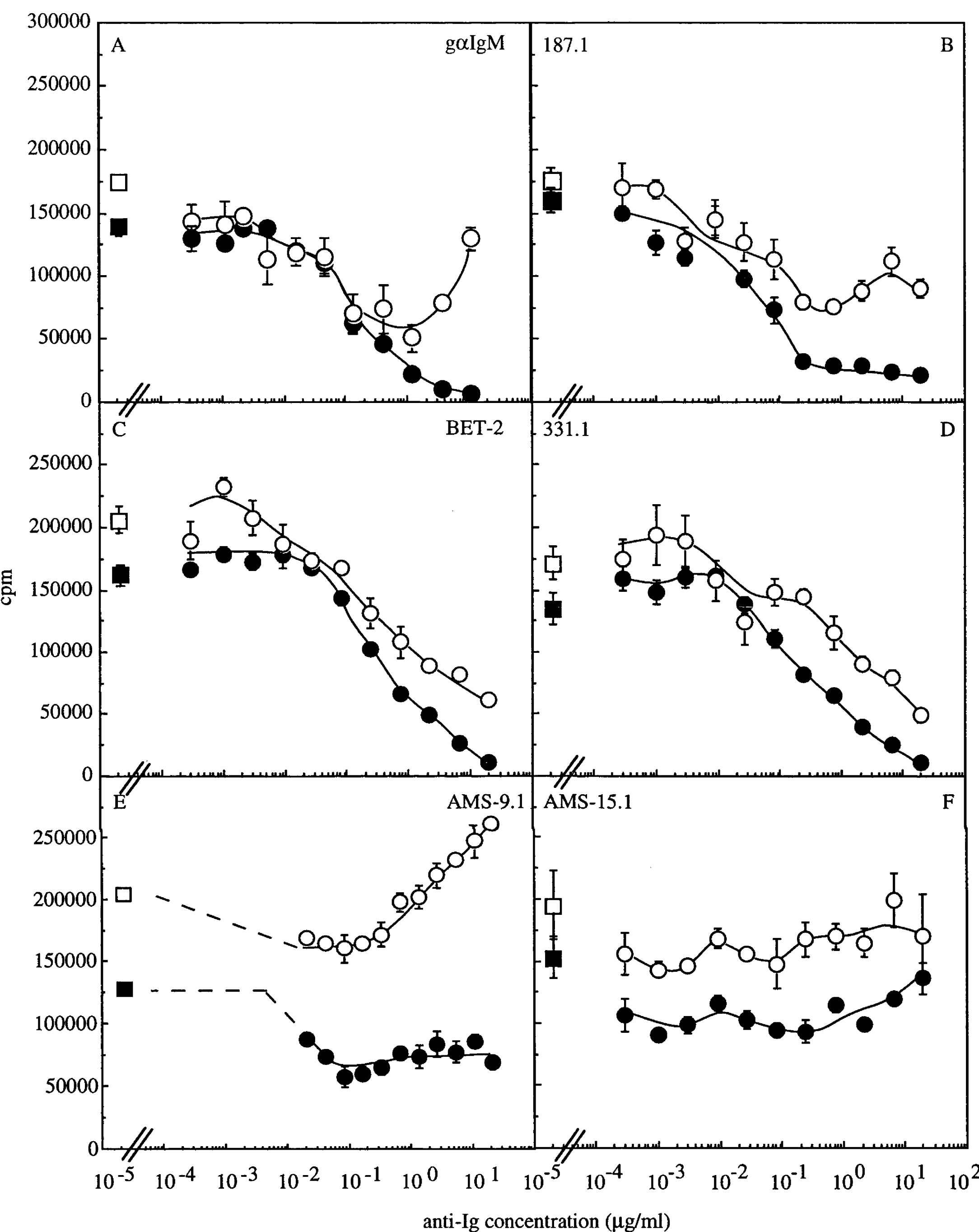

Figure 6.3. **Anti-Ig antibodies have different effects on LPS-induced B cell proliferation.** B cells were cultured as described in *Figure 6.1* in BCM containing LPS (50 μg/ml) and varying concentrations of anti-Ig antibodies (○). Replicate cultures were also prepared which contained anti-Ig antibodies, LPS and CsA (40 ng/ml) (●). After 3 days B cell proliferation was measured by ^{3}H-TdR incorporation as described in chapter 3. Background proliferation: LPS only (□), and LPS and CsA (■) is indicated. The data illustrated represents the mean of triplicate cultures ± 1 x SE.

In contrast to the other mAbs, the inclusion of AMS-9.1 to B cell cultures containing LPS resulted in a dose-dependent increase in the level of ^{3}H-TdR incorporation (Figure 6.3E). The positive effect of moderate to high doses (> 1 μg/ml) of AMS-9.1 on LPS-induced proliferation was CsA sensitive (Figure 6.3E). Thus, the effect of AMS-9.1 was similar to that obtained with gαIgM over the same dose range (Figure 6.3). In addition to the enhancing effect of AMS-9.1 on LPS-induced proliferation, low doses (< 1 μg/ml) of this mAb weakly inhibited ^{3}H-TdR incorporation (Figure 6.3E). In the presence of CsA inhibition by AMS-9.1 was more severe, however, as observed for 187.1 the level of inhibition did not increase with dose, but instead formed a plateau which extended over a two log increase in antibody concentration (Figure 6.3). Finally, although the presence of AMS-15.1 in cultures containing LPS, or LPS and CsA, did result in a slightly lower level of ^{3}H-TdR incorporation, the response was not dose-dependent (Figure 6.3F). Isotype matched control mAbs had no influence on LPS-induced proliferation (data not shown).

6.2.3. Determining the effect of co-ligation of sIg and FcγRIIB on anti-Ig induced proliferation

The results described above clearly establish the different effects of anti-Ig reagents and imply that sIg can mediate positive and negative signals. However a complication with the use of these reagents is the possibility of co-ligation of sIg and FcγRIIB, which has been shown to inhibit B cell activation (Sinclair and Chan 1971, Ryan and Henkart 1976, Sidman *et al.,* 1979, Phillips *et al.,* 1983, Phillips and Parker 1984). Given the inhibitory effects of Fc-mediated signalling it was important to determine their influence on the B cell response to anti-Ig antibodies. One way to examine the effect of Fc-mediated signals on the B cell response to anti-Ig reagents was through the use of the Fc blocking antibody, 2.4G2, which has been shown to be effective at preventing Fc-mediated inhibition (Unkeless 1979, Muta *et al.,* 1994, Ashman *et al.,* 1996). An alternative method was through the use of F(ab')$_2$ fragments, which lack the Fc region but retain the ability to influence B cell behaviour (Andersson *et al.,* 1974, Sidman *et al.,* 1979, Boyd and Schrader 1981, Leptin 1985). Both experimental approaches were examined.

To determine the effect of 2.4G2 on anti-Ig-induced B cell proliferation 30 μg/ml of 2.4G2 was included in B cell cultures containing anti-Ig reagents (Figure 6.4). This concentration was chosen as it had been shown to be in excess of the dose required to saturate Fc receptors without having an effect on B cell proliferation under various culture conditions (data not shown). In addition, similar doses of 2.4G2 have been shown to effective at preventing Fc-mediated inhibition (Wilson *et al.,* 1987, Ashman *et al.,* 1996) Co-culture of 2.4G2 with anti-Ig reagents revealed that it could enhance

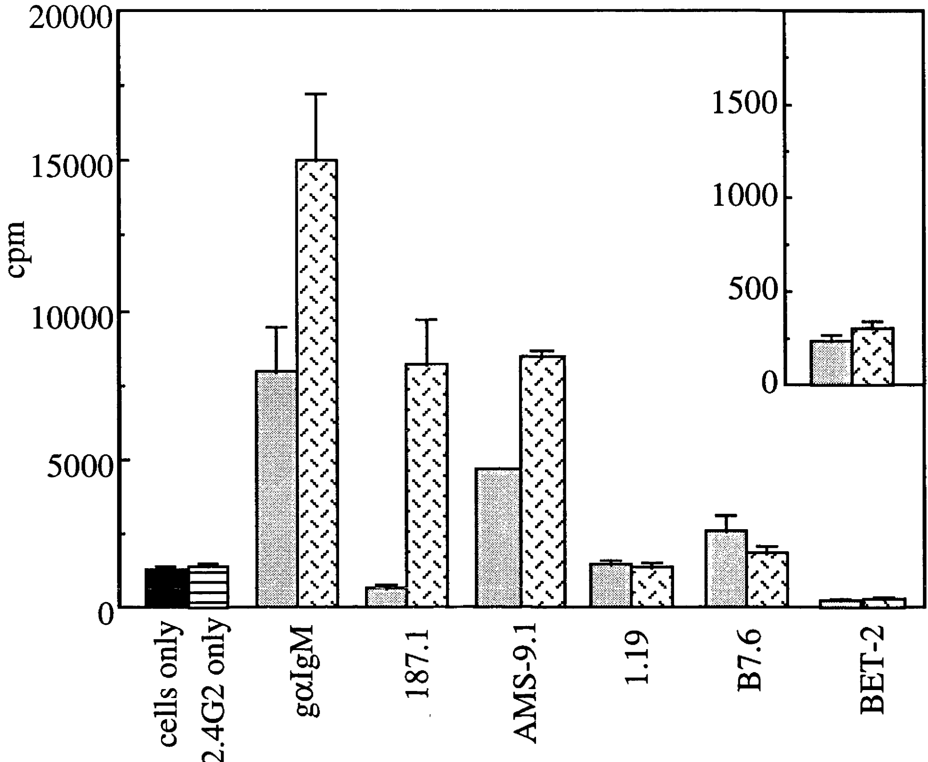

Figure 6.4. **The effect of 2.4G2 on the B cell proliferative response to various anti-Ig antibodies.**

B cells were cultured of BCM (5 x 10^4 cells / 200 µl well) containing 20 µg/ml anti-Ig antibody alone (▒) or in combination with 30 µg/ml of 2.4G2 (▨). After 3 days the level of proliferation was determined by a ^{3}H-TdR incorporation assay as described in the chapter 3. The illustrated data represents the mean of triplicate cultures ± 1 x SE. Inset shows detail of the response to BET-2.

B cell ^{3}H-TdR incorporation in response to gαIgM, 187.1 and AMS-9.1, while having no apparent affect on the response to BET-2, 1.19 or B7.6. It was of interest to note that in the presence of 2.4G2, the previously non-mitogenic 187.1, induced a level of ^{3}H-TdR incorporation which was equivalent to that obtained with gαIgM alone, this result suggests that 187.1 could induce sufficient co-ligation of sIg and FcγRIIB which masked its mitogenic potential (Figure 6.4).

6.2.4. Blocking Fc binding has different affects on anti-Ig inhibition of LPS-induced B cell proliferation and differentiation

In subsequent experiments the role of Fc-mediated inhibition in determining the B cell response to co-culture with anti-Ig antibodies and LPS was examined (Figures 6.5.1 and 6.5.2). The presence of 2.4G2 increased proliferation to varying degrees in response to five out of six anti-Ig reagents (Figures 6.5.1 and 6.5.2A-C). However in cultures containing BET-2, the addition of 2.4G2 did not have any apparent affect on proliferation. Thus, the two BET-2-induced dose response curves virtually overlayed each other (Figure 6.5.1B). In addition to determining the effect of 2.4G2 on anti-Ig and LPS-induced proliferation, its influence on ASC formation was also examined. The presence of 2.4G2 did not induce a marked change in the dose response curves for ASC formation, irrespective of the anti-Ig reagent used however, cultures which contained 2.4G2 had slightly higher numbers of ASCs (Figures 6.5.1 and 6.5.2D-F). These results demonstrate that a number of mAbs are able to inhibit LPS-induced ASC formation in a manner which is independent of the co-ligation of sIg and FcγRIIB (Figures 6.5.1 and 6.5.2D-F). Further, the results obtained with BET-2 indicate that inhibition of LPS-induced proliferation can occur in the absence of Fc-mediated inhibitory signals.

Preventing co-ligation of sIg and FcγRIIB does not affect the sIg-mediated inhibitory signal

Similar experiments to those described above were also conducted in the presence of CsA, which does not affect Fc-mediated inhibitory signals (Gottschalk *et al.,* 1994). The dose response curves obtained for proliferation and ASC formation in cultures containing anti-Ig, LPS and CsA were unaltered by the addition of 2.4G2 (Figures 6.6.1 and 6.6.2). These results indicate that preventing co-ligation of sIg and FcγRIIB had no affect on the negative sIg-mediated signal. However in the absence of CsA, 2.4G2 had a marked positive effect on proliferation in some cultures (Figures 6.5.1 and 6.5.2). Thus, the results illustrated in Figures 6.5.1-6.5.2 suggest that co-ligation of sIg and FcγRIIB have different effects on the two sIg-mediated signals resulting in the inhibition of the positive signal for growth without affecting the negative signal.

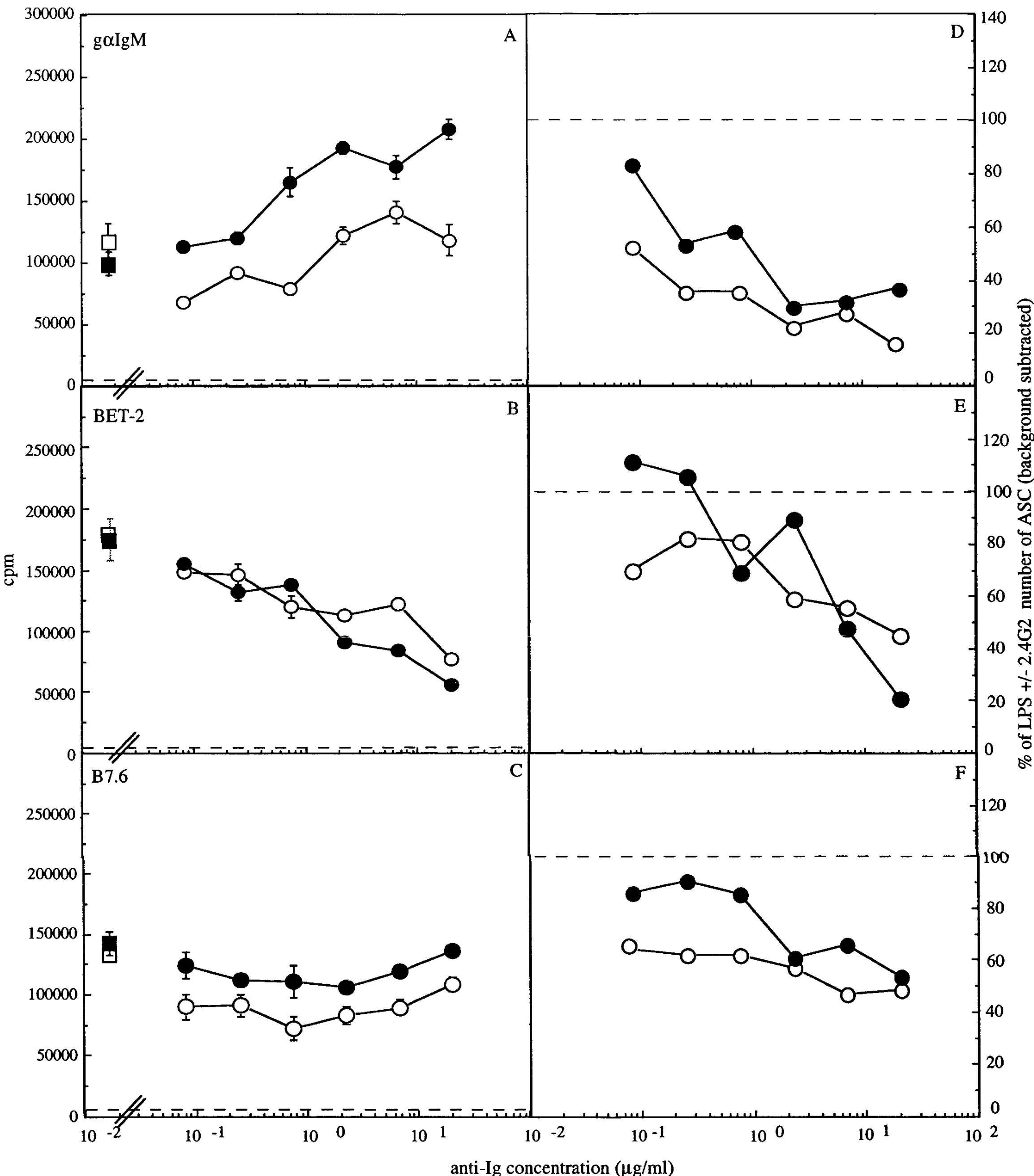

Figure 6.5.1. **The influence of the Fc blocking antibody, 2.4G2, on the B cell response to co-culture with different anti-Ig antibodies and LPS.**

B cells were cultured in BCM (5 x 10^4 cells/ 200 μl well) the presence 50 μg/ml LPS and various concentrations of anti-Ig antibodies (O). Replicate B cell cultures were also prepared which contained anti-Ig antibodies, LPS and 30 μg/ml 2.4G2 (●). After 3 days the level of B cell proliferation was determined by ^{3}H-TdR incorporation, and the numbers of ASCs determined by Elispot assay, as described in chapter 3. For proliferation each point represents the mean of triplicate cultures ± 1 x SE, whereas the number of ASCs was expressed as a percentage of the control (LPS or LPS & 2.4G2) response with the background (cells only or 2.4G2 only) subtracted. LPS-induced proliferation with (□) and with out 2.4G2 (■), background proliferation (– – -) is indicated.

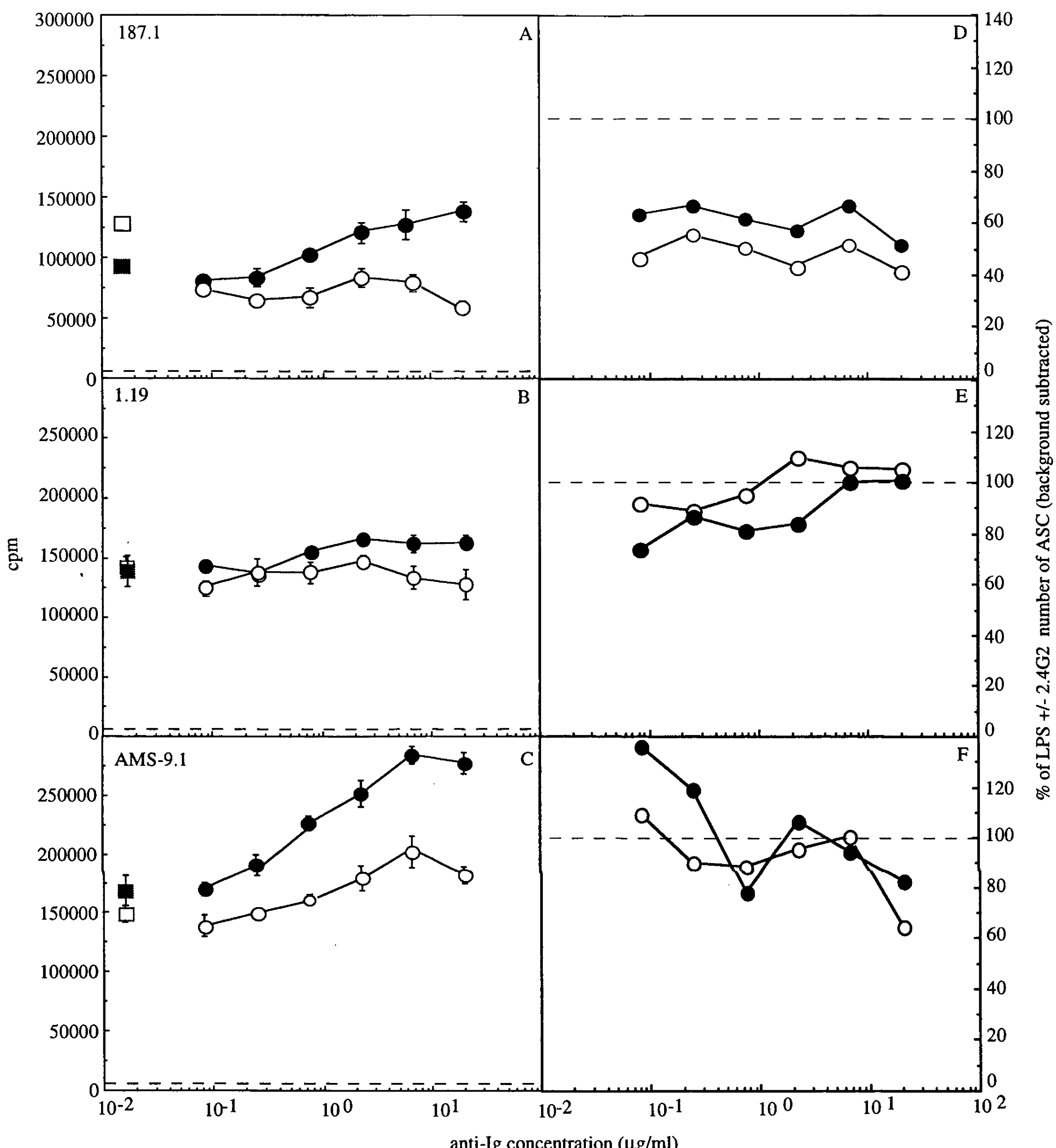

Figure 6.5.2. **The influence of the Fc blocking antibody, 2.4G2, on the B cell response to co-culture with different anti-Ig antibodies and LPS.**

B cells were cultured in BCM (5 x 10^4 cells/ 200 μl well) the presence 50 μg/ml LPS and various concentrations of anti-Ig antibodies (O). Replicate B cell cultures were also prepared which contained anti-Ig antibodies, LPS and 30 μg/ml 2.4G2 (●). After 3 days the level of B cell proliferation was determined by ^{3}H-TdR incorporation, and the numbers of ASCs determined by Elispot assay, as described in chapter 3. For proliferation each point represents the mean of triplicate cultures ± 1 x SE, whereas the number of ASCs was expressed as a percentage of the control (LPS or LPS & 2.4G2) response with the background (cells only or 2.4G2 only) subtracted. LPS-induced proliferation with (■) and with out 2.4G2 (□), background proliferation (– – –) is indicated.

