Cytokine Control of Human Immunoglobulin Class and IgG Subclass Production

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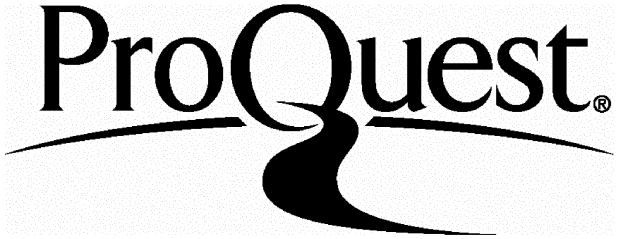

ABSTRACT

Cytokines are important regulators of Ig class production. In mice, IL4, IFNγ and TGFβ control IgE and IgG1, IgG2a and IgG3, and IgA and IgG2b respectively. In humans, IL4 regulates IgE and IgG4, and TGFβ regulates IgA production, but cytokines which regulate the other human IgG subclasses are unknown. A system was developed for the polyclonal activation of human B cells using Epstein Barr Virus (EBV). The kinetics of this system were optimised for the addition of cytokines.ELISA assays were developed for the measurement of human IgA, IgM, IgE, IgG, IgG1, IgG2, IgG3 and IgG4 in cell culture supernatants.

The effects of selected cytokines and antibodies to functional B cell surface antigens were studied. IL2 and IL6 were BCDFs increasing all Ig classes and IgG subclasses with the exception of IgE. Low concentrations of TGFβ increased IgA but high concentrations inhibited B cell proliferation and Ig class production. IL5, IFNα and IFNγ were found to have no effect. IL4 showed dual BCDF activity. Low doses of IL4 (1-5 U/ml) induced IgM, IgA, IgG1, IgG2 and IgG3 production by up to ten fold above control levels while high doses of IL4 (100 U/ml or greater) specifically increased IgE and IgG4. Low doses of IL4 also induced IgG and IgA production by $mIgM^-$ and $mIgM^+$ B cells. These results were consistent with heavy chain class switching and BCDF activity by IL4.

Anti-CD40 mAbs increased B cell proliferation and Ig production. Addition of low dose IL4 (5 U/ml) suppressed anti-CD40 induced proliferation and enhanced Ig production whereas high dose IL4 (100 U/ml) inhibited anti-CD40 induced Ig production and enhanced proliferation. Anti-CD23 antibodies inhibited EBV and IL4 induced IgA, IgM and IgG secretion as well as IL4 induced IgE secretion.

These results show that IL4 has a dual role in the regulation of human Ig production. These functions may be mediated through two IL4Rs and signalling through these receptors can differentially control the effects of CD40 ligation. In addition, ligation of CD23 can inhibit the effects transduced through both receptors and prevent IL4 dependent IgE and low dose IL4 dependent IgM, IgA and IgG production.

ACKNOWLEDGEMENTS

To Mary Kotowicz who never pushed. To Iain who did all the washing up, ran the taxi service and was always there. To Doc. Robin Callard, for persuasive arguments on the nonexistence of God, but most of all for his fortitude, forbearance and suffering in the making and writing of this Thesis. B cells don't wish and Duel = pistols at dawn!

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CHAPTER 1

INTRODUCTION

1.1. CELLS OF THE IMMUNE SYSTEM.

Pluripotent stem cells in the bone marrow give rise to all haematopoietic cells involved in the immune system (185,200) (Figure 1.1.). An immune response to antigen requires complex interactions between bone marrow derived immune cells as well as non bone marrow derived cells such as epithelial cells. Co-operation between the multiple cell types allows the immune system to identify the nature of antigen and mount an immediate specific response, utilising the most efficient means of defence, as well as retaining specific memory.

1.2. CELLULAR AND HUMORAL IMMUNITY.

Cellular immunity (cell mediated) and humoral immunity (antibody mediated) work together during an immune response to antigen. The immune system is able to recognise antigen, recruit appropriate cells and produce the most effective immune response to combat the invading pathogen. Cellular immunity involves cell mediated killing and clearance by leucocytes such as neutrophils, macrophages, natural killer (NK) cells, cytotoxic T cells (thymus derived lymphocytes), eosinophils and basophils. Humoral immunity involves B cells (bone marrow derived lymphocytes) which secrete immunoglobulin (Ig) molecules or antibodies. Igs bind to antigen and facilitate phagocytosis (opsonisation) of immune complexes and complement lysis by activation of the classical pathway. Immunoglobulins also bind to Ig receptors on monocytes, neutrophils and macrophages to facilitate removal of immune complexes. Pre-T cells originate in the bone marrow and travel to the thymus where they develop into three classes of mature T cell, cytotoxic (Tc), suppresser (Ts) and helper (Th). Tc cells, as mentioned above, take part in the cellular immune response. Ts cells act by suppressing a cellular or humoral immune reaction, and Th

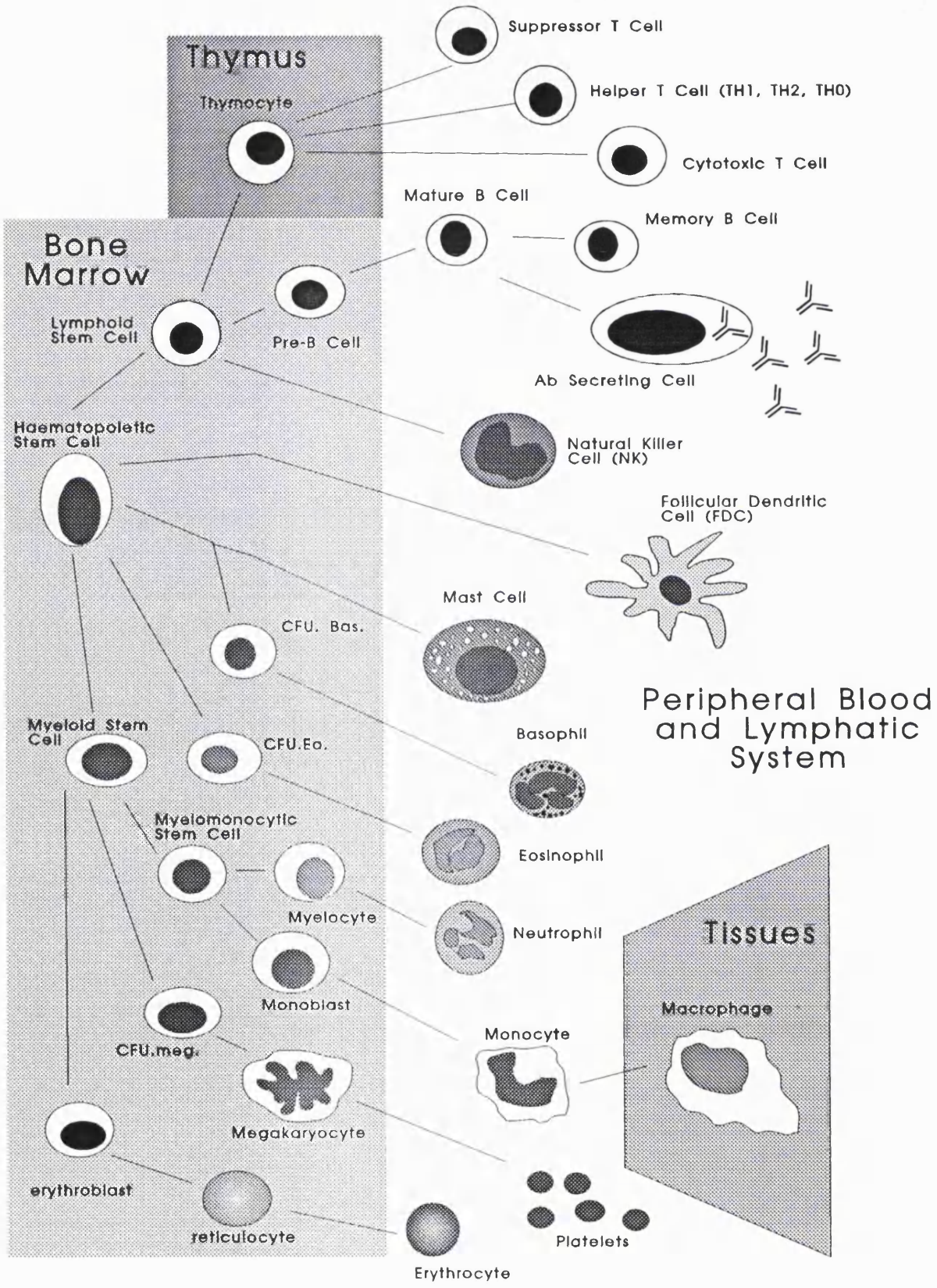


Figure 1.1. Bone Marrow Derived Immune Cells.

cells provide help to B cells during a humoral immune response and to Tc during a cellular immune response.

1.3. B LYMPHOCYTES.

In adults, B lymphocytes develop in the bone marrow and are defined as those cells which undergo rearrangements of the immunoglobulin gene loci and express membrane bound immunoglobulin. Mature B cells, released from the bone marrow, become the major players of the humoral immune response. Their surface bound immunoglobulin molecules are antigen specific, and with help given by antigen specific Th cells (cognate interaction), B cells clonally expand by proliferation and differentiate into antibody forming cells (AFC). Humoral immunity is effected by immunoglobulins which may be membrane bound or secreted.

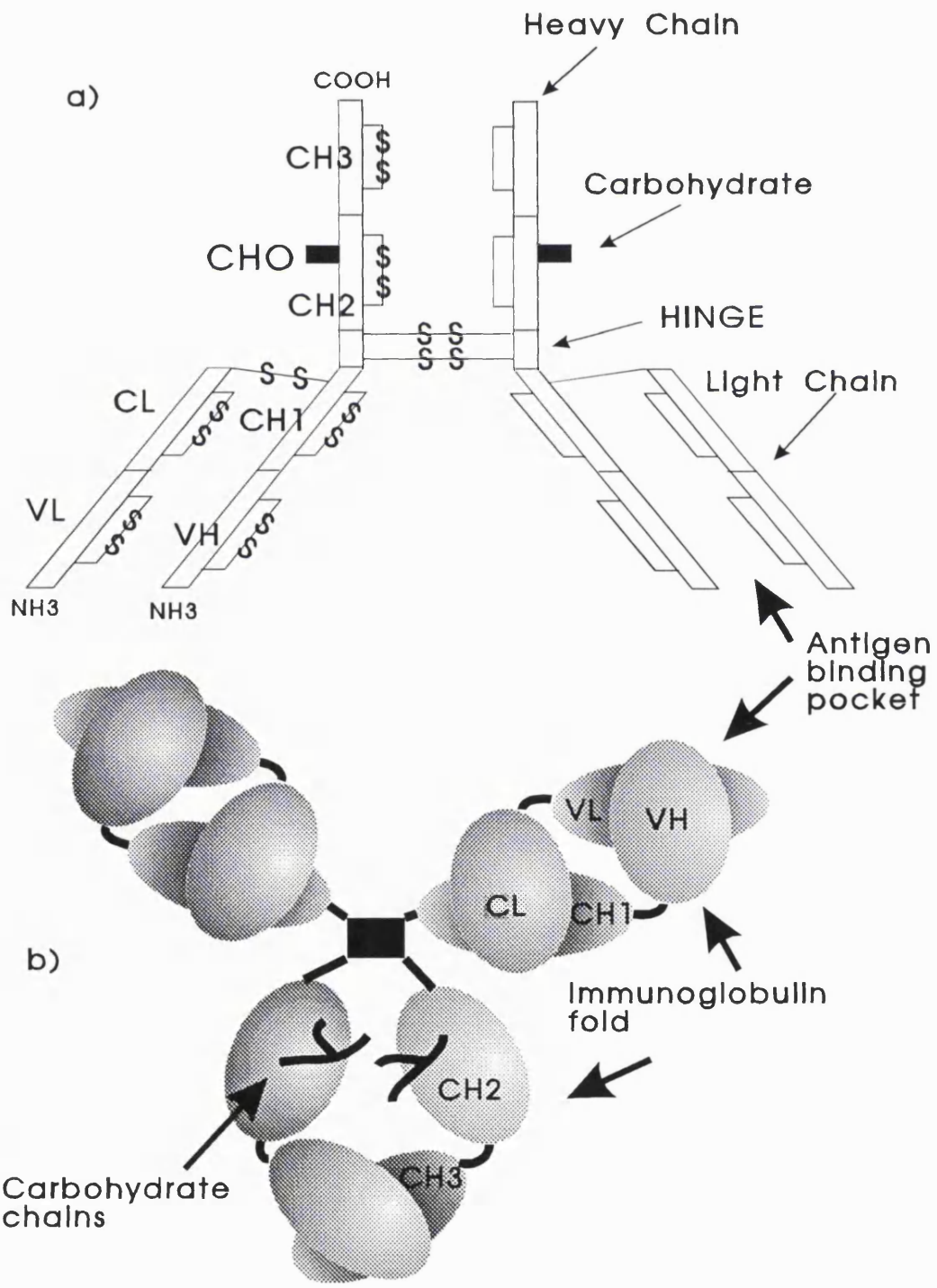
1.3.1. The Immunoglobulin Molecule, Mediator of Humoral Immunity.

The basic immunoglobulin unit is a tetrameric glycoprotein composed of two identical light and two identical heavy polypeptide chains which are approximately 220aa (25 kDa) and 440aa (50 kDa) each respectively. Although the average size of an Ig molecule is 150 kDa. The actual molecular weight depends on the Ig heavy chain class, carbohydrate content and whether the Ig molecule is the membrane bound or monomeric/multimeric secreted form.

The immunoglobulin light chain, consists of two domains, a constant (CL) and a variable (VL) domain. The heavy chain consists of a variable domain (VH) and three or four constant domains (CH1, CH2, CH3, CH4), depending on the Ig class or subclass. In some Ig molecules there is also a region called the hinge which is

Figure 1.2. Secreted human IgG1

a) Skeleton structure of IgG1 showing inter and intra chain disulphide bonds. b) Globular structure of IgG1 showing immunoglobulin domains.



interposed between the CH1 and CH2 domains. In addition, depending on whether the molecule is membrane bound or secreted, a transmembrane region or a joining region may be situated next to CH3 or CH4. One light chain is bound by a disulphide bond (S-S) in the CL domain to the CH1 domain of a heavy chain and two heavy chain/light chain dimers are bound together at the hinge by between 1 and 11 disulphide bridges to create the tetrameric unit. IgM is an exception, it does not have a hinge and uses rigid CH2 domains in close association as a hinge-like structure, its heavy/light chain dimers are disulphide bonded below the CH2 between the CH3 domains.

Each heavy and light chain C or V domain also contains an internal disulphide bond which stabilises the characteristic tertiary "immunoglobulin fold" structure. The gene sequence coding for the immunoglobulin fold structure is conserved and other cell surface molecules using it are said to be members of the immunoglobulin gene super family. In addition to covalent bonds, the Ig heavy and light chains are held by non-covalent interactions between the Ig domains (Figure 1.2.). N-linked carbohydrate chains are also linked to the immunoglobulin domains at various positions according to the Ig class, and these play a part in the structure and function of the molecule. Human IgG subclasses have one N-linked carbohydrate chain bound to each CH2 domain. This interferes with the non covalent interaction of the CH2 domains and the molecule bows out. This change in 3-D structure may influence the effector function of the heavy chain.

Enzyme digestion splits the immunoglobulin molecule into functional components (Figure 1.3.). There are two identical Fab fragments (antigen-binding), which give the molecule a bi-valent antigen binding capacity. The V domains of heavy and light chains contain hypervariable regions, CDR1, CDR2 and CDR3. When the V domain immunoglobulin fold is arranged these form extra polypeptide loops which associate non-covalently to form the antigen binding pocket. The CH1 domain of the Fab also contains a binding site for complement C4b/C3b. The Fc region

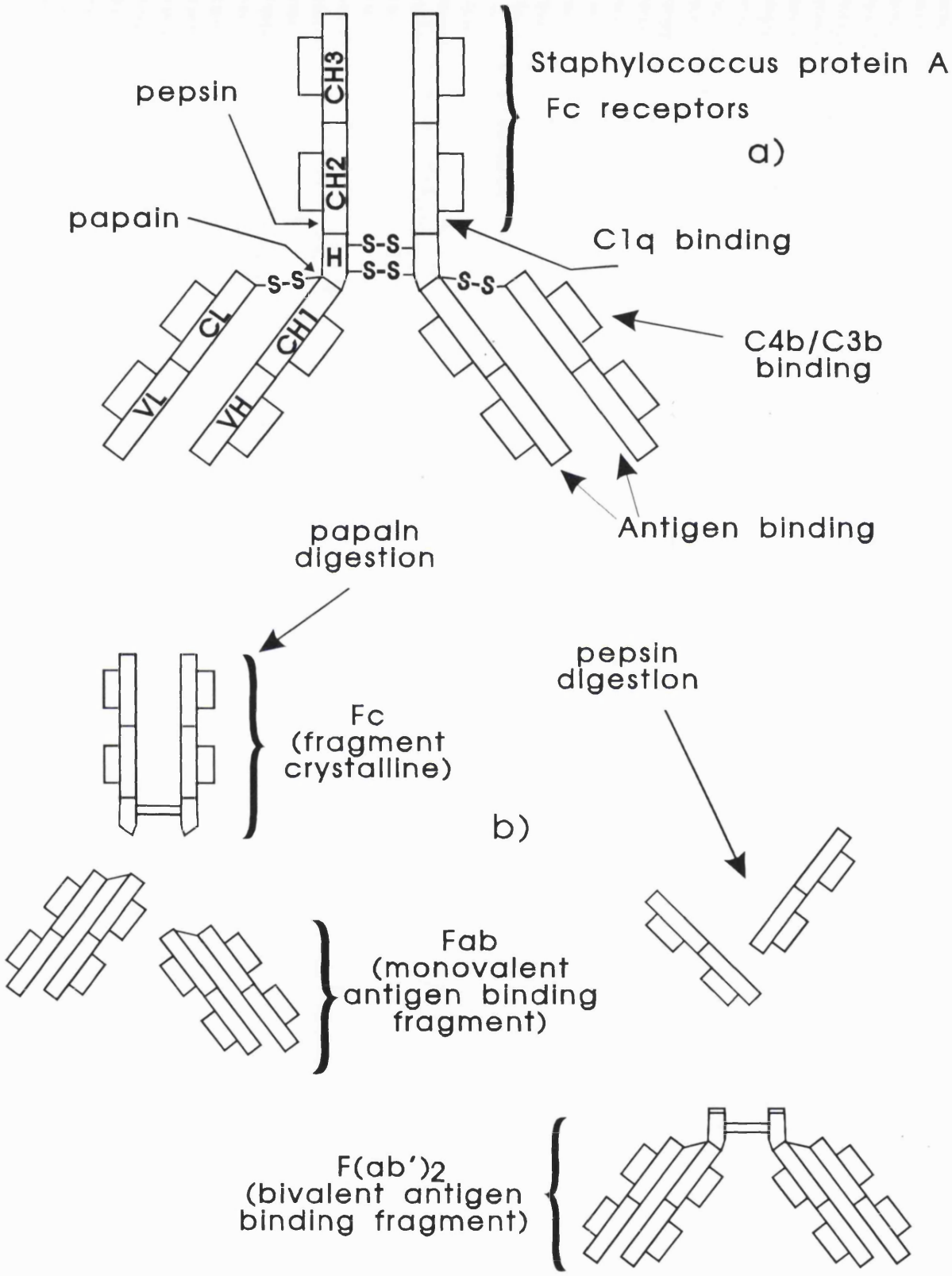


Figure 1.3. Structure Function of The Ig Molecule.

a) Skeleton diagram of Ig molecule showing various ligand binding sites and positions for enzyme cleavage.

(fragment crystalline) contains binding sites for complement C1q (CH2), Fc receptors on macrophages and monocytes (CH3), Fc receptors on neutrophils and NK cells (CH3+CH2) and staphylococcus protein A (Figure 1.3.).

1.3.2. Immunoglobulin Heavy Chain Classes.

Different heavy chain classes allow an immunoglobulin molecule of high antigen specificity to gain access to different effector functions. In the human and in the mouse there are five major classes of immunoglobulin heavy chain constant regions, M, D, G, A and E. The G class is further divided into G1, G2, G3 and G4 in the human and G1, G2a, G2b and G3 subclasses in the mouse. The human also has two subclasses of A, A1 and A2.

IgD.

In mature B cells the CHδ genes are transcribed with the CHμ genes in one long mRNA transcript (226,254). Differential or alternative splicing of the long mRNA allows IgD to be co-expressed with IgM in the membrane, but never alone (see Section 1.3.8.). IgD is present in human serum at a very low concentration (approx. 30ug/ml), but as IgD is not secreted, this may be due to proteolytic cleavage or loss from the cell surface of activated B cells. The function of IgD is controversial. It is thought to play a role in tolerance mechanisms, activation and memory (424). However, in the IgD^- mouse it is not essential for B cell maturation (328). Instead, it may act as a more efficient antigen capture molecule than IgM because of its greater hinge flexibility. Its presence with IgM on the surface of mature B cells may be for enhanced antigen capture and consequent affinity maturation, a critical advantage in defence against rapidly growing and mutating pathogens (329). IgD is

TABLE 1.1. PHYSICO-CHEMICAL AND FUNCTIONAL PROPERTIES OF THE HUMAN IMMUNOGLOBULIN CLASSES.

Property	IgM	IgD	IgA	IgE	IgG
Ig Heavy chain class	μ	δ	α1 or α2	ε	γ1, γ2, γ3 or γ4
Molecular weight (kDa)	970[a]	184	385[b]	188	150
Molecular weight of heavy chain (kDa)	65	69.7	56 or 52	72.5	53
Number of heavy chain domains	5	4	4	5	4
Ig carbohydrate content (%)	12	9-14	7-11	12	2-3
Ability to fix complement	+	-	-	-	+
Ability to cross placenta	-	-	-	-	+
Staphylococcal protein A binding	-	-	-	-	+
Binding to mononuclear cells	-	-	+	+	+
Binding to neutrophils	-	-	+	-	+
Binding to mast cells and basophils	-	-	-	+	-
Binding to T and B cells	+	-	+	+	+
Binding to platelets	-	-	-	?	+

a pentameric form, b dimeric form.

not secreted because the δ heavy chain gene lacks a secretory protein coding region. In spite of this, some myelomas (1%) secrete IgD. This may be explained by unequal crossing over of sister chromatids and insertion of the Cμ secretory exon to the 3' of Cδ (429).

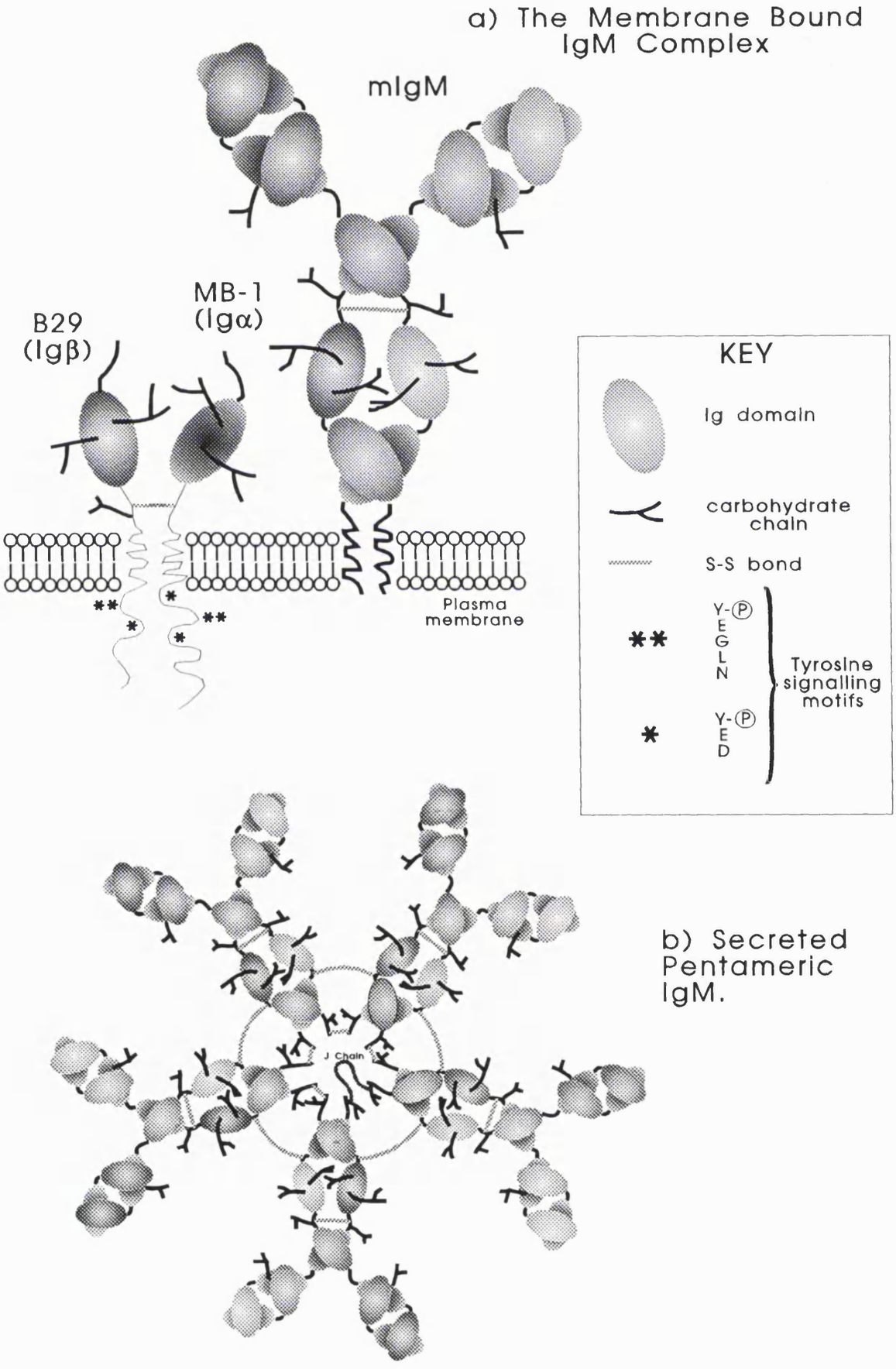
IgM

IgM is the first Ig isotype expressed on the surface of B cells. It is the first antibody to be produced during an immune response before the switch to IgG and is present in human serum at approximately 1.5-2.0 mg/ml. Secreted IgM is a pentameric molecule. IgM molecules are joined by disulphide bonds between CH3 and CH4 domains, and an additional protein called the J (joining) chain. Although individual IgM molecules are often of low affinity, their pentameric structure compensates for this, and avidity for antigen is high (Figure 1.4b.). IgM is also the only Ig molecule apart from IgG which can fix complement.

IgA.

Secreted IgA is present in normal human serum at approximately 1.4-4 mg/ml. The IgA molecule is present predominantly as a monomer in serum, but can be secreted into body fluids (e.g. milk, intestinal juice, saliva, tears) as dimers where it binds to invading pathogens as a first line of defence. Dimeric IgA is disulphide bonded by one CH3 domain to a J (joining) protein chain. An additional protein polypeptide called the secretory component is wound around the dimer and disulphide bonded at each end to one CH2 domain of each IgA molecule. IgA antigen complexes can bind to FcαRs on human monocytes, granulocytes, neutrophils and subpopulations of B and T cells (256) and mediate phagocytosis and super oxide production for bacterial

Figure 1.4. Membrane Bound and Secreted IgM.



cell lysis and killing. The human IgA subclasses IgA1 and IgA2 are found at a ratio of 9:1 in normal human serum. This concentration difference could be due to the spatial distance between the Cα2 gene and the VDJ genes, Cα2 being the most 3' of the Ig heavy chain constant region genes (Figure 1.6b.). In external secretions however, IgA2 can increase to be 50% of total IgA. This may be accounted for by the susceptibility of IgA1 to proteolysis. IgA2, which has lost a large part of the IgA hinge region (13aa), is resistant to a number of bacterial proteases which attack IgA1 in external secretions especially the gut (268). Interestingly, the rabbit has evolved 13 IgA subclasses which differ from each other in their hinge and CH1 regions, perhaps endowing them with protection against a wide range of bacterial proteases. The rabbit is a herbivore with a very large bacterial harbouring caecum and appendix and would gain considerable selective advantage from developing protective IgA (373).

IgE.

IgE is present in normal human serum at very low concentrations approximately 17-450 ng/ml. Binding to the high affinity IgE receptor (FcεR1) on basophils and mast cells causes degranulation and release of histamine, production of prostaglandins and leukotrienes which aid the killing of parasites. Binding to the low affinity IgE receptor (FcεRII, CD23) on B-cells, T-cells and a number of other immune cells increases antigen processing (284) and induces IgE dependent cellular cytotoxicity, lysis and clearance (384). IgE is also an activator of allergic responses. Loss of control of IgE production leads to excessive serum IgE and binding to FcεRI on mast cells and FcεRII (CD23) on B cells. Cross linking with allergen on FcεRI leads to immediate type hypersensitivity reactions, release of mediators and allergic responses (1,269,319,384). IgE complexes binding to FcεRII increases antigen

processing by non specific B cells and prolongs the allergenic immune response (284,309,402).

IgG and IgG Subclasses.

The emergence of the human IgG subclasses has given a selective advantage in host defence against invading pathogens. The human IgG subclass heavy chain constant region genes are coded for by distinct gene loci. Their functional differences are a direct result of structural changes in the constant and hinge regions brought about by changes in the amino acid sequence (53). The hinge is extremely important in effector function. Segment flexibility correlates with the ability to bind complement. If the hinge is deleted, the IgG molecule becomes a rigid T shape which is unable to activate complement or bind to monocytes (170). IgG1 has an extended hinge region with non co-planar Fab arms. IgG3 has an extremely long hinge region of some 90 Å which has arisen by multiple duplications of the original hinge exon. Both IgG1 and IgG3 have flexible hinge regions and are good mediators of effector function, that is complement fixation and binding to Fcγ receptors. Both IgG2 and IgG4 have short hinge regions and are poor mediators of effector function. The IgG2 hinge is particularly rigid and inflexible due to lack of glycine residues (Figure 1.5.). The classical pathway of complement is activated by the binding of C1q to aggregated or associated IgG in the CH2 domain. The flexibility of the IgG1 and IgG3 Fab arms allows optimal binding for C1q while the rigidity of IgG2 increases steric hindrance. IgG4 does not bind C1q and does not activate complement. This is probably because the IgG4 Fab arms are too close to the CH2 binding site on the Fc. Staphylococcal protein A binds to the CH2 and CH3 domains of IgG1, IgG2 and IgG4 but does not bind to IgG3 (Caucasian) due to loss of the contact residue in the CH3.

IgG subclasses bind to Fcγ receptors with variable affinity (IgG1 > IgG3 > IgG4 > > IgG2). This is not due to the structure of the hinge or flexibility of the Fab arms, as all Fcs bind with the same affinity. There are three Fcγ receptors, FcγRI (CD64), FcγRII (CDw32) and FcγRIII (CD16) (319). CD64 is the high affinity Fcγ receptor. It is found on macrophages, monocytes, neutrophils and is able to bind both monomeric and polymeric IgG. The CH2 domain of IgG1 binds to CD64 and mediates antibody dependent cytotoxicity and clearance of immune complexes. CD32 has a low affinity for aggregated IgG and exists as A, B and C forms. All three are found on monocytes and endothelial cells, the B form occurs on B cells and A and C forms on neutrophils. IgG binding to this receptor mediates phagocytosis and the oxidative burst in monocytes and phagocytes, transport of IgG across epithelial placenta and delivers a negative signal to B cells in combination with anti-Ig. CD16 (FcγRIII) also has low affinity for aggregated IgG and occurs in two forms. It is a transmembrane molecule on NK cells and macrophages, but is glycosylphosphatidylinositol (GPI) linked on neutrophils. The GPI-linked CD16 is thought to work in concert with CD32 as a capture molecule for aggregated IgG (319). IgG-antigen complexes binding to the transmembrane form mediates phagocytosis and cellular cytotoxicity.

In vivo, IgG subclasses are increased against particular antigens, and confer optimum protection. In the human. IgG1 and IgG3 subclasses are raised against protein and viral antigens (364). IgG2 is the major IgG subclass raised against carbohydrate antigens especially bacterial polysaccharides. Protection by IgG2 is seen in adults, but in young children IgG1 is protective against both protein and carbohydrate until the IgG2 response matures and takes over. IgG1 is less efficient than IgG2 at combating encapsulated bacteria with respect to Ab affinity. (172). Protection against acute and early parasitic infection is given by IgG1, IgG2 and IgG3 (normal responses to larval antigens) followed by IgE and IgG4 in response to long term exposure e.g. Schistosomiasis (70). IgG4 also increases in cases of

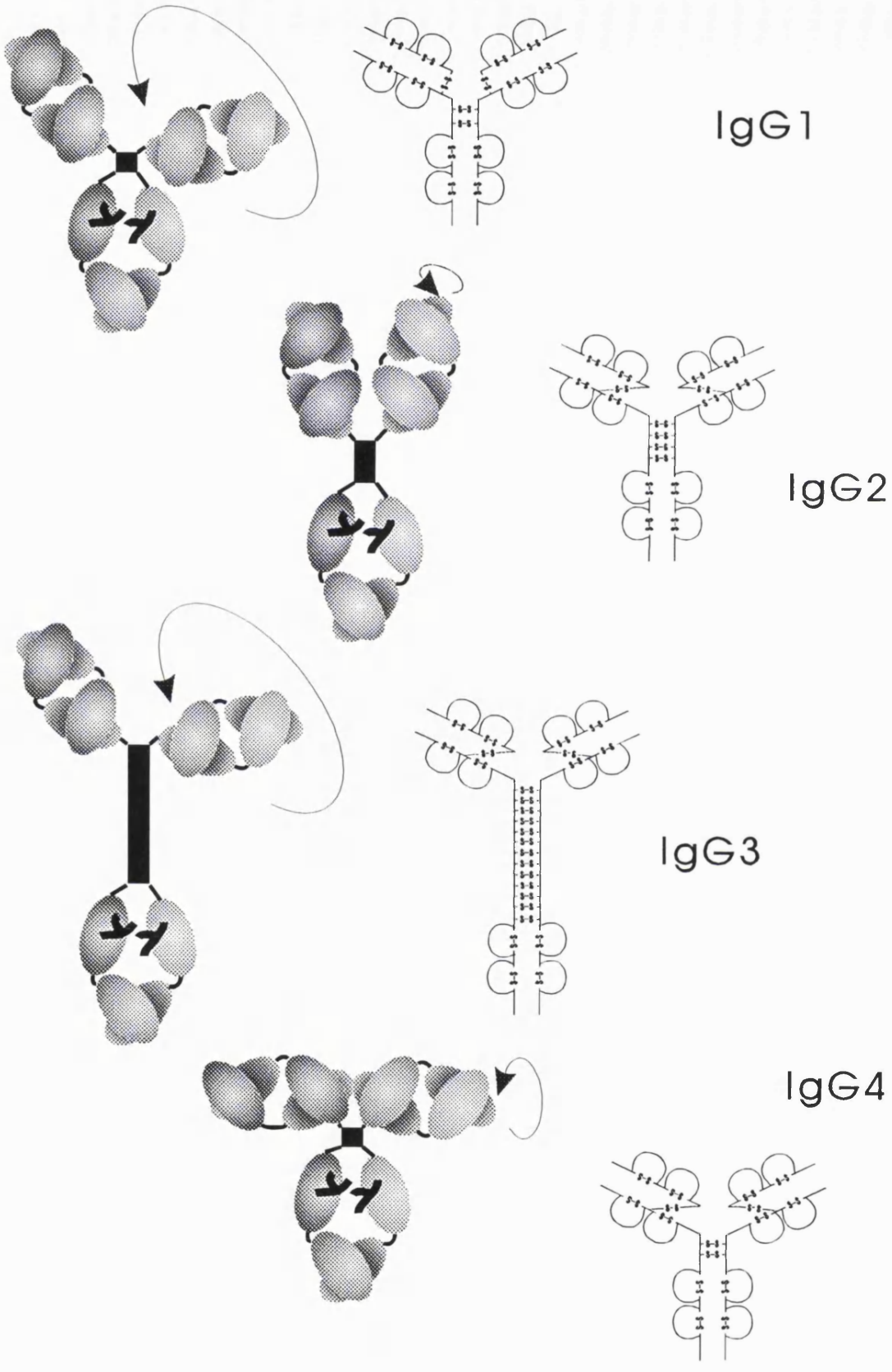


Figure 1.5. Human IgG Subclasses.

Skeleton structures on the right show the position of inter and intra chain disulphide bonds. Structures on the left show the globular Ig domains and arrows show the degree of flexibility of the Fab arms.

TABLE 1.2. PHYSICO-CHEMICAL AND FUNCTIONAL PROPERTIES OF THE HUMAN IgG SUBCLASSES.

Property	IgG1	IgG2	IgG3	IgG4
Heavy Chain class	γ1	γ2	γ3	γ4
κ:λ light chain ratio	2.4	1.1	1.4	8
Number of aa in hinge	15	12	62	12
Molecular weight (kDa)	146	146	170	146
Number of S-S bonds in hinge	2	4	11	2
% total serum IgG	60-70	20-30	5-8	0.7-4
Serum concentration (mg/ml)	5-12	2-6	0.5-1	0.2-1
Serum half life (days)	21-23	20-23	7	21
Placental transfer	+	±	+	+
Complement fixation	+++	+	+++	-
Response to carbohydrate antigens	+	+++	-	-
Response to protein antigens	+++	-	+++	-
Response to viral antigens	++	-	+++	+
Staphylococcal protein A binding	+	+	-	+
FcγRI binding (macrophages, monocytes, neutrophils)	++++	+	++++	++
FcγRII binding (monocytes, endothelial cells, B cells, neutrophils)	++++	+	++++	+
FcγRIII binding (NK cells, macrophages, neutrophils)	++++	-	++++	-

chronic antigenic stimulation (allergy) (115,287,354). The function of allergen induced IgG4 could be as a blocking antibody for IgE responses (379), but this is in dispute (213). Replacement of high affinity IgG1 with IgG4 may confer long term immunity to antigen (35,114).

1.3.3. The Immunoglobulin Gene Loci.

TABLE 1.3. CHROMOSOME LOCATION OF HUMAN AND MURINE IMMUNOGLOBULIN HEAVY CHAIN, LIGHT CHAIN AND PRE-B CELL PSEUDO-LIGHT CHAIN COMPONENTS.

	MAN	MOUSE
Lambda light chain (λ)	22q11	16
Kappa light chain (κ)	2p12	6F1
Heavy chain	14q32	12C2
Pseudo-light chains (mouse: VpreB, λ5) (human: VpreB, λ14.1)	22q11	16

1.3.4. Immunoglobulin Light Chains.

There are two light chain loci in the human and mouse genomes, κ and λ (Table 1.3). These have multiple V (variable) and J (joining) genes which code for the VL domain and C regions for the CL domain (the κ locus has only one C gene). V and

J genes are rearranged at random so that in a given number of B cells no combination is likely to occur twice and the repertoire for antigen recognition is as wide as possible. The hypervariable regions, CDR1, CDR2 and CDR3 are encoded within the V genes, and undergo somatic mutation after antigenic stimulation of the B cell. This is important in the process of Ig affinity maturation in the germinal centre where antigen binding specificity can be increased (23,212,259).

1.3.5. Immunoglobulin Heavy Chains.

There is only one locus for the immunoglobulin heavy chain in the humans and mice (Table 1.3.). The locus consists of multiple V, D (diversity) and J genes which make up the VH domain, and C genes which code for the CH domains (Figures 1.6a. and 1.6b.). V, D and J genes are rearranged at random in a similar way to the light chains, except that the presence of D regions creates an even greater diversity for Ag recognition. In the heavy chain, CDR1 and CDR2 regions are encoded in the V genes and the CDR3 region in the D genes.

1.3.6. The Heavy Chain Constant Region Genes.

There are eight functional heavy chain constant region genes in the mouse encoded by Cμ, Cδ, Cγ3, Cγ1, Cγ2b, Cγ2a, Cε and Cα (Figure 1.6a.). The human has nine functional and two non functional pseudo genes encoded by Cμ, Cδ, Cγ3, Cγ1, ψε, Cα1, ψγ, Cγ2, Cγ4, Cε, and Cα2 (Figure 1.6b.). Heavy chain constant region genes are not rearranged in immature B cells, but can be changed later in B-cell maturation by a process called Ig class switching which is under antigen dependent control. This allows retention of an antigen binding site of high affinity while changing the effector function of the Ig molecule.

Figure 1.6.a. The Mouse IgH Locus.

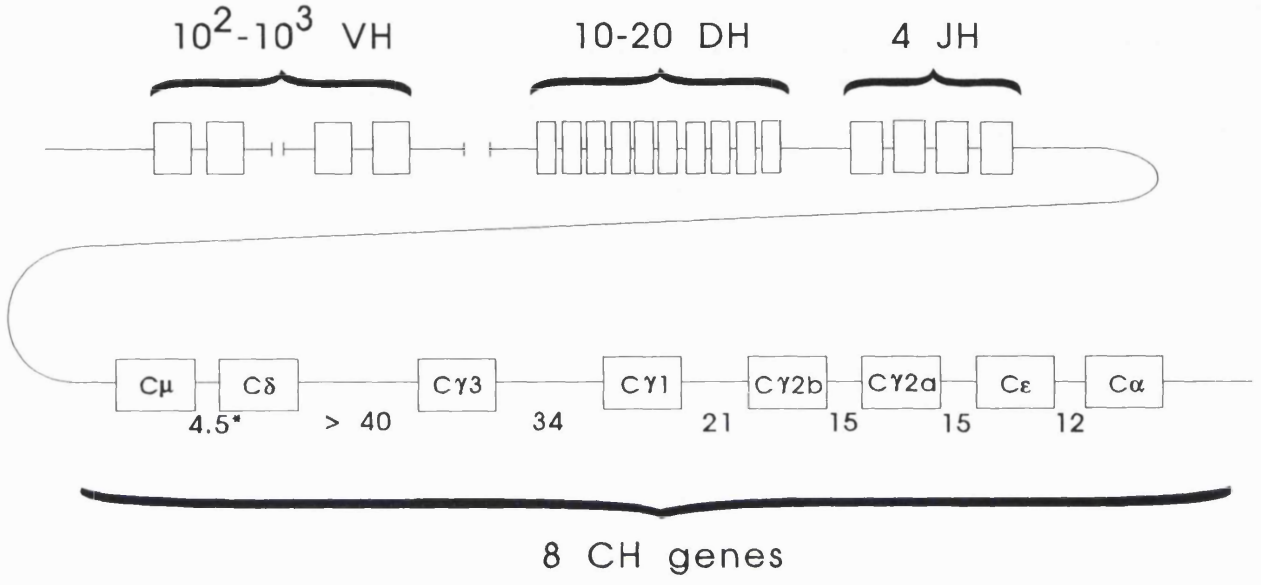
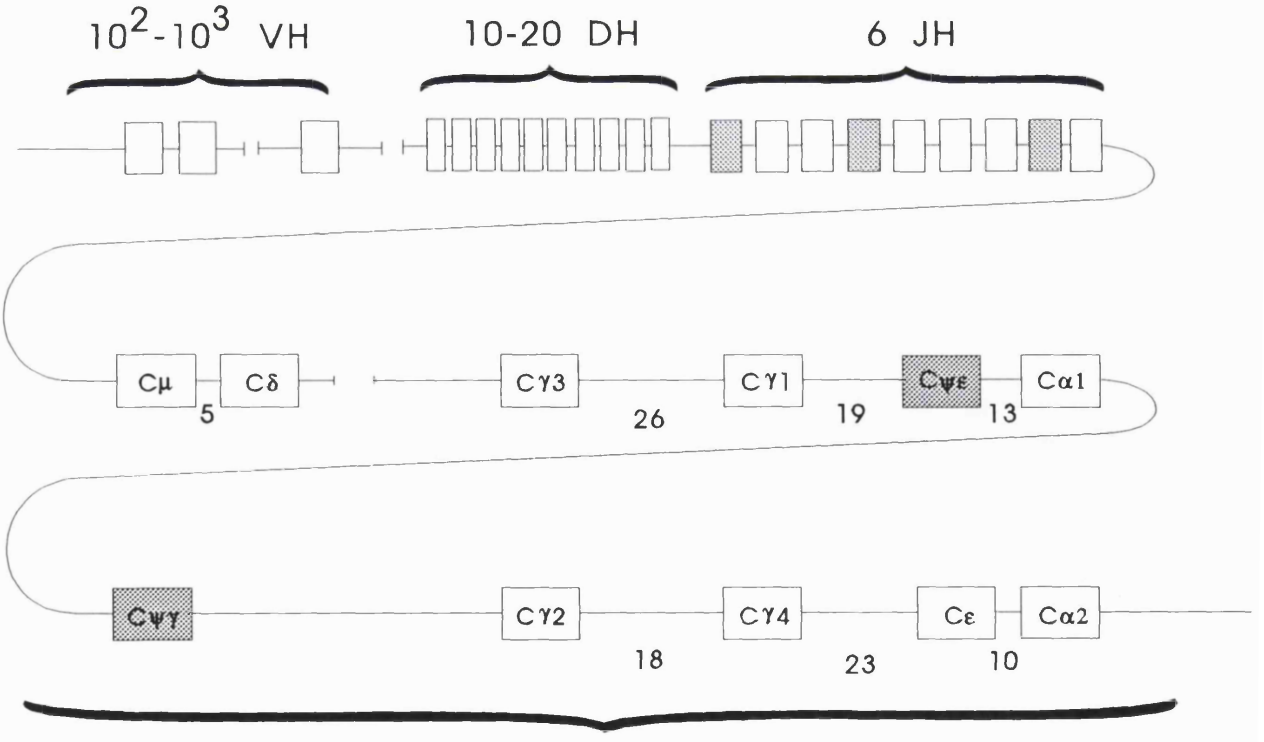


Figure 1.6.b. The Human IgH Locus.



Pseudogenes are shown as shaded boxes, *intron lengths as given in kb. Figure adapted from Owen and Lamb 1988.

1.3.7. B Cell Ontogony and Antigen Independent Maturation.

B-cell progenitor maturation is signalled by an antigen independent ordered cascade of immunoglobulin gene rearrangements accompanied by the differentially controlled expression of cell surface antigens (Reviewed by Uckun 1990 (397)) (Figures 1.7. and 1.8.). Pro-B cells are the first identifiable B cell precursors. They express MHC Class II, CD10 and CD34 but their heavy chain genes still retain germline configuration on both chromosomes.

Heavy chain rearrangements first take place on both maternal and paternal chromosomes. The sequence of gene rearrangement has been clarified by the use of murine B cell lines, plasmacytomas, hybridomas and transformation of mouse pre-B cells by AMuLV (232,298,432). Firstly, D to J gene joining takes place. This is followed by V to DJ joining to give a VDJ rearranged gene (3) which can be transcribed with the heavy chain constant region gene Cμ to give a productive VDJCμ mRNA. A productive mRNA, with VDJ transcribed in a viable reading frame, is able to produce a protein product which can be expressed, first as μ-heavy chain in the cytoplasm, and later on the cell surface with the pseudo-light chain or κ and λ (317,331).

Pseudo-light chain genes, which do not rearrange, are expressed early in the pre-B cell (VpreB and λ5 in the mouse and VpreB and λ14.1 in the human, (Table 1.1)) (171). Their protein products may associate with the newly synthesised μ heavy chains to form a cytoplasmic or membrane complex. This occurs before the appearance of immunoglobulin light chain rearrangements. (171,267,349,392). VpreB has sequence homology to the light chain V regions and λ5 and λ14.1 to the light chain J and C regions (267). The protein product of λ5, ω, is disulphide bonded to the μ-heavy chain and the protein product of Vpre-B, ι, is held in non covalent association (73), to form a heavy/pseudo light chain heterocomplex (204).

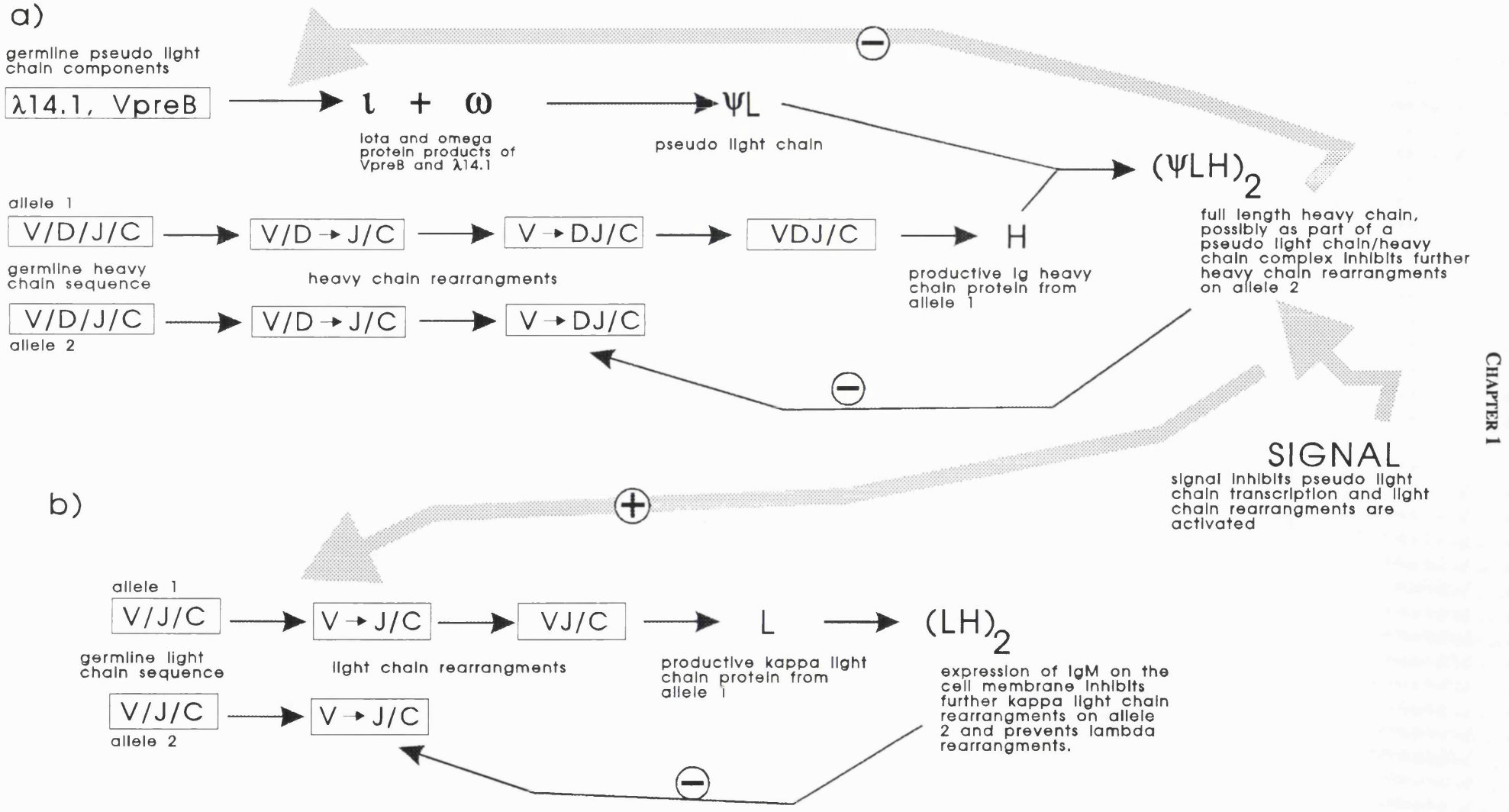


Figure 1.7. Events leading to IgM expression on the immature B Cell.
a) Heavy chain rearrangments and expression of pseudo light chain components.
b) Light chain rearrangments and expression of the mature IgM molecule.

Appearance of the heavy chain protein product on the B cell surface, either alone, or possibly in combination with the pseudo light chain components, prevents further heavy chain gene rearrangements on the other allelic chromosome (Figure 1.7a.). The B cell may be held in stasis at this point in maturation as a self renewing pool. When further maturation is required, a signal, possibly through a surface pseudo light chain heavy chain complex, may inhibit further transcription of ι and ω and induce light chain rearrangements (73) (Figure 1.7b.). κ light chain genes rearrange first in a similar fashion to the heavy chains. If the κ light chain product is non functional then λ genes rearrange. Light chain products become associated with heavy chains in the endoplasmic reticulum (ER) and are transported to the cell membrane. When functional antigen receptors are expressed further light chain rearrangements are inhibited (317,331,357,392).

1.3.8. Antigen Dependent Maturation

As soon as functional IgM (antigen receptor) is expressed at the B cell surface, B cells are subject to antigen controlled development (Figure 1.8.). Immature B cells express only IgM, and it may be this stage which is important in tolerance to self antigens. Mature B cells express both IgM and IgD. This stage is marked by a change in DNA transcription. mRNA is not terminated at the μ stop codon to the 5' of the Cδ gene, but read-through occurs creating a primary mRNA transcript with both Cμ and Cδ constant regions. Alternative splicing of the primary transcript deletes either Cμ or Cδ RNA and either a μ or δ heavy chain protein is synthesised and expressed with light chains on the surface (226,254,298). At this stage B cells may take part in the primary immune response.

Cross linking of the antigen receptor with antigen signals to the B cell and activates it. Site specific mutation in either the transmembrane region or the cytoplasmic tail

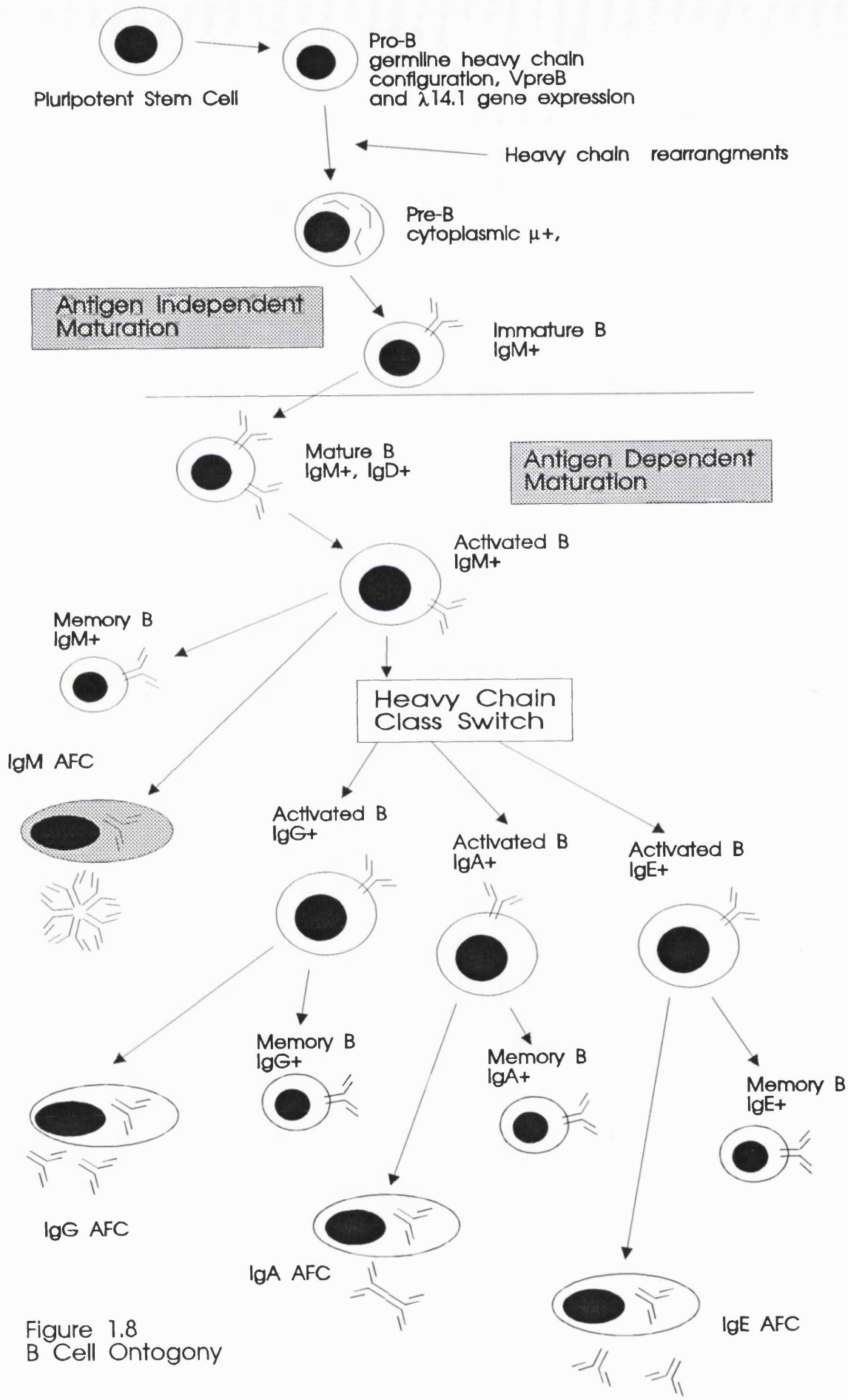


Figure 1.8
B Cell Ontogony

of IgM indicates that these sections are critical for signal transduction. The IgM associated phosphoproteins, Igα or MB1 (coded for by the mb1 gene) and Igβ or B29 (coded for by the b29 gene) are associated to form a linked heterodimer and are responsible for signal transduction of signals passed through the antigen receptor (323,324,341)(Figure 1.3a.). The Igα and Igβ heterodimer may also be involved in signalling through the pseudo-light chain complex in pre-B cells (180). The MB1 protein may be required for transport and insertion of these complexes into the membrane (426). Although the IgM and IgD complexes on the mature B cell share the same associated proteins, other proteins may be present which confer different biological responses for tolerance, activation and memory mechanisms (424).

Cross-linking of the Ig molecule with antigen and activation by T cells through cell/cell interaction and also by cytokines, causes B cell activation, proliferation and loss of IgD (44,277). Under the correct conditions B cell clones will differentiate into antibody forming cells (AFCs) or plasma cells. The shift from membrane IgM (mIgM) to secretory IgM (sIgM) is achieved at the RNA processing level and by post translational regulatory control (298).

Activated, mature B cells primarily secrete IgM, the first immunoglobulin to be secreted in an immune response. IgM^+ B cells can also undergo heavy chain class switching to become IgA, IgG and IgE AFCs and to generate IgA^+, IgE^+ and IgG^+ memory cells (Figure 1.8.). The function of immunoglobulin heavy chain class switching is to transfer an antigen binding site to a class of heavy chain which gives maximum protection against a specific pathogen (see Section 1.3.2.). Immunoglobulin class switching is controlled by Th cells and immune cells by cell/cell interaction and also by cytokines.

1.3.9. Mechanisms of Immunoglobulin Heavy Chain Class Switching.

Immunoglobulin heavy chain class switching allows an Ig molecule to change effector function whilst retaining specific high affinity antigen binding sites. Ig heavy chain class switching can take place at either the DNA or RNA level. The most common mechanisms are outlined below.

Class Switching at the RNA Level.

1)Alternative Splicing. Transcription of part or the entire heavy chain locus takes place producing a very long primary RNA transcript containing the VDJ and some or all of the heavy chain constant region genes. Alternative splicing of the large primary transcript, controlled by receptor/ligand binding responsive elements, produces a truncated mRNA with the VDJ region spliced onto the appropriate constant region exons, and deletion of the intervening RNA (128,175,226,254). This mechanism is used by B cells for the dual expression of IgM and IgD (226,254).

2) *Trans*-Splicing. Transcription of the heavy chain VDJ locus and one heavy chain C region produces a complete heavy chain pre-RNA. In addition there is transcription of one or more heavy chain constant region genes, possibly controlled by receptor signalling response elements. The pre-RNA VDJ region may be trans-spliced onto another pre-RNA C region to produce a heavy chain mRNA of alternative isotype (128,173,175,297,360,366).

Class Switching at the DNA Level

1) Unequal Sister Chromatid Exchange and Homologous Chromosome Recombination. Both these mechanisms involve unequal crossing over and recombination of DNA at sites of homology upstream of each heavy chain constant region gene. Sister chromatid exchange involves recombination of sister or duplicated chromosomes during metaphase. Homologous chromosome, or trans-switch, recombination involves recombination between the active and inactive allelic chromosomes either prior, or during, mitosis. Both mechanisms result in the removal or transfer of C genes to the productive allele, changing the class of heavy chain transcribed (128,175).

2) Site Specific Recombination: The Accessibility Model of Ig Heavy Chain Class Switching (Figure 1.9.). Signalling through a cell surface receptor activates a responsive element which initiates the opening of, or accessibility to, the DNA at specific S (switch) sites. S sites are tandemly repeating core oligonucleotide sequences stretching for 1kb (Sε) to 10kb (Sγ) which are located 5' to each heavy chain constant region gene except IgD (128,175,263,362,370). Initial opening of DNA at the S sites allows transcription of individual constant region genes, producing non productive, germline RNA (sterile transcripts). Switch site recombination may occur via a common switch recombinase enzyme, whose access to the DNA is carefully controlled, instead of site specific switch recombinases controlled individually. All heavy chain class switching may be effected by a common recombinase but under specific control by cytokine responsive elements (175). Active cell division or mitosis, involving the unwinding of chromatin during chromosome replication, may be needed to allow coming together, homologous recombination of the S sites and deletion of the intervening circular DNA (27,156,175,263).

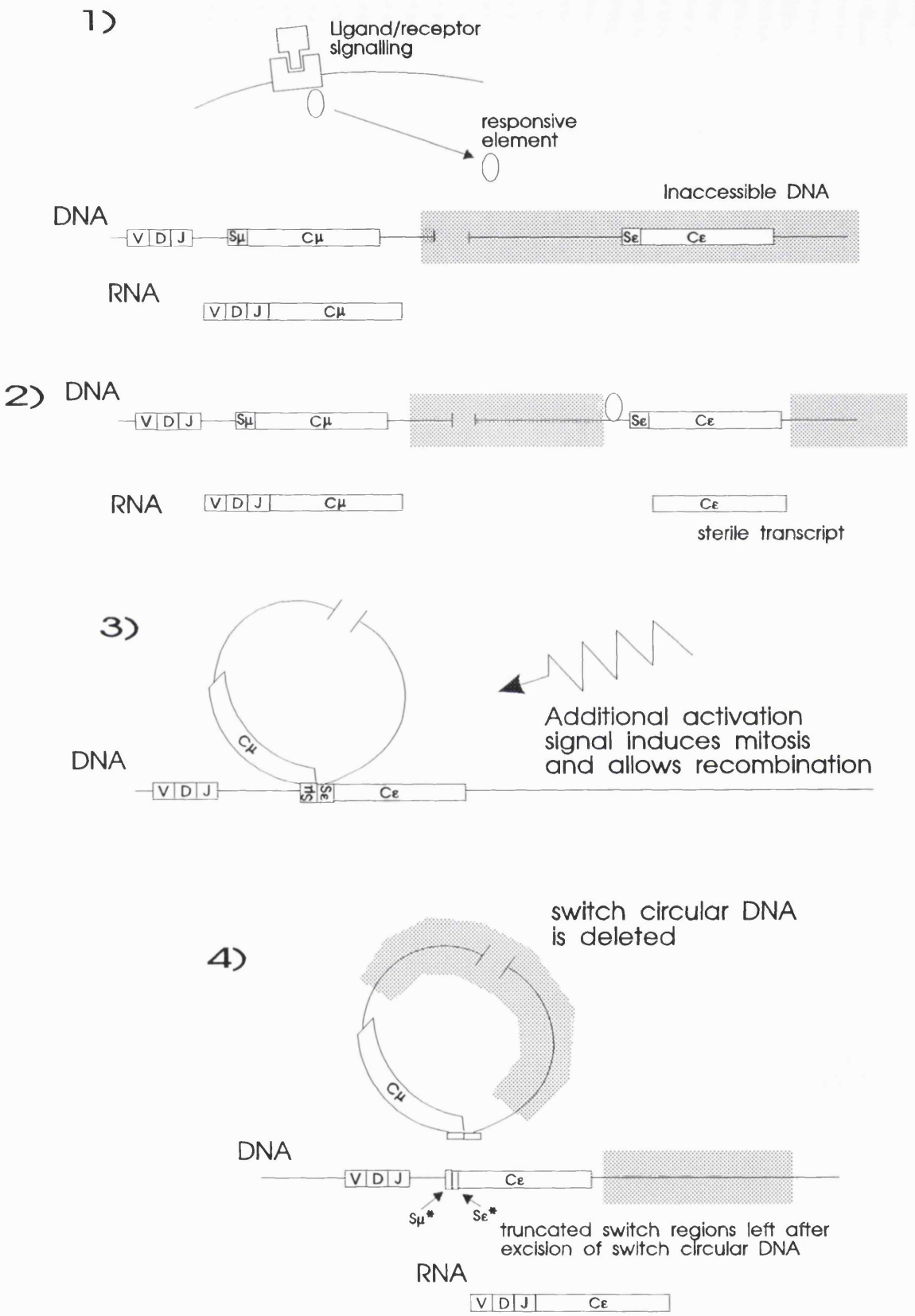


Figure 1.9. Steps involved in the accessibility model for human Ig heavy chain class switching.

