THE REGULATION OF SPECIFIC ANTIBODY ~~RESPONSES~~ SECRETION BY HUMAN B CELLS THROUGH CONTACT AND NON-CONTACT DEPENDENT MECHANISMS

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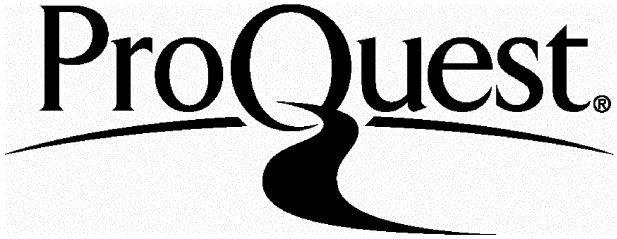

ABSTRACT

Antibody responses by human B cells to T-dependent antigens requires both cell contact-dependent signals and signals mediated by soluble molecules (cytokines) secreted by activated lymphocytes and accessory cells. This project utilised a well-established experimental system to study the role of CD40 and its ligand (CD40L) and selected cytokines in the regulation of specific antibody production *in vitro.*

Cross-linking of CD40 expressed on human tonsil B cells with monoclonal antibodies or exogenous CD40L profoundly inhibited specific antibody production. Inhibition was an early event and was lost if CD40 ligation occurred beyond the first 24 - 48 hours during a seven day culture period. We propose that following antigenic challenge, ligation of CD40 on antigen-specific B cells prevents immediate terminal differentiation into antibody forming cells and favours clonal expansion and memory cell formation.

The importance of CD40-CD40L in immune responses is highlighted by patients with hyper-IgM syndrome. In the X-linked form of the disease, the defect is caused by a mutation in the CD40L gene but non-X-linked patients may have a B cell defect. It was shown that, in some X-linked patients, IgG or IgA secretion could be restored *in vitro* by the addition of a functional CD40L and IL-10.

Studies with cytokines showed that IL-4 inhibited antibody secretion without any preferential effect on any isotype or subclass whilst IL-13 had no consistent effect. In

contrast, IL-15 was able to support specific antibody secretion in the absence of T cells indicating that it may have a similar role to IL-2 in specific antibody responses.

These findings are consistent with a vital role for CD40 and cytokines (IL-2 and IL-15) in regulating specific antibody responses but highlights the fact that biological activity cannot always be predicted from polyclonal (mitogenic) activation of human B cells.

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CONTENTS

ABSTRACT **2**

ACKNOWLEDGEMENTS **4**

CONTENTS **5**

CHAPTER 1 INTRODUCTION **15**

1.1 OVERVIEW **16**

1.2 B CELL ONTOGENY 17

1.2.1 The surrogate light chain 21

1.2.2 IgM is the B cell receptor on mature B cells 23

1.3 STUDYING B CELL RESPONSES: ANTIGENS AND MITOGENS 26

1.3.1 Mitogens 26

1.3.2 Activation by antibodies to cell surface molecules 28

1.3.3. Antigen-specific B cell responses 28

1.4 RESPONSE OF HUMAN B CELLS TO T-DEPENDENT ANTIGENS 29

1.4.1 Antigen Presentation 29

1.4.2 Activation and proliferation 31

1.4.3. Germinal centre reactions 30

Isotype switching 32

Somatic Hypermutation and affinity maturation 33

Apoptosis 34

1.4.4 Generation of antibody-forming cells and memory cells 35

1.5 CELL SURFACE MOLECULES THAT REGULATE B CELL RESPONSES 36

1.5.1 The B7 family, CD28 and CTLA-4 37

1.5.2 CD11a/CD18 and CD54 38

1.5.3. CD21 and CD23 38

1.5.4 CD58 and CD59 39

1.5.5 CD72 and CD5 39

1.6 CD40 AND CD40 LIGAND 40

1.6.1 CD40 40

Other members of the NGFR Superfamily 40

Distribution of CD40 42

Functional properties of CD40 on human B cells 43

Signal transduction through CD40 43

1.6.2 CD40L 44

1.6.3 CD40 and CD40L in T-dependent B cell responses 45

1.6.4 CD40 and CD40L interactions in class switching and antibody secretion 45

1.6.5 Hyper-IgM syndrome and CD40-CD40L interactions 46

Clinical characteristics 46

X-linked and non X-linked HIM 47

XHIGM 48

1.6.6 CD40-CD40L interactions on other cell types 49

1.7 ROLE OF CYTOKINES IN T-DEPENDENT B CELL DIFFERENTIATION 50

1.7.1 Th cell subsets are defined by their cytokine profile 51

1.7.2 Cytokines acting on human B cells 52

1.8. SPECIFIC ANTIBODY SECRETION IN T-DEPENDENT B CELL RESPONSES 61

1.9 AIMS OF THE STUDY 64

CHAPTER 2 MATERIALS AND METHODS 65

2.1 TISSUE CULTURE 66

2.1.1 Culture Medium 66

2.1.2 Holding Medium 66

2.2 CYTOKINES 66

2.3 ANTIBODIES USED IN FLOW CYTOMETRY 67

2.4 CD40 AND CD40 LIGAND 68

2.4.1. CD40 and CD40L antibodies 69

2.5 INFLUENZA VIRUS 70

2.6 CELL PREPARATION 70

2.6.1 Preparation of tonsil mononuclear cells (TMC) 70

2.6.2 Preparation of peripheral blood mononuclear cells (PBMC) 71

2.6.3 Separation of B and T cells by E-rosetting 72

Preparation of Percoll 72

Preparation of AET-treated sheep red blood cells (AET-SRBC) 72

Preparation of Gey's haemolytic balanced salt solution 73

Rosette formation 73

2.6.4 Preparation of heavy and light B cells 74

2.6.5 Monocyte depletion of E- preparations from TMC 74

2.7 FACS STAINING PROTOCOL 75

2.8 ASSAY FOR B CELL PROLIFERATION 76

2.9 ASSAYS FOR B CELL DIFFERENTIATION 76

2.9.1 Specific antibody responses 76

2.9.2 Polyclonal antibody responses by peripheral blood B cells 77

2.10 ELISA FOR THE DETECTION OF SECRETED IMMUNOGLOBULINS 78

2.10.1 Buffer solutions used for ELISA 78

2.10.2. Reagents used for ELISA 78

2.10.3 ELISA for specific antibody secretion 79

ELISA for specific IgM, IgG and IgA 79

ELISA for specific IgG subclasses 80

2.10.4 ELISA for polyclonal antibody secretion 81

Measurement of total IgG, IgM and IgA 81

Measurement of total IgE secretion 82

Measurement of IgG subclasses 83

CHAPTER 3 THE ROLE OF CD40-CD40L INTERACTIONS IN SPECIFIC ANTIBODY PRODUCTION 84

3.1 INTRODUCTION 85

3.2 CHARACTERISTICS OF THE SPECIFIC ANTIBODY RESPONSE TO INFLUENZA VIRUS 87

3.3 EFFECT OF CD40L ON SPECIFIC ANTIBODY PRODUCTION 88

3.4 EFFECT OF CD40 LIGATION ON SPECIFIC ANTIBODY RESPONSES BY T-DEPLETED B CELLS 89

3.5 INHIBITION OF SPECIFIC ANTIBODY RESPONSES BY SOLUBLE TRIMERIC CD40L 93

3.6 EFFECT OF sCD40L ON SPECIFIC AND POLYCLONAL ANTIBODY SECRETION BY ANTIGEN-STIMULATED B CELLS 96

3.7 TIMECOURSE OF ACTION OF CD40L ON SPECIFIC ANTIBODY SECRETION 101

3.8 COMPARISON OF mAbs TO CD40 WITH DIFFERENT EPITOPE SPECIFICITIES ON SPECIFIC ANTIBODY SECRETION AND B CELL PROLIFERATION 105

3.9 DISCUSSION 111

CHAPTER 4 CD40L AND THE HYPER-IgM SYNDROME 122

4.1 INTRODUCTION 123

4.2 PATIENTS, CARRIERS AND PARENTS INVOLVED IN THE CLINICAL STUDY 124

4.3 CD40L EXPRESSION OF ACTIVATED T CELLS 126

4.4 EXPRESSION OF CD40 AND CD19 128

4.5 PROLIFERATION AND ANTIBODY SECRETION BY B CELLS FROM HIM PATIENTS 129

4.5.1 XHIGM patients 129

4.5.2 Non-X-linked patients 133

4.6 EFFECT OF IL-10 AND CD40L ON ANTIBODY SECRETION 136

4.7 IgE SECRETION BY b CELLS FROM NON-X-LINKED PATIENTS STIMULATED WITH IL-4 AND CD40L 138

4.8 DISCUSSION 139

CHAPTER 5 EFFECT OF IL-4 ON HUMAN SPECIFIC ANTIBODY RESPONSES 147

5.1 INTRODUCTION 148

5.2 CHARACTERISATION OF THE ISOTYPES AND IgG SUBCLASSES SECRETED BY HUMAN TMC IN THE SPECIFIC ANTIBODY RESPONSE TO INFLUENZA VIRUS 149

5.3 EFFECT OF IL-4 ON SPECIFIC ANTIBODY ISOTYPE PRODUCTION BY UNSEPARATED TMC 151

5.4 COMPARISON OF HIGH AND LOW DOSES OF IL-4 ON SPECIFIC IMMUNOGLOBULIN ISOTYPE AND IgG SUBCLASS ANTIBODY PRODUCTION BY TMC 153

5.5 EFFECT OF HIGH AND LOW DOSES OF IL-4 ON THE SPECIFIC IMMUNOGLOBULIN ISOTYPE AND IgG SUBCLASS ANTIBODY RESPONSES BY PURIFIED B CELLS STIMULATED WITH ANTIGEN 156

AND TRF

5.6. COMPARISON OF THE EFFECTS OF IL-13 AND IL4 ON SPECIFIC ANTIBODY PRODUCTION 158

5.7. DISCUSSION 160

CHAPTER 6 COMPARISON OF IL-2 AND IL-15 IN SPECIFIC ANTIBODY PRODUCTION 166

6.1 INTRODUCTION 167

6.2 EFFECT OF IL-15 ON B CELL PROLIFERATION 168

6.3 T-CELL REPLACING FACTOR ACTIVITY OF IL-15 170

6.4 EFFECT OF IL-2 AND IL-15 ON DIFFERENT SUB-POPULATIONS OF B CELLS 173

6.5 DISCUSSION 175

CHAPTER 7 GENERAL DISCUSSION 178

BIBLIOGRAPHY 188

TABLES

2.1 Panel of mAbs to CD40 69

3.1 Specific antibody production by tonsil B cells 87

3.2 Effect of G28.5 and CD40L expressed on CV1/EBNA cells on specific antibody production by TMC 90

3.3 Effect of CD40L expressed on CV1/EBNA cells on specific antibody production by purified B cells in the presence of a TRF. 91

3.4 Inhibition of specific antibody response with a soluble form of CD40L 95

3.5 Proliferation of human B cells in response to CD40L or mAbs to CD40 108

3.6 Proliferation of human B cells in response to soluble trimeric CD40L 108

3.7 Summary of the effects of a panel of mAbs to CD40 on proliferation and specific antibody production by B cells 110

4.1 Patients, carriers and parents involved in the clinical study 125

4.2 Proliferation of XHIGM B cells in response CD40 ligation 130

4.3 Immunoglobulin secretion by XHIGM B cells stimulated with CD40L and IL-2 132

4.4 Proliferation of B cells from patients with non-X-linked HIM in response to CD40L and IL-2 133

4.5 Immunoglobulin secretion by non-X-linked HIM B cells stimulated with CD40L and IL-2 135

4.6 Effect of IL-10 and CD40L on immunoglobulin secretion 137

4.7 IgE secretion by B cells from non-X-linked patients stimulated with IL-4 and CD40L 139

5.1 Antibody isotypes and IgG subclasses produced in the specific antibody response to influenza virus by human TMC 150

5.2 Effect of high and low doses of IL-4 on specific antibody secretion by TMC 154

6.1 IL-15 is a T-cell replacing factor 170

6.2 Effect of the presence of both IL-2 and IL-15 on TRF activity in the specific antibody response 172

6.3 Comparison of TRF activities of IL-2, IL-15 and $BCGF_{lmw}$ on different B cell populations 174

FIGURES

1.1 Early events in murine and human B cell development 19

1.2 Signal transduction mechanisms in human B cells 25

1.3 Structure of CD40 41

3.1 Effect of sCD40L on polyclonal and specific IgG secretion by antigen-stimulated B cells 98

3.2 Effect of sCD40L on polyclonal IgM secretion by antigen-stimulated B cells 100

3.3 Timecourse of action of CD40L in the specific antibody response by TMC 103

3.4 Timecourse of action of CD40L inhibition of the specific antibody responses by purified B cells cultured with antigen and TRF 104

3.5 Effect of CD40mAbs on specific antibody production by human TMC 106

3.6 Effect of a panel of mAbs to CD40 on B cell proliferation 109

4.1 Expression of CD40L on activated T cells from patients with HIM 127

5.1 Effect of IL-4 on the secretion of specific total IgG and IgA by TMC 152

5.2 Effect of high and low doses of IL-4 on specific antibody secretion by B cells 157

5.3 Comparison of the effects of IL-13 and IL-4 on specific antibody production 159

6.1 IL-15 is a B cell growth factor 169

CHAPTER 1

INTRODUCTION

1.1. Overview

All vertebrates with jaws (Gnathostomata) have B lymphocytes. These cells are unique in their ability to secrete a highly diverse and specific repertoire of immunoglobulins - the soluble proteins which mediate humoral immunity. B cells are derived from pluripotent haemopoietic stem cells which become committed at an early stage to the B lineage. In humans, this complex series of developmental processes occurs within the microenvironment of the bone marrow (adults) or foetal liver. From there, the B cell matures and enters the secondary lymphoid tissues (peripheral blood, tonsil, spleen, Peyer's Patches and lymph nodes) where it is capable of recognising foreign and potentially pathogenic antigens. If it then receives appropriate signals from T cells and accessory cells, the B cell will proliferate and mature into an antibody forming cell (AFC) and other B cells will become memory cells. The whole process of development from stem cell to plasma or memory cell is tightly regulated and successful passage through each phase is dependent on the B cell receiving specific signals mediated via contact with other cells and soluble factors.

There are several 'themes' that occur throughout the various stages of B cell development: i) contact-mediated signals between the B cell and other principle effector cells e.g. T lymphocytes and dendritic cells. ii) Cytokine-mediated signals that regulate growth and differentiation at each stage of the B cell response. iii) The microenvironment in which the B cell resides, (e.g. bone marrow, germinal centre) that provides further essential signals via interactions with accessory cells for

successful maturation during each stage iv) Antigen-dependent and antigen-independent phases are another crucial aspect in development.

Research into B cell physiology is an area of intense activity and new developments are constantly being made. The research for this thesis has focused on signals required for differentiation into AFCs - the ultimate goal of the B lymphocyte.

1.2. B cell ontogeny - from stem cell to mature B cell

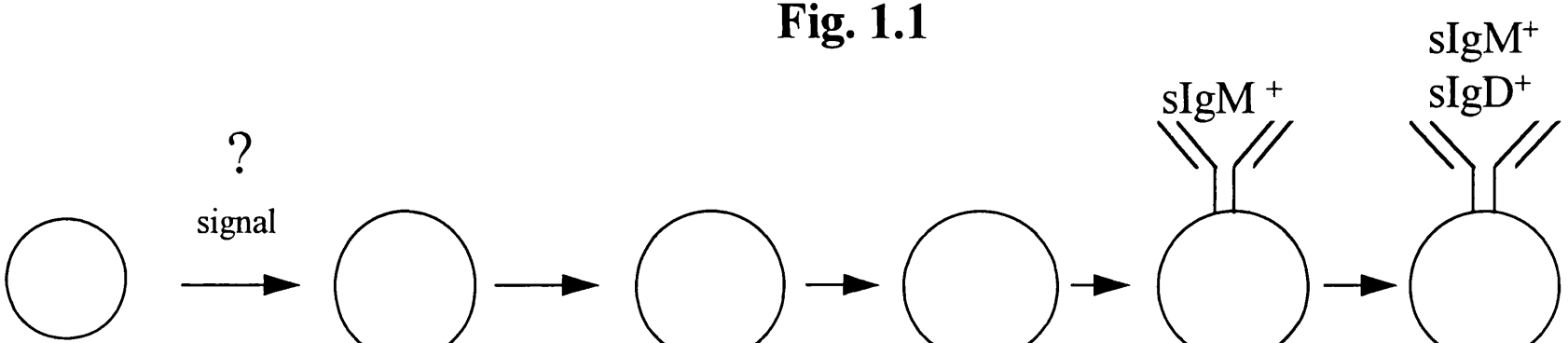
The major outcome of the early phase of B cell development is the expression of IgM with a unique specificity for foreign antigen on the surface of the mature B cell. The immunoglobulin molecule consists of two identical light (L) chains (either λL or κL chains) and two identical heavy (H) chains. The carboxy-terminal regions of the H and L chains form the constant (C) region of the molecule which defines the isotype and is primarily concerned with effector functions such as Fc receptor binding and complement fixation. The amino-terminal ends (V region) have variable amino acid sequences including three hypervariable regions known as the complementarity determining regions (CDRs) which interact with antigen and confer the specificity of the antibody and four less variable framework regions (FR). The H, κL and λL immunoglobulin polypeptide chains are encoded by multiple gene segments situated in humans on chromosomes 14, 2 and 22 respectively. The diversity of the antibody repertoire is generated during the pre-B stage onwards via successive rearrangements

of these gene segments at the H chain loci followed by rearrangement of the L-chain (see below).