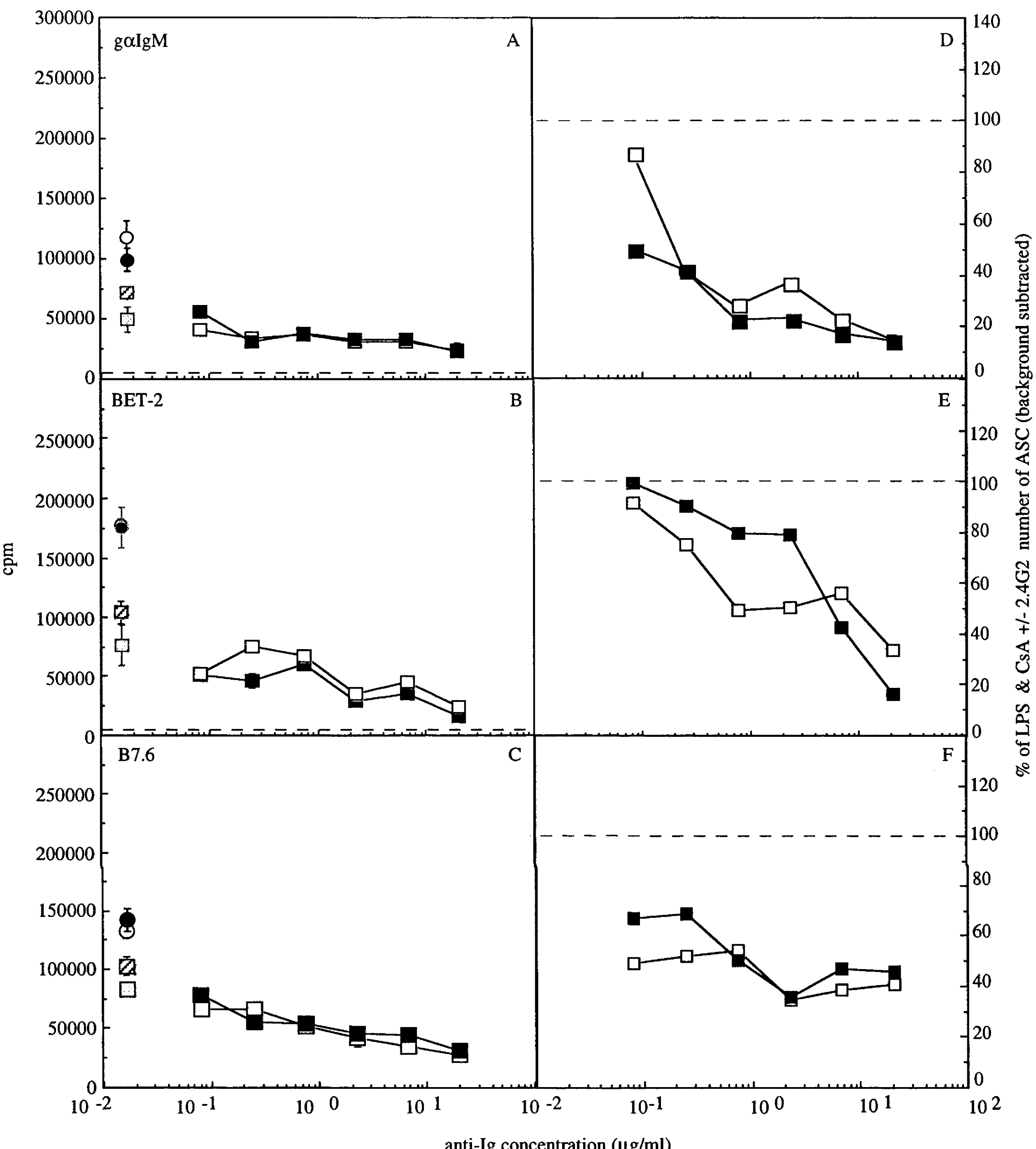

Figure 6.6.1. **The influence of the Fc blocking antibody, 2.4G2, on the B cell response to co-culture with anti-Ig antibodies, CsA and LPS.**

B cells were cultured in BCM (5 x 10^4 cells/ 200 μl well) the presence LPS (50 μg/ml), CsA (40 ng/ml) and various concentrations of anti-Ig antibodies (□). Replicate B cell cultures were also prepared which contained anti-Ig antibodies, LPS, CsA and 30 μg/ml 2.4G2 (■). After 3 days the level of B cell proliferation was determined by ^{3}H-TdR incorporation, and the numbers of ASCs determined by Elispot assay, as described in chapter 3. For proliferation each point represents the mean of triplicate cultures ± 1 x SE, whereas the number of ASCs was expressed as a percentage of the control (LPS & CsA or LPS, CsA & 2.4G2) response with the background (cells only or 2.4G2 only) subtracted. Proliferation: LPS only (○) LPS and CsA (●), LPS and 2.4G2 (▨), LPS, CsA and 2.4G2 (▢), background (– – –) is indicated.

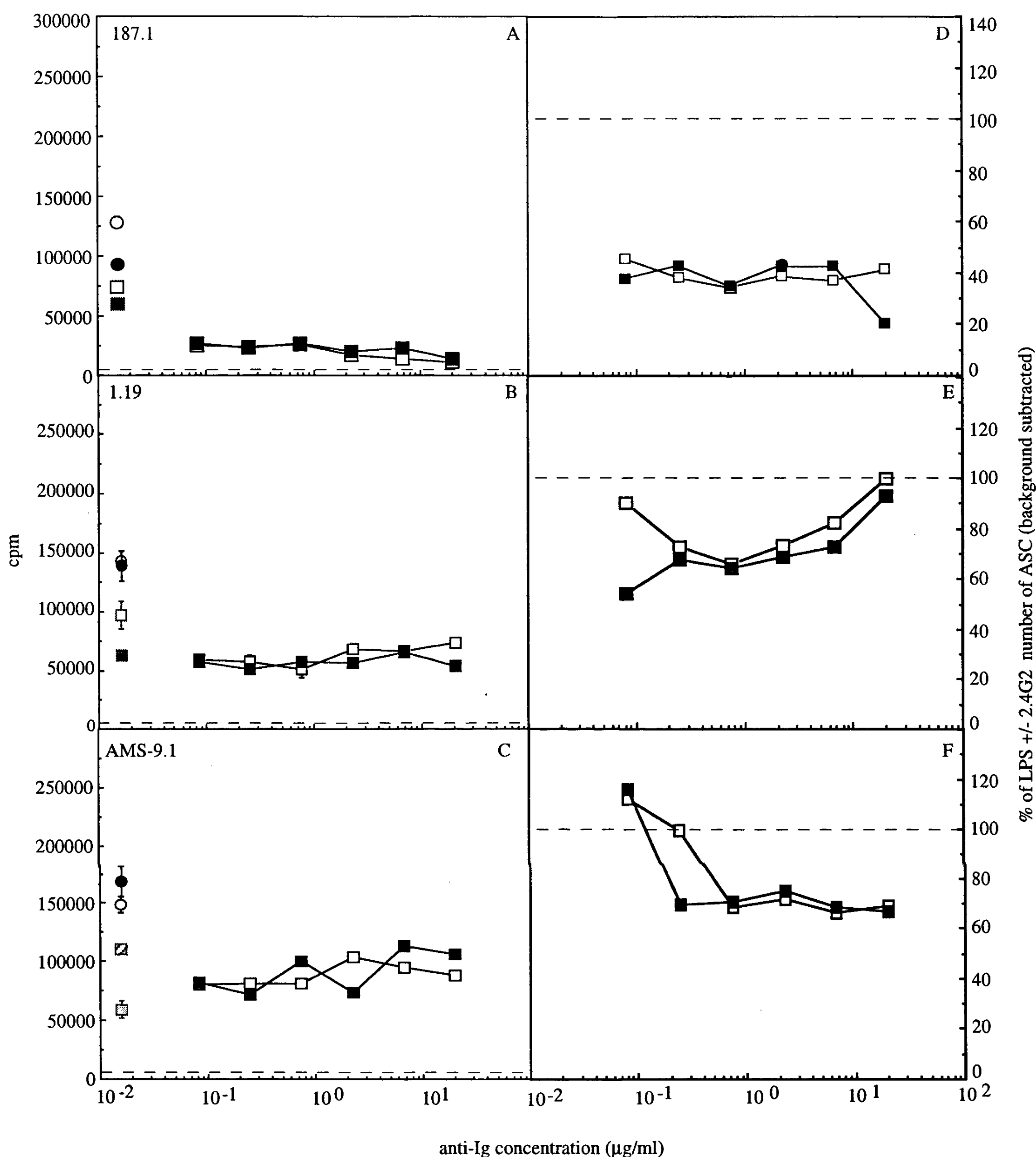

Figure 6.6.2. **The influence of the Fc blocking antibody, 2.4G2, on the B cell response to co-culture with anti-Ig antibodies, CsA and LPS.**

B cells were cultured in BCM (5 x 10^4 cells/ 200 µl well) the presence LPS (50 µg/ml), CsA (40 ng/ml) and various concentrations of anti-Ig antibodies (□). Replicate B cell cultures were also prepared which contained anti-Ig antibodies, LPS, CsA and 30 µg/ml 2.4G2 (■). After 3 days the level of B cell proliferation was determined by ^{3}H-TdR incorporation, and the numbers of ASCs determined by Elispot assay, as described in chapter 3. For proliferation each point represents the mean of triplicate cultures ± 1 x SE, whereas the number of ASCs was expressed as a percentage of the control (LPS & CsA or LPS, CsA & 2.4G2) response with the background (cells only or 2.4G2 only) subtracted. Proliferation: LPS only (○) LPS and CsA (●), LPS and 2.4G2 (▨), LPS, CsA and 2.4G2 (□), background (– – –) is indicated.

6.2.5. A comparison of B cell proliferation in response to intact anti-Ig antibodies and their F(ab')2 fragments

Having determined the effect of 2.4G2 on anti-Ig induce B cell behaviour the influence of Fc-mediated signals on these response was examined through the use of $F(ab')_2$ fragments of anti-Ig antibodies. This alternative experimental approach has the advantage of being able to compare the B cell response to the same antibody, with and without the presence of an Fc region. In these experiments the effect of intact rabbit anti-mouse IgG (rαIgG) and its $F(ab')_2$ fragment on B cell proliferation and differentiation to ASCs were compared (Figures 6.7 and 6.8). When B cells were cultured with rαIgG or its $F(ab')_2$ fragment, only the $F(ab')_2$ fragment-induced ^{3}H-TdR incorporation above the background (Figure 6.7A and B). In the presence of IL-4 however, both the intact rαIgG and its $F(ab')_2$ fragment-induced proliferation with cultures containing the $F(ab')_2$ fragment-inducing more than twice the level of ^{3}H-TdR incorporation than the intact rαIgG at a similar dose (Figure 6.7A and B).

Anti-Ig F(ab')2 fragments inhibit LPS-induced proliferation and ASC formation

When used in combination with LPS both rαIgG and its $F(ab')_2$ fragment inhibited proliferation, generating very similar dose response curves at antibody concentrations below 10 μg/ml (Figure 6.7C and D). In response to a higher concentration of $F(ab')_2$ rαIgG the level of ^{3}H-TdR incorporation increased, whereas in cultures containing rαIgG the high dose enhancement of proliferation was not observed (Figure 6.7D and C respectively). The inclusion of CsA in these cultures lowered the over all level of ^{3}H-TdR incorporation in response to either antibody (Figure 6.7C and D). While CsA did not alter the shape of the dose response curve for rαIgG, it did inhibit the increase in proliferation observed at a high dose of $F(ab')_2$ rαIgG. The dose response curve induced by $F(ab')_2$ rαIgG in combination with LPS and CsA was reminiscent of that observed with 187.1 and AMS-9.1 under similar culture conditions (Figure 6.3) in that at high concentrations of antibody the degree of inhibition formed a plateau (Figure 6.7D).

In addition to determining the effect of rαIgG and its $F(ab')_2$ fragment on LPS-induced proliferation the formation of ASCs was also examined. Both rαIgG and its $F(ab')_2$ fragment cause a dose-dependent inhibition of LPS-induced ASC formation generating dose response curves which overlay each other (Figure 6.8). This result suggests that the inhibitory sIg-mediated signal responsible for the decline in ASC number generated by the two anti-Ig reagents was similar. The similarity of the response to rαIgG and its $F(ab')_2$ fragment supports the conclusion that co-ligation of sIg and FcγRIIB is not required for inhibition of LPS-induced ASC formation.

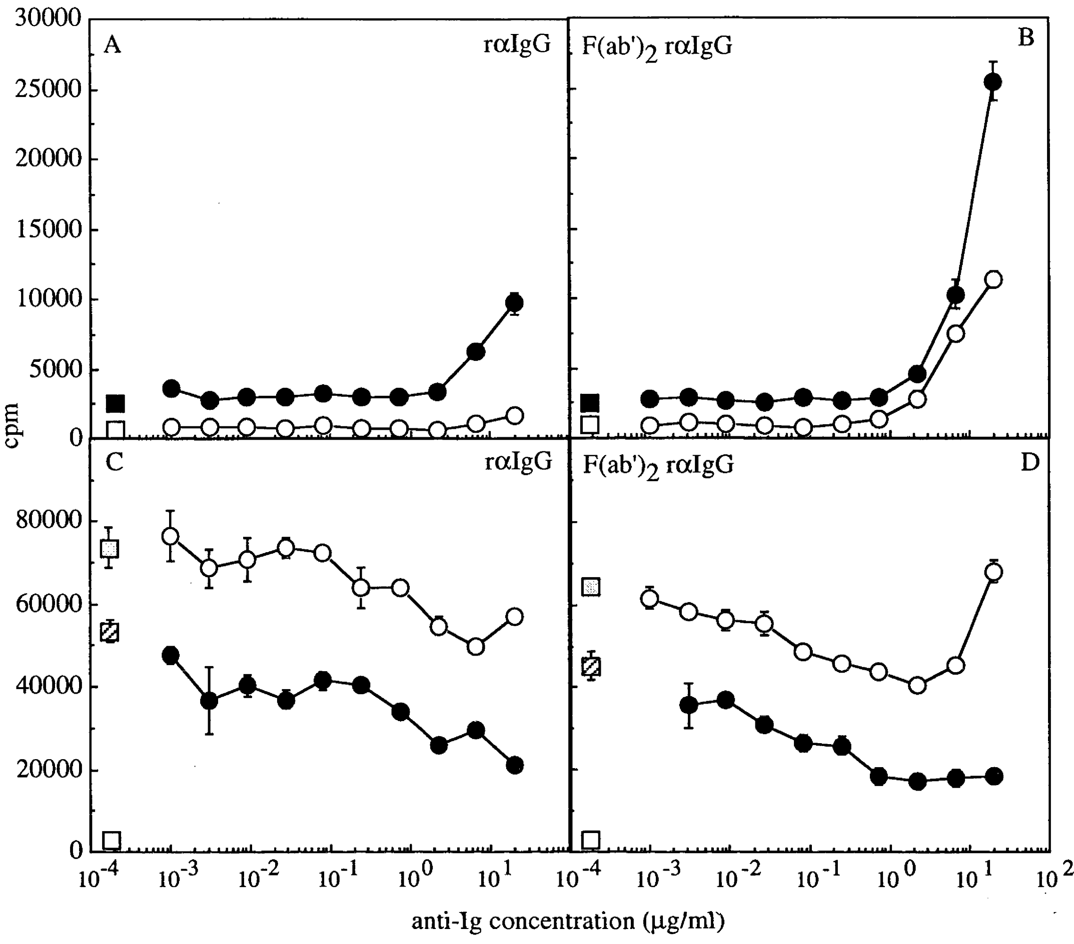

Figure 6.7. **Rabbit αIgG and its F(ab')$_2$ fragment generated similar inhibitory dose response curves but differed in their ability to induce B cell proliferation.**

B cells were cultured in BCM (5 x 10^4 cells/ 200 μl well) containing various concentrations of rαIgG or its F(ab')$_2$ fragment in the presence (●) or absence of 100 U / ml IL-4 (O) (panels A and B). Replicate cultures were prepared which contained rαIgG or F(ab')$_2$ rαIgG, in combination with 50 μg/ml LPS (O), or LPS and 40 ng/ml CsA (●) (panels C and D). After 3 days B cell proliferation was measured by ^{3}H-TdR incorporation, as described in chapter 3. Background proliferation: cells only (□), IL-4 only (■), LPS only (▣), LPS and CsA (▨) is indicated. The data illustrated represents the mean of triplicate cultures ± 1 x SE.

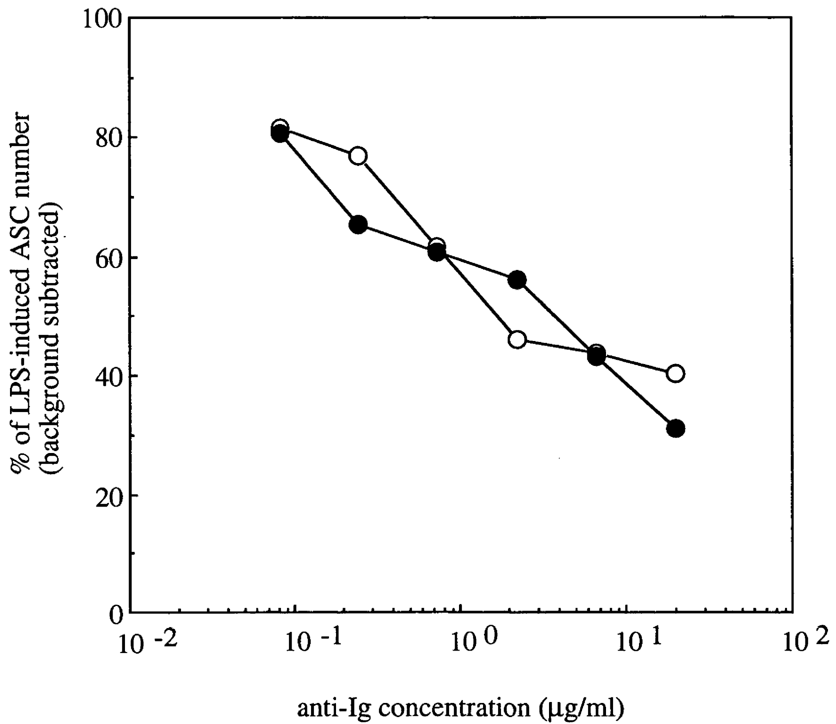

Figure 6.8. **Inhibition of LPS-induced ASC formation by rαIgG and its F(ab')$_2$ fragment generated virtually identical dose response curves.**

B cells were cultured in BCM (5 x 10^4 cells/ 200 µl well) containing various concentrations of rαIgG (O) or its F(ab')$_2$ fragment (●) in combination with 50 µg/ml LPS. After 3 days the number of ASCs was determined by Elispot assay, as described in chapter 3. The number of ASCs was expressed as a percentage of the LPS only response with the background subtracted. The data represents the mean of triplicate cultures.

6.3. Discussion

The results presented in this chapter further illustrate the diverse range of responses that anti-Ig reagents are capable of producing when used to stimulate B cells. A hypothesis which might provide a consistent interpretation of anti-Ig induced B cell behaviour, suggests that the antigen signal consist of a number of different signalling components. The components of the antigen signal appear to be activated independently of each other, can vary in strength and regulate different B cell responses. According to this model the behaviour of the B cell is determined by the integration of the components of the antigen signal within the B cell, however, it not yet understood how this might occur. Thus far, the results suggest that sIg-mediated signals can induce positive or negative effects on B cell behaviour, which are dependent on the strength and / or ratio of the different components of the antigen signal (Figure 5.10). The results described in this chapter are consistent with such a model (Figure 6.9). In addition, they suggest that it is the precise physical interaction between the surrogate antigen and its receptor that determines both the signal strength and which components of the antigen signal are activated.