Cytokine responsive elements could control heavy chain class switching by increasing DNA accessibility upstream of specific heavy chain C region genes. In humans, a novel IL4 responsive element has been found 5' to the ε switch site which appears to play an important role in sterile transcript induction and switch recombination (187). Accessibility can be induced by creating a) nucleosome free DNase I hypersensitive sites for recombinase and RNA polymerase access (28,142), or b) demethylation of the DNA at switch sites. The Ig and T cell receptor (TcR) genes are methylated in germline and lymphoid cells before activation and become demethylated after demethylation of the lymphoid recombinogenic sequences (e.g. Ig switch regions). This phenomenon is highly specific to cells of the lymphoid lineage and such sequences are demethylated normally in other lineage cells such as hepatocytes and kidney. There is significant correlation between demethylation and Ig gene transcription in B-lymphocytes (52,128).

Finally, it is thought that sterile transcripts themselves may play more than a bystander role in immunoglobulin heavy chain class switch mechanisms. Sterile transcripts occur in both mouse and human B cells (153,196,239,241) and may be important regulators of heavy chain class switching (214,335,362). Sterile transcripts may be *trans*-spliced onto the VDJ of the current heavy chain RNA to allow transcription of the new Ig isotype alongside the old before DNA recombination takes place (366,385). This would create intermediary double isotype cells (72,297). Sterile transcripts may also a) interact with factors important in targeting the locus for recombination and/or b) interact with the DNA and stabilise chromosome structure for recombination and other DNA binding proteins involved in recombination (335).

1.4. ANTIBODY RESPONSES ARE CONTROLLED BY T CELLS.

Mature T lymphocytes direct both the humoral and cellular immune responses. There are three major species;

1) Cytotoxic T cells (Tc). These are $CD3^+$,$CD8^+$ T cells which recognise antigen presented in combination with the MHC class I heterocomplex. They destroy cells which express foreign antigen, such as virally infected self and engrafted cells. Once activated these cells can develop their cytotoxic activity, proliferate and generate memory.

2) Helper T cells (Th). These cells are $CD3^+$,$CD4^+$ and recognise antigen presented by APCs in combination with the MHC class II complex. Once activated, Th cells can activate either B cells or Tc cells by T/B or T/T cell contact and secrete cytokines which can favour the humoral or the cellular immune response.

3) T suppressor cells (Ts). These $CD3^+$ T cells carry no specific Ts phenotype in the human and can be $CD8^+$ or $CD4^+$. Their mechanism of antigen recognition is unclear, but they suppress Tc and Th responses.

1.4.1. Helper T Cells.

Th cells can be further subdivided into three groups, Th1, Th2 and Th0 in the mouse (281,381) and the human (332,425). These numbered groupings relate to the T cell derived factors or cytokines secreted by each grouping. Differential cytokine production by Th1 and Th2 cells can determine the host's defence mechanism to antigen and regulate that response (352). Generally, Th1 cells secrete pro-inflammatory and cellular immune response promoting cytokines such as TNFβ (lymphotoxin), IL2 and IFNγ. Th2 cells secrete humoral immunity promoting cytokines such as IL4 and IL10. Th0 cells may secrete some or all of these factors and may mark a pre-Th1/Th2 stage in T cell development.

The T cell receptor (TcR/CD3) recognises Ag peptide presented in the groove of MHC class II by an APC. Macrophages and monocytes, although not as efficient as B cells, are generally the first APCs to process Ag by early contact in the periphery or on mucosal surfaces. It is thought that Ag presentation can determine Th1 and Th2 cell responses and recent work with human lymphocytes indicates that this may be partially directed through IL12 (155,258). For example, *Listeria monocytogenes* ($Gram^+$ bacteria) induces IL12 production when taken up by macrophages, which promotes Th1 cells and production of IFNγ (182). IFNγ also promotes pro-inflammatory cytokine production by inhibiting Th2 cell expansion (130) and IL10 production by monocytes (75). Hence IL12 promotes mobilisation of the cellular immune response. Promotion of the Th2 phenotype and humoral immune response may be controlled by IL10. Monocytes and macrophages also secrete IL10. IL10 selectively prevents antigen presentation by Mϕ (but not B cells), to T cells, by inhibition of MHC class II expression (97,98,181,389). It also specifically inhibits the Th1 product IFNγ (138,183,389) as well as monocyte and macrophage derived IL12 and pro-inflammatory cytokines. IL4, a major Th2 product, also inhibits IFNγ production (412).

The cellular and humoral immune systems are carefully controlled (352). The humoral immune system, once activated, must quickly provide high numbers of specific antibody with a heavy chain class which gives optimum protection. The signals which induce B cells to undergo heavy chain class switching may be given by immune cell derived cytokines and cell/cell contact. *In vitro*, substitution of polyclonal B cell activators for T cells provides a system in which to determine the properties of individual cytokines and functional antigens.

1.5. POLYCLONAL B CELL ACTIVATORS

In order to investigate the signals which regulate Ig class and IgG subclass responses it is important to develop a suitable system for *in vitro* Ig secretion. Activation of resting B cells *in vivo* comes about through signalling by T cell-B cell contact followed by further progression signals delivered through other cell surface molecules or soluble factors, such as cytokines. B cells are induced to enter the cell cycle, proliferate, then differentiate into antibody forming cells (AFC). A B cell activation system in which the direct effect of cytokines on B cells can be examined without the interference of T cells is important. In this Section the most common B cell mitogens are summarised and the rationale for the use of EBV as a T cell independent polyclonal B cell activator put forward.

1.5.1. Chemical Mitogens.

Chemical mitogens such as the phorbol esters, PMA and TPA, and the calcium ionophore, ionomycin, mimic natural B cell signalling pathways by bypassing the B cell membrane and directly activating protein kinase C and inducing intracellular Ca^{2+} flux. PMA alone is mitogenic at high concentrations of > 2ng/ml and induces B cell proliferation. Submitogenic doses of PMA (0.5 ng/ml) in combination with ionomycin deliver a much greater signal inducing dramatic B cell proliferation rather than differentiation into antibody secreting cells (66,83,330). However, the addition of cytokines to this system can induce Ig production (141,250). Chemical mitogens have the considerable advantage of being completely T cell independent and stimulating all B cells. However activation of PKC may interfere with other signal transduction pathways. Two other mitogens which might be included in this Section are the man-made polymers, dextran and ficoll. These

are so called T-independent antigens, and although they do not need antigen specific T cell help, are still T-cell dependent through non cognate interactions (50).

1.5.2. Plant and Bacterial Mitogens.

Plant and bacterial mitogens include Pokeweed Mitogen (PWM), Lipopolysaccharide (LPS), *Staphylococcus aureus* Cowan strain (SAC), Staphylococcal protein A (SPA), *Branhamella catarrhalis** (BC), Bacto-streptolysin O (STO) and *Nocardia* water soluble mitogen (NWSM).

PWM, an extract of Pokeweed (*Phytolacca americana*), is strongly mitogenic in mixed lymphocyte cultures inducing B cell proliferation and high antibody titres. Stimulation of purified B cells with PWM has no effect showing it to be T cell and accessory cell dependent. It also has the tendency to stimulate high IgG1 and IgM secretion (211,312,351,417). LPS is commonly used for murine B cell activation. It has an extensive polysaccharide structure with many side chains and may act by cross linking membrane immunoglobulin. It is T cell independent and induces both proliferation and Ig production by mouse B cells but does not have comparable effects on human B cells. LPS may also influence the Ig isotype secreted by B cells by activating a specific subset of B cells (61,127,131,351). SAC preparations are inactivated bacteria carrying large polysaccharide structures in their outer coat and may cross link the antigen receptor in a similar way to LPS. SAC induces B cell proliferation and Ig secretion in the absence of T cells, and may possibly influence the Ig isotypes secreted by activation of a B cell subset (61,207,351). BC is a bacterial preparation consisting of whole organisms or their cell walls. It is T cell dependent, tends to induce IgM secretion and acts predominantly on small resting B cells (62). SPA is a protein taken from the coat of the *Staphylococcus* bacteria which can bind to some but not all classes of human Ig and hence would be

* *Branhamella catarrhalis* is now classified as *Moraxella catarrhalis*.

restricted to B cells carrying these Ig. It is T cell dependent (333). SLO and NWSA, also bacterial products, are also T cell dependent mitogens (351,417).

1.5.3. Antibodies and Ligands to B Cell Surface Antigens.

Anti-Ig and anti-CD40 are most frequently used. Anti-Ig is usually anti-IgM and induces B cell proliferation by cross linking of IgM on the B cell surface. Activation is T cell independent but B cells do not differentiate into AFCs and stimulation is restricted to $mIgM^+$ cells. The best results are obtained with anti-Ig conjugated to sepharose beads or dextran (50,62,83,162,207). Anti-CD40 antibodies have also been used as polyclonal B cell activators (206,242). The best system for activation uses anti-CD40 antibodies held on the surface of CD32 (FcγRII) transfected mouse fibroblast L cells. The multiple cross linking which occurs when B cells bind via CD40 induces T cell independent B cell proliferation and Ig production (14,336). The natural ligand for CD40, CD40L has similar activities to anti-CD40, is T cell independent and induces human and mouse B cell proliferation and differentiation (4,238,255). C3dg, the complement ligand for CD21 and anti-CD21 do not induce polyclonal activation alone but co-stimulate with anti-IgM and PMA to augment proliferation (39,67,68).

1.5.4. Epstein Barr Virus (EBV).

EBV is a B-cell lymphotropic human (gamma) herpes virus 4 and was first recognised by Epstein, Achong and Barr in 1974 (126). It is an icosahedral encapsulated dsDNA virion of 172.2 Kbp encoding some 50-200 different proteins. The virus itself is pan-endemic in the human population, possibly having infected up to 90% of the worlds population at some time. It is largely asymptomatic, but may

cause Infectious Mononucleosis (glandular fever) and post viral fatigue syndrome as well as the human cancers African Burkitt's Lymphoma and nasopharyngeal carcinoma. *In vitro*, EBV is a completely T cell independent mitogen for human B cells (33,218). However, due to its pan-endemic frequency all T cell activity must be removed from B cell cultures because memory T cells become activated and inhibit EBV induced proliferation and secretion of immunoglobulin by killing virally infected B cells (417).

EBV gains entry to the host cell by binding to CD21/CR2, the B-cell receptor for the complement C3d fragment and CD23 (165,188). Binding initiates rapid internalisation and decapsulation. The virus uses host RNA polymerase to make encoded proteins involved in nucleotide metabolism which stimulate the host into cell cycle. The viral dsDNA replicates in the nucleus using the host DNA replication equipment. B-cells activated by the virus *in vitro* display EBV nuclear antigen (EBNA) on the cell surface followed by increased RNA production then DNA replication and proliferation and finally differentiate into Ig producing cells (34). EBV binds to and activates a size heterogeneous population of B cells (11,12,71,275). It also binds to B cells at all stages of cell cycle except for cells in S-G2 due to loss of CD21, (69,188) and activates B cells without preference for B cell subsets expressing different immunoglobulin classes and subclasses (69,275,289). Most importantly B cells which have been activated with EBV are not prevented from further maturation and response to cytokines or other additional added factors and retain the ability to undergo heavy chain class switching *in vitro* (11,71,275,380).

Early events in EBV infection involve polyclonal activation giving B cell proliferation and Ig secretion. Only a small number of B cells subsequently become transformed into B-lymphoblastoid cell lines (BLCLs). EBV preferentially transforms resting $IgM^{+}IgD^{+}$ B cells suggesting that these cells are more likely to

supply the correct conditions for EBV transformation and why most BLCLs are IgM secreting rather than IgG, or IgA (71).

1.6. CYTOKINE REGULATION OF B CELL ACTIVATION AND DIFFERENTIATION.

B cells have specialised immune functions. They present antigen, undergo somatic mutation during antibody affinity maturation, generate memory, re-circulate to tissues requiring specific responses (e.g. IgA in the mucosa) and are involved in tolerance mechanisms. B cell mitogens do not induce Ig isotype switching (e.g. EBV), but provide an environment in which the signals responsible for directing B cell responses can be studied. These signals may be delivered by cytokines such as IL4 and homologous or heterologous cell/cell ligand interaction (eg.CD40/CD40L, CD23/IgE or CD23/CD21).

Many different recombinant cytokines have been shown to function at strategic points in B-cell maturation. Interleukin 2 (IL2), Interleukin 4 (IL4), Interleukin 5 (IL5), Interleukin (IL6), Interleukin 10 (IL10), Interleukin 13 (IL13), interferon gamma (IFNγ), interferon alpha (IFNα), transforming growth factor beta (TGFβ) and nerve growth factor (NGF) are involved in the control of mature B-cell responses to antigen. These cytokines have been implicated in Ig production control either in the human or the mouse or both. (Figure 1.10.). Other cytokines are involved in early B cell maturation such as IL3 (a colony stimulating factor involved in differentiation of lymphoid progenitors) and IL7 (a pre-B cell growth factor) (101,422).

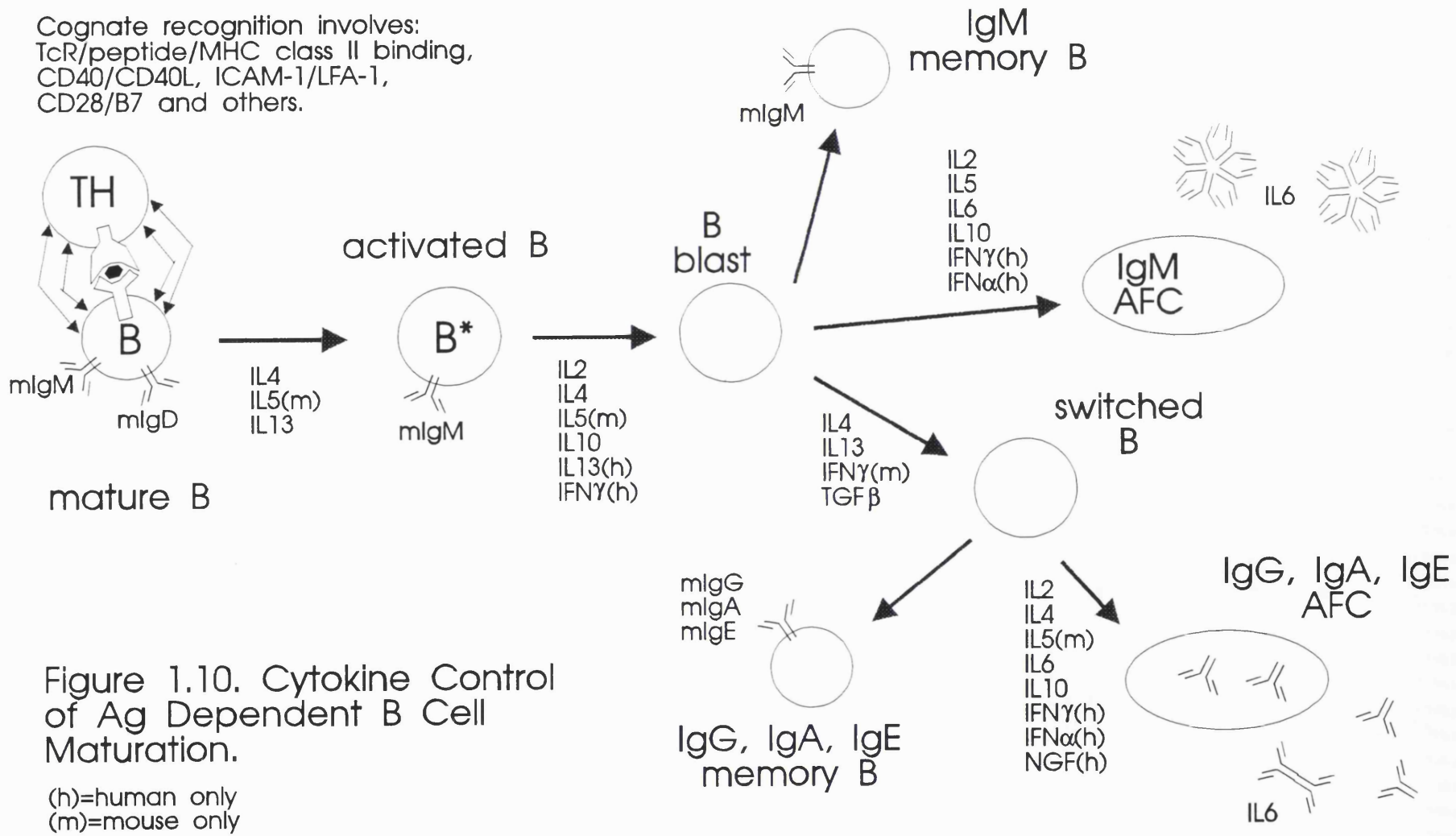


Figure 1.10. Cytokine Control of Ag Dependent B Cell Maturation.

(h)=human only
(m)=mouse only

TABLE 1.4. PHYSICO-CHEMICAL PROPERTIES OF HUMAN CYTOKINES INVOLVED IN B CELL REGULATION.

Cytokine.	aa number, expressed (pre-cursor).	Molecular weight (kDa), expressed (pre-cursor).	N or O linked poly-saccharide.	Cysteine residues (disulphide bonds).	Chromosome location/exon number.	Homology to mouse (%).	Cross-reactivity between human and mouse responses.
IL2	133 (153)	15-20 (15.4)	O	3 (1)	4q26-q27/4	60	yes
IL4	129 (153)	15-19 (15)	2N	6 (3)	5q23-q31/4	46	none
IL5	115 (134)	45 homodimer (13.1)	2N	2 (1 interchain)	5q23-q31/4	71	yes
IL6	184 (212)	21-28 (20.8)	2N	4 (2 potential)	7p21-p14/5	41	human in mouse only
IL10	160 (178)	35-40 homodimer (18)	1N potential	?	located on Ch.1/?	73	human in mouse only (vIL10 in both)
IL13	131 (112)	9-17 (12.3)	4N	4 (2)	5q23-q31/4	58	?
TGFβ1 TGFβ2 TGFβ3	112 (391) 112 (412) 112 (412)	25 homo or heterodimer (12.5)	-	9 (1)	19q13.1/? 1q41/? 14q24/?	99	yes
IFNα	165-172 (188-195)	16-27 (19.2-20.1)	0-1N	4/5 (2)	9p22-p13/? (24 genes)	62	yes
IFNγ	143 (166)	20-25 monomer, 40-70 dimer/ multimer (17)	2N	2 in pre-protein, 0 in expressed	12q24.1/4	40	none
NGF	120 (241)	26 homodimer (13.5)	3N	6 (3 inter chain)	1p13/2	90	yes

Table compiled from 13, 16, 56, 57, 96, 102, 130, 178, 236, 265, 266, 272, 278, 342.

1.6.1. IL2.

The IL2 gene exists as one copy in the human genome coding for a mature protein of 133aa of 15-20 kDa (Table 1.4.). The protein has one intramolecular disulphide bond between cysteine residues C58 and C105 which is essential for activity, and one O-linked polysaccharide at position 3. The IL2 receptor is composed of 3 chains, the α-chain (p55, Tac, CD25), β-chain (p75) and γ-chain (p64). Tac and p75 bind IL2 with low affinity (Kd=1.4 x 10^{-8}M and 1.2 x 10^{-7}M). The β-chain is a member of the Type-1 cytokine receptor super family (originally the haemopoietic growth factor receptor super family) which includes IL3R, the high affinity IL4R, IL5R, IL6R, IL7R, G-CSFR (granulocyte colony stimulating factor receptor), EPO (erythropoietin receptor) and GM-CSFR (granulocyte-macrophage-CSF receptor) (274). The γ-chain does not bind IL2 but increases the affinity of the other chains for IL2 (414). The Tac/p75 heterodimer forms the intermediate affinity IL2R (Kd=10^{-9}M) and the Tac/p75/γ-chain heterotrimer forms the high affinity IL2R (Kd=1.3 x 10^{-11}M). The γ-chain is also a shared component with the IL7R (295) and high affinity IL4R, also members of the cytokine receptor super family (230,340). IL2 is produced by activated $CD4^+$ and $CD8^+$ T cells and its receptor is found on B cells, T cells, NK cells, monocytes and macrophages (16,56,286,418). The IL2R γ-chain is critical for T cell thymic development and a deletion or mutation in its gene results in X-linked severe combined immunodeficiency (XSCID) (63,294,296,313).

Although IL2 was first defined as a T cell growth factor (TCGF-1), it has been shown to stimulate B cell growth and differentiation. IL2 is a BCGF of SAC (198,271), anti-Ig (45,273,288) and PMA (387) activated human peripheral blood and tonsillar B cells. It is an early acting factor, losing its BCGF activity when added later than 3 days after activation (273). Activated B cells, however, express a much lower number of IL2 receptors in comparison to activated T cells and do not proliferate as readily in response to IL2 (387).

IL2 is also a BCDF for normal B cells and some B cell lines. It induces the B lymphoblastoid cell line 5B4 to secrete IgM (418) but is not able to induce Ig secretion by CESS (288). IL2 increases IgM IgG1, IgG3 and some IgA from $PdBu_2$ and ionomycin activated tonsillar B cells (141). A more comprehensive study of the effect of IL2 on human IgM and IgG subclass secretion by Calvert *et al.* (61) showed that overall, IL2 stimulated IgM and IgG1, IgG2, IgG3 and IgG4 subclasses from SAC and LPS activated spleen, tonsil and blood B cells but that production of certain Ig isotypes was tissue and activator specific. There is no evidence that IL2 can direct Ig heavy chain class switching or selectively induce secretion of particular Ig isotypes.

IL2 is also a T cell replacing factor in the specific secondary antibody response to influenza, inducing specific IgM and IgG1 production. Interestingly, IL2 is only a TRF for low buoyant density activated B cells and not high buoyant density resting B cells (58,59). This may be due to the presence of the IL2 receptor on activated B cells only.

1.6.2. IL4

The IL4 gene codes for a mature protein of 129aa of 15-19 kDa (Table 1.4.). It is localised on 5q23-q21 and very close to the genes for IL5, IL3, IL13 and GM-CSF. The protein has three intramolecular disulphide bonds between cysteine residues C3-C127, C24-C65 and C46-C99, and two N-linked polysaccharides. IL4 is produced by activated $CD4^+$ T cells, mast cells, bone marrow stromal cells and B cells. There may be two receptors for IL4, a high affinity receptor, gp140, ($Kd=10^{-10}$-$10^{-11}M$) which has been cloned (147), and a proposed low affinity receptor ($Kd=10^{-8}M$) (129). The high affinity receptor is a member of the type I cytokine receptor super family and has the IL2R γ-chain as a functional component

(230,274,340). There is evidence that the two IL4 receptors may use different signal transduction pathways (327). The high affinity IL4R receptor is found on precursor and mature B cells, T cells, NK cells, monocytes, macrophages, granulocytes, eosinophils, endothelial cells and fibroblasts (16,56) and is homologous with the mouse IL4R (280).

IL4 is a mouse and human B cell activation factor. In the absence of B cell activators, IL4 induces resting B cells to enter the early G1 stage of the cell cycle and increase human B cell surface CD23, IgM, CD40 and MHC class II expression (36,83,358) and mouse CD23 expression (186).

IL4 is also a mouse and human B cell growth factor. It cannot induce proliferation alone but co-stimulates with anti-Ly6A/E (86), PMA or LPS (223,388) to induce murine B cell proliferation and with anti-Ig (83,408), anti-CD40 (14,15,164,336) PMA and ionomycin (83,163) or SAC (103) to induce human B cell proliferation.

IL4 is a mouse B cell differentiation factor. *In vitro*, it specifically increases IgE and IgG1 in cultures of LPS activated murine B cells, by Ig heavy chain class switching and not clonal expansion (24,239,334,353). The Ig heavy chain class switch mechanism used by IL4 in the mouse is deletional switch site recombination. Normal murine B cells stimulated with IL4 and LPS produce Cε and Cγ1 germline transcripts which precede switching and transcription of the whole Ig heavy chain (127,334,353). IL4 induces the transcription at an initiation site 5' to the switch region (127) possibly by demethylation of a 300bp DNase I hypersensitive site (316). Although IL4 can induce germline Cε and Cγ1 transcription, it is unable to induce switch site recombination (27,156). A second activation signal given by T-cells, LPS or some other activator is needed, possibly requiring at least one round of replication to open the DNA sufficiently for recombinases.

IL4 is also a human B cell differentiation factor. Initial work with mixed lymphocyte cultures showed that IL4 specifically increased IgE and IgG4 production by human B cells (149,191,410,411). Human IgE production from human mixed lymphocyte cultures is IL4 and T cell contact dependent (79,306,307) and may involve IL6 (196,411). The use of B cell activators such as PMA (141,250), EBV (394) and anti-CD40 (149,195,336,433) in place of T cell contact has shown that human IL4 consistently increases IgE and often IgG4. Its role in the control of the other Ig classes and IgG subclasses is less clear. IL4 with EBV increases IgM, IgG and IgA (394), IL4 with SAC increases IgG and IgM (105), IL4 with PMA and ionomycin increases IgM, IgG1, IgG2, IgG3 but no IgG4 or IgA (141) and anti-CD40 with IL4 either increases IgM and IgG (15,195,336) or does not (433). IL4 also induces IgM, IgA and IgG production by established EBV cell lines as a late acting BCDF (359). Similar to murine IL4, human IL4 induces IgE secretion by Ig heavy chain class switching (149). IL4 induces a DNA binding protein which binds at a site upstream of the Cε gene initiation site and induces transcription of Cε germline RNA (187). IL4 alone cannot induce Ig class switching to IgE, although Cε transcripts are detected. Additional activation by T cells, anti-CD40 or polyclonal activators is needed for switch site recombination (153,196,315,355). The mechanism which increases IgG4 production is unknown, but may also be by Ig heavy chain class switching (150,203,250).

In vivo, IL4 controls murine IgE production. Anti-IL4 or anti-IL4R antibodies inhibit IgE responses to the parasitic worm *Nippostrongylus brasiliensis*, nematode parasite *Heligmosomoides polygyrus*, Keyhole Limpet haemocyanin (KLH) and goat-anti-mouse IgD (135,137,399). Although murine IgE production appears to be controlled by IL4, IgG1 regulation in the mouse may be controlled by either IL4 or another unknown pathway (106). It is obviously difficult to determine the role of human IL4 *in vivo*, but studies on patients with hyper IgE syndrome, atopic dermatitis and allergy show that elevated IgE levels are often caused by over

production of IL4. In some of these diseases, human IgG4 production is also elevated along with IgE but may be a normal response to prolonged antigenic stimulation rather than induction by IL4 (1,49,70,321,322,339,347,403,421)

1.6.3. IL5

The human IL5 gene codes for a mature protein of 115aa and the active protein is expressed as a homodimer of 45kDa (Table 1.4.). One copy of the gene is localised at 5q23-q21, very close to the genes for IL4, IL13, IL3 and GM-CSF. The monomer protein has two cysteines at residues 44 and 86 at least one of which may be involved in a disulphide linkage in the dimer. There are two N-linked polysaccharides (three in murine IL5) which are not required for activity. IL5 is produced by T cells, mast cells, eosinophils and possibly B cells. The IL5 receptor has an α-chain which binds IL5 ($Kd=10^{-9}M$) and a non IL5 binding β-chain (KH97) (220). It is a member of the type I cytokine receptor super family (274) and is found on human eosinophils and basophils and on mouse eosinophils and B cells (16,56,342).

Both murine and human IL5 have potent eosinophil growth factor activity (74,343). IL5 is also a mouse BCDF (89,90,106,255) and specifically increases IgA production in cultures of LPS activated murine B cells by clonal expansion of $mIgA^+$ cells and not immunoglobulin class switching (262,318,372,431). A comprehensive study by Clutterbuck *et al* in 1987 (84) which tested human IL5 in a wide number of B cell activation assays concluded that human IL5 had no effect on human B cells. In other studies however, IL5 can enhance IL4 induced IgE production and sCD23 release by PBMCs and TMCs (304,305,308,433) and increase IL4 and anti-CD40 induced IgE production by purified B cells (151). IL5

may also increase human IgA (308) and IgM production (26), but its role as an important human BCDF seems unlikely.

1.6.4. IL6

The human IL6 gene codes for a mature protein of 184aa of 21-28 kDa (Table 1.4.) and is localised at 7p21-p14. It has four cysteine residues with 2 potential disulphide bonds between cysteine residues 43-49 and 72-82. IL6 is produced by nearly all cells types including T cells, fibroblasts, endothelial cells, keratinocytes, B cells, mast cells and a number of tumour cell lines but the main source is from monocytes and macrophages. The high affinity IL6R ($Kd=10^{-11}M$) is composed of an 80 kDa α-chain which binds IL6 ($Kd=10^{-9}M$) (219) and a β-chain, gp130, which acts as a signal transducer (177). Both chains are members of the type I cytokine receptor super family (274). Gp130 is also a shared component with other receptors including the oncostatin M (OSM), leukaemia inhibition factor (LIF), IL11 and ciliary neurotrophic factor (CNTF) receptors (143,154,220). The IL6R is expressed on haematopoietic stem cells, plasma cells, most leucocytes, epithelial cells, fibroblasts, hepatocytes, neural cells and macrophages (16,56).

IL6 (BSF-2, BCDF II, IFNβ) is a pleiotropic factor, secreted by most cells and involved directly or indirectly in most immune responses. This can be illustrated by the multiple roles of IL6 in the immune response to infection or injury. It a) synergises with IL3 and M-CSF to induce proliferation of haematopoietic progenitors in the bone marrow and maturation of megakaryocytes, b) induces T cell activation and thymocyte proliferation in combination with IL1 c) stimulates Ig production d) stimulates the hypothalamo-pituitary axis from which ACTH (adrenocorticotropic hormone) is released to enhance release of acute phase proteins in the liver, e) directly stimulates acute phase protein release from hepatocytes (e.g.

fibrinogen), and f) may regulate blood pressure by induction of angiotensin (2,178,405).

Although IL6 is a growth factor for B cell lines and myelomas (224,361,396) it does not induce proliferation of normal mouse or human B cells. It is however a BCDF for both murine and human B cells. Murine IL6 is a late acting factor which enhances IL1 induced IgM secretion (235) and IL5 induced IgA secretion, by increasing Ig production from differentiated B cells (20,235). Human IL6 is also a terminal differentiation factor (179,221,285) and increases IgM, IgG and IgA production by Ag, SAC plus IL2 and PWM activated B cells (124,221,222,285,376), and Ig production by EBV transformed B cells (22,285). It is also enhances IL4 induced IgE production by PBMC (194,304,411) but not by increasing IL4 induced Cε germ-line transcription (151). IL6 is thought to enhance Ig secretion by increasing mRNA for secretory rather than membrane bound Ig, either by post transcriptional processing or mRNA stability (320).

Although IL6 is involved in most immune responses, the IL6 transgenic mouse suggests that enhancement of Ig production may be a major function *in vivo*. These animals produce high concentrations of human IL6 and have very high levels of IgM secreting B cells which switch class to IgG1 (382). Holding in mind the nature of the beast, this may or may not indicate a novel IgG1 heavy chain class switch mechanism for IL6. It is likely that IL6 is required or closely involved in Ig secretion by murine and human B cells. Murine Th2 cells which secrete IL4 and humoral immune response promoting cytokines also secrete IL6 and human IL4 induces IL6 secretion by human B cells (365). It is also interesting to note that IL6 is not required for a specific IgG secondary antibody response to influenza virus in humans (93). This suggests that IL6 may not be required by tonsillar B cells for some memory responses.

1.6.5. IL10

The IL10 gene codes for an immature protein of 160aa and the mature expressed protein is approximately 35-40 kDa (homodimer) (Table 1.4.). It is localised on chromosome 1 and has high homology to the mouse IL10 gene and the EBV open reading frame BCRF-1 (279). The active molecule is expressed as a non-covalently linked homodimer and is a member of the 4 α-helix bundle family of molecules which includes IL5 and GM-CSF (278). IL10 is produced by human $CD4^+$ T cells, monocytes, macrophages, keratinocytes, activated B cells and some B cell lymphomas (16,51,56,278). The IL10 receptor is a member of the type II cytokine receptor family, which includes the IFNγ and IFNα/β receptors, and is expressed on B cells, macrophages, monocytes, thymocytes and T cells (56,274).

IL10 is a regulator of murine and human humoral and cellular immune responses (see also Section 1.1.4.). In mice IL10 is produced by Th2 cells and impedes promotion of the cellular immune response and Th1 cell function by inhibiting IL2, IL3, TNFβ, GM-CSF, TGFβ secretion and decreasing IFNγ mRNA production (138,183,278,390). IL10 has similar control *in vivo*. When given to mice intravenously it inhibits IL1, IL6 and TNFα production and if neutralised, in similar mice, levels of these cytokines become raised (181). Human IL10 has similar functions to its murine counterpart. It suppresses human T cell proliferation by inhibiting the antigen presenting capacity of monocytes and production of pro-inflammatory cytokines IL1α, IL1β, IL6, IL8, TNFα, GM-CSF and G-CSF but does not reduce the APC potential of B cells (97,98). IL10 also inhibits human T cell growth and IL2 production (389,390) and is antagonistic with the Th1 product IFNγ (75).

IL10 is a also a BCGF and BCDF for activated human B cells. It co-stimulates with anti-CD40, SAC and anti-Ig to increase B cell proliferation, and increases IgM,

IgG, and IgA production of SAC and anti-CD40 activated B cells (336,337). It also co-stimulates with TGFβ to induce Ig heavy chain switching of anti-CD40 activated human B lymphocytes to IgA (104).

1.6.6. IL13

The IL13 gene codes for a mature protein of 113aa of 9-17 kDa (Table 1.4.). It is localised at 5q23-q31 very close to the genes for IL3, IL4, IL5 and GM-CSF. The IL13 molecule is highly α-helical (435,436). It has four cysteine residues and has a potential for 2 disulphide bonds (266). Its aa sequence has 25-30% homology with IL4 and is concentrated in the first and last α-helices of both proteins which are required for activity (272). IL13 is produced by $CD8^+$ and $CD4^+$ T cells (435,436). Its receptor has not been identified but IL13 has biological activity on macrophages, monocytes and B cells (435).

The presence of IL13 was discovered when an $IL4^-$ human CD4+ T cell clone, A3, was found to induce IgE and IgG4 germline transcripts (149,151,153). IL13 has since been found to mimic some of the effects of IL4. It increases MHC class II and CD23 (102), and co-stimulates with anti-Ig, SAC and T cells to increase B cell proliferation (265,314). It also induces Ig class switching to IgE and IgG4 in activated IgD^+ B cells. Interestingly, there is no additive or synergistic response in combination with IL4, and both cytokines may share the same signalling pathway (314). There is evidence that IL13 may also share a receptor component with an IL4 receptor (9,435,436). This unknown component, possibly part of the low affinity IL4 receptor (129), is unrelated to the high affinity IL4 receptor protein (436). Interestingly, IL13, unlike IL4, has no activity on human T cells (435,436).

1.6.7. TGFβ.

Transforming growth factors are a family of conserved mammalian signalling peptides and have an anti-proliferative effect on human and murine T and B cells *in vitro* (87,415). They were originally isolated as products from virally transformed cells and caused the phenotypic transformation of non neoplastic cells in culture.

There are two TGFs, TGFα and TGFβ. TGFβ has three isoforms TGFβ1, TGFβ2 and TGFβ3 which are disulphide bonded to form 25kDa homodimeric or heterodimeric polypeptides. The TGFβ genes code for a mature protein of 112aa of 12.5 kDa although the precursor peptides are of different sizes (Table 1.4.). TGFβ1 is localised at 19q13.1-13.2, TGFβ2 to 1q41 and TGFβ3 to 14q24. TGFβs are produced by a wide variety of cells including platelets, T cells, fibroblasts, endothelial cells, keratinocytes, B cells, mast cells and a number of tumour cell lines but the main source is monocytes and macrophages. There are three classes of TGFβ receptor. Class I (55 kDa) and class II (80 kDa) receptors bind TGFβs with high affinity (I: TGFβ1=TGFβ2>TGFβ3, II: TGFβ1>TGFβ2>TGFβ3) and are serine/threonine kinases. Class III receptors (250-350 kDa) bind TGFβ with low affinity (TGFβ1=TGFβ2=TGFβ3) and are disulphide linked proteoglycans which may interact with the extracellular matrix. Receptors are found on many cell types including haematopoietic progenitor cells, B cells, fibroblasts and epithelial cells (56,184,415).

Although TGFβs are predominantly immunomodulators of the inflammatory immune response and tissue repair (184,415), TGFβ1 has a B cell regulatory role in both mice and humans. TGFβ stimulates IgA production from IgA^- cells but inhibits production of all other Ig isotypes from LPS activated murine B cells (88,208,209,215,240,372). TGFβ induces production of Cα germ-line transcripts before IgA^+ cells or secreted IgA are detectable and induces IgA production by Ig

heavy chain class switching (241,370). TGFβ also selectively stimulates the production of IgG2b in mice (264,370). In humans, TGFβ also selectively increases IgA production from IgD^+ human B cells activated with anti-CD40 (104). The mechanism used by TGFβ to increase human IgA is also by Ig heavy chain class switching. TGFβ induces Cα1 and Cα2 germline transcription in activated human B cells before productive αmRNA (192,292).

1.6.8. IFNγ AND IFNα.

The human IFNγ gene is localised at 12q24.1 and codes for a mature protein of 143aa and 17 kDa (Table 1.4.) which has an expressed mw. of 20-25 kDa (monomer) after glycosylation (2 potential sites). Biologically active IFNγ is a homodimer and as no cysteine residues are present in the expressed protein, the monomers are held together by non-covalent interaction. This is why IFNγ is highly labile and sensitive to changes in temperature and pH (130). IFNγ is produced mainly by activated $CD8^+$ and $CD4^+$ T cells, but also NK cells. The IFNγ receptor is a member of the type II cytokine receptor family which includes the IL10 and IFNα/β receptors (130,274) and is expressed on T and B cells, macrophages, monocytes, B cells, fibroblasts, epithelium and endothelium (16,56). In mice and humans IFNγ is a Th1 cell product which promotes the cellular immune response and is antagonistic of IL4 and Th2 cell mediated responses (see Section 1.1.4. and (130,133)). Strangely, murine IFNγ is also a BCDF and specifically increases IgG2a while inhibiting IgG1, IgG2b, IgG3 and IgE production by LPS activated mouse B cells (134,371). This BCDF activity of IFNγ appears to be important *in vivo* for regulation of the IgG2a response against the inactivated bacteria *Brucella abortus* (134). There is also evidence that IFNγ may promote IgG3 class switching in mice (369,370).

IFNγ is antagonistic of IL4 induced IgE and IgG1 production in mice (86,87) and IgE in humans (76,77,122,151,305,315,394), but in humans no IFNγ Ig class switching function has been discovered. IFNγ does however increase proliferation of SAC and anti-IgM activated human spleen, lymph and peripheral blood lymphocytes (100,197,249), and induce IgM and IgG production from IL2 and SAC activated B cells (288).

The human IFNα genes exist as approximately 24 copies, including several pseudo genes, localised on 9p22-p13. The mature proteins are 16-27 kDa molecules of 166-172aa. There are four to five cysteine residues and two disulphide bonds (Table 1.4.). Unlike IFNγ, IFNα is produced by most leucocytes including macrophages and monocytes. The IFNα receptor also binds IFNβ, is a member of the type II cytokine receptor family and is expressed on most cells (56,274). The human CD21 antigen has also been identified as a receptor for IFNα (108), and is present on B cells and T cells .

IFN-α inhibits IL4 induced IgG1 and IgE production by activated murine B cells (136), and IL4 induced IgE production by activated human B cells (111,151,305,306,339). It has been suggested that monocyte derived IFN-α may favour Th1 cell responses and secretion of IFN-γ in the mouse by inhibiting Th2 cells and transcription of IL4 mRNA (136). IFN-α has human BCDF activity and increases Ig secretion from Ig secreting human B cell lines (199), IgG and IgM secretion by purified blood B cells (291,303) and IgM, IgA and IgG1 by human B cells stimulated with PMA and ionomycin (141).

1.6.9. NGF.

The NGF gene is localised at 1p13 and codes for a mature protein monomer of 120aa with six cysteine residues, 3 intra chain disulphide bonds and a mw. of 13.5 kDa. The biologically active molecule is expressed as a non covalently linked homodimer (26kDa). NGF is secreted by the prostate, brain and nervous system. NGF receptors are present on sensory and sympathetic neurones, melanocytes, Schwann cells, mast cells, B cells and monocytes. There are two receptors for NGF. The high affinity NGFR ($Kd=10^{-11}M$) mediates neurotrophic activity on neuronal cells and the low affinity NGFR or LNGFR ($Kd=10^{-9}M$) does not mediate neurotrophic activity and is expressed on non neuronal cells. LNGFR is the founder member of the NGFR super family or type III cytokine receptors which include CD40, CD30, TNFR1 and TNFR2 (257,274).

NGF enhances neuronal growth and survival and stimulates human T cell proliferation and haematopoietic colony growth and differentiation. NGF also has BCGF and BCDF activities. It induces proliferation and IgG4 secretion by SAC and T cell activated B cells, and enhances IgM and IgA secretion with no effect on IgG1, IgG2, IgG3 and IgE production. Specific IgG4 production is due to expansion of $IgG4^{+}$ B cells and not Ig class switching (216,217).

1.7. FUNCTIONAL B CELL SURFACE ANTIGENS.

It has become obvious in the past ten years or so, that ideas about the control of humoral and cellular immune responses by cytokines alone are rather simplistic. Although there is no doubt that cytokines play a major role in controlling B cell function and directing Ig isotype production, B cell surface antigen interaction with ligands on other cells may be just as important if not more so. Two particularly

interesting B cell surface antigens which are important in B cell growth, differentiation, Ig secretion and Ig heavy chain class switching are CD40 and CD23.

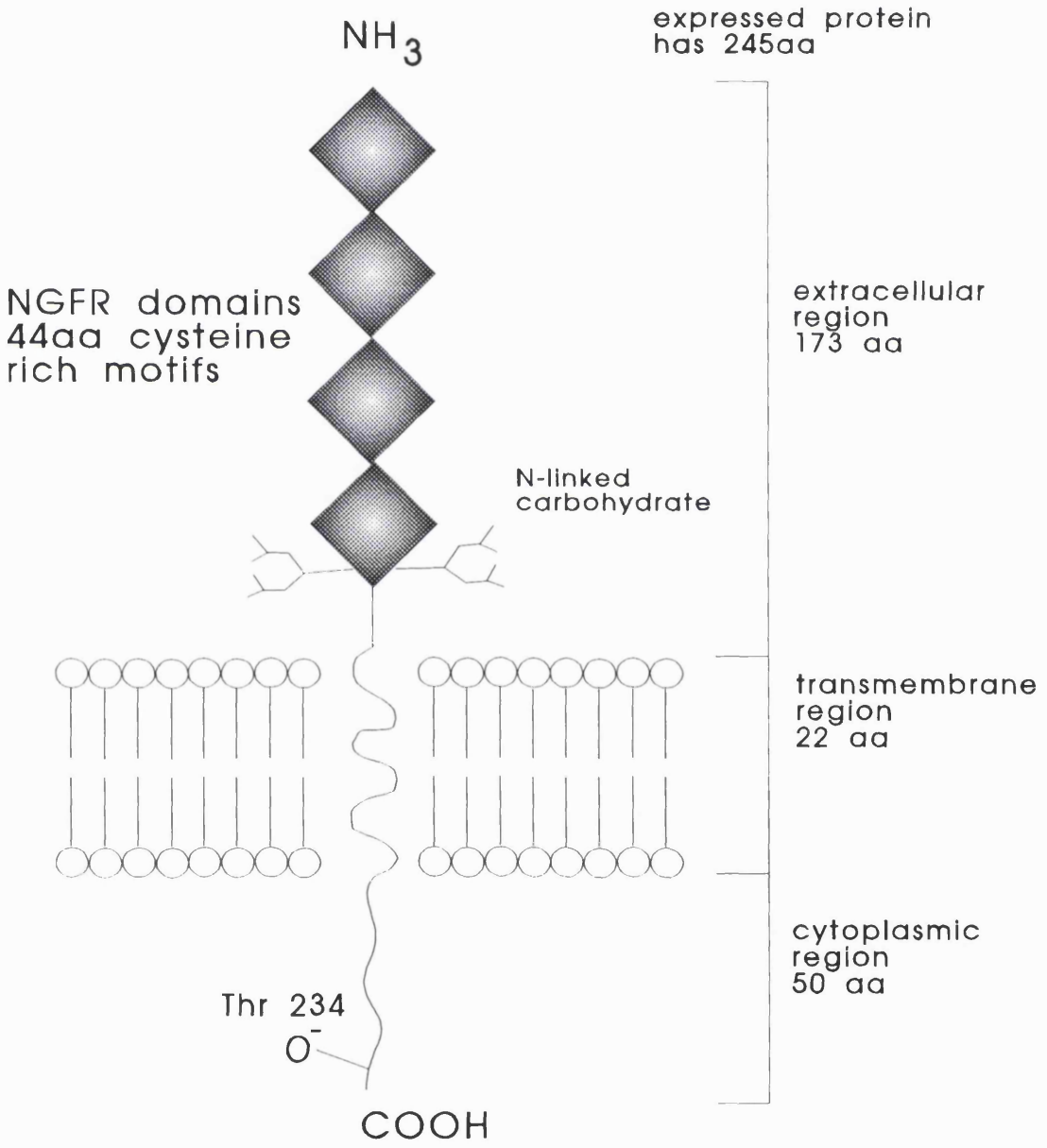
1.7.1. CD40 (Figure 1.11.).

CD40 is an integral membrane glycoprotein of approximately 45-50 kDa which may be expressed on the cell surface as an 85 kDa dimer (47,293). It is a marker for immature and mature B cells, expressed after the CD10 and CD19 antigens but before CD20, CD21, CD22, CD24 and Ig heavy chain rearrangement (398). It is also expressed on follicular dendritic cells (FDCs), some carcinomas, interdigitating cells in the T cell zones of secondary lymphoid organs and thymic epithelial cells (16,293,378).

CD40 is a member of the NGFR type III cytokine receptor family (274). This family is characterised by the presence of 2 to 5 cysteine rich motifs of approximately 40 amino acids in the extracellular domain originally identified in the LNGFR (see Section 1.6.7. and (293)). Other members of this family include LNGFR, OX-40 (rat), TNFR-I, TNFR-II, CD30 (257), CD27 (95) and FAS (193). The mechanism of signalling through CD40 is largely unknown, but it has been shown that phosphorylation at threonine residue 234 in the cytoplasmic tail is essential for activity (Figure 1.11.) (82) and that activation of PKC (189) and with IL6 (82) cause this residue to be phosphorylated.

Cross linking of CD40 with anti-CD40 antibodies induces B cell activation. This is characterised by an increase in cell volume, induction of homotypic adhesion and increased ICAM-1 (CD54) expression (164,401). Stimulation of B cells with anti-CD40 and co-activators, anti-IgM, anti-CD20, TPA and IL4 induces considerable and prolonged B cell proliferation (242,401). Long term cultures of normal human B

Figure 1.11. Structure of the Human CD40 Cell Surface Antigen.



cells have been obtained by stimulation with anti-CD40 antibodies held on the surface of CD32 (FcγRII) transfected mouse fibroblast L-cells (336). Human B cells stimulated with anti-CD40 in the mouse-L cell system also differentiate and secrete IgM, IgG and IgA, and the addition of IL4 stimulates IgE production (14,15). Soluble anti-CD40 antibodies are unable to induce Ig secretion alone, but in combination with IL4 or IL2 can induce IgM, IgG, IgA, IgE and IgG4 production (149,195). Interestingly, anti-CD40 and IL4 induced IgE and IgG4 production is IFNγ independent (14,15,433). This suggests an alternative pathway for IgE and IgG4 control (149). Soluble and L-cell bound anti-CD40 provides the second signal needed by IL4 to complete Sμ to Sε deletional switch recombination (355) and may also deliver a similar signal to allow TGFβ induced Ig heavy chain class switching of human B cells to IgA (104).

A ligand for CD40 has been recently cloned in the mouse (mCD40L) (4,6) and in the human (hCD40L) (169). CD40L is a type II integral membrane protein found on activated murine and human $CD4^+$ and some $CD8^+$ T cells with homology to TNFα (type II Integral membrane protein and soluble trimer) and TNFβ (soluble trimer) (293). CD40L has a transient expression on T cells, appearing as soon as 1 hour after T cell activation, reaching maximal expression after 8 hours but falling away rapidly by 12 hours (169,238). HCD40L (38kDa) has 62% amino acid sequence homology to mouse CD40L (36kDa) along the whole molecule and 78 % in the cytoplasmic domain (395). The gene for hCD40L (TNF-related activation protein (TRAP) or gp39) is located on the X chromosome (Xq26.3-q27.1.) and may have a soluble form in addition to the membrane bound molecule (169) although there is no biological evidence for this. MCD40L may also have membrane bound (noncovalently linked homo-trimer) and secreted forms (4) similar to the TNFs.

CD40L has so far been able to reproduce effects on B cells seen with anti-CD40 antibodies. CV1/EBNA cells transfected with and expressing the human CD40L can

induce B cell proliferation in the absence of other activators (4,237,377). Addition of IL10 or IL2 to this system induces IgM, IgA, IgG1, IgG2, IgG3 and IgG4 secretion (55,267,377). CD40L can also provide the second signal needed by IL4 to complete Ig heavy chain switching to IgE, and promote IgE secretion (5,151,355).

Human CD40L has been identified as the gene responsible for X-linked hyper IgM syndrome (XLhIgM or HIGM1). Patients with HIGM1 have no germinal centres but normal numbers of B cells, with normal or elevated IgM and little or no IgG, IgA or IgE in their serum. This may be due to a defect in their B cell Ig heavy chain class switch, or antibody affinity maturation mechanisms (55,118). HIGM1 patients have non-functional or defective expression of CD40L on T cells. Restoration of CD40L by addition of CD40L/CV1 cells to SAC activated HIGM1 PBMC with IL10 restores IgG and IgA secretion while addition of CD40L/CV1 cells with IL4 induces IgE production (231). *In vitro* activation with anti-CD40 and cross linking of the Ag receptor rescues centrocytes or $CD38^+$ germinal centre B cells from apoptosis (245-247). This suggests that CD40/CD40L signalling may be required for rescue of switched B cells after somatic mutation and expression of high affinity antibody.

Signalling through CD40 prevents other negative signals given to B cells. Anti-CD40 reduces anti-IgM induced apoptosis of the human B cells line RAMOS (400). Anti-CD40 can also prevent inhibition of IL4 induced CD23 expression by TGFβ, anti-CD19 and IFNγ (161), glucocorticoid and the PKC inhibitor staurosporin (205). Signals transduced through CD40 have extremely potent effects on human B cells. They induce positive effects, inducing proliferation and Ig production and overriding or modifying signals given by other stimuli. These findings suggest that CD40/CD40L interactions are extremely important particularly during Ig class switching, somatic mutation and affinity maturation of B cells *in vivo*.

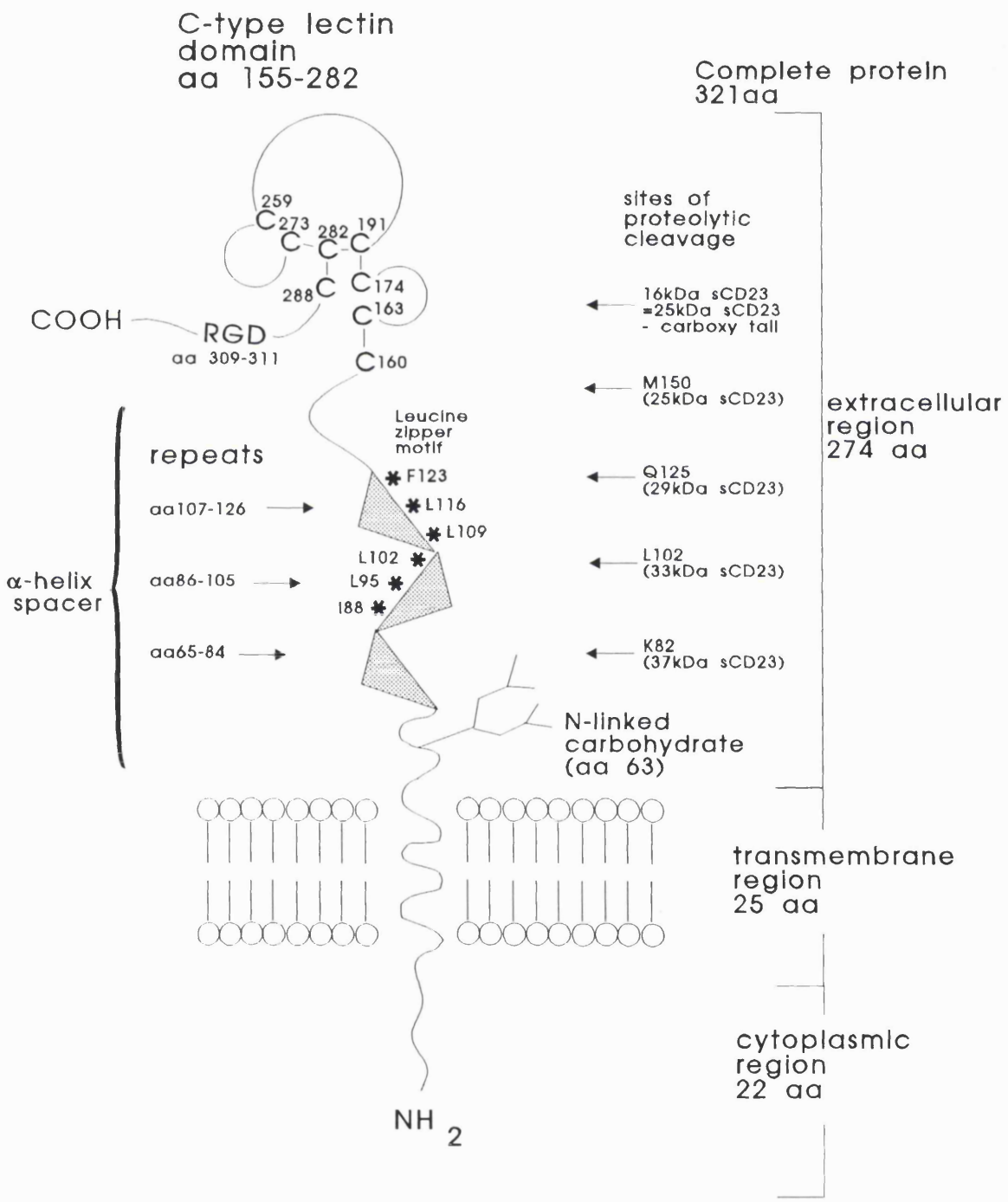
1.7.2. CD23 (Figure 1.12.).

The CD23 gene consists of 10 exons (approximately 13Kb) and is situated on human Ch19p13.3. The human molecule has two forms, a and b. The difference occurs at the tip of the cytoplasmic region, where the amino acid sequence NH_2-MEEGQYSEIE (a) is changed to NH_2-MNPPSQEIEE (b). The a and b sequences are encoded by two separate exons with different promoters. Expression of CD23a or b is achieved by alternative splicing of cytoplasmic region transcripts, onto a common mRNA (384,430). The CD23 molecule is a type II integral membrane protein (extracellular COOH terminal) of between 45 and 50 kDa with one N-linked glycosylation site at aa63 as shown in Figure 1.12. The membrane bound molecule can also undergo successive autocatylitic cleavage to form a soluble cytokine (sCD23) (243). The CD23 molecule has homologies to a number of other proteins because of its many different structural features.

1) Leucine Zipper Motif. The CD23 molecule has 3 identical repeat sequences of 21aa between the membrane and lectin head, probably a result of exon triplication (91). These repeat sequences contain a leucine zipper motif. This motif consists of heptad hydrophobic repeats (I[88]-L[95]-L[102]-L[109]-L[116]-F[123]) (112,225) and is predicted to form an α-helical coiled coil structure and mediate CD23 and sCD23 trimer formation (21,117,168,384). Recently, Klein *et al.* 1992 (225) have described homology between the cytoskeletal component vimentin and CD23. Both proteins share the leucine zipper motif, which in vimentin may be used in binding to the fibronectin receptor (225).

2) Ca^{2+} Dependent C-type Lectin Domain. The lectin domain contains 8 conserved cysteines and 3 intra chain disulphide bonds which give the characteristic tertiary structure of a lectin binding head. CD23 has particular homology to a subgroup of C-type lectins (120), of which the rat asialoglycoprotein receptor

Figure 1.12. Structure of the Human CD23 Cell Surface Antigen.



(RAsgpR) may be the archetypal gene (427). This subgroup also forms dimers and trimers by virtue of an α-helix coiled coil structure, and RAsgpR is also a trimer like CD23 (21,384). Other members of this group include human CD72 (Lyb-2), mouse CD23, rat Kupffer cell receptor (RKcR) and chicken hepatic receptor (ChR) (21). CD23 also has homology to the selectins, proteins with a selective function and a lectin domain. Although the selectins are type I transmembrane proteins (COOH terminal intracellular) they share properties with CD23. The selectins which have most homology to CD23 are granule membrane protein-140 (GMP-140/CD62), lymphocyte homing receptor (MEL-14) and endothelial leucocyte adhesion molecule-1 (ELAM-1). These selectins, like CD23, undergo proteolytic cleavage and are lost from the cell surface within minutes of activation (123).

3) RGD Integrin Recognition Motif. CD23 also contains an inverted RGD (arginine-glycine-aspartic acid) integrin recognition motif. Integrins with this domain include VLA-3 and VLA-5 (very late adhesion molecules) which bind to fibronectin, laminin, and collagen and direct leukocyte homing and adhesion (31,123).

Human CD23 is expressed in B cells, T cells, thymocytes, thymic epithelium, monocytes, macrophages, eosinophils, platelets, follicular dendritic cells, NK cells, Langerhan's cells, endothelial cells, early human myeloid precursors and some human B cell lines. FcεRIIa mRNA is constitutively produced in B cells and B cell lines FcεRIIb is expressed on the other cell types but is increased on B cells in response to IL4 (91,113,160,225,282,283,383). Murine CD23 also has a leucine zipper motif, but this is contained within 4 not 3 consensus repeats. There are 2 N-linked polysaccharides and no RGD sequence (91). Mouse CD23 also has a and b forms which are only present on B cells. Only the a form is homologous to human CD23, and only to human CD23a (326).

In the III (Oxford 1986) and IV (Vienna 1989) International Workshops on characterisation of leucocyte antigens, CD23 was confirmed as the low affinity IgE receptor (FcεRII). It is totally unrelated to FcεRI in both structure and affinity for IgE (FcεRI Kd=10^{-9}M, FcεRII Kd=10^{-6}-10^{-7}M), and is the only Ig receptor discovered to date which is not a member of the Ig super family (91,384). IgE binds to CD23 in the lectin head domain and aa165-190 and 224-256 within this structure as well as Ca^{2+} ions are required for binding (29,30,384). In 1992, CD21 was also confirmed as a ligand for CD23 (8,310). It binds to CD23 very near to the IgE binding site in the lectin head (344,384).

Signalling through CD23 by IgE-anti-IgE cross linking or anti-CD23 antibodies stimulates phosphoinositide (PIP_2) hydrolysis leading to Ca^{2+} mobilisation from intracellular stores, increased cAMP, and tyrosine phosphorylation of intracellular proteins (229). CD23 may be associated with p59 fyn a member of the src family of tyrosine kinases (227-229,253,383). Monomeric IgE will not deliver a Ca^{2+} signal unless cross linked, indicating that only IgE immune complexes can deliver signals through CD23 (21). The cytoplasmic tail of CD23 is also quite short and CD23 may also require associated proteins for signalling. CD23 may form part of an associated protein signalling complex in the cell membrane, possibly the counter structure to the CD19/CD21 complex (260). This may also explain the spatial association of CD23 with HLA-DR in the B cell membrane, and why IL4 induces CD23 and HLA-DR together (40,140,338).

CD23 is involved in protective immunity against parasites and the inflammatory immune response. It mediates IgE-dependent cytotoxicity and phagocytosis by inflammatory cells such as monocytes, macrophages, eosinophils and platelets, by promoting the uptake of IgE antibody-antigen complexes. Aggregated or cross linked IgE bound to CD23 causes activation and release of inflammatory cytokines (e.g. TNFα and IL1α) from eosinophils, platelets and macrophages playing a role

in parasitic immunity and IgE dependent lysis of parasitic larvae (43,64,65,201). Binding of IgE immune complexes to CD23 on B cells is important for Ag capture processing and presentation during an immune response to allergen. In the human, the focusing of antigen by IgE-immune complex/CD23 interaction may be responsible for the ongoing immune response against persistent parasites and allergens (1,284,309,402). This process in the mouse appears to be the primary function for CD23 (210).

The CD23 molecule undergoes autoproteolytic cleavage at the cell surface to give rise to soluble CD23 (sCD23). The first cleavage at aa81/82 produces a 37kDa molecule, sCD23 then undergoes successive cleavage into 33kDa, 29kDa, 25kDa and 16kDa molecules (112,243,344)(marked arrows in Figure 1.12.). All products except the 16kDa protein retain their IgE binding capacity (344). IgE has a 10 fold higher affinity for membrane bound CD23 than the soluble form and this may be due to a destabilisation of the trimeric head structure in sCD23 (91,384). Binding of IgE to membrane bound CD23 also prevents initial cleavage, possibly by stabilisation and protection of the trimeric coiled structure (384).

IL4 increases murine and human B cell CD23 expression and release of sCD23. IL4 also increases CD23 expression on human monocytes, eosinophils, platelets and Langerhan's cells. Of these, monocyte CD23 also gives rise to sCD23 indicating that sCD23 is not released specifically by B cells (91). CD23 is also induced by activation with other factors including PMA, EBV, anti-IgM, IL7 and IL13 (162,202,314,358).

sCD23 is a multifunctional cytokine (245). It induces T cell growth (25), B cell growth and differentiation (110,112), inhibits monocyte migration (139) triggers IgE independent histamine release from mast cells (157) and in combination with IL1α is able to rescue germinal centre B cells from apoptosis by growth induction

and differentiation into AFCs (41,246,247). It is now thought that sCD23 functions may be mediated through its second ligand CD21 (152). It has been shown that anti-CD21 and IL1α also rescue GC cells from apoptosis in a similar way to sCD23 (41). This suggests that growth factor activity of sCD23 in other systems may also due to signalling through CD21 (8).

sCD23 controls IgE production with IL4. sCD23, induced by high concentrations of IL4 can feed back and increase IL4 induced IgE production (428). It is thought that anti-CD23 specifically inhibits IgE production with no effect on IgM, IgG or IgA by binding to sCD23 and preventing its function (42,306,345,356). However, it is very difficult to determine which effects rely on sCD23 and which on signalling or prevention of signalling through the membrane bound molecule. Not all anti-CD23 antibodies which prevent sCD23 release, inhibit IgE synthesis (42). Also, IgE-anti-IgE cross linking of membrane bound CD23, which delivers a signal through CD23 (228), inhibits B cell proliferation and Ig production (251). Inhibition of IgE production could be due to CD23 signalling, and not inhibition of sCD23 release and CD21 signalling. Interestingly, triggering of CD21 with anti-CD21 or rsCD23 specifically increases IL4 driven IgE production (8). Therefore CD21 and CD23 could both be involved in IgE regulation. *In vivo*, sCD23 is not thought to play a part in disease. Although sCD23 is raised in the blood of patients with atopic disease, it is thought to be a marker for IL4 over production rather than a mediator of elevated IgE and allergenic symptoms (158). In addition to their inhibitory effects, anti-CD23 antibodies, which are known to transduce a signal through CD23 (228), can induce proliferation of human B cells activated with TPA (166,167). In humans, signalling through CD23 has both inhibitory and enhancing effects. This may be connected to the expression of a or b isoforms which may be connected to different signalling pathways.