Much of the knowledge gained so far about B cell ontogeny has relied on mouse models. Progress in the human system has been much slower due to a lack of appropriate assays and the low frequency of progenitor cells but it appears that murine and human systems are very similar in many aspects. The process of early B cell development can be divided into several distinct stages defined by the expression of specific cell surface markers (56,273,345,346). Different researchers use their own nomenclature for the various stages. In this thesis, I will use the nomenclature of Rolink and Melchers *et al* which, although specifically describes mouse B cell development, is also appropriate for human cells (Fig.1.1) (345). Their scheme of B cell development identifies distinct stages from multipotent stem cells through pro and pre-B stages (which are further subdivided into pre-BI and pre-BII), immature B and finally mature B cells.

In mice, B cell development in embryos occurs in 'waves' starting, initially, from the placenta and embryonic blood and later the foetal liver and spleen and finally in the bone marrow. In humans, B cells originate pre-natally from foetal liver. In adults all B cells originate from the bone marrow. The early stages of development are dependent upon interactions with the stroma for regulatory signals (217,243). All the cell lineages of the haemopoietic system (i.e. lymphoid, erythroid, myeloid) are considered to be derived from a population of pluripotent stem cells which can be defined as self-

Fig. 1.1



Murine:					
Thy 1.1^{lo}	B220(murine)	B220	B220	RAG-1	RAG-1$^-$
Sca. 1$^+$	RAG-1,RAG-2	RAG-1,RAG-2	RAG-1,RAG-2	RAG-2	RAG-2$^-$
Fall-3$^+$	TdT, CD43	TdT, CD43	CD25, cyto-μ		
Lin$^-$	c-kit, IL-7R	c-kit, cyto-μ	TdT, c-kit		
	cyto-μ	ψL	CD43, ψL		
Human:					
Thy-1^{lo}	**CD34**	**CD34**	**CD19**		
Lin$^-$	**CD19?**	**CD19**	**CD10**		
CD34^{hi}	**ψL?**	**CD10,**	**cytoμH**		
			CD34$^-$ TdT$^-$		
H:	G, D - J,DJ	VDJ productive	VDJproductive	VDJ productive	
L:	G	$G_{(early)}$ - Vκ -Jκ,Vλ - Jλ	Vκ ,Jκ, Vλ ,Jλ Vκ -Jκ,Vλ - Jλ	Vκ ,Jκ, Vλ ,Jλ	

Fig. 1.1 Early events in murine and human B cell development

Model of normal murine B cell development showing expression of major cell surface markers and gene rearrangements in the heavy (H) and light (L) chains. G denotes germline configuration. Cell surface markers specific for human B cells are also indicated.

renewing cells capable of re-populating lethally irradiated or SCID mice (207,379). Murine stem cells are defined phenotypically by expression of the surface markers Thy-1.1^{lo}, Sca-1^{+} and also Fall-3 but lack any lineage markers (Lin^{-})(398,399). In human foetal bone marrow, a small population (0.05 - 0.1%) of cells with stem cell activity has been identified. Phenotypically they are Thy-1^{lo}, Lin^{-} and CD34^{hi} and give rise to donor-derived B and T cells when engrafted into SCID mice (24,113). A similar population has also been identified in human peripheral blood (287).

In both mouse and human systems, the signals that direct a stem cell towards a specific lineage remain elusive, but once the stem cell is committed to the B lineage it is known as a (progenitor) pro-B cell. The earliest murine B cells express the antigen B220 which persists until the terminally differentiated plasma cell stage. In humans, CD19 is recognised as a pan-B cell marker but the precise time at which it is first expressed has not been confirmed (20). Murine pro-B cells express recombination activation genes, RAG1 and RAG2 (304,365) and the enzyme terminal deoxynucleotidyl transferase (TdT) that increases antibody diversity by the insertion of non-specific 'N' nucleotides at VDJ junctions during recombination (4). They also express CD43 and receptors for IL-7 and Sc factor (c-kit) (399). The VpreB and λ5 gene products are also present, but there is no surface expression at this stage and both the H and L chain genes remain in germ-line configuration. Pro-B cells are capable of populating bone marrow in irradiated mice (305,346) and are dependent on the stroma for contact and growth factor-mediated signals (341,346).

At the next stage, murine pre-B-I cells express B220, c-kit, CD43, RAG-1, RAG-2, TdT and VpreB and are capable of long term proliferation *in vitro* in the presence of stromal cells and IL-7 (344). H-chain rearrangements begin with D_H to J_H segments but the L chain remains in germ-line configuration (345). Early human pre-B cells express CD19, CD34 CD10, TdT and VpreB (20,56,169). Isolated human $CD34^+$ $CD19^+$ cells from bone marrow will grow in culture in the presence of IL-7 resulting in increased CD19 expression and decreased TdT, RAG-1 and RAG-2 (275).

The transition of pre-B-I cells to pre-B II cells is defined by the presence in the cytoplasm of productively rearranged $V_HD_HJ_H$ H-chain locus in >80% of the cells (346). Additionally, CD25 expression is induced (343) while expression of c-kit, CD43 and TdT is decreased. Pre-B-II cells retain expression of B220, RAG1 and RAG2 but no longer have the capacity for long-term proliferation on stromal cells (346). A population in humans which expresses cytoplasmic μH, CD19 and CD10 but lacks CD34 and TdT may correspond to pre-B II cells in mice. Early or large pre-BII cells retain their L-chain genes in germline configuration whereas at the end of this stage of development, 70% of cells have productively rearranged light chains (396) that marks the transition to immature B cells.

1.2.1. The surrogate light chain.

Pre-B cells were originally considered to be lacking surface antigen-receptor as they only expressed the μH-chain which undergoes intracellular degradation unless associated with the L-chain. More recently, the products of the λ5 and VpreB genes (pseudo L-chains, ψL) (231,358) in mice and their human counterparts (40,76) were found to associate with the μH to form a surrogate immunoglobulin which was then

expressed on the cell surface (reviewed in 274). Surface expression of the ψL on normal mouse B cells seems to be restricted to the pro-/pre-BI and large pre-BII cells (213) whereas in cell lines transformed by the Abelson leukaemia virus expression occurs at various stages of development. In humans, expression was reported to occur relatively late during the pre-B cell stage in conjunction with Igα and Igβ as well as μH (242,291), but a population of early B cells that express ψL on their surface without μH has also been identified that may correspond to the pro-B cell stage in mice (169).The precise function of the surrogate light chain remains undefined but it is likely to have a positive role in B cell development as mice that are deficient for the λ5 gene have severely reduced numbers of both pre-BII and mature B cells (221). It is a possibility that the complex transduces signals from adhesion molecules and/or cytokines to regulate further steps of development. This is supported by the observation that pre-B cells in both mice and humans trigger Ca++ mobilisation and phosphorylation patterns distinct from mature B cells upon cross-linking with anti-μ (41,52,278).

In immature B cells, productive rearrangements of $V_HD_HJ_H$ and V_LJ_L are complete and most cells express sIgM. It is possible that some surrogate light chain is still expressed (80), but sIgD is absent. At this stage, the developing B cell is no longer stromal-dependent and becomes responsive to stimulation by antigen. Immature B cells display clonal anergy and are unable to proliferate or secrete immunoglobulin upon exposure to antigen (261). These B cells retain RAG1 and RAG2 and can undergo secondary L-chain rearrangements (receptor editing) a mechanism that may function to reduce autoreactivity (342,397).

1.2.2. IgM is the B cell receptor on mature B cells

Pro-B, pre-B and immature sIg^+ cells have a high rate of production and turnover and only a very small percentage are then recruited into the mature B cell pool. Membrane-bound IgM captures antigen, but its short intracytoplasmic tail of only three amino acid residues renders it unsuitable for efficient signal transduction. sIgM is non-covalently associated with two molecules, Igα and Igβ (CD79α and β) (70,77,194,311) (320),(review) which are the products of the Ig-superfamily genes mb1 and B29 (182,357) respectively, to form the B cell receptor (BCR). A conserved amino acid motif termed ARH1 (antigen receptor homology 1)(89) has been identified in the cytoplasmic regions of a number of receptor molecules and viral proteins (335) including Igα and β which is rapidly phosphorylated following antigen-binding to sIgM. This event leads to increased binding and activation of multiple tyrosine kinases associated with the BCR including the *src*- family kinases Fyn, Lyn and Blk and the more distantly related Syk (55,199,200,424,425).

At least three biochemical signalling cascades have been identified that follow the initial tyrosine kinase activation events: i) phosphatidylinositol 4,5-bisphosphate specific phospholipase C (PLC) pathway (72,91), ii) the activation of a G-protein $p21^{ras}$ and iii) the activation of phosphatidylinositol 3-kinase (PI3-k) (Fig. 1.2). These pathways are considered to have an important role in the downstream events that regulate gene transcription. Briefly, the hydrolysis of PIP_2 by PLCγ2 (the predominant isoform of PLC present in B cells) yields inositol 1,4,5-trisphosphate (InsP3) and diacylglycerol (DAG) which act as second messengers to regulate the activation of various protein kinase C molecules (293). InsP3 also regulates calcium

influx and the release of Ca^{++} from intracellular stores in the endoplasmic reticulum. The activation of G-protein, p21ras occurs within 1-2 mins. of BCR ligation and initiates a cascade of downstream events including the activation of c-raf and the serine/threonine phosphorylation of microtubule-associated protein 2 kinase and also microtubule-associated kinase 2 (MAPk)(133,175,316). Finally, the activation of PI3-k phosphorylates the inositol ring of inositol phospholipids which has been suggested to regulate cellular activity through the platelet-derived growth factor receptor (71,404).

In addition to the components of the BCR, a number of co-receptor molecules have been described that are associated with the BCR and which modify BCR function. These include FcγRIIb which, when cross-linked, results in premature termination of extracellular Ca^{++} influx and InsP3 generation and has a negative effect on activation (33,423). CD20 may also regulate Ca-mediated signalling, and is itself a calcium channel under regulation by PKC and calmodulin-dependent protein kinase II(CaM-KII)(394). In contrast, the CD19/CD21 complex and also CD22 act to lower the threshold of B cell activation by recruiting additional *src*- and *syk*- family PTKs and enhancing PLC and PI3-kinase activity (215,244,395). Igα/β, CD19 and CD22 are all substrates for the tyrosine phosphatase CD45 and reflect the importance of signals

Fig. 1.2

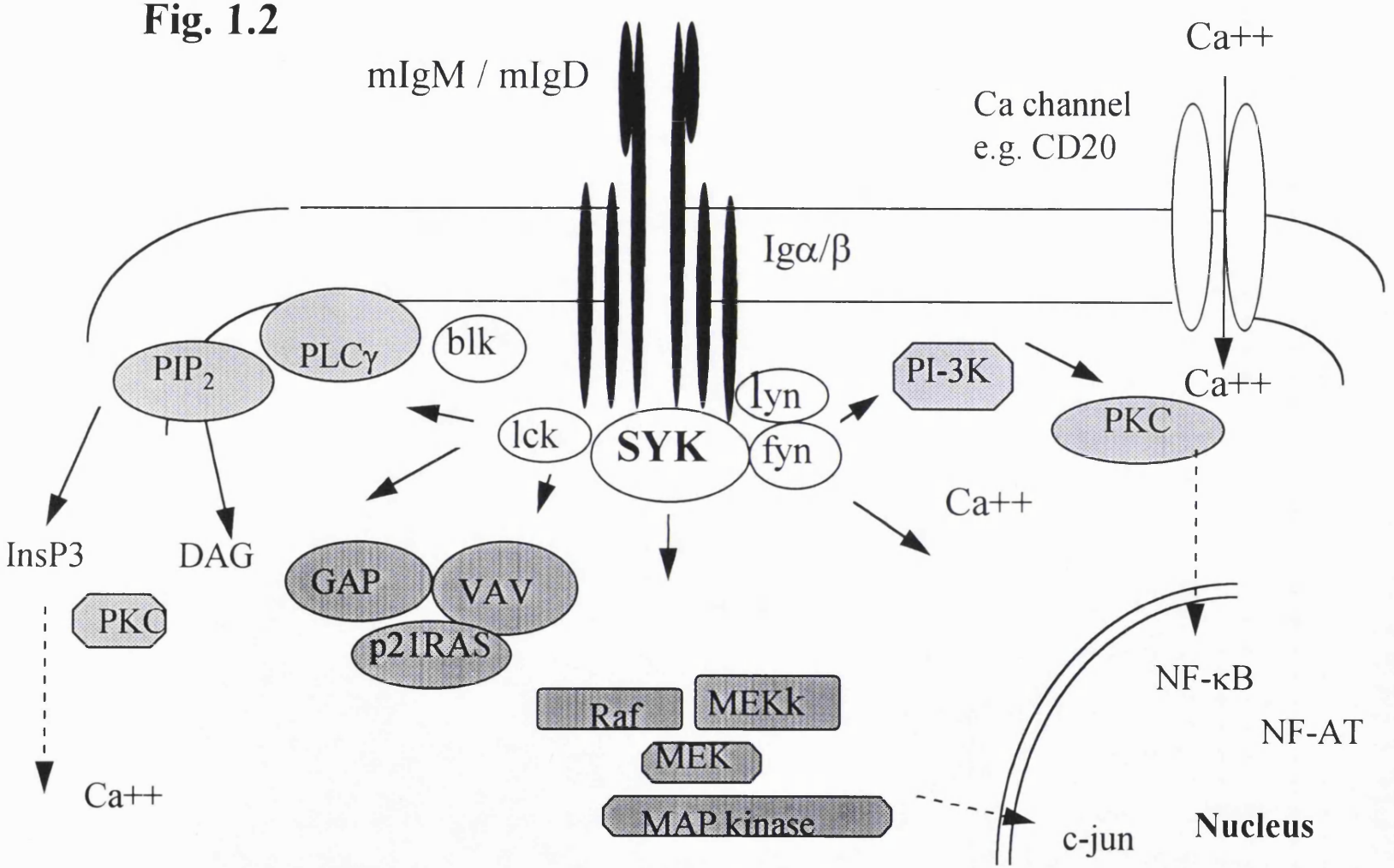


Fig. 1.2 Transmembrane signalling in mature B cells
Summary of main signalling events mediated by cross-linking sIg.
Adapted from Cambier et al 1994 (68).

mediated by phosphatase and kinase activities between the BCR and other co-receptors (210).

1.3. Studying B cell responses - antigens and mitogens

In order to elucidate the mechanism of regulation of B cell responses, a number of *in vitro* assays have been developed which are assumed to mimic *in vivo* events such as activation, proliferation and antibody secretion. A considerable array of 'agents' are commonly employed to stimulate B cell activity and will be described briefly here.

1.3.1..Mitogens

Chemical mitogens include calcium ionophore and ionomycin that induce an intracellular calcium flux and phorbol esters such as PMA which directly activate PKC (322,329). High concentrations of PMA (>2ng/ml) are directly mitogenic for B cells, while co-stimulation with ionomycin and sub-mitogenic doses of PMA delivers a stronger proliferative signal and the addition of cytokines can induce antibody secretion. The synthetic polymers, Ficoll and dextran also have mitogenic activity (54). These are large molecules with a large number of repeated antigenic determinants which induce B cell activation via extensive cross-linkage of surface immunoglobulin.

Other mitogens are derived from plants or bacteria and include lipopolysaccharide (LPS- mainly used for murine B cell activation) (132), *Staphylococcus aureus* protein

A (SPA) (349), Bacto-streptolysin O (SLO) (416), *Nocardia* water soluble mitogen (NWSM) (416) and *Staphylococcus aureus* Cowan I (SAC) and the commonly used pokeweed mitogen (PWM) (216,322). PWM is strongly mitogenic for human B cells and induces proliferation and secretion of IgM, IgG and IgA, but not IgE (359,416). The failure to induce IgE may reflect an inability to recruit appropriate Th cells or activate IgE-specific B cell precursors. The IgG subclasses are induced in approximately the same proportions as occur in normal serum suggesting that PWM is a crude B cell activator with no immunoglobulin isotype selectivity (apart from the absence of IgE) (283,368,417). In contrast, LPS and *Staph. aureus* preferentially induce IgG2 and IgG3 (417). SAC is a bacterial preparation and an efficient stimulator of proliferation and differentiation. The bacterial outer coat is extensively glycosylated which may cause cross-linking of the B cell receptor. SAC has recently been used in an assay that cross-links CD40 in the presence of cytokines where it appears to amplify the CD40 and cytokine-mediated signals resulting in enhanced immunoglobulin secretion (103,227).

A number of the mitogens described above are able to stimulate B cells in the absence of MHC II-restricted T cell help and are classed as T-independent (TI). They have the ability to deliver prolonged and persistent signals to the B cell. They tend to have a large molecular weight, a high number of repeated antigenic determinants, can activate the complement cascade and are resistant to degradation. Mitogens such as PMA and SAC are described as T-independent but they still require T cells or T cell products for optimal immunoglobulin secretion (329,338).

The Epstein Barr Virus (EBV), on the other hand, is a true T cell independent mitogen for human B cells (34,218). EBV binds to CD21 receptors on B cells and the early stages of infection are characterised by polyclonal proliferation and immunoglobulin secretion (120,218). Subsequently a small number of B cells may undergo transformation. These have been found to be mainly a resting population of IgM+/IgD+ cells. EBV has proved a useful tool for the study of B cells *in vitro* (prior to transformation) as the B cells remain sensitive to signals from cytokines and other molecules and retain the ability to undergo isotype switching (385).

1.3.2. Activation by antibodies to cell surface molecules

It is possible to activate B cells using antibodies to sIg - usually anti-IgM antibodies, which may be soluble or coupled to dextran or Sepharose beads (54). Antibodies to other cell surface antigens e.g. CD20, CD40 or CD19 also induce B cell activation (85,157,162,336). All these agents are only weakly mitogenic by themselves and require co-stimulatory signals, for example from cytokines such as IL-4, to induce proliferation and differentiation (160).