Like gαIgM, a number of mAbs were able to inhibit proliferation and LPS-induced differentiation to ASCs through a CsA insensitive signal (Figures 6.1, 6.3, 6.5.1-6.8). In addition, certain mAbs were also able to induce proliferation in a manner which was sensitive to CsA (Figures 6.3 and 6.7). The CsA sensitive signal seems to be related to the ability of anti-Ig reagents to promote B cell proliferation independently of LPS (Figures 6.1 and 6.8), a property previously determined to be a correlate of TI-2 antigenic stimulation (Brunswick *et al.,* 1988, Peçanha *et al.,* 1991). Further, the results describe in this chapter also illustrate the effect of IL-4 and co-ligation with FcγRIIB on sIg-mediated signalling. While IL-4 enhances anti-Ig induced proliferation (Figures 6.1 and 6.2) as previously described (Howard *et al.,* 1982, Hodgkin *et al.,* 1991a), the results presented in this chapter demonstrate that it cannot prevent the inhibition of proliferation by anti-Ig reagents (Figure 6.1). In addition, although Fc-mediated signals have been shown to inhibit anti-Ig induced proliferation (Figures 6.4-6.5.2 and 6.7) (Sinclair *et al.,* 1971, Sidman *et al.,* 1979, Phillips *et al.,* 1983), co-ligation of sIg and FcγRIIB had no affect on the sIg-mediated negative signal (Figures 6.5.1-6.8). Finally, the ability of a mAb to transmit the negative signal for growth does not predict whether it will also be able to provide the positive signal, as illustrated by the results obtained for BET-2 and AMS-9.1. While BET-2-induced a strong negative signal it was apparently unable to generate the positive signal required to stimulate proliferation (Figure 6.3 and 6.5.1). Conversely AMS-9.1 predominantly generated a positive signal for growth in the absence of a strong negative signal

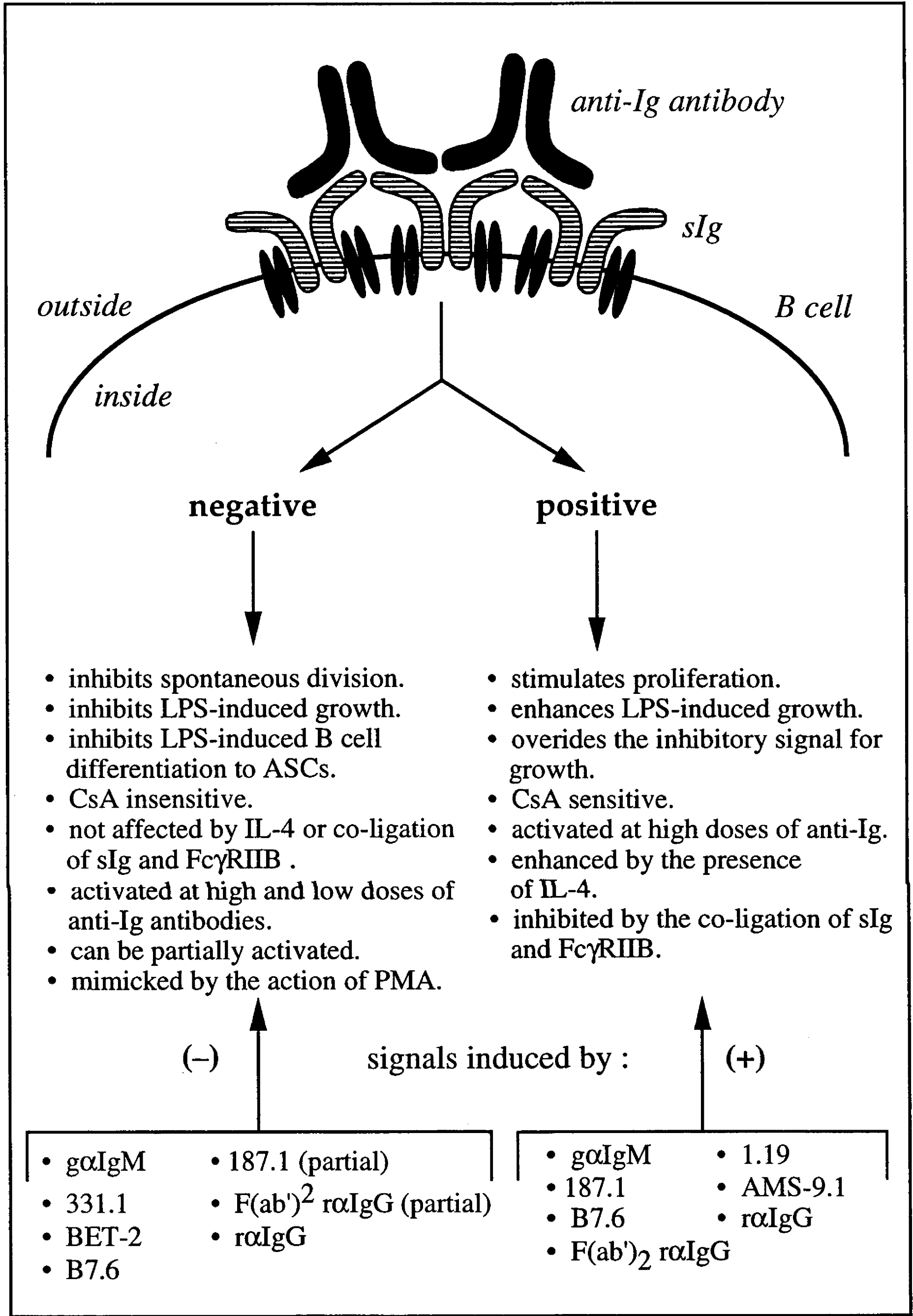

Figure 6.9. **A summary of the behaviour and charateristics of the different sIg-mediated signals .**

(Figures 6.1, 6.3 and 6.5.1). These results support the view that the two sIg-mediated signals are independently regulated, as proposed in chapter 5.

6.3.1. Differences in anti-Ig antibodies influence sIg-mediated signalling

While the experiments described do not explore the biochemical events involved in sIg-mediated signalling, the data clearly supports the hypothesis that sIg can mediate a number of different signals. Given this possibility the question arises as to what causes the activation of the different signals from the same receptor. As each of the anti-Ig reagents differ physically from one another, it is likely these differences affect the interaction between the anti-Ig antibodies and sIg, resulting in different patterns of signalling and subsequent B cell behaviour. The anti-Ig reagents used in these experiments differ in: affinity, isotype, epitope specificity, flexibility, ability to induce cross-linking of sIg, and co-ligation of sIg and FcγRIIB. Each of these differences may contribute to the way that the antibody binds to sIg, however, as discussed below, no single factor can be identified as causing the variation in the B cell response. Thus, each of the surrogate antigens could be considered to have an intrinsic ability to induce an antigen signal(s), based on the sum of the influences of each factor on its interaction with sIg.

The dose response curves for proliferation suggest that anti-Ig antibodies can be full or partial agonists for the sIg-mediated negative signal.

One of the most obvious influences on the B cell response to anti-Ig reagents was that of antibody concentration. For the mitogenic antibodies, B cell proliferation increased with dose, indicating that above a certain threshold the onset of proliferation was related to the strength, or frequency, of the sIg-mediated positive signal which appeared to be proportional to antibody concentration (Figures 6.1 and 6.2). A similar relationship between signal strength and dose was also observed for inhibition of spontaneous and LPS-induced proliferation by 331.1 and BET-2 (Figures 6.1, 6.3, and 6.5.1). The dose response curve for these antibodies suggests that they are full agonists for the negative signal. However for 187.1 the relationship between dose and the strength of inhibitory signal was not as simple, as the degree of inhibition induced by 187.1 formed a plateau at moderate-high antibody concentrations (Figure 6.3). Significantly the plateau of inhibition was also observed in the presence of CsA (Figure 6.3), excluding the possibility that this response was due to the generation of a positive signal. A similar inhibitory dose response curve was also generated by the $F(ab')_2$ fragment of rαIgG (Figure 6.7D). Thus, it appears that 187.1 and the $F(ab')_2$ fragment of rαIgG represent partial agonists for the negative signal as the level of inhibition was not directly

proportional to antibody concentration. The proposition that anti-Ig reagents may only be able to induce the partial activation of the various components of the antigen signal is discussed further in chapter 8.

The nature of the sIg-mediated signal(s) can also be influenced by the efficiency with which the antibody induces cross-linking of sIg. Anti-Ig reagents which are effective at cross-linking could potentially induce a positive signal for growth at a lower concentration than those that are poor cross-linkers. Increasing the level of sIg cross-linking by coupling anti-Ig reagents to insoluble matrixes has been shown to induce B cell proliferation by previously non-mitogenic soluble antibodies (Parker 1975, Brunswick *et al.,* 1988, Peçanha *et al.,* 1991, Udhayakumar *et al.,* 1991). However increased cross-linking of sIg can also have an inhibitory effect on B cell growth as demonstrated by Parry *et al.,* (1994) who showed that mitogenic soluble anti-Ig reagents when immobilised would induce apoptosis (Parry *et al.,* 1994b). Thus, while increasing the degree of cross-linking induced by an anti-Ig antibodies frequently enhances the mitogenic potential of these reagents, it can also have deleterious consequences for the cell.

Epitope specificity and affinity alone cannot account for the different effects of anti-Ig antibodies.

Another factor which may influence the nature (inhibitory or stimulatory) of sIg-mediated signalling is the epitope specificity of the anti-Ig reagents. It is possible that anti-Ig binding to different epitopes could induce specific patterns of sIg-mediated signalling. In addition, epitope specificity may in part determine how effective an anti-Ig antibody is at inducing cross-linking of sIg. However as with the effect of increased cross-linking, epitope specificity alone was insufficient to explain the different B cell responses, as illustrated by the results obtained for BET-2 and B7.6. Although these antibodies bind to the same epitope (Udhayakumar *et al.,* 1991), they induce different B cell behaviour (Figures 6.1-6.3 and 6.5.1). One possibility which could account for the variation in the B cell response to these two anti-Ig reagents is that they differ in affinity; B7.6 binds with high affinity, whereas BET-2 is a low affinity antibody (Udhayakumar *et al.,* 1991). However, although a high affinity for its epitope may be necessary for a mitogenic antibody, it is not sufficient, as illustrated by AMS-15.1. AMS-15.1 binds to its epitope with high affinity (Goroff *et al.,* 1986), however, as a soluble antibody it is not mitogenic (Figure 6.1). As discussed above, the coupling of anti-Ig reagents to an insoluble matrix can alter the B cell response. Thus, when coupled to sepharose beads AMS-15.1 was shown to induce B cell proliferation (Goroff *et al.,* 1986). Not surprisingly these results indicate that the ability of an anti-Ig reagent to induce a positive signal for growth requires the interaction of a number of factors, and that high affinity

and extensive cross-linking of sIg promote the induction of this signal. Given these apparent constraints on the induction of the positive signal, it is of interest to note that low affinity does not prevent BET-2 from inducing a potent inhibitory signal. Thus, the physical characteristics of the surrogate antigens required to induce either sIg-mediated signal appear to differ.

In addition the B cell response to anti-Ig reagents may be influenced by the isotype of the anti-Ig antibody. However differences in isotype are an unlikely explanation for the results described here, as 187.1, B7.6 and BET-2 are the same isotype but induce different responses (Figures 6.1-6.3, 6.5.1 and 6.5.2). In contrast, 331.1 and BET-2 are different isotypes but produce almost identical dose response curves in the presence of LPS (Figure 6.3). Further, isotope matched controls do not induce a response or alter LPS-induced B cell behaviour (data not shown).

6.3.2. The sIg-mediated positive signal for growth is inhibited by co-ligation of sIg and FcγRIIB.

It was also important to examine the possibility that the inhibitory dose response curves may be the result of Fc-mediated inhibition, rather than inhibitory signals from sIg. The results obtained with 2.4G2 reveal that the influence of Fc-mediated inhibition on the dose response curves varied between the different anti-Ig reagents (Figures 6.5.1 and 6.5.2). This could be because anti-Ig antibodies differ in their ability to induce the co-ligation of sIg and FcγRIIB. Some antibodies may have lacked the flexibility to bind both receptors at once and therefore, the presence of 2.4G2 would have no affect on the B cell response.

Blocking Fc binding of gαIgM, B7.6 and 187.1 enhanced LPS-induced proliferation without substantially altering the inhibition of ASC formation (Figures 6.5.1 and 6.5.2). Within the framework of the model, the increase in proliferation in the presence of 2.4G2 suggests that the B cell is receiving a stronger positive signal for growth. As 2.4G2 does not induce any measurable effect of ^{3}H-TdR incorporation, it may be assumed that it promotes proliferation by preventing the co-ligation of sIg and the FcγRIIB, and subsequent the inhibition of the sIg-mediated positive signal for growth. The sIg-mediated positive signal was shown to be sensitive to CsA (Figures 5.1 and 6.3), and is therefore, likely to be dependent on the mobilisation of calcium (Figure 5.10). This is consistent with the discovery that co-ligation of sIg and FcγRIIB inhibits calcium mobilisation induced by sIg cross-linking (Wilson *et al.,* 1987). As illustrated in Figure 6.10, co-ligation of sIg and FcγRIIB can result in the closing of a membrane ion channel which is required for influx of extracellular calcium

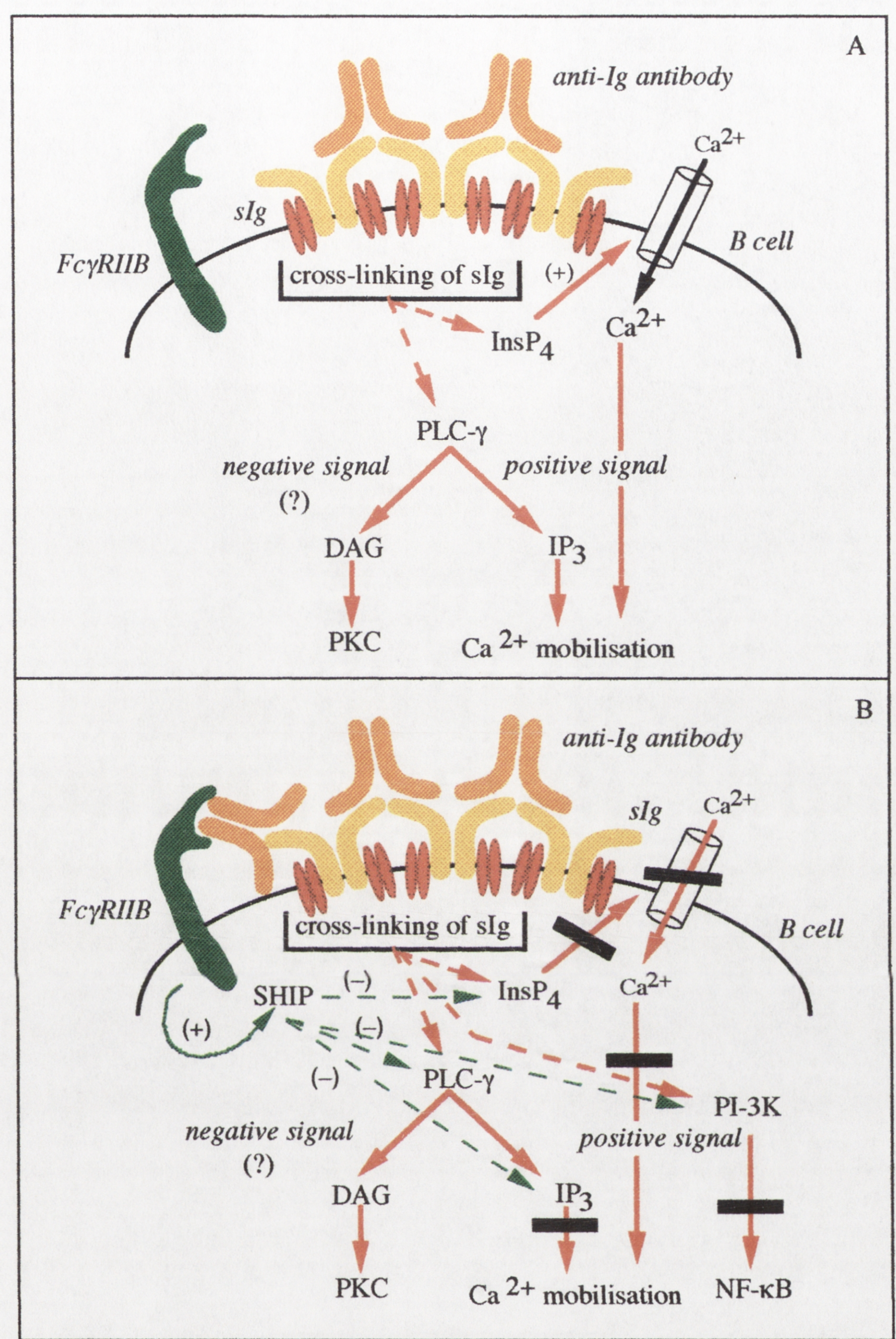

Figure 6.10. **The interaction between sIg and FcγRIIB signals that could induce Fc-mediated inhibition.**

According to the model proposed in chapter 5, cross-linking of sIg can result in the generation of a positive signal for growth which appears to involve the mobilisation of calcium (A). Co-ligation of sIg and FcγRIIB has been shown to inhibit the mobilisation of calcium, possibly as a the result of the activation of SHIP and the subsequent dephosphorylation of components of intracellular signalling pathways (B). In this way Fc-mediated signals may inhibit the generation of the sIg-mediated positive signal.

ions in response to sIg cross-linking (Choquet *et al.,* 1993, Diegel *et al.,* 1994). Although the mechanism by which this occurs has not been completely elucidated, the activation of the phosphatase SHIP in response to co-ligation of sIg and FcγRIIB (D'Ambrosio *et al.,* 1995, Chacko *et al.,* 1996, Ono *et al.,* 1996), can prevent the influx of extracellular calcium by dephosphorylating inositol 1,3,4,5-phosphate which is required to open the ion channel (Damen *et al.,* 1996, Ono *et al.,* 1996) (Figure 6.10). Thus, by inhibiting the co-ligation of sIg and FcγRIIB, 2.4G2 may prevent intracellular signalling events which inhibit calcium mobilisation in response to cross-linking of sIg (Figure 6.10).

6.3.3. Co-ligation of sIg and FcγRIIB does not affect the sIg-mediated inhibitory signal

While the effect of 2.4G2 on B cell proliferation indicates that co-ligation of sIg and FcγRIIB can prevent the generation of mitogenic sIg-mediated signals, a similar conclusion cannot be made for the generation of inhibitory signals from sIg (Figure 6.11). The results obtained for LPS-induced B cell differentiation in the presence of 2.4G2 (Figures 6.5.1 and 6.5.2) indicate that anti-Ig antibodies are able to induce a sIg-mediated signal which inhibits LPS-induced ASC formation in a manner which is independent of co-ligation of sIg and FcγRIIB. This conclusion is supported by the inhibitory effect of the $F(ab')_2$ fragment of rαIgG (Figures 6.7 and 6.8) and TNP-Ficoll (Figure 4.9) on LPS-induced B cell activation. Similar results have also been reported in the literature. $F(ab')_2$ antibody fragments have been shown to inhibit spontaneous and LPS-induced proliferation (Boyd *et al.,* 1981, Leptin 1985) and ASC formation (Andersson *et al.,* 1974, Warner and Scott 1991). In these experiments the inhibitory response induced by the $F(ab')_2$ fragments was equivalent to that obtained with the intact antibody. Further, in transgenic mice "natural" antigens have been shown to the inhibit LPS-induced ASC formation generating dose response curves which were similar to that obtained for anti-Ig mediated inhibition (Grandien *et al.,* 1993). Combined all of these results indicate that Fc-mediated signals cannot account for inhibitory responses induced by anti-Ig reagents, and support the proposition that cross-linking of sIg can generate a negative signal which is not dependent on co-ligation of sIg and FcγRIIB (Figure 6.11).