CD23 is involved in the spontaneous aggregation of B cell lines, BJAB, RAMOS, Namalva and CESS, (91) as well as the homotypic B cell adhesion induced by anti-CD40 and IL4 (38). Although this interaction is thought to be CD23/CD21 mediated (38) it is not unlikely that CD23 may have other ligands. Human CD23 has a lectin binding domain which binds IgE and CD21 by protein interaction (31) and, unlike its murine counterpart, an integrin recognition motif. It is possible that another two potential ligands may bind CD23, one an unknown lectin and second an unknown integrin.

Final Note.

Cytokines are required at all stages of human B cell activation, growth and differentiation. Very little is known about the mechanisms which control Ig heavy chain class switching, especially of the human IgG subclasses. We know that Human TGFβ induces Ig heavy chain switching to IgA, NGF clonally expands $IgG4^+$ B cells and IL4 and IL13 induce Ig heavy chain class switching to IgE. IL4 may also be involved in regulation of other Ig classes and IgG subclasses but conflicting data from different groups makes the picture unclear. Apart from these four cytokines no other human factor is known to specifically induce Ig classes either by Ig heavy chain class switching or clonal expansion. As outlined in Section 1.3.2., specific human IgG subclasses are produced against protein and polysaccharide antigens, and the sites of action for IgA and IgE are in the gut, lungs and skin. There are mechanisms *in vivo* which ensure that the correct Ig class is produced quickly at the right time and in the correct situation. These mechanisms probably come about through controlled cell/cell interaction and release of specific cytokines. To investigate these mechanisms *in vitro*, a T-cell independent B cell activation system using EBV was developed and defined to test the roles of cytokines and the functional B cell surface antigens CD40 and CD23 on human B cell Ig class and IgG subclass production.

CHAPTER 2

REAGENTS, MATERIALS AND METHODS

2.1. REAGENTS AND MATERIALS.

Reagents are listed below with suppliers' name and order number. Details of suppliers can be found in APPENDIX I.

2.1.1. General Reagents.

^{3}H-thymidine-deoxyribonucleotide (^{3}H-TdR.).	Amersham, TRA.120.
BSA, Fraction V.	Sigma, A-4503.
Cyclosporin.	Sandoz, 5013054,190035.
Foetal Calf Serum.	Sera-lab, S-0001a.
Gelatin	Difco, 0143-02-6.
Gentamicin, (80mg/2ml).	Roussel.
L-glutamine, (29.23 mg/ml/200mM).	Flow, 16-801-49.
H_2O_2.	BDH, 10128.
Preservative free Heparin, (1000 U/ml).	Fisons, PL0495/5015.
Lymphocyte separation medium.	Flow, 16-922-54.
p-Nitrophenyl Phosphate disodium (pNPP).	Sigma, 104-105.
Normal mouse serum.	Serotec, C11SD.
Penicillin/Streptomycin (10,000U/100ml).	Gibco, 043-05140H.
Percoll.	Pharmacia, 17-0891-01.
O-Phenyl-diamine (OPD).	Sigma, P1526.
Phytohaemaglutinin (PHA).	Wellcome Diagnostics, HA16/17.
S-2-aminoethylisothiouronium bromide hydrobromide (AET).	Aldrich, A5460-1.
20% Sheep Blood in Alsevers.	TCS Ltd.
Scintillation fluid, Optiscint II Hi-Safe.	Pharmacia, SC 9195-21.
Tween 20.	BDH, 66368.

2.1.2. General Equipment.

Cell Harvester, Dynatech AutoMash 2000.	Dynatech.
ELISA Plate Reader, Dynatech MR4000.	Dynatech.
FACScan Flow Cytometer.	Becton Dickinson.
FACScan Research and Lysis II Software.	Becton Dickinson.
LKB 1218 RackBeta scintillation counter.	LKB.
MACS Magnet.	Miltenyi Biotech.
Mikrotek ELISA Software.	Dynatech.

2.1.3. Tissue Culture Vessels.

96 well round bottomed sterile microtitre plates.	Gibco NUNC, 163320.
96 well flat bottomed sterile microtitre plates.	Gibco NUNC, 167008.
24 well flat bottomed sterile microtitre plates.	FLOW LINBRO, 76-033-05.
Tissue culture flasks $50cm^3$- $500cm^3$.	Falcon.
5ml tissue culture tubes.	Falcon.

2.1.4. Media.

Culture Media.

RPMI 1640 with 25mM Hepes and L-glutamine (Gibco, 041-02400M (liquid) or 079-03018 P (powder)) and RPMI 1640 with L-glutamine (Gibco, 041-01875M (liquid)) were supplemented with 10% FCS, 2 mM L-glutamine and 50 mg/ml gentamicin.

Iscoves Modified Dulbeccos Medium with L-glutamine (Gibco, 041-01980M) was supplemented with 10% FCS, 2 mM L-glutamine and 50 U/ml penicillin/streptomycin.

Holding Media.

In order to maintain cells at pH 7.2 during preparation before culture, holding medium, RPMI 1640 with L-glutamine but without $NaHCO_3$ (Gibco, 07401800P) was made up with the addition of Hepes to a final concentration of 25 mM and supplemented with 5% FCS and 50 μg/ml gentamicin.

For biotin free holding of cells during magnetic cell separation (MACS), Dulbeccos Modification of Eagles Medium (DMEM) without sodium bicarbonate or glutamine (Flow, 12-334-54) was used. This medium was supplemented with 20mM Hepes, 5% FCS, 2mM L-glutamine, 50 μg/ml gentamicin and 0.01% NaN_3.

2.1.5. Preparation of AET Sheep Red Blood Cells.

12.5ml of 20% sheep red blood cells (SRBCs) in Alsever's solution were washed four times in sterile saline (0.14 M NaCl) by centrifugation at 200g for 5 minutes and the buffy coat removed. AET solution was prepared immediately before use by dissolving 40.2 mg/ml of AET in water and adjusting to pH 9.0 with 4M NaOH. The solution was then filter sterilised using a 0.2μm filter. 2.5ml (1 vol) packed SRBCs was incubated with 4 volumes of AET solution for 15 minutes at 37°C. The SRBCs were then washed four to five times in sterile saline and resuspended in RPMI 1640 holding medium with no additions. The resulting 10% AET-SRBC suspension was stored at 4°C for a maximum of four weeks before use.

2.1.6. Preparation of Gey's Haemolytic Balanced Salt Solution.

Solution A: 35g NH_4Cl, 1.85g KCl, 1.5g $Na_2HPO_4.12H_2O$, 0.119g KH_2PO_4, 5g glucose, 0.005g phenol red and 25g gelatin were made up to 1 litre with double distilled water.

Solution B: 4.2g $MgCl_2.6H_2O$, 1.4g $MgSO_4.7H_2O$ and 3.4g $CaCl_2$ were made up to 1 litre with double distilled water.

Solution C: 22.5g $NaHCO_3$ was made up to 1 litre with double distilled water.

Solutions A, B and C were sterilised by autoclaving and stored at 4°C.

When required, Gey's haemolytic balanced salt solution was made up by mixing 7 volumes water with 2 volumes solution A (warmed at 37°C), 0.5 volumes solution B and 0.5 volumes solution C.

2.1.7. Preparation of Percoll Discontinuous Density Gradients.

An iso-osmolar stock solution of percoll was obtained by adding 1 volume of x10 PBS (Dulbecco's A) to 9 volumes of Percoll (specific gravity 1.130). A diluent was also prepared using RPMI 1640 holding medium supplemented with 10% FCS and 50 μg/ml gentamicin. The specific gravity (SG) of stock percoll and diluting medium was determined with a 10 ml specific gravity bottle. The actual SG of stock percoll and diluent was calculated as follows:

$SG_{percoll}$ = weight Percoll (gms)/weight of an equal volume of H_2O (gms)

$SG_{diluent}$ = weight diluent (gms)/weight of an equal volume of H_2O (gms)

The percentage of stock percoll required to create a particular discontinuous gradient ($SG_{required}$) was calculated as follows:

$$\% \text{ percoll} = [(SG_{required} - SG_{diluent})/(SG_{percoll} - SG_{diluent})] \times 100$$

2.1.8. Recombinant Human Cytokines.

Cytokine	Source	Specific Activity
IL2	Amersham International.	2×10^6 U/mg.
IL4	Sterling Drug Company.	$1.6\text{-}0.942 \times 10^7$ U/mg.[a]
IL5	Glaxo.	not known
IL6	Sterling Drug Company and Immunex.	not known
IFNγ	NIBSC. and Genzyme.	$2\text{-}2.5 \times 10^7$ U/mg.
IFNα	NIBSC.	not known
TGFβ	NIBSC.	6×10^6 U/mg.

a The activity of IL4 was determined by proliferation of human tonsillar B cells stimulated with 10 ug/ml of rabbit anti-human IgM conjugated immunobeads (Biorad, 170-5120). 100 U/ml IL4 was defined as that giving maximum ^{3}H-TdR uptake, 3 days after stimulation of B cells with anti-IgM beads. The specific activity of IL4 was calculated as $1.6\text{-}0.942 \times 10^7$ U/mg.

2.1.9. Antibodies for Flow Cytometry and Tissue Culture.

Mouse Monoclonal Antibodies.

Human T cells were detected by anti-CD3 mAbs Leu-12 (FITC-conjugated, Becton Dickinson, 349211) or UCHT1 (Prof. Peter Beverly, ICRF, UK.).

T helper cells were detected by the FITC-conjugated anti-CD4 mAb Leu-3a and cytotoxic T cells were detected by the PE-conjugated anti-CD8 mAb Leu-2a (Becton Dickinson, 340039).

Monocytes and macrophages were detected by the anti-CD14 mAb UCHM1 (Prof. Peter Beverly, ICRF, UK.).

B cells were detected with anti-CD19 mAbs Leu-18 (PE-conjugated, Becton Dickinson, 349211) and BU12 (Prof. Noel Ling, Birmingham University, UK.).

B cell surface antigens CD20 and CD21 were detected by mAbs B1 (Coulter, 6602140) and OKB7 (Ortho Diagnostic Systems Ltd.).

B cell surface antigens CD40 and CD23 were detected by mAbs G28.5 (CD40: Prof. Ed Clark, Seattle, WA., U.S.A.), MHM6 (CD23: Dako, M 763) and B-G6 (CD23: Serotec, MCA 665). These antibodies were also used in tissue culture experiments.

The human IFNγ receptor was detected by a mAb GIR-208 obtained from the V International Workshop on Leukocyte Differentiation Antigens, Boston 1993.

The human high affinity IL4 receptor was detected by a mAb m57 (Dr. Richard Armitage, Immunex Corporation).

Polyclonal Antibodies.

B cell surface immunoglobulins IgG and IgM were detected by FITC conjugated $F(ab')_2$ goat anti mouse IgG (Tago, 4350), FITC conjugated goat anti-human IgM (Sigma, F-5384) and FITC conjugated $(Fab')_2$ rabbit anti-human IgM (Dako, F-317).

2.1.10. Antibodies and Beads for Magnetic Cell Separation (MACS).

Biotin conjugated goat anti-human IgM (Fcμ), anti-human IgD (Fcδ) and FITC conjugated Streptavidin (Southern Biotechnology Associates, 2020-08, 2030-08, 7100-02).

Biotin conjugated paramagnetic beads (Becton Dickinson, 221-02).

2.1.11. Antibodies for ELISA.

Mouse Monoclonal Antibodies.

Biotin conjugated mouse anti-human IgG1 (mAb HP6069), IgG2 (mAb HP6002), IgG3 (mAb HP6047) and IgG4 (mAb HP6025). (Zymed, 05-3340, 05-3540, 05-3640, 05-3840).

Biotin conjugated mouse anti-human IgG4 (mAb HP6025). (ICN, 63-475-1).

Polyclonal Antibodies.

goat anti human: IgG(Fcγ), IgM(Fcμ), IgA(Fcα), IgE(Fcε). (Sigma, I-3382, I-0759, I-0884, I-0632).

Horse radish peroxidase conjugated goat anti-human IgG (Fcγ), IgM (Fcμ), IgA (Fcα). (Sigma, A-6029, A-6907, A-7032).

Horse radish peroxidase conjugated goat anti-rabbit IgG (whole molecule). (Sigma, A-0407).

rabbit anti-human IgE (Fcε). (MIAB, Ab105).

rabbit anti-human IgG (H+L). (Jackson Immuno Research, 309-005-082).

sheep anti-human IgG4 . (The Binding Site, AB009).

Streptavidin Conjugates for ELISA.

Horse radish peroxidase conjugated Streptavidin. (Amersham, RPN 1231).

Alkaline phosphatase conjugated Streptavidin. (Amersham, RPN 1234).

2.1.12. ELISA Buffers.

ELISA Coating Buffer, pH 9.6.

1.59g Na_2CO_3, 2.93g $NaHCO_3$ and 0.2g NaN_3 were made up to 1 litre in double distilled water and pH adjusted to 9.6.

Phosphate Buffered Saline (Dulbecco's A)/tween 20, pH 7.2 (PBS Tween).

8.0g NaCl, 0.2g KCl, 1.15g $NaHPO_4$ (anhydrous) and 0.2g KH_2PO_4 were made up to 1 litre with double distilled water and the pH adjusted to 7.2. Tween 20 (0.5ml) was added to give a solution of 0.05% v/v.

Phosphate Citrate Buffer for Horse Radish Peroxidase, pH 5.0.

1M solutions of Na_2HPO_4 (28.4 g/l) and citric acid (21.0 g/l) were made up separately in double distilled water. Equal volumes were mixed immediately prior to use.

Buffer for Alkaline Phosphatase pH 9.6.

1.59g Na_2CO_3, 2.93g $NaHCO_3$ and 9.52mg $MgCl_2$ were made up to 1 litre in double distilled water and pH adjusted to 9.6.

2.1.13. ELISA Plates.

Sterilin, 96 well round bottomed non sterile plates.	Marathon, MN350-10.
Linbro EIA II, 96 well flat bottomed plates.	Flow, 76-181-04.
Immulon II, 96 well flat bottomed plates.	Dynatech, M1298.

2.2. METHODS.

2.2.1. Isolation of Human Tonsillar B Lymphocytes.

Excised human tonsils were obtained fresh from the 'Royal National Ear, Nose and Throat Hospital', London and from 'Great Ormond Street Hospital for Sick Children', London. The tonsils were washed for 10 seconds in 70% alcohol (IMS) to kill surface micro-organisms then rinsed in RPMI holding medium to remove the alcohol. Tonsillar cells were obtained by teasing into holding medium, and then centrifuged over lymphocyte separation medium for 20 minutes at 1000g. The tonsillar mononuclear cells (TMC) were removed from the interface, washed and counted in a haemocytometer. Viability was determined by trypan blue exclusion. T cells were removed by E rosetting with AET treated SRBCs. AET/SRBC (2.5 mls of 10% suspension) were added to 5 x 10^7 TMC in 10 mls of RPMI 1640 holding medium and the FCS concentration increased to 10%. The cells were then pelleted gently at 200g for 15 minutes at 4^oC and incubated for one hour on ice. The E rosettes were gently resuspended and then layered onto percoll (SG 1.080) and centrifuged at 1000g for 20 minutes. The E^- cells were collected from the interface, washed, counted and stained for B cell, T cell, and monocyte/macrophage content. E^- cells were routinely <0.5% $CD3^+$ (T-cell), <0.5 % $CD14^+$ (monocyte/macrophage) and > 97% $CD19^+$, $CD20^+$ (B-cell).

E^+ (T cells) were recovered from the pellet by lysis of SRBCs with Geys haemolytic buffered salt solution.

2.2.2. Preparation of Mononuclear Cells and B Cells from Peripheral and Cord Blood.

Fresh human umbilical cord blood or peripheral blood was collected over preservative free heparin (20 IU/ml whole blood) and diluted with an equal volume of RPMI 1640 holding medium supplemented with 20 IU/ml heparin but without FCS or antibiotics. Mononuclear cells were separated by centrifugation over lymphocyte separation medium at 1000g for 20 minutes. Cells at the interface were washed twice in RPMI 1640 holding medium and excess human erythrocytes lysed by pipetting for 15-30 seconds with sterile water. Osmolarity was corrected immediately by the addition of x10 PBS and cells were washed and counted. After staining, PBMCs were routinely 5-20% $CD19^+$ (B cell), 60-80% $CD3^+$ (T cell) and 5-20% $CD14^+$ (monocyte/macrophage). T cells were removed by two rounds of E rosetting. Monocytes and macrophages were removed by adherence to petri dishes previously coated with FCS and incubated at 37°C in an atmosphere of 5% CO_2 in air for 30 minutes. T-depleted PBMC were incubated twice at 30 x 10^6 cells/plate for 1 hour at 37°C in an atmosphere of 5% CO_2 in air. After each incubation non adherent cells were resuspended by gentle rocking. B cell enriched PBMC were routinely 50-70% CD19+ (B), 1-8% CD3+ (T) and 5-20% CD14+ (Monocyte/Mϕ).

2.2.3. Preparation of High and Low Buoyant Density B Cells.

Tonsillar B cells (5 x 10^7) were resuspended in 5mls of holding medium, layered onto 10mls of percoll (SG 1.074) in 15ml falcon tubes and centrifuged at 1000g for 20 minutes. High density B cells (SG > 1.074) were pelleted and low density B cells (SG < 1.074) remained at the interface. Both populations were washed to remove remaining percoll and counted.

2.2.4. Separation of Sub Populations of Human B Cells by Magnetic Cell Separation (MACS).

Tonsillar B cells (1.5 x $10^{8)}$ were pelleted and resuspended in 0.5 ml DMEM supplemented with 5% FCS, 2mM L-glutamine and 0.01% NaN_3. Biotinylated anti-IgM at a pre-determined concentration was added and the cells incubated on ice for 15 minutes. The cells were then washed twice in DMEM, resuspended with streptavidin conjugated FITC and incubated on ice for 15 minutes. The cells were then washed twice, resuspended with biotinylated paramagnetic beads and incubated for a further 15 minutes on ice. After a final 2 washes, the cells were layered onto a MACS column of appropriate matrix size (as stipulated in the manufacturers' instructions) outside the magnetic field, and allowed to run in. When all cells had entered the column matrix, the column was fixed into the magnetic field and washed through with DMEM. Cells positive for membrane IgM were retained on the column and the negative fraction collected. The bound cells were collected from the column by removal from the magnetic field and washing off in DMEM. After separation, the positive and negative cells were washed immediately in RPMI holding medium to remove azide, and returned to room temperature. Both fractions were analysed on the FACScan flow cytometer to determine purity. The IgM positive fraction was routinely >95% IgM^+ and the negative fraction < 5% IgM^+.

2.2.5. Preparation and Staining of Cells for Flow Cytometry.

For each test, 0.25-0.5 x 10^6 cells were pelleted and resuspended in 50 ul of a previously determined concentration of antibody diluted in RPMI holding medium with 0.01% NaN_3 and incubated on ice for 30 minutes. Where appropriate, the cells were washed and a second layer of FITC or PE conjugated goat anti-mouse

IgG $(Fab')_2$ was added for 30 minutes on ice. The cells were then washed and resuspended to 1-2 x 10^6 cells/ml for analysis on a FACScan flow cytometer (Becton Dickinson) using FACScan Research and Lysis II software.

2.2.6. Preparation of EBV.

The marmoset B lymphoblastoid cell line B95-8 secretes viable transforming Epstein-Barr virus at high concentration. B95-8 cells were seeded at 1 x 10^6 cells/ml initially and grown in RPMI 1640 culture medium with 10% FCS, 2mM L-glutamine and 50μg/ml gentamicin at 37°C in an atmosphere of 5% CO_2 in air. Cultures were split into 250 ml tissue culture flasks at 5 x 10^5 cells/ml and left for four weeks with no feeding. Supernatants were centrifuged at 400g for 20 minutes to remove cells and debris, and then passed through a 1.2μm filter. The virus containing cell free supernatant was aliquoted and stored at -70°C.

2.2.7. Titration of EBV Supernatants.

Five EBV containing cell free supernatant batches were tested (A, B, C, D and E). Virus from each batch was diluted in RPMI 1640 culture medium in serial 10 fold dilutions from 10^{-1} to 10^{-5}. Cord blood mononuclear cells were resuspended to 12.5 x 10^6/ml in each dilution of B95-8 supernatant and incubated for 1 hour at 37°C in an atmosphere of 5% CO_2 in air. Virus was removed by washing and the cells resuspended in RPMI 1640 culture medium with 10% FCS, 2 mM L-glutamine and 50μg/ml of gentamicin to give a final concentration of 10^6 cells/ml. Each test was plated out in eight replicate 200μl cultures in 96 well flat bottom plates. Cells were incubated for 4 weeks at 37°C in an atmosphere of 5% CO_2 in air and fed once a week by removal of half the supernatant and replacement with an

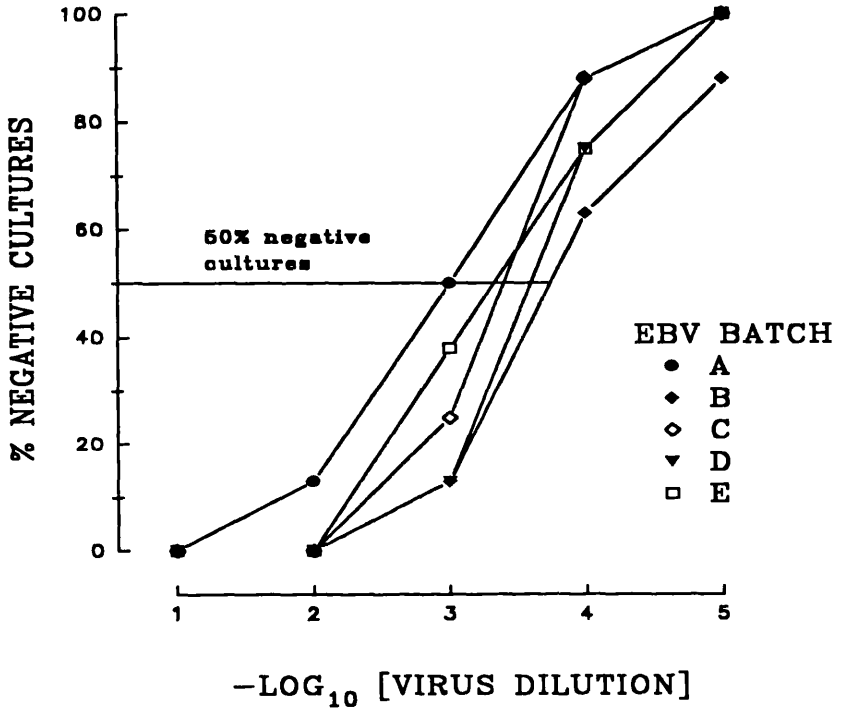
equal volume of culture medium with additions as before. At the end of four weeks the cultures were scored positive or negative by the presence or absence of proliferating foci. The transforming efficiency of each virus batch was determined (table 2.1.). Virus was diluted accordingly and used at a TD50 = 3 in all experiments.

TABLE 2.1. VIRAL TRANSFORMING EFFICIENCY:

Virus batch	TD_{50}[a]
A	3
B	3.75
C	3.4
D	3.6
E	3.3

[a]Transforming efficiency (TD50) = $\log_{10}$ [dilution giving 50% negative cultures]

Figure 2.1.



Determination of EBV Transforming Efficiency.

Five batches of EBV containing supernatant (A, B, C, D and E) from the marmoset B lymphoblastoid cell line B95-8 were titrated to determine their transforming efficiency on naive human cord blood B cells as described in section 2.2.7.

2.2.8. Activation of Human Tonsillar B Cells and B Cell Enriched PBMCs with EBV.

Tonsillar B cells and B cell enriched PBMCs were activated with EBV containing B95-8 cell supernatant with an established viral titre (TD_{50}) of 3. B cells or B cell enriched PBMCs (12.5 x 10^6 cells per ml of virus containing supernatant) were incubated for 1 hour at 37°C in an atmosphere of 5% CO_2 in air. Tonsillar B cells were then washed and resuspended in RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine and 50 μg/ml of gentamicin unless otherwise stated. B cell enriched PBMCs were resuspended in IMDM supplemented with 10% FCS, 2 mM L-glutamine and 50 IU/ml penicillin/streptomycin with the addition of cyclosporin to a final concentration of 1 ug/ml, to prevent *in situ* cytotoxic T cell killing of EBV infected B cells.

2.2.9. Assay for Induction of B Cell Surface Antigens.

Tonsillar B cells (2.5 x 10^5) were cultured in 250μl volumes in 96 well flat bottom microtitre tissue culture plates for 3 days. B cells were then washed in RPMI holding medium with 0.01% NaN_3 and stained with mAbs or polyclonal antibodies to particular B cell surface antigens or receptors for FACS analysis as in section 2.2.5.

2.2.10. Assay for B Cell Proliferation.

Tonsillar B cells (2.5 x 10^5) were cultured in 250μl volumes in 96 well round bottom microtitre tissue culture plates. 1 μCi ^{3}H-TdR. was added for the last 8 hours of culture. The cells were then harvested on a Dynatech AutoMash 2000 cell

harvester, dried at 100°C for 2-4 hours and counted in 4mls of scintillation fluid on an LKB 1218 RackBeta scintillation counter. Results were calculated in dpm. Unless otherwise stated, all cultures were set up in triplicate or greater.

2.2.11. Assay for Immunoglobulin Secretion.

Tonsillar B cells or B cell enriched PBMCs, at a concentration of 1 x 10^6 cells/ml were cultured in 0.5ml volumes in 5 ml round bottom tissue culture tubes for 13 days. In some experiments, tonsillar B cells were also cultured in 2ml volumes in 24 well flat bottom tissue culture plates, also for 13 days. Supernatants were removed and assayed for immunoglobulin content by ELISA. All cultures were set up in triplicate or in some cases replicates of 4 to 6.

For long term cultures of greater than 13 days, 300 µl supernatant samples were removed from 2ml B cell cultures for assay of immunoglobulin content at the times indicated, and replaced with an equal volume of fresh culture medium. Results were adjusted to compensate for dilution of immunoglobulin content.

2.2.12. Toxicity Assays.

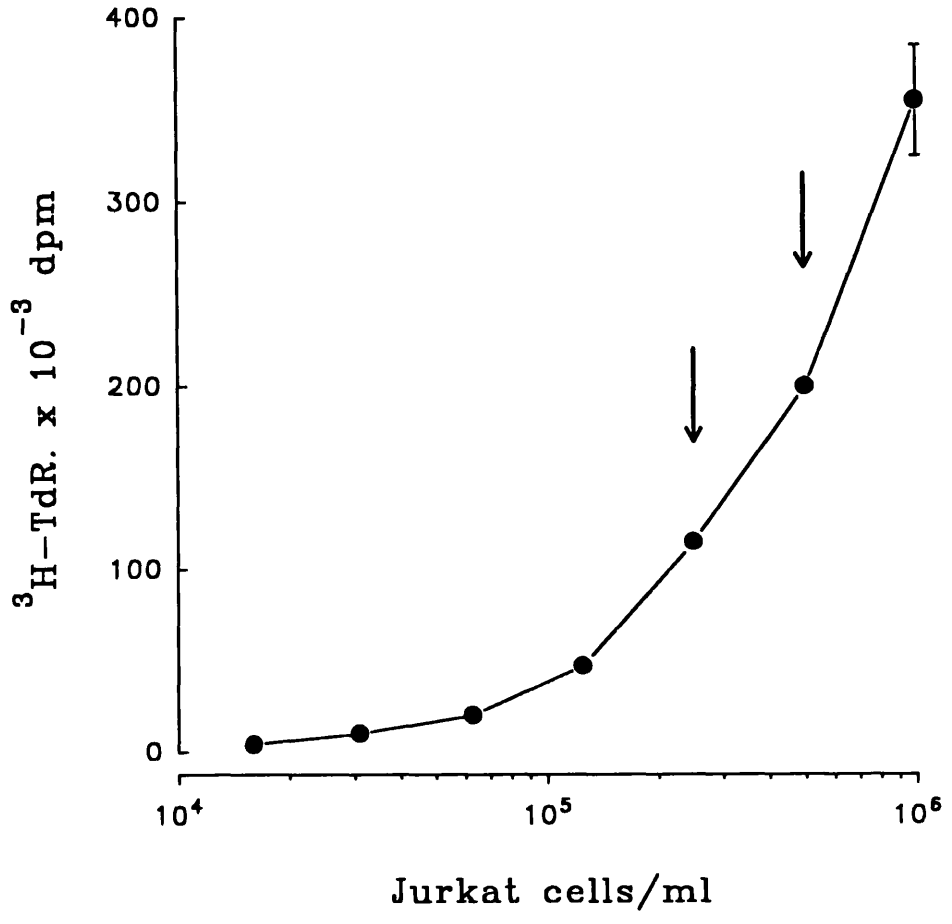
Human T cell PHA Response.

T cells or PBMC at a concentration of 10^6 cells/ml in 200ul volumes were cultured with 1ug/ml of PHA in 96 well tissue culture plates. Proliferation was measured by the addition of 1uCi of ^{3}H-TdR for 8 hours on day 3 of culture before harvesting and counting as in section 2.2.10.

Proliferation of the Jurkat T cell Line.

Cells of the Jurkat human leukaemic T cell line, grown in RPMI 1640 without Hepes, were washed, resuspended in the same culture medium and seeded in decreasing cell concentrations into 96 well round bottom tissue culture plates in 200 ul volumes. Proliferation was assayed by the addition of 1uCi of ^{3}H-TdR for 8 hours on day 3 of culture. The cell growth titration curve shown in figure 2.2. shows that Jurkat concentrations of 0.25 and 0.5 x 10^{6} cells/ml are in exponential growth phase on day 3 of culture, and being on the linear part of the curve, a small inhibition of growth would give the greatest deflection from the normal and be easily identifiable. Antibodies to be tested for toxicity were added to Jurkat cells at these two concentrations at the start of culture and proliferation measured on day three for detection of inhibitory effect.

Figure 2.2.



Jurkat Cell Growth Titration Curve.

Jurkat cells were grown to maximum density, then seeded at the cell concentrations shown in 200 ul volumes. Proliferation was measured on day 3 by ^{3}H-TdR incorporation.

2.2.13. ELISA Assays for the Detection of Human Immunoglobulin Classes and IgG Subclasses.

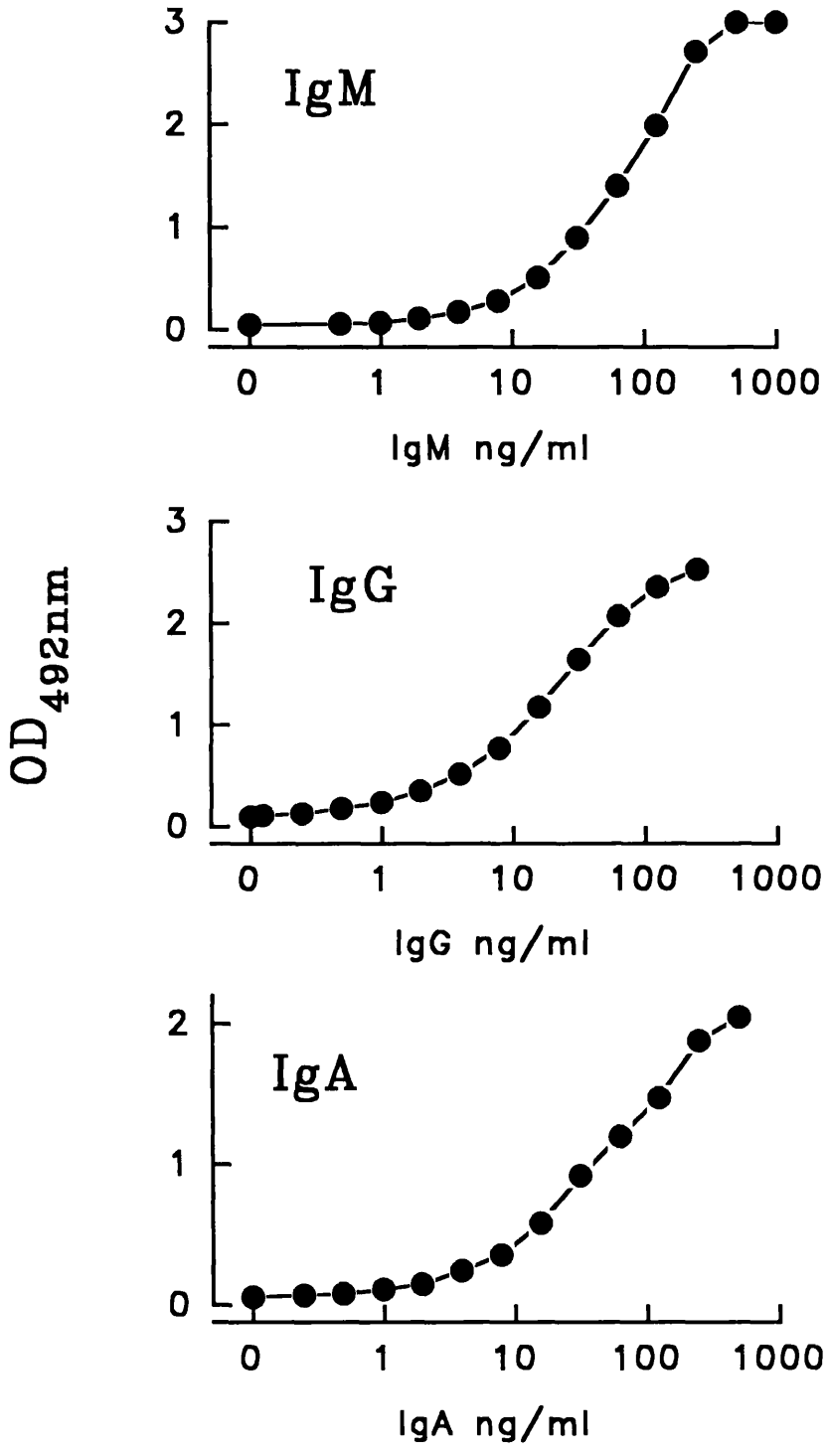
IgM, IgA and IgG.

1. Sterilin 96 well round bottomed plates were incubated over night with 75µl of goat anti-human IgG, IgA or IgM (1ug/ml) in coating buffer at room temperature.
2. The plates were washed x3 and then blocked for 1 hour at 37°C with 100µl PBS/0.05% Tween 20/1% BSA.
3. After washing x3, samples (75µl) were added to the plates and incubated for 1 hour at 37°C.
4. The plates were then washed x3 and horse radish peroxidase conjugated goat anti-human IgG, IgA or IgM (75µl of 1µg/ml) added and incubated at 37°C for 1 hour.
5. After a final x3 wash, 75µl OPD substrate (0.5mg/ml) dissolved in phosphate/citrate buffer and H_2O_2 (0.015% v/v) was added to each well and developed at room temperature.
6. Colour development was stopped with 40µl 4N H_2SO_4 and plates read at 492nm with a Dynatech ELISA plate reader.

All washing steps were in PBS/0.05% Tween 20. Samples and antibodies were diluted in PBS/0.05% Tween 20/1% BSA.

Each ELISA plate included serial dilutions of a standard serum of known human IgA, IgG or IgM concentration, for construction of a standard curve. Results were calculated in ng/ml against this standard curve using Mikrotek ELISA software.
The detection ranges were 1-1000, 1-500 and 5-500 ng/ml for IgA, IgG and IgM respectively as shown in figure 2.3.

Figure 2.3.



Standard Titration Curves for Human IgM IgG and IgA ELISAs.
The range of detection for IgM, IgG and IgA is shown in ng/ml.

IgE.

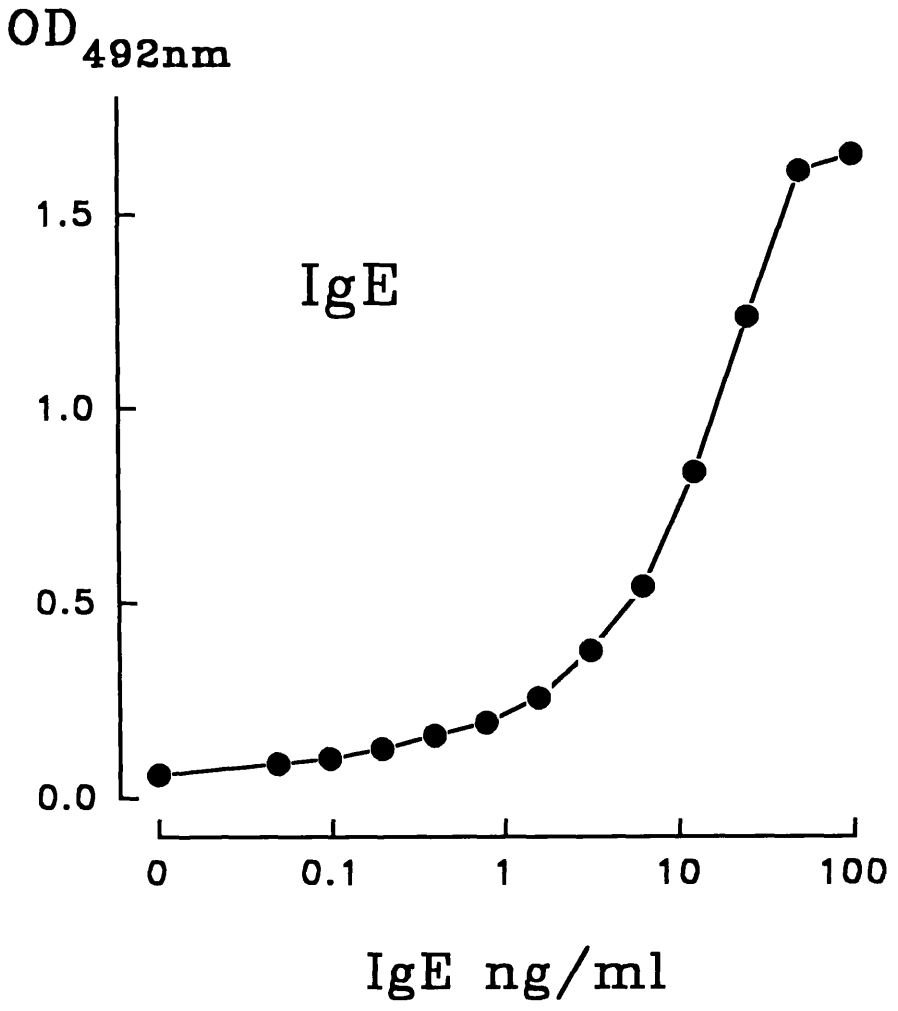
1. Flow LINBRO EIA II flat bottomed ELISA plates were incubated over night with 75µl of goat anti-human IgE (2ug/ml) in coating buffer at room temperature.
2. The plates were washed x3 and then blocked for 6 hours at room temperature with 100µl PBS/0.05% Tween 20/1% BSA.
3. After washing x3, samples (75µl) were added to the plates and incubated over night at room temperature.
4. The plates were then washed x3 and rabbit anti-human IgE (75µl of 0.02 µg/ml) added and incubated at room temperature for 4 hours.
5. The plates were then washed x3 and horse radish peroxidase conjugated goat anti-rabbit IgG (75µl of 1µg/ml) added and incubated at room temperature for 2 hours.
6. After a final x3 wash, 75µl OPD substrate (0.5mg/ml) dissolved in phosphate/citrate buffer and H_2O_2 (0.015% v/v) was added to each well and developed at room temperature.
7. Colour development was stopped with 40µl 4N H_2SO_4 and plates read at 492nm with a Dynatech ELISA plate reader.

All washing steps were in PBS/0.05% Tween 20. Samples and antibodies were diluted in PBS/0.05% Tween 20/1% BSA.

Each plate included serial dilutions of a standard serum of known human IgE concentration for construction of a standard curve. Results were calculated in ng/ml against this standard curve using Mikrotek ELISA software.

The range for detection of IgE was 0.1-100 ng/ml as shown in figure 2.4.

Figure 2.4.



Standard Titration Curve for Human IgE ELISA
The range of detection for IgE is shown in ng/ml.

Human IgG Subclasses, IgG1, IgG2, IgG3 and IgG4.

1. Dynatech flat bottomed Immulon II ELISA plates were incubated overnight with 75µl of either rabbit anti human IgG (H+L) (1µg/ml) or sheep anti-human IgG4 (2µg/ml) in coating buffer at room temperature.
2. The plates were washed x3 and then blocked for 6 hours at room temperature with 100µl PBS/0.05% Tween 20/1% NMS.
3. After washing x3, samples (75µl) were added to the plates and incubated over night at room temperature for the assay of IgG1, IgG2 and IgG3 and overnight at 37°C for the assay of IgG4.
4. The plates were then washed x3 and biotinylated mouse monoclonal anti-human IgG1 (75µl of 0.5µg/ml), IgG2 (75µl of 1µg/ml) and IgG3 (75µl of 2µg/ml) added and incubated for 2 to 4 hours at room temperature. Anti-human IgG4 (75µl of 2µg/ml) was added and incubated for 4 hours at 37°C.
5. The plates were then washed x3 and alkaline phosphatase (75µl of 1/2000) or Horse Radish Peroxidase (75µl of 1/1000) conjugated streptavidin added and incubated for 1-2 hours at room temperature.
6. After a final x3 wash, either pNPP (75µl of 1.0 mg/ml) in alkaline phosphatase substrate buffer (pH 9.6) or OPD substrate (75µl of 0.5mg/ml) dissolved in phosphate/citrate buffer and H_2O_2 (0.015% v/v) was added to each well and developed at room temperature in the dark.
7. Colour development of pNPP was stopped with 40µl 3M NaOH and plates read at 410nm and development of OPD was stopped with 40µl 4N H_2SO_4 and plates read at 492nm with a Dynatech ELISA plate reader.

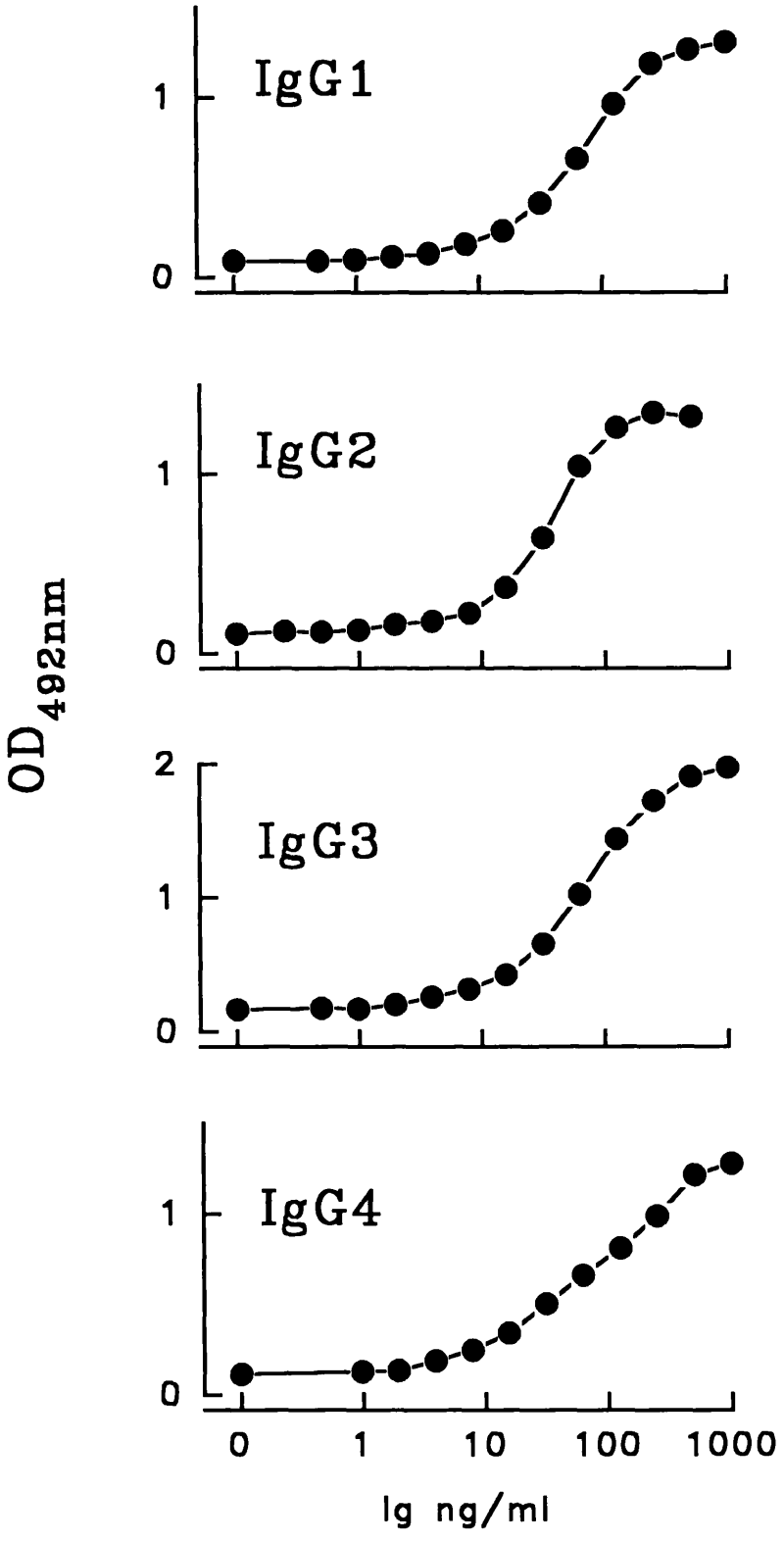
All Washing steps were in PBS/0.05% Tween 20. Samples and antibodies were diluted in PBS/0.05% Tween 20/0.2 % normal mouse serum.

Each plate included serial dilutions of a standard serum of known human IgG1, IgG2, IgG3 or IgG4 concentrations for construction of a standard curve. Results were calculated in ng/ml against this standard curve using Mikrotek ELISA software.

The detection ranges were 1-500, 1-200, 1-1000, and 1-500 ng/ml for IgG1, IgG2, IgG3 and IgG4 respectively as shown in figure 2.5.

Assay specificity was shown by non binding to B72.3 chimeric monoclonal antibodies given by kind permission of Ray Owens (Celltech Ltd., Slough, UK.). These antibodies are comprised of mouse V regions against the TAG72 human colon carcinoma mucin antigen and either the human γ1, γ2, γ3 or γ4 C regions (1b). B72.3 IgG1 mAb was purified by protein G chromatography from supernatants of the mouse myeloma NSO cell line and B72.3 IgG2, B72.3 IgG3 and B72.3 IgG4 mAbs from Chinese hamster ovary cell lines.

Figure 2.5.



Standard Titration Curves for Human IgG subclass ELISAs.
The range of detection for IgG1, IgG2, IgG3 and IgG4 is shown in ng/ml.

CHAPTER 3

POLYCLONAL ACTIVATION OF HUMAN B LYMPHOCYTES BY EPSTEIN BARR VIRUS

3.1. INTRODUCTION.

In vivo, Epstein Barr Virus (EBV) (125,126) is able to infect a number of different cell types expressing the EBV receptor (CD21/CR2), including B cells, follicular dendritic cells (FDC) and pharyngeal and cervical epithelium (244). This is reflected in a number of EBV attributed diseases such as infectious mononucleosis (glandular fever) and nasopharyngeal carcinoma in addition to the EBV derived B cell cancer, African Burkitt's Lymphoma. Recently, EBV has been found to infect human thymocytes (419) perhaps implying more roles for the virus in disease than previously thought.

In vitro, EBV is a T cell independent mitogen for human B cells (33,218), activating purified human B cells to proliferate and then differentiate into immunoglobulin secreting cells. The presence of activated T cells in culture inhibits EBV induced proliferation and secretion of immunoglobulin by killing virally infected B cells (417). After a prolonged period of culture, EBV transforms a small sub-population of infected B cells giving rise to lymphoblastoid cell lines (34). EBV has been used by many groups for the study of human B cell activation and to examine the human immune repertoire in age and disease (69,71,119,121,165,276,393).

There are several characteristics of polyclonal B cell activation with EBV that make it suitable for the study of cytokine action on B cells.

1) EBV activation of B cells does not require the presence of T cells.

2) EBV activates all B cells without marked preference for subsets expressing different immunoglobulin classes and subclasses (69,275,289).

3) EBV will bind to and activate B cells of varying cell sizes (71), and at all stages of the cell cycle except for cells in S-G2 due to loss of CD21 (69,188).

4) B cells which have been activated with EBV are not arrested at any particular stage in maturation or differentiation and retain the ability to undergo heavy chain class switching *in vitro* (11,71,275,380).

In this Chapter, the kinetics and cell culture conditions for the polyclonal activation of human B cells by EBV were established and B cells activated with EBV were shown to secrete all immunoglobulin classes and subclasses.

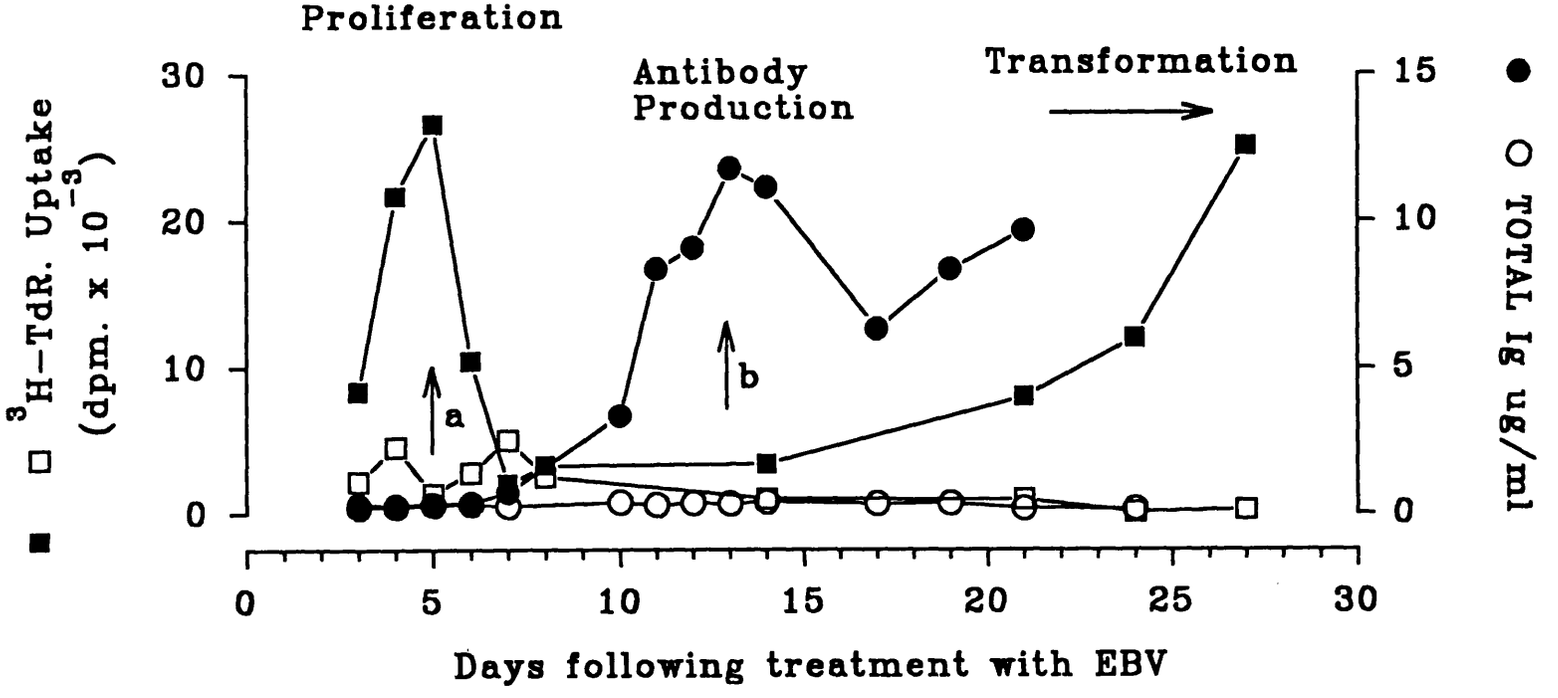
3.2. TIME COURSE OF PROLIFERATION AND ANTIBODY SECRETION BY B CELLS ACTIVATED WITH EBV.

In order to establish the kinetics of EBV induced B cell responses, EBV activated and non activated tonsillar B cells were examined for proliferation and immunoglobulin secretion over a period of 1 to 30 days.

3.2.1. Proliferation.

The proliferative response of EBV activated B cells was biphasic. An initial peak occurred on day 4/5 after activation which decreased rapidly to background levels by day 7. After a lag period of 10-15 days, proliferation again increased in log phase growth, characteristic of rapidly dividing transformed clones. Observation of the cultures by phase contrast during the log phase proliferation showed foci of rapidly dividing cells. The results from a typical experiment are shown in Figure 3.1.

Figure 3.1.



Kinetics of EBV Induced B Cell Activation.

Tonsillar B cells were either activated with EBV (filled symbols) or incubated with medium alone (open symbols). Proliferation (■ □) was assayed by uptake of ^{3}H-TdR. and Ig production (● ○) by ELISA.

3.2.2. Immunoglobulin Secretion.

Immunoglobulin secretion by EBV activated tonsillar B cells was also biphasic. Immunoglobulin levels remained similar to those obtained from non activated B cell cultures until day 7 and then rose steadily to a maximum at day 12/13. Maximum secretion was maintained until day 14/15 and then remained steady, or dropped away in some experiments. The decrease which occurred in some experiments may have been due to degradation by lysosomal enzymes released into the culture as a result of cell death. A second increase in antibody secretion was seen late in culture after day 21. These cultures were predominantly IgM secreting and microscopic examination showed that they had the morphology of rapidly dividing foci characteristic of EBV transformed cells. Typical results for the kinetics of EBV induced immunoglobulin secretion are shown in Figure 3.1.

In all subsequent experiments proliferation was measured on day 5 and immunoglobulin secretion on day 13 unless otherwise stated.

3.3. EBV INDUCES SECRETION OF ALL HUMAN IMMUNOGLOBULIN CLASSES AND IgG SUBCLASSES EXCEPT FOR IgE.

To confirm that EBV was able to stimulate production of all human immunoglobulin classes and IgG subclasses, B cells were activated with EBV and assayed for IgM, IgA, IgG, IgE, IgG1, IgG2, IgG3 and IgG4 on day 13 of culture.

3.3.1. Results.

In six experiments, EBV stimulated secretion of all immunoglobulin classes and IgG subclasses with the exception of IgE. In all cases IgE was below the detection limit of assay (< 0.5 ng/ml). Values obtained on day 13 from two experiments are shown in Table 3.1.

TABLE 3.1. IMMUNOGLOBULIN CLASS AND IgG SUBCLASS SECRETION INDUCED BY ACTIVATION WITH EBV.

Ig Isotype	Experiment 1		Experiment 2	
	Medium	EBV	Medium	EBV
IgM	158 ± 25[a]	10903± 308	11 ± 1.7	2744± 532
IgA	26 ± 8.2	1076 ± 291	151 ±12.5	1365± 151
IgE	< 0.5	< 0.5	< 0.5	< 0.5
total IgG	446 ± 35	8325± 2992	61 ±10	3059±374
IgG1	351 ± 25	4841± 1243	44 ± 2.5	1967±333
IgG2	60 ± 3.0	106 ± 7.0	24 ± 7.7	114 ± 34
IgG3	56 ± 14	205 ± 33	6 ± 1.3	89 ± 17
IgG4	< 1.0	6.34 ± 1.4	134 ± 53	293 ± 24

[a] Ig secretion expressed as ng/ml ± 1SE

3.4. REFINEMENT OF CULTURE CONDITIONS FOR B CELLS ACTIVATED WITH EBV.

In the process of determining the optimal culture conditions for EBV activated B cells, the effects of B cell concentration, exposure to EBV and type of culture vessels, were examined.

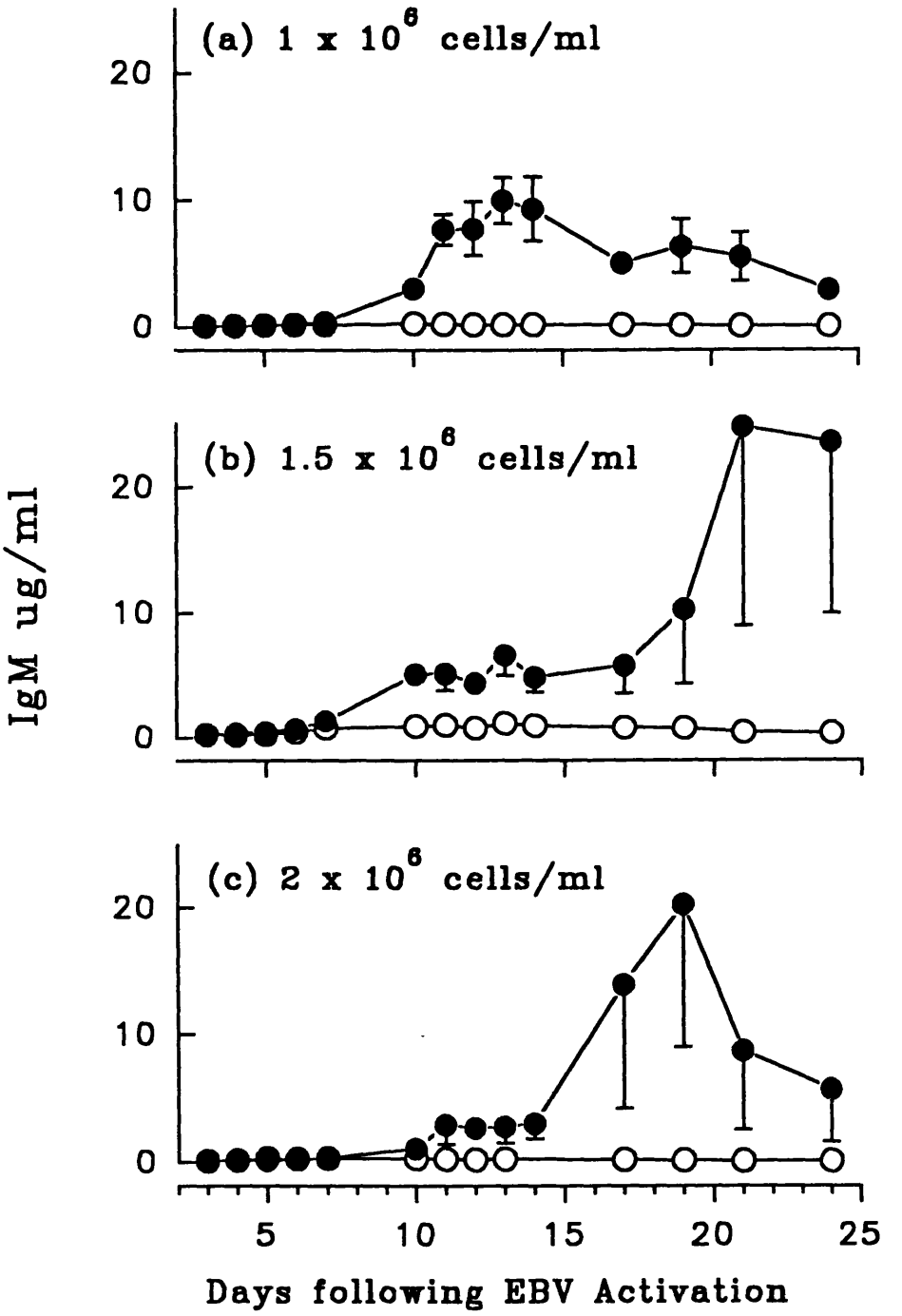
3.4.1. Effect of Cell Density.

Tonsillar B cells were activated with EBV and cultured as in Section 3.3. at varying cell densities from 1 - 4 x 10^6 cells/ml. Proliferation and immunoglobulin secretion was determined over 1 - 30 days.

Proliferation was optimal at a cell density of 10^6 cells per ml. Cell concentrations higher than this gave inconsistent results and were more likely to produce transformed foci late in culture. Moreover, at higher cell concentrations, background proliferation of non activated B cells was high (results not shown).

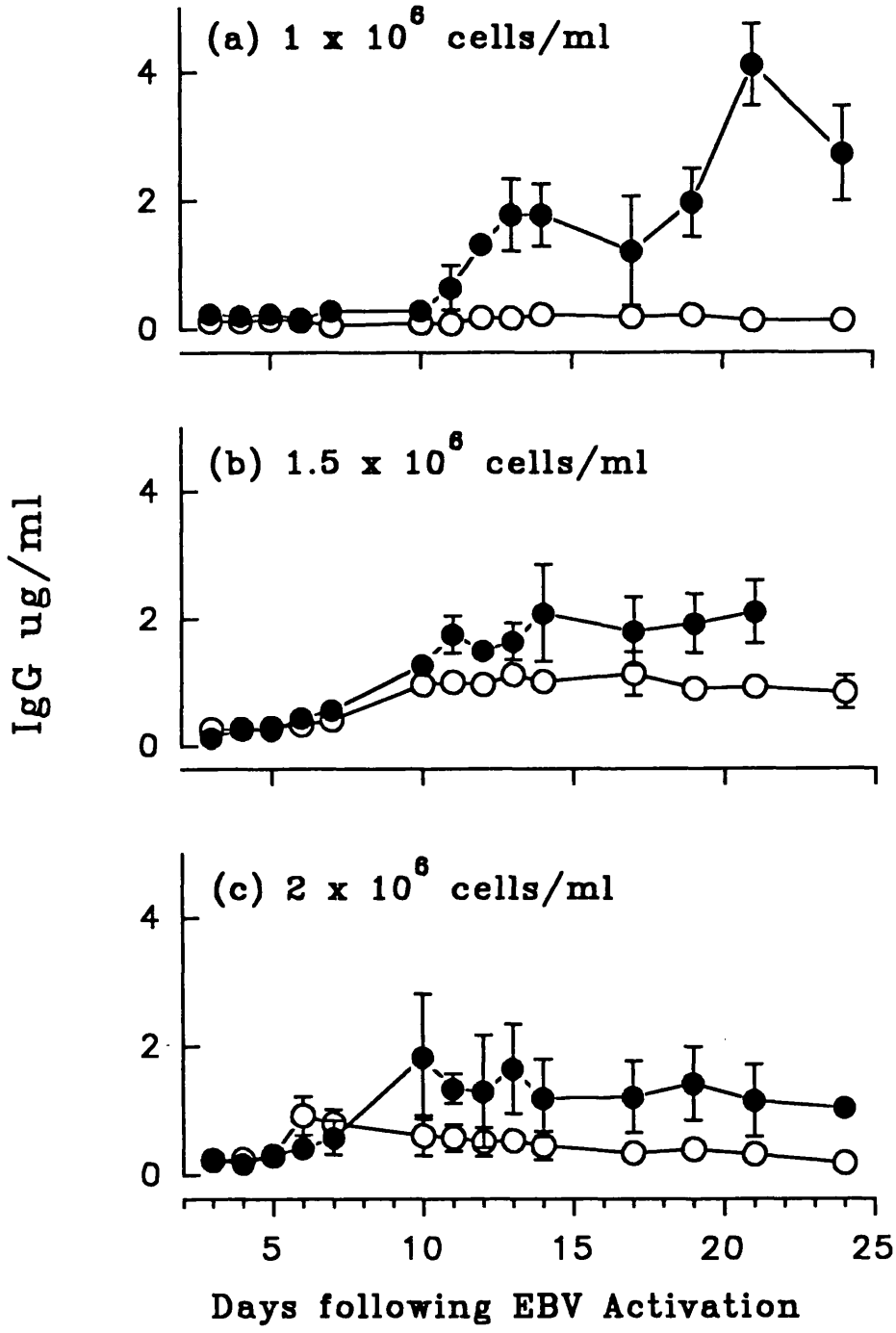
A cell density of 10^6 cells/ml gave good reproducible peaks of immunoglobulin secretion at day 13, and background secretion by non activated B cells was consistently low (Figures 3.2a and 3.3a). Higher cell concentrations of 1.5 - 2 x 10^6 cells/ml produced less IgM antibody during the period of polyclonal secretion from day 1 to day 13 and were more susceptible to transformation. This was indicated by the emergence of IgM secreting clones after day 16 (Figure 3.2b and c). In addition, although IgG secreting transformed clones were not so evident, the higher cell densities gave unacceptably high background IgG secretion (Figure 3.3b and c). Interestingly, the higher cell densities yielded fewer IgG secreting transformed clones (data not shown).