1.3.3. Antigen-Specific B cell responses

Human B cell responses can also be obtained *in vitro* to a number of specific antigens (reviewed in 59). These include tetanus toxoid (46), keyhole limpet hemocyanin (KLH) (233), sheep red blood cells (SRBC) (188) and several viruses including influenza virus (58), polio (401) and herpes (422). By and large these are memory responses, e.g. to influenza virus, and the predominant immunoglobulin secreted is IgG, although other antigens such as SRBC and KLH have been reported to induce IgM secretion only and may represent a primary response.

1.4. Response of human B cells to T-dependent antigens

The majority of antigen-driven human B cell responses are T-dependent (with the possible exception of TNP-Brucella abortus). B cells stimulated through the BCR require contact and cytokine- mediated help from activated Th cells to drive B cell proliferation and antibody secretion (reviewed in 310).

1.4.1. Antigen presentation

The first stage in the antibody response is antigen processing and presentation (239). Antigen is taken up by antigen-presenting cells (APC) and undergoes internal proteolytic degradation. Processed peptide fragments are then re-expressed on the cell surface in association with MHC II and the peptide-MHC complex is recognised by a Th cell with complementary TcR. The interaction may be strengthened via antigen-independent interactions between MHC II and CD4 (119). Antigen presenting cells include dendritic cells, monocytes and macrophages which take up antigen in a non-specific manner. Alternatively, a high degree of specificity may be achieved by antigen binding to the BCR on B cells which also function as APCs. This scenario is desirable as specific interactions have been demonstrated to be more efficient in initiating an immune response particularly, if antigen concentrations are limiting (240). Such an interaction may, however, be more relevant in secondary responses as B cells are not thought to be able to present antigen to naive T cells (241).

1.4.2. Activation and Proliferation

A successful interaction between antigen-MHC II complex and the TcR results in T cell activation which is followed by both the expression of cell surface molecules such as CD40L and the release of soluble cytokines such as IL-2. Activated T cells proceed to enhance B cell activation via contact-dependent mechanisms. B cell activation, following cross-linking of sIg is characterised by an increase in cell volume and total cellular RNA accompanied by expression of c-*myc* (67,222,286) and increases in the concentration of Ca^{++} (45) and the attachment of PKC to the cell membrane (78). The expression of cell surface molecules is increased (156) (e.g. MHC II, CD40) or induced (e.g. CD80[B7-1], CD86 [B7-2] and CD23) and the B cell proceeds from G_0 to G_1 in a cytokine-independent fashion. *In vitro*, efficient activation can be achieved through cross-linking s.IgM (67,222,236), MHC II (236) CD40 (162) or CD20 (85,87,157). Other competence signals have been identified including IL-4 (98,102), or chemical activators such as PMA (5), however these signals fail to induce a calcium influx. B cell proliferation *in vitro* is normally measured by the uptake of tritiated thymidine (^{3}HTdR). Studies into the requirements for proliferation have shown that following activation, progression to S phase requires additional signals e.g. from cytokines. Thus mAbs to s.IgM or CD40 acting alone are poor stimulators of proliferation, but can synergise with IL-4 to induce a strong proliferative signal (88,161).

1.4.3. Germinal centre reactions

In a primary response, an activated, naive, B cell may either become a plasma cell secreting IgM, or it may be recruited to B cell follicles giving rise to germinal centre formation where it is subject to class switching, affinity maturation and memory cell

development (reviewed in 262). A resting follicle consists largely of a follicular dendritic cell (FDC) network and a number of re-circulating B cells. FDCs and interdigitating cells (IDC's, also called dendritic cells) are two types of accessory cell with an important role in the microenvironment in B cell responses (82,386). Their origin is still uncertain (74,178,211). Dendritic cells appear to have haemopoietic origins but FDCs may be derived from mesenchymal and/or haemopoietic precursors. Both dendritic cells and FDCs are extremely efficient antigen-presenting cells. They express a large number of cell surface molecules including lymphocytic (e.g. MHC II, CD25) and epithelial cell markers (82). Dendritic cells also exhibit strong expression of functional CD40 (75). The characteristic phenotype of the FDC with elongated extensions (82) allows it to retain unprocessed antigen on its surface for long periods of time and it is thought to play a role in affinity maturation and memory cell formation.

Germinal centres arise in the first three weeks following exposure to antigen (193,257). In a typical response, the resting follicle is colonised, on average, by three activated B blasts (230,257). These cells then undergo rapid clonal expansion with a doubling time of only 6-7 hours and by 72 hours following immunisation the follicle takes on its characteristic appearance of a germinal centre with dark and light zones. Each compartment can be distinguished by the expression of various cell surface markers such as CD38, IgD/IgM, CD44, CD77 and CD23 (173,262). The B blasts, now termed centroblasts, migrate through the dark zone, a region packed with lymphoid cells and few FDCs, and give rise to centrocytes, a non-proliferating population that occupies the dense network of FDCs known as the light zone. It is

likely that isotype switching, somatic hypermutation and affinity maturation occur in this region

Isotype switching

There are five immunoglobulin classes (IgM, IgD, IgG, IgA and IgE), four IgG subclasses and two IgA subclasses that are associated with different effector functions. For example, IgE binds to FcεRI receptors on mast cells and basophils stimulating histamine release and is a mediator in allergic reactions (388). IgG1 is effective for combating viral protein antigens (374) and IgG2 is associated with responses to polysaccharide antigens and encapsulated bacteria (171). The process by which B cells undergo recombination of the VDJ region to other CH genes is isotype switching and is thought to occur in the germinal centre. Several mechanisms for switching have been proposed both at RNA and DNA levels (reviewed by Harriman *et al* 174) but the most likely one, for which there is some direct evidence, involves the looping out and deletion of segments of DNA followed by recombination (205). The precise mechanisms that direct and control class switching and the role of sterile transcripts and binding proteins are still to be clarified (174). The cutting and ligating of the DNA is assumed to be performed by a recombinase similar to VDJ recombinase. A recognition sequence (corresponding to the heptamer-nonamer sequences associated with VDJ-recombinase) is expected to initiate the binding of such a recombinase and may exist within the switch regions that precede each C gene segment at the H chain locus (except Cδ). Switching usually occurs after a committed B lymphocyte has been stimulated by antigen or mitogen and requires signals from cytokines and cell surface molecules (for example the regulation of IgE production by IL-4 and CD40). In humans, a novel IL-4 responsive element has been identified

upstream of the ε switch sequence which appears to have a role in sterile transcript induction and switch recombination (201). IL-4 alone does not stimulate IgE secretion but is thought to facilitate unwinding of the DNA to allow transcription and it does induce germline transcription of the ε gene (143,152,204).

The precise timing of switching events within the germinal centre is unclear, but a recent study by Feuillard *et al* (134) demonstrated that both centroblast-enriched (CD44-) and centrocyte-enriched ($CD44^{lo}$) fractions of human tonsil germinal centre B cells had deleted most of their IgCμ genes. This indicated that switching occurs well before follicular cells are induced to leave the follicle and differentiate into memory or plasma cells.

Somatic hypermutation and affinity maturation

Affinity maturation is the increase in antibody affinity observed after immunisation. The mechanism by which it occurs is the somatic mutation of V-region genes followed by selection by antigen for high affinity BCR. In human tonsil B cells, it begins in centroblasts when they lose IgD and CD23 expression (312) by day 6 and continues well into the second week following antigenic challenge (420). Hypermutation is targeted to the rearranged immunoglobulin V gene and occurs throughout its length via the stepwise introduction of successive single nucleotide exchanges (53,57,198,260,411). The exchanges are introduced randomly at a high rate of approximately 10^{-3} per base per generation (28,271), however mutations are more frequent in the CDR's than in the framework region (419). In addition a number of 'hotspots' with a higher than average frequency of mutation that may be

characteristic of the particular antigen have been identified and maybe associated with features of DNA primary or secondary structure (29).

Apoptosis

Somatic hypermutation gives rise to B cells expressing immunoglobulin with altered affinity for antigen that die by apoptosis unless they are rescued through high affinity binding to unprocessed antigen presented by FDC's and other signals e.g. CD40. Apoptosis removes from the B cell pool those cells which might lead to an impaired immune response e.g. by secretion of antibodies with too low an affinity for antigen. A second hypothesis suggests that apoptosis also serves to remove potentially autoreactive clones. (323,373). The centrocytes that survive express switched immunoglobulin with optimal affinity for antigen and may then be selected for development into either memory or a plasma cells.

Centrocytes exhibit a high death rate through apoptosis and much research has been focused on elucidating the signals which determine survival or suicide. In the classic experiments by Liu *et al* it was shown that apoptosis of CD39/IgD negative centrocytes could be inhibited *in vitro* through antibody cross-linking of sIg and CD40 (255). Addition of IL-4 maintained the rescued cells in cycle for several days. More recent data confirmed that GC cells can also be rescued *in vitro* by the addition of exogenous CD40L (190). A role for the 26kDa protein product of the oncogene *bcl-2* has also been postulated in the rescue of germinal centre B cells. *bcl-2* was first identified in the neoplastic cells of patients with follicular lymphoma but is not expressed by the majority of germinal centre B cells. Those signals (e.g. CD40-ligation) which rescue centrocytes from apoptosis also induce the expression of *bcl-2*

and suggests a link between the two (256). However, *bcl-2* expression does not occur until 2 days following CD40 ligation and may provide a secondary survival signal (190). CD40L has been identified on human germinal centre T cells (248) and it is probable that CD40-CD40L interactions are required for the formation of germinal centres (139). This is also highlighted by the lack of germinal centres in patients with HyperIgM syndrome due to defective CD40L expression. Several other signals which may help prevent apoptosis have also been identified including CD21 interactions with CD23 (38) and engagement of CD20 (403).

1.4.4. Generation of antibody forming cells and memory cells

Some of the signals have been identified that induce isolated tonsillar centrocytes to become plasma blasts. For example, IL-1α and soluble CD23 stimulate maturation to IgG-secreting plasma blasts (254) and IL-2 can induce a subset of CD5+ sIgM+ cells to secrete IgM (205). When the plasmablasts leave the follicles they are still in cell cycle, however they differentiate further into non-dividing cells which reside in the bone marrow or the lamina propria of the gut.

In the rat, the life-span of plasma cells in the bone marrow is approximately 1 month and in the gut is one month or less (338). Thus, in established T- cell-dependent responses which may last several months, there must be continuous plasma cell production, presumably derived from memory cells. Memory cells may be defined as switched cells that have proliferated in response to antigen but are no longer in cycle. They can proliferate, however, in follicles in response to antigen held on the FDC network (54,177,269) and they can give rise to plasma cells and further memory cells without forming germinal centres (257) or undergoing further somatic mutation

(132). Debate remains however whether memory B cells can undergo germinal centre formation and somatic mutation (263).

The exact signals and interactions that direct the differentiation of memory and plasma cells from centrocytes in the germinal centre are still unknown. However, a recent study by Arpin *et al* proposed a model for memory cell vs plasma cell formation driven by CD40-CD40L interactions (13). Prolonged stimulation via CD40-CD40L *in vitro* in the presence of IL-2 and IL-10 was suggested to induce the formation of CD38-/CD20+ memory cells as shown by their ability to proliferate but not to secrete immunoglobulin. In contrast, limited stimulation of CD40 (3 days) induced differentiation to CD38-/CD38+ plasma cells shown by secretion of antibody but their inability to proliferate upon further stimulation.

1.5. Cell Surface Molecules that Regulate B cell Responses

A physical interaction between B and T_h cells has long been recognised to be vital in antibody responses (279). A number of experimental studies demonstrated the requirement for contact-mediated signals in which resting B cells stimulated with fragments from activated T cell membranes or T cell associated molecules became activated and could proliferate (22,23,47,296,361). Full B cell activity, however, measured by the synthesis of mature immunoglobulin transcripts and immunoglobulin secretion, required further cytokine-mediated signals (187,295,298). These

observations underline the mutual importance of both cognate and soluble factors in Th-B interactions.

Some of the molecules involved in Th-B interactions are constitutively expressed, for example, CD40 on B cells and CD28 on Th cells, although their expression is increased upon activation. Each receptor-ligand pair does not act in isolation but as a cascade of bi-directional signals resulting in the reciprocal dialogue between B, Th and accessory cells that is essential for the precise regulation of the response (84). In some cases there are well defined receptor-ligand pairs e.g. CD40 and CD40L, while others are still 'searching' for a ligand e.g.CD20 and CD19. Major cell surface molecules that are expressed on activated B and Th cells and which merit a brief description here are the B7 family and their counterparts CD28 and CTLA-4, CD21 and CD23, CD11a/18 (LFA-1) and CD54, CD58 (LFA-3) and CD2, CD72 and CD5, Finally I will focus on the CD40 and CD40L receptor-ligand pair.

1.5.1. The B7 family, CD28 and CTLA-4

Th cells activated through the TcR by antigen require further stimulation via CD28. This regulates IL-2 expression and prevents T cell anergy and unresponsiveness to further stimulatory signals (209). In addition IL-4 production is increased and T cell proliferation is enhanced. The absence of a CD28-mediated signal leads to impaired immune function *in vitro* and *in vivo*. CTLA-4 has 75% nucleotide sequence with CD28 and its transient expression is detected 2-3 days following T cell activation. CD28 and CTLA-4 both bind B7-1/BB1 and B7-2/B70 (CD80 and CD86 respectively) (141,176,362) expressed on activated B cells (238). B7-2 is an early activation antigen expressed rapidly following B cell activation (92,142). B7-1

expression occurs much later, after approx. 24-48hrs. CD28-cross-linking is likely to have a positive feedback effect in the regulation of T-dependent B cells responses as stimulation enhances CD40L expression (223) which in turn can enhance CD80 expression (426). The role of CTLA-4, was less clear but CTLA-4-deficient mice have been found to die at an early age from a severe lymphoproliferative disorder which suggests a role as a negative regulator of T cell activation (3,418).

1.5.2. CD11a/CD18 and CD54

CD11a/CD18 (LFA-1) and its ligand, CD54 (ICAM 1), are adhesion molecules constitutively expressed on both T and B cells (382). Following cross-linking of the T cell receptor complex, an active form of CD11a/CD18 is induced and CD54 expression is increased (21,124). In response to antigen-specific activation, a CD11a/CD18-CD54-dependent signal is transmitted to the antigen-presenting B cell (237). Interaction between CD11a/CD18 and CD54 is induced following cross-linking of CD40 and promotes homotypic adhesions and allogeneic T cell proliferation (21). A requirement for CD11a/CD18-CD54 in T-B cell interactions is highlighted by patients with leukocyte adhesion defect who do not express CD11a/CD18 (303). These patients are able to produce antigen-specific IgG, but antibody titres are low indicating an impairment in the production of memory cells.

1.5.3. CD21 and CD23

CD23 is expressed on a large number of cell types including activated B and T cells, monocytes and follicular dendritic cells (93,109). On B cells, it is an early activation marker induced in response to stimulation by IL-4, IL-13 and CD40 cross-linking (98,100,324). CD23 is the low affinity receptor for IgE (FcεRII) and is postulated to

have a role in the regulation of IgE and allergic responses (137,147,183,387). CD23 also binds CD21 and functions as both a signalling molecule and an adhesion molecule (16,39,224,225). CD21-CD23 interactions may promote homotypic and heterotypic interactions following activation (36) and may regulate T-dependent B cell responses, such as IgE production and germinal centre cell survival (38). CD21 is also the receptor for EBV and the iC3b C3dg and C3d fragments of the third component of complement (94). In addition it can form a non-covalent association with CD19, CD81 and Leu-13 to form a signal transduction complex (395).

1.5.4. CD58 and CD59

CD58 (LFA-3) and CD59 on activated B cells both bind to CD2 on T cells (170,268). Their role in T-dependent B cell activation, although not precisely understood, is indicated by the inhibition of specific antibody responses by blocking mAbs to CD2 or CD58 (126,268).

1.5.5. CD72 and CD5

CD72 is expressed on human pre-B and mature B cells but is absent from plasma cells. Cross-linking with mAbs induces B cell proliferation - either with or without other growth factors and increases both MHC II expression and concentrations of intracellular Ca^{++} suggesting that the molecule is a receptor. The ligand for CD72 was reported to be CD5 (although this is not firmly established) (407) which is expressed on all mature T cells and a subset of B cells. mAbs to CD5 can activate T cells and stimulate CD3- and TcR- mediated T cell proliferation, and also stimulate the secretion of IL-2, expression of IL-2R and increase intracellular Ca^{++}. CD5-CD72 interactions may mediate early signals in T-B cell reponses.

1.6. CD40 and CD40L

The role of CD40 in the control of specific antibody production was a major focus for this thesis and therefore CD40 and its ligand merit a more detailed introduction here.