In the previous chapter negative signalling by sIg was proposed to involve the activation of PKC (Figure 5.6). Within the literature there is some debate as to whether or not co-ligation of sIg and FcγRIIB affects the activation of PKC or PLCγ, which is required to activate PKC (Figure 1.4) (Gottschalk *et al.,* 1994, Ono *et al.,* 1996, Sarkar *et al.,* 1996). However although co-ligation of sIg and FcγRIIB inhibits the propagation of certain sIg-mediated signals it does not block them completely (Wilson *et al.,* 1987),

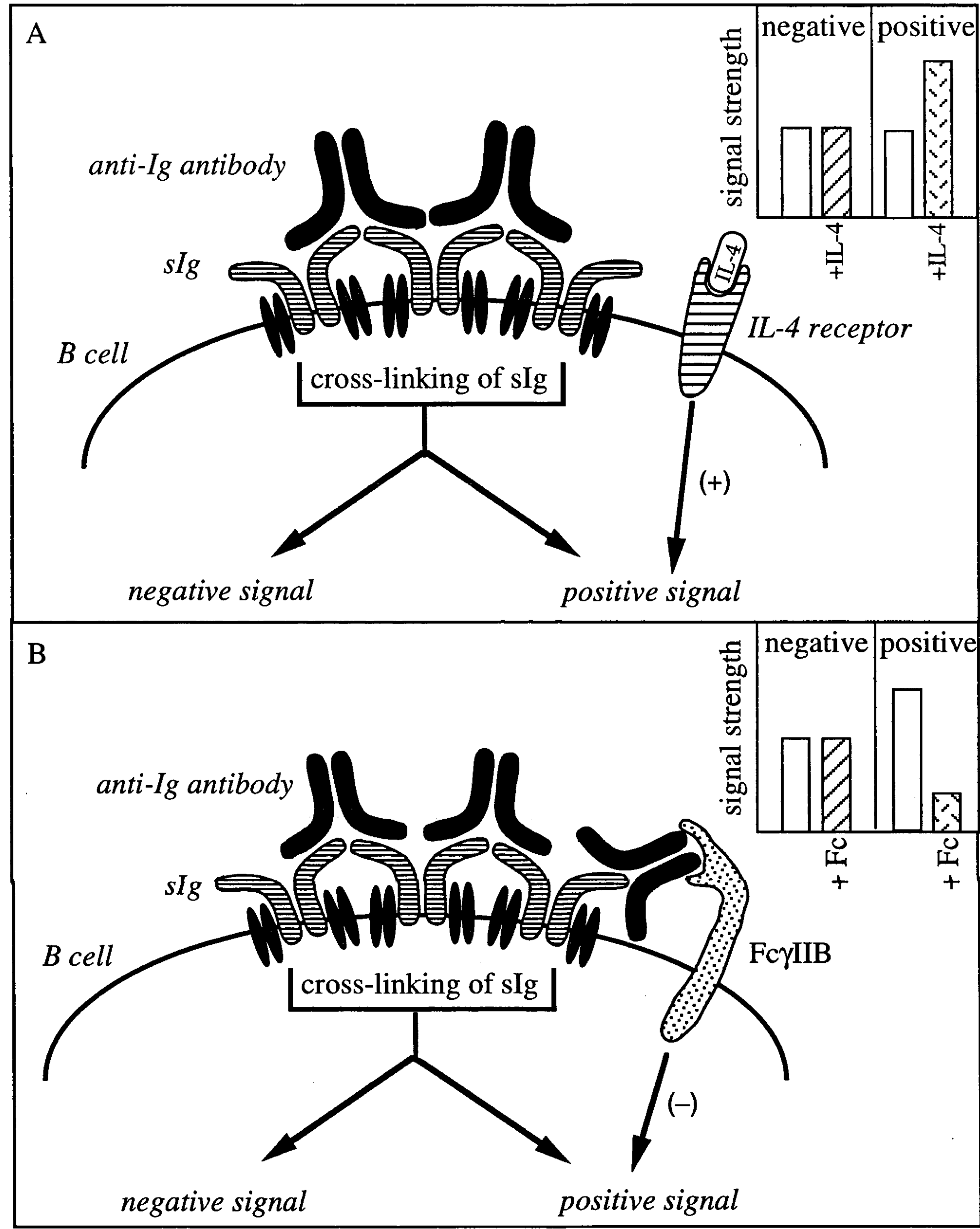

Figure 6.11. **The influence of IL-4 or co-ligation with FcgRIIB on sIg mediated signalling.**

This Figure illustrates how IL-4 or co-ligation of sIg and FcγRIIB could influence the two sIg-mediated signals. Panel A illustrates the influence of IL-4, which appears to enhance the sIg-mediated positive signal without effecting the negative signal. In contrast co-ligation of sIg with FcγRIIB appears to inhibit the positive signal but does not affect the negative signal (panel B). Inset shows the relative signal strength of both signals and the influence of either IL-4, or co-ligation of sIg and FcγRIIB on the two sIg-mediated signals.

it is therefore, conceivable that sufficient PKC could still be activated to provide an inhibitory signal. Alternatively the negative sIg-mediated signal may be delivered by another, as yet unidentified, component(s) of the antigen signal. Thus, co-ligation of sIg and FcγRIIB promotes the inhibition of B cell activation by preventing the generation of the sIg-mediated positive signal for growth, without altering the induction of the negative signal (Figure 6.11).

6.3.4. Does cross-linking of sIgM or sIgD induce different patterns of intracellular signalling and subsequent B cell behaviour ?

Although the number of mAbs tested in these experiments was small the results imply there may be some difference in the sIg-mediated signalling generated by sIgD and sIgM. The anti-IgD antibodies appeared to be more capable of inducing a positive signal than generating negative signals (Figures 6.1-6.3 and 6.5.2). In contrast, the anti-IgM reagents were clearly able to induce both signals, but appeared to be more inhibitory than the anti-IgD mAbs (Figures 6.1, 6.3 and 6.5.1). Within the literature there is evidence to support the proposition that cross-linking of sIgD or sIgM mediate different responses. However no consistent interpretation of the roles of sIgD or sIgM has been reached. While some authors have found evidence to indicate sIgD-mediated signalling tends to have a positive effect on the B cell behaviour and that cross-linking of sIgM had a predominantly negative effect (Brines *et al.,* 1992, Carsetti *et al.,* 1993), others have suggested that the converse is true (Phillips and Klaus 1993). There is also data to support the counter argument; that signals generated by either isotype are functionally equivalent and capable of inducing the same range of B cell behaviour (Leptin 1985, Goroff *et al.,* 1986, Harnett *et al.,* 1989, Brink *et al.,* 1992, Roes and Rajewsky 1993, Parry *et al.,* 1994a, Snapper *et al.,* 1995a, Norvell and Monroe 1996). Given the contradictory nature of the experimental evidence it is not possible to come to a definitive conclusion about the role of sIg isotype in determining B cell response. However as discussed above, the diversity of the B cell behaviour could be due to differences in the interaction between the (surrogate) antigen and sIg, which could be translated into distinct patterns of intracellular signalling and thereby induce B cell responses specific to this interaction. Therefore, while the answer to the question whether or not sIgM and sIgD have different functions remains elusive, the results can be interpreted an supporting a model in which variations in the antigen signal determine the B cell response.

6.3.5. IL-4 enhances the positive sIg-mediated signal without affecting the negative sIg-mediated signal

Finally B cell behaviour in response to anti-Ig antibodies was also influenced by the presence of IL-4. Cultures stimulated with anti-Ig antibodies and IL-4 produced a range

of behaviour from; enhancing an existing proliferative response; to inducing strong ^{3}H-TdR incorporation from previously non-mitogenic antibodies; to having no measurable affect on the B cell response (Figures 6.1, 6.2 and 6.7). These results support the view that there is a qualitative difference in the type of signalling induced by each antibody. In addition, they reveal that IL-4 enhances the positive sIg-mediated signal without having any apparent affect on the inhibitory signal (Figures 6.1 and 6.11). IL-4's effect on proliferation was probably as a result of is ability to enhance the strength of positive signal (Hodgkin *et al.,* 1991a), and counter the negative effects of Fc-mediated signals (Phillips *et al.,* 1988). However, IL-4 could not alter the dose response curves of anti-Ig reagents which were only able to induce a sIg-mediated negative signal, as illustrated by the response to 331.1 (Figure 6.1D, inset). Thus, these results support a model where cross-linking of sIg initiates different signals within the B cell, and where the presence of IL-4 enhances only one arm of the signalling pathway (Figure 6.11).

The results described in this chapter are consistent with a model where sIg mediates a number of independently regulated signals with different functional consequences. The ratio of these signals appears to be dependent on the type of interaction between sIg and the surrogate antigen. In addition, the relative strengths of the antigen signals can be altered by a number of factors including Fc binding and the presence of cytokines such as IL-4. Further, the results suggest that "natural' antigens could also be able to induce a similar variation in sIg-mediated signals. If correct, this model represents a mechanism by which the B cell can regulate its behaviour so that the most appropriate response is generated for a particular antigen. A variable antigen signal would allow for a precise regulation of the B cell response. This hypothesis and its implications for B cell behaviour are discussed further in chapter 8.

Chapter 7

Sensitivity of B cell populations to multiple sIg-mediated signals

7.1. Introduction.

The results obtained in the previous chapter indicate that anti-Ig reagents initiate a number of sIg-mediated signals which have different effects on B cell behaviour. While antibodies such as the polyclonal gαIgM and monoclonal 187.1 appear to be able to induce both stimulatory and inhibitory effects on B cell proliferation, other anti-Ig reagents seem to predominantly either inhibit or stimulate ^{3}H-TdR incorporation (Figures 6.1, 6.5.1 and 6.5.2). Given the ability of AMS-9.1 and BET-2 to predominantly deliver a positive or negative signal for growth, respectively (Figures 6.1, 6.5.1 and 6.5.2), and the fact that they bind to IgD and IgM, it was possible to examine the effect of the two opposing sIg-mediated signals when delivered by independent sources. The ability to mix the ratio of the positive and negative signals could provide some insight into the relationship between the two signals, and how they jointly regulate B cell behaviour.

Further, as the results in chapters 5 and 6 indicated that the two sIg-mediated signals were independently regulated it was of interest to examine the response of B cells from CBA/N mice. B cells from these immunodeficient mice do not proliferate in response to mitogenic anti-Ig antibodies (Sieckmann *et al.,* 1978a, Sieckmann *et al.,* 1978b), and are therefore, presumably, unable to generate the positive sIg-mediated signal. However, it has not been determined as to whether or not CBA/N B cells are susceptible to the sIg-mediated inhibitory signal. If the two sIg-mediated signals are independent then it is possible that anti-Ig antibodies may be able to induce the negative signal in CBA/N B cells.

7.2. Methods

7.2.1. Electronic sorting of B cells

B cells were prepared as described in section 3.2.2, washed in cold PBS containing 0.1% BSA (0.1% BSA), and resuspended at approximately 10^5 cells/50 µl in cold 0.1% BSA. Hybridoma supernatant containing the anti-FcγRIIB antibody, 2.4G2, was added to the cells and incubated on ice for 30 minutes. The cells were then washed and resuspended in 0.1% BSA at approximately 10^5 cells/50 µl. B cells were then stained for sIgD and CD23 expression with the anti-IgD antibody, AMS15.1, coupled to PE and the anti-CD23 antibody, B3B4.1, directly conjugated to FITC, using dilutions of antibody which had previously been determined to be optimal. After a 30 minute incubation on ice, the cells were washed twice in cold 0.1% BSA and passed though 50 µm membrane, to exclude aggregated cells. The B cells were counted and resuspended at 5 x 10^6 cells/ml in 0.1% BSA All cell manipulations were performed under sterile conditions and the cells were stored on ice before and after sorting.

B cells were sorted using a FACStar plus flow cytometer (Becton and Dickenson, San Jose, CA). Cells were gated on forward and side scatter to distinguish the live cell population. Cells falling within the live cell gate were further divided into different populations based on sIgD and CD23 expression. The B cells were sorted into $sIgD^{hi}/CD23^{hi}$ and $sIgD^{lo}/CD23^{lo}$ populations. The selected cells were sorted into tubes containing 1 ml of HI FCS and stored on ice until sorting was completed. The cells were then washed in BCM, and the viable cell number determined by vital dye exclusion.

Antibodies used for electronic sorting

The AMS-15.1-PE conjugate was prepared and provided by Dr J. Hasbold (Centenary Institute, Sydney). Sn containing the anti-FcγRIIB antibody, 2.4G2 was prepared from a hybridoma (ATTC HB 197) . The anti-CD23 antibody B3B4.1 (Rao *et al.,* 1987) was a gift from Dr P Lalor (WEHI, Melbourne).

7.3. Results.

7.3.1. Anti-Ig reagents that induce opposing sIg-mediated signals stimulate B cell behaviour that can be approximated by the sum of the response to the two independent signals.

From the experiments described in chapters 5 and 6 it is clear that anti-Ig reagents can induce both positive and negative sIg-mediated signals. Although a number of antibodies are able to induce both signals, others seem to only induce a predominantly negative or positive signal (Figures 6.1, 6.5.1 and 6.5.2). Thus, it appeared that the two sIg-mediated signals could be delivered independently.

To experimentally test this possibility the effect of varying the ratios of the two signals on proliferation was determined. B cells were stimulated with different combinations of BET-2 and AMS-9.1 and the level of ^{3}H-TdR incorporation measured. This was done in the presence of LPS so that the influence of either signal could be more clearly seen (Figure 7.1). The effect of BET-2 and AMS-9.1 on LPS-induced proliferation has been described in chapter 6, and as previously observed BET-2 inhibited, whereas AMS-9.1 enhanced LPS-induced ^{3}H-TdR incorporation in a dose-dependent manner (Figure 7.1A). Figure 7.1, panels B and C, illustrates the effect of two different concentrations of BET-2 on the dose response curve for AMS-9.1 in the presence of LPS. As before, BET-2 inhibited proliferation, however, the response was not equivalent to that obtained with BET-2 and LPS alone. Thus, while the level of ^{3}H-TdR incorporation was substantially reduced by BET-2, the positive effect of high concentrations AMS-9.1 was still apparent (Figure 7.1B and C). In a similar manner, the inclusion of a high concentration of AMS-9.1 increased the level of ^{3}H-TdR incorporation in cultures where BET-2 was titrated in the presence of LPS (Figure 7.1D).

One possible explanation for the results illustrated in Figure 7.1 was that the inhibitory and stimulatory dose response curves represented the behaviour of different B cell populations. Previously it has been shown that distinct B cell populations differ in their response to various stimuli. Separation of splenic B cells on the basis of size into large and small B cells, has produced B cell populations which respond differently to anti-Ig reagents and lipoprotein (Peçanha *et al.,* 1991, Snapper *et al.,* 1995b). Furthermore, sorting B cells based on their expression of sIgM or CD23 has resulted in populations that have different responses to LPS and mitogenic mAbs (Waldschmidt *et al.,* 1992, Snapper *et al.,* 1993, Oliver *et al.,* 1997). Thus, a hypothesis was formed in which the effects of BET-2 and AMS-9.1 were independent and operated on distinct B cell populations. This hypothesis predicts that although both populations respond to LPS

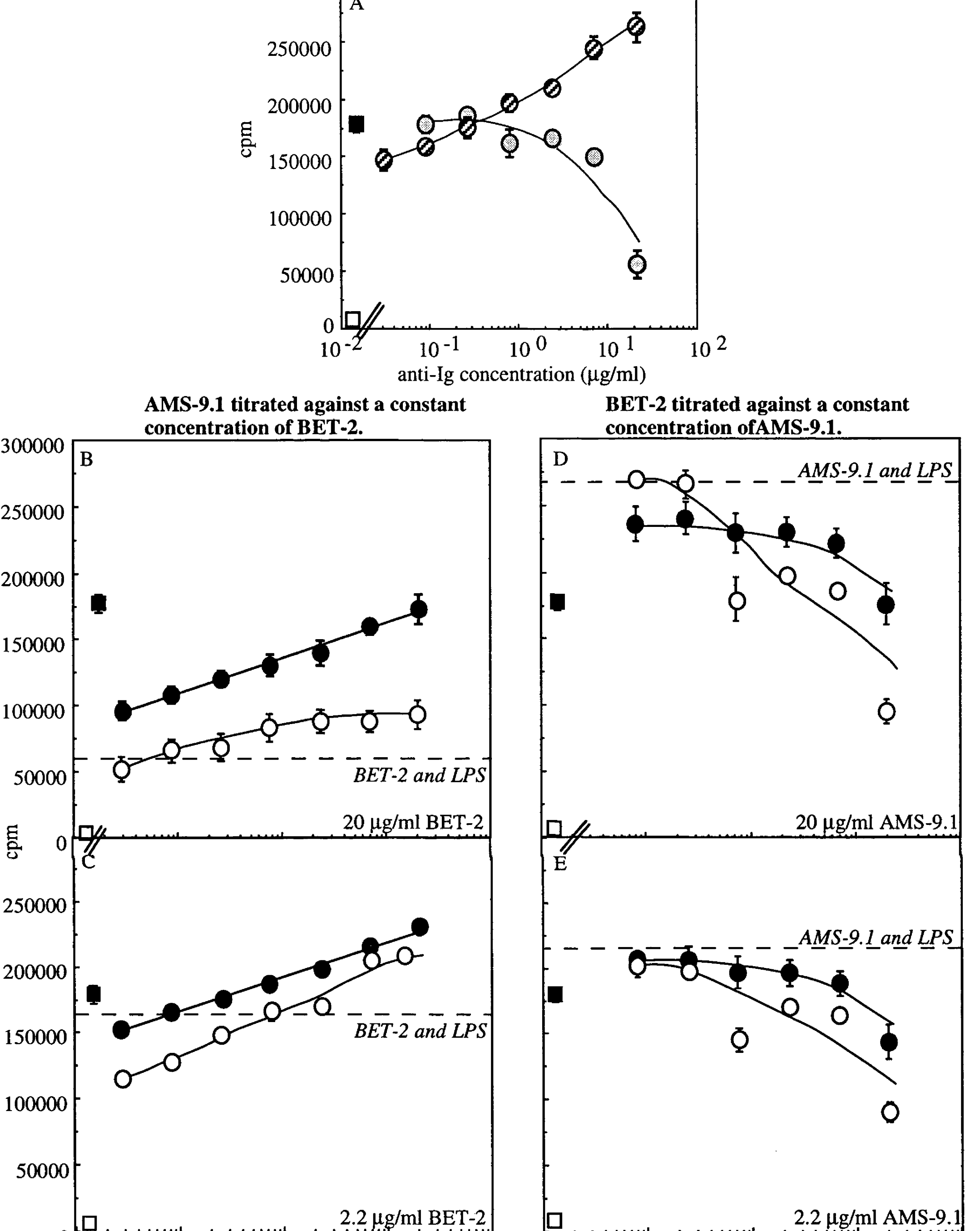

Figure 7.1. **The combined effect of different anti-Ig reagents on LPS-induced B cell proliferation may be approximated by the sum of the response to each antibody.**

B cell were prepared as described in chapter 3 and cultured in BCM (5 x 10^4 cells/ 200 μl well) with AMS-9.1 (⊘) or BET-2 (◍) in combination with 50 μg/ml LPS (panel A). Additional cultures were also prepared containing LPS (50 μg/ml) and a fixed concentration of one antibody (lower right corner), titrated against a second antibody (O). After 3 days the level of proliferation was determined by ^{3}H-TdR incorporation assay, as described in chapter 3. The data illustrated represents the mean of triplicate cultures ± one standard error. The second set of dose response curves represent the sum of the response to AMS-9.1 and BET-2 as illustrated in panel A (●). Background proliferation: cells only (□), LPS only (■), and LPS in combination with either AMS-9.1 or BET-2 at the concentration which appears in the lower right corner of panels B-E (— — —).

one will be insensitive to BET-2 inhibition but enhanced by AMS-9.1, whereas the other population would be inhibited by BET-2 and unaffected by AMS-9.1. Therefore, if correct the behaviour of cells stimulated with a combination of BET-2 and AMS-9.1 would represent the sum of the response of distinct B cell populations. Following this prediction the effect of combining the two sIg-mediated signals was determined arithmetically by adding means and dividing by two. The dose response curves generated in this way are illustrated in Figure 7.1, panels B-E, and reveal that this treatment of the data generated dose response curves that were a fair approximation of those obtained experimentally. The similarity between the experimentally and arithmetically derived dose response curves provides support for the hypothesis that the response represented the sum of the behaviour of different B cell populations and justified an attempt to explore this issue experimentally. This hypothesis was examined in two ways Initially B cells were separated into different populations as described in sections 3.3.2 and 7.2.1. The second approach was to examine the response of B cells from CBA/N mice. The B cell population from these immunodeficient is dominated by the sIgD^{lo} population, whereas the sIgD^{hi} population is virtually absent (Scher 1982a, Hardy *et al.,* 1983). In each case the B cell response to anti-Ig reagents alone or in combination with IL-4, LPS and CsA was examined.

7.3.2. The response of small and large B cells to anti-Ig reagents imply that the two populations differ in their sensitivity to the different sIg-mediated signals.