Figure 3.2.



IgM Secretion by EBV Activated Tonsillar B Cells Cultured at Different Cell Densities. Tonsillar B cells were either activated with EBV (●) or incubated in medium alone (O), then cultured at cell densities from 1-2 x 10^6 cells/ml. IgM secretion was measured by ELISA.

Figure 3.3.



IgG Secretion by EBV Activated Tonsillar B Cells Cultured at Different Cell Densities. Tonsillar B cells were either activated with EBV (●) or incubated in medium alone (○), then cultured at cell densities from 1–2 x 10^6 cells/ml. IgG secretion was measured by ELISA.

A cell concentration of 10^6 B cells per ml was used for both proliferation and immunoglobulin secretion assays in all subsequent experiments unless otherwise stated.

3.4.2. Inclusion of EBV in Culture Inhibits Both B Cell Proliferation and Immunoglobulin Secretion.

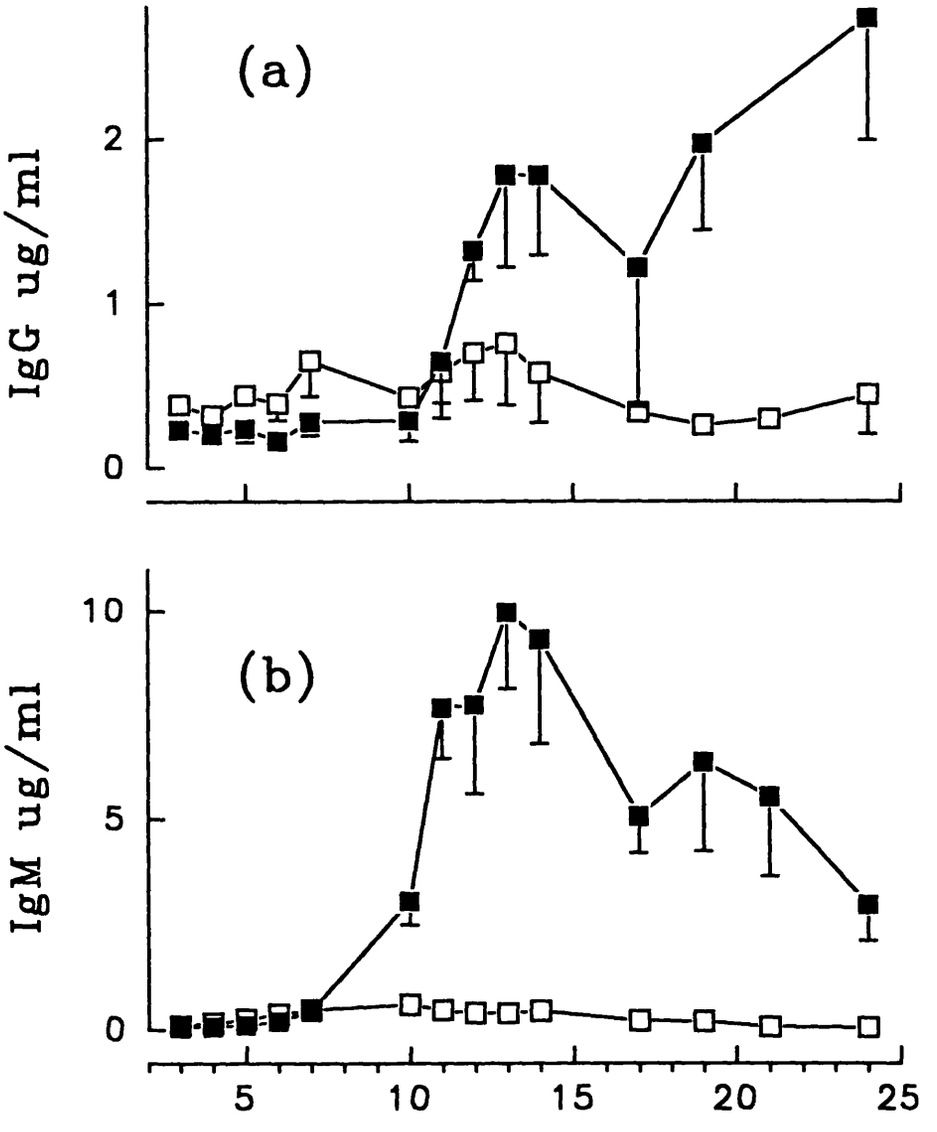
It was possible that prolonged viral exposure during culture might increase polyclonal proliferation and immunoglobulin secretion and further optimise EBV activation. In order to test this, B cells were activated with EBV then split into two aliquots. One sample of activated B cells was washed to remove the virus before culture, another sample was cultured without removing the virus. B cells were cultured at a final cell concentration of 10^6 cells/ml in RPMI 1640. Proliferation and immunoglobulin secretion were measured as before.

When cells were not washed to remove the virus before culture, both proliferation and antibody secretion were significantly reduced, sometimes by 75% or greater. Results from a typical experiment showing IgM and IgG secretion are shown in Figure 3.4.

3.4.3. Establishment of B Cell Micro-Culture Conditions for the Addition of Cytokines.

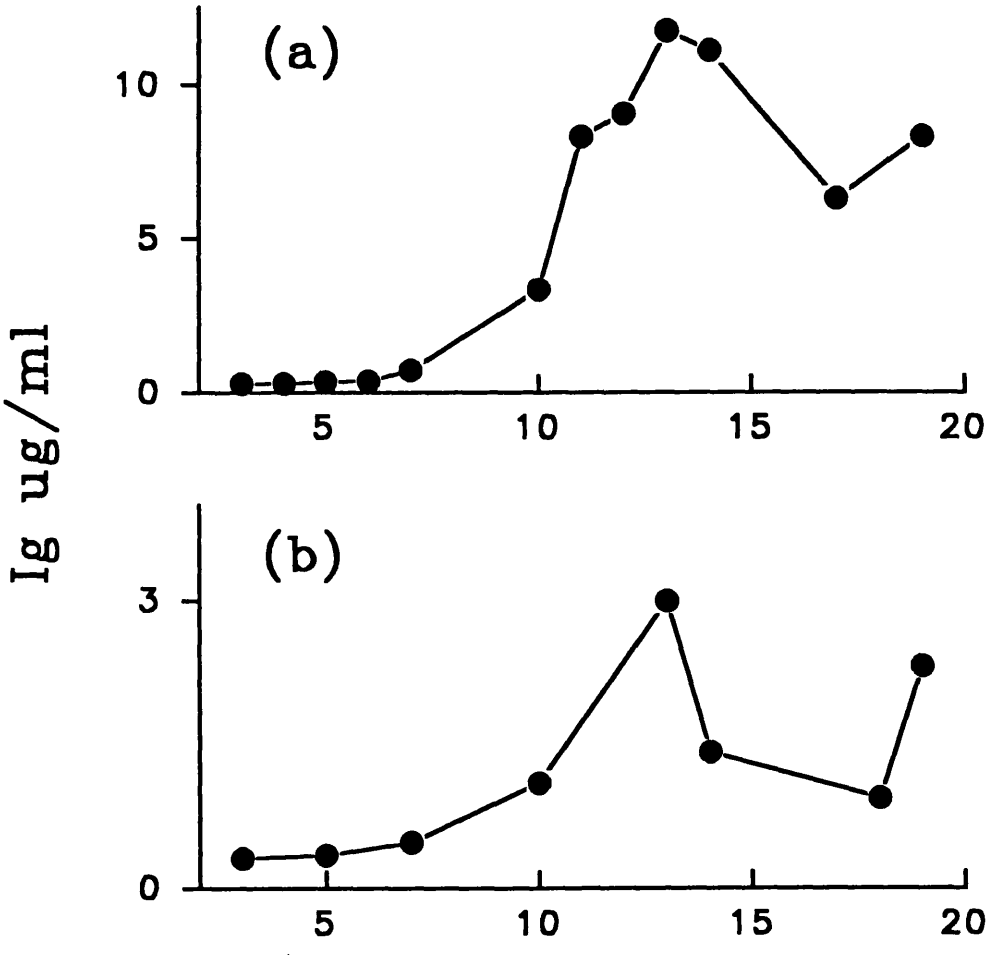
Initial large scale experiments to determine the kinetics of EBV polyclonal immunoglobulin secretion used 2ml cultures in flat bottom plates, but it was not possible or economical in all experiments to use cultures of such a large volume for the addition of cytokines. Proliferation and immunoglobulin secretion by EBV

Figure 3.4.



Inhibitory Effect of EBV when Included in Culture.
Tonsillar B cells were incubated with EBV then either washed (■) to remove the virus or not washed (□). IgG (a) and IgM (b) production was measured by ELISA.

Figure 3.5



Comparison of Macro and Micro Culture Conditions for Ig Production from EBV Activated Tonsillar B Cells.
Tonsillar B cells at a concentration of 10^6 cells/ml were cultured in (a) macro cultures of 2mls and (b) micro cultures of 0.5mls. Ig production was measured by ELISA.

activated B cells in macro (2mls) cultures were therefore compared to micro (0.5mls) cultures in falcon tubes (set up as in the materials and methods) and found to give comparable results. An example of immunoglobulin secretion is shown in Figure 3.5.

3.5. DISCUSSION

EBV activation of human tonsillar B cells was found to be biphasic, consisting of initial polyclonal mitogenic stimulation of B cells and a later more selective transformation of a very small, predominantly IgM secreting, population. After initial activation with EBV, the primary peak of B cell proliferation occurred on day 4-5, falling back to control levels by day 7. After day 7 B cells differentiated into immunoglobulin secreting cells and the antibody content of the cell culture supernatants rose steadily to optimal concentrations on days 12-14. Only after a considerable lag period did a small percentage of cells undergo a second proliferative and immunoglobulin secretory phase consistent with transformation. Examination of cultures at this late stage showed the presence of a small number of rapidly dividing clones characteristic with EBV LCLs. These clones were predominantly IgM secreting.

It was found that EBV activation of human B cells in the absence of T lymphocytes induced secretion of all Ig classes and IgG subclasses with the exception of IgE. The absence of IgE in EBV induced Ig secretion may have been due to very low numbers of IgE^+ B cells in the original culture or indicate that IgE may need very specific conditions for its production and regulation (104,107,410,411,413).

Interestingly, IL10 has sequence homology to the EBV gene BCRF-1 whose recombinant protein has been termed viral IL10 (vIL10) (183). IL10 is a BCGF and

a BCDF for human B cells and vIL10 can replace the natural molecule with equal potency (337). EBV activated B cells may produce vIL10, and this in combination with EBV as a polyclonal activator may potentiate B cell responses. Recently it has been shown that EBV activation of human B cells induces human IL10 production (51). The kinetics of IL10 production after EBV activation shows only low amounts present by day 7 but a maximum peak at day 14 followed by complete reduction at the B cell transformation stage (51). The day 14 peak of IL10 production is parallel to the peak of Ig production induced by EBV found in this Chapter. IL10 is known to increase IgA, IgM and IgG strongly (337), and may be acting in combination with EBV to induce polyclonal Ig secretion. However, as little IL10 is produced by day 7, it is almost certainly not involved in EBV induced proliferation.

The optimal cell density and culture conditions for EBV induced polyclonal proliferation and immunoglobulin secretion were established and may now be used to investigate the effects of human recombinant cytokines.

CHAPTER 4

EFFECT OF HUMAN RECOMBINANT CYTOKINES ON IMMUNOGLOBULIN PRODUCTION BY EBV ACTIVATED B CELLS

4.1. INTRODUCTION.

A number of key cytokines have been implicated in the control of B cell immunoglobulin class and IgG subclass production. From studies with LPS activated mouse B cells, IL4 has been shown to induce IgE and IgG1 production by heavy chain class switching (127,334). IFNγ specifically induces IgG2a secretion and promotes Ig class switching to IgG3 (134,370,371) and TGFβ specifically induces IgG2b secretion and Ig class switching to IgA (241,264,370). Murine IL5 enhances IgA production, but by clonal expansion of IgA^+ B cells rather than induction of heavy chain class switching (262).

Although IL4 (151,196,355) and TGFβ (104,192,292) have been found to control production of IgE and IgA respectively from human B cells, the role of cytokines in the regulation of IgG subclass production is much less clear. NGF has been reported to enhance the production of IgG4 by clonal expansion of pre-committed B cells rather than by induction of class switching (216). IL2 control of human IgG subclass production differs with respect to the source of B cells and the method of activation (61), while the evidence for IL4 control of IgG subclasses is conflicting. In some cases IL4 stimulates production of IgA, IgM, IgG1, IgG2 and IgG3 with no effect on IgE (141). In other cases IL4 either induces secretion of IgE only or of IgE and IgG4 with either no effect on IgA, IgM or total IgG (149,150,250), or an increase in these classes (394). Humans and mice have similar immunoglobulin heavy chain constant region genes, but the IgG subclasses appear to have arisen independently (60). Accordingly, it is likely that the cytokine network controlling IgG subclass production in both species may be dissimilar.

In addition to IL2, IL4 and NGF, a number of different cytokines stimulate human B cell proliferation and differentiation into antibody secreting cells, but their effect on immunoglobulin class and IgG subclass production is largely unknown. One

reason for the lack of information has been the paucity of truly T cell independent mitogens able to stimulate Ig production by human B cells in a similar way to LPS activation of mouse B cells. In this Chapter, the effect of different cytokines on immunoglobulin class and IgG subclass responses obtained by polyclonal activation of human B cells with EBV has been examined.

4.2. EXPERIMENT DESIGN.

Tonsillar B cells were polyclonally activated with EBV and washed and cultured in RPMI 1640 as described in Chapter 2 (Section 2.2.8.). Recombinant human cytokines IL2, IL4, IL5, IL6, TGFβ, IFNγ or IFNα were added to the cultures at time 0 at various concentrations as indicated. Non activated B cells were also cultured under identical conditions. Proliferation (^{3}H-TdR. uptake) was measured on day 5, and immunoglobulin secretion was measured on day 13 as described in Sections 2.2.10. and 2.2.11. All experiments were repeated a minimum of three times.

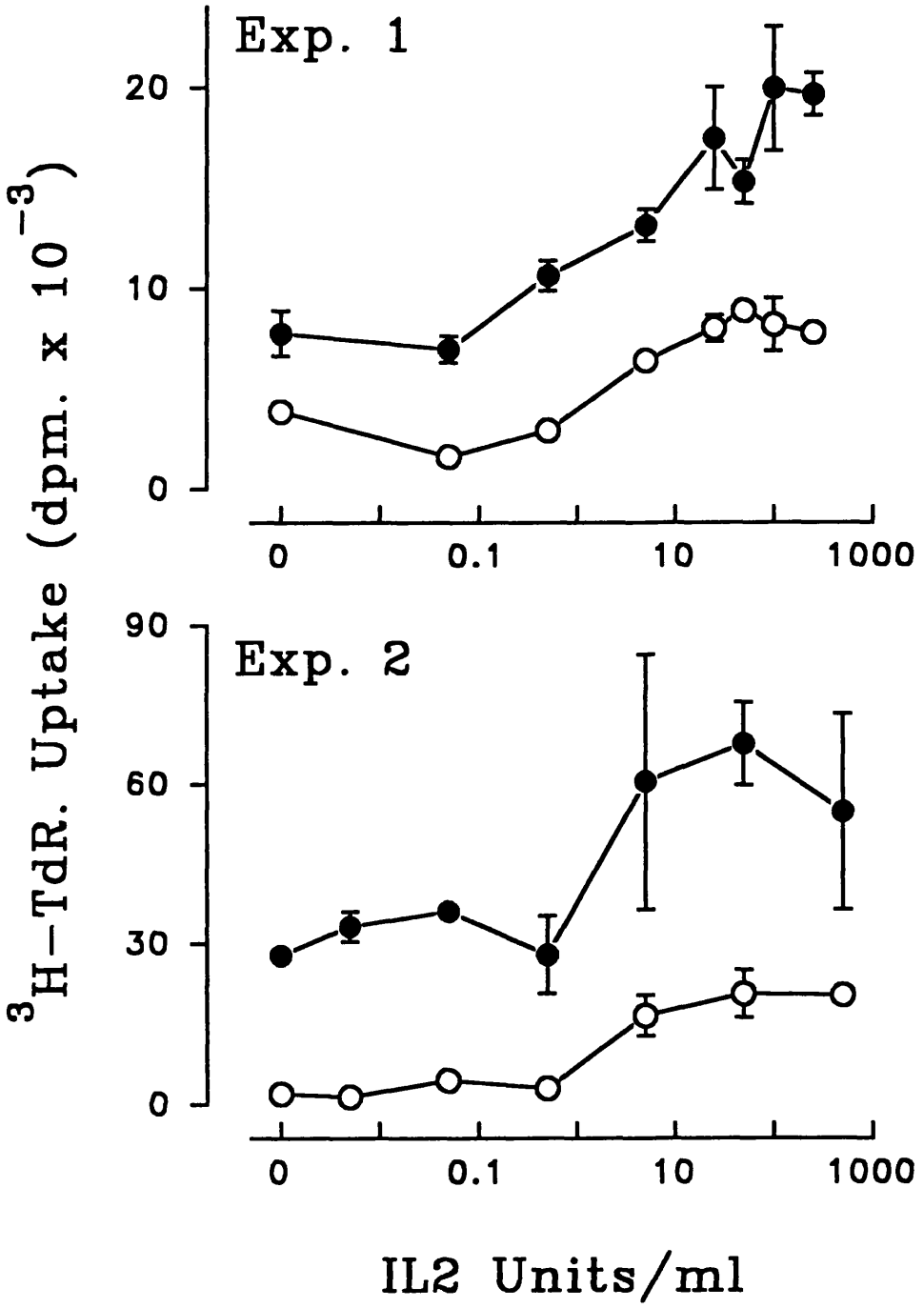
4.3. INTERLEUKIN 2.

IL2 is a potent human B cell growth and differentiation factor. This Section examines its role in specific immunoglobulin class and/or IgG subclass regulation.

4.3.1. Effect of IL2 on Proliferation of B Cells Activated with EBV.

At concentrations below 0.1 U/ml, IL2 had no effect on B cell proliferation. Concentrations of IL2 greater than 0.1 U/ml enhanced proliferation of EBV

Figure 4.1.



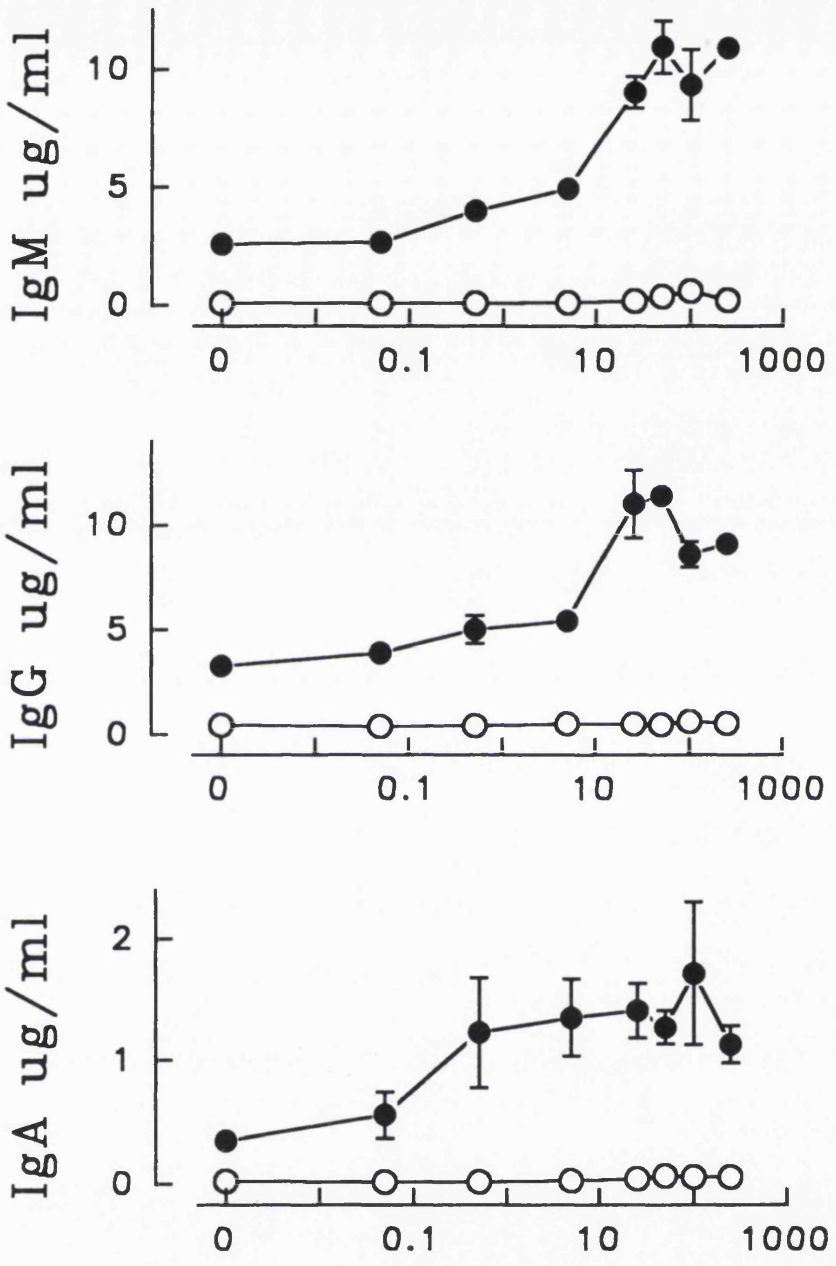
Effect of IL2 on EBV Induced B cell Proliferation.
Tonsillar B cells, activated with EBV (●) or incubated in medium alone (O), were cultured with IL2 at centrations from 0-500 U/ml. Proliferation (^{3}H-TdR. uptake) was measured on day 5.

stimulated B cells in a dose dependent manner with maximum proliferation attained at 25-50 U/ml of IL2. Doses of IL2 in excess of this had no additional effect. Results from a typical experiment are shown in Figure 4.1. IL2 also enhanced proliferation of unstimulated B cells in some experiments, albeit to a much lesser extent.

4.3.2. Effect of IL2 on Immunoglobulin Production by B Cells Activated with EBV.

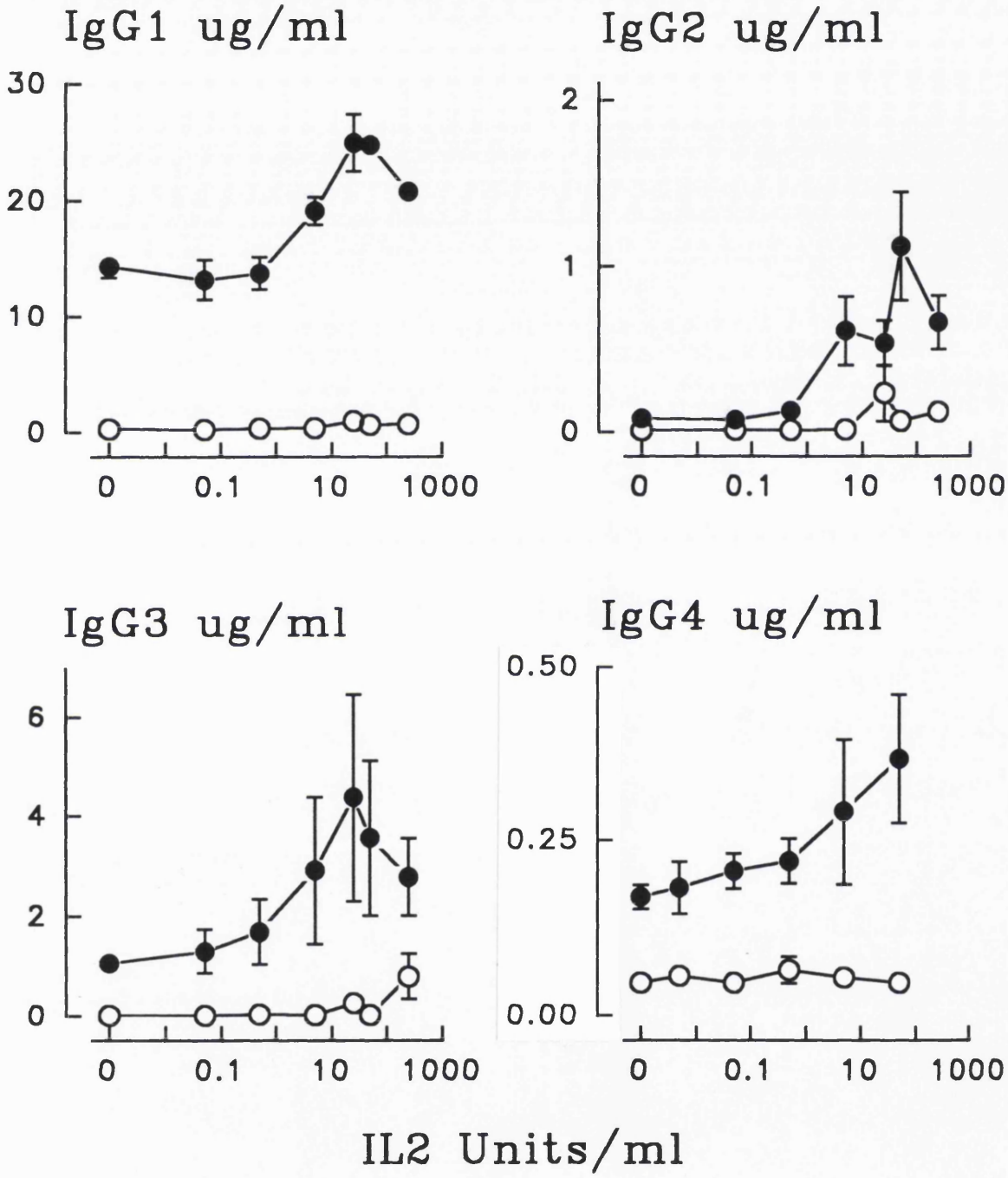
At concentrations of IL2 greater than 0.1 U/ml, immunoglobulin secretion by EBV activated B cells increased in a dose dependent manner with maximum production of immunoglobulin at 25-50 U/ml IL2. No further increase was observed with concentrations of IL2 up to 500 U/ml. Total IgM, IgG, IgA and all IgG subclasses (IgG1, IgG2, IgG3 and IgG4) were increased in a similar dose dependent manner. Typical results are shown in Figure 4.2. for IgM, IgG and IgA and in Figure 4.3 for IgG1, IgG2, IgG3 and IgG4. IL2 had no effect on IgE production. Levels of IgE remained below the level of detection in all experiments. Immunoglobulin secretion in control cultures of B cells without EBV was very low, and was not increased by IL2 over the concentration range 0-500 U/ml (Figures 4.2. and 4.3.).

Figure 4.2.



Effect of IL2 on EBV Induced IgM, IgA and IgG Secretion.
Tonsillar B cells, activated with EBV (●) or incubated in medium alone (O), were cultured with IL2 at concentrations from 0–500 U/ml. Ig secretion was measured on day 13. *Similar results were obtained in 3 separate experiments.*

Figure 4.3.



Effect of IL2 on EBV Induced IgG Subclass Secretion.
Tonsillar B cells, activated with EBV (●) or incubated in medium alone (O), were cultured with IL2 at concentrations from 0–500 U/ml. IgG subclass secretion was measured on day 13. Similar results were obtained in 3 separate experiments.

4.4. INTERLEUKIN 4.

IL4 induces a class switch to IgE and IgG1 in LPS activated murine B cells (127,334). IL4 also induces class switching of activated human B cells to IgE and IgG4 (150,196), but there is conflicting evidence for its role in regulation of other immunoglobulin classes and IgG subclasses (105,141,250). In this Section the role of IL4 in regulation of EBV induced B cell proliferation and immunoglobulin secretion was investigated.

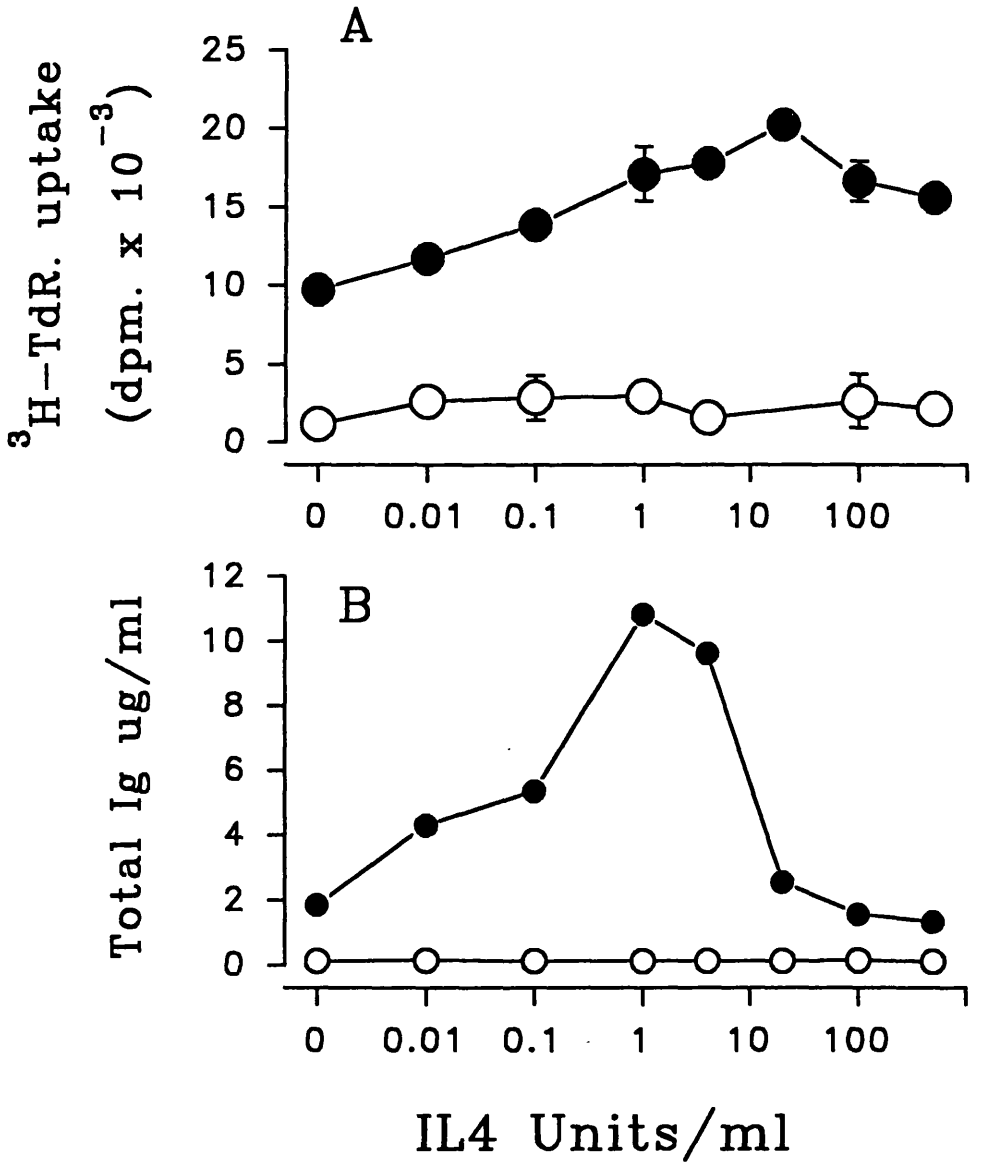
4.4.1. Effect of IL4 on Proliferation by EBV Activated B Cells.

At concentrations below 0.01 U/ml IL4 had no effect on B cell proliferation. Concentrations of IL4 greater than 0.01 U/ml enhanced proliferation of EBV activated B cells in a dose dependent manner with maximum proliferation at concentrations between 50-100 U/ml IL4. Doses of IL4 in excess of 100 U/ml had no additional effect. Results from a typical experiment are shown in Figure 4.4A. In some experiments, unstimulated B cells incorporated low levels of ^{3}H-TdR in response to high doses of IL4, but this was not significant compared with the response to IL4 by EBV activated B cells.

4.4.2. Effect of IL4 on Immunoglobulin Production by EBV Activated B Cells.

Concentrations of IL4 below 0.01 U/ml had no effect on Ig secretion by EBV activated B cells. At doses higher than this, IL4 significantly increased immunoglobulin secretion in a dose dependent manner with maximum secretion occurring at 0.5-5 U/ml IL4. At concentrations of IL4 greater than 5 U/ml, immunoglobulin production was diminished, and at 100 U/ml of IL4 or greater, was

Figure 4.4.



Effect of IL4 on EBV Induced Proliferation and Ig Production.
Tonsillar B cells, activated with EBV (●) or incubated in medium alone (○), were cultured with IL4 at concentrations from 0–500 U/ml. Proliferation (^{3}H-TdR. uptake) was measured on day 5 (A) and Ig secretion was measured on day 13 (B).

reduced to background levels or less. Results from a typical experiment are shown in Figure 4.4B. Non activated B cells did not usually respond to IL4, but in some experiments a small increase was observed with high doses of IL4.

The increase in immunoglobulin secretion with low doses (0.5-5 U/ml) and inhibition at high doses (> 100 U/ml) of IL4 was unexpected. Furthermore, it was interesting that maximum proliferation occurred at tenfold higher doses of IL4 than maximum immunoglobulin secretion. These unusual results prompted further investigation of the effects of IL4 on the production of human immunoglobulin classes and IgG subclasses as described in detail in the next Chapter.

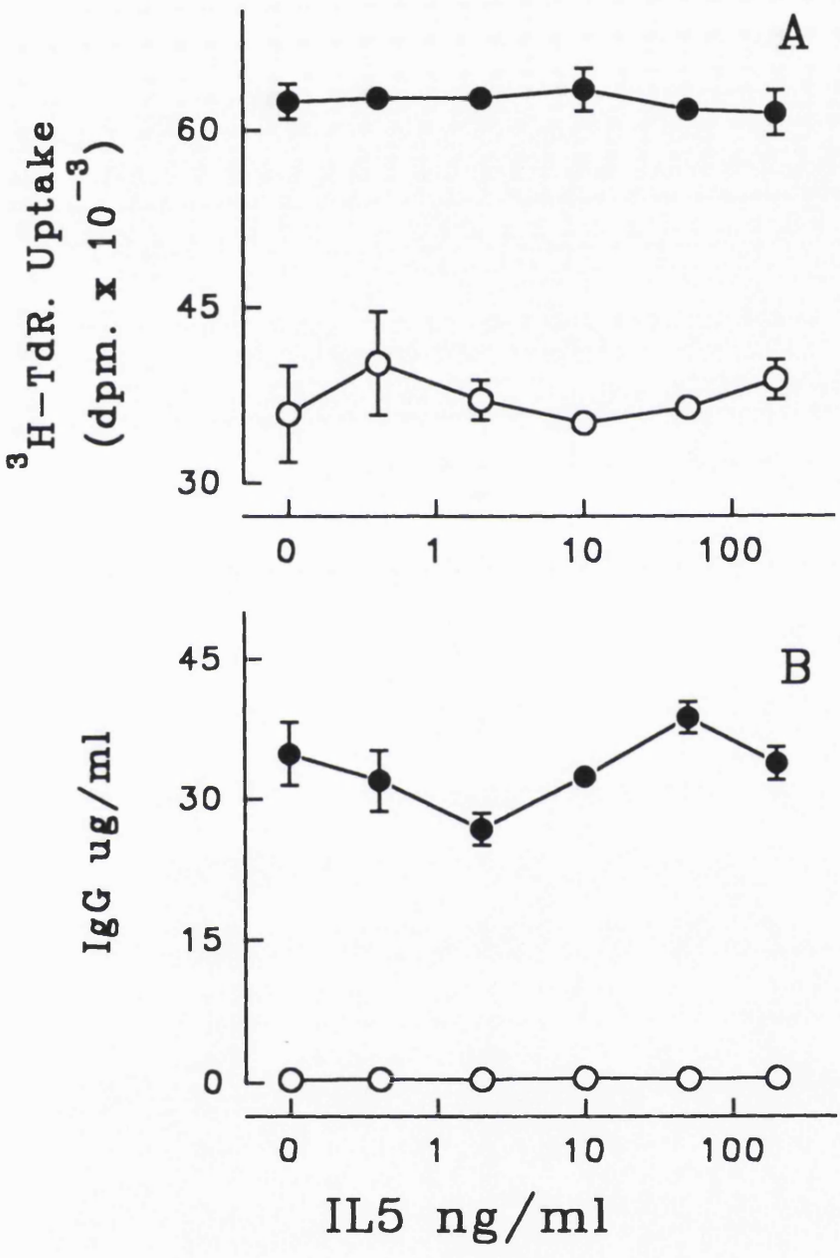
4.5. INTERLEUKIN 5.

IL5 clonally expands LPS activated IgA^+ murine B cells to secrete IgA. In humans, IL5 is an eosinophil growth factor (84) with no clear activity on human B cells. However, there are reports that IL5 supports IL4 induced IgE production (304,305,308,433) and induces IgM secretion from SAC activated human B cells (26). In this Section the role of IL5 in regulation of EBV induced B cell proliferation and immunoglobulin secretion was investigated.

4.5.1. Effect of IL5 on Proliferation by EBV Activated B Cells.

At a range of concentrations from 0 to 200 ng/ml, IL5 had no effect on the proliferation of EBV stimulated B cells or on unstimulated control B cells. Typical results are shown in Figure 4.5A.

Figure 4.5.



Effect of IL5 on EBV Induced Proliferation and Ig Production.
Tonsillar B cells, activated with EBV (●) or incubated in medium alone (O), were cultured with IL5 at concentrations from 0-250 ng/ml. Proliferation (^{3}H-TdR. uptake) was measured on day 5 (A) and Ig secretion was measured on day 13 (B). This experiment was repeated 3 times with similar results.

4.5.2. Effect of IL5 on Immunoglobulin Production by EBV Activated B Cells.

IL5 between 0 and 200 ng/ml had no effect on EBV induced human immunoglobulin secretion or background secretion by unstimulated control cells. No increase was seen in IgA, IgM or IgG secretion and in all experiments IgE was below the detection limit of assay (< 500 pg/ml). A typical experiment is shown in Figure 4.5B.

4.6. INTERLEUKIN 6.

IL6 is a growth factor for B cell lines and myelomas (224,361,396) and in other systems has B cell maturation factor activity (124,376). In this Section, the role of IL6 in regulation of EBV induced human B cell proliferation and immunoglobulin class and IgG subclass production was investigated.

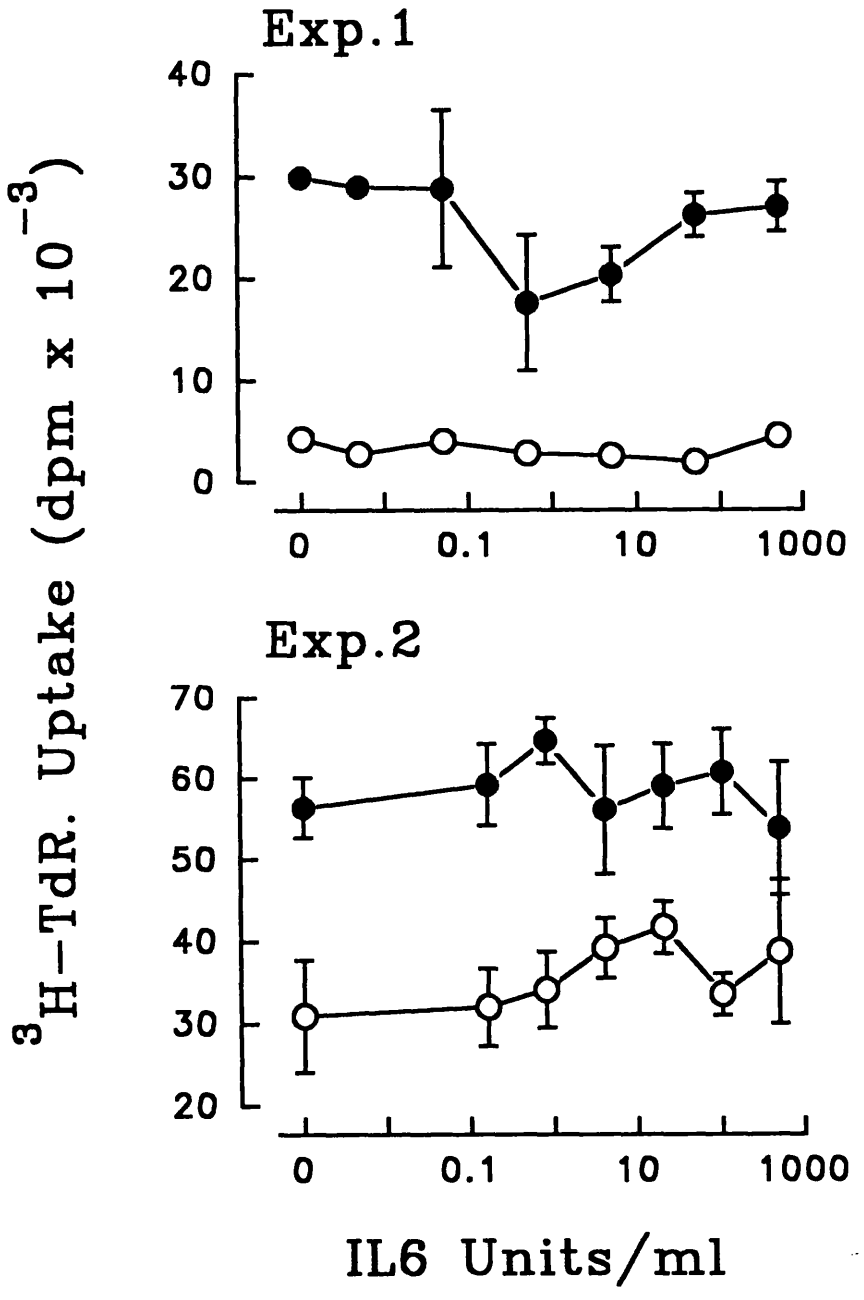
4.6.1. Effect of IL6 on Proliferation by EBV Activated B Cells.

IL6 consistently had no effect on EBV induced B cell proliferation or on unstimulated B cells. Typical results are shown in Figure 4.6.

4.6.2. Effect of IL6 on Immunoglobulin Production by EBV Activated B Cells.

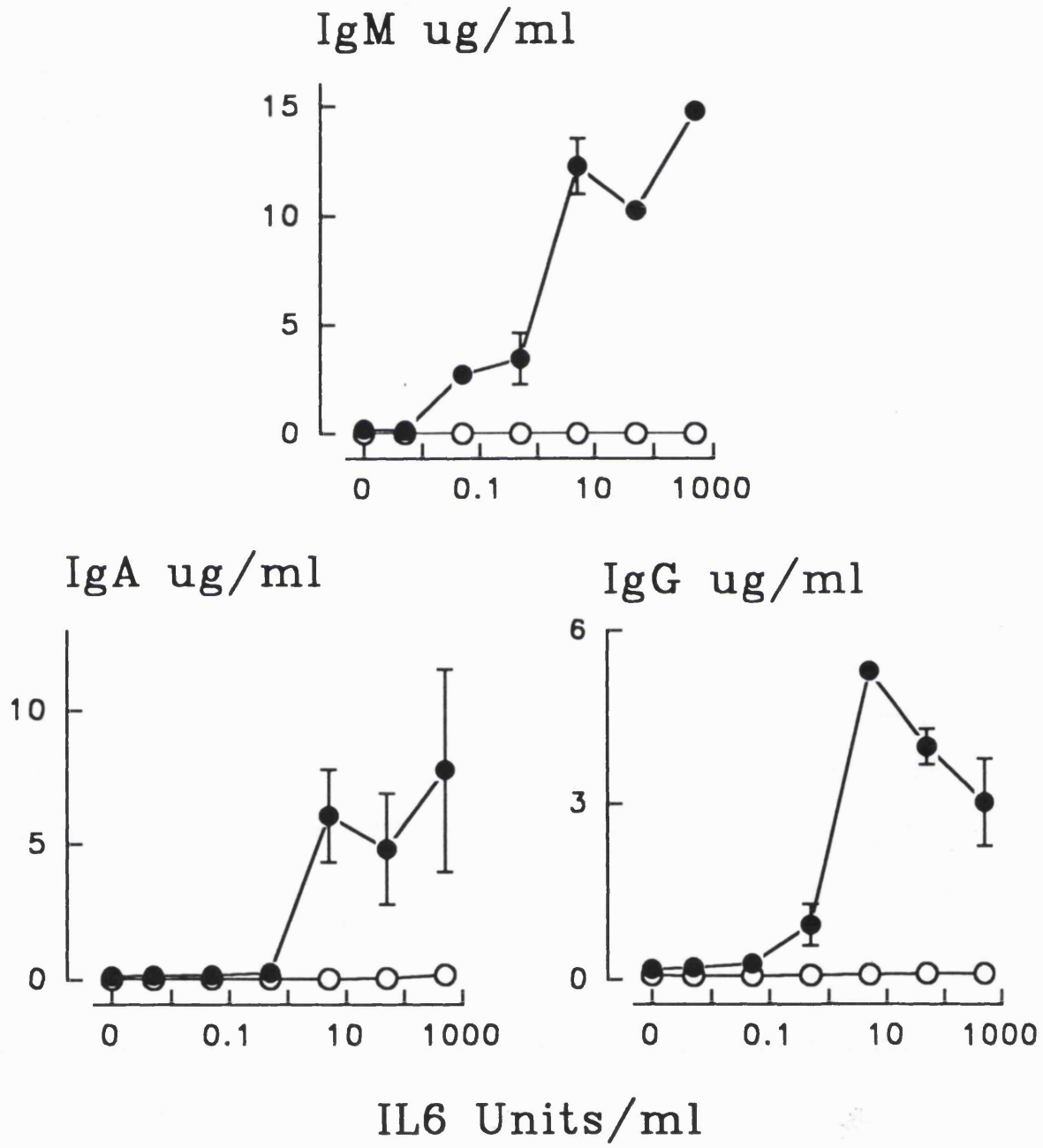
At concentrations of IL6 greater than 0.05 U/ml, immunoglobulin secretion was increased in a dose dependent manner attaining maximum secretion with concentrations of IL6 between 5 and 10 U/ml. No further increase was observed with IL6 up to 500 U/ml. IL6 increased total IgM, IgG and IgA. Analysis of the

Figure 4.6.



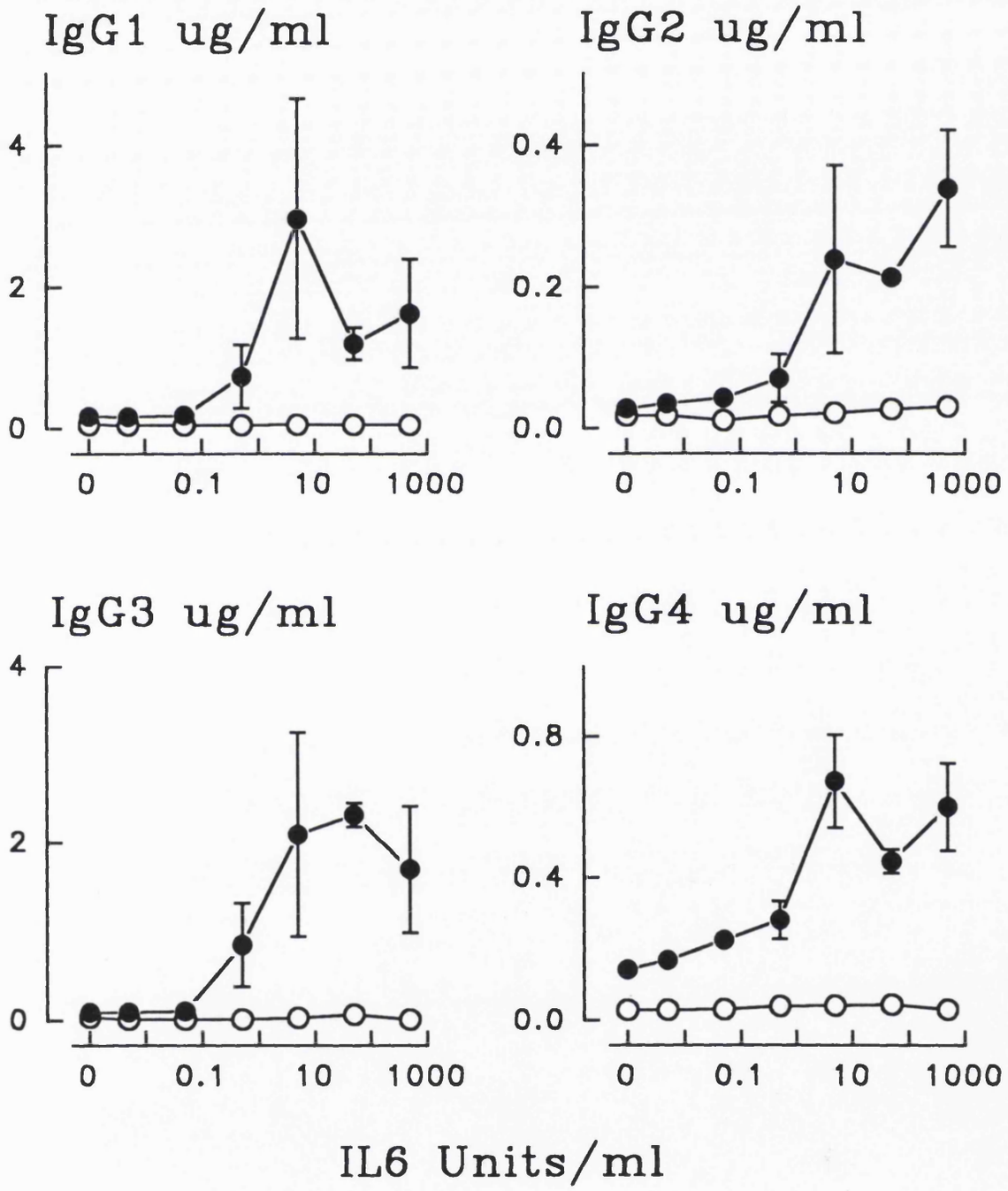
Effect of IL6 on EBV Induced B cell Proliferation.
Tonsillar B cells, activated with EBV (●) or incubated in medium alone (O), were cultured with IL6 at centrations from 0-500 U/ml. Proliferation (^{3}H-TdR. uptake) was measured on day 5.

Figure 4.7.



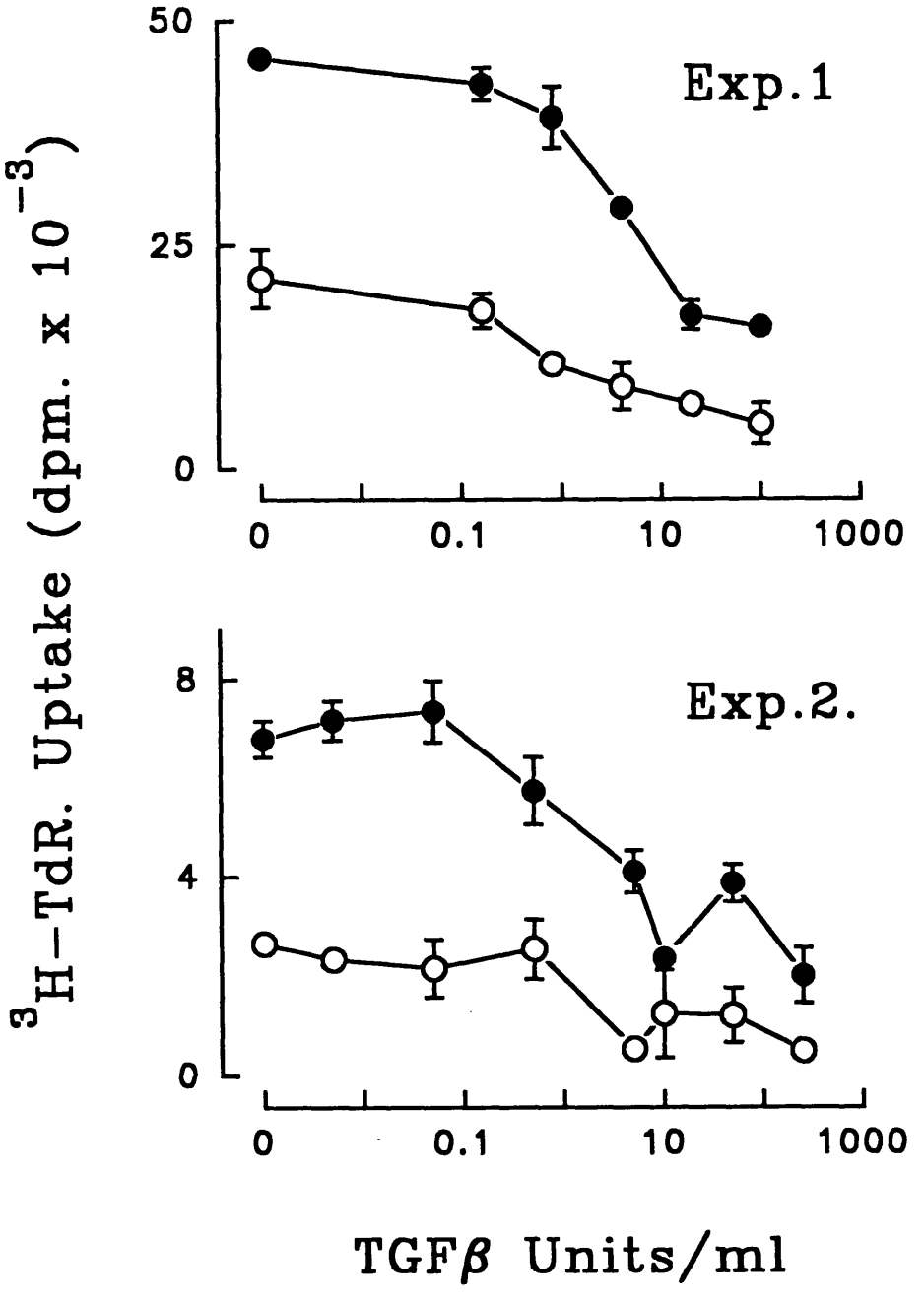
Effect of IL6 on EBV Induced IgM, IgA and IgG Secretion.
Tonsillar B cells, activated with EBV (●) or incubated in medium alone (O), were cultured with IL6 at concentrations from 0–500 U/ml. Ig secretion was measured on day 13. Similar results were obtained in 3 separate experiments.

Figure 4.8.



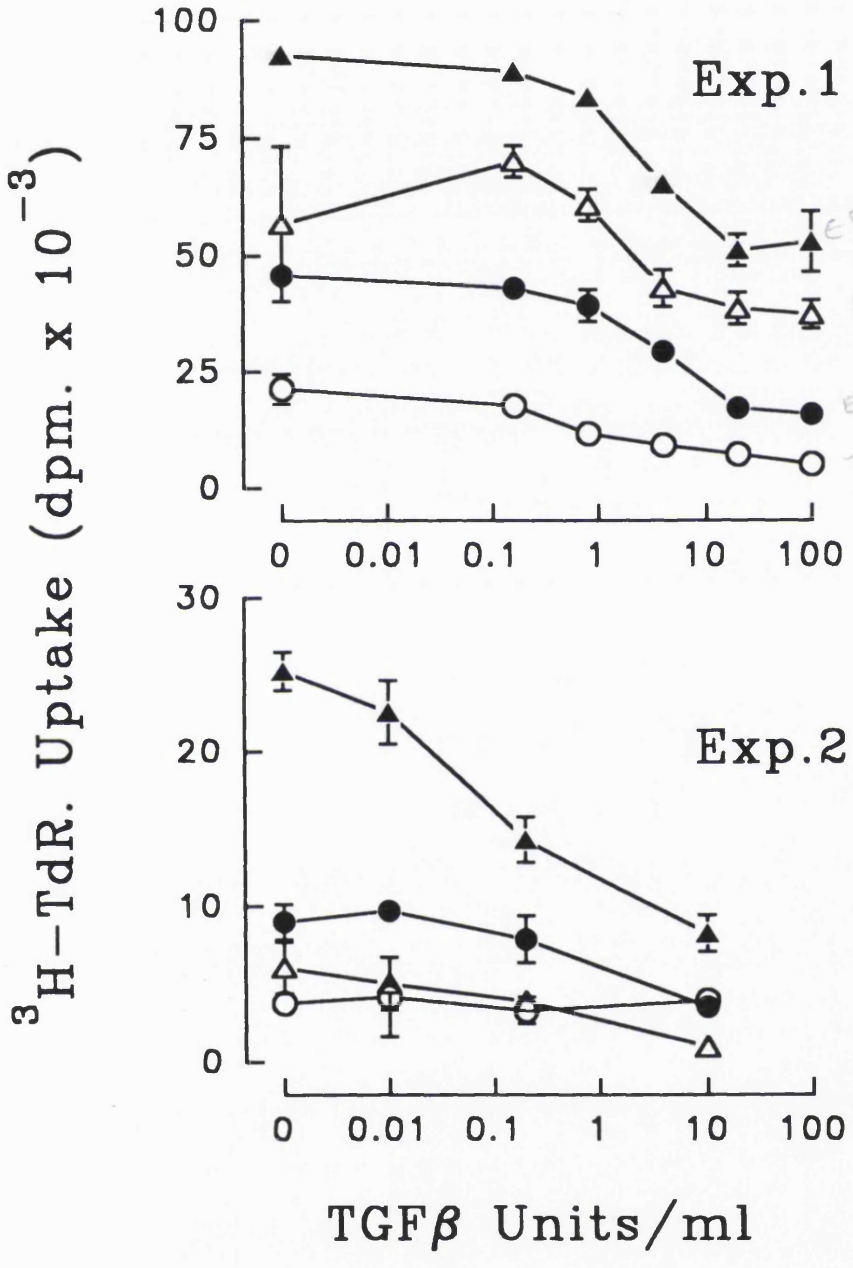
Effect of IL6 on EBV Induced IgG Subclass Secretion.
Tonsillar B cells, activated with EBV (●) or incubated in medium alone (O), were cultured with IL6 at concentrations from 0–500 U/ml. IgG subclass secretion was measured on day 13. Similar results were obtained in 3 separate experiments.

Figure 4.9.



Effect of TGFβ on EBV Induced B cell Proliferation.
Tonsillar B cells, activated with EBV (●) or incubated in medium alone (O), were cultured with TGFβ at centrations from 0-250 U/ml. Proliferation (^{3}H-TdR. uptake) was measured on day 5.

Figure 4.10.



Effect of TGFβ and IL2 on EBV Induced B Cell Proliferation.
Tonsillar B cells, activated with EBV (▲ ●) or incubated in medium alone (△ ○), were cultured with TGFβ at concentrations from 0-100 U/ml, with (▲ △) or without (● ○) 20 U/ml IL2. Proliferation (^{3}H-TdR. uptake) was measured on day 5.

Typical results from two experiments are shown in Figure 4.9. In some experiments, unstimulated B cells gave a low background proliferative response which was also inhibited by TGFβ (shown in Figure 4.9).

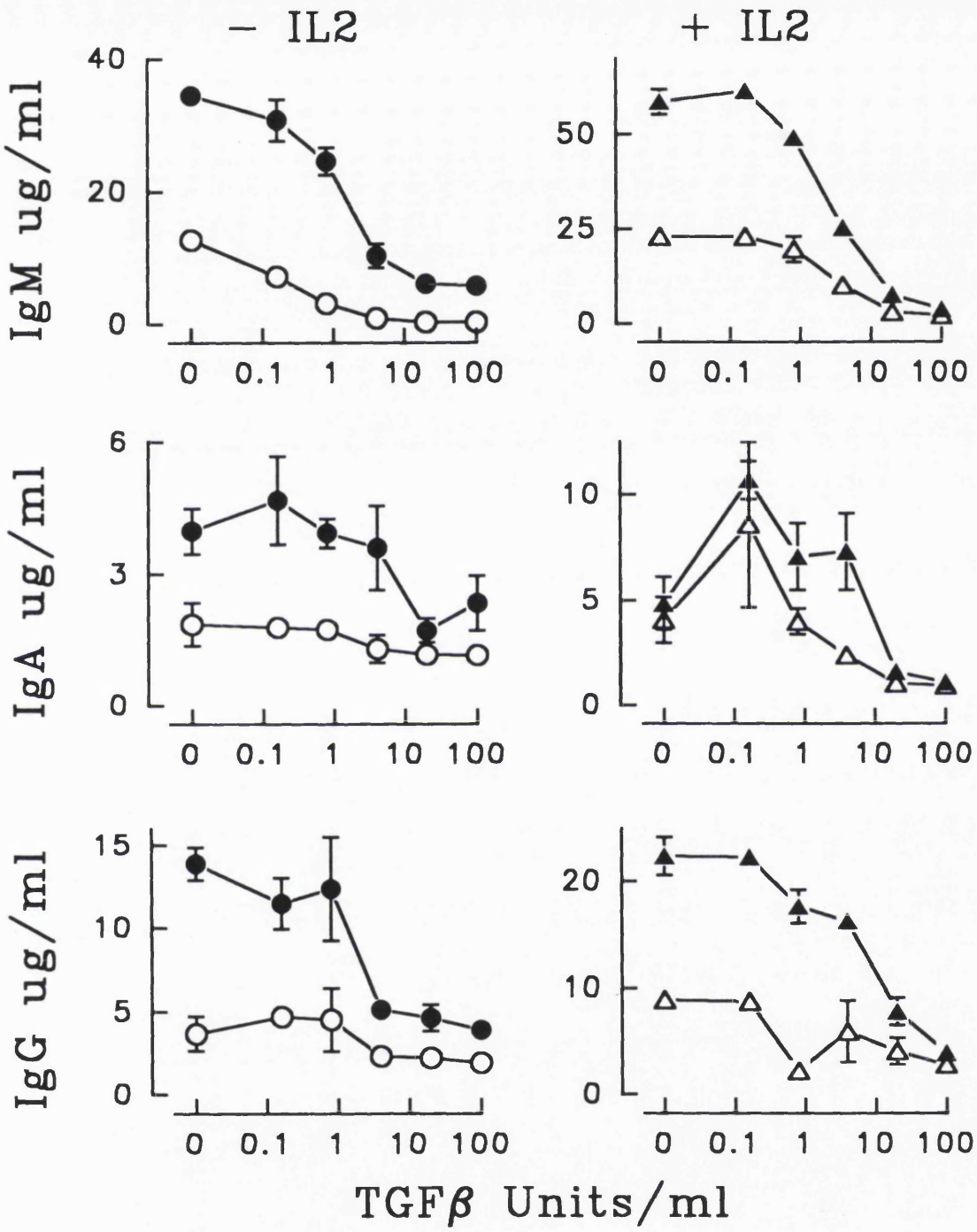
B cell proliferation in response to EBV and IL2 was also inhibited with TGFβ at concentrations greater than 10 U/ml. Typical results from two experiments are shown in Figure 4.10.