1.6.1.CD40

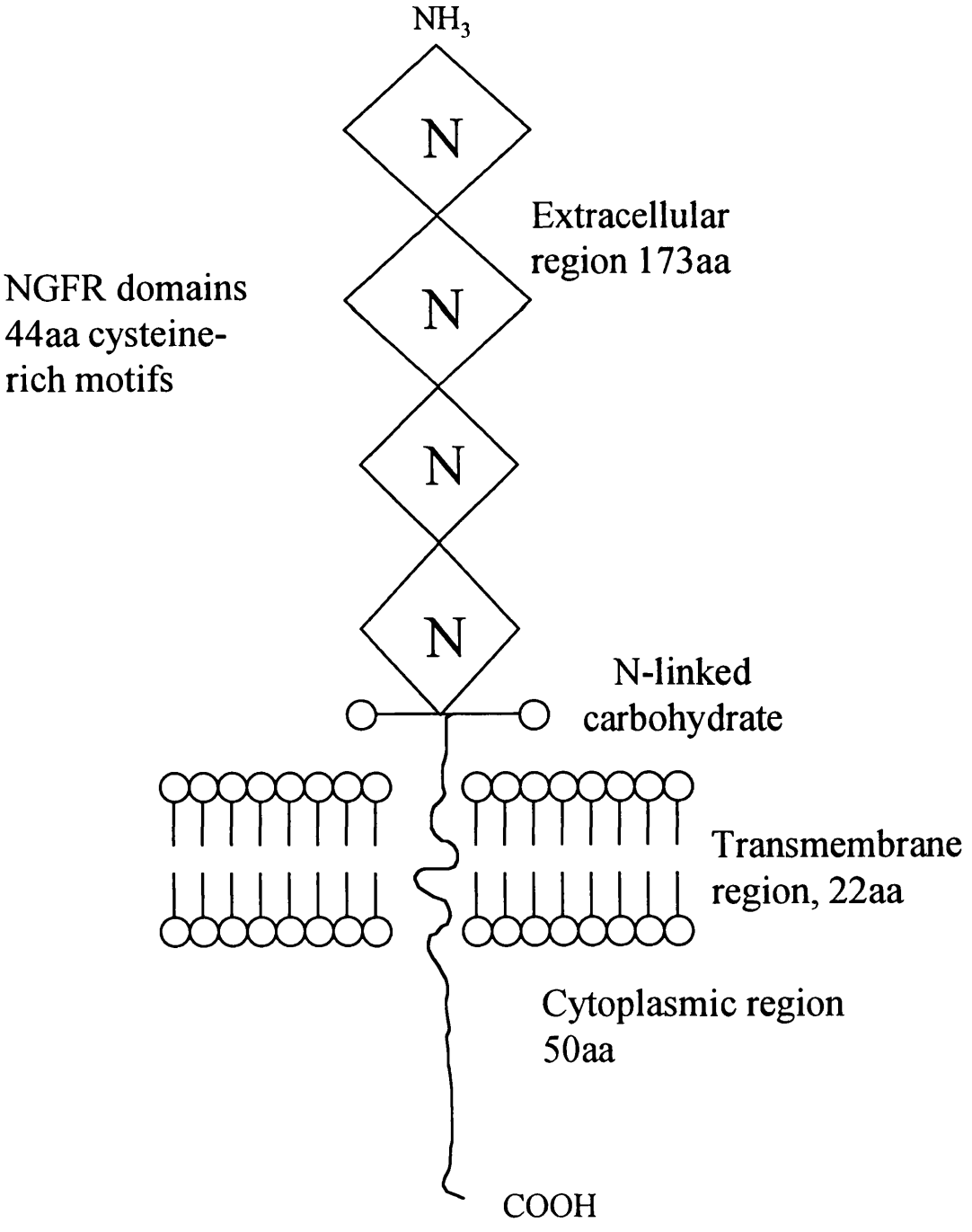
Identification

The CD40 molecule was identified independently by two groups in 1985 and 1986. Paulie *et al* identified a molecule, p50 present on bladder carcinoma cells using the mAb S2C6 (314). Clark and Ledbetter, using mAb G28.5 identified Bp50 on B cells which was involved in early activation events (83). Both mAbs were submitted to the Third International Workshop of human leucocyte antigens in Oxford in 1986 where the molecule was designated CDw40 (245,313). At the fourth workshop in Vienna, its specificity was confirmed as CD40 (118). Cloning CD40 revealed a 277 amino acid type I transmembrane protein (Fig. 1.3)(383,384). The N-terminal domain was found to be rich in Cysteine residues and the molecule displayed a high degree of homology to the nerve growth factor receptor (NGFR) and the tumour necrosis factor receptor TNFR as well as a number of other growth factor receptors which are termed the NGFR or TNFR superfamily.

Other members of the NGFR Superfamily

Members of the nerve growth factor superfamily share homology in their extracellular domains in the form of a number of conserved cysteine residues. This family comprises the low affinity NGFR (206) which is expressed widely on neural cells as well as lymphocytes. Neural cells expressing NGFR undergo apoptosis unless the

Fig 1.3 Structure of CD40



receptor is engaged by NGF or mAbs (327) - in this sense it acts in a similar way as cD40 in the germinal centre. TNFRI, TNFRII (259,364,375,393) are well established mediators of immune function, CD27 (69,97), CD30, a marker for Hodgkins disease (123), FAS (Apo 1/CD95) which can induce apoptosis (203), OX40 (265) and 41BB (167).

All the members of this family of molecules are emerging as having regulatory effects on lymphocyte growth and development, e.g., FAS (110,208), CD27 (97,185). Mutations in the genes for receptor or ligand are implicated in disease, for example, mutations in FAS or FAS ligand result in lymphoproliferative disease and a link has been suggested between CD30 and HIV infection (30,107,288,367). Non-human homologues of the NGFR superfamily include several pox virus gene products (196,197,371,375,376,400) and the ECP1 protein from the tomato fungus *Cladosporium fulvum* (408).

Distribution of CD40

CD40 can be detected on B cells at an early stage of development and is present throughout development although it may be absent from terminally differentiated plasma cells (reviewed in 17). Original descriptions of CD40 also identified it on B cell malignancies and several carcinomas in which the corresponding normal tissue was CD40 negative (245,314). Expression is thus not limited to B cells and CD40 with functional activity has now been detected on monocytes (2), dendritic cells (75) and thymic epithelium (149). Further, endothelial cells isolated from human umbilical vein (HUVEC) have been found to be strongly CD40 positive ((192) and K. Kotowicz, personal communication).

Functional properties of CD40 on human B cells

Cross-linking of CD40 on B cells with mAbs leads to activation, characterised by an increase in cell size and homotypic adhesion which is partially LFA-1-dependent (21). CD40 mAbs stimulate progression from G_0 - G_1 and the cells, thus primed, are receptive to further signals. CD40mAbs alone are not sufficient to induce significant levels of B cell proliferation, although they provide a strong co-stimulatory signal with anti-Ig or IL-4 (161,162,246,406). Further research showed that CD40 is important in the rescue of B cells from apoptosis in the germinal centre (255,403), in the maintenance of long-term proliferation of B cells (18,19) and also as a second signal with IL-4 in the isotype switch to IgE secretion (204,370).

Signal transduction through CD40

The precise biochemical signalling mechanisms which are induced on engagement of CD40 have not yet been definitively characterised. There is some disagreement between different groups of researchers on various observations which may in part be due to the different mAbs used for cross-linking of CD40 (143). A threonine residue at position 234 which lies within the intracytoplasmic region of the molecule was found to be essential for signalling (202). Early studies with various inhibitors (212) found that homotypic adhesions between human B cells following triggering of CD40 involved tyrosine kinases but not protein kinase C. Further investigations have revealed that cross-linking CD40 on human B cell lines or tonsil B cells leads to rapid phosphorylation of the *src*- type kinases *lyn, fyn* and *syk* (129,333). This is accompanied by the activation of phosphatidylinositol-3-kinase and phospholipase Cγ2 and the appearance of a 28kd phosphorylated protein. Induction of the

transcription factors NF-κB, NF-AT and AP-1 in response to CD40 ligation has been observed (27,140). More recently, two other molecules have been identified that have a role in CD40-mediated signalling. BSAP, B cell specific transcription factor, exhibits increased expression following engagement of CD40 and CRAF1 which interacts with the cytoplasmic tail of CD40. (79,415).

1.6.2. CD40L

The homology of CD40 to the NGFR superfamily and its role in B cell responses supported the concept of a receptor-ligand mode of action. The ligand for CD40 was first identified on mouse and human by Armitage *et al* at the Immunex Corp. in 1992 (8,11) and independently by several other groups (165,181,235,249,297). At Immunex, a fusion protein was constructed which consisted of the extracellular domain of CD40 linked to the Fc region of human IgG1. A number of cell lines were screened for binding of the construct and the murine thymoma line, EL4, was selected for the isolation the CD40L gene (8). Human CD40L was isolated from an activated peripheral blood T cell library (381) as a 261 amino acid type II membrane protein with 78% overall amino acid identity to the murine molecule. CD40L was found to display homology to TNF, thus revealing another family of molecules that are structurally related (130) and which include ligands for CD30 (377) and CD27 (158). As a consequence of this homology, it has been predicted that CD40L could exist as a trimeric molecule (315) on the cell surface, similar to TNF although, as yet, there is no direct evidence. CD40L is expressed on activated T cells (mainly CD4+ and a small subset of CD8+ cells) with similar distribution between Th1 and Th2 subsets (381). CD40L has also been identified on B cells (166); the implications of this finding will be addressed in Chapter 7. Expression on T cells is maximal between 8-16 hours

following activation, but is still detectable up to 36hrs. The ligand is not detected on neonatal T lymphocytes but normal expression is obtained approximately four weeks after birth. Its ability to stimulate B cell proliferation and cytokine-dependent antibody secretion confirmed initial observations using CD40 mAbs (10,11,381).

1.6.3.CD40 and CD40L in T-dependent B cell responses

CD40L expressed on the surface of fixed CV1/EBNA cells or as a soluble trimer was found to be directly mitogenic for both resting and activated B cells (10). The proliferative signal is augmented in the presence of IL-2, IL-4 or IL-10. CD40-CD40L interactions alone do not, however, provide sufficient signals necessary for progression of the B cell in response to antigen. Additional signals, possibly provided by cytokines or by cell-cell interactions for example between the B7 family and CD28 /CTLA-4 or CD11/18a and CD54, are likely to be required for optimal B cell activation (21,209). The role of CD40-CD40L interactions in the rescue of GC centrocytes from apoptosis has already been described and is supported by substantial experimental evidence. In addition to this, CD40-CD40L interactions are also likely to be required for the formation of germinal centres as shown patients with Hyper IgM syndrome (HIM) and also CD40 or CD40L-deficient mice that lack germinal centres (73,139,301,334).

1.6.4. CD40-CD40L interactions in class switching and antibody secretion

The requirement for a CD40L -CD40 interaction in immunoglobulin secretion was noted by several researchers. A number of papers describe experiments which either

blocked the CD40-CD40L interaction with soluble CD40 (127) or with mAbs to CD40L or by reconstituting T cell-B cell interactions using recombinant CD40L or CD40-fusion proteins (191,234). Although CD40L or mAbs to CD40 alone do not significantly enhance immunoglobulin secretion, the addition of cytokines or a polyclonal activator e.g. SAC is a powerful stimulus for B cell differentiation (10,103,164). Cross-linking CD40 in the presence of IL-4 or IL-13 results in the secretion of IgE due to H-chain switching (10,90,324,381). IL-10 is a potent differentiation factor and stimulates the production of IgM, IgG, including IgG1, IgG2 and IgG3 and IgA from CD40-activated B cells (10,353). In addition CD40L and TGF-β can induce class switching in IgD- B cells to secrete IgA (103). By far the biggest indication that CD40L was necessary for immunoglobulin secretion was the discovery of its involvement in the immunodeficiency XHIGM (301).

1.6.5. Hyper-IgM syndrome and CD40-CD40L interactions

Clinical characteristics

Hyper-IgM syndrome is a rare immunodeficiency disorder characterised by low or absent IgG, IgA and IgE detected in the serum but normal or elevated levels of IgM and increased susceptibility to opportunistic bacterial infections. The disease can occur as either a primary or acquired disorder. Patients with primary HIM present by the end of the 1st or early in the 2nd year of life with recurrent infections including upper respiratory tract infections, otitis media, diarrhoea and pneumonia. The diarrhoea and pneumonia are often caused by infection with *Cryptosporidium* and *Pneumocystis carinii* respectively - two organisms which are commonly associated with T cell abnormalities. Autoimmune reactions are also common resulting in

arthritis, nephritis and haemolytic anaemia. Some patients also have an increased susceptibility to certain tumours e.g. lymphomas. Treatment of HIM is by regular intravenous administration of immunoglobulin (IVIG). This has the dual effect of reaching near normal levels of serum IgG while at the same time decreasing serum IgM. Infections, when they occur, are controlled with antibiotic, anti-viral or anti-fungal drugs whereas steroid treatment may be needed to counter autoimmune reactions.

Patients are able to mount primary responses to antigens but secondary responses, such as vaccination boosters, are seriously impaired. This suggested a defect in the heavy chain switching mechanism in the B cells and gave rise to early assumptions that HIM was due to a B cell defect. However, later studies showed that H-chain switching mechanism in the B cells was intact (179). Lymphoid hyperplasia and neutropaenia are two further common characteristics of HIM and also abnormalities in myeloid differentiation. The number and distribution of T cells is generally normal and the numbers of re-circulating B cells normal or elevated. However, lymph node architecture is frequently abnormal and patients show thymic atrophy and lack follicles and germinal centres.

X-linked and non X-linked HIM

In primary HIM, there is a well documented familial pattern of inheritance which affects mostly males and thus suggests an X-linked immunodeficiency. However in one study, about 22% of affected patients were female indicating the existence of a non-X-linked form of the disease. This could be due to an autosomal dominant or recessive mode of inheritance. Furthermore, some 'sporadic' cases of HIM occur in

male patients with no previous family history of the disease which could be due either to the occurrence of a new mutation within the X chromosome or by an autosomal mechanism.

XHIGM

Early genetic studies into the X-linked form of HIM relied on a few families with a well documented pedigree of the disease. Using linkage analysis the XHIGM gene was assigned to Xq24-27 and later, more accurately, to Xq26 (307). CD40L was also mapped on the X chromosome to q26.3 - 27.7 (96,125). Its close proximity to the HIM site suggested a link between the two and the gene for CD40L was named as the candidate gene responsible for X-linked HIM. This was quickly confirmed by several groups who demonstrated the presence of various mutations in the CD40L gene of patients with XHIGM (14,96,125,145,227,330). An indication of impaired CD40L expression is obtained initially by flow cytometric staining of activated peripheral blood T cells with either polyclonal or monoclonal antibodies to CD40L (60). The specific mutation is identified by SSCP and sequencing. To date, a large number of different mutations have been identified (14,96,116,144,227, summarised in 229). These include point mutations caused by amino acid substitutions, or deletions resulting in frameshift mutations. A large number of these mutations have mapped to the extracellular domain of the molecule, mostly within the TNF-homology domain. One mutation has been found in the transmembrane region of CD40L which results in the insertion of a positively charged Arg residue in place of Met and prevents insertion of the molecule into the membrane (227). Finally, an insertion mutation has been identified which results in a frameshift (229).

A number of studies have confirmed that B cells from XHIGM patients are able to function normally with respect to proliferation and differentiation via CD40 stimulation. Isotype switching can be induced *in vitro* by cross-linking CD40 and the addition of cytokines. Secretion of IgG and IgA is obtained in the presence of IL-10 (122,227,410) and addition of IL-4 induces IgE production (14,96,122,144,227).

XHIGM is the result a single gene defect which ultimately may be a target for gene therapy. Currently however, the increasing knowledge of the structure of the CD40L gene and its regulation has allowed researchers to focus upon improved carrier detection methods and prenatal diagnosis of the disease with some encouraging early results. A highly polymorphic dinucleotide repeated sequence was identified situated in the 3` untranslated region of the CD40L gene which has proved informative in the determination of carrier status (117).

1.6.6 CD40-CD40L interactions on other cell types

CD40 expression is not restricted to B cells and continued research into CD40-CD40L interactions has demonstrated a pleiotropic role for the receptor-ligand pair. Functional CD40 has been identified on dendritic cells which is likely to have a role in germinal centre reactions and memory cell responses. Some endothelial cells e.g. HUVECs are strongly CD40+ve although its function on this cell type is not fully understood. Human monocytes express CD40 which is enhanced in the presence of GM-CSF, IL-3 or IFNγ (2). The CD40 on these cells is functional and mediates adhesion to cells expressing CD40L. Cross-linking CD40 stimulates cytokine-dependent secretion of TNFα and IL-6 and increased secretion of IL-8. In addition

monocytes show tumouricidal activity against human melanoma in the absence of a co-stimulus.

CD40 has also been identified on myeloma cells where cross-linking induces IL-6 production and proliferation (421). This suggests a mechanism whereby CD40 may play a role in tumour expansion, possibly through the stimulation of autocrine growth factors. In contrast, several B lymphomas exhibited significant growth inhibition when their CD40 was cross-linked (146). Taken together, these and all the other observations described above project clearly the importance of CD40-CD40L interactions in multiple aspects of responses by haemopoietic cells.

1.7. Role of cytokines in T-dependent B cell differentiation

The requirement for cytokine-mediated signals in B cell responses has long been recognised. Many cytokines exhibit pleiotropic activities in immune responses: IL-4 stimulates activation, proliferation and differentiation of B cells; others appear to act largely on populations of B cells at a particular stage of development, for example IL-3 is involved early in B cell maturation and IL-7 is a pre-B cell growth factor (180). On the other hand, IL-5 has well documented activity on murine B cells but no parallel role in human systems, although it acts on other human cells.

1.7.1. Th cell subsets are defined by their cytokine profile

The concept of Th subsets evolved from the observation by Mosmann (284) that mouse CD4+ T cell clones could be subdivided according to their profile of secreted cytokines. Th1 cells secrete IL-2 and IFN-γ but not IL-4 or IL-5 whereas Th2 cells secrete IL-4 and IL-5. Other cytokines are secreted by both Th1 and Th2 cells. This concept has also been applied to human T cells (347) where it is thought that the different subsets originate post-thymically from a precursor cell, (Thp), which secretes only IL-2 (409). After antigen activation, Thp develop into Th0 effector cells that secrete a range of cytokines including IL-3, IL-4, IL-5, GM-CSF and IFN-γ. The precise stimulus received by Th0 cells then seems to direct the development of Th1 or Th2 cells. Th0 stimulated with IL-4 mature into Th2 cells which secrete IL-3, IL-4, IL-5, IL-6, IL-10, IL-13, TNF-α and GM-CSF. In contrast, stimulation by IFN-γ and IL-12, stimulates the generation Th1 cells that secrete, IL-2, IFN-γ, TNF-α and β and under certain conditions IL-3, IL-6, IL-10 and GM-CSF. Although there is considerable overlap in the cytokines secreted by each Th subset, some appear exclusive to one type e.g. IL-4 and Th2. Generally, in mice, Th1 cells and the cytokines IL-2 and IFN-γ are associated with cellular immune response and inflammation whereas Th2 cells secrete cytokines which regulate humoral immunity e.g. IL-4. A number of diseases can also be classified as showing predominantly Th1 or Th2 type responses for example in humans responses to allergens are associated with Th2 cells and cytokines
(347,348).