B cells were separated by percoll purification into large and small B cell populations and the effect of anti-Ig reagents on proliferation determined (Figure 7.2). As previously described for unseparated B cell cultures, different anti-Ig antibodies varied in their ability to induce or inhibit proliferation (Figures 6.1 and 6.2) with similar differences observed in small and large B cell cultures (Figure 7.2). In addition, the effect of each antibody on small or large B cell proliferation was consistent with the response induced in unseparated B cell cultures. However,, there were some differences between the responses of the two populations. As illustrated in Figure 7.2, mitogenic anti-Ig antibodies induced a greater proportional increase in ^{3}H-TdR incorporation in cultures containing small B cells, than in large B cell cultures. Thus, high doses (5-20 μg/ml) of 187.1-induced an increased ^{3}H-TdR incorporation over the background level in cultures containing small B cells (Figure 7.2A). In contrast 187.1 failed to induce proliferation above the background in large B cell cultures (Figure 7.2A). A similar pattern of proliferation was obtained with B7.6, where in response to high doses of B7.6 small B cells incorporated ^{3}H-TdR above the

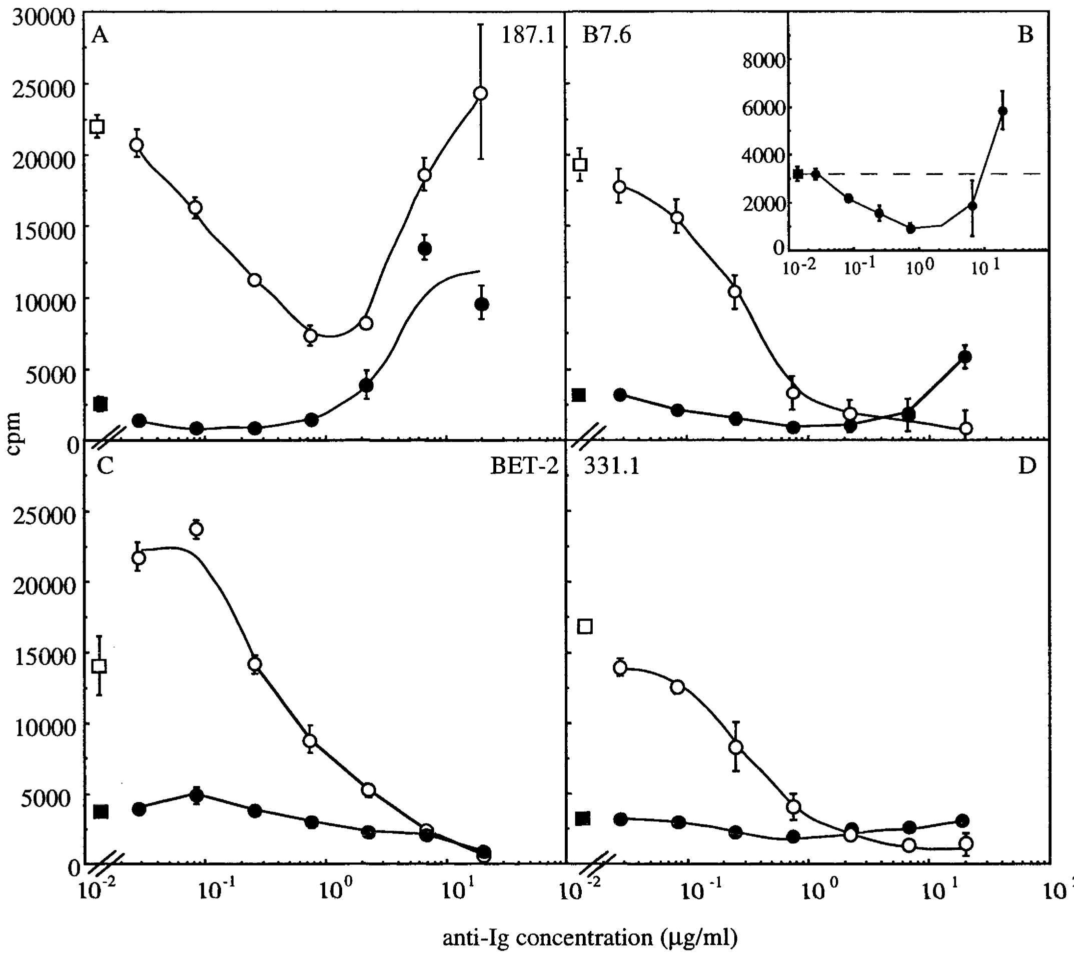

Figure 7.2. **Anti-Ig induced proliferation in small and large percoll purified B cells .**

Large (O) and small (●) percoll purified B cells were prepared as described in section 3.2 and cultured in BCM ($5x10^4$ cells/ 200 μl well) containing varying concentrations of different anti-Ig reagents and 2.4G2 (30 μg/ml). After 3 days the level of proliferation was determined by a ^{3}H-TdR incorporation assay as previously described in chapter 3. Background proliferation: large cells (□), and small cells (■), Each point represents the mean of triplicate cultures ± one standard error. Inset panel B illustrates the detail of the small B cell response to B7.6.

background, albeit weakly (Figure 7.2B). In addition, it is of interest to note that separation of splenic B cells into small and large populations revealed that cultures containing large B cells had a high background rate of proliferation when compared to small B cells. This observation suggests that the high background for proliferation seen in unseparated B cell cultures (Chapters 4 to 6) was due to ^{3}H-TdR incorporation by the large less dense B cells present in these cultures.

In contrast to 187.1 and B7.6, stimulation with BET-2 and 331.1 did not induce proliferation in either B cell population, but rather inhibited the background level of ^{3}H-TdR incorporation of the large B cells (Figure 7.2C and D). In cultures containing small B cells BET-2 inhibited background proliferation, whereas 331.1 had little appreciable effect (Figure 7.2C and D). Inhibition of the background was also apparent at low doses (<1 μg/ml) of 187.1 in both large and small B cell cultures (Figure 7.2A). In addition, B7.6-induced a dose-dependent inhibition of ^{3}H-TdR incorporation in large B cell cultures, and at low doses (< 7 μg/ml) inhibited the proliferation of small B cells (Figure 7.2D inset). These results suggest that small B cells may be more sensitive to the positive signal for growth than the large B cells. Conversely the large B cells appear to be more sensitive to anti-Ig mediated inhibition of the background proliferation.

7.3.3. IL-4 is more effective at enhancing the sIg-mediated positive signal in the small B cells than in large B cells.

The addition of IL-4 to small and large B cell cultures containing 187.1 or B7.6 enhanced the mitogenic signal delivered by these mAbs (Figure 7.3A and B, respectively). The positive effect of IL-4 on 187.1-induced proliferation was apparent on both small and large B cells, and generated almost identical dose response curves which were off set by an approximately three fold difference in antibody concentration (Figure 7.3A). As the proliferative response of small B cells was initiated at lower antibody concentrations it appeared that these cells were more sensitive to the positive signal for growth than the large B cells. Another feature of the response to 187.1 and IL-4 was that in large B cell cultures moderate doses (~1 μg/ml) of 187.1-induced a weak inhibition of proliferation (Figure 7.3A). The apparent greater sensitivity of small B cells to mitogenic anti-Ig antibodies was also evident in the response B7.6. Stimulation of small B cells with B7.6 and IL-4 resulted in a dose-dependent increase in proliferation, whereas in large B cell cultures low doses (< 1 μg/ml) of B7.6 inhibited the proliferation to approximately 50% of the background (Figure 7.3B). However,, at higher concentrations of B7.6 the level of ^{3}H-TdR incorporation increased but remained below the background (Figure 7.3B). In contrast to 187.1 and B7.6, the addition of IL-4 did not result in B cell proliferation in response to BET-2 or 331.1, nor did it prevent

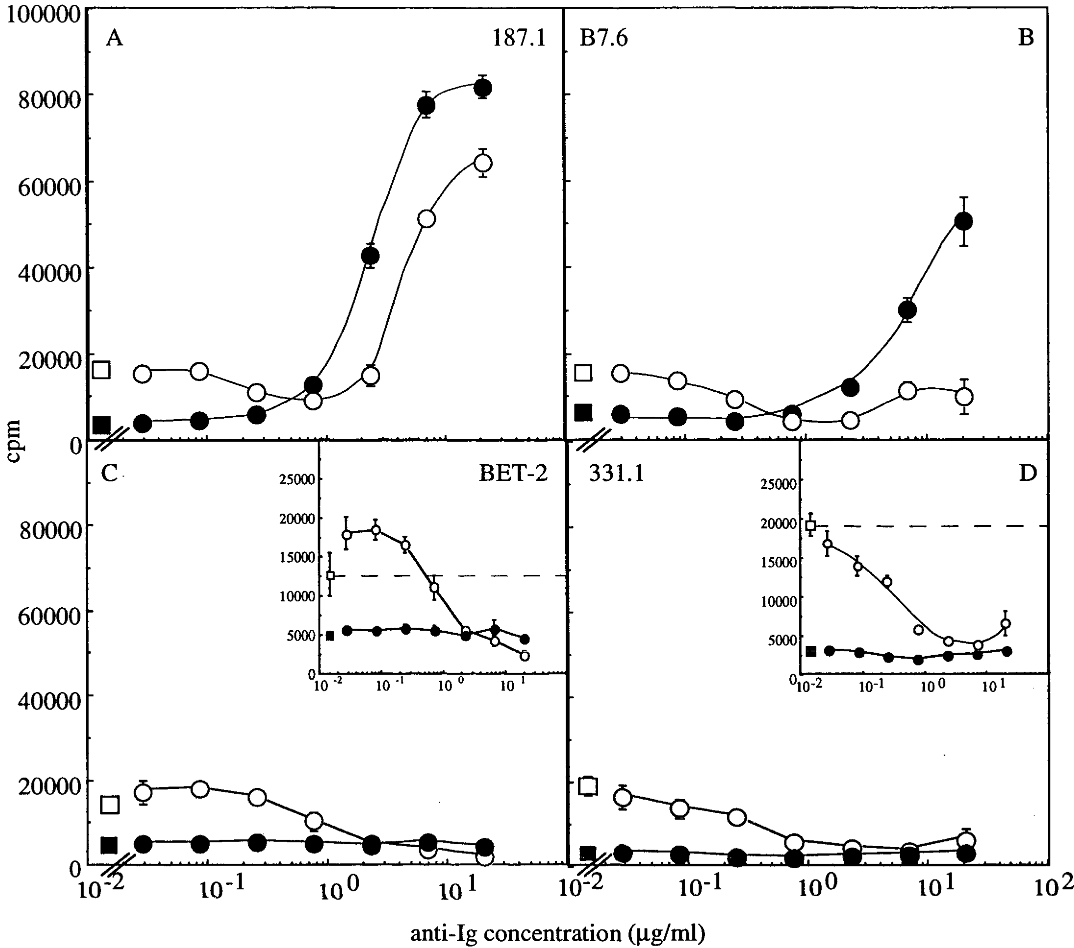

Figure 7.3. **Variation in the anti-Ig induced proliferative response of small and large percoll purified B cells in the presence of IL-4.**

Large (O) and small (●) percoll purified B cells were prepared as described in section 3.2 and cultured in BCM ($5x10^4$ cells/ 200 μl well) containing varying concentrations of anti-Ig reagents, 2.4G2 (30 μg/ml) and IL-4 (100 U/ml). After 3 days the level of proliferation was determined by a ^{3}H-TdR incorporation assay as described in chapter 3. Background proliferation: large cells and IL-4 (□), small cells and IL-4 (■). The data illustrated represents the mean of triplicate cultures, ± one standard error. Insets in panels C and D illustrate the detail of the B cell response to BET-2 and 331.1, respectively.

the inhibition of the background proliferation of large B cells by these antibodies (Figure 7.3C and D). Surprisingly the highest dose of 331.1-induced a slight increase in ^{3}H-TdR incorporation in large B cells (Figure 7.3D, inset). The results obtained with 331.1 in this experiment differ from those described previously (Figure 6.1). The differences in the two dose response curves could be attributed to 2.4G2 which was present in the small and large B cell cultures (Figure 7.3), but not in the unseparated B cell cultures (Figure 6.1). The results indicate that the effect of IL-4 was to enhance the sIg-mediated positive signal for growth without influencing the inhibitory signal, as previously described (Figures 6.1 and 6.11).

Combined, the results illustrated in Figures 7.2 and 7.3 are approximately consistent with the hypothesis that the inhibitory and stimulatory dose response curves represent the behaviour of different B cell populations. These results suggest that small B cells are more sensitive to the positive signal for growth, whereas large B cells appear to have a higher sensitivity to the sIg-mediated inhibitory signals. Alternatively the large B cells may have a lower sensitivity to the mitogenic signal which could allow the inhibitory signal to dominate the response. However, is clear that there is considerable overlap in the behaviour of the two populations which, if the hypothesis is correct, may reflect a lack of purity of the two putative B cell populations. Having determined the pattern of behaviour of these two populations of B cells in response to stimulation with anti-Ig antibodies in the presence and absence of IL-4, the effect of these reagents on LPS-induced proliferation was examined.

7.3.4. In combination with LPS anti-Ig reagents-induce very similar dose response curves for small and large B cell proliferation.

The proliferative response of large and small B cells to anti-Ig antibodies in the presence of LPS is illustrated in Figure 7.4. In this experiment the two B cell populations had an almost identical response to LPS alone, and generated very similar dose response curves following culture with LPS and various anti-Ig reagents. The dose response curves illustrated in Figure 7.4 were consistent with the results obtained with the same antibodies in unseparated B cell cultures (Figures 6.3, 6.5.1 and 6.5.2). Thus, as previously described (Figure 6.3) 187.1 and gαIgM-induced dose response curves which provided evidence of inhibitory and stimulatory effects, whereas BET-2 only induced an inhibitory effect on proliferation (Figure 7.4, panels A, E and C respectively). However, while 331.1 generated a similar response in small and large B cells, the dose response curve differed somewhat to that described for unseparated B cell cultures (Figure 6.3). Previously co-culture with 331.1 and LPS resulted in a profound inhibition of proliferation (Figure 6.3), whereas in this experiment 331.1 only induced a

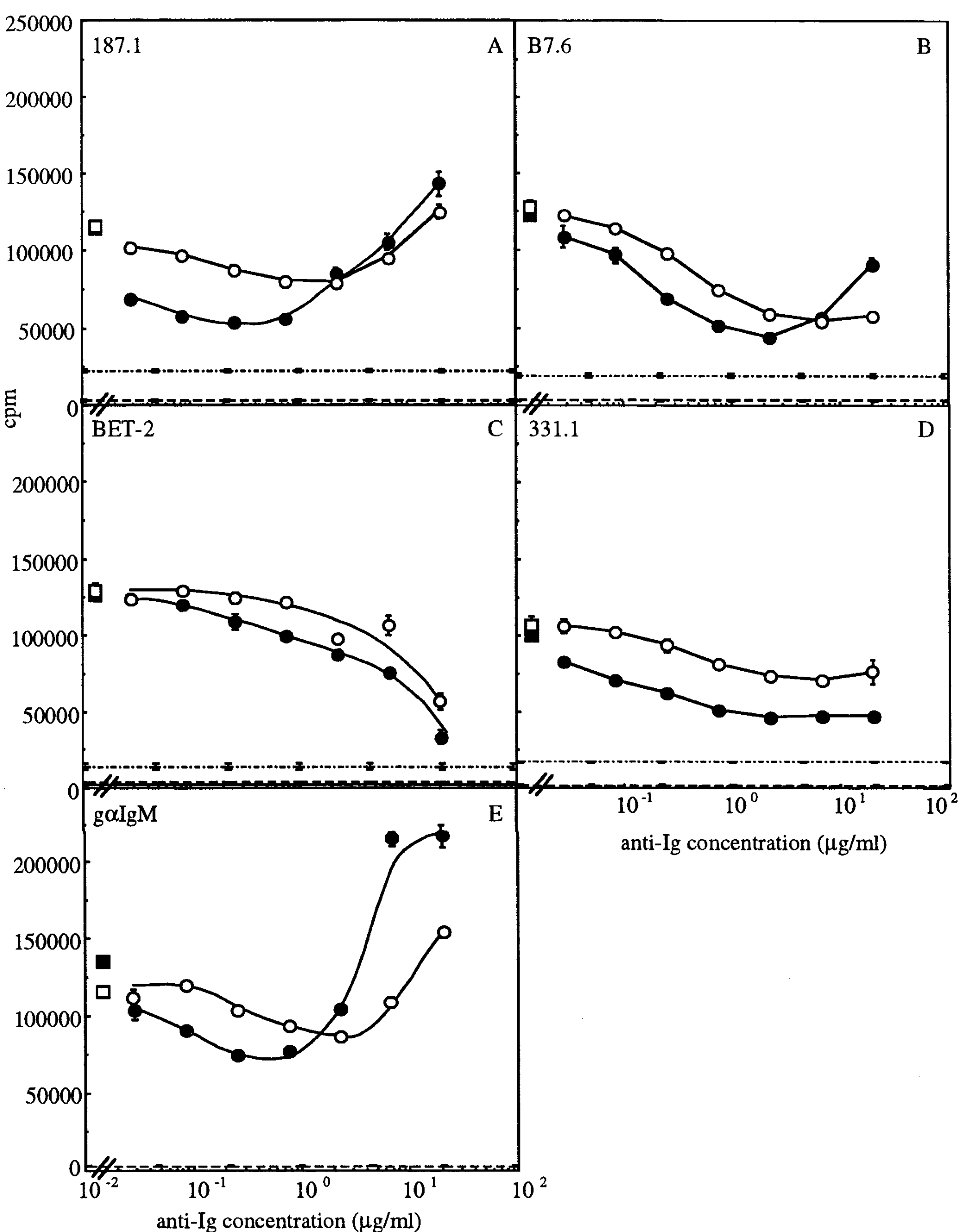

Figure 7.4. **The influence of anti-Ig reagents on LPS-induced proliferation of small and large percoll purified B cells.**

Large (O) and small (●) percoll purified B cells were prepared as described in section 3.2, and cultured in BCM ($5x10^4$ cells/ 200 μl well) containing varying concentrations of different anti-Ig reagents, 2.4G2 (30 μg/ml) and LPS (50 μg/ml). After 3 days the level of proliferation was measured by ^{3}H-TdR incorporation as described in chapter 3. Background proliferation: LPS only large cells (□), small cells (■). Cells only - large (·······) and small (- - - -). The data illustrated represents the mean of triplicate cultures ± one standard error.

mild inhibition which did not continue to increase with antibody concentration at doses greater than 2 μg/ml (Figure 7.4D). The decline in the severity of inhibition by 333.1 could have been due to the presence of 2.4G2 in these cultures. Finally although each B cell population appeared to be susceptible to the stimulatory or inhibitory sIg-mediated signal, the results suggest that small B cells are more sensitive to both sIg-mediated signals than large B cells. This result is in contrast to those illustrated in Figures 7.2 and 7.3 which suggest that large B cells are more sensitive to the inhibitory sIg-mediated signals.

7.3.6. Electronic sorting of B cells into two distinct B cell populations

As discussed previously, the anti-Ig induced dose response curves could have been affected by the purity of the percoll purified B cell populations. Thus, in these cultures it is possible that the dose response curve obtained for one population was influenced by contaminating cells from the other population. This may be particularly important when examining differences in proliferation in the presence of a potent mitogen like LPS. Given the differences in the response of percoll purified small and large B cells it appeared possible that the variation in the response might be more clearly defined in highly purified B cell populations defined by cell surface phenotype. Snapper *et al.,* (1993) have reported that B cells which express $sIgM^{lo}/sIgD^{hi}/CD23^{hi}$ proliferate more vigorously in response to anti-δ– or anti-μ-dextran than B cells with a $sIgM^{hi}/sIgD^{lo}/CD23^{lo}$ phenotype. In contrast, B cells which express low levels of CD23 B cells have been shown to be more responsive to LPS than $CD23^{hi}$ B cells (Snapper *et al.,* 1993, Oliver *et al.,* 1997). As the small B cell population has been shown to principally consist of B cells which are $sIgM^{lo}/sIgD^{hi}$, and the large B cell fraction to be dominated by $sIgM^{hi}/sIgD^{lo}$ B cells (Bartell and Hodgkin, unpublished data), it was feasible that the variation in the response of large and small B cell populations reflected a difference in the behaviour of $sIgM^{lo}/sIgD^{hi}$ and $sIgM^{hi}/sIgD^{lo}$ B cells. To determine if the variation in the response to anti-Ig reagents became more obvious when comparing purified B cell populations, splenic B cells were electronically sorted into $sIgD^{hi}/CD23^{hi}$ and $sIgD^{lo}/CD23^{lo}$ B cell populations and their response to anti-Ig antibodies examined.

From the results obtained in chapter 6 it was clear that some anti-Ig reagents can alter B cell proliferation. As the aim of this experiment was to determine the effect of anti-Ig antibodies on the proliferative response of $sIgD^{hi}/CD23^{hi}$ and $sIgD^{lo}/CD23^{lo}$ B cells it was necessary to use an antibody which would not influence B cell proliferation. AMS-15.1 was chosen, as this antibody had been shown to have little effect on B cell proliferation (Figures 6.1 and 6.3). B cells were sorted into $sIgD^{hi}/CD23^{hi}$ and $sIgD^{lo}/CD23^{lo}$, as illustrated in Figure 7.5. Post-sort analysis revealed that 82% of the

$sIgD^{hi}/CD23^{hi}$ and 85% of the $sIgD^{lo}/CD23^{lo}$ fell within the sort gates, and that the two populations did not overlap (Figure 7.5B and C). When the two populations were examined on the basis of sIgD expression alone the purity of the $sIgD^{lo}$ and $sIgD^{hi}$ B cell populations was greater than 95% (Figure 7.5D and E). Thus, the sorted B cell populations have a high degree of purity but maybe more appropriately described as $sIgD^{hi}$ and $sIgD^{lo}$. Following sorting the $sIgD^{hi}$ and $sIgD^{lo}$ B cells populations were placed in culture with anti-Ig antibodies in combination with IL-4 or LPS and the level of ^{3}H-TdR incorporation determined after 2.5 days (Figures 7.6 and 7.7).