4.7.2. Effect of TGFβ and TGFβ + IL2 on Immunoglobulin Production by EBV Activated B Cells.

In cultures of human B cells activated with EBV, TGFβ at concentrations greater than 0.1 U/ml decreased secretion of immunoglobulin in a dose dependent manner (Figure 4.11.). Maximum inhibition was obtained with 10 U/ml or greater. In contrast to its effects on proliferation, at higher doses (>10 U/ml) TGFβ inhibited EBV induced immunoglobulin secretion to background levels equivalent to those obtained from unstimulated B cells. Although IgA, IgM, IgG, IgG1, IgG2, IgG3 and IgG4 were inhibited at high doses, an increase in IgA was seen at low doses of TGFβ (0.2 U/ml) in some experiments (Table 4.1. and Figure 4.11.). Typical results for the concentration dependent inhibition of IgM, IgG and IgA production by TGFβ are shown in Figure 4.11. All four IgG subclasses were similarly inhibited (data not shown).

Although the presence of IL2 enhanced immunoglobulin secretion of EBV activated B cells, it had no effect on the inhibition of IgA, IgM, IgG and IgG subclass secretion by TGFβ. Typical results for IgM, IgG and IgA secretion are shown in Figure 4.11. Similar results were obtained for the IgG subclasses (data not shown).

Figure 4.11.



Effect of TGFβ and IL2 on EBV Induced B Cell Ig Production.
Tonsillar B cells, activated with EBV (● ▲) or incubated in medium alone (○ △), were cultured with TGFβ at concentrations from 0–100 U/ml, with (▲ △) or without (● ○) 20 U/ml IL2. Ig secretion was measured on day 13. Similar results were obtained in 3 separate experiments.

In contrast, the increased secretion of IgA obtained with 0.2 U/ml TGFβ was increased further in the presence of IL2 (Table 4.1. and Figure 4.11.).

TABLE 4.1. EFFECT OF TGFβ ON IgA PRODUCTION BY TONSILLAR B CELLS ACTIVATED WITH EBV.

	IgA secretion (ng/ml)		
Additions	Exp.1	Exp.2	Exp.3
Medium.	<1[a]	230 ± 130	1860 ± 490
EBV	4 ± 0	13890 ± 3330	3980 ± 500
EBV + TGFβ (0.2 U/ml).	**16**[b] ± 2	**20260** ± 6970	**4680** ± 1010
EBV + TGFβ (10 U/ml).	<1	450 ± 130	1720 ± 290
EBV + IL2 (20 U/ml).	140 ± 20	31360 ± 5300	4840 ± 1250
EBV + IL2 (20 U/ml) + TGFβ (0.2 U/ml).	**350** ± 140	**76170** ± 14760	**10680** ± 910
EBV + IL2 (20 U/ml) + TGFβ (10 U/ml).	40 ± 30	1960 ± 680	1570 ± 160

a Values are expressed as ng/ml IgA ± 1 SE of the mean.

b Values picked out in bold shown an increase in IgA due to TGFβ.

4.8. INTERFERON γ AND INTERFERON α.

IFNγ specifically increases IgG2a secretion by LPS activated murine B cells while suppressing IgG1, IgG2b, IgG3 and IgE production (134,371). It may also induce Ig heavy chain class switching by murine B cells to IgG3 (369,370). IFNγ does not appear to control any specific immunoglobulin class or IgG subclass in humans, but

it inhibits IL4 induced IgE production (76,151,305,394) and co-stimulates with IL2 to induce IgM and IgG production in cultures of SAC activated B cells (288).

IFNα inhibits IL4 induced IgG1 and IgE production by LPS activated mouse B cells, but it has no reported class switching function. IFNα increases IgG and IgM in cultures of human B cells purified from peripheral blood (303)and IgM, IgA and IgG1 from human B cells activated with PMA and ionomycin (141). There is therefore some confusion as to its role in human immunoglobulin isotype regulation.

In the following Section, the action of IFNγ and IFNα on EBV induced B cells proliferation and immunoglobulin production was investigated.

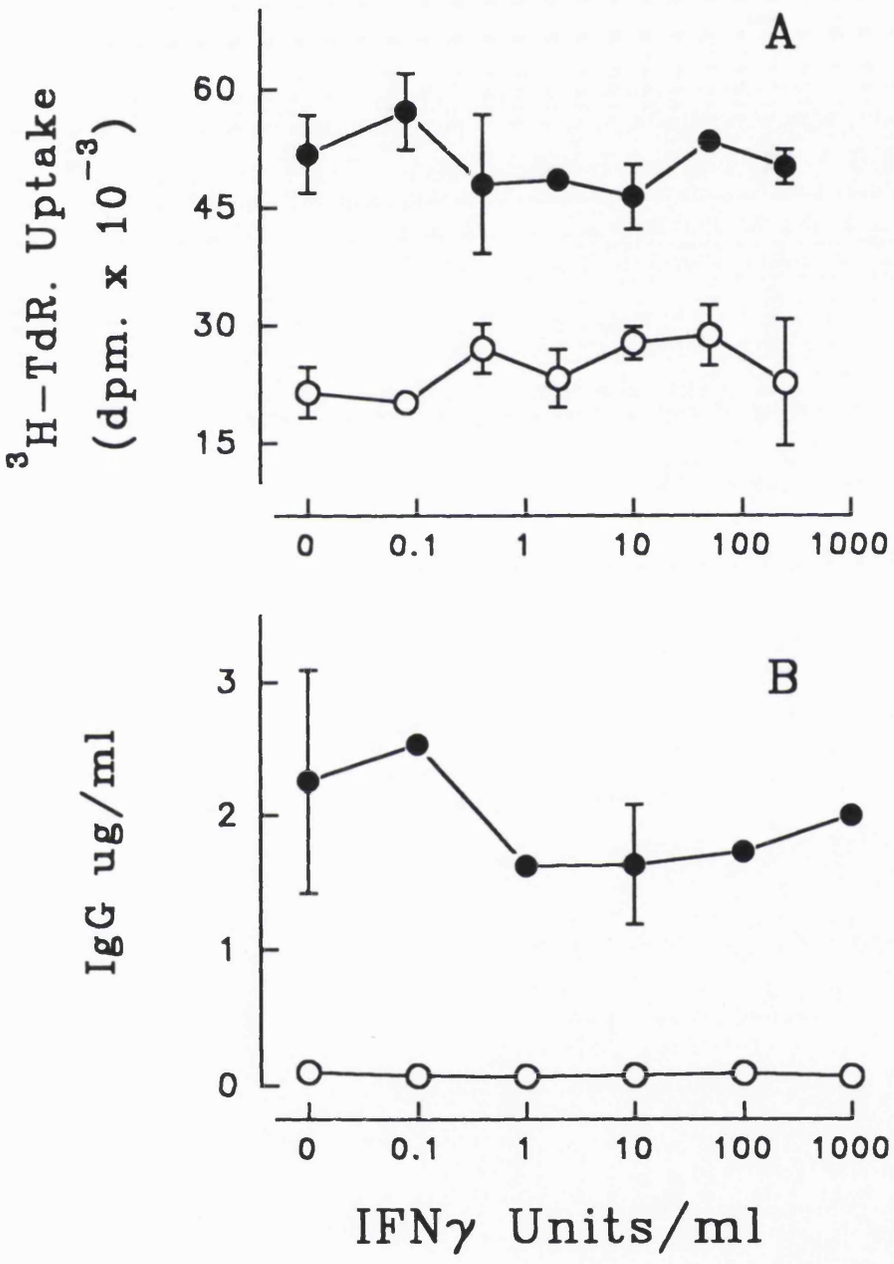
4.8.1. Effect of IFNγ and IFNα on Proliferation by EBV Activated B Cells.

IFNγ and IFNα had no consistent effect on EBV induced human B cell proliferation at concentrations between 0 and 1000 U/ml and 0 and 500 U/ml respectively. Typical results are shown in Figures 4.12. and 4.13.

4.8.2. Effect of IFNγ and IFNα on Immunoglobulin Production by EBV Activated B Cells.

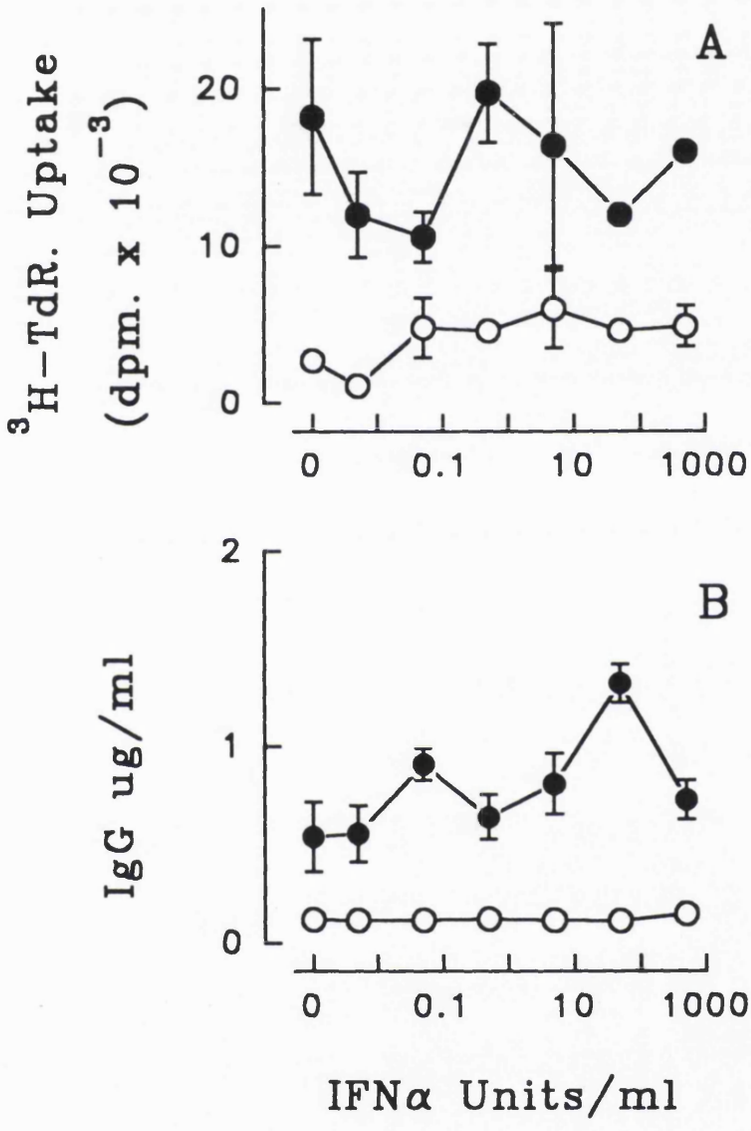
IFNγ at concentrations between 0 and 1000 U/ml had no effect on immunoglobulin secretion by B cells stimulated with EBV. There was no preferential effect on human Ig classes or IgG subclasses and IFNγ was not able to induce IgE production as levels were below the detection limit of assay in all experiments (500 pg/ml). Typical results for IgG are shown in Figure 4.12.

Figure 4.12.



Effect of IFNγ on EBV Induced Proliferation and Ig Production.
Tonsillar B cells, activated with EBV (●) or incubated in medium alone (O), were cultured with IFNγ at concentrations from 0–1000 U/ml. Proliferation (^{3}H-TdR. uptake) was measured on day 5 (A) and Ig production was measured on day 13 (B). This experiment was repeated 3 times with similar results.

Figure 4.13.



Effect of IFNα on EBV Induced Proliferation and Ig Production.
Tonsillar B cells, activated with EBV (●) or incubated in medium alone (○) were cultured with IFNα at concentrations from 0–500 U/ml. Proliferation (^{3}H-TdR. uptake) was measured on day 5 (A) and Ig secretion measured on day 13 (B). This experiment was repeated 3 times with similar results.

Similarly, IFNα at concentrations between 0 and 500 U/ml had no effect on IgM or IgA secretion by EBV activated B cells (results not shown), but in some experiments total IgG was increased slightly with doses of IFNα greater than 1.0 U/ml. This was not a consistent result however and its significance is unclear. A typical experiment is shown in Figure 4.13.

4.9. DISCUSSION.

Many cytokines have been shown to stimulate B cell proliferation and/or differentiation into antibody secreting cells. Some appear to have preferential effects on Ig classes (IgA, IgM, IgA, IgE) and IgG subclasses but these vary with different B cell activation systems. In this Chapter, IL2, IL4, IL5, IL6, TGFβ, IFNγ and IFNα, cytokines with known BCGF and/or BCDF activity on human B cells, were examined for their effect on EBV induced human B cell proliferation and IgG, IgM, IgG, IgE, IgG1, IgG2, IgG3 and IgG4 secretion.

IL2.

IL2 increased immunoglobulin secretion in cultures of EBV activated B cells in a dose dependent manner clearly showing BCDF activity (Figures 4.2. and 4.3.). These results are similar to the findings of other groups where IL2 acts, at similar doses, in synergy with the B cell activators SAC and LPS (61,198,273) and $PdBu_2$ and ionomycin (141). When individual isotypes were measured, IL2 was found to increase secretion of IgM, IgA, IgG1, IgG2, IgG3 and IgG4 with no preference for any one isotype (Figures 4.2. and 4.3.) and with no detectable effect on IgE secretion. There was no evidence for Ig heavy chain class switching to specific Ig isotypes by IL2. These results are consistent with those of Van Vlasselaer *et al.*

where IL2 induced IgG and IgA secretion from mIgG$^+$ and mIgA$^+$ but not IgM$^+$ B cells (406). There was also no evidence for selective expansion of Ig isotypes from pre-switched B cells for this corresponded with the results of Calvert *et al* (61), where IL2 was shown to increase secretion of IgM, IgG1 and IgG2 by SAC activated tonsillar B cells and IgM, IgG1, IgG2, IgG3 and IgG4 by LPS activated tonsillar B cells. Another study by Flores-Romo *et al* 1990 (141), using phorbol ester and calcium ionophore as B cell activators, showed that IL2 increased IgM, IgG1, IgG2 and IgA with no effect on IgG3 (IgG4 was not looked at in this study).

In addition to its BCDF activity, IL2 was also a potent BCGF for tonsillar B cells treated with EBV (Figure 4.1). Maximum proliferation was obtained with 50 U/ml IL2 or greater. Similar concentrations of IL2 induced proliferation of B cells activated with PMA and ionomycin (144,386), SAC (198,271) and anti-Ig (45,273). The BCGF activity of IL2 on B cells activated with T-cells, SAC, PMA or anti-Ig may be dependent on an increase in expression of the IL2 receptor Tac chain (CD25) induced by those activators (273,286,406). Although induction of the IL2 receptor by polyclonal activation with EBV was not investigated, some EBV transformed lines express CD25 and respond to IL2 (286), and the EBV gene EBNA-2 has the potential to increase expression of CD25 in human B cell lines (174). In some experiments, unstimulated B cells were found to proliferate in response to IL2. This was probably due to IL2 receptor expression on B cells which had been pre-activated *in vivo*.

Maximum immunoglobulin secretion and proliferation were obtained with similar concentrations of IL2. This indicated that the IL2 induced increase in Ig production may result from IL2 expansion of EBV activated B cells rather than maturation of B cells *per se*. It follows that the increase in all isotypes with the exception of IgE may be due simply to further expansion of precursor B cells activated with EBV. On the other hand, treatment of B cells with anti-Ig and IL2 promotes proliferation

but not immunoglobulin secretion (45,288), and pre-treatment with PMA and ionomycin completely inhibits immunoglobulin secretion but permits enhanced proliferation of B cells to IL2 (144). These results show that proliferation and differentiation may not be coupled and that one can be induced without the other. Therefore, the IL2 increase in Ig secretion of EBV activated B cells may not be due to proliferation. The results shown in this Chapter found no evidence for selective expansion of Ig isotypes by IL2. IL2 has the potential to induce all Ig classes and IgG subclasses but its effects can depend on the activation signals used.

IL4.

In the initial studies described in this Chapter, low doses of IL4 (0.5-5 U/ml) were found to increase Ig secretion whereas at high doses of IL4 (> 100 U/ml), secretion was unaffected or inhibited below values obtained with EBV alone (Figure 4.4.). Enhanced immunoglobulin secretion obtained with low doses of IL4 was not due to IL4 induced proliferation of EBV activated B cells as optimum BCGF activity occurred at higher doses of IL4. These low dose effects of IL4 on immunoglobulin production have not been previously described and are examined in more detail in the next Chapter.

IL5.

In mice, IL5 is an eosinophil growth factor and a B cell growth and differentiation factor (5,255,343). As a BCDF, it preferentially increases IgA secretion by clonally expanding IgA^+ B cells (90,262,431). In humans it has been reported to be an eosinophil growth factor with no activity on B cells (84), although it has been reported to augment IL4 induced IgE production at sub optimal levels of IL4

(308,409,411). These experiments however were undertaken with unseparated TMC and PBMC where the contribution of T cells and monocytes was not defined. In this Chapter, IL5 had no BCGF or BCDF activity on EBV activated B cells (Figure 4.5.). The absence of any effect on human B cells is consistent with an extensive multi-Centre study by Clutterbuck *et al* 1987 (84) which showed that IL5 had no effect on human B cells activated with anti-IgM, PMA, SAC, $PdBu_2$ and ionomycin or on a number of B cell lines at different maturational stages. However, Bertolini *et al* 1993 (26), have reported that IL5 induced IgM, but not IgA or IgG secretion, by SAC activated B cells. This activity was totally dependent on the time of pre-incubation with SAC. An activation period longer than 24 hours resulted in a rapid loss of IL5 responsiveness possibly due to loss of the IL5 receptor. This may account for the lack of activity on B cells described by Clutterbuck *et al* (84). The negative results found in this Chapter could be explained likewise. That is, activation with EBV may result in loss of the IL5 receptor rendering B cells insensitive to this cytokine.

IL6.

Although IL6 is known to be a growth factor for B cell lines and myelomas (224,361,396) it had no growth factor activity on EBV activated B cells. This agrees with previous work with murine (20) and human B cells (221,376) where IL6 alone or in combination with B cell mitogens, T cells and other cytokines was unable to induce or augment B cell proliferation. The lack of BCGF activity could be explained by an absence of the IL6 receptor in early B cell activation. For example, the IL6 receptor may only be expressed on Ig secreting B cells or B cell lines. This could be tested by phenotypic studies with antibodies to the IL6 receptor, but these experiments have not been done.

In contrast to its inability to promote B cell growth, IL6 has potent BCDF activity on B cells costimulated with SAC or with SAC plus IL2, and on PBMC stimulated with PWM (124,219,285,376). It also increases secretion of antibody by B plasma cells suggesting that it is a late acting differentiation factor (221). IL6 has been shown to enhance IgG, IgM and IgA secretion in combination with SAC, SAC and IL2 and PWM (124,219,285,376). The results in this Chapter confirm that IL6 is a potent BCDF for activated B cells. Moreover, it increased secretion of all immunoglobulin classes and IgG subclasses by EBV activated B cells with the exception of IgE. The absence of IgE may be due simply to the absence of IgE^+ B cells in the activated cultures as IL6 is able to amplify IgE production by human B cells activated with anti-CD40 and IL4 (151,195).

TGFβ.

TGFβ is an important cytokine in immune regulation especially in immune suppression and inflammatory cell recruitment and activation. It is also involved in the control of B and T cell function. TGFβ specifically induces IgG2b secretion by murine B cells (264,370). Low concentrations of TGFβ induce LPS activated murine B cells to secrete IgA, while high concentrations of TGFβ inhibit secretion of all Ig isotypes (88,372). In other experiments, the presence of IL2 was found to increase the effect of low dose TGFβ on IgA secretion by murine B cells ten fold (215,240). In our experiments, TGFβ at high concentrations (> 10 U/ml) completely inhibited EBV and EBV plus IL2 induced immunoglobulin production of IgA (Figure 4.11.). This agrees with results from SAC and IL2 activated human B cells where IgG and IgM secretion were inhibited at high dose TGFβ by greater than 90% (208,209). The production of IgA induced by TGFβ in the mouse may not be so easily achieved with human B cells. This has been addressed by Defrance *et al* (104) who showed a specific increase of IgA with low doses of TGFβ (0.6

ng/ml) in populations of human B cells activated with IL10 in the presence of anti-CD40. Although such complicated activation conditions were not used in our studies, secretion of IgA by B cells activated with EBV and EBV and IL2 was increased by TGFβ at doses similar to those used by Defrance *et al* (104) (Table 4.1.). The regulation of IgA secretion in mouse B cells by TGFβ is known to be by induction of heavy chain class switching (241). TGFβ has also been shown to induce class switching to IgA in human B cells activated with *Branhamella catarrhalis* (192,292). Defrance *et al* (104) has suggested that TGFβ in the presence of IL10 and anti-CD40 also induces IgA production by an Ig class switch mechanism. The specific increase of IgA secretion by TGFβ shown in this Chapter may also be due to Ig heavy chain class switching. EBV may also be able to deliver the signal required by B cells to induce heavy chain class switching to IgA in response to TGFβ. EBV activated B cells may produce vIL10 (183,337), or hIL10 (51) and this in combination with EBV as a polyclonal activator, may give a signal similar to that of IL10 and anti-CD40 in combination with TGFβ to increase IgA.

B cell proliferation is also inhibited with TGFβ in other systems at concentrations similar to those used here (Figure 4.9.). It inhibits proliferation of LPS activated mouse B cells (94) and proliferation of human B cells activated with SAC plus IL2 (208)), anti-Ig plus BCGF, anti-CD20 plus BCGF and PMA plus anti-CD40 (18). Unlike its effect on immunoglobulin secretion, TGFβ did not totally inhibit proliferation of EBV plus IL2 activated B cells (Figure 4.10). Similar results were seen by Kehrl *et al* (208) in cultures of SAC activated B cells where high doses of IL2 (>100 U/ml) rescued human B cells from TGFβ inhibition of proliferation but not immunoglobulin secretion. A number of explanations are possible. For example, loss of the TGFβ receptor on EBV infection rendering B cells resistant to TGFβ (234), may be accelerated by IL2. This is not consistent however, with the increased IgA secretion with IL2 and TGFβ. Another possible explanation is that IL2 may act differently on a subset of B cells, pre-activated *in vivo*, and already carrying the IL2

receptor. This subset of B cells may be able to proliferate in response to IL2, but have no reactivity to TGFβ. More simply, proliferation of EBV activated B cells is not as sensitive to TGFβ as differentiation into Ig secreting cells.

IFNγ and IFNα.

In the mouse IFNγ inhibits B cell proliferation and Ig production induced by supernatants from Th2 (IL4 secreting) cell lines (325). It also inhibits IgG1, IgG2b, IgG3 and IgE production while specifically inducing IgG2a production by LPS activated murine B cells (134,371). Recently IFNγ has also been shown to induce heavy chain class switching to IgG3 by mouse B cells (369,370). IFNγ is also an antagonist of human IL4 induced IgE production and class switching (76,151,394), but does not by itself induce specific human Ig isotypes. Although IFNγ has been reported to increase proliferation of SAC and anti-IgM activated human spleen, lymph and peripheral blood lymphocytes (249,324) and increases IgM and IgG secretion by SAC activated B cells (288), our results show that IFNγ has little, if any, activity on EBV activated human B cells (Figure 4.12.). This was not due to the absence of the receptor for IFNγ as human tonsillar B cells express the IFNγ receptor (data not shown). It was also not due to loss of receptors by activating with EBV because IFNγ was able to inhibit IL4 induced CD23 upregulation (personal observation). Perhaps EBV activation of B cells is not sufficient for IFNγ to induce Ig secretion. Another explanation could be inhibition of IFNγ induced responses by the presence of vIL10 or IL10 produced by EBV activated B cells *in vitro*. IL10 is known to inhibit IFNγ mRNA production and IFNγ induced responses (130,363).

Like IFNγ, IFNα also inhibits IL4 induced IgG1 and IgE by LPS activated murine B cells, but with less potency than IFNγ (136). It has been suggested that IFNα secreted by monocytes may direct the immune response to antigen in favour of Th1

cells and the secretion of IFNγ by inhibiting Th2 cells and transcription of IL4 mRNA (136). In humans IFNα inhibits the growth of myelomas, but not lymphoblastoid cell lines and high doses of IFNα (50-100 U/ml) increase Ig secretion from Ig secreting human B cell lines (199). No antagonistic activity of IFNα against IL4 induced responses has been reported in the human. It has however been shown to increase IgG and IgM secretion by purified blood B cells (303) and to increase IgM, IgA and IgG1 by human B cells stimulated with PMA and ionomycin (141). In contrast to these other B cell activation systems, IFNα did not have any notable effect on immunoglobulin secretion or proliferation of human B cells activated with EBV (Figure 4.13.). This could be due to loss of the IFNα receptor on activation with EBV, or the unfavourable activation state of B cells treated with EBV rendering them insensitive to IFNα. Unlike IFNγ, IFNα is produced in small amounts by most cells. It plays an important role in host resistance to viral infections by enhancing cytotoxic T cells and macrophage killing. Its stimulation of Ig production in other systems may be a bystander enhancing effect and require particular activation conditions.

CHAPTER 5

INTERLEUKIN 4: REGULATION OF IMMUNOGLOBULIN CLASS AND IgG SUBCLASS RESPONSES

5.1. INTRODUCTION

IL4 induces immunoglobulin class switching to IgG1 and IgE in cultures of LPS activated murine B cells. It was shown by Rothman *et al* (334) that IL4 induced Cε germ line transcripts in the cytoplasm of mouse B lineage cells, and by Esser and Radbruch (127) that IL4 also induced Cγ1 germ line transcripts. IL4 can also induce Cε germ line transcripts and class switching to IgE in activated human B cells (150,196). However, although IL4 alone can induce Cε germ line transcripts, a second signal provided by T cells, LPS, EBV or anti-CD40 is needed to accomplish switch site recombination and production of the IgE isotype. Evidence of control by IL4 of other human immunoglobulin classes, especially the IgG subclasses, is somewhat conflicting (105,141,250,306,307).

In Chapter 4, preliminary results obtained with IL4 and B cells activated with EBV revealed two quite distinct effects on immunoglobulin secretion by IL4 (Figure 4.4.). Low concentrations of IL4 (0.5-5 U/ml) significantly increased EBV induced immunoglobulin secretion while at high concentrations (100 U/ml or greater) IL4 had no effect or inhibited secretion below levels obtained with EBV alone. Interestingly, the dose of IL4 required for maximum proliferation (20-100 U/ml IL4) was at least 20 fold greater than that giving maximum Ig secretion (1-5 U/ml IL4). This indicated that maximum levels of immunoglobulin secretion induced by low concentrations of IL4 could not be accounted for by proliferation alone. In this Chapter the role of IL4 on human immunoglobulin class and IgG subclass responses by EBV activated human B cells was investigated in detail.

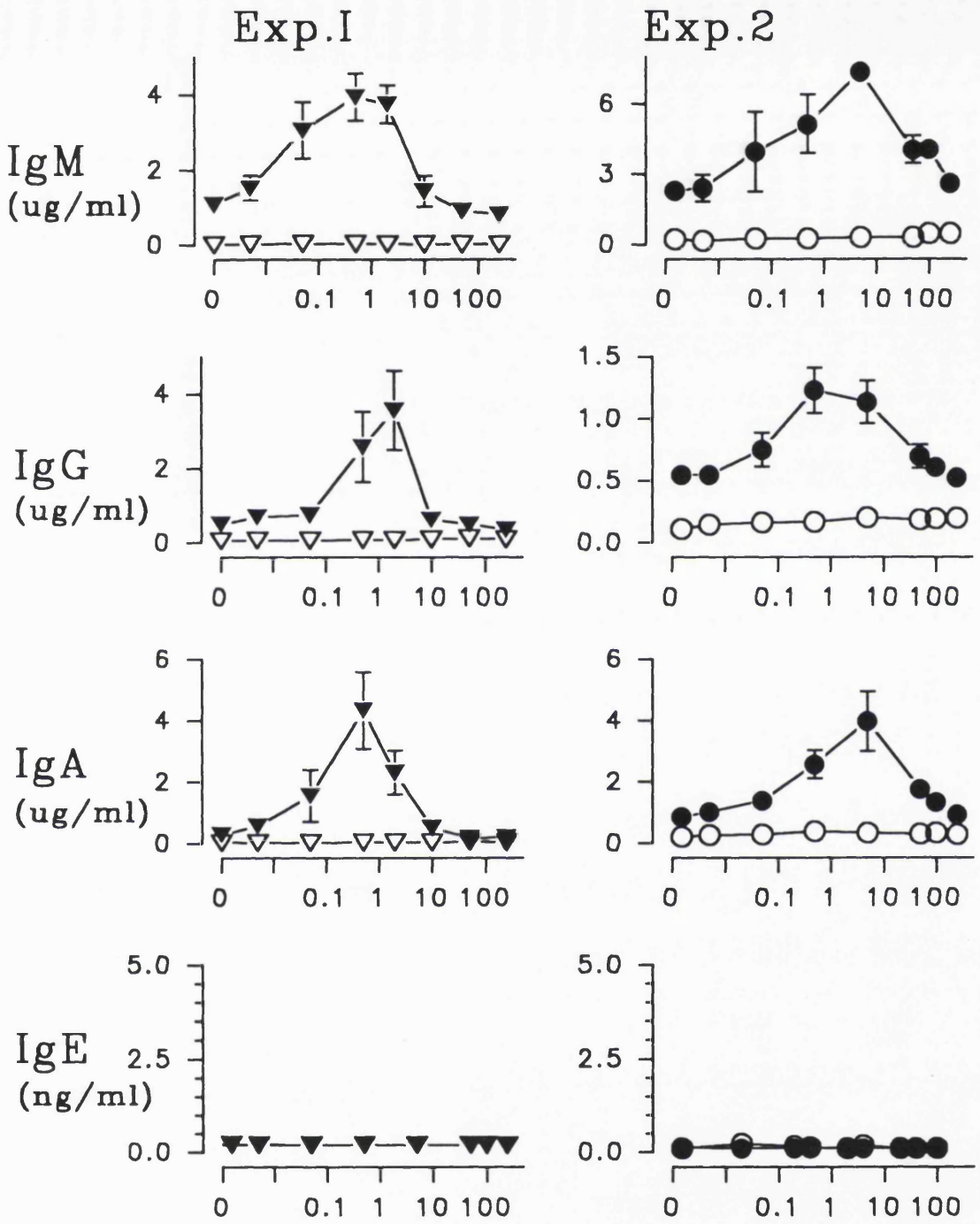
5.2. EFFECT OF IL4 ON EBV INDUCED POLYCLONAL IMMUNOGLOBULIN CLASS AND IgG SUBCLASS SECRETION.

Most effects of IL4 on B cell immunoglobulin production reported in the literature have been obtained with doses of IL4 of 100 U/ml or greater and the low dose IL4 increase in immunoglobulin secretion seen in Chapter 4. (Figure 4.4.) was unexpected. In this Section, immunoglobulin class and IgG subclass production by EBV treated and unstimulated tonsillar B cells cultured in RPMI 1640 with concentrations of IL4 from 0 to 500 U/ml was investigated. Immunoglobulin secretion was assayed on day 13.

Low doses of IL4 (0.5 - 5 U/ml) maximally increased production of IgG, IgM, IgA, IgG1, IgG2 and IgG3 in cultures of EBV activated B cells (Figures 5.1. and 5.2.). This increase was synergistic and not additive. At high doses of IL4 (100 U/ml or greater) the increase in IgG, IgM, IgA, IgG1, IgG2 and IgG3 production was either reduced back to, or inhibited below, levels obtained from EBV activated B cells alone. No IgE secretion was observed with any concentration of IL4 and levels remained below the detection limit of assay. Similarly, IL4 had no effect on the levels of IgG4 which remained at concentrations induced by EBV alone. In other experiments using IMDM rather than RPMI 1640, IgE and IgG4 responses were obtained with IL4 (see Section 5.5. below).

IL4 had no significant effect on immunoglobulin secretion by unstimulated B cells. Results from two typical experiments are shown in Figures 5.1. (IgG, IgA, IgM and IgE secretion) and Figure 5.2. (IgG1, IgG2, IgG3 and IgG4 secretion).

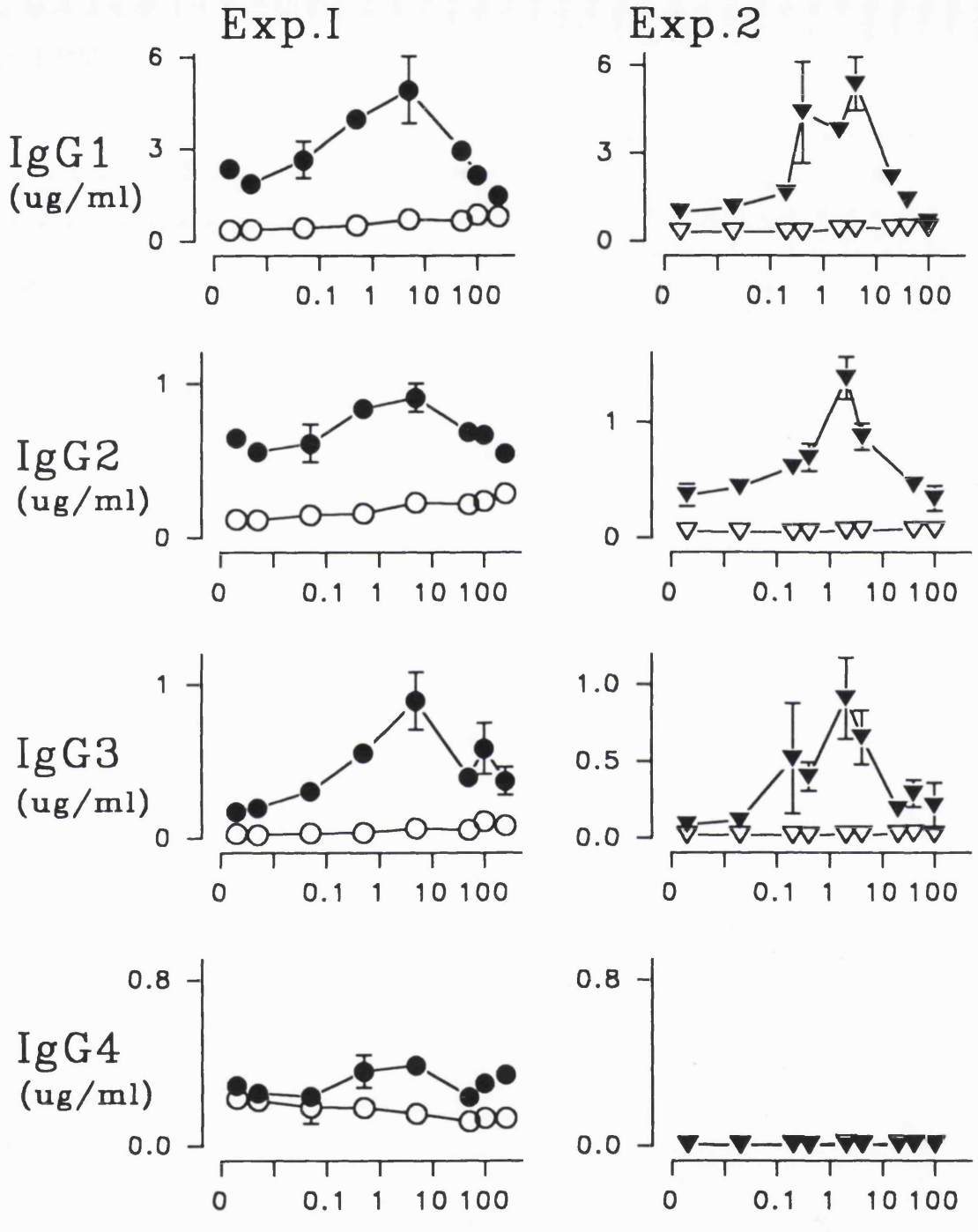
Figure 5.1.



Effect of IL4 on EBV Induced B Cell Ig Secretion.

Tonsillar B cells, activated with EBV (▼ ●) or incubated in medium alone (▽ ○), were cultured with IL4 at concentrations from 0–250 U/ml. IgM, IgG, IgA and IgE secretion was measured on day 13 by ELISA. Results from two experiments are shown. This experiment was repeated 6 times.

Figure 5.2.



Effect of IL4 on EBV Induced B Cell IgG Sub-Class Secretion.
Tonsillar B cells, activated with EBV (● ▼) or incubated in medium alone (○ ▽), were cultured with IL4 at concentrations from 0-250 U/ml. IgG1, IgG2, IgG3 and IgG4 secretion was measured on day 13 by ELISA. Results from two experiments are shown. This experiment was repeated 6 times.

5.3. IL4 DOES NOT ALTER THE KINETICS OF B CELL ACTIVATION WITH EBV.

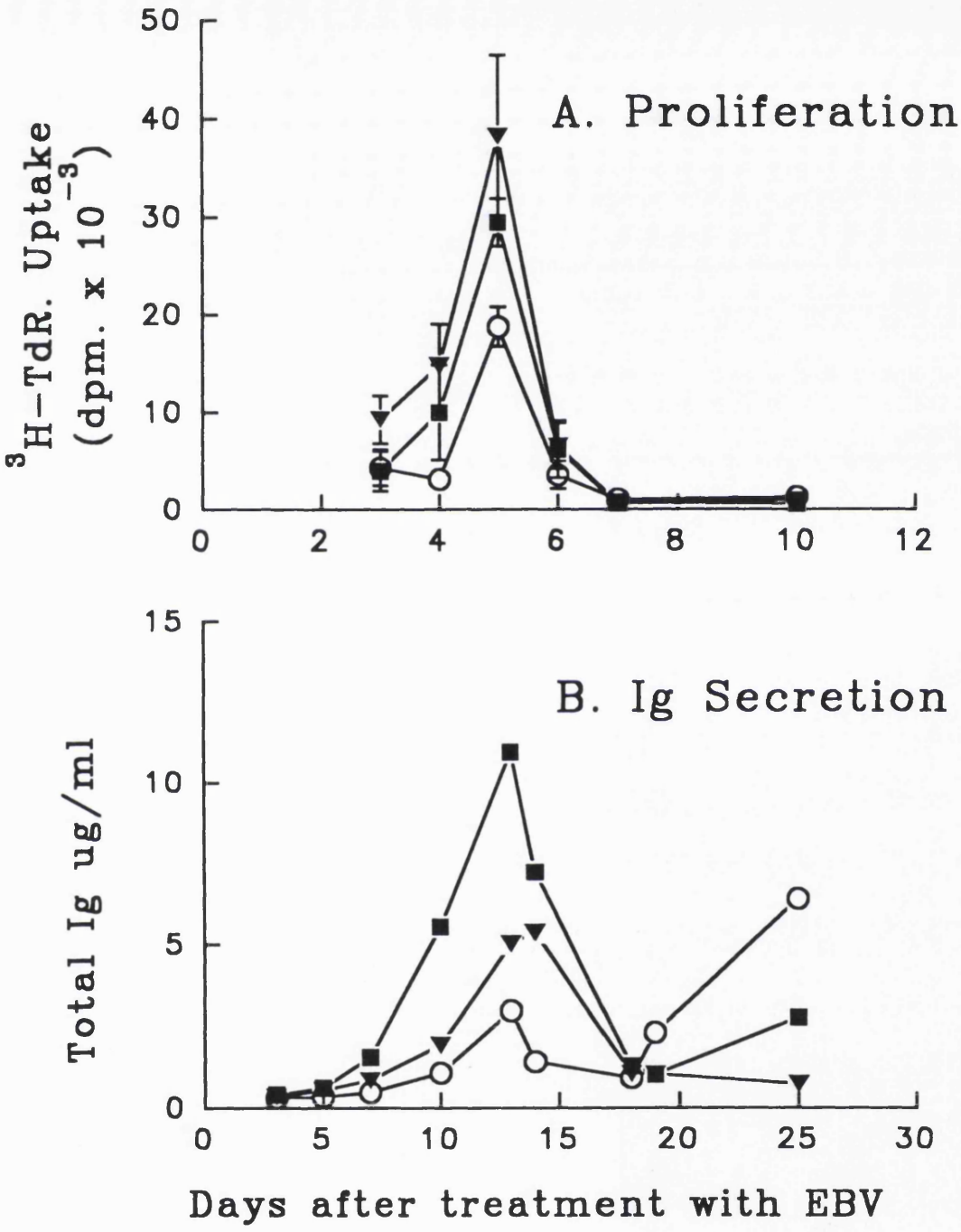
The difference in effect of high and low doses of IL4 on immunoglobulin secretion could in part be due to an alteration in the kinetics of the EBV response. This was investigated by a culture of EBV activated B cells in the presence of high (100 U/ml) and low (5 U/ml) doses of IL4 and by measuring proliferation and immunoglobulin secretion over a period of 1 to 30 days.

5.3.1. Effect of IL4 on The Kinetics of Polyclonal B Cell Responses to EBV.

The presence of IL4 either at low or high doses did not alter the time course kinetics of EBV activation and the peaks of polyclonal proliferation and immunoglobulin production occurred at 5 days and 13 days respectively (Figure 5.3. A and B) as previously determined in Chapter 3. The kinetics of IgG, IgA and IgM examined individually gave similar results (Figure 5.4).

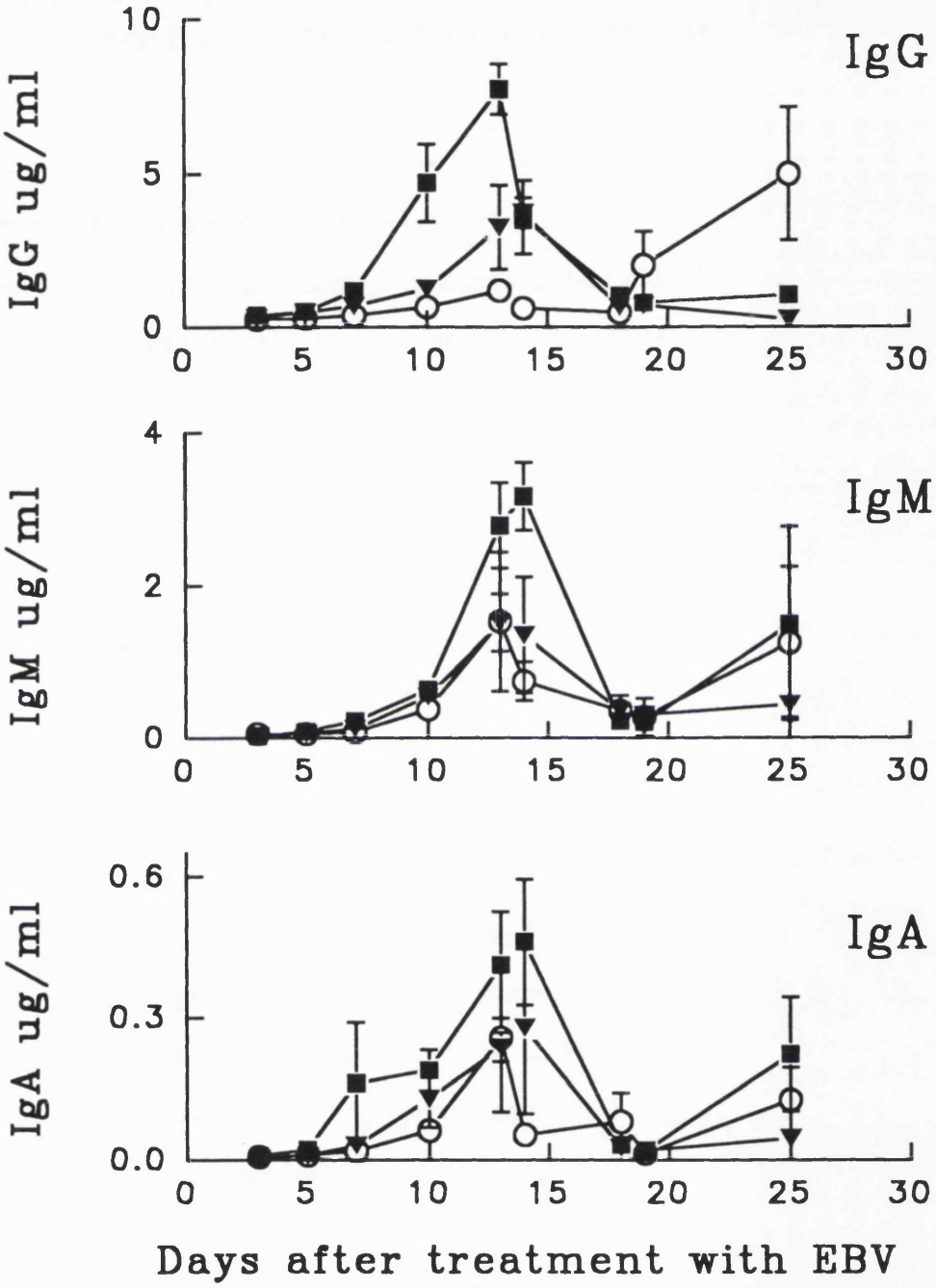
It was also noted in these experiments that high concentrations of IL4 not only inhibited polyclonal Ig secretion but also appeared to prevent the emergence of transformed B cell clones. This was evident by the reduced Ig secretion at 100 U/ml of IL4 after 20 days of culture (Figures 5.3.A and 5.4.) and was confirmed by visual inspection of the culture wells (data not shown). This potentially interesting finding was not investigated further.

Figure 5.3.



Effect of High and Low Dose IL4 on the Kinetics of EBV Induced B Cell Proliferation and Ig Production.
Tonsillar B cells were activated with EBV and cultured without IL4 (○), with 5 U/ml IL4 (■) or with 100 U/ml IL4 (▼). Proliferation (^{3}H-TdR. uptake)(A) and Ig secretion (B) were measured on the days indicated.

Figure 5.4.



Effect of High and Low Dose IL4 on the Kinetics of EBV Induced B Cell IgG, IgM and IgA Secretion.

Tonsillar B cells were activated with EBV and cultured without IL4 (○), with 5 U/ml IL4 (■) or with 100 U/ml IL4 (▼). Ig secretion was measured on the days indicated.

5.4. HIGH BUT NOT LOW DOSES OF IL4 INDUCE IgE PRODUCTION BY EBV TREATED B CELLS WHEN CULTURED IN IMDM.

In the experiments described so far no IgE production was observed even in the presence of high doses of IL4. It has been reported previously that IgE secretion by human B cells may be optimised by culture in IMDM (78,79). In this Section, the response of human B cells to EBV and IL4 in IMDM and RPMI 1640 was compared.

5.4.1. Comparison of Immunoglobulin Secretion and Proliferation by B Cells Cultured in RPMI 1640 or IMDM.

EBV treated B cells cultured in IMDM, without IL4, produced up to 10 fold more immunoglobulin than B cells cultured in RPMI 1640. Results from a typical experiment are shown in Table 5.1. Production of all Ig classes and IgG subclasses (with the exception of IgE which was below the detectable range of assay (<0.5 ng/ml) was much greater with IMDM as culture medium (see Table 5.1). Secretion by unstimulated control B cells was similar in both RPMI and IMDM giving a signal to background ratio of 9.6_{RPMI} compared with 61.2_{IMDM} for IgM and 4.9_{RPMI} compared with 18_{IMDM} for IgG.

Proliferation (^{3}H-TdR uptake) by EBV activated B cells cultured in IMDM was the same as for those cultured in RPMI 1640 (Table 5.2.). Moreover, cells cultured in RPMI 1640 and IMDM showed similar viability by trypan blue exclusion and propidium iodide exclusion on FACS analysis (results not shown). The increase in Ig secretion in IMDM was therefore not due to an increase in cell numbers.

TABLE 5.1. COMPARISON OF IMMUNOGLOBULIN SECRETION BY B CELLS CULTURED IN RPMI 1640 AND IMDM.

	Immunoglobulin Secretion (ng/ml ± 1SE)			
	RPMI 1640		IMDM	
Isotype	Medium	EBV	Medium	EBV
IgM	240±40	2295±477	139±37	8513±1167
IgA	220±27	856±103	179±7.0	2919±748
IgE	< 0.5	< 0.5	< 0.5	< 0.5
IgG	111±6.0	544±27	115±14	2169±232
IgG1	364±11	2369±237	399±90	9622±1275
IgG2	118±20	647±13	180±20	1713±208
IgG3	27±7.0	171±40	81±10	1554±357
IgG4	229±53	293±24	98±2.0	434±16

TABLE 5.2. COMPARISON OF PROLIFERATION BY B CELLS CULTURED IN RPMI 1640 AND IMDM.

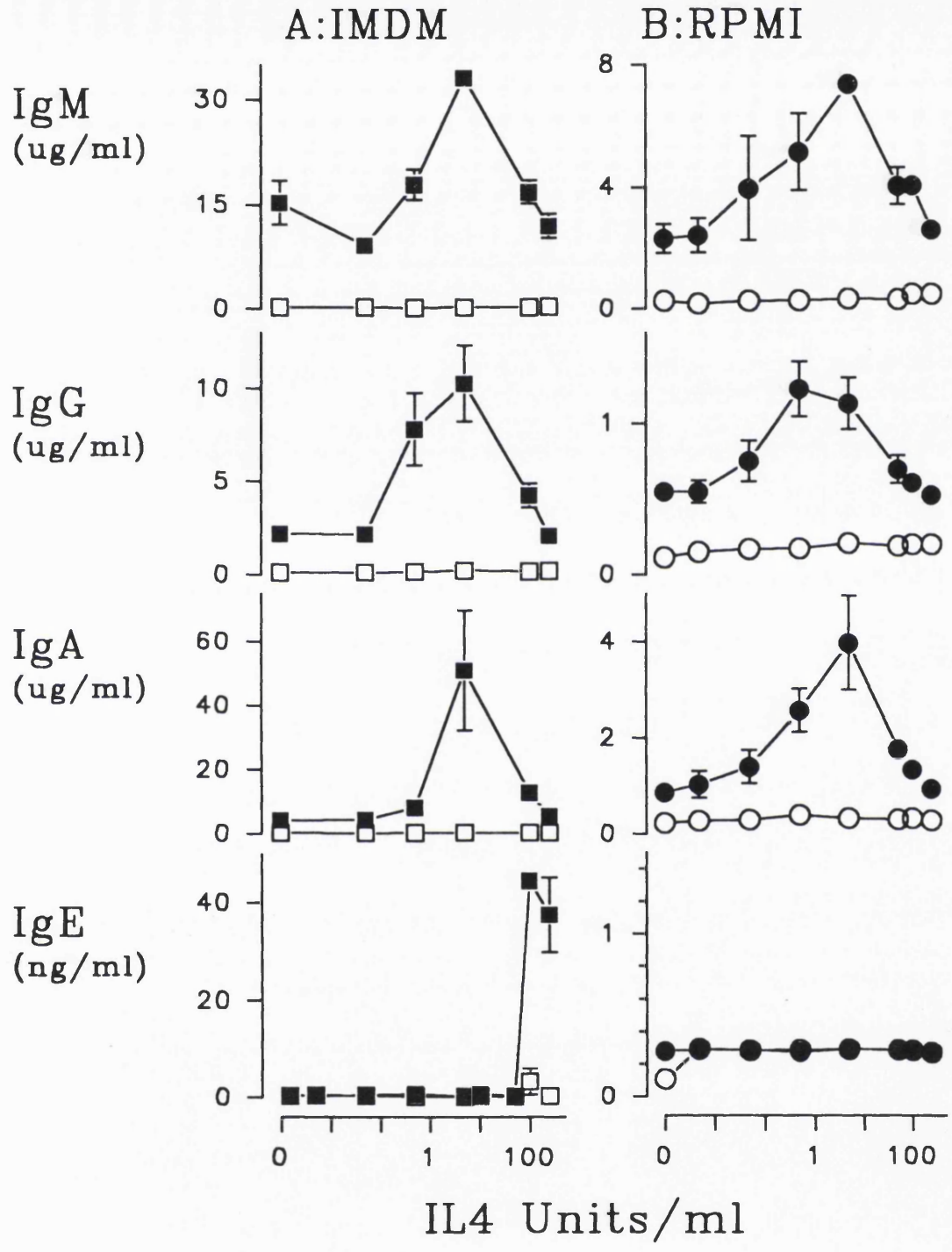
Proliferation (^{3}H-TdR uptake (dpm x 10 $^{-3}$) ± 1SE)			
RPMI 1640		IMDM	
Medium	EBV	Medium	EBV
7.65±0.41	26.98 ± 4.07	8.76 ± 1.96	25.80 ± 4.32

5.4.2. Effect of High and Low Doses of IL4 on Immunoglobulin Secretion and Proliferation of B Cells in IMDM Compared with RPMI 1640.

At low doses of IL4 (5 U/ml), secretion of IgG, IgM, IgA, IgG1, IgG2 and IgG3 by EBV activated B cells was increased in both RPMI 1640 and IMDM, but the response in IMDM was approximately 10 fold greater (Figures 5.5. and 5.6.). At these low doses of IL4, levels of IgE remained undetectable (< 500 pg/ml) and there was no increase in IgG4 in either RPMI 1640 or IMDM (Figures 5.5. and 5.6.). At high doses of IL4 (100 U/ml or greater) a significant increase was seen in levels of IgE and IgG4 when B cells were cultured in IMDM but not in RPMI 1640. Secretion of IgG, IgM, IgA, IgG1, IgG2 and IgG3 was reduced to, or inhibited below, values obtained from B cells treated with EBV alone in both IMDM and RPMI 1640 cultures. Typical results are shown for IgM, IgA, IgG and IgE in Figure 5.5. and for IgG1, IgG2, IgG3 and IgG4 in Figure 5.6.

At all doses of IL4, proliferation of EBV activated B cells and unstimulated B cells cultures was similar in RPMI 1640 and IMDM (Figure 5.7.). The increase in immunoglobulin secretion seen when B cells were cultured in IMDM therefore, could not be explained by an increase in cell numbers.

Figure 5.5.

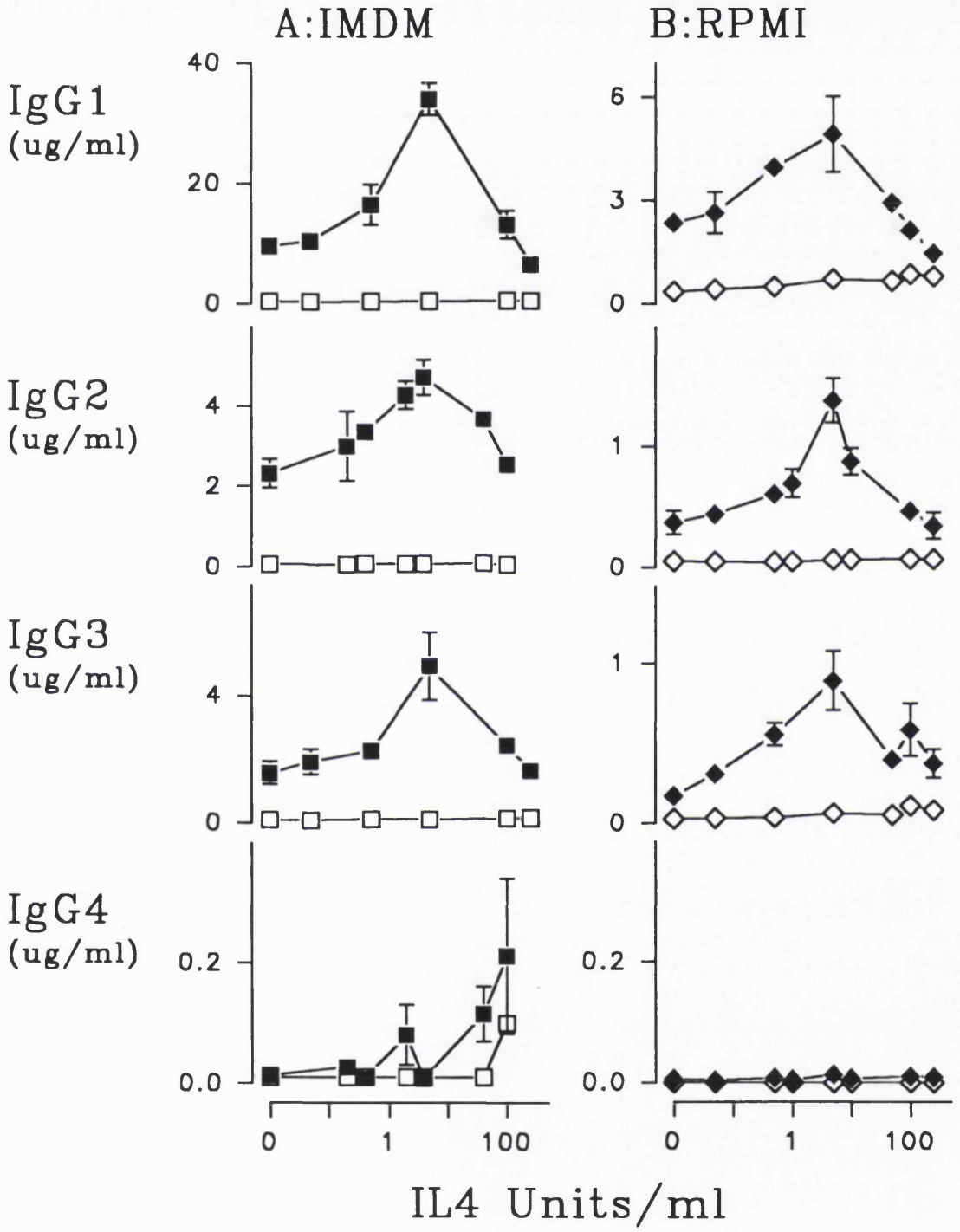


Effect of IL4 on EBV induced B Cell Ig Secretion in Different Culture Media.

Tonsillar B cells, activated with EBV (■ ●) or incubated in medium alone (□ ○), were cultured with IL4 at concentrations from 0–250 U/ml in IMDM (A) or RPMI 1640 (B). Ig secretion was measured on day 13 by ELISA.

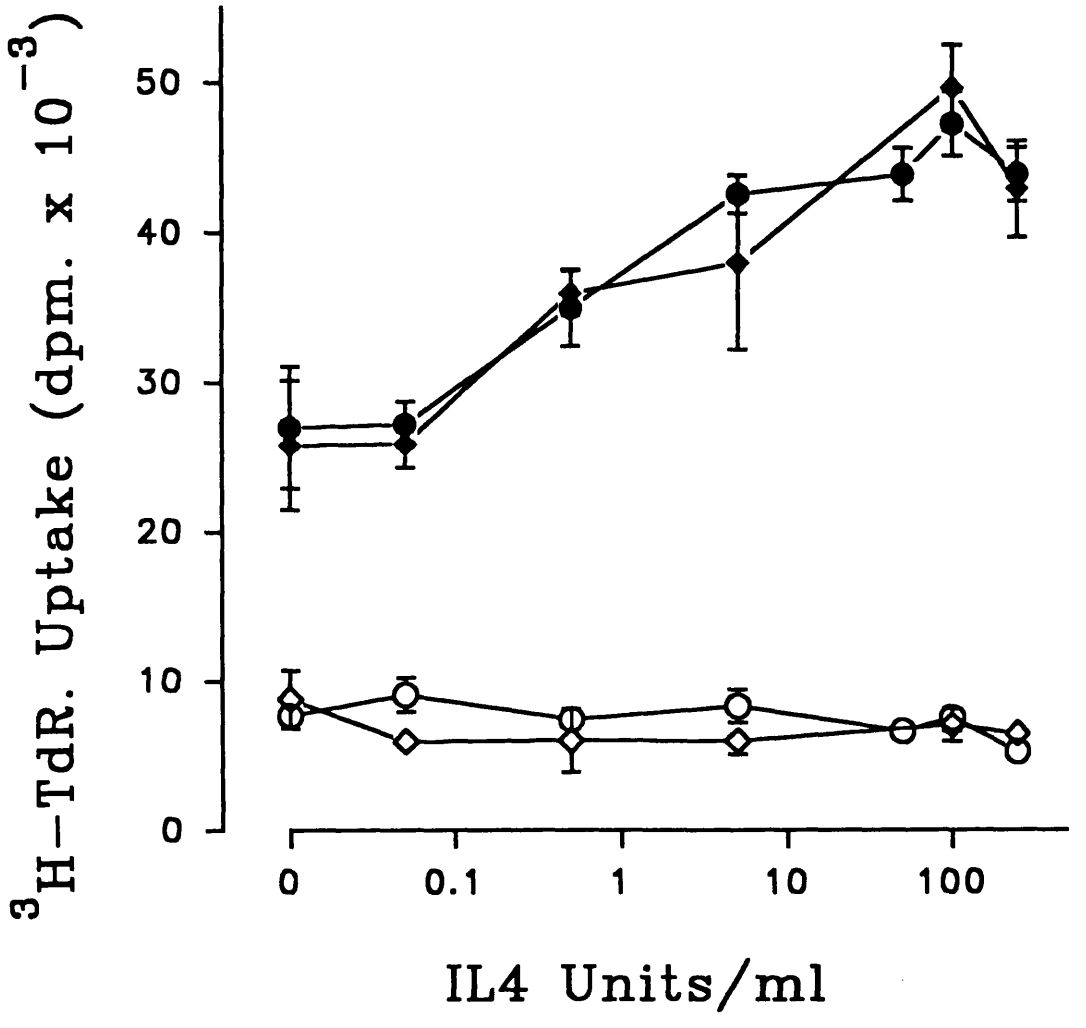
Experiments in IMDM were repeated 6 times. Results for IgM, IgG and IgA were consistent but IgE secretion was seen in 3/6 experiments.

Figure 5.6.



Effect of IL4 on EBV Induced B Cell IgG Subclass Secretion in Different Culture Media.
Tonsillar B cells, activated with EBV (■ ◆) or incubated in medium alone (□ ◇), were cultured with IL4 at concentrations from 0–250 U/ml in IMDM (A) or RPMI 1640 (B). IgG Subclass secretion was measured on day 13 by ELISA. Experiments in IMDM were repeated 6 times. Results for IgG1, IgG2 and IgG3 were consistent but IgG4 secretion was seen in 2/6 experiments.

Figure 5.7.



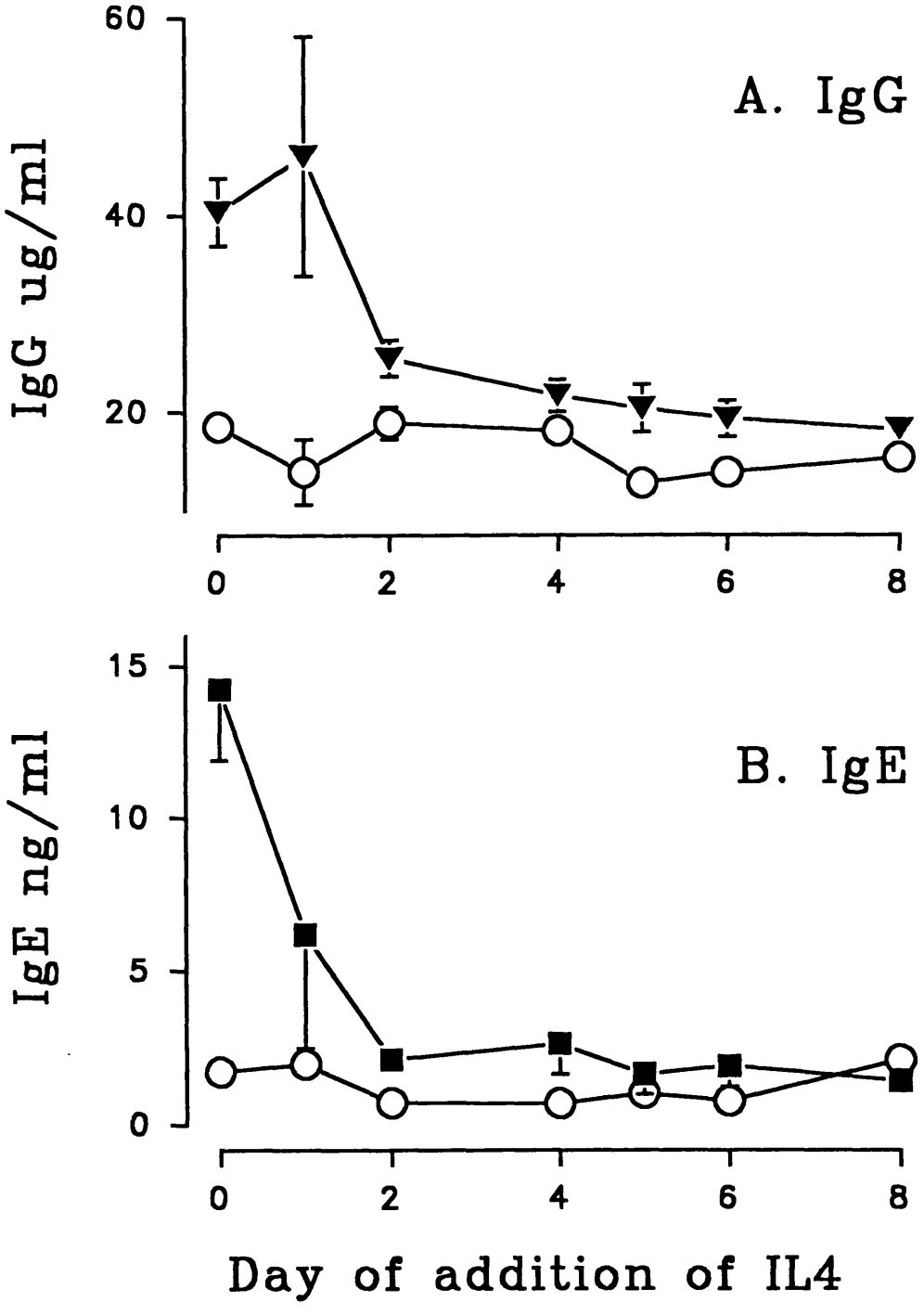
Effect of IL4 on EBV Induced Tonsillar B Cell Proliferation in IMDM or RPMI 1640.
Tonsillar B cells, activated with EBV (● ◆) or incubated in medium alone (○ ◇), were cultured with IL4 at concentrations from 0–250 U/ml in IMDM (◆ ◇) or RPMI 1640 (● ○). Proliferation (^{3}H-TdR. uptake) was measured on day 5.