1.7.2. Cytokines acting on human cells

The list of characterised cytokines is extensive and expanding and there are many books and reviews that extol their virtues. The following section comprises a brief overview of the major cytokines that act on B cells, with emphasis on those whose activities were investigated during this project, namely, IL-2, IL-4, IL-10, IL-13 and IL-15.

IL-1

IL-1 has a range of biological activities on a number of cell types including B and T lymphocytes and monocytes. Two different forms of IL-1 exist, IL-1α and IL-1β that are encoded by different genes. Both forms bind the same receptor and have similar biological effects. IL-1 is primarily an inflammatory cytokine. Its effects on human B cells include activation and induction of cytokine secretion. It is also implicated in a number of diseases e.g. osteoporosis and diabetes and autoimmune disorders such as arthritis (111,114,115).

IL-2

IL-2 is a 15-20kDa glycoprotein secreted by activated CD4+ and CD8+ cells. The 3-D structure of IL-2 is a 4α-helix bundle and it belongs to the family of molecules known as the helical cytokines (25,270). This family includes IL-4, GM-CSF, and IL-15 which are structurally related but exhibit little sequence identity. All these cytokines bind to receptors belonging to the cytokine receptor superfamily (CKR-SF) (25,280). IL-2 was originally thought to be a T cell growth factor but was subsequently found to have potent effects on human B cells and also NK cells. It can

co-stimulate with anti-Ig to induce B cell proliferation (43,289) and enhances antibody secretion by cells activated with anti-Ig or SAC (186,328). It also has an important role in specific antibody secretion and can replace T cell help (64). There is no evidence that IL-2 can induce heavy-chain switching in B cells. Secretion of IL-2 is regulated via CD28 and CTLA-4 interactions with the B7 family of molecules (reviewed in 209). Cross-linking of CD28 and CD3 on both CD4+ and CD8+ T cells induces secretion of IL-2 and IL-4 and enhances T cell proliferation.

IL-2 binds to a multi-subunit receptor (IL-2R) expressed on B cells , T cells, NK cells monocytes and macrophages. The IL-2R consists of at least three distinct components, the IL-2Rα (p55, CD25, Tac), IL-2Rβ (p75, CD122) and IL-2Rγ (p64) (reviewed (276,392). IL-2Rβ and IL-2Rγ are members of the cytokine receptor superfamily. Expression of different combinations of the three receptor chains gives rise to the high, intermediate and low affinity forms of IL-2R. The γ-chain has recently been shown also to be a component of receptors for IL-4 (226,355), IL-7 (299), IL-9 (356) and IL-15 (154) and has been designated the common gamma chain (γ_c). The γ_c is required for a variety of functions including T-B interactions in response to cytokines (267). Its importance was highlighted recently by the discovery that X-linked severe combined immunodeficiency (X-SCID) is the result of mutation in the γ_c gene (300).

IL-4

IL-4 is a pleiotropic cytokine secreted by activated Th2 cells, mast cells, bone marrow stromal cells and possibly B cells which exhibits a wide range of effects on human B cells. Its activities include activation of resting B cells (326) resulting in the increased

expression of sIgM(372), CD23(98), MHC II(351), LFA-1 (350) and CD40 (162,406) and induced expression of B7-1(CD80) (405). In the presence of co-stimulatory factors such as anti-IgM antibodies, IL-4 enhances proliferation of B cells (102). In addition, IL-4 stimulates IgG and IgM secretion by B cell blasts (105) but it is probably most noted as a switch factor for the production of IgE and IgG4 (IgG1 in mice) and its role in atopic allergy (150,151,318,413). IL-4 induces the expression of Cε germline transcripts (370), but a second signal provided by CD40 is required for secretion of IgE (430). IL-9(121) and CD28 (250) may also regulate IgE secretion. Recently, a CD40-independent mechanism for IgE production has been reported in which CD58 mAb (LFA-3) co-stimulated with IL-4 to induce IgE secretion (112). This is supported by observations of an XHIGM patient with defective CD40L whose T cells can stimulate IgE secretion *in vitro* (251).

The IL-4 receptor complex is composed of a high affinity IL-4-binding chain, gp140 (IL-4Rα/CD140) (148), associated with the non-IL-4-binding γ_c (267). The nature of IL-4 binding to its receptor has generated much interest. Studies in this laboratory have demonstrated a dual action of IL-4 on human B cells when present at different concentrations (337). First, it was shown that a ten-fold higher concentration of IL-4 was required for CD23 expression than for sIgM expression and that separate signal transduction pathways were responsible. It was also found that cross-linking of CD19 could inhibit IL-4- induced CD23 expression but not sIgM expression. Together these observations indicated the existence of two, independently regulated IL-4 receptors. Subsequently, a second low affinity receptor for IL-4 has been identified for which a partial sequence has been obtained but not cloned (128). Another study supported the existence of high and low affinity receptors involved in B cell differentiation. In

human B cells activated with EBV, low doses of IL-4 were found to enhance secretion of IgM, IgG1-3 and IgA while high doses induced secretion of IgG4 and IgE (228). The experiments designed to investigate the role of IL-4 by influenza-stimulated human B cells are described in Chapter 5.

IL-6

IL-6 is secreted by lymphoid and non-lymphoid cells and acts on a number of cell types including B and T lymphocytes and haemopoietic cells. IL-6 can stimulate activation, proliferation and differentiation of B cells but it does not appear to act as a switch factor (219,220). In addition, IL-6 was shown to induce phosphorylation of CD40 on B cells (86). Receptors for IL-6 are expressed on activated but not resting B cells, plasma cells, T cells monocytes and some non-lymphoid cells including epithelial cells and fibroblasts. The high affinity receptor is formed by the non-covalent association between an IL-6-binding chain (CD126) and gp130 (CD130) that does not bind IL-6 but which is esssential for signal transduction (220,390).

IL-10

IL-10 was first identified in murine (135) and then human cells (414) and belongs to the 4α- helix bundle family. Both mIL-10 and hIL-10 have a high degree of homology to the EBV BCRF1 gene product which is expressed late in the lytic cycle (282). hIL-10 is secreted by a wide variety of cell types including activated Th0, Th1 and Th2 cells and CD8+ T cells (106). It is also secreted by monocytes, macrophages, keratinocytes, activated B cells, B lymphomas and EBV-activated Burkitt lymphoma cells.

IL-10 regulates many aspects of cellular and humoral immunity by its action on a number of different cell types (reviewed in 281). These include monocytes, macrophages and dendritic cells, both directly and in their capacity as APCs, T cells, mast cells and thymocytes. Most notably it acts as a cytokine synthesis inhibitory factor (CSIF) (414) and can reduce IFN-γ production by Th1 cells. In human, IL-10 also inhibits IL-4 and IL-5 production by Th2 cells (106). IL-10 is also a potent growth and differentiation factor for human B cells (353). It can co-stimulate with anti-IgM or SAC to induce proliferation. Alone, it is a less effective BCGF than IL-2 or IL-4, but proliferation is significantly increased if CD40 is cross-linked and it is synergistic with IL-4. IL-10 also has BCDF- activity for SAC or anti-CD40 activated B cells, and induces secretion of IgM, IgG and IgA by increasing the number of immunoglobulin -producing clones. In the presence of TGF-β, IgA secretion by naive cells is increased, most likely by H-chain switching (49,103). Finally cross-linking of CD40 in the presence of IL-10 was found to induce IgG1 and IgG3 secretion from δ+ cells and supports the role of IL-10 as a switch factor (50). The IL-10 receptor belongs to a cytokine receptor family that includes receptors for IFN-γ, IFNαB and tissue factor (26). Human IL-10R mRNA has ben detected in haemopoietic cells and cell lines. Signal transduction is likely to involve the JAK family of kinases (253).

IL-13

IL-13 was first identified by Minty *et al* (277) and found to be secreted by activated human T cells. IL-13 mRNA can be detected as early as 1 hr following T cell activation and peaks after 2hrs and is still observed after 72hrs (431). This contrasts to the pattern of IL-4 expression which occurs later and is transient (428). IL-13 is

structurally similar to IL-4, and belongs to the 4α-helix cytokine family. The gene for IL-13 is closely linked to the IL-4 gene and is found on chromosome 5q23-31. Early studies showed that rIL-13 synergises with IL-2 in the regulation of IFN-γ synthesis in large granular lymphocytes. In addition it inhibited IL-6 production by LPS-stimulated human PBMC and thus appears to regulate inflammatory responses.

IL-13 has similar actions on human B cells as IL-4 and enhances CD23, MHC II, sIgM, CD71 and CD72 expression on resting cells (277,325). It co-stimulates with anti-IgM or anti-CD40 to induce proliferation, and also stimulates IgE secretion (90,100) and IgG4 secretion (325). In addition to its effects on B cells, IL-13 has profound effects on monocytes (reviewed in 431). Human monocytes cultured with IL-13 develop long processes, form homotypic aggregates and become strongly adherent to their substrates. In addition, they show increased expression of a large number of adhesion molecules (such as Cd11b, CD11c, CD18, CD29 and CD49e) appear to show enhanced survival *in vitro*. In contrast, it does not have any activity on human T cells (431,433).

Studies of IL-13 binding indicated the IL-13R was a complex of subunits with an IL-13 specific IL-13Rα but also a common receptor subunit shared by IL-4 and IL-13 (433). This shared component is unlikely to be γ_c as human SCID B cells that do not express γ_c are responsive to IL-13 (267). Although IL-4 appears to be specific for IL-4Rα, neutralising antibodies to IL-Rα inhibit both IL-4 and IL-13 activities on B cells suggesting that the shared component of the two receptors may be the IL-4Rα (432). A model for this was put forward by Lin *et al* (252) that suggested that IL-4 initially

binds to the IL-4Rα followed by association with either γ_c or the IL-13Rα. On the other hand, binding of IL-13 to the IL-13Rα would lead to recruitment of the IL-4Rα resulting in the formation of a functional receptor. The recent cloning of the murine IL-13Rα is consistent with this model (184).

IL-14

IL-14 was originally described as high molecular weight B cell growth factor (6,7) and acts predominantly on mature and malignant B cells. IL-14 induces proliferation of activated B cells, but inhibits immunoglobulin secretion. It can selectively expand certain B cell sub-populations such as IgD- cells and has a proposed role in memory cell function.

IL-15

IL-15 is a recently described novel cytokine with activities similar to those of IL-2 (163). IL-15 was first purified from the simian kidney epithelial line, CV1/EBNA (272), and shown to support proliferation of an IL-2-dependent line, CTLL. The human homologue was cloned from a stromal cell line (IMTLH) and exhibits 97% amino acid identity with simian IL-15. IL-15 has little sequence homology with IL-2, but it belongs to the 4α-helix bundle family of cytokines and displays a high degree of structural homology with IL-2. Unlike IL-2, IL-15 is not secreted by peripheral blood T cells, but is produced by monocyte-enriched PBMC, epithelial and fibroblast lines. Strong IL-15 mRNA expression was also detected in placenta and skeletal muscle with lower levels in heart, lung, liver and kidney.

IL-15 has many biological activities in common with IL-2 including the generation of cytolytic effector cells *in vitro* and the activation of non-antigen-specific LAK cells (163). Some functional differences between the two cytokines have been described. For example an IL-3-dependent line 32D, that expresses IL-2Rα and γ chains, proliferates in response to IL-2 but not IL-15 (163). IL-15 also has stimulatory activity for human B cells and co-stimulates with anti-IgM or phorbol ester to induce proliferation. In the presence of CD40L, IL-15 stimulates the secretion of polyclonal IgM, IgG1 and IgA but not IgG4 or IgE. (9), (Jones *et al*, in preparation). In this B cell system, however, the activities of IL-2 and IL-15 are much more comparable and as yet no significant differences between the two have been observed.

The IL-15R utilises the IL-2β and γ_c but not IL-2Rα (154). A third α-like component has now been cloned and reported (in mice) which is unique to IL-15 (155).

Interferon-γ (IFN-γ) and Interferon-α (IFN-a)

IFN-γ is secreted by Th1 cells and is associated with the promotion of cellular immune responses and the inhibition of IL-4-mediated responses (81,131). IFN-γ increases the proliferation of B cells activated with SAC or anti-IgM and induces IgM and IgG secretion from IL-2 and SAC-activated B cells (99,289). It does not, however, direct isotype switching. The receptor for IFN-γ is acomplex consisting of a high affinity IFN-γ -specific component (CD119) and an accessory protein that is required for signal transduction (366). IFN-γ receptors are expressed on most haemopoietic cells except erthrocytes and also on epithelial, endothelial and tumour cells.

IFN-α has similar activities in human as IFN-γ although it is encoded by a different set of genes. It stimulates antibody secretion from B cells and B cell lines (136,290,319), but inhibits IL-4 -induced IgE secretion (317). Two IFN-α receptors have been identified; one that is specific for IFN-αB and a second that binds IFN-α and -β (302,402). Both receptors are expressed on most cell types.

Nerve Growth Factor (NGF)

NGF is secreted by the brain and nerve tissue and prostate and acts upon cells of the central and peripheral nervous system. NGF is also secreted by B cells (363) and it has an effect on B and T cells, monocytes and mast cells. It has BCGF activity and a regulatory effect on immunoglobulin secretion (51,306). NGF binds to both high affinity and low affinity receptors. The high affinity receptor is expressed exclusively on NGF-responsive cells and regulates neurotrophic activity. The low affinity receptor for nerve growth factor ($p75^{LNGFR}$) is also expressed on non-neuronal cells and most notably has homology with CD40 (384) and is the founder member of the nerve growth factor receptor superfamily (264).

Transforming growth factor-β (TGF-β)

TGF-β is a pleiotropic cytokine whose primary effect is the inhibition of cell growth. It therefore exerts a regulatory effect in tissue remodelling, wound repair, development and haematopoiesis(339). TGF-β has a regulatory effect on pre-B cells and *in vitro* selectively inhibits the acquisition of surface λ light chains (332). In mature B cells it tends to block B cell activities such as apoptosis and IL-4-induced

CD23 expression (159,189). It is also a switch factor for IgA and, in the presence of IL-10, it induces the production of Cα1 and Cα2 transcripts and IgA transcription in IgD+ B cells activated via cross-linking CD40 (103). Receptors for TGF-β are expressed on most cell types. Three types of receptor have been identified; high affinity type I and type II receptors and a low affinity type III receptor (266). The type I and type II receptors are thought to associate to mediate signal transduction.

Tumour necrosis factors α and β

TNFα and TNFβ (lymphotoxin,LT) are members of a cytokine family that includes CD40L (30,130). They have paracrine and endocrine activity and are associated with inflammatory responses and induction of shock and are implicated in disease e.g. multiple sclerosis (354). They also regulate other aspects of immune responses and TNF-α is an autocrine growth factor for normal human B cells (42). Recently a mouse lacking one of the genes for TNF–β was found to have no lymph nodes although lymphocyte numbers were normal suggesting that it played a role in normal lymph node development. The two molecules share 35% sequence identity and have a trimeric quaternary structure. They bind to receptors that are members of the NGFR superfamily and that show some cross-reactivity with respect to receptor binding.

1.8. Specific antibody secretion in T-dependent B cell responses

The properties of cytokines and cell surface molecules in Th-dependent B cell responses are based mainly on observations from experimental systems which utilise

polyclonal activators e.g. SAC and EBV. Relatively little is known about the regulatory effects of such molecules in responses to specific antigens which was the focus of this project.

In this thesis project, I utilised a well established assay for *in vitro* production of specific antibodies by human B cells in response to stimulation by influenza virus. This system was first described by Callard in 1979 (58) using PBMC. It was found that PBMC cultured with human influenza virus secreted antibodies which were specific for the stimulating strain of virus (as shown by the use of two non-cross reacting strains). The response was Th cell dependent, with help confined to the CD4+/CD45Ra- CD45Ro+ subset (32). Expression of CD45Ro has subsequently been shown to accompany the generation of memory T cells. (1,31,360). This is consistent with the nature of the specific antibody response to a recall antigen and is supported by the observation that specific antibody responses are not obtained from naive donors. Furthermore, the predominant isotype secreted is IgG with only low levels of IgM which is typical of a secondary response. Measurement of specific antibody secretion was originally made by RIA but this has been superseded by a specific ELISA (429). The response requires histocompatability between co-operating B and T cells but this was found to be due to the activation of suppressor T cells acting directly on B cells rather than genetic restriction as removal of CD8+ cells from allogeneic populations restored T cell help (66,378).