7.3.7. Anti-Ig reagents induce different patterns of proliferation in $sIgD^{hi}$ and $sIgD^{lo}$ B cell populations

As previously described, the B cell response to mAbs was dependent on the antibody used and its concentration and as illustrated in Figure 7.6 the response was also determined by the B cell population. Although the background proliferation of both $sIgD^{lo}$ and $sIgD^{hi}$ B cells was inhibited to varying degrees in response to a low dose of each of the mAbs, the pattern of proliferation differed between antibodies when used at high doses (Figure 7.6A and B). Thus, the high dose of B7.6 or AMS-9.1 induced an increase in the level of ^{3}H-TdR incorporation by approximately two fold over the background in cultures containing $sIgD^{hi}$ B cells, whereas the same dose of 187.1 or 331.1 did not induce proliferation (Figure 7.6A). In the cultures containing $sIgD^{lo}$ B cells only a high dose of AMS-9.1 was able to induce proliferation above the background (Figure 7.6B). Stimulation with the 331.1, B7.6 or 187.1 inhibited the background proliferation of $sIgD^{lo}$ B cells (Figure 7.6B). As the $sIgD^{hi}$ B cells predominantly represent small B cells, and the $sIgD^{lo}$ B cells characterise large B cells (Bartell and Hodgkin, unpublished data), these results are in agreement with those described in Figure 7.2, which indicate that small B cells are more sensitive to the positive sIg-mediated signal.

The inclusion of IL-4 in cultures containing $sIgD^{hi}$ or $sIgD^{lo}$ B cells enhanced anti-Ig induced proliferation, as previously described (Figures 7.3, 6.1 and 6.2). However, the effect of IL-4 on anti-Ig induced proliferation was considerably more dramatic in cultures containing $sIgD^{hi}$ B cells (Figure 7.6C). In these cultures the combination of IL-4 and the high dose of B7.6, 187.1 or AMS-9.1 resulted in a more than ten fold increase in ^{3}H-TdR incorporation over that obtained with antibody or IL-4 alone (Figure 7.6). While the enhancing affect of IL-4 on proliferation was also observed in cultures containing $sIgD^{lo}$ B cells, its influence was much weaker. Thus, stimulation of $sIgD^{lo}$ B cells with a high dose of 187.1 or AMS-9.1 in combination with IL-4 caused a two fold increase in ^{3}H-TdR incorporation over the background (Figure 7.6D). So far, the

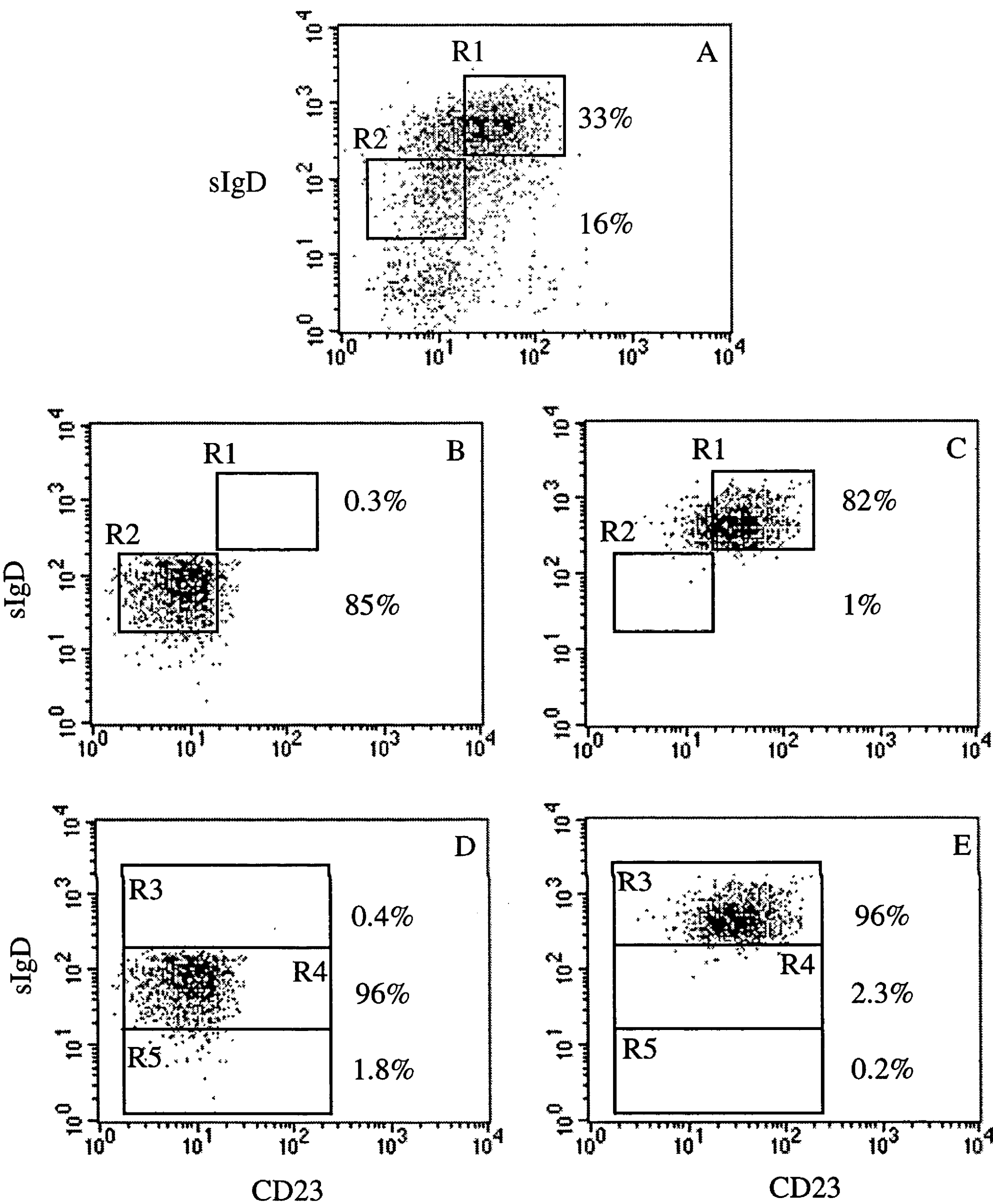

Figure 7.5. **Purity of B cell populations following electronic sorting based on sIgD and CD23 expression.**

Spleen cells were prepared form CBA/H mice and depleted of T cells as described in section 3.2. These cells were then stained for sIgD and CD23 expression as outlined in section 7.2.1, gated on live cells and sorted into two populations $sIgD^{lo}/CD23^{lo}$(R2 - panel B) and $sIgD^{hi}/CD23^{hi}$ (R1 - panel C), Panels D and E illustrate the purity of the two populations when assessed on sIgD expression alone. Finally panel A shows the expression of sIgD and CD23 on live T cell depleted spleen cells prior to sorting.

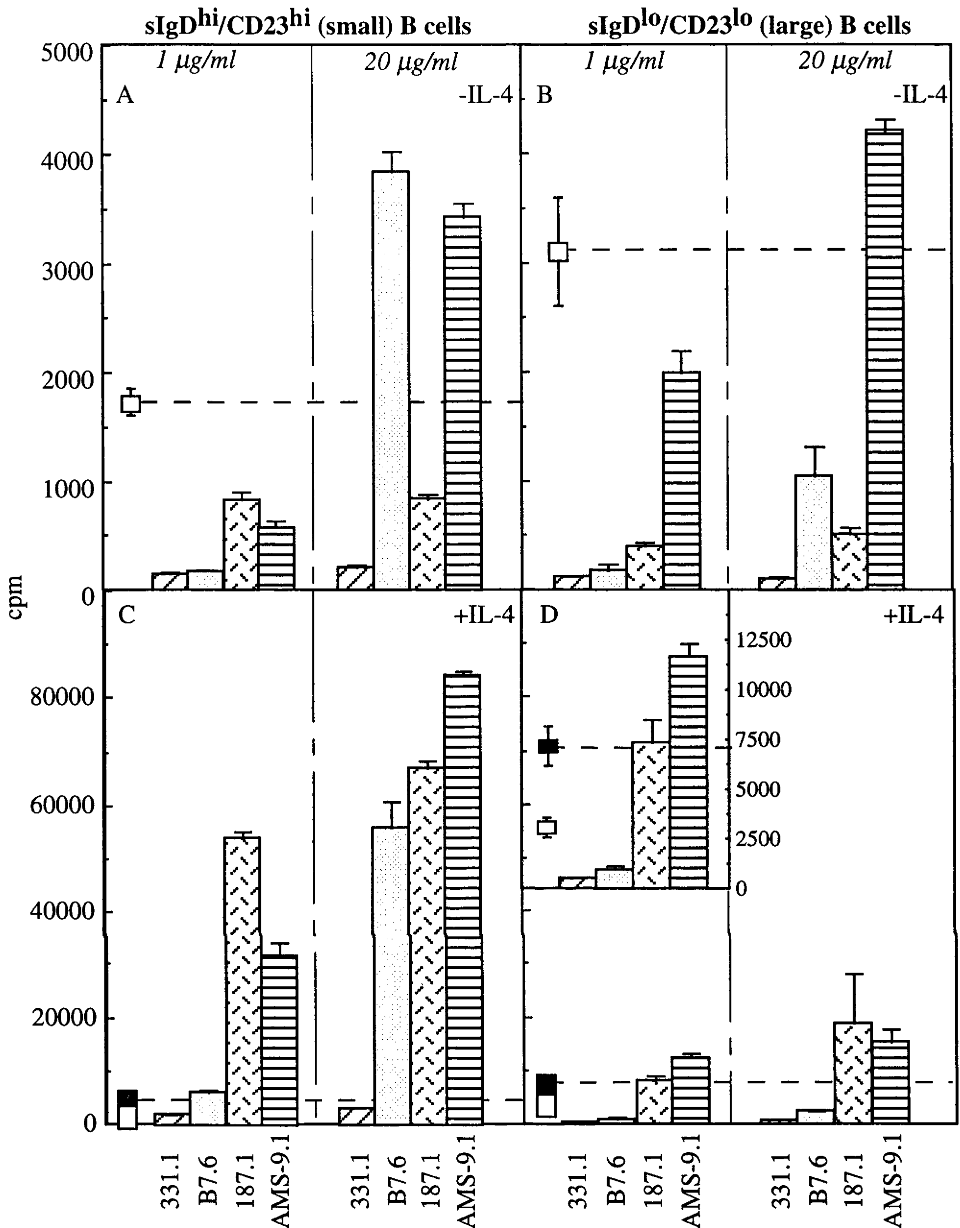

Figure 7.6. **sIgD^{hi}/CD23^{hi} and sIgD^{lo}/CD23^{lo} B cells differ in their proliferative response to anti-Ig reagents, irrespective of the presence of IL-4.**

B cells were purified by flow cytometry based on sIgD and CD23 expression into IgD^{hi}/CD23^{hi} and IgD^{lo}/CD23^{lo} B cell populations as described in section 7.2.1. These cells were cultured in BCM (4 x 10^4 cells/ 200 μl well) containing different anti-Ig antibodies alone (panels A and B), or in combination with 100 U/ml IL-4 (panels C and D). After 60 hours the cells were pulsed with ^{3}H-TdR to determine the level of proliferation as described in chapter 3. The data illustrated represents the mean of triplicate cultures ± one standard error. Background proliferation: cells only (□) and IL-4 only (■). Inset in panel D illustrates the detail of the response of IgD^{lo}/CD23^{lo} B cells to 1 μg/ml of 331.1, B7.6, 187.1 and AMS-9.1 in the presence of IL-4.

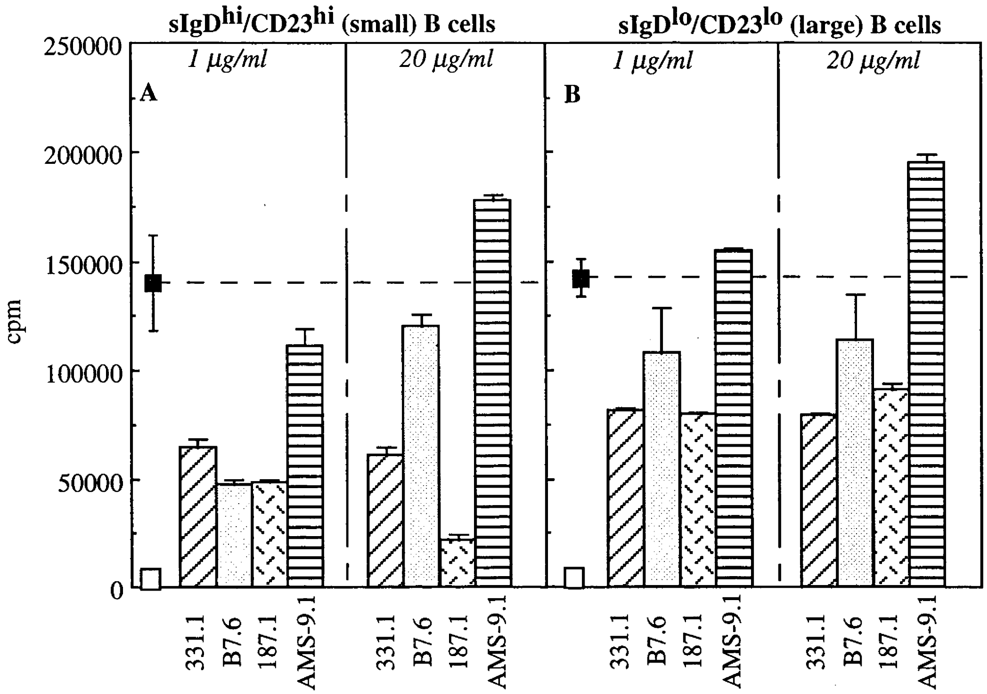

Figure 7.7. **Anti-Ig antibodies have a similar effect on LPS-induce proliferation of both sIgD^{hi}/CD23^{hi} and sIgD^{lo}/CD23^{lo} B cells .**

B cells were purified by flow cytometry based on sIgD and CD23 expression into IgD^{hi}/CD23^{hi} and IgD^{lo}/CD23^{lo} B cell populations as described in the section 7.2.1. These cells were cultured in BCM (4 x 10^4 cells / 200 μl well) containing different anti-Ig antibodies and LPS (50 μg/ml). After 60 hours the cells were pulsed with ^{3}H-TdR to determine the level of proliferation as described chapter 3. The data illustrated represents the mean of triplicate cultures ± one standard error. Background proliferation: LPS only (■) and cells only (□).

results obtained with highly purified IgD^{hi} and IgD^{lo} B cell populations mirror the results described for small and large B cells (Figures 7.2 and 7.3), indicating that while the two B cell populations are susceptible to both sIg-mediated signals that they differ in their sensitivity to these signals.

7.3.8. Anti-Ig antibodies have similar affects on LPS-induced proliferation of $sIgD^{hi}$ and IgD^{lo} B cells

In contrast to the differences in the response of $sIgD^{hi}$ and IgD^{lo} B cells illustrated in Figures 7.5 and 7.6, in the presence of LPS the two populations behaved in a similar manner (Figure 7.7). Thus, $sIgD^{hi}$ and IgD^{lo} B cells incorporated equivalent amounts of ^{3}H-TdR in response to stimulation with LPS alone, and the inclusion of 331.1, B7.6 or 187.1 to these cultures inhibited the LPS-induced proliferation of both B cell populations (Figure 7.7A and B). Only the response to AMS-9.1 differed from this inhibitory pattern, as the addition of a high dose of AMS-9.1 enhanced LPS-induced proliferation (Figure 7.7A and B). When the two B cell populations were compared the increase in LPS-induced ^{3}H-TdR incorporation in response to AMS-9.1 was slightly higher in cultures containing IgD^{lo} B cells (Figure 7.7A and B). These results, and those described in Figure 7.6, indicate that both B cell populations were susceptible to the stimulatory and inhibitory affects of anti-Ig reagents. Although there appear to be some differences in the sensitivity of the two populations to the sIg-mediated signals in cultures containing anti-Ig alone, or anti-Ig and IL-4, in the presence of LPS the overall response of the both $sIgD^{hi}$ and IgD^{lo} B cells was remarkably similar (Figure 7.7).

7.3.9. The response of B cells from CBA/N mice

In addition to examining the anti-Ig induced behaviour of different B cell populations, it was of interest to determine the response of B cells from CBA/N mice to these antibodies. In CBA/N mice B cell development is impaired which results in a decline in the number of $sIgD^{hi}$ B cells (Hardy *et al.,* 1983) and the loss of the $CD5^{+}$ B cell population (Hayakawa *et al.,* 1986). Thus, the B cell compartment is principally made up of $sIgM^{hi}/sIgD^{lo}$ B cells (Scher 1982a, Hardy *et al.,* 1983). The results illustrated in Figure 7.6 indicate that anti-Ig reagents alone or in combination with IL-4 can induce proliferation in $sIgD^{lo}$ B cells from normal mice. However, similar stimulation of CBA/N B cells does not induce proliferation (Sieckmann *et al.,* 1978a, Sieckmann *et al.,* 1978b). Despite the absence of proliferation in response to mitogenic antibodies, anti-Ig stimulation of B cells from CBA/N mice has been shown to induce the up-regulation of MHC class-II (Hawrylowicz *et al.,* 1984) and an increase in cell size (DeFranco *et al.,* 1982a). Thus, sIg on CBA/N B cells can clearly activate intracellular signalling pathways. However, the extent of activation of these signalling pathways

appears to be reduced in CBA/N B cells when compared to B cells from normal mice (Rigley *et al.,* 1989, Lindsberg *et al.,* 1991). Given the impaired sIg-mediated signalling ability in CBA/N B cells, it was of interest to determine if anti-Ig reagents could induce the sIg-mediated negative signal which was proposed in chapters 5 and 6 to inhibit LPS-induced B cell proliferation and differentiation.

LPS-induced proliferation is impaired in B cells from CBA/N mice

Initially LPS-induced proliferation of B cells from CBA/H and CBA/N mice were compared (Figure 7.8). While B cells from both mouse strains proliferated in response to LPS, B cells from CBA/N mice incorporated less ^{3}H-TdR at each dose of LPS than CBA/H controls (Figure 7.8). In addition, the concentration of LPS required to induce proliferation above the background was higher in CBA/N B cells than in the controls. These results suggest that the two B cell populations differ in their sensitivity to LPS. The lower level of LPS-induced proliferation in B cells from CBA/H mice when compared to normal controls has been previously observed by Scher *et al.,* (1975) and Klaus *et al.,* (1986) (Scher *et al.,* 1975, Klaus *et al.,* 1986).

Anti-Ig antibodies inhibit LPS-induced proliferation of B cells from CBA/N mice

Having determined the response of B cells from CBA/N mice to LPS, the influence gαIgM on proliferation was examined. Stimulation of B cells with gαIgM and IL-4 induced proliferation in B cells from CBA/H mice, as previously described (Chapter 6), but did not induce proliferation in B cells from CBA/N mice (Figure 7.9B). The inability of mitogenic anti-Ig reagents to induce proliferation of CBA/N B cells was consistent with the results previously obtained by Sieckmann *et al.,* (1978a and b). When combined with LPS, gαIgM-induced a U shaped dose response curve in the control B cells (Figures 7.9C, 5.4 and 6.3). In contrast, in cultures containing B cells from CBA/N mice, gαIgM inhibited LPS-induced proliferation but did not produce an increase in ^{3}H-TdR incorporation at high doses of antibody (Figure 7.9C). Inhibition of LPS-induced proliferation by gαIgM inhibition was dose-dependent up to 2 μg/ml of antibody, however, at higher antibody concentrations the level of proliferation formed a plateau (Figure 7.9C), generating a dose response curve which was similar to that obtained for 187.1 and LPS (Figure 6.3). Finally in the presence of CsA, gαIgM inhibited LPS-induced proliferation of the B cells from both mouse strains (Figure 7.9D). Thus, while gαIgM was unable to induce ^{3}H-TdR incorporation in B cells from CBA/N mice, it was able to inhibit LPS-induced proliferation in these B cells.

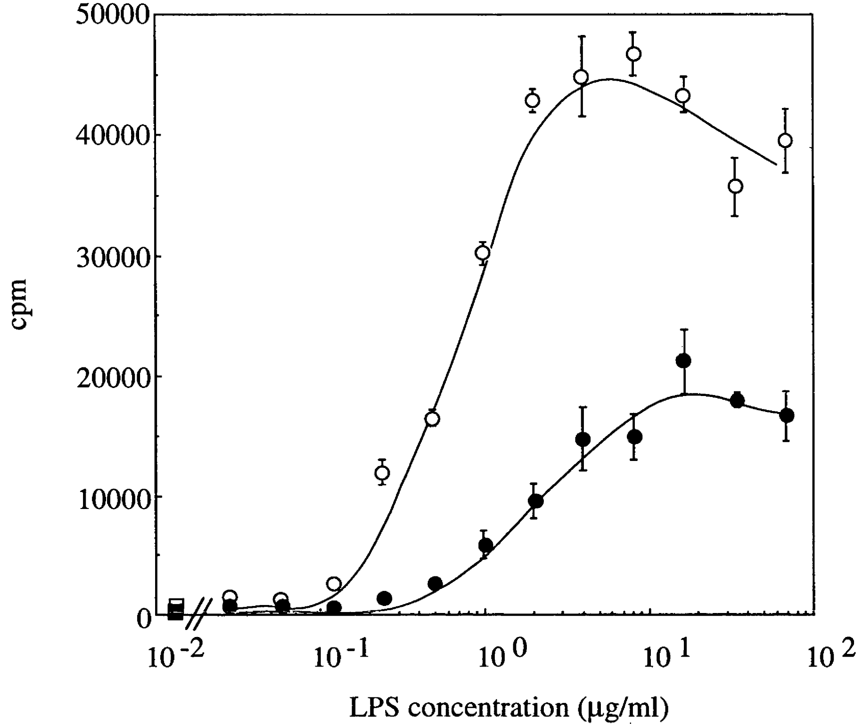

Figure 7.8. **LPS-induced proliferation in B cells from CBA/H and CBA/N mice.**

B cells were prepared from either CBA/H (O) or CBA/N (●) mice as described in section 3.2 and cultured in BCM (5 x 10^4 cells / 200 μl well) containing various concentration of LPS. After 3 days the level of proliferation was determined by ^{3}H-TdR incorporation assay as described in chapter 3. The data illustrated represents the mean of triplicate cultures, ± one standard error. Background proliferation: CBA/H cells only (□) and CBA/N cells only (■).