5.5. HIGH AND LOW DOSES OF IL4 ACT EARLY TO INCREASE IMMUNOGLOBULIN SECRETION.

IL4 is a B cell activator and has also been reported to be a late acting BCDF by increasing Ig secretion in B cell lymphoblastoid cell lines (359). In an attempt to distinguish between these two possible actions of IL4, EBV treated B cells were cultured in IMDM with either high (100 U/ml) or low (5 U/ml) doses of IL4 added at different times from day 0 to 8 as indicated. Culture supernatants were harvested on day 13 and assayed for immunoglobulin content.

Maximum increases in immunoglobulin secretion occurred when low dose IL4 (5 U/ml) was added during the first 24 hours of culture. Results for IgG are shown in Figure 5.8A. Similar results were obtained for IgA and IgM. After the first 24 hours, IL4 ceased to have an effect on antibody production. The increase in IgE was also at an optimum when high dose IL4 (100 U/ml) was added during the first 24 hours (Figure 5.8.B).

Figure 5.8.



Effect of IL4 Added at Different Times on EBV Induced B Cell Ig Secretion.
Tonsillar B cells were activated with EBV then cultured without IL4 (○) or with with (A) 5 U/ml IL4 (▼) or (B) 100 U/ml IL4 (■) added into the cultures at the the times indicated. IgG (A) and IgE (B) secretion was assayed on day 13 by ELISA.

5.6. IL4 ACTS PREDOMINANTLY ON HIGH AND NOT LOW DENSITY B CELLS.

The results seen in Section 5.5. suggested that both high and low doses of IL4 acted early to induce Ig secretion, perhaps by signalling B cells in the very early stages of EBV activation. This was tested in another way by looking at the action of EBV and IL4 on resting (high density) and activated (low density) tonsillar B cells. High density and low density B cells separated as described in Section 2.2.2, were incubated with EBV or a medium control and then cultured in RPMI 1640 with low dose IL4 (5 U/ml) for immunoglobulin secretion and high dose IL4 (100 U/ml) for proliferation. Proliferation was measured on day 5 and immunoglobulin secretion on day 13.

5.6.1. Effect of IL4 on EBV Induced Proliferation by High and Low Density B Cells Activated with EBV.

IL4 (100 U/ml) significantly increased proliferation by unseparated and high density B cells activated with EBV. In contrast, although low density B cells proliferated in response to EBV alone, the addition of IL4 had little or no effect. Background proliferation of unstimulated high density B cells was very low in comparison to unseparated and low density B cells indicating the absence of pre-activated B cells. Results from a typical experiment are shown in Figure 5.9. and Table 5.3.

TABLE 5.3. PROLIFERATION OF UNSEPARATED, HIGH AND LOW DENSITY B CELLS ACTIVATED WITH EBV AND IL4.

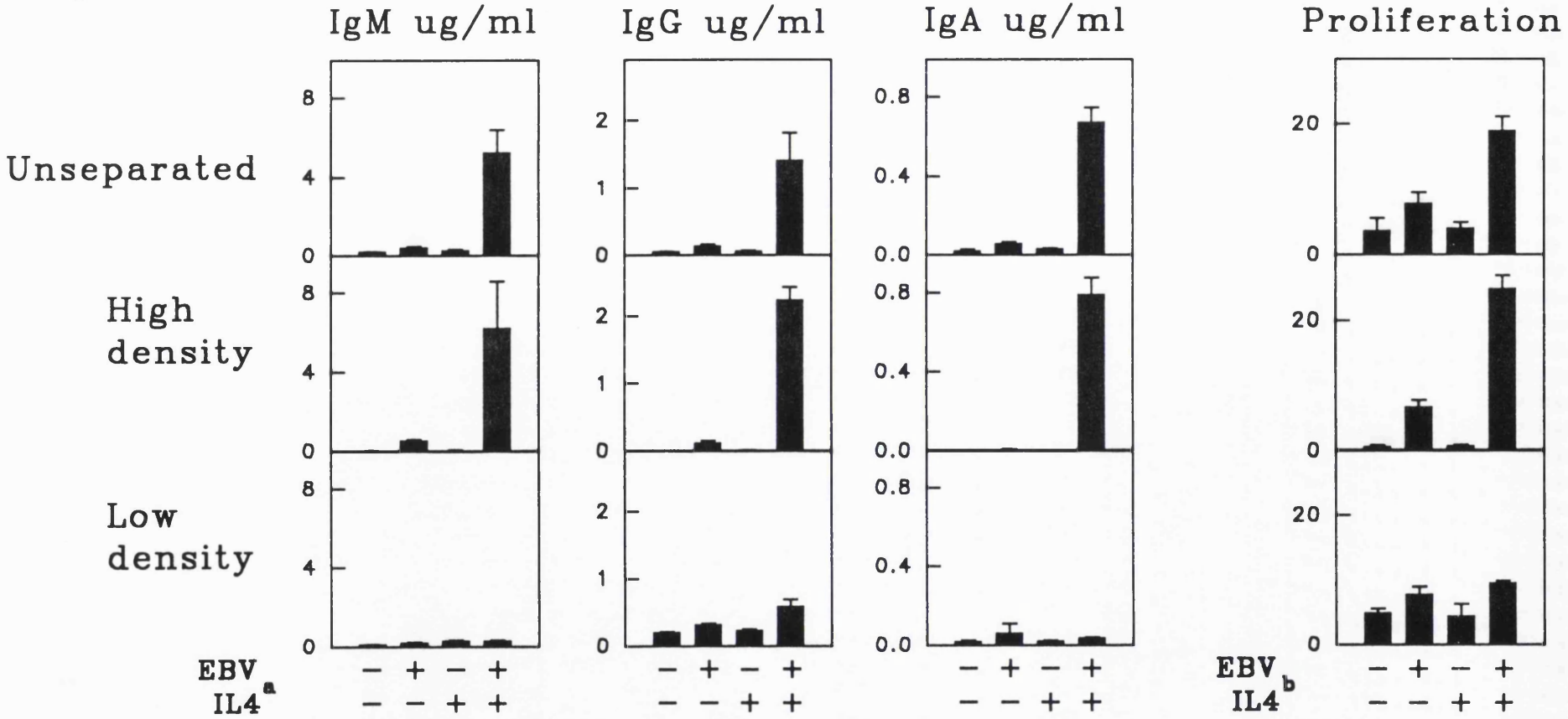
	No additions		100 U/ml IL4	
	Medium	**EBV**	**Medium**	**EBV**
Unseparated	3.65 ± 1.92[a]	7.92 ± 1.68	4.10 ± 0.84	18.97 ± 2.15
High Density	0.61 ± 0.23	6.75 ± 1.04	0.74 ± 0.16	24.88 ± 1.96
Low Density	4.91 ± 0.65	7.83 ± 1.08	4.41 ± 1.85	9.46 ± 0.35

a ^{3}H-TdR uptake (dpm x 10^{-3}) ± 1SE).

5.6.2. Effect of IL4 on EBV Induced Immunoglobulin Secretion by High and Low Density B Cells.

Activation of unfractionated B cells with EBV induced low levels of polyclonal immunoglobulin secretion. Addition of low dose IL4 (5 U/ml) greatly increased secretion of IgM, IgG and IgA by 10 to 12 fold, as illustrated in Figure 5.9. and Table 5.4. Unstimulated B cells gave background immunoglobulin secretion when cultured alone or in response to IL4.

Figure 5.9.



Effect of IL4 on High and Low Density EBV Activated B Cells.
Tonsillar B cells were separated into high and low density fractions, activated with EBV or incubated with medium alone, then cultured without IL4, with 5 U/ml IL4 ($IL4^a$) or with 100 U/ml IL4 ($IL4^b$). Proliferation (^{3}H−TdR. uptake/dpm. x 10^{-3}) was measured on day 5 and IgM, IgG and IgA secretion was assayed on day 13 by ELISA.
This experiment was repeated 3 times with similar results.

Activation of high density B cells with EBV also induced polyclonal immunoglobulin secretion. This was increased by between 10 and 80 fold with the addition of IL4 (5 U/ml) (Figure 5.9. and Table 5.4.). The increase in secretion by high density B cells with IL4 was often greater than that from unfractionated B cells. Non stimulated high density B cells gave extremely low levels of Ig secretion when cultured alone or with low dose IL4. Results for IgM, IgG and IgA are shown in Figure 5.9. and Table 5.4.

EBV alone induced low levels of Ig secretion by low density B cells and the addition of IL4 (5 U/ml) did not have any significant effect (Figure 5.9. and Table 5.4.). Background immunoglobulin secretion by unstimulated low density B cells was high in comparison to unseparated and high density B cells indicating the presence of B cells pre-activated *in vivo*.

These results are consistent with IL4 activation of resting but not activated B cells.

TABLE 5.4. Ig SECRETION OF UNSEPARATED, HIGH AND LOW DENSITY B CELLS ACTIVATED WITH EBV AND IL4.

		No additions		5 U/ml IL4	
		Medium	EBV	Medium	EBV
IgM	Unseparated	200 [a]	420	260	5250
		± 10	± 60	± 60	± 1110
	High Density	20	540	40	6260
		± 10	± 40	± 10	± 2330
	Low Density	110	300	210	300
		± 20	± 40	± 20	± 20
IgG	Unseparated	50	140	60	1410
		± 4	± 20	± 10	± 400
	High Density	3	120	4	2240
		± 0.2	± 30	± 2	± 180
	Low Density	200	320	240	590
		± 10	± 10	± 10	± 100
IgA	Unseparated	20	60	30	670
		± 10	± 10	± 3	± 70
	High Density	2	10	2	790
		± 0.1	± 4	± 0.1	± 90
	Low Density	20	60	20	40
		± 10	± 50	± 1	± 4

a ng/ml ± 1SE.

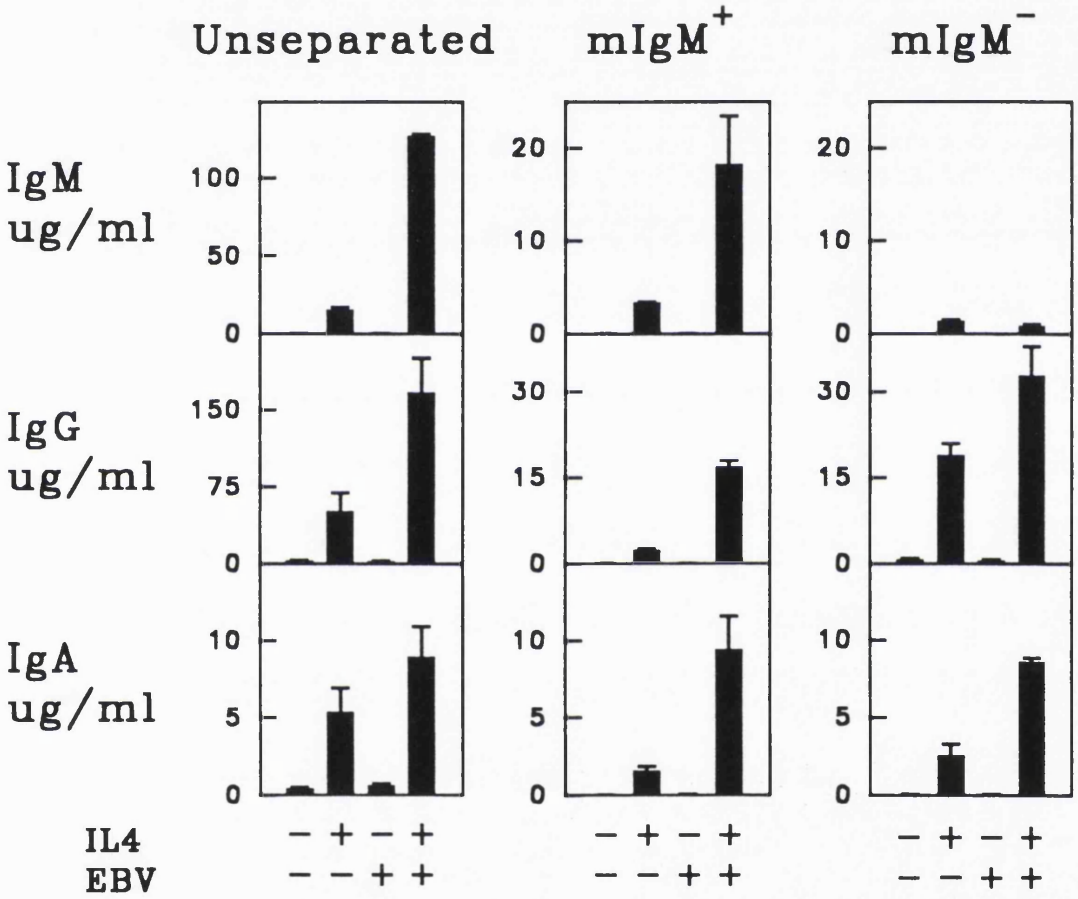
5.7. LOW DOSE IL4 INCREASES IMMUNOGLOBULIN SECRETION BY mIgM$^+$ B CELLS.

The increase in IgE secretion by IL4 (100 U/ml) by EBV activated B cells has been shown by others to be by Ig heavy chain class switching rather than clonal expansion of pre-committed cells (196). However, the mechanism by which low doses of IL4 induce production of IgG, IgM and IgA is unknown. The finding that IL4 acts early and induces an increase in these isotypes which cannot be accounted for by proliferation, raises the question that IL4 at low concentrations may be able to induce class switching to IgA, IgG1, IgG2 and IgG3. If this were true, membrane IgM positive B cells (mIgM$^+$) could be induced to make IgA, IgG1, IgG2 and IgG3 in the presence of low doses of IL4. To test this, tonsillar B cells were separated by MACS (magnetic cell separation), as described in Chapter 2 (Section 2.2.4.), into mIgM$^+$ and mIgM$^-$ populations. Unseparated, mIgM$^+$ and mIgM$^-$ B cells were incubated with EBV, washed and cultured with high (100 U/ml) and low (5 U/ml) doses of IL4. Proliferation assays were harvested on day 5 and culture supernatants were removed for immunoglobulin secretion assays on day 13.

5.7.1. Response of mIgM$^+$ and mIgM$^-$ B Cells to Low Doses of IL4.

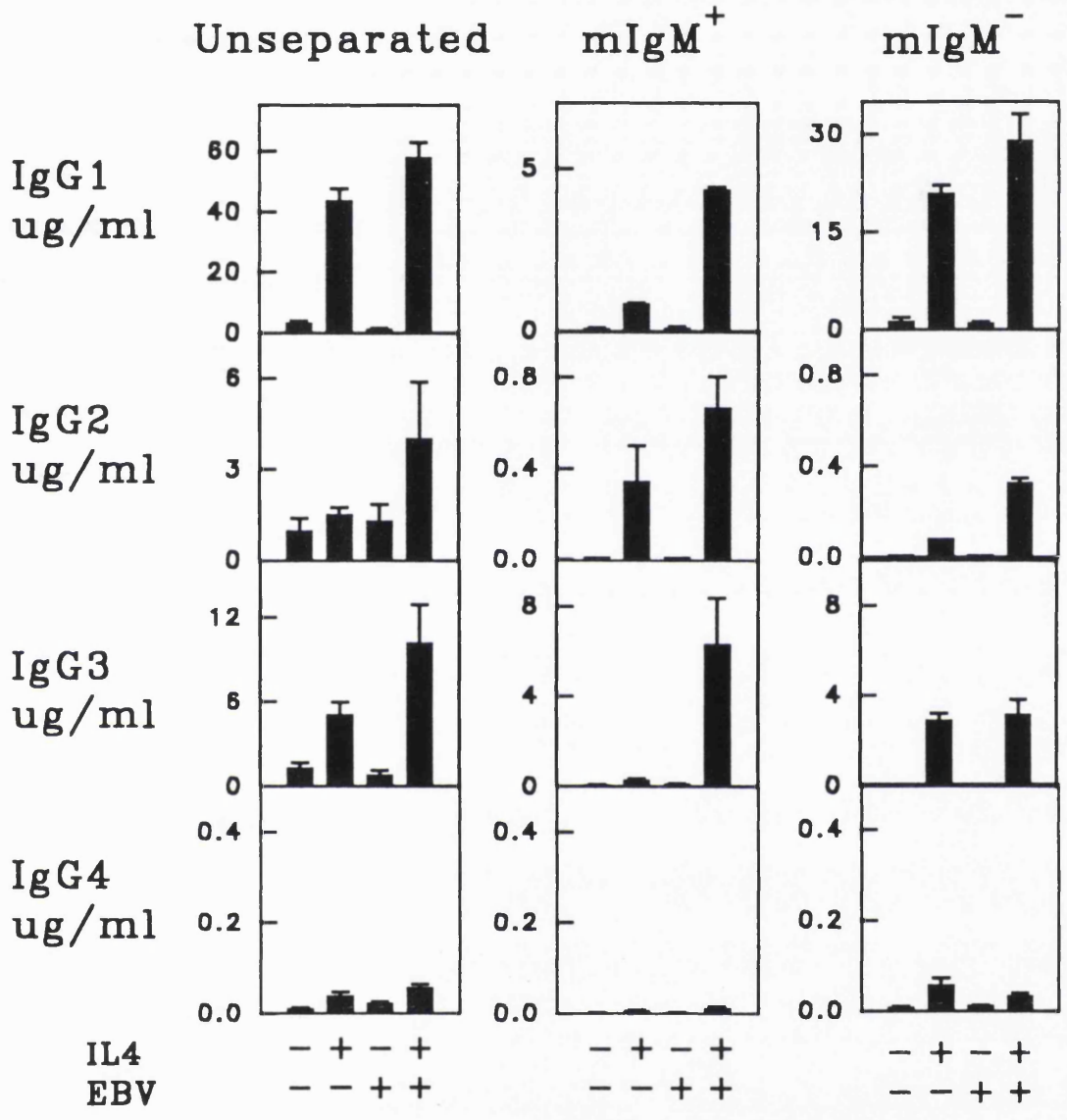
IL4 (5 U/ml) significantly increased IgA, IgG1, IgG2 and IgG3 production by mIgM$^+$ B cells activated with EBV (Figure 5.10 and Figure 5.11). This was not due to contamination with mIgM$^-$ B cells as the method used gave mIgM$^+$ and IgM$^-$ fractions of up to 98% purity, and no IgM was secreted by the mIgM$^-$ fraction (Figure 5.10). These results are consistent with heavy chain class switching by mIgM$^+$/mIgG$^-$/mIgA$^-$ B cells. IgG4 secretion by mIgM$^+$ B cells and low dose IL4 was always very low or below the detection limit of assay ($<$ 1 ng/ml) and did not

Figure 5.10.



Effect of IL4 on Ig Isotype Secretion by EBV Activated mIgM$^+$ and mIgM$^-$ B Cells.
After separation of tonsillar B cells by MACS, unseparated, mIgM$^+$ and mIgM$^-$ B cells were activated with EBV or incubated in medium alone then cultured with or without 5 U/ml IL4. IgM, IgG and IgA secretion was assayed on day 13 by ELISA. Similar results were obtained in 2 separate experiments.

Figure 5.11.



Effect of IL4 on IgG Subclass Secretion by EBV Activated mIgM$^+$ and mIgM$^-$ B Cells.
After separation of tonsillar B cells by MACS, unseparated, mIgM$^+$ and mIgM$^-$ B cells were activated with EBV or incubated with medium alone then cultured with or without 5 U/ml IL4. IgG1, IgG2, IgG3 and IgG4 secretion was assayed on day 13 by ELISA. Similar results were obtained for 1991, 1992 and 1993 in two separate experiments. Results for 1994 were less consistent.

significantly increase above values obtained with EBV alone (Figure 5.11. and Table 5.5.). Similarly, IgE secretion was below the detectable limit of assay (<0.5 ng/ml) in all experiments and no increase was seen in IgE secretion with low dose IL4 (Table 5.6).

In some experiments, low doses of IL4 increased IgG1, IgG2, IgG3 and IgA in the $mIgM^-$ population (Figures 5.10. and 5.11.), presumably by activation of $mIgG1^+$, $mIgG2^+$, $mIgG3^+$ and $mIgA^+$ B cells in the IgM^- fraction, but the relative increase was usually much less than that of $mIgM^+$ B cells. Values obtained for IgG4 were always low and did not significantly increase above those obtained with EBV alone (Table 5.5.). IgE was not detectable (<0.5 ng/ml) (Table 5.6.). These results, together with the enhancing effect of IL4 on IgM secretion by $mIgM^+$ B cells (Figure 5.10), are consistent with IL4 also acting as a BCDF.

5.7.2. Response of $mIgM^+$ and $mIgM^-$ B Cells to High Doses of IL4.

High doses of IL4 (100 U/ml) inhibited IgA, IgM, IgG1, IgG2 and IgG3 production by unseparated, $mIgM^+$ and $mIgM^-$ B cells activated with EBV (results not shown). In contrast, IgE production was significantly increased in cultures of unseparated B cells and IgM^+ B cells. There was generally no IgE production in $mIgM^-$ B cell cultures, but in some experiments where the purity of the IgM^- fraction was less than 95%, IgE was also increased in this B cell fraction by high doses of IL4 (Table 5.6.). IgG4 secretion was always very low in these experiments. IgG4 secretion by the unseparated and $mIgM^-$ fractions although low, tended to be decreased further with high doses of IL4 (100 U/ml) (Table 5.6.), but in the $mIgM^+$ fraction IgG4 secretion was usually increased with high doses of IL4 (Table 5.6.). These results are consistent with heavy chain class switching to IgE and possibly IgG4 by $mIgM^+/mIgG^-/mIgA^-$ B cells.

TABLE 5.5. IgG4 PRODUCTION BY EBV ACTIVATED mIgM$^+$ AND mIgM$^-$ B CELLS. *

		IgG4 (ng/ml) ± 1SE.	
	Additions	Medium	EBV
Unseparated	Medium	11 ± 2	39 ± 8
	5 U/ml IL4	23 ± 3	58 ± 7
	100 U/ml IL4	6 ± 1	11 ± 1
mIgM$^+$	Medium	2 ± 0.1	8 ± 1.3
	5 U/ml IL4	3 ± 0.2	13. ± 3·6
	100 U/ml IL4	8.5 ± 1.7	114 ± 12
mIgM$^-$	Medium	9 ± 1.7	58 ± 15
	5 U/ml IL4	13 ± 0.6	35 ± 5
	100 U/ml IL4	4 ± 0.4	10 ± 2

TABLE 5.6. IgE PRODUCTION BY EBV ACTIVATED mIgM$^+$ AND mIgM$^-$ B CELLS. *

		IgE (ng/ml) ± 1SE.	
	Additions	Medium	EBV
Unseparated	Medium	< 0.5	< 0.5
	5 U/ml IL4	< 0.5	< 0.5
	100 U/ml IL4	< 0.5	63.5 ± 29
mIgM$^+$	Medium	< 0.5	< 0.5
	5 U/ml IL4	< 0.5	< 0.5
	100 U/ml IL4	< 0.5	39 ± 31
mIgM$^-$	Medium	< 0.5	< 0.5
	5 U/ml IL4	< 0.5	< 0.5
	100 U/ml IL4	< 0.5	5.36 ± 4

* Although the results seen for IgG, IgM, IgA, IgG1, IgG2 and IgG3 were consistent in 3/3 experiments. IgE and IgG4 secretion was seen in 1/3 experiments.

5.8. EFFECT OF HIGH AND LOW DOSES OF IL4 ON B CELLS FROM A PATIENT WITH X-LINKED HYPER IgM SYNDROME (HIGM1) .

X-Linked Hyper IgM Syndrome (HIGM1) is characterised by normal or raised levels of serum IgM and low or absent IgA, IgG and IgE. Patients' B cells are unable to switch to other Ig isotypes from IgM due to a defect in the T cell activation antigen, CD40L (169) and consequently have large numbers of circulating non switched IgM^+ B cells (55,231). The B cells are normal and can be induced to switch class *in vitro* to other Ig isotypes (145,231). As circulating B cells are in these patients are non switched and IgM^+, they may be used to investigate Ig heavy chain class switching in the absence of the expansion of pre-switched cells. In this Section, HIGM1 B cells were used to investigate Ig heavy chain class switching to IgA and IgG by low doses of IL4.

B cell enriched PBMCs from a patient (AT) with HIGM1 syndrome and from a normal control (SS) were activated with EBV and cultured with high dose (100 U/ml) and low dose (5 U/ml) IL4. Cyclosporin (1 ug/ml) was included in the cultures to prevent cytotoxic T cell killing of EBV infected B cells. Supernatants were assayed for immunoglobulin secretion on day 13.

5.8.1. Immunoglobulin Secretion by HIGM1 B Cells in Response to EBV Activation.

EBV increased IgM, IgG and IgA secretion from B cell enriched PBMCs from the normal donor by between 25-55 fold (Table 5.7). In contrast, EBV alone induced little IgG and non detectable levels of IgA (< 1 ng/ml) from B cell enriched PBMCs from the HIGM1 patient although IgM secretion was increased 9 fold. IgE secretion by both normal and HIGM1 cells was below the detectable range of assay

(< 0.5 ng/ml). Interestingly, although unstimulated normal and HIGM1 cells both secreted similar background levels of IgM, when activated with EBV, normal B cell secretion was increased by 26 fold whereas HIGM1 B cell secretion increased by only 9 fold (Table 5.7).

5.8.2. Immunoglobulin Secretion by HIGM1 B Cells in Response to EBV and IL4.

Low dose IL4 (5 U/ml) significantly increased IgM, IgA and IgG secretion by EBV activated B cells from the normal donor. However, IgG secretion by HIGM1 B cells was only slightly enhanced with IL4 and IgA remained below the detection range of assay (< 1 ng/ml) (Table 5.7). IgM secretion was significantly increased by low dose IL4. These results are in line with IL4 acting as a BCDF and not an Ig heavy chain class switch factor.

High dose IL4 (100 U/ml) significantly increased IgE secretion by EBV activated normal PBMC B cells and reduced levels of IgM, IgG and IgA. High dose IL4 also reduced IgM secretion from HIGM1 B cells back to levels obtained with EBV alone. No increase in IgE was obtained from these cells. Even with the addition of IL2, which increased IgE in normal B cell cultures by 3 fold, IgE secretion by HIGM1 B cells remained below the detection range of assay (< 0.5 ng/ml) (Table 5.7).

TABLE 5.7. EFFECT OF IL4 ON Ig SECRETION BY NORMAL AND HIGM1 B CELLS.

		Normal PBMC B Cells		HIGM1 PBMC B Cells	
	Additions	Medium	EBV	Medium	EBV
IgM ng/ml	Medium	250 ± 10	6530 ± 1430	330 ± 30	2640 ± 220
	5 U/ml IL4	220 ± 70	15500 ± 1440	330 ± 20	5550 ± 810
	100 U/ml IL4	270 ± 20	9600 ± 410	320 ± 30	2570 ± 550
IgA ng/ml	Medium	100 ± 10	5780 ± 1130	< 1	< 1
	5 U/ml IL4	250 ± 40	13300 ± 2770	< 1	< 1
	100 U/ml IL4	110 ± 40	6490 ± 3420	< 1	< 1
IgG ng/ml	Medium	50 ± 10	1510 ± 790	14 ± 1	23 ± 1
	5 U/ml IL4	80 ± 10	6210 ± 2490	14 ± 2	31 ± 6
	100 U/ml IL4	90 ± 10[a]	3730 ± 1240	15 ± 1	29 ± 4
IgE ng/ml	Medium	< 0.5[b]	< 0.5	< 0.5	< 0.5
	Medium + IL2 (25 U/ml)	< 0.5	< 0.5	< 0.5	< 0.5
	5 U/ml IL4	< 0.5	< 0.5	< 0.5	< 0.5
	5 U/ml IL4 + IL2 (25 U/ml)	< 0.5	< 0.5	< 0.5	< 0.5
	100 U/ml IL4	< 0.5	1.467 ± 0.765	< 0.5	< 0.5
	100 U/ml IL4 + IL2 (25 U/ml)	< 0.5	3.181 ± 1.007	< 0.5	< 0.5

5.10. DISCUSSION.

IL4 does not induce B cell proliferation or immunoglobulin production. It does however activate B cells from resting G0 into early G1 of the cell cycle. This is characterised by an increase in cell volume and a dose dependent increase in B cell surface antigens, mIgD (personal observation), mIgM, CD23, CD40 and HLA-DR (36,83,358). Although IL4 can induce the production of murine Cε and Cγ1 (127,334) and human Cε germline transcripts (151,196,315), switch site recombination, deletion of intervening circular DNA and Ig heavy chain class switching requires an additional activation signal. *In vivo*, additional signals are provided by T cells and monocytes (107,150,191,250,300,410,412) involving cognate cell cell interaction. One such T cell antigen is CD40L. This delivers its signal through CD40 (145,231). *In vitro*, the murine B cell activator LPS (367) and human B cell activators PMA and ionomycin (141,163,250), anti-CD40 (15,149), anti-IgM (103,358), SAC (105) and EBV (196,394) can replace T cells and provide the activation signals needed for IL4 mediated B cell responses. However, although IL4 is an undisputed regulator of human IgE and IgG4 production, its regulation of the other immunoglobulin classes, especially the IgG subclasses, is unclear.

Although EBV has been used in previous studies to look at the effects of high doses of IL4 on Ig isotype regulation, culture conditions and the timing of the response showed that B cell transformation rather than polyclonal activation may have been investigated (394). Careful definition of EBV activation kinetics (see Chapter 3) provided ideal conditions for the study of IL4 regulation of Ig class and IgG subclass responses. In this system IL4 was shown to act not only as a BCGF but also as a BCDF, with two quite distinct effects on immunoglobulin secretion.

IL4 is a BCDF for EBV Activated B Cells.

Low doses of IL4 (0.5-5 U/ml) significantly increased IgM, IgA, IgG, IgG1, IgG2 and IgG3 secretion by EBV activated B cells, but at high doses (> 100 U/ml) these isotypes were reduced or inhibited below values obtained with EBV alone (Figures 5.1. and 5.2). EBV activated B cells cultured with high doses of IL4 in RPMI 1640 were unable to increase either IgE or IgG4 but when B cells were cultured in IMDM, IgE was consistently increased by high doses of IL4 and less frequently, IgG4 (Figures 5.5. and 5.6.). In parallel with these results, secretion of the other Ig isotypes in the same cultures was sometimes increased by up to 10 fold, but this was not due to increased proliferation or viability in IMDM (Figure 5.7., Tables 5.1. and 5.2.). IMDM medium supported IgE and IgG4 production as well as increased secretion of the other Ig isotypes. It was possible that this increase was achieved by supplying the culture environment needed for optimal antibody production (79) possibly by relief of oxidative stress (54).

The increase of IgE and IgG4 at high doses of IL4 (> 100 U/ml) was similar to previous results obtained with PBMC alone, PBMC and anti-CD40 (149,191) and PMA and EL4 cells (250). IL4 induced IgE and IgG4 secretion is well documented and has been shown to be due to Ig heavy chain class switching (196,315,355)). The effects of IL4 on production of the other Ig classes and IgG subclasses is not clear with inconsistent reports from different laboratories. IL4 has been reported to be a BCDF for human B cell lymphoblastoid cell lines, increasing IgG, IgM and IgA secretion (359) as well as normal B cells, increasing IgM, IgG1, IgG2 and IgG3 by B cells activated with PMA and ionomycin (141), IgE, IgG, IgM but not IgA by B cells activated by anti-CD40 L-cells (15) and IgG and IgE but not IgM or IgA by PBMC (306). However, these effects of IL4 on immunoglobulin secretion were all induced by high doses of IL4 of 100 Units/ml or greater. Low dose effects of IL4 on antibody production by human B cells described in this Chapter have not

been reported elsewhere. These findings suggest that IL4 may have another distinct role in the control of immunoglobulin class and IgG subclass production which is separate and different to high dose IL4 responses.

Low doses of IL4 are also known to optimally increase expression of mIgM while high doses of IL4 optimally increase CD23 (327). Rigley *et al* (327) showed that different signalling pathways were used by low and high doses of IL4 for the increase of mIgM and CD23 on purified tonsillar B cells, contributing functional evidence for the presence of two IL4 receptors. The high affinity receptor for IL4 has been cloned (147) and recently evidence for the presence of a second low affinity receptor for IL4 has been published (129). These findings contribute to the possibility that the control of B cell immunoglobulin production by IL4 may also be mediated through two separate receptors with different signalling mechanisms. One receptor, with high affinity, may increase IgM, IgA IgG1, IgG2 and IgG3 at low doses of IL4 (1-5 U/ml), and a second receptor, of low affinity, may inhibit or override high affinity receptor responses and increase IgE and IgG4 with high doses of IL4 (> 100 U/ml). The low affinity receptor may conduct the class switching activity of IL4 for control of IgE and IgG4, but the mechanism through which IL4 increases IgM, IgG, IgA, IgG1, IgG2 and IgG3 via a high affinity receptor is unknown.

IL4 is a BCGF for EBV Activated B Cells.

IL4 optimally increased EBV induced B cell proliferation with 20-100 U/ml IL4. These results were consistent with the BCGF action of IL4 in combination with anti-IgM (408), anti-CD40 (14,15) and PMA and ionomycin (163). Interestingly, the concentration of IL4 which induced maximum proliferation was 10 fold greater than the dose of IL4 which induced maximum immunoglobulin production (Figures 5.7.,

5.5. and 5.6. Chapter 4, Figure 4.4.). In fact, doses of IL4 which gave optimum proliferation inhibited immunoglobulin secretion. These findings show that the increase in Ig production with low doses of IL4 was not due to a proliferation of B cells. The effect was also not an alteration of EBV activation kinetics by IL4 (Figures 5.3. and 5.4.) High doses of IL4 also appeared to prevent transformation of IgG, IgA and IgM clones into lymphoblastoid cell lines late in culture. It may be that the signals given by IL4 create unfavourable conditions for transformation, but have no effect on EBV kinetics during polyclonal activation.

Both High and Low Doses of IL4 Act Early in B Cell Activation.

In this Chapter, the time at which IL4 is added to EBV activated B cells was found to be critical for IL4 induced responses. IL4 added to B cells later than 48 hours after activation ceased to have any effect in both the low dose (5 U/ml) increase in IgM (data not shown) and IgG, and the high dose (100 U/ml) increase in IgE (Figure 5.8). IL4 is therefore an early acting factor in its control of Ig production by EBV activated B cells. IL4 has been classed as an early acting factor in other systems. High doses of IL4 are needed during the first 24 hours of culture for IgE secretion by PBMC (76), and IL4 is required during the first 72 hours after exposure of TMC to antigen to inhibit the specific antibody response to influenza virus (59). IL4 does not appear to be a late acting BCDF in the same way as IL6 which enhances Ig production from pre-committed plasma cells. Rather, IL4 signals to B cells during or shortly after initial activation.

Although both high density and low density B cells activated with EBV were able to proliferate and produce immunoglobulin, B cell responses to low and high doses of IL4 were largely confined to the resting, high density B cell population (Figure 5.9., Tables 5.4. and 5.5.). Low density B cells are said to be pre-activated *in vivo*,

and IL4 may not be able to induce responses from these cells either because they may have passed beyond the 48 hour threshold for responses to IL4 (see Figure 5.8.), or because the intracellular conditions are unsuitable for IL4 signalling. In contrast, high density resting B cells responded well to IL4.

Recently, a monoclonal antibody to the high affinity IL4 receptor (m57), was used to examine IL4 receptor expression in non activated and PMA stimulated tonsillar B cells. Expression of this receptor was detectable on a sub population of unseparated, non activated B cells and was either dramatically decreased or non-detectable within 72 hours of stimulation with PMA (personal observation). IL4 may be an early acting factor in low dose responses due to the transience of its receptor. This could also conveniently explain why IL4 responses are confined largely to high density resting B cells, low density B cells having been pre-activated and no longer expressing the IL4 receptor. Whether this is also the case with high doses of IL4 binding to a low affinity receptor has yet to be determined.

Action of IL4 on mIgM$^+$ and mIgM$^-$ B Cells.

Separation of tonsillar B cells into mIgM$^+$ and mIgM$^-$ populations gave interesting and conflicting results. mIgM$^-$ B cells, activated with EBV, produced IgG, IgA, IgG1, IgG2, IgG3 and IgG4, but no IgM. These isotypes were significantly increased with low dose IL4 (5 U/ml) (Figures 5.10. and 5.11.). These results were consistent with IL4 acting as a BCDF similar to the effect of IL4 on SAC activated B cells (105) and IL4 on B cell lines (359).

EBV activation of mIgM$^+$ B cells induced IgM secretion which was significantly increased with low doses of IL4 (Figure 5.10.). IgA, IgG1, IgG2 and IgG3 were also significantly increased suggesting that IL4 might also be inducing Ig class

switching (Figures 5.10. and 5.11.). As EBV alone induced a small increase of IgA, IgG1, IgG2, IgG3 and IgG4, some pre-switched or contaminating $mIgM^-$ B cells may be present in the $mIgM^+$ cultures. However, low doses of IL4 induced a greater increase of IgA, IgG1 and IgG3 in $mIgM^+$ B cell cultures when compared with $mIgM^-$.

With IL4 at high doses, IgM, IgA, IgG1, IgG2 and IgG3 secretion was significantly inhibited in unseparated, $mIgM^-$ and $mIgM^+$ cell cultures (data not shown). IgE production however, was significantly increased in unseparated and $mIgM^+$ B cell cultures with 100 U/ml IL4 (Table 5.6.). In some experiments, a small increase in IgE was seen in $mIgM^-$ cultures probably due to $mIgM^+$ cell contamination. IgG4 secretion was also increased with high dose IL4 in unseparated and $mIgM^+$ B cell cultures but not in $mIgM^-$ cultures (Table 5.6.). These results are consistent with the those of Gascan *et al* where highly purified $mIgM^+$ B cells activated with anti-CD40 and IL4 (149) and with activated T cell membranes and IL4 (148) secreted high levels of IgE and IgG4.

These results suggest that in addition to the specific increase of IgE and IgG4 secretion at high doses by Ig heavy chain class switching, IL4 may switch $mIgM^+$ cells to IgA and IgG subclasses at low concentrations as well as act as a BCDF.

Hyper IgM syndrome is an immunodeficiency characterised by high levels of circulating IgM but little or no IgG, IgA or IgE antibodies in the patients' serum. The X-linked form of the disease (HIGM1) is due to a malfunction in the ligand for CD40 (CD40L) on T cells which stops it from binding to the patients B cells and effecting Ig class switch mechanisms. Consequently, patients B cells are normal but have an unswitched IgM^+ phenotype. These HIGM1 B cells provide an excellent model for examining the possibility of Ig class switching to IgG and IgA by low

doses of IL4. Although 5 U/ml IL4 was able to induce significant increases in IgM, IgG and IgA from normal B cells, only IgM was increased in cultures of HIGM1 B cells. These results indicate that at low doses, IL4 acts as a BCDF and not an Ig heavy chain switch factor. In the same experiment, normal B cells were induced to produce IgE by 100 U/ml IL4, but there was no response by HIGM1 B cells. In other studies, IL4 was found to induce IgE secretion by B cells from some HIGM1 patients (145,231). The fact that IgE was not detected in our patient, and that IgM secretion was lower in cultures of EBV activated HIGM1 B cells and IL4 suggests that conditions for class switching and optimal Ig production might not have been met even though experiments were in IMDM, or this was simply due to patient variation, as not all HIGM1 patients can be induced to produce IgE (145,231). Therefore, the mechanism by which IL4 induces IgG and IgA from IgM^+ B cells remains unanswered. However, these experiments confirm that IL4 is a BCDF for IgM. Further investigation of these responses must now be determined by limiting dilution analysis and examination at the molecular level.

CHAPTER 6

CONTROL OF EBV INDUCED IMMUNOGLOBULIN PRODUCTION BY LIGATION OF THE B CELL SURFACE ANTIGEN CD40

6.1. INTRODUCTION

CD40 is an integral membrane glycoprotein of approximately 50kDa, and a member of the NGFR superfamily (293,378). A human ligand has been cloned for CD40 (hCD40L) which shows homology to TNFα and β (293) and is expressed on activated T cells (169,238). Most experimental work with CD40 has been carried out with mouse monoclonal anti-CD40 antibodies which cross link CD40 and induce B cell activation (169). Anti-CD40 antibodies stimulate significant B cell proliferation in combination with anti-IgM, anti-CD20, TPA and IL4 (14,15,242,401). Binding to CD40 by anti-CD40 antibodies also supplies the second signal required by IL4 to effect class switch recombination to IgE (149,151) and for TGFβ to induce class switching to IgA (104). CD40 signalling also rescues germinal centre B cells from apoptosis (245-247) and may be critical for T cell B cell interactions during antibody affinity maturation and Ig class switching in germinal centres (55).

In previous work, designed to investigate the role of CD40 in IL4 dependent IgE production, the concentrations of IL4 used were always high (> 100 U/ml). Two independent dose effects of IL4 on immunoglobulin class production by EBV activated B cells have been examined in Chapter 5. In this Chapter, the effect of anti-CD40 monoclonal antibodies on EBV induced B cell proliferation and Ig isotype production was examined in the presence of IL4.

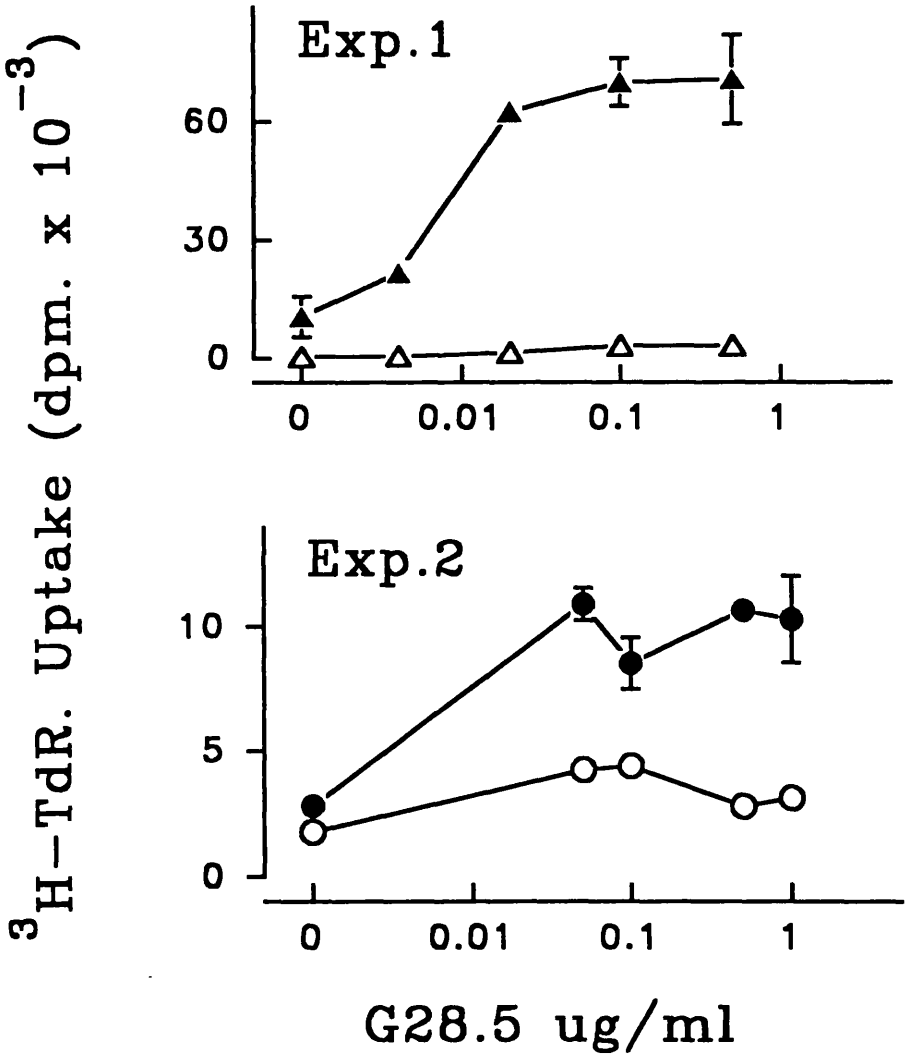
6.2. EFFECT OF G28.5, A MONOCLONAL ANTIBODY TO HUMAN CD40, ON EBV INDUCED B CELL PROLIFERATION AND IMMUNOGLOBULIN SECRETION.

Anti-CD40 antibodies in combination with a number of different co-activators deliver strong growth signals to human B cells resulting in proliferation, and in some cases, immunoglobulin production (15,149,433). Signalling through CD40 is also a second signal required for Ig heavy chain class switching (151,355). In this Section the effect of G28.5, a mouse mAb to human CD40 (80), on proliferation and immunoglobulin secretion by EBV stimulated B cells was investigated. G28.5 was titrated at concentrations between 0.005 and 1.0 ug/ml into cultures of EBV activated and unstimulated B cells. Proliferation was assayed on day 5 and immunoglobulin secretion was assayed on day 13.

G28.5 increased proliferation of EBV stimulated B cells in a dose dependent manner rising from levels obtained with EBV alone at 0.01 ug/ml to a maximum with 0.1-0.5 ug/ml. Concentrations of G28.5 greater than 0.5 ug/ml had no further effect. G28.5 alone did not induce proliferation of unstimulated B cells. Typical results from two experiments are shown in Figure 6.1.

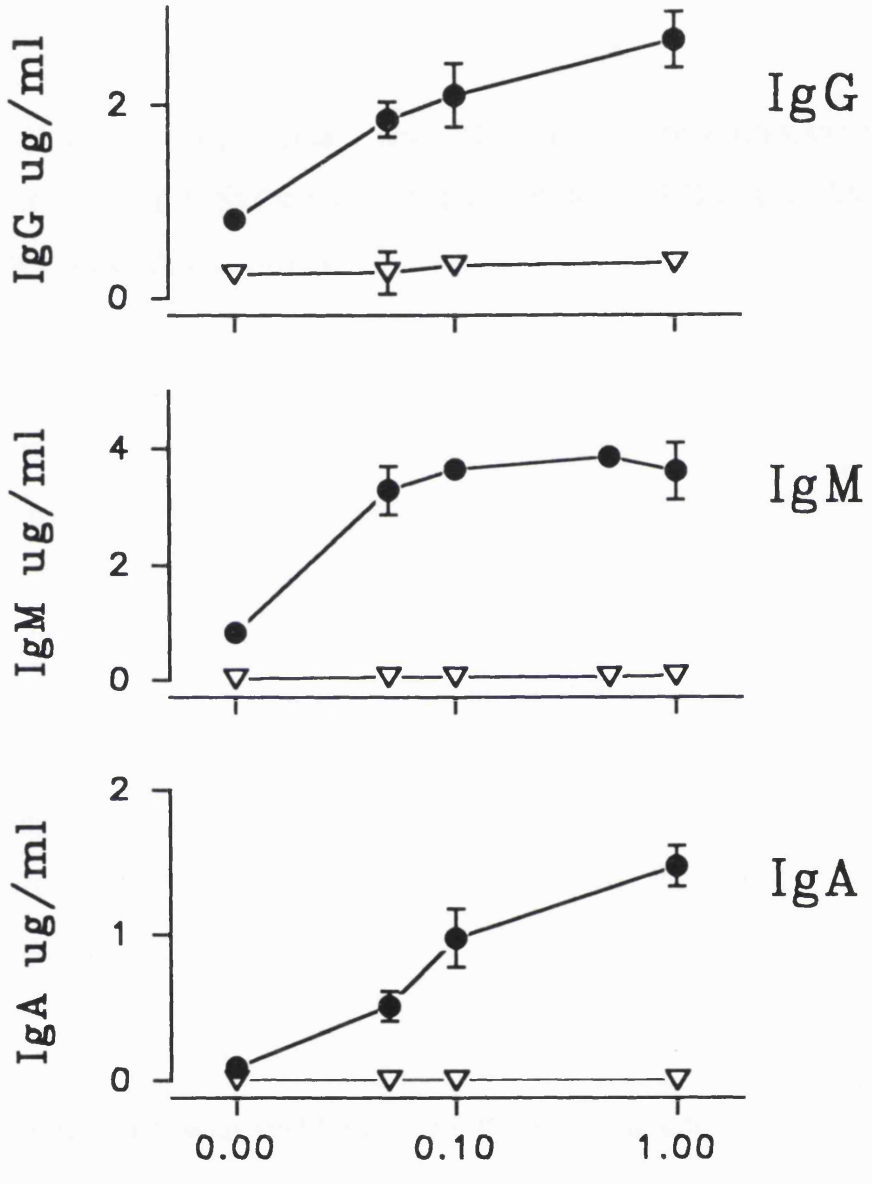
G28.5 also increased Ig secretion by EBV stimulated B cells in a dose dependent manner rising from levels obtained with EBV alone to a maximum with 0.1-0.5 ug/ml of G28.5. Concentrations of G28.5 greater than 0.5 ug/ml had no further effect. It was not possible to discern any effect of anti-CD40 on IgE production as IgE remained below levels of detection (< 0.5 ng/ml) in all experiments. G28.5 had no significant effect on immunoglobulin secretion of unstimulated B cells. Typical results for IgM, IgG and IgA are shown in Figure 6.2. G28.5 also

Figure 6.1.



Effect of G28.5 mAb on EBV Induced B Cell Proliferation.
Tonsillar B cells, activated with EBV (▲ ●) or incubated in medium alone (△ ○), were cultured with G28,5 mAb at concentrations from 0 to 1 ug/ml. B cell proliferation (^{3}H-TdR. uptake) was measured on day 5. Results from two experiments are shown.

Figure 6.2.



Effect of G28.5 mAb on EBV Induced B Cell Immunoglobulin Secretion.
Tonsillar B cells, activated with EBV (●) or incubated in medium alone (▽), were cultured with G28,5 mAb at concentrations from 0 to 1 ug/ml. B cell IgG, IgM and IgA secretion was measured on day 13 by ELISA.
Similar results were seen in 3 separate experiments.

increased the IgG subclasses, IgG1, IgG2, IgG3 and IgG4 in a similar dose dependent manner (results not shown).

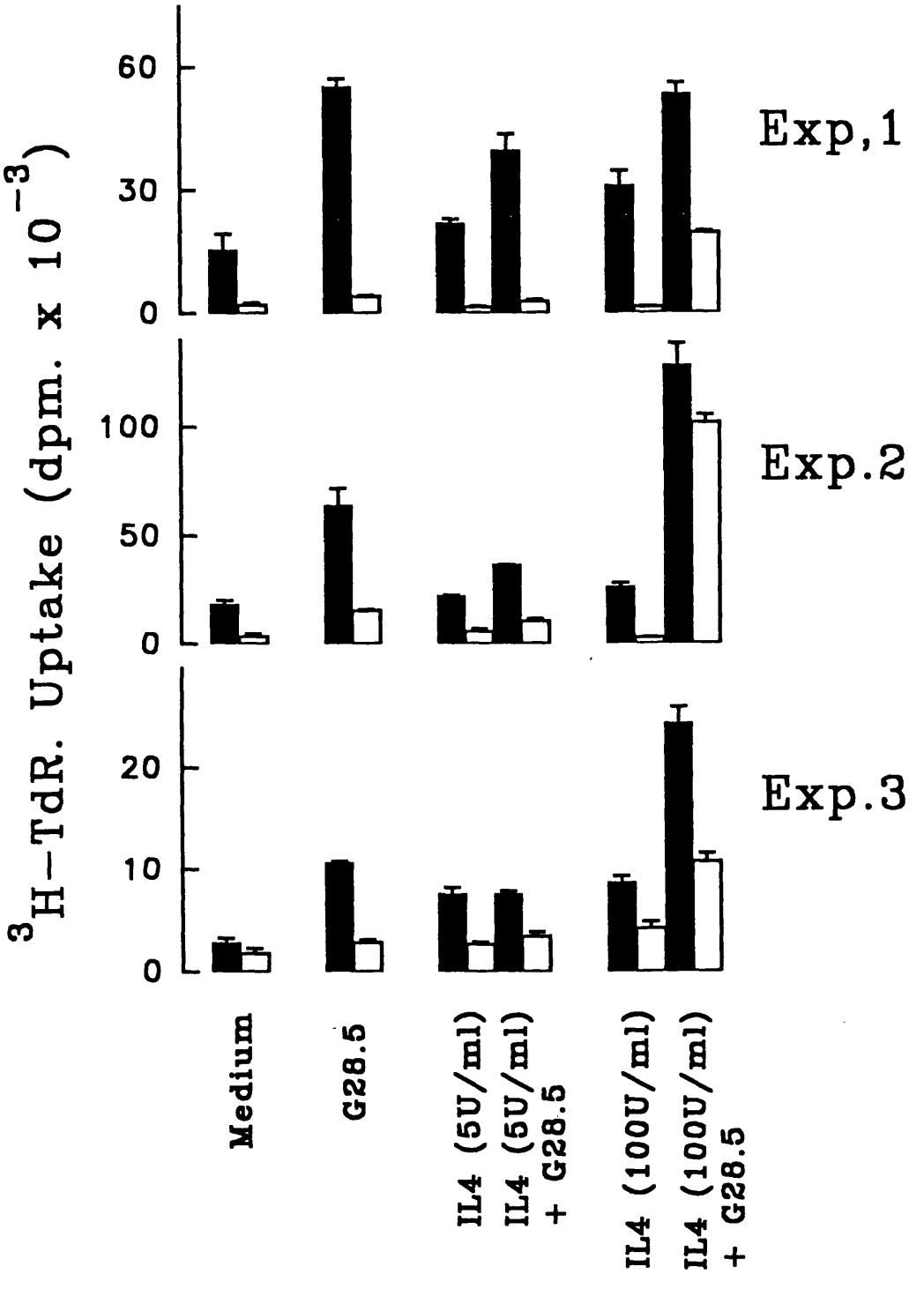
6.3. EFFECTS OF IL4 ON B CELL PROLIFERATION AND IMMUNOGLOBULIN SECRETION IN RESPONSE TO EBV AND ANTI-CD40 MONOCLONAL ANTIBODIES.

High concentrations of IL4 are known to co-stimulate with anti-CD40 to induce human B cell proliferation and production of IgE and IgG4 (149,195,433). In this Section the effects of high and low doses of IL4 on anti-CD40 and EBV induced B cell proliferation and Ig production was investigated. As established in Section 6.2., 0.5 ug/ml G28.5 induced both optimum immunoglobulin production and proliferation of EBV activated B cells. In this Section, G28.5 (0.5 ug/ml) was added at the start of culture to EBV activated and unstimulated B cells together with either high dose IL4 (100 U/ml), low dose IL4 (5 U/ml), or a titration of IL4 with doses from 0-200 U/ml. Proliferation was assayed on day 5 and immunoglobulin secretion was assayed on day 13.

6.3.1. Effect of IL4 on Proliferation by B Cells Stimulated with EBV and Anti-CD40 Monoclonal Antibodies.

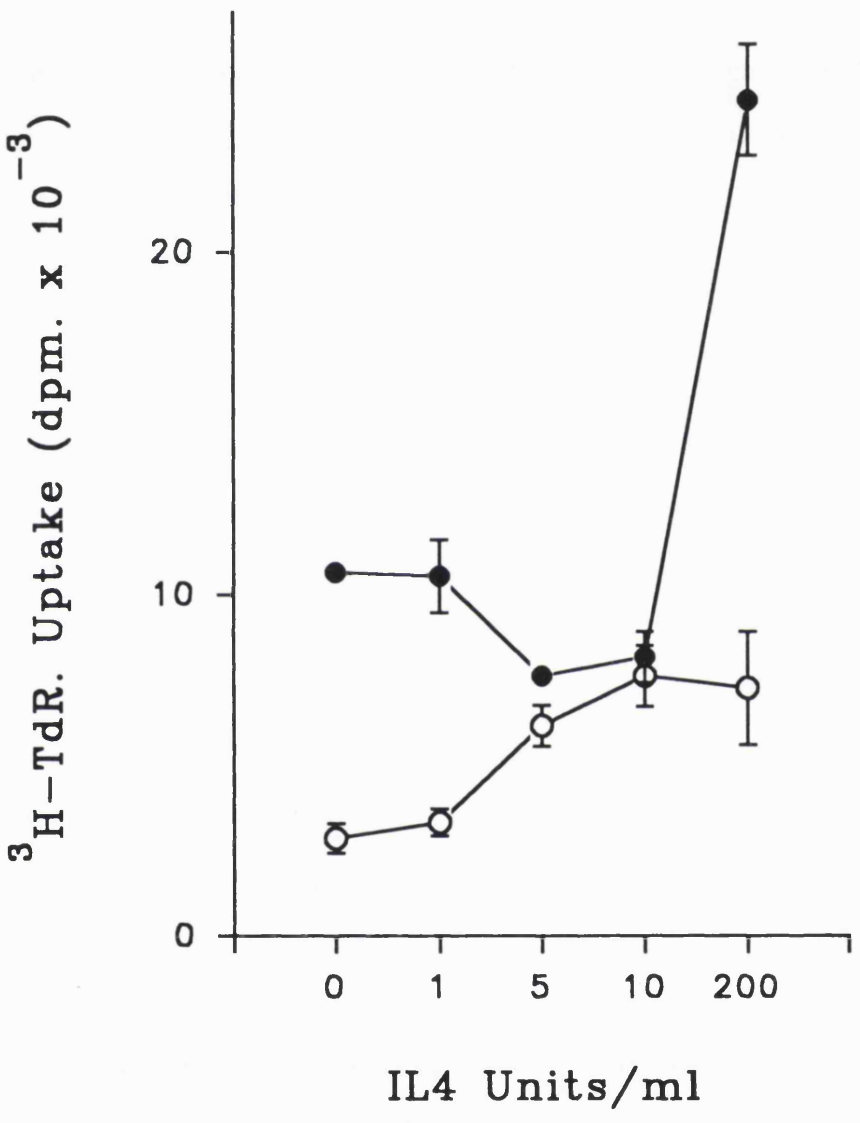
Proliferation of EBV treated B cells was significantly increased by G28.5 alone (Figure 6.3.). Addition of low (0.5 U/ml) and high dose IL4 (100 U/ml) to EBV treated B cells also increased proliferation but the growth signal delivered by IL4 was always much less than with G28.5. Results from three experiments are shown in Figure 6.3. When low dose IL4 and G28.5 were added together, proliferation, although increased above levels obtained with EBV alone, was always less than with

Figure 6.3.



Effect of IL4 on G28.5 Induced B Cell Proliferation.
Tonsillar B cells, activated with EBV (■) or incubated with a medium alone (□), were cultured with IL4 or G28.5 as indicated. Proliferation (^{3}H-TdR. uptake) was measured on day 5. Three separate experiments are shown.

Figure 6.4.



Effect of IL4 Titration on G28.5 Induced B Cell Proliferation.
Tonsillar B cells were activated with EBV and cultured with (●) or without (○) 0.5 ug/ml G28.5 mAb and IL4 at concentrations from 0-200 U/ml. Proliferation (^{3}H-TdR. uptake) was measured on day 5.
This experiment was repeated 3 times with similar results.

G28.5 alone, and in some experiments did not increase above levels obtained with EBV and low dose IL4. This result is consistent with inhibition of G28.5 induced proliferation by low dose IL4. In contrast, the addition of G28.5 and high dose IL4 together increased proliferation above that obtained with IL4 or G28.5 alone in 2/3 experiments and was the same in 1 experiment. There was also significant B cell proliferation in response to high dose IL4 and G28.5 in the absence of EBV. Similar synergy between G28.5 and high doses of IL4 has been reported by other groups in the absence of other B cell activators (401).

Titration of IL4 into cultures of EBV treated B cells increased proliferation in a dose dependent manner as seen in Chapter 5. G28.5 also increased proliferation and this response was always greater than the response by EBV activated B cells cultured with high dose IL4 alone. The addition of IL4 to B cells activated with EBV and G28.5 reduced proliferation in a dose dependent manner. Maximum inhibition occurred at about 5 U/ml IL4, where proliferation was reduced to levels obtained with IL4 alone. At concentrations of IL4 above 5 U/ml, the inhibitory effect was diminished until the response was dramatically increased with 100 U/ml IL4 or greater. Results from a typical experiment are shown in Figure 6.4.