Further characterisation of the specific antibody response demonstrated that T cells could be replaced by soluble factors present in the supernatants of PHA-activated T cells. The assay therefore provided a good model for assessing directly the effects of

cytokines and other factors in the regulation of specific antibody production by human B cells. rhIL-2 and $BCGF_{lmw}$ were both found to have TRF activity. IL-2 was found to have TRF activity only for low density (activated) B cells (64,65) whereas $BCGF_{lmw}$ could stimulate specific antibody secretion from both low and high density B cells (62). The reason for the difference in action of these two cytokines has never been fully elucidated. In the original experiments, $BCGF_{lmw}$ was obtained as a partially purified commercial preparation derived from activated T cell supernatants. Although it contained some IL-2, this was not the sole source of the TRF activity as shown by experiments using antibodies to block the IL-2 receptors. An anti-Tac antibody was not capable of blocking the effect of $BCGF_{lmw}$, but the addition of mAb to the IL-2β chain did abrogate the response. $BCGF_{lmw}$ was also found to contain small amounts of IFN-γ, TNF-α, and GM-CSF. It is possible that a combination of cytokines acted synergistically to induce a response. Finally, the possibility of the preparation containing T cell membrane fragments with stimulatory activity (e.g. CD40L, CD28?) has never been completely excluded. By no means do all cytokines display TRF activity as demonstrated in one study which tested twelve recombinant cytokines (380). Out of the cytokines tested, namely, IL-1α and β, IL-2, IL-3, IL-4, IL-5, IL-6, IFN-α and -γ, GM-CSF and TNF-α only IL-2 had any TRF activity although stimulation was obtained from a combination of IFN-γ, TNF-α, and TNF-β with a suboptimal dose of IL-2.

1.9. Aims of the study

The aim of this project was to study the role of soluble cytokines and contact-dependent signals in the regulation of specific antibody production to influenza virus. The role of three cytokines in specific antibody production was studied - IL-4, IL-13 and IL-15. The studies with IL-4 aimed to address its effect with respect to IgG isotype secretion and were complementary to studies previously undertaken in this laboratory. The cytokines IL-13 and IL-15 were newly described during the course of the study and I compared and contrasted their activities with those of the more well-defined cytokines. In addition I studied the effect CD40-CD40L interactions in the regulation of the specific antibody response as the assay provided a unique experimental system for studying the effects of this receptor-ligand pair. Finally, the importance of CD40 and CD40L and cytokines in humoral responses was addressed again in a study with HIM patients.

CHAPTER 2

MATERIALS AND METHODS

2.1. Tissue Culture

2.1.1. Culture Medium

Cells were cultured in either RPMI 1640 or Iscove's Modified Eagle's Medium (IMDM) purchased from Gibco Life Technologies Ltd., (Paisley,U.K), supplemented with foetal calf serum (FCS) (Gibco, Tissue Culture Services, Slough, UK or Sigma, Poole, UK). For specific antibody responses, cells were cultured in RPMI 1640 supplemented with 10% horse serum (Gibco). All media were also routinely supplemented with 50μg/ml Gentamicin solution (Roussel Laboratories) and 2mM glutamine (Sigma).

2.1.2. Holding medium.

Cells were prepared in RPMI 1640 containing 25mM Hepes but without bicarbonate which would otherwise equilibrate with atmospheric CO_2 and increase the pH of the medium. Holding medium was also routinely supplemented with 50μg/ml gentamicin and 5% FCS.

2.2 Cytokines

IL-2. *E. coli*-derived human recombinant IL-2 (rhIL-2) was a generous gift from Amersham International (Amersham, Bucks. UK). One unit/ml of IL-2 is defined as the concentration required to stimulate half maximal ^{3}H-TdR incorporation by CTLL-2.

IL-4. *E. coli*-derived rh IL-4 was a generous gift from Stirling Drug Inc. Malvern, PA., US. One unit/ml of IL-4 is defined as the amount required to induce half maximal ^{3}H-TdR incorporation by human peripheral blood T lymphocytes co-stimulated with PHA.

IL-10. *E-coli*-derived rhIL-10 was either obtained as a gift from DNAX, Palo Alto, USA, or purchased from Genzyme Diagnostics, West Malling, Kent, UK.

Low molecular weight BCGF ($BCGF_{lmw}$) was purchased from Sera-lab Ltd., Sussex, UK.

hIL-13 was a generous gift from Dr.Adrian Minty, Sanofi Biorecherches, Labege, France.

IL-15. Recombinant simian IL-15 was kindly provided by Dr. Richard Armitage, Dr. Kenneth Grabstein and colleagues at the Immunex Corporation, Seattle, WA, USA.

2.3 Antibodies used in flow cytometry and cell culture

Purified UCHT1 (CD3) and UCHM1, (CD14) monoclonal antibodies (mAbs) used to check the purity of T and B cell populations were kindly provided by Professor Peter Beverley, University College, London.

8EB1 (CD19) mAb was kindly provided by Dr. Noel Ling, University of Birmingham. The IgG fraction was purified from ascites by FPLC.

G28.5 (CD40) mAb was a gift from Prof. Ed Clarke, University of Washington, Seattle, US.

CD3/CD19 'Simultest' antibodies were purchased from Becton Dickinson Immunocytometry Systems and used to check the purity of B cell populations from peripheral blood. This product was preferred to UCHT1 which tended to cross-react with the large number of monocytes present in preparations from blood.

2.4 CD40 and CD40 Ligand

CD40L preparations were generously provided by Richard Armitage, Immunex Corporation, Seattle,WA. CD40L expressed as a cell surface molecule was generated by transfection of the Simian CV1/EBNA cell line with human CD40L cDNA. Control CV1/EBNA cells were transfected with empty vector alone (272). The cells were fixed three days post-transfection in 1% paraformaldehyde and used in assays at a concentration of 10^5 cells/ml.

Soluble trimeric human CD40L (s.CD40L) was obtained by transfecting COS cells with a construct containing the extracellular domain of CD40L fused to a modified leucine zipper motif which results in spontaneous trimerization of secreted CD40L

(9,172). sCD40L was obtained as a day 4 supernatant and used in assays at a 1/4 - 1/10 (final) dilution of a 10X concentrated solution.

CD40.Fc soluble fusion protein consisting of the extracellular domain of CD40 fused to the Fc region of human IgG1 (127) was also provided by Richard Armitage, Immunex Corporation, Seattle.

2.4.1. CD40 and CD40L antibodies

A panel of eight CD40 mAbs submitted to the 5th International Workshop and Conference on Human Leucocyte Differentiation Antigens were used in this study (Table 2.1)

Table 2.1 Panel of mAbs to CD40

mAb/Clone	Isotype	Supplied by:
EA5	IgG1	Dr. Tucker Lebien, Minneapolis,US.
G28.5	IgG1	Prof. Ed. Clark/Dr. Jeffrey Ledbetter, University of Washington Medical School, Seattle, WA.
BL-OGY/C4	IgM	Dr. Helmut Fiebig, Reinbek, Germany.
5C3	IgG1	Dr. Hitoshi Kikutani, Osaka University, Japan.
HB14	IgG1	Prof. T. Tedder, Duke University Medical Centre, Durham, US.
14G7	IgM	DR. R.A.W. Van Lier, Transfusion Service, Amsterdam.
M2-CD40	IgG1	Dr.William Fanslow/Dr.Richard Armitage, Immunex Corp.
M3-CD40	IgG1	Seattle, WA.

Notes: Information was compiled from LDAD database.
All antibodies were raised in mice and were known to be specific to CD40.

5c8 monoclonal antibody to human CD40L (249) was a kind gift from Dr. Leonard Chess, Columbia University, New York.

Anti-TRAP, a polyclonal antibody to human CD40L, (165), was kindly provided by Dr. Richard Kroczek, Molecular Immunology, Robert Koch Institute, Berlin.

2.5 Influenza Virus

Influenza virus, used as antigen for generating specific antibody responses, was very generously provided by Dr. John Wood (NIBSC, South Mimms, UK) as a sucrose gradient purified preparation. The strains most commonly used were *X97* (H3N2) and *NIB24* (H5N2). The concentration of the original suspension was approx. 20mg/ml and was stored at 4°C in the presence of 0.01% NaN_3. A working stock solution was prepared from the original suspension containing 100 μg/ml virus in holding medium containing 50μg/ml gentamicin but without FCS which was stable at 4°C for several months. Influenza virus was then diluted further in culture to give a final concentration of approx. 0.2μg/ml.

2.6 Cell Preparation

2.6.1. Preparation of tonsil mononuclear cells (TMC)

Freshly excised human tonsils were obtained from the Royal National Throat, Nose and Ear Hospital, London. Each lobe was sterilised in 70% alcohol for 5 secs. and

then washed three times in RPMI 1640 holding medium before placing in a Petri dish where the cells were teased gently into holding medium. The resulting cell suspension was layered onto Ficoll-sodium diatrizoate (Flow Laboratories, Rickmansworth,UK) at a ratio of approximately 15mls of cell suspension and 8-10mls Ficoll, and then centrifuged at 1000*g* for 15 mins. at room temperature. The interface layer was then removed together with a small amount of the Ficoll and the cells diluted with at least an equal volume of wash medium and centrifuged at 200*g* for 10 mins. Washed cells were resuspended in medium and counted and checked for viability by exclusion with Trypan Blue (Flow Laboratories). The cells were washed once more and resuspended in culture medium. Cells not required immediately were frozen and stored in liquid nitrogen in Nunc cryotubes at 100 x 10^6 cells/ml in 50% FCS, 10% DMSO (Merck-BDH, Poole, Dorset,UK) and 40% holding medium. Freshly prepared TMC were routinely screened for their ability to respond to influenza virus by the production of specific antibodies and those which gave good responses were used in subsequent experiments. For recovery from liquid nitrogen, aliquots of frozen cells were removed, thawed quickly at 37°C and washed in warmed medium. Cells were then resuspended in holding medium, ready for use.

2.6.2 Preparation of peripheral blood mononuclear cells (PBMC)

Blood was taken from volunteer donors or patients into a syringe wetted with preservative-free heparin and then diluted with an equal volume of holding medium containing 10 IU/ml preservative-free heparin but without FCS. The blood was layered onto Ficoll as for the preparation of TMC and centrifuged @ 1000*g* for 20 mins. The interface layer was recovered and washed in medium with heparin for 15 mins. at 200*g* and treated as for TMC.

2.6.3 Separation of B and T cells by E-rosetting

The best method for depleting T cells was by formation of E^- rosettes with AET-treated SRBC which were then separated by centrifugation over Percoll.

Preparation of Percoll

An iso-osmolar stock of Percoll (Pharmacia, Milton Keynes, U.K.) was prepared by addition of 1 volume of 10X concentrated PBS to 9 volumes of Percoll. The specific gravity (SG) of this stock solution and also of the RPMI 1640 containing 25mM Hepes and 10% FCS used to dilute the Percoll was measured in a specific gravity bottle and calculated by:

$$SG_{percoll/medium} = \frac{\text{weight of known volume of Percoll}}{\text{weight of an equal volume of water}}$$

Percoll was then diluted to the required specific gravity using the following formula:

$$\% \text{ Percoll} = \frac{(\text{SG required} - \text{SG medium})}{(\text{SG Percoll} - \text{SG medium})} \times 100$$

Percoll with SG 1.080 was routinely used for the separation of B cells from whole lymphocyte preparations and SG 1.074 for the separation of high and low density B cells.

Preparation of AET-treated Sheep red blood cells (SRBC)

SRBC for rosetting were treated with Alsever's solution as described by Kaplan and Clark. In brief, SRBC in Alsever's solution (Tissue Culture Services Ltd, Botolph Claydon, Buckingham, U.K.) were washed three times in 0.9% sterile saline removing all supernatant and any buffy coat after each wash. A sterile solution of S-2-aminoethylisothiouronium bromide hydrobromide (AET), (Sigma) at 40.2 mg/ml, pH

9 was prepared and 4 volumes of AET incubated with 1 volume packed SRBC at 37°C for 15 minutes. Cells were then washed five times in sterile saline and resuspended in RPMI 1640, without FCS, to give a 10% SRBC suspension which was stored at 4°C for up to three weeks.

Preparation of Gey's haemolytic balanced salt solution

Three solutions were prepared:

Solution A 650mM NH_4Cl, 25mM KCl, 5.6mM $Na_2HPO_4.12H_2O$, 0.9mM KH_2PO4, 28mM glucose, 0.0005% Phenol Red, 25g/L gelatine (Difco) in double distilled water.

Solution B 21mM $KgCl_2.6H_2O$, 5.6mM $MgSO_4.7H_2O$, 31mM$CaCl_2$ in double distilled water.

Solution C 267mM $NaHCO_3$ in double distilled water.

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

Rosette Formation

10ml of TMC or PBMC at 5 x 10^6cells/ml in holding medium were mixed with 2.5ml of 10% AET-SRBC and 1ml FCS in a plastic Universal tube and centrifuged at 100*g* for 15 mins. at room temperature with the brake off. Cells were incubated on ice for 60 mins. after which the rosettes were resuspended by gentle rotation of the tube. The resulting suspension was layered onto 10ml of Percoll (SG = 1.080) and centrifuged

at 1000*g* for 20 mins. at room temperature with the brake off. The interface layer containing non-rosetting E^- cells (i.e. mostly B cells) was removed and washed before counting and resuspending in culture medium. E^- cells from tonsil were routinely found to contain >95% CD20+ B cells, <3%CD3+ (T) cells, <2% CD14+ (monocytes) and <2% HNK1+ (NK) cells. E^- preparations from blood after one cycle of rosetting would typically contain 20% B cells, 5% T cells, 60% monocytes 10% NK cells and 5% null cells. A further round of rosetting was performed if necessary to further reduce the T cell proportion to <1%.

To recover the E^+ fraction, the remaining Percoll was removed without disturbing the pellet. The pellet was then resuspended in 5ml of Gey's haemolytic solution for 1-2 minutes to lyse the red cells. After red cell lysis, the cells were washed twice.

2.6.4 Preparation of heavy and light B cells

E^- cells were separated into heavy and light populations by discontinuous density gradient centrifugation over Percoll. Up to 3 x 10^7 E^- cells in 2-3 mls of holding medium were layered onto Percoll (SG = 1.074) in a 10ml conical centrifuge tube and centrifuged at 1000*g* for 20 mins. The interface layer of cells was collected, most of the remaining Percoll discarded, and the pellet of small dense B cells recovered. Both fractions were washed twice and counted. The dense fraction consisted mostly of resting B cells whereas the light fraction consisted of activated B cells.

2.6.5 Monocyte Depletion of E^- preparations from TMC

10mls of FCS was placed into tissue culture plastic Petri dishes and incubated at 37^0C for 30 mins. The FCS was then removed and 2 x 10^7 E^- cells in 10mls of holding

medium containing 10% FCS were added to the plates and incubated at 37°C for a further 45 mins. Non-adherent cells were resuspended by gently rocking the dishes and removed with a pipette. This procedure was repeated twice more with 10ml warm medium added to the dish. This method reduced the monocyte contamination of E^- preparations from TMC 10-fold giving a final monocyte concentration of 0.2% checked by staining with UCHM1, a monoclonal antibody to CD14. Alternatively, monocyte depletions were carried out using anti-CD14 coated Dynabeads. In this procedure, E^- cells were incubated with Dynabeads at a ratio of approx. 1:5 in capped Falcon tubes, at 4°C for 30 mins. on a rotating mixer. After this time, the rosetted cells were isolated by placing the tubes in the Dynal MPC magnet for 2-3 mins. The supernatant containing monocyte-depleted E^- cells was removed and washed and the remaining monocyte-bead fraction discarded.

2.7 FACS staining protocol

To check the purity of E^- cells, preparations were stained with a panel of mAbs. 0.5 x 10^6 E^- cells were incubated with 50µl of 8EB1, UCHT1 or UCHM1 or 5µl of the 'Simultest' in LP3 FACS tubes for 30 minutes on ice. Cells were washed twice in cold medium containing 2% FCS and 0.01% sodium azide and incubated with a FITC-conjugated goat anti-Ig antibody for a further 30 minutes on ice. Cells were then washed twice more and resuspended in 0.5ml medium and analysed on a Becton Dickinson Facscan using Lysis II Software.

2.8 Assay for B cell proliferation

Purified B cells were cultured in 96-well flat-bottomed plates at 10^6 cells/well in RPMI 1640 containing 5% FCS with 2mM glutamine and 50μg/ml gentamicin as previously described. The cytokine and/or mAb to be tested was added to triplicate wells at the optimum concentration or over a range of doses if a titration was being performed. A control was included consisting of E^- cells cultured in medium alone. The final volume of each well was adjusted to 200μl and the plates were incubated at 37°C in 5% CO_2 for 72hrs. After this time, 1μCi of ^{3}H-TdR, (Amersham International Amersham, Bucks., UK.) in RPMI 1640 was added to each well and the plates incubated at 37°C overnight. Labelled cells were harvested onto glass-fibre discs using a Dynatech Automash cell harvester and incorporated ^{3}HTdR counted on a liquid scintillation counter and expressed as disintegrations per minute (dpm) after correction for quenching and efficiency of counting. Optiphase 'HiSafe' for liquid scintillation counting was purchased from Fisons Chemicals, (Loughborough UK).

2.9 Assays for B cell differentiation

2.9.1 Specific antibody responses

For unseparated TMC, triplicate cultures were set up in 12 x 75mm capped Falcon tubes in 1ml total volume containing 2 x 10^6 TMC/ml in medium RPMI 1640 containing 25mM Hepes, 50μg/ml gentamicin, 10% horse serum and influenza virus at a final concentration of 0.2μg /ml. A negative control consisting of TMC in

medium alone was always included. Cultures were incubated at 37°C, in a humidity chamber, for seven days. At the end of the culture period, the cells were washed, resuspended in 0.5ml RPMI 1640 containing 5% FCS and incubated for a further 18 - 24 hours. Culture supernatants were then collected and analysed by ELISA for the presence of specific antibodies.

Supernatants could be frozen at -20°C and thawed up to four times without loss of activity.

For T cell-depleted E^- (B) cells, triplicate cultures were set up in Falcon tubes at 0.6 - 0.75 E^- cells/ml in RPMI 1640 + 10% horse serum. Influenza virus was added to give a final concentration of 2μg/ml. 5U/ml of IL-2, 10% $BCGF_{lmw}$ or 20 -100ng/ml IL-15 were added as T cell replacing factors (TRF). Two negative controls were included consisting of E^- cells alone and E^- cells plus antigen and a positive control which contained E^- cells, antigen and 10^6 E^+ cells. Cultures were incubated as described for whole TMC.