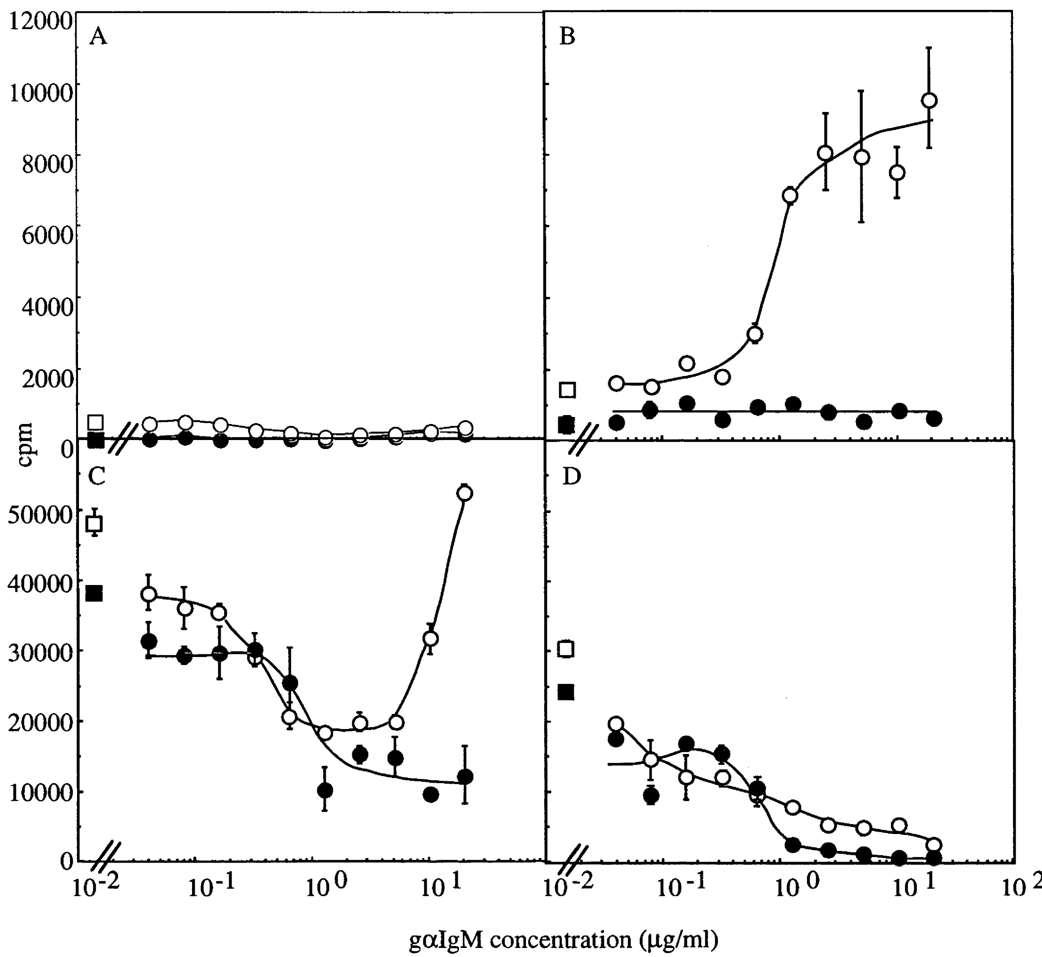

Figure 7.9. **GαIgM inhibits LPS-induced proliferation of CBA/N B cells but fails to deliver a detectable positive sIg-mediated signal.**

B cells were prepared from either CBA/H (O) or CBA/N (●) mice as described chapter 3 and cultured in BCM (5 x 10^4 cells/ 200 μl well) which contained varying concentrations gαIgM alone (panel A) or in combination with: 100 U/ml IL-4 (panel B); 50 μg/ml LPS (panel C); or LPS and 40 ng/ml CsA (panel D). After 3 days the level of proliferation was determined by ^{3}H-TdR incorporation assay as described in chapter 3. The data illustrated represents the mean of triplicate cultures ± one standard error. Background: panel A - cells only CBA/H (□) and CBA/N (■); panel B - IL-4 only CBA/H (□) and CBA/N (■); panel C - LPS only CBA/H (□), and CBA/N (■); panel D - LPS and CsA CBA/H (□) and CBA/N (■). Inset in panel A illustrates the detail of the gαIgM-induced response of B cells from CBA/H and CBA/N mice.

LPS-induced differentiation of CBA/N B cells is inhibited by gαIgM

The ability of gαIgM to inhibit LPS-induced CBA/N B cell responses was also apparent when the ASC response was examined (Figure 7.10). While the dose response curves for LPS-induced differentiation into ASCs was very similar between the two strains of mice, cultures containing B cells from CBA/N mice generated fewer ASCs (Figure 7.10A). The low number ASCs obtained from CBA/N B cell cultures, when compared to cultures containing CBA/H B cells, is consistent with the view that CBA/N B cells are less sensitive to LPS. The addition of CsA to these cultures did not significantly alter the number of ASCs obtained from cultures containing B cells from CBA/N mice, whereas the addition of CsA resulted in a decline in ASC number in cultures containing CBA/H B cells (Figure 7.10B), as previously observed (Figure 5.3). Thus, although B cells from CBA/N mice are unable to generate the positive signal for growth as a result of cross-linking sIg, it is clear that they are susceptible to the inhibitory signals delivered by gαIgM which cause the downturn in LPS-induced proliferation and ASC formation (Figures 7.9 and 7.10).

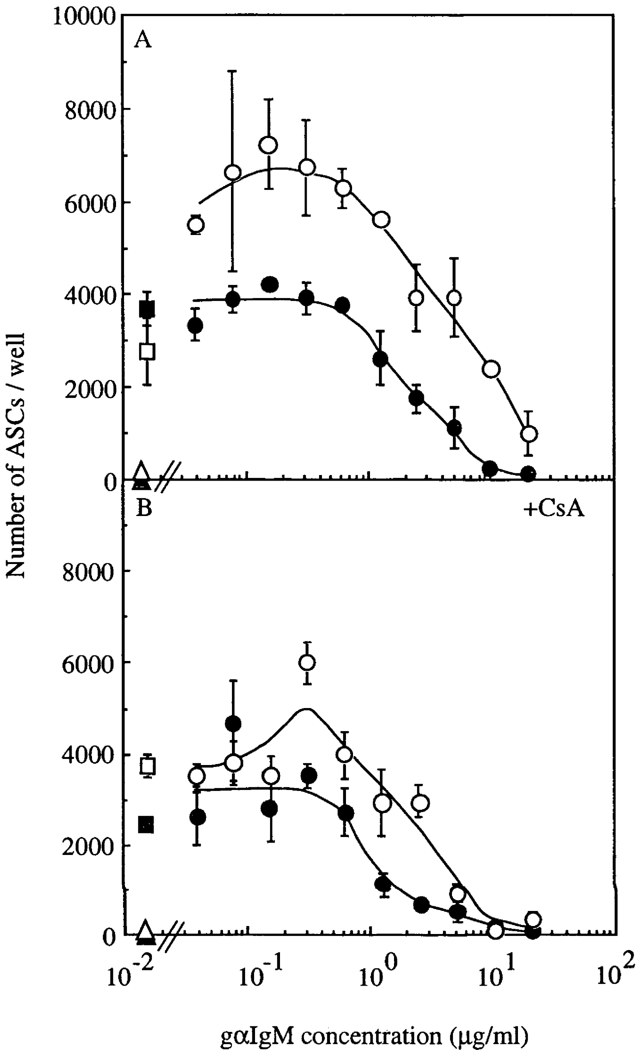

Figure 7.10. **GαIgM inhibits LPS-induced ASC formation in B cells from both CBA/H and CBA/N mice, irrespective of the presence of CsA.**

B cells were prepared from either CBA/H (O) or CBA/N (●) mice as described in chapter 3, and cultured in BCM containing LPS (50 μg/ml) and various doses of gαIgM. Additional cultures were also prepared which contained LPS, gαIgM and CsA (40 ng/ml). After 3 days cells were washed and transferred to Elispot plates which had been coated with sαIg to determine the total number of ASCs by Elispot assay as described in chapter 3. The data illustrated represents the mean of triplicate cultures ± one standard error. Background: CBA/H - panel A LPS only, and panel B LPS and CsA (□), cell only (Δ), CBA/N -panel A LPS only and panel B LPS and CsA (■), and cells only (▲).

7.4. Discussion

The experiments described in chapter 6 indicated that cross-linking of sIg can mediate a number of different signals which appear to be independent of each other, and have diverse functional consequences for the B cell. A simple explanation for the data was that the different sIg-mediated signals were acting on distinct B cell populations. In this chapter this hypothesis was examined through the use of AMS-9.1 and BET-2. Stimulation of unseparated B cell cultures with various combinations of BET-2 and AMS-9.1, in the presence of LPS, generated dose response curves which could be roughly approximated by the sum of the response to each antibody (Figure 7.1). The results illustrated in Figure 7.1 support the hypothesis that each sIg-mediated signal was acting on a different B cell population. This hypothesis was pursued by purifying B cells into distinct populations and examining the proliferation of each population in response to similar stimuli (Figures 7.2-7.4, 7.6 and 7.7).

Initially the results obtained with percoll and electronically purified B cell populations appeared to provide additional support for the hypothesis that the inhibitory and stimulatory responses were coming from different B cell populations (Figures 7.2, 7.3 and 7.6). However, these results also revealed that each of the B cell populations did not exclusively respond to one or other of the sIg-mediated signals. Instead each B cell population was shown to be susceptible to both sIg-mediated signals with varying degrees of sensitivity (Figures 7.2-7.7). Despite the differences in the response of the distinct B cell populations to anti-Ig reagents alone, or in combination with IL-4 (Figures 7.2, 7.3 and 7.6), in the presence of LPS each of the B cell populations behaved in a similar manner (Figures 7.4 and 7.7). This was particularly evident when comparing the response of electronically purified $sIgD^{hi}$ and $sIgD^{lo}$ B cell populations (Figure 7.7). Given the high degree of purity of the electronically sorted B cells (Figure 7.5) and the similarity of the response of the two populations (Figure 7.7), it appears that the initial hypothesis that the different effects of anti-Ig reagents represented the response of distinct B cell populations was not correct.

7.4.1. An alternative model of B cell behaviour suggests that the two sIg-mediated signals are delivered to the same cell

The similarity in the results obtained for the $sIgD^{hi}$ and $sIgD^{lo}$ B cell populations (Figure 7.7) imply that the two sIg-mediated signals are transmitted to the same cell, and that the response will depend on the mode of signal integration with in the B cell. As before it is possible to develop a hypothesis about the mode of integration, and to test it by comparing the predicted dose response curves to the experimental data. The sIg-mediated signals could be integrated in a number of different ways, however, one simple way would be if the negative signal reduced by a constant proportion (dependent on

dose) the positive signal induced by LPS alone, or LPS in combination with mitogenic anti-Ig antibodies. The dose response curves generated by this mode of signal integration were plotted against the experimental data. As illustrated in Figure 7.11, the predicted dose response curves provide a good but not exact fit for the experimental data. Dose response curves generated by a proportional model of signal integration did however, provide a better fit for the experimental data than those generated by using the sum of the two opposing sIg-induced responses (Figures 7.11 and 7.1, respectively). While further experiments will be required to asses the precise mode of signal integration, the important conclusion can be drawn that the two signals are being transmitted independently to the same B cell and that the consequence may have a simple mathematical solution.

7.4.2. B cells from CBA/N mice retain the ability to respond to the sIg-mediated negative signal

Although sIgD^{lo} B cells from non-immunodeficient mice respond to both sIg-mediated signals (Figures 7.6 and 7.7), B cells from CBA/N mice, which principally consist of sIgD^{lo} B cells (Scher *et al.,* 1976, Scher 1982a, Hardy *et al.,* 1983), appear to only be susceptible to the inhibitory sIg-mediated signal (Figures 7.9. and 7.10). The inability of B cells from CBA/N mice to proliferate in response to mitogenic anti-Ig reagents, while retaining their susceptibility to anti-Ig mediated inhibition, could be due to the point mutation within the *btk* gene (Rawlings *et al.,* 1993, Thomas *et al.,* 1993). This mutation has been shown to influence sIg-mediated calcium mobilisation (Rigley *et al.,* 1989, Lindsberg *et al.,* 1991). Thus, although anti-Ig reagents induce calcium mobilisation in B cells from CBA/N mice, the response is much lower than that observed in B cells from normal mice (Rigley *et al.,* 1989, Lindsberg *et al.,* 1991). These results imply that CBA/N B cells do not proliferate in response to anti-Ig reagents because of a defect in calcium signalling. In addition, as CBA/N B cells respond to the negative sIg-mediated signal in the absence of any detectable positive signal (Figure 7.9) the results support the conclusion that the two sIg-mediated signals are independently regulated.

In addition to inhibiting LPS-induced proliferation and differentiation to ASCs of B cells from CBA/N mice (Figures 7.9 and 1.10), anti-Ig antibodies have also been shown to induce an increased rate of cell death these cells (Anderson *et al.,* 1996). The decline in viable CBA/N B cell number was shown to occur at antibody concentrations which were mitogenic in small percoll purified B cells isolated from non-immunodeficient mice (Anderson *et al.,* 1996). In the presence of CsA the previously mitogenic antibodies induced a decline in viability normal B cells, but did not alter the response of B cells from CBA/N mice (Anderson *et al.,* 1996). These results imply that the CsA sensitive

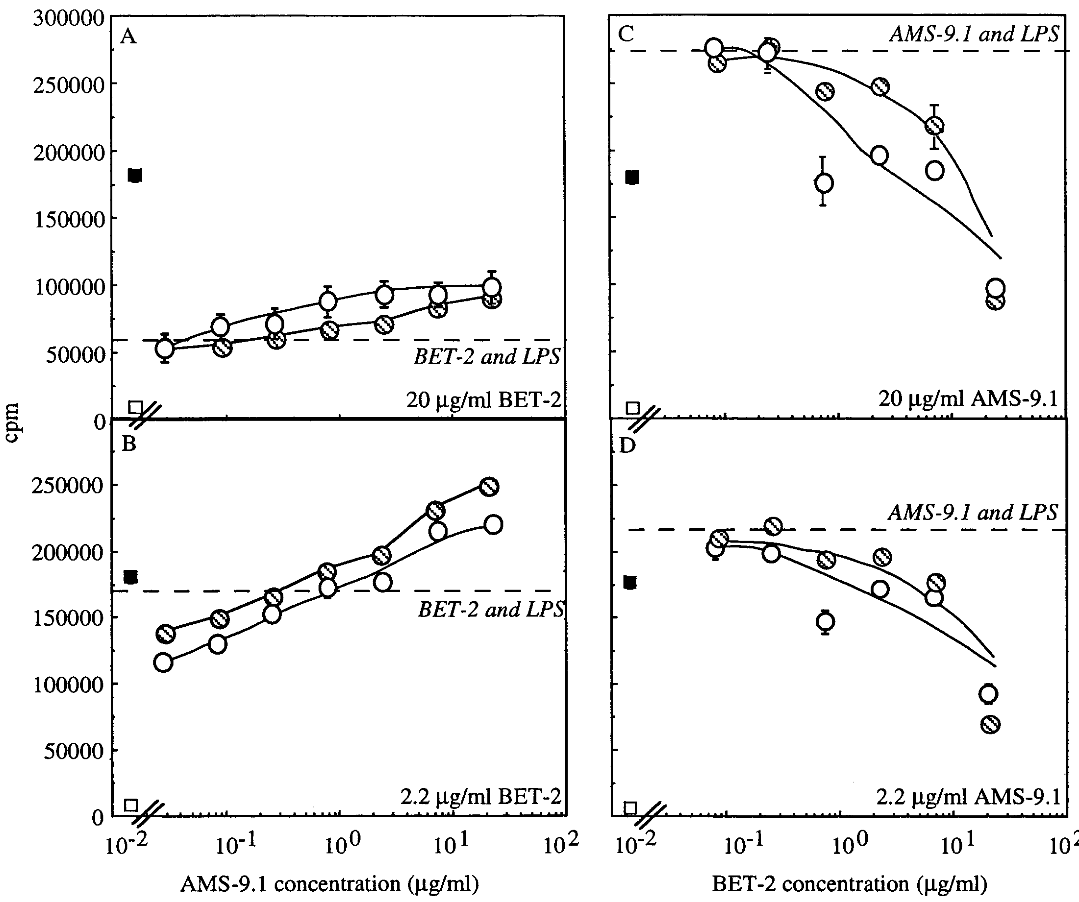

Figure 7.11 **A model of the effect of AMS-9.1 and BET-2 on LPS-induced proliferation suggests that the B cell response is the product, rather than the sum of the two independent signals.**

This figure illustrates three dose response curves for LPS-induced B cell proliferation in the presence of different combinations of AMS-9.1 and BET-2. The experimental dose response curves (○), and an arithmetically derived dose response curves based on the sum of the B cell response to either antibody (●) have been previously described in *Figure 7.1*. In addition this figure illustrates a second arithmetically obtained dose response curve based on the product of B cell response to either antibody (⊘). The method used to generate the second set of arithmetically derived dose response curves is described in section 7.4. This second model of B cell behaviour generated dose response curves which provided a better fit for the experimental data. Thus, these results indicate that the B cell response to co-culture with AMS-9.1 and BET-2 represents a proportional response to each signal in all B cells, rather than the behaviour of distinct B cell populations. Background: LPS only (■) and cells only (□).

sIg-mediated signal may also help maintain the viability of B cells. This could occur through the increased expression of the cell survival gene *bcl-x$_L$*. Mitogenic anti-Ig reagents have been shown to up regulate the expression of *bcl-x$_L$* in normal, but not CBA/N, B cells (Anderson *et al.,* 1996, Solvason *et al.,* 1998). In addition increased expression of *bcl-x$_L$* has been shown to be blocked by CsA (Anderson *et al.,* 1996). Combined these results suggest that the sIg-mediated positive signal which induces proliferation could also promote B cell survival.

7.4.3. Further speculation on the role of sIg isotype in determining the B cell response

In comparing the responses of the sIgD^{hi}/CD23^{hi} and sIgD^{lo}/CD23^{lo} B cells, it was apparent that mAbs which induced proliferation in both populations where able to bind sIgD (Figure 7.6). In contrast, anti-IgM mAbs only induced proliferation in cultures containing B cells with a sIgD^{hi}/CD23^{hi} phenotype (Figure 7.6C and D). These results are consistent with those obtained by Snapper *et al.,* (1993) who found that anti-IgM or -IgD reagents induced equivalent amounts of proliferation in B cells with a sIgM^{lo}/sIgD^{hi}/CD23^{hi} phenotype, but that anti-IgD reagents induced a more vigorous response in cultures containing B cells with a sIgM^{hi}/sIgD^{lo}/CD23^{lo} phenotype (Snapper *et al.,* 1993). It was of interest to note that in both experiments the mitogenic effects of anti-IgD mAbs were not dependent on a high level of sIgD expression. These results imply that cross-linking of sIgD may be prone to inducing the positive signal for growth, as discussed in chapter 6. However, given the variability in the B cell response to anti-Ig reagents it is also possible that the ability of AMS-9.1 to induce proliferation was not dependent of the isotype to which it bound. Thus, as before no definitive conclusion can be drawn about the different patterns of intracellular signalling activated by cross-linking sIgD or sIgM.

The experiments described in this chapter examined the possibility that the two sIg-mediated signals acted on different B cell populations. However, the results indicate that each of the B cell populations examined was susceptible to both negative and positive sIg-mediated signals. These results indicate that the behaviour described for the unseparated B cells (Chapters 5 and 6) represented a reasonable approximation of the response of distinct B cell populations. The experiments with B cells from CBA/N mice indicate that although these cells do not proliferate in response to mitogenic anti-Ig reagents, they are still susceptible to inhibitory sIg-mediated signals. Thus, these results support the conclusion that the positive and negative sIg-mediated signals are independently regulated. In addition, the results described in this chapter suggest that these is an additional level of specificity in the B cell response to antigen, which is determined by the sensitivity of distinct B cell populations to different signals.