6.3.2. Effect of IL4 on Immunoglobulin Production by B Cells Stimulated with EBV and Anti-CD40 Monoclonal Antibodies.

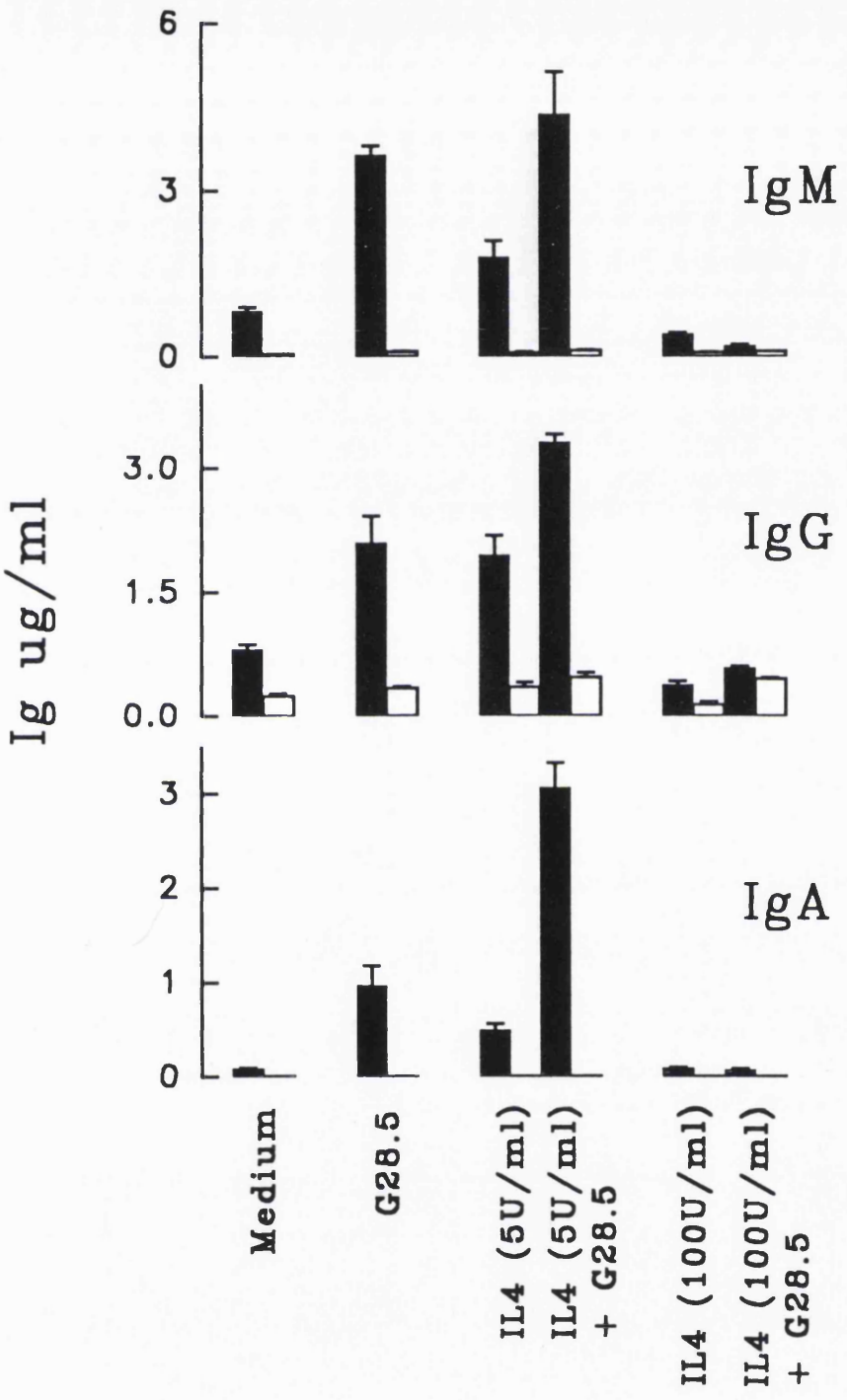
Immunoglobulin production by EBV activated B cells was greatly increased by either G28.5 (0.5 ug/ml) alone or low dose IL4 (5 U/ml) alone, but the effect of G28.5 was always greater than IL4 (Figure 6.5.). When low dose IL4 and G28.5 were added together, Ig secretion was increased above that obtained with either G28.5 or low dose IL4. In contrast, addition of high dose IL4 to B cells stimulated with EBV and G28.5 completely inhibited the increase of immunoglobulin secretion

below that obtained with G28.5 alone. Typical results for IgM, IgG and IgA are shown in Figure 6.5. IgE levels remained below the detection range of assay in all experiments (< 0.5 ng/ml). This result is similar to the inhibition of EBV induced Ig secretion with high dose IL4 (Figures 5.5. and 5.6.).

Titration of IL4 into cultures of EBV activated B cells in the absence of G28.5, increased Ig secretion optimally with low dose IL4 (5 U/ml), and inhibited Ig secretion below levels obtained with EBV alone at high doses of IL4 (>100 U/ml). G28.5 alone also increased immunoglobulin secretion by EBV activated B cells. G28.5 induced Ig secretion was increased further with low concentrations of IL4 (5 U/ml or less) but was inhibited with concentrations of IL4 greater than 100 U/ml. Typical results for IgM, IgG, IgA and IgG1 are shown in Figure 6.6. Results for IgG2 and IgG3 were similar (not shown). In unstimulated cultures, neither IL4 nor G28.5 alone or in combination had an effect on immunoglobulin production (results not shown).

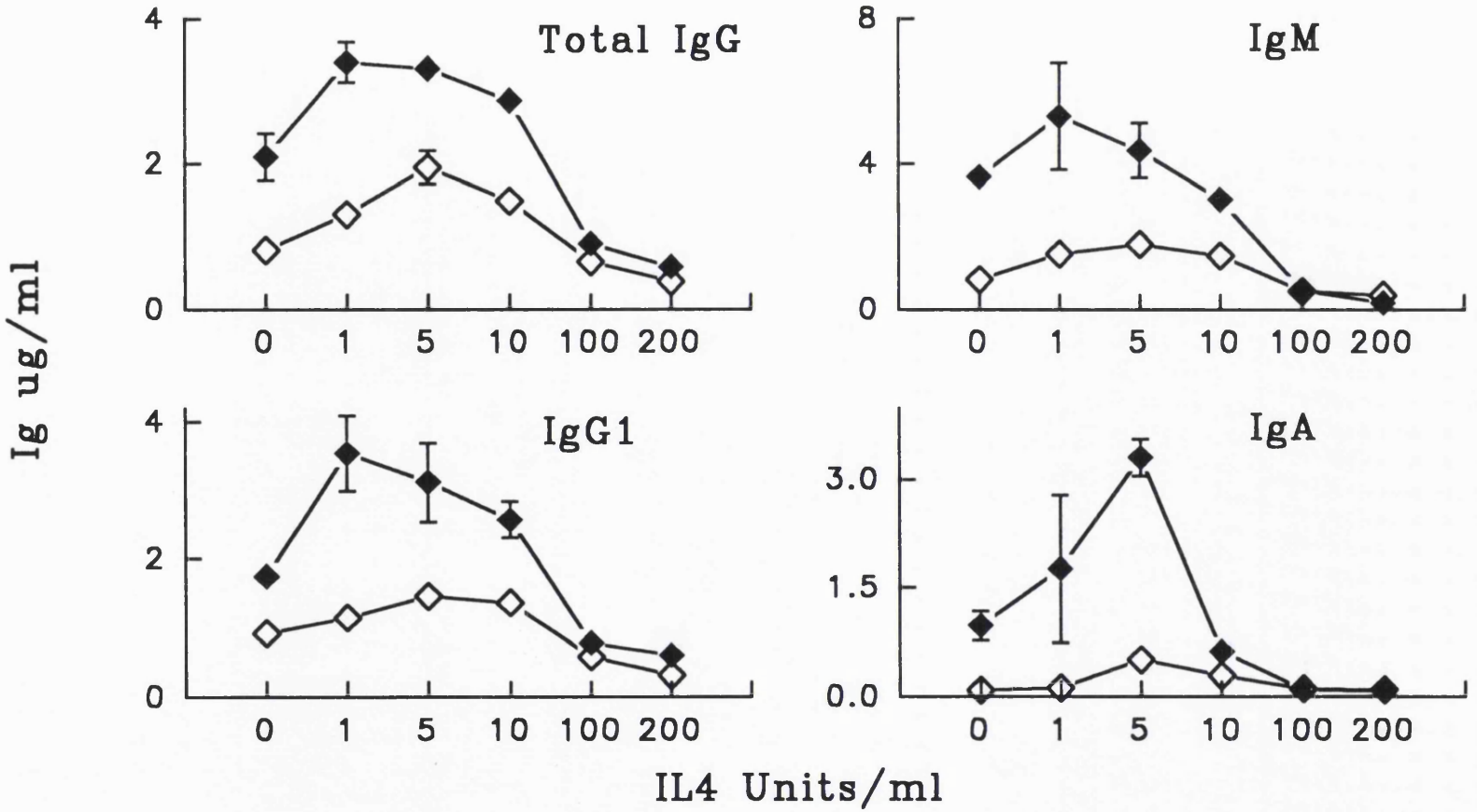
In these experiments no IgE was detected in supernatants of B cells stimulated with G28.5 and high dose (100 U/ml) IL4, even when B cells were cultured in IMDM.

Figure 6.5.



Effect of IL4 on G28.5 Induced B Cell Immunoglobulin Secretion.
Tonsillar B cells, activated with EBV (■) or incubated with a medium alone (□), were cultured with IL4 or G28.5 as indicated. IgM, IgG and IgA secretion was measured on day 13 by ELISA. *This experiment was repeated 3 times with similar results.*

Figure 6.6.



Effect of IL4 Titration on G28.5 Induced B Cell Immunoglobulin Secretion. Tonsillar B cells were activated with EBV then cultured with (◆) or without (◇) 0.5 ug/ml G28.5 mAb and IL4 at concentrations from 0–200 U/ml. Ig secretion was measured on day 13 by ELISA. This experiment was repeated 3 times with similar results.

6.4. DISCUSSION.

Anti-CD40 monoclonal antibodies alone cannot generally induce B cell proliferation, although anti-CD40 in the L-cell system is able to do so (15). Work with the CD40L has shown that the membrane bound recombinant molecule and soluble trimer are also able to induce B cell proliferation alone (4,55,237,377). The strength of CD40 signalling may be related to the degree of its cross linking by ligand. CD40 may be a homodimer in the B cell membrane (47,293) and thus may require a polyvalent ligand. This is demonstrated by work with anti-CD40 antibodies where whole Fc or $F(ab')_2$ but not monovalent Fab anti-CD40 co-stimulates human B cell proliferation (80). This cross linking requirement may be optimised by membrane bound CD40L, soluble CD40L trimer and membrane bound anti-CD40 in the mouse L-cell system. The signal given by stimulation with anti-CD40 L-cells and IL4 can induce prolonged B cell proliferation and is able to maintain cultures of normal B cells indefinitely (14).

Anti-CD40 mAbs, although unable to induce B cell proliferation alone, will induce B cell activation. This is characterised by an increase in cell volume, homotypic adhesion and increased ICAM-1 (CD54) expression (17,80,401). Anti-CD40 co-activates with IL4 (15,336,401), phorbol esters (80,242,302), anti-IgM (237,242,302,401) and anti-CD20 (242) to induce B cell proliferation.

Anti-CD40 antibodies also co-stimulate with cytokines to induce human Ig secretion. Anti-CD40 L-cells alone induce IgG, IgM and IgA secretion, and co-stimulate with IL4, IL2 or IL10 induce B cells to secrete IgE IgM, IgG and IgA (15,336,337). Other groups have reported that anti-CD40 and IL4 promote secretion of IgE (195,433,434), IgM, IgE, total IgG and IgG4 (149), IgM, IgG1, IgG2, IgG3, IgG4 and IgE (375). Anti-CD40 is known to induce IgE secretion with IL4 by supplying

the second signal needed for IL4 Ig heavy chain class switching to IgE (151,195,355,434) and possibly for IgG4 (148,149,203).

In recent years, it has become obvious that cytokines alone do not regulate the immune response. The role of functional B cell surface antigens in B cell responses such as growth, differentiation and maintenance of memory has become more and more apparent. Signalling through CD40 induces profound effects on human B cells which suggests that this signal may be very important for human B cell growth and differentiation. Previous work has shown that anti-CD40 antibodies co-stimulate with cytokines to enhance the production of Ig. In this Chapter anti-CD40 antibodies were used to stimulate B cells which had been polyclonally activated with EBV to investigate whether signalling through CD40 itself could promote specific Ig class or IgG subclass secretion.

Anti-CD40 Increases Proliferation and Antibody Production by EBV Activated B Cells.

The results described in this Chapter confirmed that the cross-linking of CD40 in combination with another B cell activation signal (EBV) induces impressive B cell proliferation. Doses of anti-CD40 (0.5 ug/ml), which optimally increased EBV induced B cell proliferation, were similar to doses which induced B cell proliferation with phorbol esters and ionomycin (164), phorbol esters alone (80,242,302), anti-IgM (237,242,302,401) anti-CD20 (242) and IL4 (401). Anti-CD40 also greatly enhanced EBV induced IgG, IgM, IgA, IgG1, IgG2, IgG3 and IgG4, but not IgE, secretion. There was no evidence for preferential induction of individual Ig isotypes. These results were comparable to those of Banchereau and co-workers (15) where anti-CD40 L-cells were shown to stimulate human B cell IgG, IgA and IgM production in the absence of cytokines.

Anti-CD40 increased EBV induced B cell proliferation and Ig secretion at similar doses. This suggested that anti-CD40 signalling may enhance Ig secretion by expanding EBV activated precursors thus increasing the number of Ig secreting B cells *per se*, rather than acting as a late acting BCDF like IL6. The absence of IgE may have been due to low numbers of IgE precursors in the original B cell population. Although IgE was not increased in EBV activated B cell cultures, anti-CD40 is known to co-activate with IL4 to induce Ig heavy chain class switching to IgE and promote IgE secretion (151,355).

IL4 Modulates Anti-CD40 BCGF and BCDF Activity

It was shown in Chapter 5 that low and high doses of IL4 have quite distinct effects on EBV induced Ig secretion. Low dose IL4 (5 U/ml) enhances secretion of IgM, IgA, IgG1, IgG2 and IgG3 with no effect on IgE or IgG4, whereas high dose IL4 (100 U/ml) inhibits or has no effect on secretion of IgM, IgA, IgG1, IgG2 and IgG3 but increases IgE and IgG4. These two doses also have distinct effects on the BCGF and BCDF activity of anti-CD40 on EBV activated B cells. The considerable increase in IgM, IgA, IgG1, IgG2 and IgG3 secretion induced by anti-CD40 is enhanced still further by low dose IL4. In contrast, high dose IL4 completely abrogates the increase obtained with anti-CD40 and inhibits secretion of these isotypes back to, or below levels seen with EBV alone. The increase of Ig secretion obtained with anti-CD40 and low dose IL4 has not been previously reported, but the inhibition of the increase of Ig secretion with high dose IL4 is comparable to the findings of Zhang *et al* (433) who found that anti-CD40 and IL4 had no effect on IgG, IgM or IgA but selectively increased IgE secretion by PBMC. These results suggest that low doses of IL4 enhance anti-CD40 BCDF activity for IgM, IgA,

IgG1, IgG2 and IgG3 whereas high doses of IL4 required for IgE production inhibit the other Ig isotypes.

High doses of IL4 (100 U/ml) increased EBV induced B cell proliferation but this effect was always less than the BCGF activity of anti-CD40 mAbs. High dose IL4 enhanced proliferation in response to EBV and CD40 mAb. Interestingly, high doses of IL4 increased expression of the CD40 antigen on EBV activated B cells (results not shown). This upregulation of CD40 by IL4 has been noted by other groups (36,401) and the increase in anti-CD40 binding sites may contribute to the magnitude of the proliferation response seen with IL4. The increased proliferation seen with high dose IL4 and anti-CD40 was similar to the findings of other groups (15,336,401). In contrast to the high dose IL4 effects, low dose IL4 (5 U/ml) inhibited proliferation of B cells stimulated with EBV and anti-CD40 mAbs. The inhibition with low dose IL4 has not been previously reported.

Collectively, these results indicate a reciprocal relationship between low and high dose IL4 and anti-CD40 induced Ig secretion and proliferation. This reciprocal effect is only seen with B cells co-stimulated with EBV and anti-CD40 mAbs and is not observed with B cells stimulated with EBV alone. Low dose IL4 (which has been proposed to signal through a high affinity IL4R) enhances Ig production but inhibits proliferation. In contrast, high dose IL4 (which has been proposed to signal through a low affinity IL4 receptor) inhibits Ig production but enhances proliferation. It would be predicted from the results obtained in Chapter 5, that high doses of IL4 should enhance IgE and IgG4 secretion which would be increased further with anti-CD40. Anti-CD40 in other systems acts as a second signal for IL4 induced Ig class switching to IgE and IgG4 (149,151,195,355,433). In my experiments IL4 did not increase IgE secretion. This may have been a tonsil variation problem but was not followed up further.

Signalling through CD40/CD40L is critical *in vivo* for T cell/B cell interactions required for formation of B cell germinal centres, somatic mutation, Ig heavy chain class switching and rescue of high affinity antibody bearing B cells during an immune response. The importance of this interaction is highlighted by HIGM1 syndrome in humans, where loss or defective expression of the CD40L prevents the formation of B cell germinal centres and B cell Ig heavy chain class switching from IgM to IgG, IgA or IgE (55). Ig heavy chain class switching to IgE by HIGM1 B cells can be restored with CD40L and IL4 *in vitro* (55,231). This indicates that Ig heavy chain class switching *in vivo* is achieved by T cell/B cell CD40/CD40L interaction and secretion of appropriate cytokines such as IL4 (6) and TGFβ (104) *in situ*. In the mouse anti-CD40L antibodies ablate 1^{o} and 2^{o} immune responses *in vivo* showing that CD40L interactions are critical for T cell dependent cognate B cell interaction (293). *In vitro*, the cross linking of CD40 and the antigen receptor rescues centrocytes (somatically mutated, switched, Ig^{+} GC B cells) from apoptosis (245-247). CD40L and CD40 L-cells can also promote secretion of IgM, IgA, IgG1, IgG2, IgG3 and IgG4 in combination with IL2 and IL10 (15,55,267,336,377). This indicates that CD40 signalling is important for the survival of switched activated B cells as well as the initial cognate interaction with non activated virgin $IgM^{+}D^{+}$ or memory B cells for germinal centre formation and class switching.

It is hypothesised that the increase in proliferation and inhibition in Ig secretion seen in this Chapter with high dose IL4 and anti-CD40 is comparable to CD40/CD40L, IL4/low affinity IL4R interaction in the T cell zones of the germinal centre (81). IL4 released by activated T cells would be maintained at high concentration in the close microenvironment between cells and bind to a low affinity IL4R receptor on B cells. Low affinity receptor signalling, together with the signal transduced through CD40, induces rapid proliferation and induction of specific Ig heavy chain class switching, but prevents non specific IgM, IgA, IgG1, IgG2 and IgG3 secretion. On the other

hand, low concentrations of IL4 and anti-CD40 which induce Ig secretion but inhibit proliferation, might occur in the periphery, away from the microenvironment of the germinal centre, at sites of infection where rapid Ig secretion, and not B cell proliferation, is required from activated B cells.

It has recently been shown by Gascan *et al* (152) that human mast cells and basophils also express the CD40L and can induce Ig heavy chain class switching of B cells to IgE with IL4. The presence of CD40L on other cell types in the periphery in addition to T cells adds credence to the proposition that CD40 signalling and cytokines are important for B cell Ig production outside the germinal centre.

CHAPTER 7

CONTROL OF EBV INDUCED IMMUNOGLOBULIN PRODUCTION BY LIGATION OF THE B CELL SURFACE ANTIGEN CD23

7.1. INTRODUCTION

CD23 or FcεRII is a type II integral membrane protein of 45kDa and a member of the animal C-type lectin family (91). The membrane bound CD23 molecule also undergoes autocatylitic cleavage to produce sCD23, a multi-functional cytokine (110,112,245). sCD23 and CD23 are thought to specifically regulate human IgE production (8,42,306,345,356,428). Two natural ligands have been identified for CD23 and sCD23, IgE and CD21. Binding of IgE to CD23 is involved in B cell antigen focusing and presentation (210,284,309,402) and may inhibit IL4 induced IgE production as a negative feed back mechanism (251). CD21/CD23 binding may be important in B cell homotypic adhesion (38,91) and CD21/sCD23 binding is thought to be involved in rescue of germinal B cells from apoptosis (41) and IgE regulation (8).

Previous work has shown that anti-CD23 antibodies specifically inhibit IL4 dependent IgE production. However, B cell responses were examined in unseparated PBMC or TMC, or in the presence of activated T cells. Further, B cells were not polyclonally activated and the concentrations of IL4 used were always high (> 100 U/ml) (42,305,306,308,346). The aim of this Chapter is to examine the effects of anti-CD23 antibodies on proliferation and Ig secretion by purified human B cells activated with EBV, and to look at their effects on proliferation and Ig production activated with EBV and low dose IL4 (5 U/ml) and high dose IL4 (100 U/ml).

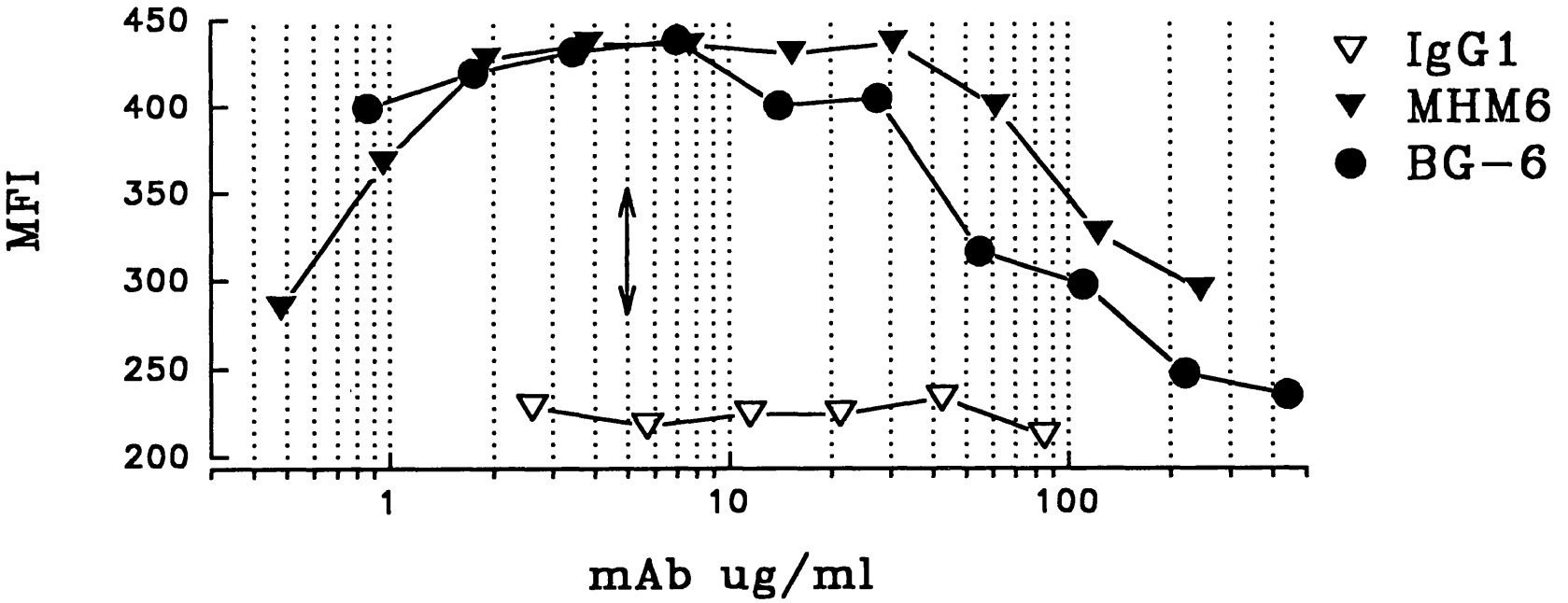
7.2. EFFECT OF LIGATION OF CD23 ON HUMAN B CELLS POLYCLONALLY ACTIVATED WITH EBV IN THE PRESENCE OF HIGH AND LOW DOSES OF IL4.

Some monoclonal antibodies to the B cell surface antigen CD23 are able to induce biological activity. Binding to CD23 can induce B cell proliferation in combination with phorbol ester (166,167) and inhibit IL4 induced IgE production (42,306,345,356). The effects of anti-CD23 antibodies on human Ig production are thought to be IgE specific, but effects of these antibodies on the other Ig isotypes have not been studied in detail. In this Section, the effect of two different monoclonal anti-CD23 antibodies, MHM6 and B-G6, on B cell proliferation and Ig production when stimulated by EBV and EBV plus IL4 were investigated.

7.2.1. Saturation Binding of Anti-CD23 Monoclonal Antibodies.

Two mouse IgG1 monoclonal antibodies to the human CD23 (FcεRII) antigen, MHM6 and B-G6 and a control mouse IgG1 mAb against the fluorophor FITC were titrated for their optimal binding efficiency to human B cells. Cells from an in house B cell lymphoblastoid cell line, KF, (100% $CD23^+$, $CD19^+$) were stained and prepared for FACS analysis and the results were expressed as the mean fluorescence intensity (MFI) calculated by Lysis II software (Figure 7.1.). Optimum binding concentrations were determined for MHM6 and B-G6 as 5 ug/ml. A mouse IgG1 anti-FITC mAb, with no discernible human B cell binding activity on FACS analysis, was used as a negative control for Fc binding (Figure 7.1.).

Figure 7.1.



Binding of Anti-CD23 Monoclonal Antibodies to the B-Lymphoblastoid Cell Line KF.
KF cells were incubated with mouse anti-human CD23 clones MHM6 (▼) and BG-6 (●) at concentrations from 0-400 ug/ml, compared with a control mouse IgG1 mAb (▽). Optimal binding for MHM6 and B-G6 was obtained at 5 ug/ml (arrow).

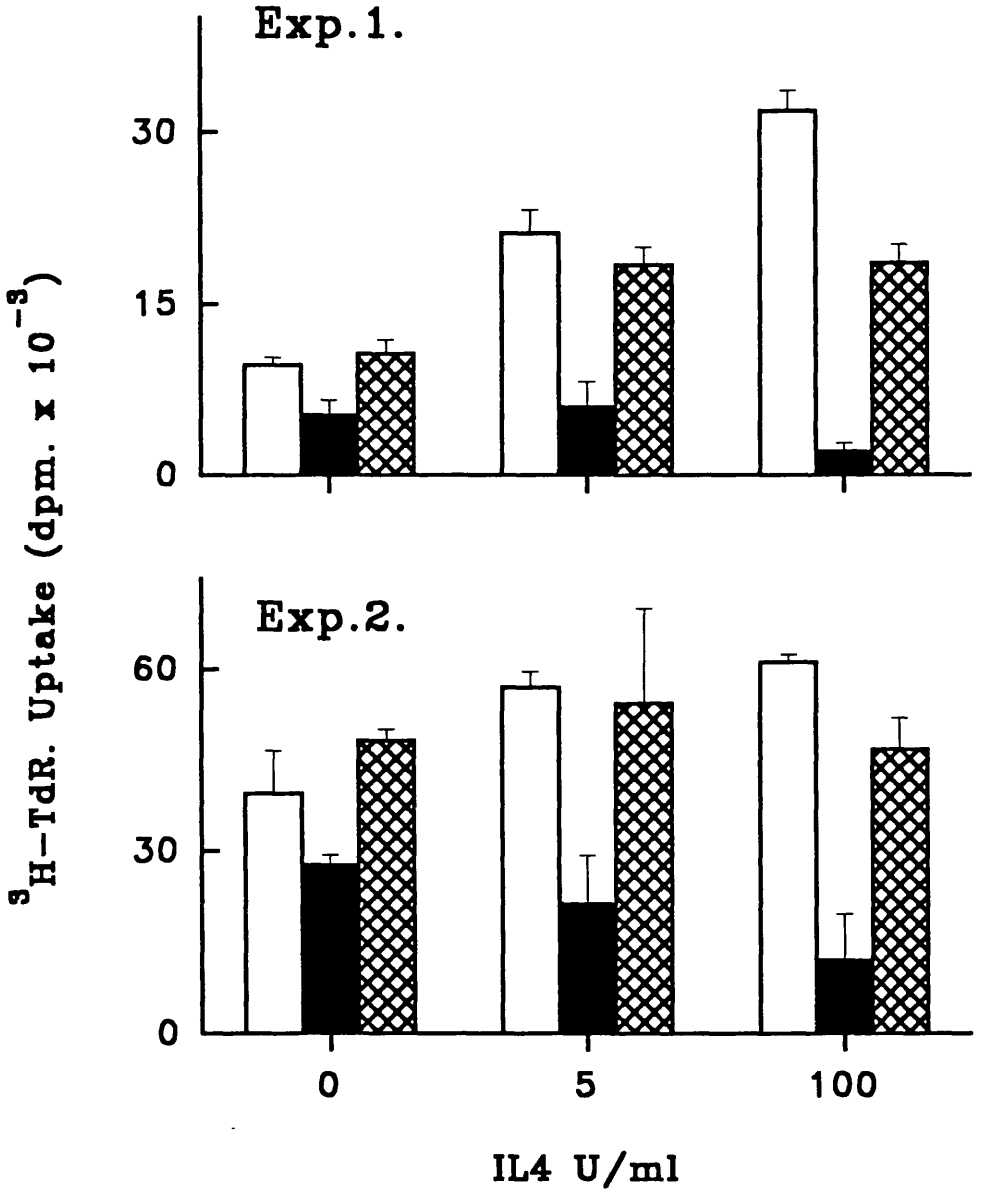
7.2.2. Effect of Anti-CD23 on B Cell Proliferation.

The anti-CD23 mAb MHM6 inhibited EBV induced proliferation by 29-44 % (range of 3 experiments). In contrast, the mAb B-G6 had no inhibitory effect, and in some experiments proliferation was slightly increased. In contrast, proliferation of EBV activated B cells cultured with low dose IL4 (5 U/ml) was inhibited by both antibodies. MHM6 reduced proliferation by 62-71% whereas B-G6 inhibited by only 5-13% (range of 3 experiments). High dose IL4 (100 U/ml) optimally increased EBV induced proliferation, and this was again inhibited by both antibodies: MHM6 by 80-93% and B-G6 by 40-42% (range of 3 experiments). Results from two experiments are shown in Figure 7.2. Interestingly, the inhibition by both anti-CD23 mAbs was greater with increasing concentrations of IL4. The mAb B-G6 was clearly less potent than MHM6, even though both antibodies were used at concentrations which allowed maximal binding to CD23. Proliferation by non stimulated B cells was also reduced in a similar fashion in some experiments (results not shown).

7.2.3. Effect of Anti-CD23 on B Cell Immunoglobulin Production.

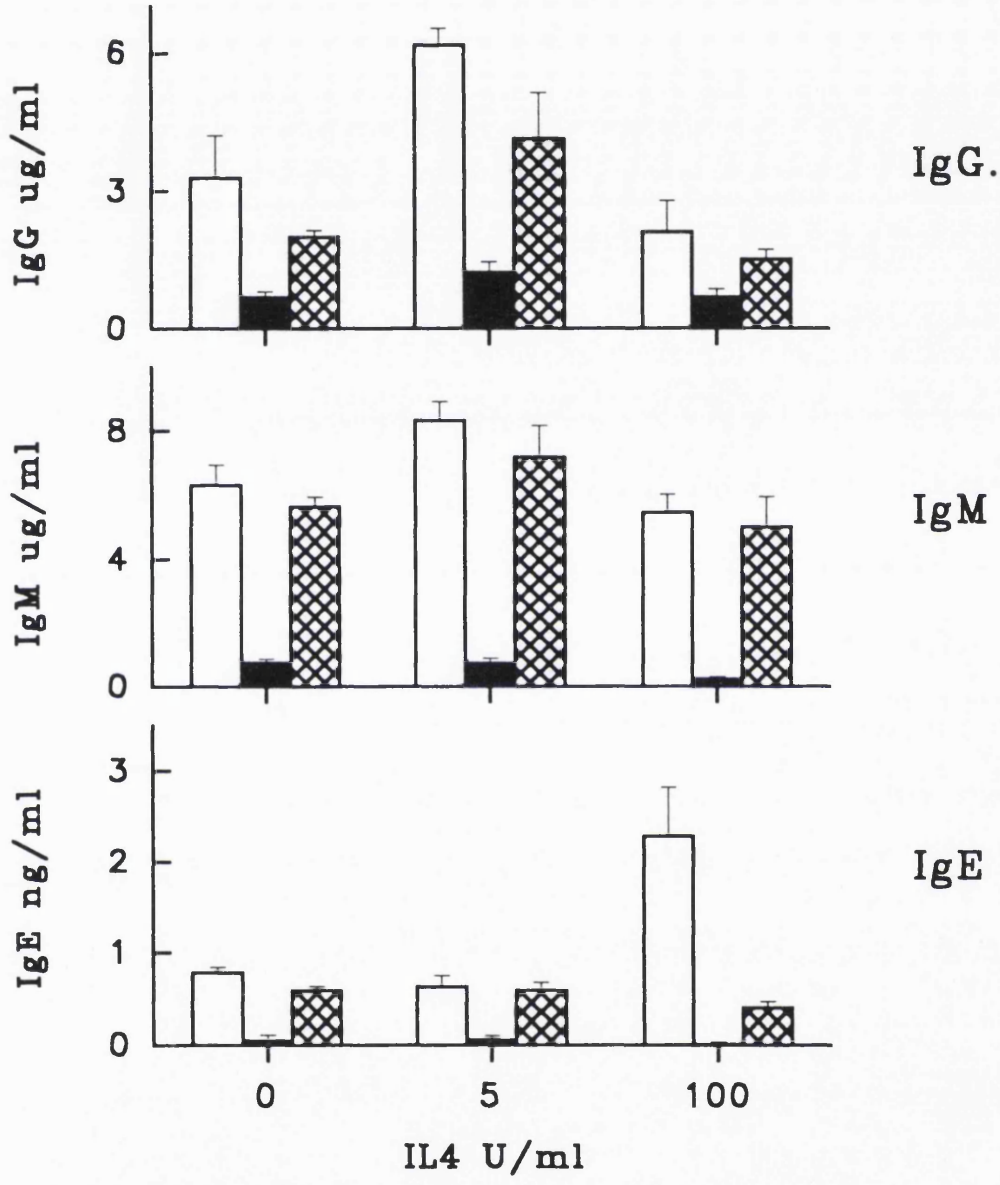
Very interesting results were obtained with anti-CD23 and EBV activated B cells. Firstly, although both antibodies were able to inhibit Ig secretion, MHM6 was again by far the most effective. Both antibodies reduced EBV induced IgM, IgG (Figure 7.3.) and IgA secretion (results not shown). MHM6 inhibited by 60-90% and B-G6 inhibited by 14-22% (range of 3 experiments). In one experiment background levels of IgE present in the EBV activated cultures were also reduced further by MHM6 but not B-G6 (Figure 7.3.).

Figure 7.2.



Effect of Anti-CD23 Monoclonal Antibodies on EBV and IL4 Induced B Cell Proliferation. Tonsillar B cells were activated with EBV and cultured with 5 ug/ml mouse anti-human CD23 mAbs MHM6 (■) or B-G6 (▩) in the presence of 0, 5 or 100 U/ml IL4. A mouse anti-FITC IgG1 mAb (□) was used as a control. Proliferation (^{3}H-TdR. uptake was measured on day 5. Results from two experiments are shown.

Figure 7.3.



Effect of Anti-CD23 Monoclonal Antibodies on EBV and IL4 Induced B Cell Ig Production. Tonsillar B cells were activated with EBV and cultured with 5 ug/ml of mouse anti-human CD23 mAbs MHM6 (■) or B-G6 (▩) in the presence of 0, 5 or 100 U/ml IL4. A mouse anti-FITC IgG1 mAb was used as a control (□). Ig production was measured on day 13 by ELISA. This experiment was repeated 2 times with similar results.

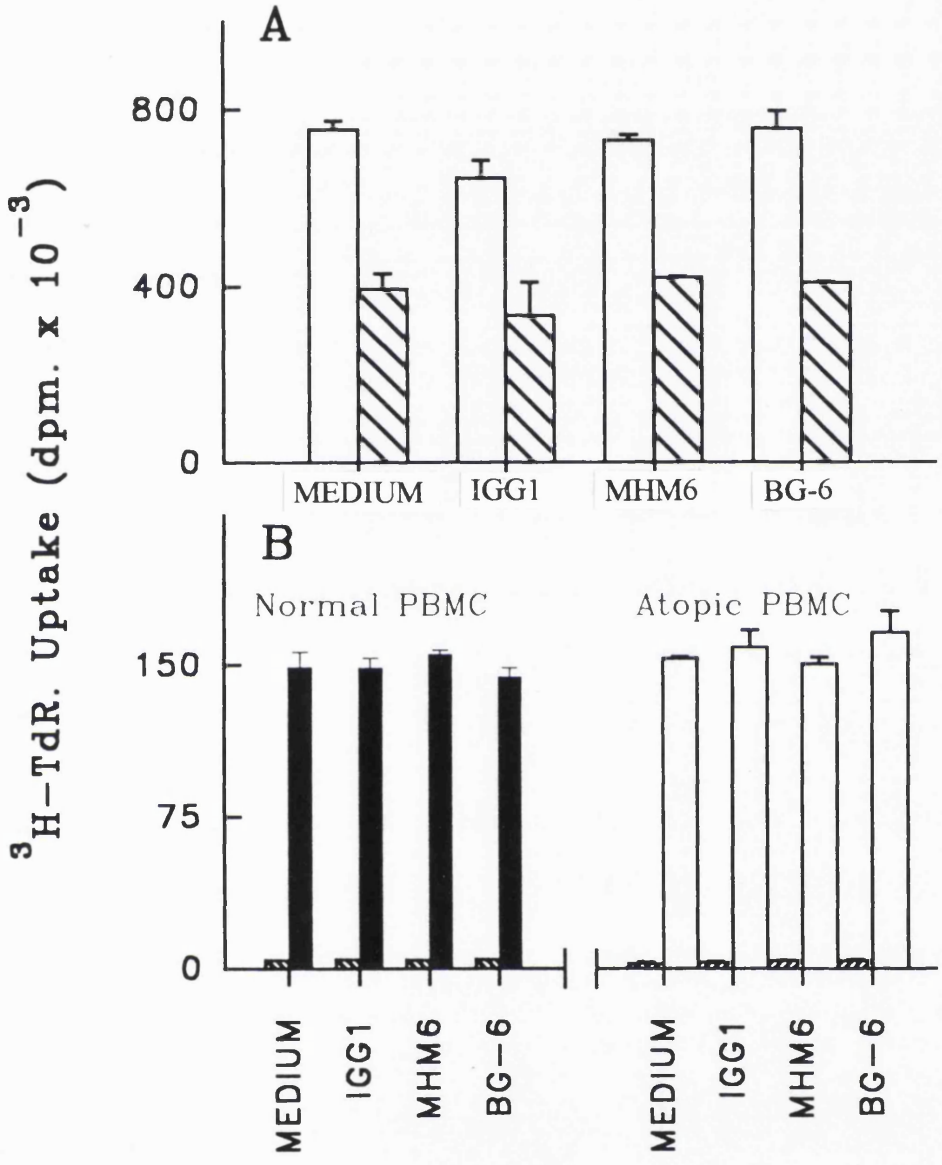
The addition of 5 U/ml IL4 to EBV activated cultures increased IgM, IgG (Figure 7.3.) and IgA (results not shown) but not IgE production. Both MHM6 and B-G6 inhibited Ig secretion obtained with low dose IL4 by 70-90% and 14-22% respectively (range of 3 experiments), an overall decrease similar to that obtained with anti-CD23 and EBV alone.

In cultures of EBV activated B cells with 100 U/ml of IL4, IgG, IgM and IgA (results not shown) production was reduced back to levels obtained with EBV alone and IgE production was increased (Figure 7.3.). Interestingly, IgG, IgM and IgA production was again reduced by MHM6 (70-90%) and B-G6 (10-35%) with a magnitude similar to that obtained with anti-CD23 and EBV alone. IgE production however was dramatically inhibited by both anti-CD23 antibodies.

7.2.4. Determination of Toxicity of Anti-CD23 Antibodies.

The anti-CD23 mAbs used in this Section had quite profound effects on B cell proliferation and Ig secretion induced by EBV and EBV plus IL4. In theory, this could be explained by non specific toxicity in the mAb preparations, and not the biological effects exerted by the antibodies themselves. To eliminate this possibility, the antibodies were tested in two non B cell activation systems: proliferation of the human leukaemic T-cell line Jurkat ($CD3^+$, $CD23^-$) and PHA responses by PBMC from normal and atopic donors. As shown in Figure 7.4. MHM6, B-G6 and the IgG1 control mAb had no effect in the activation systems tested. The results seen in Sections 7.2.2. and 7.2.3. were not, therefore, due to toxicity.

Figure 7.4.



Non Specific Toxicity Testing of Anti-CD23 mAbs in B Cell Independent Activation Systems. A:Proliferation of Jurkat cells seeded at 2.5×10^6 (□) and 1.25×10^6 (▨) cells/ml. B: Proliferation of normal PBMCs with (■) and without (▨) PHA, and atopic PBMCs with (□) and without (▨) PHA. Proliferation was assayed on day 3 after stimulation.

7.3. DISCUSSION.

MAb binding to human CD23 on B cells can have inhibitory and stimulatory effects. Some mAbs are able to signal through CD23 and induce PIP_2 hydrolysis leading to Ca^{2+} mobilisation from intracellular stores and increased cAMP as well as tyrosine phosphorylation of intracellular proteins (228,229). The known differences between activities of anti-CD23 antibodies may be due to the binding of different epitopes on the CD23 molecule. Such epitopes may be close to binding sites for natural ligands or the degradative enzymes which catalyse sCD23 release. IgE and CD21 are two natural ligands for CD23 and both stimulate B cells. IgE-immune complex binding to membrane bound CD23 potentiates antigen uptake and presentation (140,309) and inhibits IgE production by IL4 activated PBMC, TMC (42,109,112) and the IgE secreting plasmacytoma U266 (356). It also inhibits IL4 plus anti-IgM or SAC induced B cell proliferation (251). CD21-CD23 interaction is involved in the homotypic adhesion of normal B cells and some B cells lines (37). CD21 may also be a functional receptor for sCD23 (8) which induces proliferation of phorbol ester (159) and anti-IgM (110) activated B cells, and enhances IL4 induced IgE production (8,112,346).

Anti-CD23 Inhibition of B Cell Proliferation.

MHM6 inhibited B cell proliferation induced by EBV alone, EBV plus low dose IL4 and EBV plus high dose IL4 (Figure 7.2.). These results were similar to those of other groups who have shown that anti-CD23 inhibits SAC plus IL4, anti-IgM plus IL4 (416) and anti-CD40 plus IL4 (37) induced B cell proliferation. Interestingly, inhibition of proliferation with high dose IL4 was greater than with low dose IL4 or with EBV alone (Figure 7.2.) and may be related to IL4 induced increase in CD23 expression (99,327).

Anti-CD23 mAbs could inhibit B cell proliferation in two ways. By binding to the membrane bound CD23 molecule and transducing a negative signal or by sequestering sCD23, either by preventing its cleavage from the cell surface or preventing its binding to a natural ligand.

It was noted that in all experiments, the mAb B-G6 was less effective at inhibiting proliferation than MHM6. Although B-G6 and MHM6 bind to the same number of B cells with similar intensity (Figure 7.1.), the difference in functional activity may be related to the epitopes on CD23 to which these antibodies bind. MHM6 is known to induce a greater Ca^{2+} signal through CD23 than other anti-CD23 antibodies (229). This may explain why MHM6 is able to inhibit proliferation of EBV and EBV plus low dose IL4 induced proliferation where levels of CD23 are low (data not shown), as well as EBV plus high dose IL4 induced proliferation, were CD23 expression is optimal (data not shown and (99,327)). B-G6 may bind to a site which signals ineffectively and requires optimal CD23 expression in order to transduce a signal of sufficient magnitude to inhibit proliferation. In the scenario of sCD23 driven proliferation, MHM6 could either prevent release of sCD23 more effectively than B-G6, or sterically hinder the binding of sCD23 to a natural ligand better than B-G6.

Anti-CD23 Inhibition of Ig Production.

Anti-CD23 inhibition of IgE production has been shown by many groups, but these have nearly always involved direct stimulation of PBMC or TMC with IL4 in the absence of B cell activators (8,42,305,306,308,346). This has led to the idea that CD23 is a specific regulator of IL4 induced IgE production and synthesis. It is known however that IL4 induced CD23 is expressed on many B cells, not all of

which will produce IgE. In addition, anti-CD23 inhibits polyclonal B cell proliferation (37,251) suggesting its effects are not restricted to IgE^+ B cells. To date the action of anti-CD23 mAbs have not been tested on secretion of IgE and other Ig isotypes in the same activation system (8,42,306,345). The results in this Chapter show that anti-CD23 not only inhibits IL4 induced IgE production (Figure 7.3.), but secretion of IgA, IgM, IgG1, IgG2 and IgG3 induced by EBV or EBV plus low dose IL4 (IgG4 was not assayed). Although Luo *et al* (251) showed that anti-CD23 inhibits SAC plus IL4 induced IgM secretion, inhibition of IgA and IgG subclasses has not been previously reported.

Both anti-CD23 antibodies (MHM6 and B-G6) inhibited EBV and EBV plus low dose IL4 induced B cell Ig secretion, but MHM6 was more effective than B-G6. However, although B-G6 reduced IgG, IgM and IgA to a much lesser extent than MHM6, both antibodies completely inhibited IgE production by B cells stimulated with EBV and high dose IL4. This suggests that the mechanism used by high dose IL4 to induce IgE production may be different than that of low dose IL4 induced Ig secretion. High dose IL4 responses may involve sCD23 (8,76,346) while the mechanism used by low dose IL4 is unknown.

Antibody binding to CD23 on B cells does not specifically inhibit IgE production, but under the correct conditions has the potential to inhibit all Ig class and IgG subclass production, as well as B cell proliferation. CD23 is an important molecule in B cell interactions. The inhibitory results obtained in this Section are similar to the effects of IgE-immune complex binding to CD23. Cross-linked IgE signals through CD23 (229), and inhibits B cell proliferation (251,356) as well as IL4 induced IgE production by PBMC and TMC (42,91,109,112). IgE-immune complexes which bind to CD23 are important for Ag focusing and presentation by B cells to T cells (140,309). Antigen presenting B cells would not be required to proliferate or produce Ig. Anti-CD23 antibodies which inhibit B cell responses may

be acting in a similar way to IgE-immune complexes, inhibiting proliferation and antibody production of a diverse B cell population, in favour of Ag presentation.

CHAPTER 8

GENERAL DISCUSSION

8.1. DO CYTOKINES EXCLUSIVELY CONTROL HUMAN Ig CLASS AND IgG SUBCLASS PRODUCTION ?

Some cytokines are able to specifically promote production of individual immunoglobulin isotypes by either clonally expanding pre-committed B cells or inducing Ig heavy chain class switching. In the mouse, each Ig class and IgG subclass is controlled by at least one recognised cytokine. IgA is induced by TGFβ (241) and IL5 (262), IgE and IgG1 by IL4 (127,334,367), IgG2a and IgG3 by IFNγ (369,371) and IgG2b by TGFβ (264,370). In the human, IL4 and IL13 induce IgE and IgG4 (9,149,191,250,314), TGFβ increases IgA (192,407) and NGF also induces IgG4 production (216). Cytokines which control the human IgG subclasses, IgG1, IgG2 and IgG3 have not yet been identified.

It has been suggested already that B cell activation systems used in the past did not provide optimal conditions for the study of cytokine control of human B cell Ig production. Such systems were often T cell dependent, induced production of specific Ig classes themselves, or targeted B cell subsets. The T cell-independent EBV activation system used here, stimulated polyclonal B cell proliferation and differentiation of B cells into IgM, IgA, IgG1, IgG2, IgG3 and IgG4 AFCs. This system was used to reappraise the action of the recombinant cytokines IL2, IL4, IL5, IL6, TGFβ, IFNγ and IFNα, on Ig class and IgG subclass regulation.

IL2 and IL6 both have BCDF activity and the potential to increase secretion of all human Ig classes and IgG subclasses. No evidence was found to indicate that either cytokine was able to selectively induce different Ig class or IgG subclass production. This non Ig class specific BCDF activity of IL2 and IL6 may be important during an immune response *in vivo*. Production of IL2 by activated T cells during a specific immune response induces T cell growth (236), but also has the potential to increase antigen specific B cell clones by promoting B cell proliferation and

differentiation into AFCs. IL6, a pluripotent cytokine and produced by many cell types during an immune response (56,178,405) is also produced by B cells on activation through their CD40 antigen (423). This suggests IL6 may be important when produced *in situ* during B cell/T cell interaction as an autocrine BCDF.

High concentrations of TGFβ (10 U/ml) inhibited all Ig class and IgG subclass production by EBV activated B cells but IgA production was specifically increased by low doses (0.2 U/ml TGFβ). This result was similar to those described previously for the mouse (88,372) and human (104,208,209). TGFβ promotes wound healing and tissue repair by suppressing T cell proliferation and the inflammatory immune response (184,415). These T cell and B cell suppressive actions make it difficult to see why TGFβ promotes Ig class switching. Perhaps the class switching activity induced by low concentrations of TGFβ may be involved early in an immune response to promote IgA responses in the mucosal surfaces of the gut, lung and upper respiratory tract or at sites of injury. Its inhibitory function may be required late, when inflammation and mediator release caused by T cells and antibody immune complexes must be suppressed to promote wound healing.

IFNγ is now known to regulate two mouse IgG subclasses, IgG2a and IgG3 (369,371) therefore the results obtained with IFNγ and IFNα on EBV activated human B cells were disappointing. Neither cytokine had any significant effect on B cell proliferation or Ig production. In the case of IFNγ, this was unlikely to be due to the loss of IFNγ receptors on EBV activated B cells because IFNγ inhibited IL4 induced CD23 expression on EBV activated B cells (results not shown). The lack of effect of IFNγ on human B cell Ig production may be due to evolutionary divergence in IFNγ function between mice and humans. Mouse and human IFNγ have only 40% homology and have no cross species reactivity (96,130). In the human, the primary role of IFNγ may be in promotion of the inflammatory and

cellular immune response by inhibiting Th2 cell responses (130) and suppression of humoral immune response promoting cytokines (75).

Among the group of cytokines studied, IL4 has given new and interesting insights into human Ig class and IgG subclass regulation. High concentrations of IL4 induced IgE and IgG4 consistent with the results of others (149,191,250), but low concentrations of IL4 increased IgM, IgA, IgG1, IgG2 and IgG3 production with no effect on IgE or IgG4. This shows that there are two distinct and separate effects of IL4 on human Ig regulation which are concentration dependent. Low dose effects of IL4 on Ig production have not been previously reported, and could reflect IL4 signalling through a high affinity receptor. High dose IL4 promotion of IgE and IgG4 secretion could likewise reflect the down stream effects of IL4 signalling through a low affinity receptor. These results and the evidence for two IL4 receptors are discussed in more detail below.

Although human IgA, IgE and IgG4 production is controlled by cytokines, the signals required for promotion of the other human IgG subclasses have remained unknown. The cytokines which induce murine IgG subclasses have no similar activities on human B cells. This is not entirely surprising as human and mouse IgG subclasses classes arose independently in both species (60). It is also likely that the mechanisms regulating the IgG subclasses in the two species are also dissimilar. Human IgG subclasses arose by duplication of precursor Cγ, Cε and Cα genes which resulted not only in the creation of IgG subclasses, but also an extra human IgA subclass and a pseudo Cε gene which are not present in the mouse (Figures 1.6a. and 1.6b.). Human and mouse IgG subclasses also have dissimilar structures and effector functions *in vivo*. For example, murine IgG3 is protective against carbohydrate antigens and human IgG3 is protective against protein antigens.

How does the human immune system regulate IgG subclass expression and what are the roles of cytokines? It is beyond doubt that Ig heavy chain class switching to IgG subclasses takes place in the human. Molecular evidence for a directed IgG class switch program can seen by looking at IgG secreting B cells and B cells lines (248,420). Each human IgG subclass gene has its own distinct switch region (128,175), and IgG secreting B cells have undergone switch site recombination and deletion of intervening DNA (190). *In vivo*, specific IgG subclasses are consistently produced in response to particular antigens. For example: IgG1 is produced against bacterial proteins (172,364) and IgG2 is produced against bacterial polysaccharides (172); IgG1 and IgG3 are produced against viral infections (364); IgG1, IgG3 and IgG4 are produced against parasite derived proteins (70) and IgG2 is produced against parasite derived polysaccharides (70). These repeated patterns of IgG subclass production must be specifically regulated.

The lack of evidence for cytokine regulation of IgG could be due to several reasons:

1) The cytokines which regulate human IgG subclasses may not yet have been characterised. New human cytokines are being discovered and characterised with increasing rapidity in today's advanced technology. The new cytokine IL13, which is a human Ig class switch factor with properties similar to IL4, illustrates this case very well (272,314).

2) Signals for human Ig heavy chain class switching to IgG1, IgG2 and IgG3 may be mediated through functional cell surface antigens during cell/cell contact rather than cytokines, possibly during antigen specific cognate T cell/B cell interaction.

3) IgG subclass production may not be controlled by cytokines but by antibody affinity mechanisms. The affinities of mAbs against rheumatoid factor (146), LPS carbohydrate side chains (350) and bacterial polysaccharide antigens (92) are

considerably increased when one IgG subclass heavy chain is exchanged for another. In the germinal centres, B cells with antibody molecules bearing the wrong IgG subclass heavy chain could bind to antigen ineffectively and be lost by apoptosis. Those bearing antibodies with Ig heavy chains which increase the affinity of the antibody would be rescued from apoptosis and go on to provide high affinity specific IgG for the secondary immune response.

4) Immunoglobulin V regions may pre-determine the IgG subclass of antibody produced by the activated B cell. In the human, the immune system utilises particular V region families for protection against allergens (404). This could also be the case for proteins and carbohydrate antigens. These V regions may be linked with those IgG subclass heavy chains which confer protection.

8.2. IL4: A CYTOKINE OPERATING THROUGH TWO RECEPTORS?

The results in this Thesis have shown that IL4 has two distinct effects on human B cell Ig production which are dependent on IL4 concentration. High dose (100 U/ml or greater) IL4 specifically increased IgE and IgG4 production by B cells activated with EBV, with no increase or inhibition of the other Ig isotypes (Figures 5.5. and 5.6.). In contrast, low dose IL4 increased EBV induced IgM, IgA, IgG1, IgG2 and IgG3 production but had no effect on IgE or IgG4 secretion (Figures 5.5. and 5.6.). It was proposed that these two distinct effects of IL4 were mediated by two separate IL4 receptors: High dose IL4 acting through a low affinity receptor, and low dose IL4 acting through a high affinity receptor. Results with $mIgM^+$ B cells and IL4 in Chapter 5 (Tables 5.5. and 5.6.) and the findings of other groups (149,191,250,355) suggest that the high dose increase of IgE and IgG4 is by Ig heavy chain class switching. In our experiments low dose IL4 acted as a BCDF for $mIgM^+$ B cells and induced IgM secretion. However, IL4 also enhanced IgA, IgG1, IgG2 and IgG3

secretion by mIgM$^+$ and to a lesser extent by mIgM$^-$, suggesting that low dose IL4 may act as a switch factor and also as a BCDF. Preliminary studies with HIGM1 B cells have indicated that low dose IL4 acts only as a BCDF. It is proposed that low dose IL4 signalling through a high affinity receptor induces differentiation of mIgM$^+$, mIgA$^+$, mIgG1$^+$, mIgG2$^+$ and mIgG3$^+$ B cells into IgM, IgA, IgG1, IgG2 and IgG3 AFCs and that high dose IL4 signalling through a low affinity receptor induces Ig class switching to IgE and IgG4.

The results seen in Chapter 5 and the results of others (327) provide functional evidence for the existence of two IL4 receptors. An IL4 receptor called gp140, has been cloned and structurally characterised in both the mouse (280) and human (147). It is a member of the type I cytokine receptor family and forms a high affinity receptor for IL4 when associated with the IL2R γ-chain (340). A low affinity IL4 binding protein (IL4bp) with a mw. of 65-85 kDa has also been identified on pre-B cell lines which is not recognised by mAbs against gp140 (7,129). IL4bp, purified from supernatants of the pre-B cell line JM1, blocks the high dose IL4 induced increase of CD23 on PBMCs and IL4 induced proliferation of the JM1 cell line. Antibodies, raised against an N-terminal 12 amino acid peptide of the IL4bp, bind to human pre-B cells but not gp140 transfected chronic T cell lymphocytic leukaemia (CTLL) (7). This is evidence that in addition to gp140, IL4 binds to a low affinity receptor which is unrelated to gp140, and which can transduce IL4 dependent signals to B cells.

A new cytokine, IL13, shares some, but not all, functions with IL4. The human IL13 gene is on Ch5q23-q21 and thought to be another product of the gene duplication which gave rise to IL5, GM-CSF, IL3 and IL4. The amino acid sequences of IL4 and IL13 have only about 30% sequence homology but their secondary and tertiary protein structures are extremely similar. Both have 4 α-helices and 2 β-pleated sheets, and IL13 has 2 disulphide bonds in positions similar

to those within IL4 (436). The IL13 receptor (IL13R) has not been identified but may share common subunits with an IL4 receptor.

The functional effects of IL13 on B cells and B cell lines have so far been similar to those induced by IL4. IL13 increases CD23, MHC class II, CD71, and CD72 expression (102,314), co-stimulates with anti-Ig, anti CD40 and CD40L to induce proliferation (48,102,435) and increases IgM, total IgG, IgG4 and IgE by B cells activated with CD40L (85), T cells (102,265,314) and anti-CD40 L-cells (48). IL13 increases IgE and IgG4 production by Ig heavy chain class switching (9,149,314). However, unlike IL4, IL13 has no observed activity on T cells (272,435) suggesting that T cells do not carry the IL13R. The effects of IL13 on human B cell Ig production are very similar to the effects of high dose IL4 seen in Chapter 5. It is possible that IL13 and IL4 may either share the same low affinity receptor, or a functional receptor component, which is able to transduce the same signal to human B cells.

A mutant IL4 protein, IL4Y124D has been made, which has a Tyr124-Asp substitution at the final amino acid of the 4th α-helix domain of IL4. This mutation allows IL4Y124D to bind to the IL4 receptor with an affinity similar to IL4, but without signal transduction (233). Studies with IL4Y124D show that the IL4 and IL13 receptors share a common component which is not the IL6R signal transducer gp130 or the IL5R transducer KH97 (436) and may not be the IL2R γ-chain (9). IL4Y124D is a competitive inhibitor of TF-1 cell (human pre-myeloid cell line) responses to IL4 and IL13 (436), and blocks proliferation and IgE and IgG4 production by normal B cells induced by anti-CD40 and IL4 (9), or anti-CD40 and IL13 (9). IL4Y124D therefore competes with both cytokines for a receptor on TF-1 and B cells.

IL2 and IL4 also share a structural homology even though their amino acid sequences are unrelated (19). IL2 and IL4 have similar core bundle hydrophobic amino acids and are highly α-helical. This structural homology is also shared by other cytokines which bind to cytokine receptor type I molecules (19,116,299,311). The IL2R γ-chain is a functional component of the gp140 IL4 receptor and increases its binding affinity for IL4 (340). This structural homology and the shared IL2R γ-chain signalling component may account for the similar BCDF activities of IL2 and low dose IL4. It is interesting to note that the IL7R which also has the γ-chain as a functional component (295) is also a human pre-B cell and mature B cell growth and differentiation factor (57,101,202,422). Antibodies to the γ-chain inhibit IL2 binding and IL4 induced growth of CTLL cells (230), whereas antibodies to the proposed low affinity IL4R (IL4bp) do not (7). This may suggest that only a high affinity IL4 receptor is present on T cells whereas both a high affinity and a low affinity IL4 receptor may exist on B cells.

What is the purpose of two receptors for IL4? In this Thesis low doses of IL4 significantly enhance Ig secretion by EBV activated B cells. Other groups have reported that low doses of IL4 optimally increase IgM expression on B cells and increase B cell antigen presenting ability to T cells (327). IL4 is not B cell specific, but a pluripotent cytokine which acts on many other immune cells. It is a thymocyte and mature T cell growth factor (13,374), it induces proliferation of activated NK cells (290), induces the expression of adhesion molecules on endothelial cells (176,225) and increases CD23 on monocytes (301), Langerhan's cells (32) and eosinophils (391). The low dose activity of IL4 on B cells may have a role similar to IL6 and be part of a general enhancement of the immune response.

High dose IL4 induction of IgE and IgG4 by B cells may be a specific and antigen directed response which takes place in the germinal centre microenvironment or in close association with antigen primed T cells. B cell/activated T cell contact would

lead to high concentrations of IL4 released from T cells at the B cell surface where it could bind directly to low affinity receptors. IL4 signalling in combination with CD40/CD40L signalling would induce rapid B cell proliferation and Ig heavy chain class switching prior to centrocyte formation and selection in the germinal centre.

The overlapping BCDF activities of IL4 and IL2 and other cytokines which bind to the cytokine receptor type I family suggest that these cytokines derive from an ancestral molecule. The function of the precursor IL4 molecule may have been as a general Ig promoting factor and general potentiator of the humoral immune response. Later, with the evolution of different Ig classes and IgG subclasses, IL4 may have developed an IgE and IgG regulatory activity. This additional activity could have come about through the appearance of another cell surface molecule which could bind IL4 and transduce signals to induce Ig heavy chain class switching. This puts forward the possibility that the major function of IL4 *in vivo* may be through a high affinity receptor which transduces BCDF activity while IL13, which has no apparent BCDF or T cell activating activity, takes precedence in IgE regulation.

Finally, when gp140 was cloned, three additional proteins were also found to bind IL4, or were closely associated with gp140 (147). These three unknown proteins were smaller than gp140, with molecular weights of 65-75 kDa, 60-65 kDa and 45 kDa. The two higher molecular weight proteins were present in the pre-myeloid cell line TF-1 but all three proteins were detected in the B cell line Raji. These proteins may represent components of the high and low affinity IL4 receptors. It is interesting to note that the two larger proteins have molecular weights similar to the IL2R γ-chain and IL4bp.

8.3. FUTURE WORK.

It has been proposed that IL4 signals through both high and low affinity receptors on EBV activated B cells and induces either IgM, IgA, IgG1, IgG2 and IgG3 production or IgG4 and IgE production respectively. This hypothesis could be tested by using recently available antibodies to the high affinity IL4R (gp140) and the low affinity IL4bp. It would be predicted that anti-gp140 would block low dose IL4 induced IgM, IgA, IgG1, IgG2, and IgG3 production, while anti-IL4bp would specifically block high dose IL4 induced IgE and IgG4

IL13, which has some but not all activities in common with IL4 could also be a valuable tool in determining the nature of the IL4 receptor(s) and their functional effects on B cells. It would be hypothesised that IL13 signalling through a low affinity IL4 receptor, or low affinity IL4 receptor component on B cells, would mimic the effects induced by high dose IL4 on EBV activated B cells. IL13 would induce IgE and IgG4 but not IgM, IgA, IgG1, IgG2 and IgG3 secretion.

Likewise, antibodies against the IL2R γ-chain could also be used to examine functional effects transduced through the high affinity IL4 receptor. If the low dose BCDF activity of IL4 is mediated through the high affinity IL4 receptor (of which the IL2R γ-chain is a functional component), it would be predicted that anti-IL2R γ-chain antibodies would block IL4 induced IgM, IgA, IgG1, IgG2 and IgG3 but not IgE and IgG4 secretion.

Signalling to B cells through the CD40 antigen is important for normal B cell function. The early expression of CD40 in B cell ontogeny indicates that it is important for antigen independent B cell development as well as regulation of mature B lymphocyte functions (398). CD40 is now recognised as one of the pivotal molecules of the humoral immune response and critical for human B cell Ig class

switching *in vivo* (55), but TNFα/TNFαR interaction may also be involved in normal human B cell Ig class and IgG subclass regulation.

Infection of T cells with human immunodeficiency virus (HIV) *in vivo* inhibits the cellular immune response (252,270) but there is also intense polyclonal activation of B cells leading to hypergammaglobulinaemia and B cell malignancies. HIV infected $CD4^+$ T cells have no CD40L expression but a subset express membrane bound TNFα, a protein sequence and structural homologue of CD40L (293). These cells induce B cell Ig production *in vitro* by binding to B cell TNFαRs (252) which are protein sequence and structural homologues of CD40 (257). *In vitro*, the 25kDa membrane TNFα on activated T cells co-stimulates with IL4 to induce Ig heavy chain class switching to IgE and IgG4 (10). TNFα/TNFαR is a CD40/CD40L-like interaction which is also involved in Ig heavy chain class switching. It would be interesting to study the effects of ligation of B cell TNFαR in the presence of IL4 and other cytokines in the EBV system with special note taken of its effects on production of the IgG subclasses.

In Chapter 6 is was shown that anti-CD23 antibodies do not specifically inhibit IL4 induced IgE production, but can inhibit EBV and low dose IL4 the BCDF activity by inhibiting IgM, IgA and IgG secretion. If the inhibitory effects of anti-CD23 antibodies on IL4 induced Ig secretion are not restricted to IgE, anti-CD23 may also inhibit the BCDF activity of other cytokines. In this Thesis IL2 and IL6 were found to increase Ig secretion in a similar way to low doses of IL4. It would be predicted that anti-CD23 would inhibit IL2 and IL6 induced Ig production in a similar way to low dose IL4.