2.9.2 Polyclonal antibody responses by peripheral blood B cells

For polyclonal antibody responses, 0.5 x 10^6 E^- cells were cultured in capped Falcon tubes in IMDM supplemented with 10% FCS, 25mM Hepes, Penicillin and Streptomycin. Cells were cultured in medium alone and in the presence of 10^5 cells /ml of CV1/EBNA cells expressing CD40L or control CV1/EBNA cells containing the empty vector. In latter experiments, the soluble trimeric form of CD40L was used at a predetermined optimal concentration depending on the activity of a particular batch. To this basic system were added various cytokines in order to investigate their properties as differentiation or switch factors. Experiments were performed in

triplicate and incubated at 37°C for 8-10 days when the supernatants were collected for ELISA. If cell numbers were insufficient for large cultures, for example due to small blood samples from patients, experiments were performed in round-bottomed microtiter wells containing 2 x 10^5 E^- cells in 200µl medium were used instead.

2.10. ELISA for secreted immunoglobulins

2.10.1 Buffer solutions used for ELISA

Bicarbonate Buffer: 15mM Na_2CO_3, 35mM $NaHCO_3$ and 0.02% NaN_3 in distilled water, pH 9.6

PBS-Tween used for washing and incubation steps: 0.01% Tween-20 was added to a solution of Dulbecco's phophate buffered saline A and the pH adjusted to 7.5

HRP substrate buffer: equal volumes of phosphate and citrate buffer solutions were mixed immediately before use to which was added 1µl/ml of 30% hydrogen peroxide solution.

NPP substrate buffer: bicarbonate buffer pH 9.6 with 10^{-4}M $MgCl_2$.

2.10.2. Reagents used for ELISA

Immulon II and Immulon IV plates were purchased from Dynatech.

The following chemicals and antibodies were all purchased from Sigma: Tween 20, BSA Fraction V, goat anti-human IgG, IgM and IgA, alkaline phosphatase-

conjugated goat anti-human IgG, IgM and IgA, horse radish peroxidase-conjugated IgG, IgM and IgA, goat anti-human IgE, HRP-conjugated goat anti-rabbit IgG, o-nitrophenylene diamine (OPD) and o-nitrophenyl phosphate (NPP). Normal mouse serum was obtained from Serotec (Oxford, UK). Biotinylated mouse anti-human IgG1, IgG2, IgG3 and IgG4 manufactured by Zymed Laboratories and purchased through Cambridge Biosciences (Cambridge UK).

Alkaline Phosphatase-conjugated streptavidin was purchased from Amersham International. Rabbit anti-human IgE was obtained from MIAB (Uppsala, Sweden). Rabbit anti-human IgG (H+L) was obtained from Jackson Laboratories (West Grove, PA) and sheep anti-human IgG4 was purchased from The Binding Site (Birmingham, UK).

2.10.3 ELISA for specific antibody secretion

All incubation steps were carried out at room temperature unless otherwise indicated. Washing steps consisted of 2 x 3 min. washes with PBS/Tween and 1 x 3 min wash with PBS/Tween + 1%BSA.

ELISA for specific IgG, IgM and IgA

1. 75µl of a suspension of the stimulating strain of virus at 20µg/ml in PBS containing 0.02% NaN_3 was added to each well of Immulon IV plates and incubated for 2 hours. The virus was recovered from each well and saved for future use and the plate dried by inverting onto tissue paper.

2. 75µl of PBS/Tween containing 1% BSA was added to each well and incubated for 2hrs to block any non-specific binding sites.

3. Plates washed x3.

4. Samples were added to the appropriate wells. Serial dilutions of standard human serum from 1:100 to 1:100,000 for IgG and IgM and 1:10 -1:10,000 for IgA to obtain a standard curve. The plates were incubated, usually overnight, at room temperature.

5. Plates washed x3.

6. Alkaline phosphatase-conjugated goat anti-human IgG, IgM or IgA antibodies were diluted 1:1000 in PBS/Tween/BSA and 75µl added for a further two hours.

7. Plates washed x3.

8. O-nitrophenyl phosphate (NPP) at a concentration of 1mg/ml was dissolved in bicarbonate buffer containing 10^{-4}M $MgCl_2$ and 100µl added to each well. Colour development typically occurred between 10-30mins. at which time the reaction was stopped by the addition of 100µl of 3M NaOH. The absorbence at 405 nm was then read on a Dynatech automatic plate reader and the results expressed using Dynatech Microfit software as units of specific Ig secreted/ml (U/ml) where 1000U/ml was the amount detected by the highest concentration of serum on the standard curve.

ELISA for specific IgG subclasses

Steps 1 -4 As above, except that the blocking buffer contained 1% normal mouse serum (NMS).

5. Biotinylated mouse monoclonal antibodies to IgG1, IgG2, IgG3 and IgG4 were diluted in PBS/Tween containing 0.2% NMS at dilutions of 1:2000, 1:1000, 1:500 and 1:500 respectively and 75µl was added to the appropriate wells of the ELISA plate and incubated for 2 hours.

6. Plates washed x 3.

7. 75µl of a 1:1000 dilution of AP-conjugated streptavidin was added for a further 90 minutes.

8. The plates were washed again and the colour developed as described above.

2.10.4 ELISA for polyclonal antibody secretion

Measurement of total IgG, IgM and IgA

1. Immulon 2 plates were coated overnight with 75µl of a 1:1000 dilution of the appropriate goat anti-human Ig in bicarbonate buffer.

2. Plates were then blocked in PBS/Tween/1%BSA for two hours.

3. Plates washed x3.

4. Diluted samples were applied to the wells together with a standard curve of immunoglobulin of known concentration and incubated for 2 hrs.

5. Plates washed x3.

6. 75µl of a 1:1000 dilution of HRP-conjugated goat anti-human Ig was added to each well and incubated for a further 2 hrs.

7. Plates washed x3.

8. 75µl of 0.5µg/ml OPD in citrate/phosphate buffer containing 1µl/ml 30% H_20_2 was added to each well. After sufficient colour development, the reaction was stopped by the addition of 30µl $4NH_2SO_4$ before measuring the absorbance at 492nm.

Measurement of total IgE secretion

1. Immulon 2 plates were coated overnight with goat anti-human IgE at 1:500 dilution in bicarbonate buffer.

2. Plates were then blocked for 6hrs with PBS/Tween/1%BSA.

3. Plates washed x3

4. Samples were applied together with a standard curve of serum of known concentration and incubated overnight.

5. Plates washed x3.

6. 75µl of a 1:25,000 dilution of MIAB rabbit anti-human IgE in PBS/Tween/1%BSA was added to each well and incubated for a further 4hrs.

7. Plates washed x3.

8. 75µl of a 1:1000 dilution of a HRP-conjugated goat anti-rabbit IgG was added to each well and incubated for 2hrs.

9. Plates washed x3.

10. HRP substrate added as described.

Note: This assay can also be adapted to measure the production of specific antibodies by coating the plate with the stimulating virus. The greatest difficulty encountered in this assay was in constructing a standard curve, as IgE antibodies specific for influenza are not abundant in normal human serum.

Measurement of IgG subclasses

1. Immulon II plates were coated with 75µl (1µg/ml), of rabbit anti-human IgG (heavy and light chains) or 2µg/ml sheep anti-human IgG4 in coating buffer overnight at room temperature.

2. Plates washed x3.

3. Plates blocked for 6hr with PBS/Tween/1%NMS.

4. Plates washed x3.

5. Samples diluted in PBS/Tween/0.2% NMS were added and incubated overnight at room temperature.

6. Plates washed x3.

7. 75µl of biotinylated mouse anti-human IgG1 (0.5µg/ml), IgG2 (1µg/ml), IgG3 (2µg/ml) were added and incubated for 2, 2 and 4 hours respectively at room temperature. Anti-human IgG4 @ 2µg/ml was incubated for 4 hours, also at room temperature.

8. Plates washed x3.

9. 75µl of 1/2000 dilution of alkaline phosphatase-conjugated streptavidin was added to each well for 1-2hrs at room temperature.

10. Plates washed x3 and the NPP substrate for alkaline phosphatase added and colour development at 410nm measured as described.

CHAPTER 3

THE ROLE OF CD40-CD40L INTERACTIONS IN SPECIFIC ANTIBODY PRODUCTION

3.1. Introduction.

The interaction between CD40 on B cells and CD40L on activated T cells is essential for T-dependent B cell responses and has a regulatory effect on B cell development (247,292). Studies into the role of CD40-CD40L interactions in antibody production have largely relied on polyclonally activated B cell responses. Thus ligation of CD40 with mAbs or with CD40L induces B cell activation and proliferation (18,21,83,162). Furthermore, it enhances immunoglobulin production (352) and in the presence of IL-4 provides a second signal in isotype switching to IgE secretion (204,430).

Several groups of researchers have also addressed its role in specific antibody production. One study by Grabstein *et al* (164) defined a central role for murine CD40L and IL-2 in *in vitro* primary specific responses to SRBC. In these experiments, recombinant CD40L and IL-2 stimulated secretion of SRBC-specific IgM in a similar way to fixed activated Th membranes and the response could be blocked by the addition of a CD40.Fc fusion molecule. These observations were supported by an *in vivo* study by Foy *et al* in which a blocking antibody to CD40L inhibited primary and secondary specific antibody responses as well as polyclonal responses(138). Gray *et al* investigated the effect of blocking CD40-CD40L interactions *in vivo* with a CD40.Fc fusion molecule (168). Primary and secondary responses to DNP-OVA and DNP-KLH were impaired but no quantitative difference in development of germinal centres was noted which suggested that CD40L was required for memory cell formation but not germinal centre reactions. In contrast, Foy *et al* were able to inhibit both germinal centre and memory cell formation with a

blocking CD40L mAb (139). Finally, mice deficient in either CD40 or CD40L, generated by targeted gene inactivation, have been described (73,334) The CD40L-deficient mice had reduced levels of serum immunoglobulin (except IgM), and were unable to mount secondary antigen-specific responses to T-dependent antigens, although the T-independent response was intact (334). Similarly, CD40-deficient mice were unresponsive to CD40L with respect to proliferation and isotype switching and were also unable to respond to T-dependent antigens (73). To date, there is only one study addressing the role of CD40 in specific antibody production in humans. In this study, *in vitro* phage-specific antibody production was obtained by cross-linking CD40 with a mAb in the presence of IL-10 (294). However, controls in this study did not exclude polyclonal antibody production and the role of CD40 in the specific antibody response to T-dependent antigens in humans has not been fully elucidated.

This chapter describes experiments performed to investigate the effect of recombinant human CD40L on specific antibody responses to influenza virus by human B cells. The experimental system for studying specific antibody production is well defined and represents a T cell dependent, memory response to a protein antigen. The specificity was confirmed by experiments using two non-crossreacting strains of virus which stimulated secretion of antibodies specific only for the stimulating strain of the virus (58,64). In addition, T cells can be replaced by soluble factors e.g. IL-2, $BCGF_{lmw}$ which allowed investigation of the effect of CD40L directly on B cells (64,380).

3.2 Characteristics of the specific antibody response to influenza virus.

In order to characterise the specific antibody response to influenza virus, 2 x 10^6 TMC or 0.75 x 10^6 E^- cells were cultured with antigen in RPMI 1640 containing 10% horse serum as described in Materials and Methods. T helper cells (E+) or TRF (5U/ml IL-2) were added as indicted. Secretion of specific IgM, IgG and IgA was measured after 7 days by ELISA. A typical example of the results obtained is shown in Table 3.1

Table 3.1 Specific antibody production by tonsil B cells

Cells [a]	Antigen	TRF	Specific antibody secretion (U/ml) [b]		
			IgM	IgG	IgA
TMC	-	-	<5	<5	<5
TMC	+	-	<5	120 ± 12	35 ± 3
E^-	-	-	8 ± 3	<5	7 ± 4
E^-	+	-	9 ± 2	23 ± 15	11 ± 4
E^- + E+	+	-	13 ± 4	256 ± 24	120 ± 72
E^-	+	IL-2	15 ± 6	151 ± 28	96 ± 45

[a] Unseparated tonsil mononuclear cells or E^- cells purified by one round of rosetting with AET-SRBC were cultured with antigen and TRF as indicated

[b] Specific antibody secretion from triplicate cultures in units/ml ± 1 s.e.m.

TMC cultured in the absence of antigen did not secrete a significant amount of antibody showing the antigen-dependent nature of the response. TMC stimulated with the influenza virus secreted mainly specific IgG with some IgA and little, if any, IgM. Over different experiments this was variable and significant IgM production was obtained with some donors. IgG and IgA always predominated over IgM secretion which is typical of a memory response. In experiments using purified B (E^-) cells, no significant antibody secretion was obtained in the absence of either antigen or T (E^+) cells demonstrating the T cell-dependent nature of the response. The response could be restored fully by addition of T (E^+) cells. Alternatively, T cells could be replaced by the addition of 5U/ml IL-2. Experiments confirming the specific nature of the specific antibody response with T cells and with TRF are not shown here but have been detailed elsewhere (58).

3.3. Effect of CD40L on specific antibody production

In order to examine the effect of CD40 cross-linking on specific antibody responses, G28.5 and CD40L were added to cultures containing antigen-stimulated TMC or T-depleted B cells plus TRF.

In these experiments, whole TMC were stimulated with antigen in medium alone, or in the presence of 0.5μg/ml G28.5, or 10^5 CV1/EBNA cells/ml expressing CD40L ($CV1/EBNA_{CD40L}$). 10^5 CV1/EBNA cells/ml containing the empty vector were used

as controls ($CV1/EBNA_{empty\ vector}$). Table 3.2 shows the results for two such experiments. expressed as mean antibody secretion from triplicate cultures ± 1 s.e.m.

In Exp.1, TMC stimulated with antigen alone gave a good antibody response and secreted specific IgM, IgG and IgA. Secretion of all three isotypes was inhibited to almost background levels in the presence of either G28.5 or CD40L. Similar results were obtained in Exp. 2 except that no IgM was detected. CV1/EBNA cells containing the empty vector occasionally enhanced specific antibody secretion compared to control cultures containing TMC + antigen (Exp. 1).

3.4. Effect of CD40 ligation on specific antibody responses by T-depleted B cells

To examine the possibility that T cells were involved in the inhibition of the antibody response by G28.5 or CD40L, the experiment was repeated with purified B cells in the presence of either IL-2 or $BCGF_{lmw}$ as TRF. B cells were prepared and cultured with antigen and either IL-2 at a concentration of 5U/ml or $BCGF_{lmw}$ at a final concentration of 10%v/v. CD40mAb (G28.5), CD40L/CV1/EBNA cells and control cells were added as described in the previous experiment. The results of two representative experiments are shown in Table 3.3.

Table 3.2. Effect of G28.5 and CD40L expressed on CV1/EBNA cells on specific antibody production by TMC

Exp. 1

Cells[a]	Antigen	Factor	Mean specific antibody secretion (U/ml)[b] IgM	IgG	IgA
TMC	-	-	<5	14 ± 1	<5
TMC	+	-	120 ± 9	112 ± 53	>200
TMC	+	G28.5	28 ± 9	30 ± 9	18 ± 10
TMC	+	CV1/EBNA $_{CD40L}$	12 ± 3	15 ± 2	6 ± 3
TMC	+	[c]CV1/EBNA$_{ev}$	123 ± 16	63 ± 7	>100

Exp.2.

Cells[a]	Antigen	Factor	Mean specific antibody secretion (U/ml)[b] IgM	IgG	IgA
TMC	-	-	<5	21 ± 7	76 ± 14
TMC	+	-	5 ± 1	44 ± 8	75 ± 8
TMC	+	G28.5	<5	8 ± 1.5	32 ± 5
TMC	+	CV1/EBNA $_{CD40L}$	<5	6 ± 3	24 ± 10
TMC	+	[c]CV1/EBNA$_{ev}$	5 ± 1	>100	>100

[a] Unseparated tonsil mononuclear cells.
[b] Specific antibody secretion from triplicate cultures ± 1 s.e.m.
[c] CV1/EBNA cells containing only the empty vector.

Table 3.3. Effect of G28.5 or CD40L expressed on CV1/EBNA cells on specific antibody production by purified B cells in the presence of a TRF.

Exp.1

Cells[a]	Antigen	TRF	CD40 ligation	Specific antibody secretion (U/ml)[b] IgM	IgG	IgA
E^-	-	-	-	14± 0.8	<5	4 ± 3
E^-	+	-	-	20 ± 12	7 ± 0.5	20 ± 12
E^-, E+	+	-	-	> 1000	>1000	>1000
E^-	+	IL-2	-	28 ± 8	465 ± 241	>1000
E^-	+	IL-2	G28.5	104 ± 37	188 ± 42	3 ± 4
E^-	+	IL-2	CV1/EBNA $_{CD40L}$	13 ± 0.8	<5	59 ± 29
E^-	+	IL-2	[c]CV1/EBNA$_{ev}$	8 ± 0.4	9 ± 3	28 ± 8
E^-	+	$BCGF_{lmw}$	-	12 ± 0.6	87 ± 32	199 ± 70
E^-	+	$BCGF_{lmw}$	CV1/EBNA $_{CD40L}$	11 ± 1	3 ± 1	43 ± 29
E^-	+	$BCGF_{lmw}$	[c]CV1/EBNA$_{ev}$	10 ± 0.4	64 ± 22	125 ± 62

[a] E^- cells purified by one round of rosetting with AET-SRBC cultured with E+ cells or TRF as indicated.

[b] Specific antibody secretion from triplicate cultures ± 1 s.e.m.