Chapter 8

General discussion

8.1. A model in which sIg-mediates two independently regulated signals which have different effects on B cell behaviour

This thesis describes the effect of sIg-mediated signalling on TI type-1 B cell activation, as characterised by the B cell response to LPS. The results reveal that cross-linking of sIg can induce the activation of two independently regulated intracellular signalling pathways which differ in their effect on B cell proliferation and differentiation to ASCs, and in their sensitivity to CsA, IL-4 and FcγRIIB-mediated signals (Table 8.1). The type of sIg-mediated signal (positive or negative) induced appears to be dependent on the specific physical interaction between the antigen receptor and its ligand. A model in which the specific interaction between sIg and antigen can result in different patterns of intracellular signalling may provide a coherent explanation for the varied, and occasionally contradictory, B cell responses induced by both anti-Ig reagents and natural antigens. Thus far, the two sIg-mediated signals have been referred to as negative or positive, based on their effect on LPS-induced B cell behaviour. However, as these signals appear to mediate a number of effects which are not so easily defined (discussed below) they will be referred to as the CsA insensitive or CsA sensitive signal, respectively, in this discussion.

8.2. How valid is the use of anti-Ig antibodies as a substitute for natural antigens?

The conclusion that ligation of sIg can induce the activation of independently regulated signalling pathways was based on experiments using anti-Ig reagents as a substitute for natural antigen. While the use of anti-Ig antibodies has been subject to criticism (Möller 1978), both anti-Ig reagents and natural antigens have been shown to induce similar B cell behaviour (Andersson *et al.,* 1974, Hasbold *et al.,* 1990, Grandien *et al.,* 1993, Hartley *et al.,* 1993, Lenschow *et al.,* 1994, Mond *et al.,* 1995). This suggests that surrogate and natural antigens are capable of inducing similar patterns of intracellular signalling. Many of the studies using natural antigens have employed transgenic B cells that express antigen receptors with a uniform affinity for a defined antigen (Nemazee and Bürki, 1989b, Grandien *et al.,* 1993, Hartley *et al.,* 1993, Cooke *et al.,* 1994). This is in contrast to experiments that use anti-Ig reagents (Andersson *et al.,* 1974, Mond *et al.,* 1981, Hasbold *et al.,* 1990, Peçanha *et al.,* 1991, Lenschow *et al.,* 1994). In these studies the specificity and affinity of the antigen receptor differs within the B cell population, whereas the substitute antigen remains the same. Thus, while experimental systems using transgenic B cells or anti-Ig antibodies have artificial elements, both approaches can provide valid and complementary information about the B cell response to antigen. In addition, the experiments described in this thesis have identified anti-Ig reagents that predominantly induce the CsA sensitive or insensitive signal. These

Table 8.1 A summary of the characteristics of the two sIg-mediated signals

characteristics	**CsA insensitive signal** (negative)	**CsA sensitive signal** (positive)
antibody dose	• induced over a range of anti-Ig concentrations	• only induced at high anti-Ig concentrations
sensitivity to CsA	• insensitive	• sensitive
response to IL4	• no effect	• enhanced
response to Fc-mediated signals	• no effect	• inhibited
possible intracellular messengers	• inhibition of LPS-induced B cell activation PMA implies a possible role for PKC	• CsA sensitivity indicates that Ca^{2+} mobilisation and calcineurin activation are obligatory.
interaction between the two sIg-mediated signals	• the CsA sensitive has no effect on the inhibition of ASC the CsA insensitive signal	• overcomes inhibition of proliferation by the CsA insensitive signal
response of $sIgD^{hi}$ vs $sIgD^{lo}$ B cell populations	• both B cell populations are sensitive to this signal	• both B cell populations are sensitive to this signal. However, $sIgD^{hi}$ B cells appear to be more responsive than the $sIgD^{lo}$ B cells.
CBA/N mice	• inhibits LPS-induced B cell activation	• not detectable
B cell behaviour	• inhibits background and LPS-induced proliferation and ASC formation	• stimulates or enhances B cell proliferation

antibodies could be used to characterise the intracellular signalling events involved in either pathway. While such a study is beyond the scope of this thesis, it may be worth pursuing as it could provide insight into how different antigens subtly alter the pattern of sIg-mediated signalling and thereby regulate the B cell response.

8.3. Speculation on the influence of other B cell surface receptors on sIg-mediated signals

The effect of IL-4 and 2.4G2 on the B cell response to anti-Ig antibodies clearly indicates that signals from other cell surface receptors can have different effects on the two sIg-mediated signals. One obvious candidate is the CD21/CD19/CD81 complex. Previous work has shown that co-ligation of this receptor and sIg can have a synergistic effect on anti-Ig induced B cell proliferation (Carter *et al.,* 1992). This may be due to signals mediated by the CD21/CD19/CD81 complex that enhance the positive sIg-mediated signal for growth, as observed for IL-4 (Chapter 6). Support for this suggestion may be derived from the enhancing effect of co-ligation of sIg and the CD21/CD19/CD81 complex on sIg-mediated calcium mobilisation (Bradbury *et al.,* 1993, Lankester *et al.,* 1996). While it is clear that signals from the CD21/CD19/CD81 complex can promote B cell activation by anti-Ig reagents (Carter *et al.,* 1992, Matsumoto *et al.,* 1993, O'Rourke *et al.,* 1998) the role of these signals, if any, in the experiments described in this thesis has not been determined. It is possible that residual complement within the FCS may have been able to bind to anti-Ig reagents forming immune complexes capable of cross-linking sIg and the CD21/CD19/CD81 complex, thereby promoting B cell proliferation. However, this would presumably only play a minor role in the B cell response to anti-Ig antibodies as the concentration of fixable complement in heat inactivated FCS would be low.

A second B cell receptor that can influence sIg-mediated signalling is CD22. Ligation of CD22 results in the phosphorylation of its cytoplasmic tail and subsequent recruitment and activation of phosphatases such as SHP-1 that down regulate the antigen signal (Doody *et al.,* 1995, Cornall *et al.,* 1998). Thus, it is possible that intracellular signals generated by CD22 could inhibit the sIg-mediated positive signal for growth in a similar manner to that described for FcγRIIB (Chapter 6). Finally, the expression of CD45 is obligatory for sIg-mediated calcium mobilisation (Justement *et al.,* 1991), therefore, CD45 may directly promote the generation of the CsA sensitive sIg-mediated signal. Alternatively, CD45 may indirectly promote the sIg-mediated inhibitory signal by promoting the phosphorylation tyrosine residues within the cytoplasmic tail of negative regulators of the antigen signal, such as CD22 (Healy and Goodnow 1998). While discussion about the interaction between the two sIg-mediated signals and signals induced by other B cell surface receptors is speculative, it may be possible to determine

the interaction between these signals experimentally through the use of anti-Ig mAbs that predominantly induce one component of the sIg-mediated signals and B cells from knockout mice.

Irrespective of the relationship between sIg-mediated signals and those induced by other B cell surface receptors, the unique interaction between sIg and antigen appears to be able to generate different patterns of intracellular signalling that are specific for the particular sIg / antigen combination. It is proposed that these antigen-specific patterns of sIg-mediated signalling regulate the B cell response. If correct, such a system would allow for a coordinated response by the B cell that is principally driven by its interaction with antigen. This hypothesis has implications for the regulation of B cell behaviour, some of which are discussed below.

8.4. Surface Ig-mediated signals regulate TI type-1 B cell activation

According to Coutinho and Möller's (1975) one-signal model, LPS-induced B cell activation occurs in the absence of an antigen signal, and the downturn in LPS-induced ASC number is due to high dose paralysis. However, the results presented in this thesis reveal that high dose paralysis is not obligatory, and indicate that the antigen signal can promote LPS-induced proliferation while inhibiting the formation of ASCs. The inhibitory effect of anti-Ig antibodies on LPS-induced ASC formation is consistent with the work of Andersson *et al.,* (1974 and 1978), Kearney *et al.,* (1976 and 1978) and Grandien *et al.,* (1993). In addition, these results identify a previously unrecognised role for sIg-mediated signalling in regulating LPS-induced B cell activation that may result in the proliferation of antigen specific B cells and the production of low affinity antibody (Chapter 2).

A further speculative point may be made about LPS-induced B cell activation that concerns cells which express antigen receptors specific for LPS. Andersson *et al.,* (1972) have shown that within the B cell population there are cells that express sIg which specifically bind to epitopes within LPS. These cells principally recognise epitopes within the O-antigen (Rietschel *et al.,* 1994). As described for anti-Ig antibodies or natural antigens, antigen recognition by LPS-specific B cells could result in the activation of one or both sIg-mediated signals. As the O-antigen is highly variable it is possible that a large number of B cells can bind to it with varying degrees of specificity (Figure 8.1, A). Thus, the number of B cells that bind LPS via their antigen receptor would be dependent on the specificity of sIg and the concentration of LPS (Figure 8.1, B). If correct, B cells that express an antigen receptor with a high degree of specificity for LPS would be the first to receive the sIg-mediated CsA insensitive signal

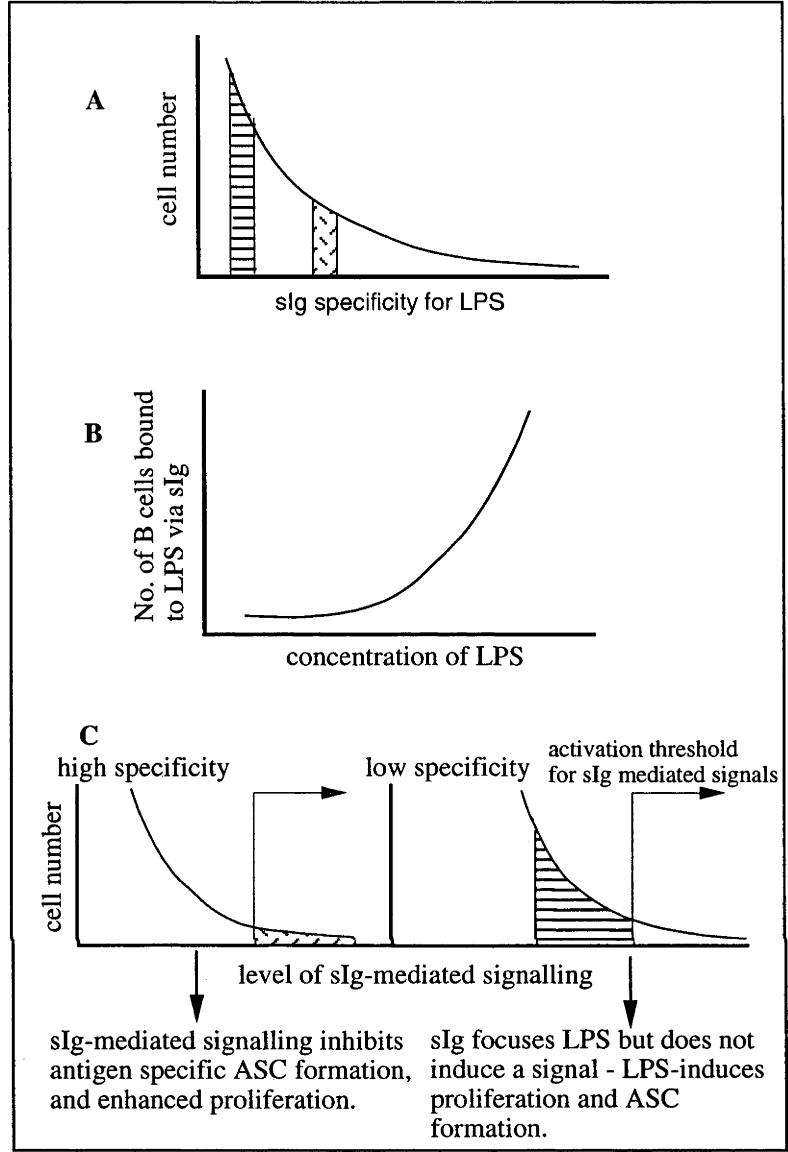

Figure 8.1. **The effect of sIg specificity for, and concentration of, LPS on the B cell response.**

This figure illustrates how sIg specificity and LPS concentration could effect LPS-induced B cell activation. Within a B cell population a number cells will express sIg that are specific epitopes within LPS. The distribution of LPS specific B cells is illustrated in Panel A. While the majority of B cells will express sIg with a low specificity (▤), some cells will have sIg with a high specificity for LPS (▨). The number of B cells that bind LPS via sIg is proportional to the concentration of LPS (B). At a given concentration of LPS B cells that express LPS-specific sIg will bind suffient LPS to induce sIg-mediated signalling that can inhibit LPS-induced ASC formation (C). In contrast, at the same LPS concentration B cells that have sIg with a low specificity for LPS may be able to focus it to the B cell surface without initiating an inhibitory signal and will proliferate and differentiate into ASCs (C). If correct, this model would result in the secretion of a high concentration of antibody with a low specificity for LPS, and the proliferation of B cells with a high degree of specificity for LPS in the absence of antibody production.

that inhibits ASC formation (Figure 8.1, C). In contrast, B cells that express antigen receptors with a low specificity for LPS may be able to focus it to the B cell surface without triggering this signal (Figure 8.1, C). These B cells could then undergo LPS-induced differentiation to ASCs as proposed by Coutinho and Möller (Coutinho *et al.*, 1975). Such a system would promote a low affinity anti-LPS antibody response which could be effective at clearing antigen due to the high number of responding cells.

8.5. What role do the two sIg-mediated signals have in TI type-2 B cell activation?

The activation of B cells by TI type-2 antigens has been shown to require extensive cross-linking of sIg (Mond *et al.*, 1979, Peçanha *et al.*, 1991). This is dependent on the physical form of the antigen, in particular its valency. Changes to the physical form of these antigens alters their ability to induce B cell activation, as illustrated by the response to different forms of flagellin (Feldmann *et al.*, 1971), and soluble anti-Ig antibodies vs anti-Ig-dextran (Peçanha *et al.*, 1991, Snapper *et al.*, 1995a). The results described in this thesis suggest that differences in the physical form of the antigen can effect its interaction with sIg in a manner that can be translated into distinct patterns of sIg-mediated signalling that result in different B cell responses . While it is clear that B cell activation by TI type-2 antigens requires the generation of the sIg-mediated CsA sensitive signal (Yamada *et al.*, 1993), the involvement of the CsA insensitive signal is less certain. As the two sIg-mediated signals can be activated independently it is possible that TI type-2 antigens do not induce the CsA insensitive signal . Alternatively, these antigens could activate both sIg-mediated signals and a particular ratio of the two may be required to induce B cell activation. As the pattern of sIg-mediated signalling induced by TI type-2 antigens that results in B cell activation is not known, elucidating the ability of these antigens to induce the activation of the different sIg-mediated signals requires further study.

8.6. The CsA insensitive sIg-mediated signal may promote T cell dependent B cell activation

In contrast to TI type-2 antigens, TD antigens are generally small protein aggregates which are unable to induce extensive cross-linking of sIg (Parker 1993). As discussed, the ability of anti-Ig reagents to induce cross-linking of sIg affects the B cell response (Chapter 6). Non-mitogenic or inhibitory soluble antibodies can induce proliferation when coupled to an insoluble matrix (Parker 1975, Goroff *et al.*, 1986, Brunswick *et al.*, 1988, Peçanha *et al.*, 1991, Udhayakumar *et al.*, 1991). Thus, for certain antibodies the ability to induce the CsA sensitive signal that promotes proliferation appears to be related to the density of sIg cross-linking they induce. However, as some soluble antibodies inhibit B cell proliferation, it is likely that the CsA insensitive signal is

not as dependent on the density of sIg cross-linking. Therefore, it is possible that TD antigens that are not able to induce extensive cross-linking of sIg are still able to induce the CsA insensitive signal. While this signal in combination with LPS results in the inhibition of proliferation and antibody production, the same signal could also promote TD B cell activation by up regulating cell surface molecules involved in T cell / B cell collaboration. This prediction is supported by experiments in which anti-Ig antibodies have been shown to induce increased expression of MHC class-II molecules in a CsA insensitive manner (Mongini *et al.,* 1992). Thus, according to the hypothesis presented in this thesis the up regulation of MHC class-II could be the induced by activation of the CsA insensitive component of the antigen signal. While exploring the role of the different sIg-mediated signals in TD B cell activation is beyond the scope of this thesis, it may be possible to examine this question through the use of mAbs that induce only one component of the antigen signal, and determining their effect on the expression of B cell surface molecules and T cell / B cell collaboration.

These examples illustrate how different sIg-mediated signals could influence B cell activation. If sIg does mediate more than one signal after interaction with antigen, its effect would not be limited to influencing B cell activation. A model in which sIg can generate multiple signals may also be applicable to B cell development in the bone marrow, affinity maturation in germinal centres and the induction of tolerance. In each instance it has been proposed that the antigen signal is involved in the positive and negative selection of B cells (Freitas *et al.,* 1991, Gu *et al.,* 1991, Goodnow 1992, Nossal 1992, Nossal 1994, von Boehmer 1994). The activation of, and the interaction between, different sIg-mediated signals could determine the fate of B cells during selection. The view that the fate of B cells is in part determined by differential sIg-mediated signalling is supported by results from Healy *et al.,* (1997) who have shown that the pattern of sIg-mediated signalling differs between tolerant and naive B cells (Healy *et al.,* 1997). In addition, the effect of the sIg-mediated signals would be dependent on the maturation state of the cell, as illustrated by the inhibitory effect of anti-Ig antibodies on immature B cells compared to their mitogenic effect on mature B cells (Sieckmann *et al.,* 1978a, Boyd *et al.,* 1981, Hasbold *et al.,* 1990, Brines *et al.,* 1991). As it may now be possible to identify the intracellular messages involved in the CsA sensitive and insensitive sIg-mediated signals, it would be of interest to examine the role of these different signalling pathways in B cell development and affinity maturation. Finally, if the antigen signal in B cells is composed of more than one independently regulated pathway, where each pathway can induce a different response, then this may also hold true for other cell types. A similar model, which is briefly described below, has been proposed for T cells.

8.7. The antigen signal regulates T cell behaviour in a similar manner to that described for B cells

The T cell and B cell antigen receptors are remarkably similar in structure, the signals they induce, and in their association with co-receptors (reviewed in Cambier 1992, Cambier *et al.,* 1994, Weiss *et al.,* 1994, DeFranco 1995, Cantrell 1996). If the B cell antigen receptor is able to induce different independent signals with varying thresholds of activation, might not the same hold true for T cells? A model has been described which suggests that the T cell antigen receptor may induce differential signalling (Sloan-Lancaster and Allen 1996). Sloan-Lancaster and Allen's model (1996) was based on experiments where the peptide antigen recognised by a specific TCR has been altered with a conservative amino acid substitution, generating an altered peptide ligand (APL). They proposed that APL binding to the TCR induced a different pattern of intracellular signalling than that induced by the original peptide, and that the pattern of signalling determined the T cell response (Sloan-Lancaster *et al.,* 1996). Thus, stimulation of T_h type-2 cells by the original peptide antigen induced: proliferation, IL-4 production, and the provision of B cell help (Evavold *et al.,* 1993, Sloan-Lancaster *et al.,* 1993). In contrast, stimulation of the same cells with an APL induced IL-4 production and the generation B cell help but not T cell proliferation (Evavold *et al.,* 1993, Sloan-Lancaster *et al.,* 1993). This phenomenon of partial T cell activation has also been described for T_h type-1 cells, where stimulation with APLs resulted in the generation of cytolytic activity and the up regulation of IL-2 receptor expression, but not proliferation or cytokine production (Evavold *et al.,* 1993, Sloan-Lancaster *et al.,* 1993). The partial activation of T cells by APLs could be considered to be a form of inhibition of the T cell response, as after interaction with APLs the T cell is rendered unresponsive to the original ligand (Sloan-Lancaster *et al.,* 1993). More recently, it has been shown that the interaction of agonist, partial agonist and antagonist peptides with the TCR can induce distinct patterns of intracellular signalling (Chau *et al.,* 1998). Thus, as the T cell responses to antigen binding the TCR can be separated from each other, it has been suggested that T cell activation should not be considered an "all or none" phenomenon, but rather a collection of possible responses (Sloan-Lancaster *et al.,* 1993).

The model proposed by Sloan-Lancaster and Allen (1996) and the model described in this thesis for sIg-mediated signalling in B cells have a number of similarities. In both models the antigen signal may differ according to the physical form of the antigen, the response of these lymphocytes represents a collection of possible responses rather than being "all or nothing", and the behaviour is governed by the pattern of the antigen signal. In addition, both B cells and T cells require the antigen signal to induce Ca^{2+} mobilisation for complete activation (Cambier *et al.,* 1994, Sloan-Lancaster *et al.,*

1996). The similarity in the behaviour of B cells and T cells suggests that the activation of both cell may be regulated in a similar manner.

8.8. Conclusion

This thesis presents a model in which the regulation of B cell responses is determined by the activation of different sIg-mediated signals. As the B cell responses can be separated from each other, it is thought that the response to antigen is not "all or nothing", but rather represents a pool of possible responses. It is proposed that the physical form of the antigen determines the pattern of sIg-mediated signalling and thereby regulates the B cell response. In this way the antigen signal(s) may represent the key regulators of B cell behaviour. This model is complementary to that proposed by Sloan-Lancaster and Allen (1996) in which the TCR can mediate different signals which induce distinct patterns of T cell behaviour (Sloan-Lancaster *et al.,* 1996). Finally, the implications of an antigen receptor that can induce different intracellular signalling following the interaction with antigen, when placed in a broader context, may ultimately lead to a better understanding of the regulation of the antibody response.

Chapter 9

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