Finally, although the mechanism by which anti-CD23 inhibits EBV and IL4 induced responses is unknown, this may involve the CD19/CD21/TAPA-1 B cell multi-

complex (46, 260, 261) which associates with mIgM and HLA-DR molecules in the B cell membrane (132, 348). Cross-linking of CD21, a CD23 ligand and integral part of this B cell signalling complex, lowers the threshold for B cell activation through mIgM (67, 68, 348). CD19/CD21/TAPA-1 complex binding to a counter complex (which could involve CD23), induces B cell adhesion and allows B cells to respond to very low concentrations of antigen (132, 261, 348). The CD19/CD21/TAPA-1 complex itself is thought to be required during cellular interactions and to support specific B cell signals. If anti-CD23 antibodies inhibit CD19/CD21/TAPA-1 interaction with a CD23 counter complex, then anti-CD19 and anti-CD21 antibodies might also inhibit EBV and IL4 induced proliferation and Ig secretion. This would provide further evidence that CD23 is not a molecule exclusively involved in IL4 dependent B cell responses.

APPENDIX I: ABBREVIATIONS

ψ: pseudo gene

Å: Ångstrom unit, 10^{-8}m

Ag: antigen

Anti-Ig: anti-immunoglobulin

AFC: antibody forming cell

APC: antigen presenting cell

BCDF : B cell differentiation factor

BCGF : B cell growth factor

BLCL(s): B cell lymphoblastoid cell line(s)

bp: base pair

BSA: Bovine serum albumin

BSF: B cell stimulatory factor

BSS: buffered salt solution

CD: Cluster determinant

CDR1, CDR2, CDR3: complementarity determining regions

CFU: colony forming unit

CH 1-4: Ig heavy chain constant domains 1-4

CHO: carbohydrate

CL: Ig light chain constant domain

CLL: chronic lymphocytic leukemia

Cμ: μ constant region genes of Ig heavy chain

CTLL: chronic T cell lymphocytic leukaemia

D: Diversity

EBNA: Epstein Barr Virus nuclear antigen

EBV: Epstein Barr Virus

Fab: fragment antigen binding

Fc: fragment crystalline

FcαR: IgA Fc receptor

FcεRI: high affinity IgE Fc receptor

FcεRII: Low affinity IgE Fc receptor

FcγRI, FcγRII, FcγRIII: IgG Fc receptors I, II and III

FCS: foetal calf serum

FDC: follicular dendritic cell

G-CSF: granulocyte colony stimulating factor

GC: Germinal centre

GM-CSF: Granulocyte macrophage colony stimulating factor

GPI: glycosylphosphatidyl inositol

HLA: human histocompatability leukocyte antigen

IFN: Interferon

Ig: immunoglobulin

IL: Interleukin

J: Joining

kDa: kilo Dalton

KLH: Keyhole Limpet haemocyanin

LPS: lipopolysaccharide

M-CSF: myeloid colony stimulating factor

MFI: mean fluorescence intensity

MHC: major histocompatabilty complex

mIg: membrane bound Ig

MNC: mononuclear cells

mAb: monoclonal antibody

mRNA: messenger ribonucleic acid

NGF: nerve growth factor

NGFR: nerve growth factor receptor

NK: natural killer cell

OD: optical density

PBL: peripheral blood lymphocytes

PBMC: peripheral blood mononuclear cells

PIP_2: phosphoinositol bis phosphate

PBS: phosphate buffered saline

$PDBu_2$: phorbol-12-13-dibutyrate

PHA: phytohaemagglutinin

PKC: protein kinase C

PWM: Pokeweed Mitogen

PMA: phorbol-12-myristate-13-acetate

S, Sμ: Switch region, μ switch region

S-S: di-sulphide bond

SAC: *Staphylococcus aureaus* Cowan I (Pansorbin)

SMC: splenic mononuclear cells

sCD23: soluble CD23

SG: specific gravity

sIgM: secreted IgM

SRBC: sheep red blood cells

Tc: cytotoxic T cell

TCDF: T cell differentiation factor

TCGF: T cell growth factor

Th: helper T cell

TK: tyrosine kinase

TMC: Tonsillar mononuclear cell

TNF: tumour necrosis factor

TNFR: tumour necrosis factor receptor

TPA: 12-O-tetradecanoyl-phorbol-13-acetate

TRF: T cell replacing factor

TcR: T cell receptor (CD3)

TRAP: TNF related protein

Ts: suppressor T cell

V: variable

VH: varible domain of Ig heavy chain

VL: variable domain of Ig light chain

APPENDIX II: COMPANY ADDRESSES.

Aldrich Chemical Company Ltd., The Old Brickyard, New Road, Gillingham, Dorset, SP8 4JL.
Amersham International Plc., Amersham Place, Little Chalfont, Amersham, Bucks, HP7 9NA.
BDH: Merck Ltd., Merck House, Poole, Dorset, BH15 1TD.
Becton Dickinson UK. Ltd., Between Towns Road, Cowley, Oxford, OX4 3LY.
Bio-rad Laboratories Ltd., Caxton Way, Watford Business Park, Watford, Hertfordshire, U.K., WD1 8RP.
The Binding Site Ltd., University of Birmingham Research Unit, 97 Vincent Drive, Edgbaston, Birmingham, B15 2SQ.
Boehringer Mannheim U.K., Bell Lane, Lewes, East Sussex, BN7 1LG.
British Biotechnology Ltd., Watlington Road, Cowley, Oxford, OX4 5LY.
Coulter Immunology, 440 West 20 Street, Hialeah, Florida, 33010, USA.
Dako Ltd., 16 Manor Courtyard, Hughenden Ave, High Wycombe, Bucks, HP13 5RE.
Difco Laboratories Ltd., P.O. Box 14B, Central Ave, West Molesey, Surrey, U.K., KT8 2SE.
Dynatech Laboratories Limited, Daux Road, Billingshurst, West Sussex, RH14 9SJ.
Falcon., supplied by; Marathon Laboratory Supplies, Unit 6, 55-57 Park Royal Road, London, NW10 7LP.
Fisons PLC., Bishop Meadow Road, Loughborough, Leicestershire, LE11 0RG.
Flow Laboratories Ltd., Woodcock Hill, Harefield Road, Rickmansworth, Herts, WD3 1PQ.
Gelman Sciences, Brackmills Business Park, Caswell Road, Northampton, NN4 OE2.
Genzyme Diagnostics, 50 Gibson Drive, Kings Hill, West Malling, Kent, ME19 6HG.
Gibco, P.O. Box 35, 3 Washington Road, Paisley, Scotland, PA3 4EF.
Glaxo Group Research Ltd., Greenford Road, Greenford, Middx, UB6 0HB.
ICN Biomedicals Ltd., Eagle House, Peregrine Business Park, Gomm Road, High Wycombe, Bucks, HP13 7DL.
Immunex Manufacturing Corporation, Seattle, WA 98101, USA.
Jackson Immuno Research Laboratories Inc., 872 West Baltimore Pike, P.O. Box 9, West Grove, Pennsylvania, 19390, USA.
LKB. supplied by; FSA Laboratory Supplies, Loughborough, Leics., LE11 0RG, UK.
Marathon Laboratory Supplies, Unit 6, 55-57 Park Royal Road, London, NW10 7LP.
MIAB, Box 97, S-74100 Knivsta, Uppsala, Sweden.
Miltenyi Biotech, GmbH: Friedrich Ebert StraBe 68, D-5060 Bergisch-Gladbach 1, W. Germany.
NIBSC., National Institute for Biological Standards and Control, South Mimms, Potters Bar, Herts, EN6 3QG. (0707 54753)

NUNC, supplied by Flow.
Ortho Diagnostic Systems Ltd., Denmark House, Denmark Street, High Wycombe, Bucks, HP11 2ER.
Pharmacia Biosystems Ltd., Davy Ave, Knowl Hill, Milton Keynes, MK5 8PH.
Roussel of Ireland Ltd., Dublin 2, Republic of Ireland.
Sandoz Pharmaceuticals (UK) Limited, Frimley Business Park, Fromley, Camberley, Surrey, GU16 5SG.
Sera-lab Limited., Crawley Down, Sussex, RH10 4FF.
Serotec, 22 Bankside, Station Approach, Kidlington, OX, OX5 1JE.
Sigma Chemical Co. Ltd., Fancy Road, Poole, Dorset, BH17 7NK.
Southern Biotechnology Associates Inc., supplied by; Laboratory Impex Ltd., 111-113 Waldegrave Road, Teddington, Middx.
Sterling Drug Company Inc., Malvern, PA 19355, USA.
Tago, P.O. Box 4463, 887 Mitten Road, Burlingame, California 94011, USA.
TCS Ltd., Botolph Claydon, Buckingham, MK18 2LR. (029 6714071/2)
Wellcome Diagnostics, Temple Hill, Dartford, DA1 5AH.
Zymed Laboratories INC., 458 Carlton Court, So. San Francisco, CA 94080. (supplied in the UK. by; Cambridge Bio Science, 25 Signet Court, Stourbridge Common Business Centre, Swann's Road, Cambridge, CB5 8LA.).

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Human immunoglobulin class and IgG subclass regulation: dual action of interleukin-4*

Epstein-Barr virus (EBV) was used as a polyclonal human B cell mitogen to investigate the regulation of immunoglobulin class and IgG subclass responses by interleukin-4 (IL-4). Activation of tonsillar B cells with EBV resulted in an early peak of polyclonal immunoglobulin secretion between days 13 and 14 consisting of IgM, IgA, and IgG1, IgG2, IgG3 and IgG4, but not IgE. Addition of IL-4 to EBV-activated B cells at concentrations of 100 U/ml or greater induced the production of IgE and enhanced IgG4 secretion, but had no effect, or more often inhibited the other isotypes. In contrast, low concentrations of IL-4 (1–5 U/ml) significantly increased the production of IgM, IgA, IgG1, IgG2 and IgG3, but had no effect on IgG4 or IgE. The increase in immunoglobulin secretion obtained with low concentrations of IL-4 was found to occur only with high-density (resting) B cells, suggesting that IL-4 was not functioning simply as a late-acting differentiation factor. Low concentrations of IL-4 significantly increased IgG1, IgG2, IgG3, and IgA production by surface (s)IgM$^+$ (sIgG$^-$/sIgA$^-$) B cells which is consistent with heavy chain switching. In some experiments, however, IL-4 enhanced IgM secretion by sIgM$^+$ B cells, and IgA, IgG1, IgG2, IgG3 by sIgM$^-$ B cells, suggesting that it may have an additional B cell differentiation factor activity which was not isotype specific. The different effect of IL-4 at high and low concentrations were similar to those observed in B cell activation experiments, and may be due to the existence of high- and low-affinity IL-4 receptors.

1 Introduction

There are nine human immunoglobulin classes and subclasses (IgM, IgD, IgG1, IgG2, IgG3, and IgG4, IgA1, IgA2, and IgE), each of which has specialized functions important for humoral immunity. Over the past few years, many studies have shown that cytokines are major regulators of immunoglobulin class and IgG subclass responses. The best known of these is interleukin-4 (IL-4) which stimulates the production of IgE and IgG1 in mice and IgE in man [1–3]. In both species, IL-4 induces IgE secretion by sIgE$^-$ B cells [4], and stimulates the synthesis of germ-line C_ε transcripts [4, 5] indicating that IgE production is due to heavy chain switching rather than selection of pre-committed B cells. In much the same way, the production of IgA in response to transforming growth factor-β (TGF-β) has also been found to be due to heavy chain switching [6, 7]. TGF-β has also been shown to act in concert with IL-10 and CD40 to stimulate IgA secretion by human tonsillar B cells [8]. In both humans and mice, IgE and IgA class switching by IL-4 and TGF-β appears to be very similar [4, 6, 9–11]. In contrast, IgG subclass regulation has little in common in the two species. In mice, secretion of IgG1 is induced by IL-4 [9, 10] and IgG2a by IFN-γ [12], in both cases by heavy chain switching rather than selection. Similar studies with human B cells, however, have shown that nerve growth factor (NGF) stimulates IgG4 production [13], whereas IL-4 has been reported to enhance IgG4 production by EBV-activated B cells [14, 15], and to enhance IgG1, IgG2 and IgG3, but not IgG4 production by B cells stimulated with PMA and ionomycin [16]. The reasons for this discrepancy, and for the differences in IgG subclass regulation in humans and mice, are not clear. Cross species comparisons between the IgG subclasses are difficult to make because they do not have homologous structures or functions. Indeed, the weight of evidence suggests that the IgG subclasses in the two species evolved independently [17], and are therefore unlikely to have developed the same regulatory mechanisms. In the present study, we have used EBV as a T cell-independent polyclonal B cell mitogen to investigate the action of IL-4 on human immunoglobulin class, and IgG subclass responses. We show that IL-4 has two quite distinct actions depending on whether it is used at low or high concentrations. Our results explain the previous conflicting data on the effects of IL-4 on IgG subclass responses, and are consistent with the existence of two distinct IL-4 B cell activation pathways coupled to high- and low-affinity receptors [18].

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Abbreviations: **BCDF:** B cell differentiation factor **SAC:** *Staphylococcus aureus* Cowan I **TGF-β:** Transforming growth factor-β **TMC:** Tonsillar mononuclear cells

Key words: Interleukin-4 / B cells / Switching / IgG subclasses / B cell differentiation

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2 Materials and methods

2.1 Reagents

Cells were prepared and cultured in RPMI 1640 medium or Iscove's modified Dulbecco's medium (IMDM) supplemented with 25 mM Hepes and 10% fetal calf serum (FCS)

(Gibco, Paisley, GB). Ficoll-sodium diatrizoate (Lymphoprep) (Flow Laboratories, Rickmansworth, GB) and Percoll (Pharmacia, Milton Keynes, GB) were used as separation media. S-2-aminoethylisothiouronium bromide hydrobromide (AET) was purchased from Aldrich Chemical Co. (Gillingham, Dorset, GB), [^{3}H]dThd (TRA 120) was purchased from Amersham International (Aylesbury, GB), and anti-human IgM conjugated to polyacrylamide beads from Bio-Rad (Watford, GB). Recombinant human IL-4 (rhIL-4) was kindly supplied by Dr. Jacques Banchereau, UNICET, Dardilly, France, or obtained from Stirling Drug Inc. (Malvern, PA). The concentration of IL-4 required for half-maximal [^{3}H]dThd incorporation by 5×10^3 T cells pre-activated with PHA is defined as 1 U/ml. Optimal [^{3}H]dThd incorporation by B cells costimulated with 1 μg/ml of anti-IgM was obtained routinely with 100–200 U/ml of IL-4. Cell preparations were checked for purity with monoclonal antibodies UCHT1 (CD3) and UCHM1 (CD14) kindly supplied by Dr. Peter Beverley (ICRF, London), and BU12 (CD19) prepared by protein A affinity chromatography of supernatants from the BU12 hybridoma kindly provided by Dr. N. Ling (Birmingham University, GB). Monoclonal antibody binding was detected with an affinity-purified FITC-conjugated $(Fab')_2$ goat anti-mouse IgG adsorbed against human serum proteins (TAGO 6250) supplied by T.C.S. (Slough, GB). Monoclonal antibodies used in the ELISA were biotinylated anti-human IgG1, IgG2, IgG3 and IgG4 made by Zymed and obtained from Cambridge Biosciences (Cambridge, GB). The polyclonal antibodies goat anti-human IgM, IgG, IgA and IgE, and horseradish peroxidase (HRP)-conjugated goat anti-human IgM, IgG, IgA, and anti-rabbit IgG were obtained from Sigma (Poole, Dorset, GB). Rabbit anti-human IgE was obtained from MIAB (Uppsala, Sweden) and rabbit anti-human IgG (H+L) was obtained from Jackson Laboratories (West Grove, PA). Sheep anti-human IgG4 was purchased from The Binding Site (Birmingham GB). Alkaline phosphatase- and peroxidase-conjugated streptavidin were purchased from Amersham International. Substrates for alkaline phosphatase p-nitrophenyl (NPP) and horseradish peroxidase, o-phenylenediamine (OPD) were purchased from Sigma.

2.2 Epstein-Barr virus preparation

Mycoplasma-free cells of the EBV-infected Marmoset B lymphoblastoid cell line B95-8 were grown in RPMI 1640 containing 10 % FCS, 2 mM glutamine, 25 mM Hepes and 80 μg/ml of gentamicin for 2–4 weeks. Freshly split cultures were then grown for a further 4 weeks without feeding. Virus containing supernatants were filtered through a 1.2-μm filter and stored at −70 °C until use. Each EBV containing supernatant was titrated and the concentration adjusted for optimal activation of B cells.

2.3 Cell preparation

Tonsillar mononuclear cells (TMC) were prepared by teasing cells from excised tonsils into RPMI 1640 medium containing 5 % FCS and 50 μg/ml of gentamicin followed by centrifugation over Ficoll-sodium diatrizoate. Sheep erythrocyte rosette-forming (E^+) cells were prepared with AET-treated sheep red blood cells (SRBC) at 4 °C [19] and separated from non-rosette-forming E^- (B) cells by centrifugation over Percoll (1.080 kg/l) as described previously [20]. E^- cells recovered from the interface routinely contained > 95 % $BU12^+$ ($CD19^+$) B cells, < 1 % $UCHT1^+$ ($CD3^+$) T cells, 1–2 % monocytes and 1–2 % null cells. To obtain B cells of different densities, tonsillar E^- cells were centrifuged at 1000 × g for 20 min over Percoll at a density of 1.074 kg/l as described previously [20]. Magnetic cell sorting (MACS) separation of tonsillar B cells into $sIgM^+$ and $sIgM^-$ populations was carried out exactly as described previously [20]. Purities of 90–98 % were obtained routinely by this method.

2.4 Activation with EBV and cell culture

To activate with EBV, isolated B cells were pelleted and resuspended in B95-8 supernatant at a concentration of 12.5×10^6 cells/ml and incubated for 1 h at 37 °C in 5 % CO_2 in air. After washing, the cells were resuspended in IMDM supplemented with 2 mM glutamine, 25 mM Hepes, antibiotics and 10 % FCS. IL-4 was added as indicated. In proliferation assays, the cells were cultured at 10^6 cells/ml in 200-μl- round-bottom microtiter wells for 5 days or the time indicated. [^{3}H]dThd (1 μCi) was added for the last 8 h of culture before harvesting. Incorporated [^{3}H]dThd was determined by liquid scintillation counting. In immunoglobulin secretion assays, the cells were cultured at 10^6 cells/ml either as 2-ml cultures in 24-well flat-bottom plates or 0.5-ml cultures in Falcon tubes for the time indicated, usually 13 days. Supernatants were then collected and assayed for immunoglobulin content by ELISA.

2.5 ELISA for secreted immunoglobulins

Flat-bottom 96-well Immulon II plates (Dynatech) were coated with goat anti-human IgM, IgG, IgA and IgE overnight at room temperature and blocked with 1 % BSA in PBS/Tween 20. Supernatants from B cell cultures were added and incubated for 1 h at 37 °C, or overnight at room temperature for IgE. IgM, IgG and IgA were detected with peroxidase-conjugated goat anti-human IgM, IgG or IgA. Human IgE in supernatants was detected with rabbit anti-human IgE followed by peroxidase-conjugated goat anti-rabbit IgG. Color development with OPD was determined on a Dynatech plate reader at 492 nm. The assays had lower detection limits of 0.5, 1.0, 1.0 ng/ml for IgM, IgG and IgA, respectively, and 300–600 pg/ml for IgE. IgG subclasses were assayed by coating plates with rabbit anti-human IgG(H+L) or sheep anti-human IgG4 capture antibodies overnight at room temperature. The plates were blocked for 4 h with 1 % normal mouse serum and incubated with culture supernatants overnight at room temperature. IgG subclasses were detected with biotin-conjugated mouse anti-human IgG1, IgG2, IgG3 and IgG4 followed by either alkaline phosphatase- or HRP-conjugated streptavidin. The IgG subclass assays had lower detection limits of between 1 and 10 ng/ml.

3 Results

3.1 Polyclonal B cell activation with EBV

EBV has important properties which make it suitable as a polyclonal mitogen for human B cells. Like LPS in the mouse, it is entirely T cell independent [21], and it elicits immunoglobulin secretion of all classes and subclasses [21, 22]. EBV has been used previously to investigate isotype regulation in human B cells [4, 23], but the culture conditions employed and the timing of the response meant that B cell transformation rather than polyclonal activation may have been examined. This important distinction is made clear by the time-response to EBV shown in Fig. 1. In this experiment, proliferation and immunoglobulin secretion were measured from days 3 to 30 after activation of tonsillar B cells with EBV. It can be seen that an early burst of [^{3}H] dThd uptake occurred with a maximum at day 4–5 which then decreased to baseline levels for a lag period of nearly 2 weeks before increasing again. The second phase of proliferation at day 21 corresponded to the emergence of a few transformed clones whereas the early phase represented polyclonal activation. Immunoglobulin secretion followed a similar pattern, albeit delayed by several days. The polyclonal immunoglobulin response peaked at day 13–14 and then declined before a second phase of secretion corresponding to the emergence of transformed B cells after day 21. This biphasic response after activation with EBV was observed in many different experiments. The levels of IgM, IgG, IgA, IgE and subclasses IgG1, IgG2, IgG3, IgG4 and were measured in supernatants collected on day 13. The results of two such experiments are given in Table 1 and show that EBV stimulated the production of all classes and subclasses except IgE. In each case, optimal levels were obtained on days 13–14 (data not given). In the rest of this study, proliferation was determined on day 5 and immunoglobulin secretion at day 13.

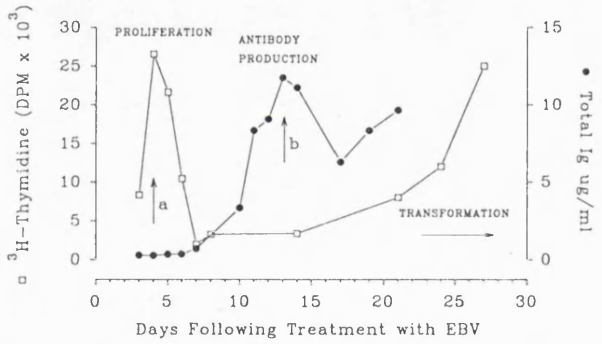

Figure 1. Time course of proliferation and total immunoglobulin secretion by EBV activated B cells. Peak polyclonal responses were obtained at day 5 for proliferation (arrow a) and day 13–14 for immunoglobulin secretion (arrow b). These times were used in all other experiments.

3.2 Dose response effects of IL-4 on EBV-induced B cell proliferation and antibody secretion

In most studies, concentrations of IL-4 used to stimulate human B cells have typically ranged from 10–300 U/ml. We have shown recently, however, that concentrations of IL-4 between 1 and 5 U/ml can have potent effects on B cells quite distinct from the effects obtained with higher concentrations [18]. The action of IL-4 on EBV-activated B cells was, therefore, examined over a range of concentrations from 0.01 to 500 U/ml. In these experiments, B cell proliferation was maximally enhanced with 10–50 U/ml of IL-4 (Fig. 2). Total immunoglobulin secretion was also increased by IL-4, but the optimal response was obtained with 10 × lower concentrations of IL-4 (1–5 U/ml; Fig. 2).

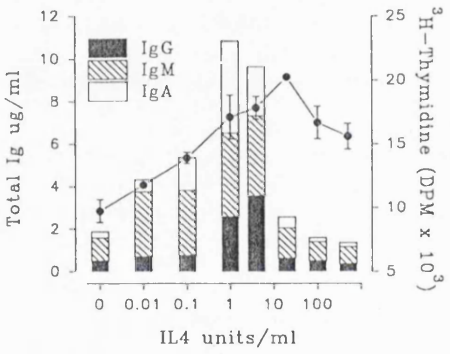

Figure 2. Effect of IL-4 on proliferation and Ig secretion by EBV activated B cells. Tonsillar B cells were activated with EBV and cultured with IL-4 at different concentrations from 0 to 500 U/ml. Proliferation ([^{3}H] dThd uptake; -●-) was measured at day 5, and IgM, IgG and IgA secretion at day 13.

3.3 Effect of IL-4 at different concentrations on IgM, IgG, IgA and IgE production by EBV-activated tonsillar B cells

Tonsillar B cells were activated with EBV and cultured with IL-4 at concentrations between 1 and 250 U/ml. The levels

Table 1. Immunoglobulin classes and IgG subclasses produced by EBV-activated tonsillar B cells[a)]

Isotype	Experiment 1		Experiment 2	
	Medium	EBV	Medium	EBV
IgM	158 ± 25[b)]	10 903 ± 308	139 ± 37	8513 ± 1167
IgA	26 ± 8	1076 ± 291	179 ± 7	2919 ± 748
IgE	< 0.5	< 0.5	< 0.5	< 0.5
IgG (total)	446 ± 35	8325 ± 2992	115 ± 14	2169 ± 232
IgG1	351 ± 25	4841 ± 1243	399 ± 90	9622 ± 1275
IgG2	60 ± 3	106 ± 7	180 ± 20	1713 ± 208
IgG3	56 ± 14	205 ± 33	81 ± 10	1554 ± 357
IgG4	< 1	6 ± 1	98 ± 2	434 ± 16

a) Tonsillar B cells were incubated with EBV containing B95-8 supernatant or medium for 1 h at 37 °C then washed and cultured at 1 × 10^6 cells/ml in IMDM. Immunoglobulin secretion was measured after 13 days in culture. The data shown are from two representative experiments.

b) Immunoglobulin class and IgG subclass secretion (in ng/ml) from triplicate cultures ± 1 SEM.

of IgM, IgG, IgA and IgE in culture supernatants were determined after 13 days. In these experiments, low concentrations (1–5 U/ml) of IL-4 significantly increased secretion of all immunoglobulin classes except for IgE (Fig. 3). In contrast, high concentrations of IL-4 (100 U/ml) induced significant IgE secretion (Fig. 3), but had no effect, or inhibited secretion of the other classes (IgM, IgG and IgA), usually below the levels obtained with EBV alone. In time course experiments, immunoglobulin secretion of each class (IgM, IgG, IgA, and IgE) peaked on days 13–14 whether IL-4 was present or not (data not given) showing that IL-4 did not alter the kinetics of the response.

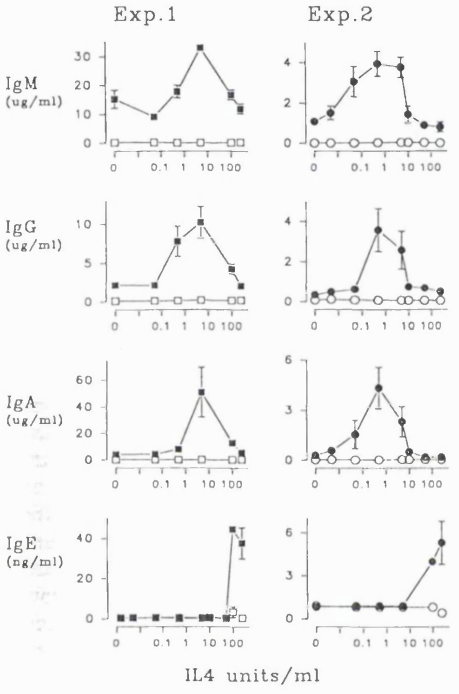

Figure 3. Effect of IL-4 on IgM, IgG, IgA and IgE secretion by EBV-activated B cells. Tonsillar B cells were activated with EBV and cultured with IL-4 at concentrations from 0 to 250 U/ml in IMDM. Immunoglobulin secretion was measured on day 13 in two separate experiments (1 and 2). Filled symbols indicate the response with EBV and IL-4, empty symbols indicate the response with IL-4 alone. The lower detection limit for IgE was 0.5 ng/ml).

3.4 Effect of IL-4 at different concentrations on IgG subclass production

High and low concentrations of IL-4 also had completely different effects on IgG subclass responses. Following the same pattern observed for IgM, IgG and IgA, significant increases in IgG1, IgG2 and IgG3 secretion were obtained with 1 to 5 U/ml of IL-4 which was reduced to base levels or less with concentrations of IL-4 > 100 U/ml (Fig. 4). Enhanced IgG4 production was also observed in some experiments, but this was usually small and inconsistent. When it did occur, it was most evident at 100 U/ml of IL-4, suggesting that IgG4 and IgE were regulated in a similar way, and independently from the other subclasses.

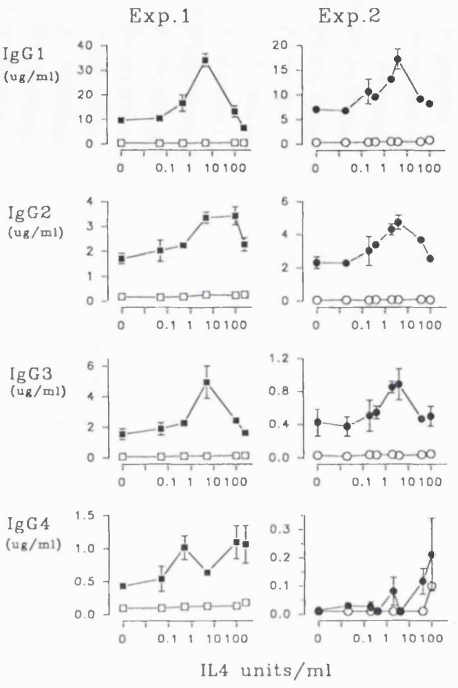

Figure 4. Effect of IL-4 on IgG subclass by EBV-activated B cells. Tonsillar B cells were activated with EBV and cultured with IL-4 at concentrations from 0 to 250 U/ml in IMDM. IgG1, IgG2, IgG3, and IgG4 secretion was measured on day 13 in two separate experiments (1 and 2). Filled symbols indicate the response with EBV and IL-4 and empty symbols indicate the response with IL-4 alone.

3.5 IL-4 increases immunoglobulin production by high-but not low-density B cells

EBV has been reported previously to stimulate and transform large, low-density activated B cells [24] suggesting that IL-4 may be acting in our experiments as a B cell differentiation factor (BCDF) for pre-activated B cells similar to that described for *Staphylococcus aureus* Cowan I

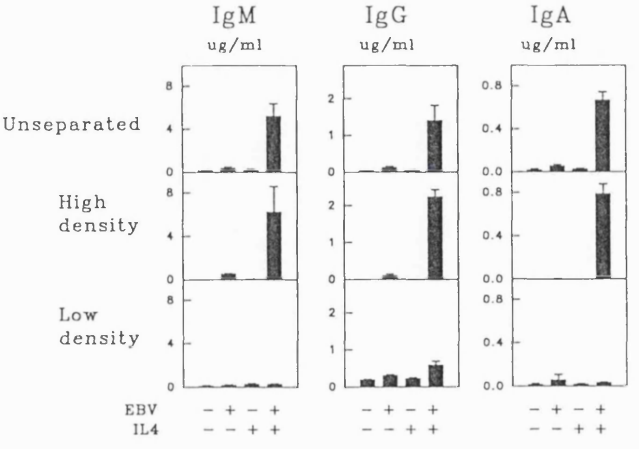

Figure 5. Effect of IL-4 on Ig secretion by heavy and light B cells activated with EBV. Tonsillar B cells separated on discontinuous Percoll gradients into high- and low-density fractions were activated with EBV and cultured with 5 U/ml of IL-4. IgM, IgG and IgA secretion was measured on day 13.

(SAC)-stimulated B cells [25]. To investigate which cells were responding to EBV and IL-4, tonsillar B cells were separated into low-density (< 1.074 kg/l) and high-density (> 1.075 kg/l) populations on discontinuous Percoll density gradients, activated with EBV, and then cultured with 5 U/ml of IL-4. Immunoglobulin secretion was measured after 13 days. The results from these experiments were clear cut (Fig. 5). EBV alone stimulated a small amount of immunoglobulin production (IgM, IgG, IgA) in both high- and low-density B cells, but this was increased by IL-4 only in the high-density "resting" B cell fraction. Similar results were obtained for the IgG subclasses (data not given).

3.6 Effect of IL-4 on immunoglobulin class and IgG subclass production by sIgM$^+$ and sIgM$^-$ B cells

Increased IgE secretion by EBV-activated B cells cultured with concentrations of IL-4 > 100 U/ml has been shown by others to be due to heavy chain switching rather than selection of pre-committed cells [4, 26]. However, the mechanism by which low concentrations of IL-4 stimulate IgM, IgG and IgA production is not known. An indication of whether switching or selection is operating can be obtained by testing B cell subpopulations isolated according to the surface expression of the isotype concerned (IgG, IgA, IgE, etc.). Rather than undertake these experiments for each class and subclass, TMC B cells were separated into sIgM$^+$ and sIgM$^-$ populations and tested for their response to IL-4. The majority of sIgG$^+$, sIgA$^+$ and sIgE$^+$ B cells are found in the sIgM$^-$ population. The results from six such experiments suggest that two mechanisms may be operating. IL-4 (5 U/ml) significantly increased IgG, and IgA (Fig. 6, Top) and IgG1, IgG2, and IgG3 (Fig. 6, Bottom) production by sIgM$^+$ B cells. This result is consistent with heavy chain switching by sIgM$^+$/sIgG$^-$/sIgA$^-$ B cells. In some experiments, however, IL-4 increased immunoglobulin secretion in the sIgM$^-$ fraction. This was not due to cross-contamination as the method used to separate these populations gave up to 98% purity, and no IgM was secreted by the sIgM fraction (Fig. 6, Top). This result together with the enhancing effect of IL-4 on IgM secretion by sIgM$^+$ cells (Fig. 6, Top) is consistent with IL-4 acting as a BCDF rather than a switch factor.

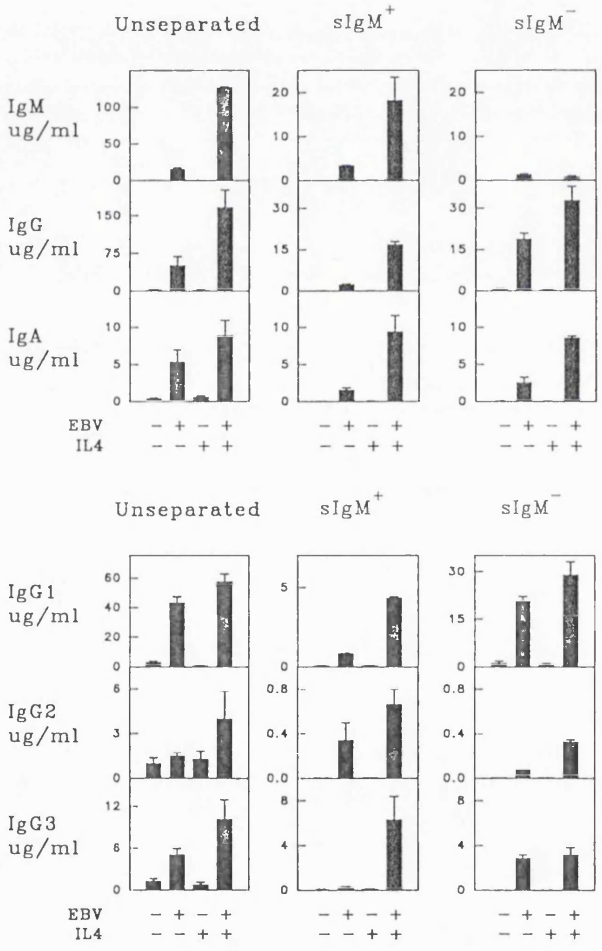

Figure 6. Response of sIgM$^+$ and sIgM$^-$ B cells to EBV and IL-4. Tonsillar B cells were separated on the MACS into sIgM$^+$ and sIgM$^-$ fractions then activated with EBV and cultured with 5 U/ml of IL-4. Each population was shown to be more than 95% pure. Production of IgM, IgG, IgA (Top) and IgG subclasses 1–4 (Bottom) was determined on day 13. No IgG4 or IgE was detected in this experiment.

4 Discussion

EBV has several important characteristics which make it particularly suitable as a B cell mitogen for investigating cytokine regulation of Ig class and IgG subclass responses by human B cells. Unlike most other human B cell mitogens, it is entirely T cell independent, and it elicits secretion of all the Ig classes and IgG subclasses except for IgE [21, 22, 24] (Table 1). These properties of EBV have been exploited previously to show that IL-4 is required for IgE production by purified human B cells [4, 23]. In this earlier work, B cells activated with EBV and cultured *in vitro* with 100 U/ml or more of IL-4 made IgE in the 10 to 100 ng/ml range. The IgE response was obtained from sIgE$^-$ B cells, and was preceded by the appearance fo germ-line C_ε transcripts [4] consistent with heavy chain switching induced by IL-4 rather than selection of pre-committed B cells.

The conditions under which EBV has been used in isotype regulation experiments has varied from one study to another. For example, responses were measured every 3 days for 24 days in one study, and after 26 days in another. This is not a trivial distinction. As shown in our experiments (Fig. 1) the response to EBV is biphasic. The first phase of proliferation (day 5) and immunoglobulin secretion (days 13–14) is due to polyclonal activation. The early polyclonal response is followed by a lag period of 1–2 weeks, and then rapid proliferation and antibody secretion by a few transformed clones after 21 days. The responses of EBV-activated B cells to IL-4 described here were measured at days 13–14, and are distinct from the later responses of transformed B cells [23].

Two quite different effects of IL-4 on immunoglobulin secretion by EBV-activated B cells were observed depending on the concentration used. At 100 U/ml, IL-4 stimulated the production of IgE and IgG4 by EBV-activated B cells, but either had no effect or inhibited production of IgM, IgG1, IgG2 and IgG3 and IgA (Figs. 3 and 4). The IgE responses were very similar to those reported previously for

B cells activated with EBV [4, 23], phorbol ester [14], and pokeweed mitogen [26], and are consistent with the known regulation of IgE responses by IL-4 in the mouse [9].

A completely different picture emerged when IL-4 was used at lower concentrations. At 1–5 U/ml, IL-4 greatly increased production of IgM, IgA, and IgG1, IgG2 and IgG3, but had no effect on IgG4 or IgE (Figs. 3 and 4). No IgE was ever detected with low concentrations of IL-4. Interestingly, the effect of low concentrations of IL-4 was very similar to the findings reported by Flores-Romo et al. [16] in which IL-4 stimulated IgM and IgG1, IgG2 and IgG3, but not IgG4 by B cells activated with phorbol ester and ionomycin. However, high concentrations of IL-4 (1000 U/ml were used by Flores-Romo, equivalent to the high doses used by Thyphronitis to generate IgE production by EBV-activated B cells [23]. Whether these conflicting results can be explained by the different means of activating B cells is unclear.

Different responses to high and low concentrations of IL-4 have also been found in B cell activation experiments [18]. At 100 U/ml, IL-4 was shown to increase expression of both CD23 and sIgM on human B cells, whereas low concentrations of IL-4 (5 U/ml) increased expression only of sIgM and not CD23. In signal transduction experiments, it was found that increased CD23 expression by high concentrations of IL-4 (> 100 U/ml) depended on hydrolysis of phosphatidyl inositol bisphosphate (PIP_2) and activation of PKC followed, after a brief lag period, by elevation of cAMP [27]. This signalling pathway was not activated by low concentrations of IL-4. It was concluded from these experiments that IL-4 binds to low- and high-affinity receptors coupled to distinct signal transduction pathways [18]. The high-affinity human IL-4 receptor has been cloned [28], and a second low-affinity receptor has recently been identified and partially sequenced [29]. This raises the interesting possibility that regulation of IgE and IgG4 by high concentrations of IL-4 (> 100 U/ml) may involve different (low-affinity) receptors and signalling mechanisms than the effects of low concentrations of IL-4 (1-5 U/ml) on IgM, IgA, and IgG1, IgG2 and IgG3. In this context, it is significant that a new human cytokine now called IL-13 has been cloned and found to increase CD23 expression, and induce IgE and IgG4 secretion by human B cells. Moreover, cross-blocking studies have suggested that the IL-13 receptor binds IL-4 (J. Banchereau, personal communication). An intriguing possibility arising from these observations is that the IL-13 receptor and the low-affinity IL-4 receptor are the same or share a common subunit.

Dose-response effects of IL-4 on different isotypes have also been shown in mice [30]. Bimodal IgG1 production was observed with peaks at 100 and 10 000 U/ml and substantial IgE secretion was obtained with LPS and 10 U/ml of IL-4. Interestingly, inhibition of IgM, IgG2a, IgG2b and IgG3 occurred with concentrations of IL-4 around 600 U/ml. There are, however, significant differences in the regulation of IgG subclasses by cytokines in humans and mice. For example, IgG1 production by murine B cells is stimulated by IL-4 [30] and IgG2a by IFN-γ [12] through introduction of heavy chain switching rather than selection of IgG subclass-specific B cells. In contrast, IgG4 production by human B cells is stimulated by IL-4 [14, 15]. Human IgG4 and murine IgG1 are not structural or functional analogues. Indeed, there is more homology between the human IgG subclasses than between human and mouse IgG subclasses, and it is probable that they evolved independently after the radiation of the mammals 60 million years ago [17]. It is, therefore, unlikely that the same mechanisms for subclass regulation would operate in the two species. With this in mind, it is interesting that IL-4 has the effect it does on IgG responses in both species, albeit on unrelated IgG subclasses. One explanation for this may be that early in evolution IL-4 was first developed as a B cell differentiation factor, and has only more recently acquired its more specialised role in IgE and IgG1 or IgG4 heavy chain switching. Indeed, IL-4 has been shown in a number of experimental systems to enhance Ig secretion without obvious preference for any class or subclass. Examples are SAC-pre-activated B cells [25, 31], CD40-activated B cells [32], and B lymphoblastoid cell lines [33]. Our results are consistent with high concentrations of IL-4 having Ig class switching activity, and low concentrations having both class switching and BCDF activity. In several different experiments, low concentrations of IL-4 were found to enhance IgM, IgA, and IgG1, IgG2 and IgG3 secretion by $sIgM^+$ B cells which is consistent with IL-4 class switching activity (Fig. 6). However, the increase in IgM secretion by $sIgM^+$ cells, and some IgA and IgG by $sIgM^-$ cells can best be explained by BCDF action of IL-4 (Fig. 6). Interestingly, the increased immunoglobulin secretion obtained with IL-4 was confined to high-density "resting" B cells (Fig. 5) suggesting that IL-4 was functioning as an early activating signal rather than a late-acting BCDF. IL-4 has also been shown to act early in IgG1 class switching of murine B cells [34]. Confirmation that Ig class switching is occurring with low concentrations of IL-4 must await limiting dilution analyses and/or detection of germ-line transcripts for each antibody class and IgG subclass.

Finally, it is interesting to speculate on the role of EBV in these experiments. Polyclonal activation of human B cells by EBV requires live virus. Binding to its receptor (CD21) is not in itself sufficient. Recently, the EBV gene product BCRF1 has been shown to have homology with the cytokine IL-10 [35] and to have similar biological properties [36]. IL-10 is a growth factor for human B cells [37], and it has been shown to cooperate with TGF-β and CD40 antibodies to elicit IgA production by human B cells [8]. It will be interesting now to determine whether IL-10 is also involved in Ig class and subclass responses regulated by IL-4.

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Originals

Interleukin-4 stimulates immunoglobulin secretion by Epstein-Barr virus (EBV)-activated tonsillar B cells, and by EBV-transformed lymphoblastoid B cell lines without increasing cell division

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Summary. Freshly prepared Epstein-Barr virus-transformed B lymphoblastoid cell lines derived from five different donors were tested for their responses to recombinant human interleukin-4 and to low molecular weight B cell growth factor. In the absence of either cytokine, all five lines secreted immunoglobulin of more than one isotype (IgM, IgG, and IgA, but not IgE). Stimulation with interleukin-4 resulted in a significant increase in immunoglobulin secretion, but did not enhance cell division measured by tritiated-thymidine uptake or cell counts. In contrast, low molecular weight B cell growth factor increased both immunoglobulin secretion and cell division. The increase in immunoglobulin secretion stimulated by interleukin-4 occurred for each of the different isotypes (IgM, IgG and IgA) produced by the unstimulated line. No IgE secretion was detected for any of the five lines. It was also found that low (5 units/ml), but not high (100 units/ml), concentrations of interleukin-4 increased IgM, IgG and IgA secretion by tonsillar B cells polyclonally activated with Epstein-Barr virus. Again, no IgE was detected at any time. These results suggest that interleukin-4 can function as a late-acting B cell differentiation factor as well as a growth factor for human B cells.

Key words: Interleukin 4 – Activated B cells – Immunoglobulin secretion – B cell growth factor – Cell division – Epstein-Barr virus

Introduction

Interleukin-4 (IL-4) is a T cell-derived cytokine with activity on cells of most haemopoietic lineages, including B cells [12], T cells [8, 27], macrophages [4], mast cells [1] and haemopoietic progenitor cells [13]. Its action on B cells is complex. Activation of small resting B cells with IL-4 results in increases in size and expression of activation antigens such as MHC class II [11, 14], CD23 [5, 9] and surface IgM [17]. In addition, IL-4 is a potent growth factor for B cells co-stimulated with anti-IgM or TI type 2 antigens [6, 12, 22]. Whether or not IL-4 is also a B cell differentiation factor (BCDF) is less clear. IL-4 promotes IgG1 and IgE secretion by lipopolysaccharide (LPS)-stimulated mouse spleen B cells [10, 25], but this is thought to be an early event in which resting B cells are primed by IL-4 for subsequent heavy chain switching and antibody secretion rather than differentiation of already activated cells [16, 19]. IL-4 is not a differentiation factor for B cells co-stimulated with anti-IgM or *Staphylococcus aureus* Cowan I (SAC), but it has been shown to enhance IgM and IgG secretion by human B cell blasts obtained by prior stimulation with SAC [7] or SAC plus IL-2 [21]. We report here that immunoglobulin secretion by freshly prepared Epstein-Barr virus-transformed lymphoblastoid B cell lines (EBV-LCL) is also enhanced by IL-4. Proliferation in the same cultures was not increased, showing that IL-4 is a BCDF but not a B cell growth factor (BCGF) for activated B cells. In addition, low but not high concentrations of IL-4 increased immunoglobulin secretion by tonsillar B cells polyclonally activated by EBV. These findings show that IL-4 is a late-acting differentiation factor as well as a growth factor for human B cells.

Materials and methods

Reagents. Medium RPMI 1640 with 25 mM HEPES and fetal calf serum (FCS) were obtained from Gibco (Paisley, UK), Ficoll-sodium diatrizoate (Lymphoprep) from Flow (Rickmansworth, UK) and tritiated-thymidine (^{3}HTdR, TRA 120) from Amersham (Aylesbury, UK). Supernatants from L cells transfected with cDNA encoding the human IL-4 gene provided by Dr. Jacques Banchereau (UNICET, France) were used as a source of recombinant human IL-4 (rhIL-4) [26]. Mock supernatants from L cells which had not been transfected with the IL-4 gene were used as controls. Highly purified rhIL-4 was also used in some experiments. One unit per millilitre of IL-4 is defined as the concentration required to give half-maximal stimulation of ^{3}HTdR uptake by phytohaemagglutinin-activated T cell blasts [6]. Optimal B cell activation and proliferation was obtained with 250 units/ml. Low molecular weight BCGF

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($BCGF_{low}$) manufactured by Cellular Products was purchased from Sera Lab (Crawley Down, UK).

Preparation of EBV-LCL. Peripheral blood mononuclear cells (PBMC) were prepared by centrifugation of heparinised blood from normal healthy donors over Ficoll-sodium diatrizoate with a density of 1.077 kg/l. Between 1×10^7 and 5×10^7 PBMC were incubated in 2 ml supernatant from the EBV-secreting marmoset cell line B95-8 for 1 h at 37 °C. The cells wee then washed once, resuspended to a concentration of 1×10^6 cells/ml in RPMI 1640 containing 25 mM HEPES, 2 mM glutamine, 10% FCS, 50 μg/ml gentamicin and 1 μg/ml cyclosporin A, and cultured in 24-well Costar plates at 2×10^6 cells/well. Clumps of cells were usually seen at the end of 2 weeks, and stable EBV-transformed lines established by the end of 4 weeks. The cultures were expanded into 25-cm^2 flasks and then subcultured until the yield approached 10^8 cells, when they were frozen in aliquots of 20×10^6 cells. When required, the cells were thawed and grown in medium supplemented with 25 mM HEPES, 2 mM glutamine and 10% FCS for 2–3 weeks. Only cells in the logarithmic phase of growth were used for experiments with IL-4 and $BCGF_{low}$. All the lines were polyclonal when used, as evidenced by secretion of more than one immunoglobulin isotype (IgM, IgG and IgA).

Stimulation of EBV-LCL. EBV-LCL from bulk cultures were washed and dispensed into flat-bottomed microtitre wells at densities of 0.7×10^3, 2.2×10^3 and 7×10^3 cells/well in 200 μl HEPES-buffered RPMI 1640 containing 2 mM glutamine, 50 μg/ml gentamicin and 5% FCS. Where indicated, IL-4 was included at a final concentration of 250 units/ml. $BCGF_{low}$ was used at a final concentration of 10%. For proliferation assays, the cultures were incubated for 4 days (unless otherwise indicated) and pulsed with ^{3}HTdR (1 μCi/well) for the last 8 h. The cells were harvested onto glass filter paper discs with a Dynatech Automash harvester (Dynatech, Billingshurst, GB) and incorporated ^{3}HTdR determined by liquid scintillation counting. The results are expressed as mean disintegrations per minute ± 1 SEM from triplicate cultures. For differentiation (immunoglobulin secretion), the cultures were incubated for 8 days (unless otherwise indicated) when 100 μl supernatant was removed from each well and assayed for IgM, IgG and IgA concentration by enzyme-linked immunosorbent assay (ELISA) as described previously [3]. In some cases, low levels of IgG and IgA were found to contaminate preparations of $BCGF_{low}$. These were subtracted from the values obtained for cultures stimulated with $BCGF_{low}$ to obtain true IgG and IgA secretion by the cell lines.

EBV activation of tonsillar B cells. Tonsillar mononuclear cells were prepared as described previously and T cells depleted by E-rosetting with AET-treated sheep red blood cells [2]. The cells were washed and resuspended to 1×10^6 cells/ml in EBV-containing B958 supernatant for 1 h at 37 °C and then cultured at 1×10^6 cells/ml in micro-titre wells with or without IL-4 at 5 units/ml or 100 units/ml. Immunoglobulin (IgM, IgG, IgA and IgE) levels in culture supernatants were determined by ELISA after 12 days.

Results

Immunoglobulin secretion by EBV-LCL is enhanced by IL-4 or $BCGF_{low}$

The effect of IL-4 and $BCGF_{low}$ on immunoglobulin secretion by EBV-LCL was investigated using freshly prepared lines from five different donors (Fig. 1). Each EBV-LCL was cultured with optimal concentrations of IL-4 (250 units/ml), $BCGF_{low}$ (10%) or in medium alone. Secretion of IgM, IgG and IgA was determined on the 8th day. This time was shown to be optimal in preliminary experiments. In unstimulated cultures, all three isotpyes (IgM, IgG and IgA) were secreted by two of the five lines, and IgM plus IgG or IgA were secreted by the other three. As expected, levels of IgM were much higher than either IgG or IgA. Stimulation with IL-4 significantly enhanced IgM secretion by all five EBV-LCL by between 130% and 300%. IgG secretion was greatly increased by IL-4 in all four lines secreting IgG, in one example from undetectable levels to over 100 ng/ml (Fig. 1 B). An increase in IgA secretion was observed with two EBV-LCL in response to IL-4. Of the other three, one did not respond, one made no detectable IgA and one was not tested. No IgE was detected in supernatants from any line with or without IL-4 activation. The sensitivity of the IgE assay was 200 pg/ml.

With most of the EBV-LCL, a greater response was obtained with $BCGF_{low}$ than IL-4, although in one line more IgG and IgM was secreted in reponse to IL-4 than $BCGF_{low}$ (Fig. 1 C). Both IL-4 and $BCGF_{low}$ enhanced secretion of all three isotypes, and there was no evidence for isotype selection by either cytokine. Mock IL-4 from L cells not transfected with the IL-4 gene had no effect on immunoglobulin secretion by EBV-LCL (Fig. 2).

IL-4 enhances immunoglobulin secretion but not proliferation by EBV-LCL

The increase in immunoglobulin secretion by EBV-LCL stimulated with IL-4 could have been due to proliferation rather than differentiation. To exclude this possibility, proliferation was measured by both ^{3}HTdR uptake and cell counts after stimulation with IL-4. No significant increase in ^{3}HTdR uptake or cell counts was observed for any of the six lines in response to IL-4 (Table 1). In contrast, $BCGF_{low}$ stimulation resulted in very significant proliferation measured by either ^{3}HTdR or cell counts with increases ranging from 200% to 1,200%. When cell numbers and immunoglobulin concentrations were mea-

Table 1. Proliferation by EBV-LCL stimulated with IL-4 or $BCGF_{low}$

EBV-LCL	^{3}HTdR incorporation (dpm/well)		
	Medium	IL-4	$BCGF_{low}$
A	3,395 ± 758	4,819 ± 1,398	35,290 ± 1,627
B	17,828 ± 8,736	25,168 ± 2,013	56,899 ± 1,707
C	17,193 ± 1,032	21,598 ± 1,296	37,475 ± 1,124
D	30,045 ± 2,103	30,546 ± 1,527	93,674 ± 3,747
E	7,943 ± 2,621	9,996 ± 2,999	45,288 ± 2,261
F	8,240 ± 2,884	3,554 ± 924	24,589 ± 1,229
	(Cells/well × 10^{-3})		
A	21	29	51
B	12	16	205
G	11	18	154

EBV-LCL, Epstein-Barr virus-transformed lymphoblastoid B cell lines; IL-4, interleukin-4; $BCGF_{low}$, low molecular weight B cell growth factor; dpm, disintegrations per minute; ^{3}HTdR, tritiated-thymidine

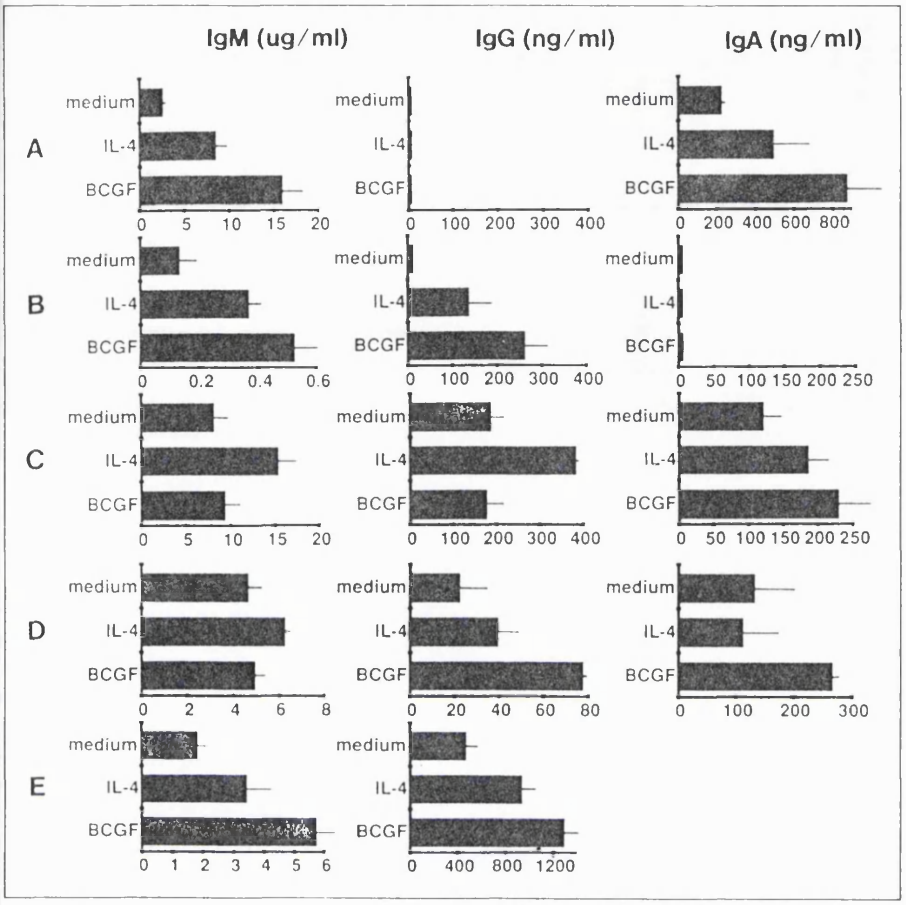

Fig. 1. Stimulation of immunoglobulin secretion with interleukin-4 (*IL-4*). Epstein-Barr virus-transformed lymphoblastoid B cell lines (*EBV-LCL*) from five different donors (A–E) were cultured with IL-4, low molecular weight B cell growth factor ($BCGF_{low}$) or medium alone, and secretion of IgM, IgG and IgA measured after 8 days. The results are expressed as mean immunoglobulin concentrations from triplicate cultures ±1 SEM

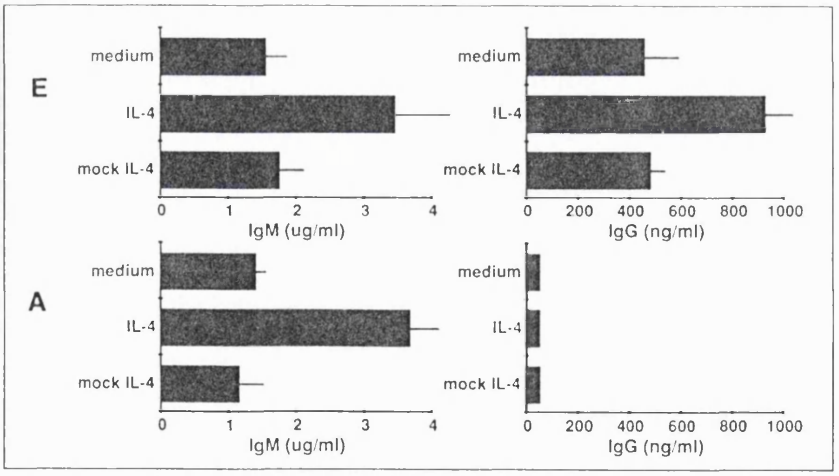

Fig. 2. EBV-LCL from two donors (A and E) were cultured with IL-4, mock IL-4 from supernatants of L cells not transfected with IL-4 cDNA or medium alone. Secretion of IgM and IgG was determined after 8 days and the results expressed as the mean immunoglobulin concentrations from triplicate cultures ±1 SEM

sured in the same culture wells, immunoglobulin secretion was shown to be enhanced by IL-4 with no increase in cell numbers (Fig. 3).

Effect of IL-4 on immunoglobulin secretion by tonsillar B cells polyclonally activated with EBV

Tonsillar B cells were stimulated with EBV and cultured with and without IL-4. Secretion of IgM, IgG, IgA and IgE was determined on the 12th day. At this time, the cells had been polyclonally activated with EBV, but no transformed B cell clones emerged. IL-4 at a concentration of 100–200 units/ml had no effect, or in some cases slightly inhibited secretion of IgM, IgG and IgA (data not given). In contrast, low concentrations of IL-4 (5 units/ml) significantly increased the production of IgM, IgG and IgA with no preferential effect on any one isotype (Table 2). No IgE secretion was detected, with or without IL-4, by an ELISA with a sensitivity of 200 pg/ml.

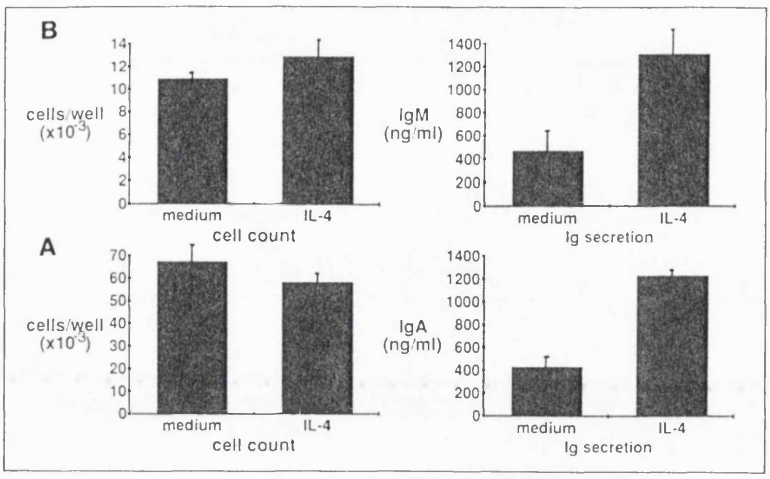

Fig. 3A, B. Relationship between cell division and immunoglobulin secretion in the same culture wells of EBV-LCL stimulated with IL-4. EBV-LCL from two different donors (**A** and **B**) were stimulated with IL-4 or medium alone. Cell counts and immunoglobulin concentrations were determined in each culture well after 8 days. The results are expressed as mean values from triplicate wells ±1 SEM

Table 2. Effect of IL-4 on immunoglobulin secretion by tonsillar B cells stimulated with EBV

Stimulation	Immunoglobulin secretion (ng/ml)			
	IgM	IgG	IgA	IgE
IL-4	265 ± 56	166 ± 17	383 ± 78	<0.2
EBV	2,295 ± 477	544 ± 27	856 ± 103	<0.2
EBV + IL-4	7,361 ± 96	1,227 ±	3,972 ± 974	<0.2

Discussion

IL-4 is known to have multiple activities on B cells. Small resting B cells activated with IL-4 increase their cell volume and expression of surface antigens such as MHC class II [11], CD23 [5] and surface IgM [17]. Co-stimulation of mouse or human B cells with IL-4 and anti-IgM (or SAC induces cell division but not immunoglobulin secretion. Moreover, IL-4 is not a T cell replacing factor in specific antibody responses by human B cells [18]. Together, these results suggest that IL-4 exerts its effect early on in normal B cell responses. Although IgE and IgG1 switching in cultures of mouse spleen cells stimulated with LPS and IL-4 is sometimes referred to as differentiation by IL-4, it is more likely to be an early (activation) rather than late (differentiation) event initiated by IL-4 [19, 20]. More recently, IL-4 has been shown to enhance IgM and IgG secretion by B cell blasts obtained by prior stimulation with *SAC* [7], or SAC plus IL-2 [21], suggesting that IL-4 may also be a late-acting BCDF.

Our results here show that immunoglobulin secretion by EBV-LCL stimulated with IL-4 is similarly increased. No significant cell division occurred in response to IL-4, and when cell counts and immunoglobulin concentrations were measured in the same wells, it was clear that IL-4 enhanced immunoglobulin secretion without increasing proliferation (Table 1, Fig. 3). IL-4 is therefore a differentiation factor but not a growth factor for activated B cell blasts.

IgM, IgG and IgA secretion by tonsillar B cells polyclonally activated with EBV was also increased with IL-4 (Table 2). Immunoglobulin secretion was measured on the 12th day in these experiments before the emergence of transformed B cells lines on about day 21. The increase in IgM, IgG and IgA occurred only with low concentrations of IL-4 (5 units/ml). When high concentrations of IL-4 (100 units/ml) were used, immunoglobulin secretion did not increase, and in some cases was inhibited. The different effects of IL-4 at low and high concentrations is consistent with our recent work indicating the existence of high- and low-affinity IL-4 receptors coupled to different signalling pathways [15].

IL-4 had no selective effect on the immunoglobulin isotype (IgM, IgG and IgA) secreted by the EBV-LCL or tonsillar B cells activated with EBV. Moreover, no IgE was detected in supernatants from any of the lines whether stimulated with IL-4 or not. These results suggest that IL-4 is unable to induce heavy chain switching in differentiated B cells (EBV-LCL), even those secreting IgM, and are consistent with previous observations that switching occurs by the action of IL-4 on resting cells prior to proliferation and immunoglobulin secretion [19, 20]. Tonsillar B cells stimulated with low concentrations of IL-4 also failed to secrete detectable levels of IgE. This finding is entirely consistent with the ability of IL-4 to enhance IgM and IgA, but not IgE secretion by SAC-stimulated B cell blasts [7]. It is however at variance with the work of others who have shown that IL-4 increases IgE secretion by long-term EBV-activated B cells [23]. The reason for this discrepancy is not known. It may be due to the absence in our B cell preparations of T cells necessary for IgE production [24], and/or to the use of RPMI 1640 medium rather than Iscoves IMDM which has been shown to be necessary for optimal IgE secretion. Taken together, our results indicate that IL-4 may be a differentiation factor as well as a growth factor and a switch factor.

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