[c] CV1/EBNA cells containing only the empty vector.

continued.....

Table 3.3 (continued)

Exp. 2

Cells[a]	Antigen	TRF	CD40 ligation	Specific antibody secretion (U/ml)[b] IgM	IgG	IgA
E^-	-	-	-	7 ± 0.3	<5	10 ± 6
E^-	+	-	-	9 ± 0.3	10 ± 1	13 ± 4
E^-, E+	+	-	-	123 ± 12	243 ± 76	>1000
E^-	+	IL-2	-	10 ± 0.3	168 ± 24	>1000
E^-	+	IL-2	G28.5	11 ± 3	30 ± 8	19 ± 13
E^-	+	IL-2	$CV1/EBNA_{CD40L}$	7 ± 1	2 ± 0.3	<5
E^-	+	IL-2	[c]$CV1/EBNA_{ev}$	7 ± 0.3	25 ± 7	6 ± 1
E^-	+	$BCGF_{lmw}$	-	13 ± 6	126 ± 35	100
E^-	+	$BCGF_{lmw}$	$CV1/EBNA_{CD40L}$	7 ± 1	<1	<1
E^-	+	$BCGF_{lmw}$	[c]$CV1/EBNA_{ev}$	13 ± 5	195 ± 33	>100

[a] E^- cells purified by one round of rosetting with AET-SRBC cultured with E+ cells or TRF as indicated.

[b] Specific antibody secretion from triplicate cultures ± 1 s.e.m.

[c] CV1/EBNA cells containing only the empty vector.

In both experiments, no significant antibody secretion was obtained in the absence of either T cells or TRF. In cultures containing T cells, a good response to antigen was observed and cells secreted IgG, IgM and IgA. Addition of either IL-2 or $BCGF_{lmw}$ as a TRF to cultures containing E^- and antigen restored the IgG and IgA but not the IgM response. Addition of mAb G28.5 or $CV1/EBNA_{CD40L}$ cells inhibited IgG and IgA secretion when either IL-2 or $BCGF_{lmw}$ were used as TRFs.

During these experiments it was observed that when IL-2 was used as a TRF, the control CV1/EBNA cells containing the empty vector also inhibited the specific antibody secretion to about the same extent as CV1/EBNA expressing CD40L. In contrast, the control CV1/EBNA cells did not inhibit the response obtained when $BCGF_{lmw}$ was used to replace T cells. This result suggests that IL-2 and $BCGF_{lmw}$ do not function as TRF in the same way and/or on the same subset of B cells. In addition this indicates the occurrence of complex interactions between the cell surface of the CV1/EBNA cells and specific antibody production which complicate the interpretation of CD40L inhibition of the specific antibody response.

3.5. Inhibition of specific antibody responses by soluble trimeric CD40L

The inhibition of specific antibody production by CD40L on CV1/EBNA cells confirmed the results obtained using mAbs to CD40. However, the observed inhibition of IL-2 TRF activity by CV1/EBNA cells containing the empty vector suggested some interaction between the CV1/EBNA membranes and B cells and complicated the results. In order to avoid this problem we used a soluble trimeric form of CD40L which has been shown to activate B cells.

TMC or E^- cells plus TRF were cultured with influenza virus and soluble trimeric CD40L was added in the form of a 10X concentrated supernatant from the COS cell line transfected with the CD40L construct. The actual concentration was equivalent to the dose required for optimal B cell proliferation (data not shown). A similar dilution of a supernatant from COS cells containing only the empty vector was used as a control. Table 3.4 shows the results for experiments using TMC and E^- + IL-2.

In this experiment, the addition of sCD40L inhibited IgM, IgG and IgA secretion by TMC to background levels. sCD40L also inhibited IgM, IgG and IgA production by E^- cells cultured with antigen and IL-2 as a TRF albeit to a lesser extent. These results were reproducible over several experiments and confirmed that the direct action of CD40L on B cells inhibits antibody secretion. The control COS s/n did not have any inhibitory effect on the IL-2 response, but rather sometimes enhanced antibody secretion. Although not known at the time, the increase in antibody secretion was probably due to the presence of IL-15 in the supernatant from the COS cell line.

Table 3.4. Inhibition of specific antibody response with a soluble form of CD40L

				Antibody secretion (U/ml)[a]		
Cells[b]	**Antigen**	**TRF**	**CD40 ligation**	**IgM**	**IgG**	**IgA**
TMC	-	-	-	<5	14 ± 1	<5
TMC	+	-	-	120 ± 21	112 ± 53	>100
TMC	+	-	cos s/n	205 ± 54	235 ± 41	>100
TMC	+	-	sCD40L	5 ±1	13 ± 2	<5
E^-	-	-	-	<5	5 ± 0.2	<5
E^-	+	-	-	20 ± 14	9 ± 2	8 ±7
E^- + E+	+	-	-	184 ± 15	131 ± 37	>100
E^-	+	IL-2	-	95 ± 53	66 ± 12	55 ± 53
E^-	+	IL-2	cos s/n	210 ± 53	310 ± 97	> 100
E^-	+	IL-2	sCD40L	22 ± 3	40 ± 1	26 ± 23

[a] Unseparated tonsil mononuclear cells or E^- cells purified by one round of rosetting with AET-SRBC.

[b] Specific antibody secretion from triplicate cultures ± 1 s.e.m.

3.6 Effect of sCD40L on specific and polyclonal antibody secretion by antigen-stimulated B cells.

Early experiments showed that CD40L was able to induce immunoglobulin secretion by B cells in the presence of cytokines. In contrast, our results show that the addition of CD40L profoundly inhibits specific antibody production. We therefore considered the possibility that CD40L was inhibiting specific antibody secretion while stimulating polyclonal antibody secretion at the same time. This was investigated by measuring the effect of CD40 on specific and poyclonal immunoglobulin produced by E^- cells cultured with antigen and TRF. In these experiments cells were cultured in IMDM rather than RPMI as this has been found previously to optimise polyclonal antibody secretion (K. Kotowicz, personal communication.). Supernatants were analysed for the secretion of both specific and polyclonal IgG and IgM secretion. The results for IgG and IgM secretion from two experiments are shown in Figs. 3.1 and 3.2 respectively.

Fig 3.1. Effect of sCD40L on total and specific IgG secretion by antigen-stimulated B cells

B cells were cultured in IMDM and stimulated with antigen and TRF (IL-2) with or without sCD40L or COS s/n control. After 7 days in culture, supernatants were analysed for the secretion of specific and total (polyclonal) IgG. Results are shown for two experiments.

Fig. 3.1

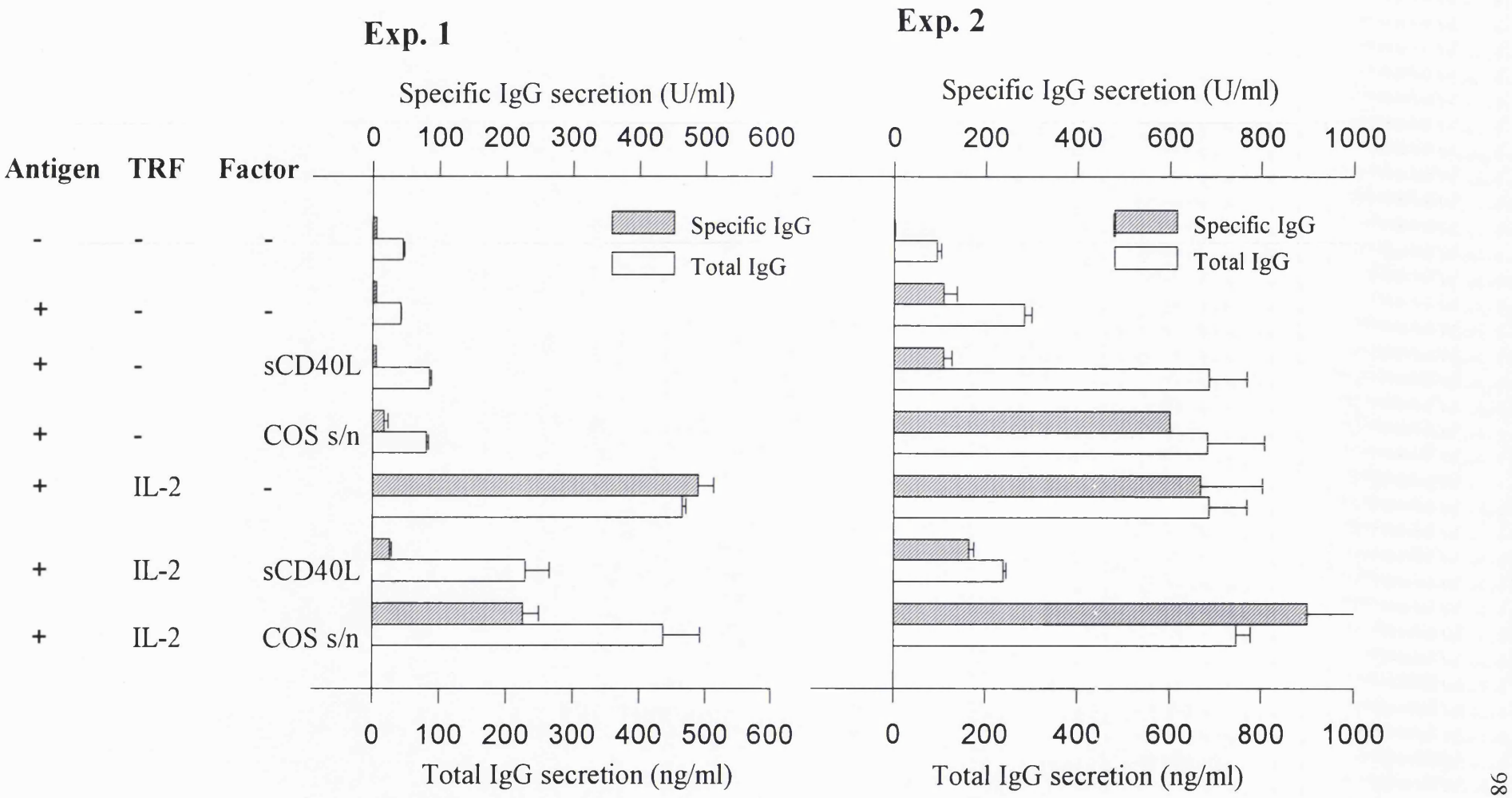
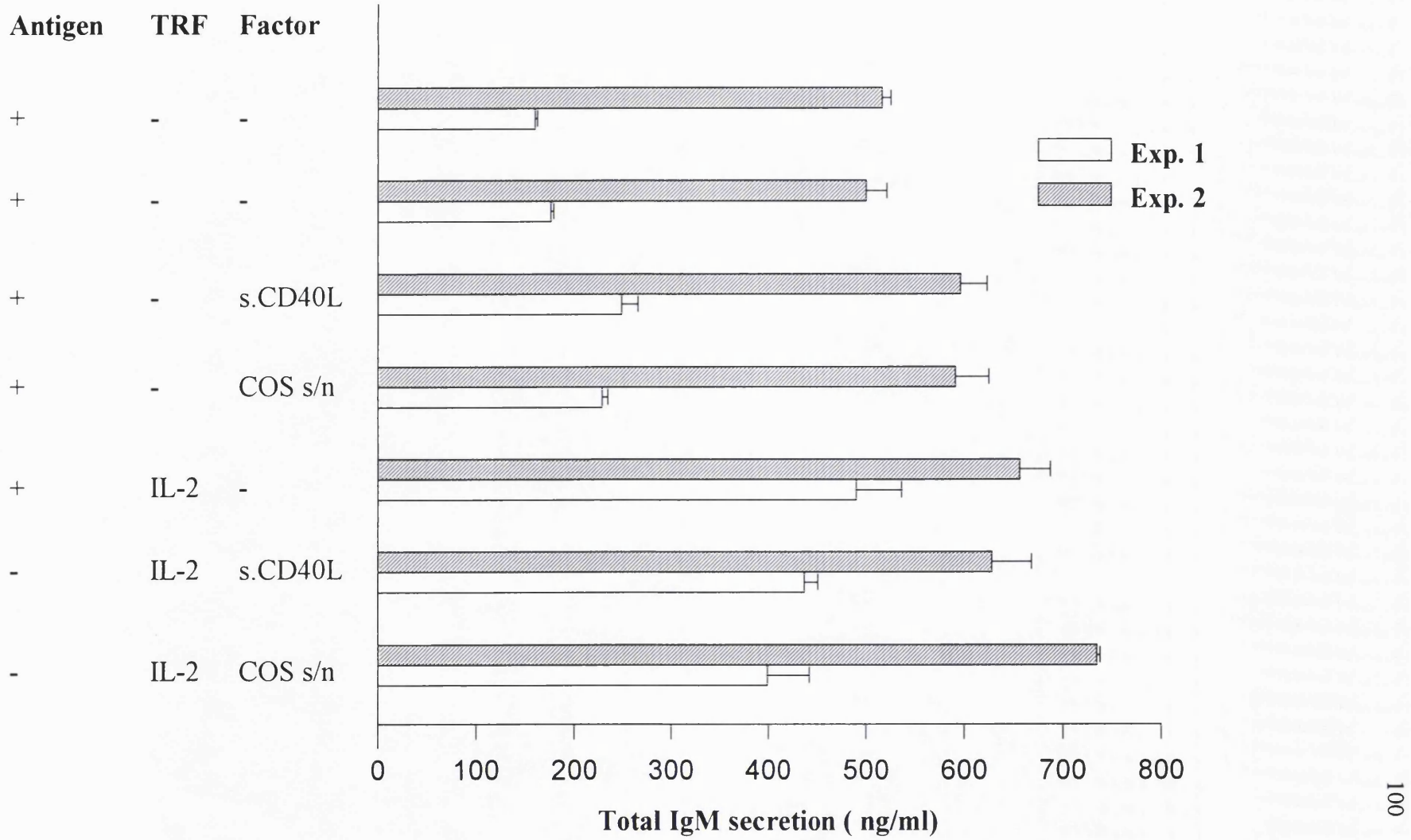

Exp. 1
Exp. 2
Specific IgG secretion (U/ml)
Specific IgG secretion (U/ml)
0 100 200 300 400 500 600
0 200 400 600 800 1000
Antigen
TRF
Factor
Specific IgG
Total IgG
- - -
+ - -
+ - sCD40L
+ - COS s/n
+ IL-2 -
+ IL-2 sCD40L
+ IL-2 COS s/n
0 100 200 300 400 500 600
0 200 400 600 800 1000
Total IgG secretion (ng/ml)
Total IgG secretion (ng/ml)

Fig 3.2. Effect of sCD40L on total and specific IgM secretion by antigen-stimulated B cells

B cells were cultured in IMDM and stimulated with antigen and TRF (IL-2) with or without sCD40L or COS s/n control. After 7 days in culture, supernatants were analysed for the secretion of specific and total (polyclonal) IgM. No significant specific IgM secretion was detected. Results are shown for two experiments.

Fig.3.2


Antigen
TRF
Factor
+ - -
+ - -
+ - s.CD40L
+ - COS s/n
+ IL-2 -
- IL-2 s.CD40L
- IL-2 COS s/n
Exp. 1
Exp. 2
0
100
200
300
400
500
600
700
800
Total IgM secretion (ng/ml)

In Exp. 1, as observed previously, B cells stimulated with antigen in the presence of IL-2 secreted specific IgG that was inhibited by the addition of sCD40L. In this experiment, the control COS s/n alone did not stimulate significant levels of specific antibody secretion. Similar effects of sCD40L and COS s/n were observed on polyclonal (total IgG) antibody secretion. The presence of IL-2 in the cultures induced the secretion of significant amounts of both polyclonal and specific IgG. In Exp. 2 the sCD40L alone increased total immunoglobulin secretion and the COS s/n increased both polyclonal and specific antibody production. Nevertheless, the addition of sCD40L to cultures stimulated with antigen and IL-2 resulted in profound inhibition of both specific and polyclonal antibody secretion. No specific IgM was obtained in either experiment but a high background level of total IgM secretion was observed (Fig. 3.2). The addition of IL-2 to the cultures enhanced IgM secretion above background levels but further addition of CD40L had no consistent effect.

These experiments did not produce easily interpreted results but seemed to indicate that cross-linking CD40 on antigen-stimulated B cells inhibits both polyclonal (total immunoglobulin) and specific antibody secretion.

3.7. Timecourse of action of CD40L on specific antibody secretion.

One possible mechanism of action for the observed inhibition of specific antibody production is that CD40L ligation of CD40 promotes proliferation exclusively of differentiation. We therefore investigated whether CD40L-mediated inhibition was an early event consistent with an effect mediated during activation/proliferation of B cells, or a late event which might indicate a direct effect on antibody secretion. In

these experiments, sCD40L was added to TMC on days 0,3,5 and on Day 6. Results for this experiment are shown in Fig 3.3.

Addition of sCD40L to cells at the start of culture (Day 0) inhibited specific antibody production to background levels. However, when sCD40L was added on Day 3, there was an apparent increase in antibody secretion to 525U/ml compared to 139 U/ml for the control cultures containing TMC and antigen alone. This high level of antibody secretion was largely sustained until Day 5. Addition of CD40L at the end of the culture period had no effect.

In a second experiment, purified E^- cells, rather than TMC, were cultured with antigen in the presence of IL-2 (5U/ml). sCD40L was added on Days 0,1,2,3,5,6 and 7(post-wash). The results are shown in Fig.3.4.

In this experiment, the addition of sCD40L on Day 0 caused inhibition of specific antibody secretion but no inhibition was observed beyond this time. Addition of the CD40L on Days 1,2,3,4 and 5 apparently increased antibody secretion to some extent, although this was not consistent or of the magnitude observed in Experiment 1. Unfortunately, the degree of variation between days 1-6 was too great to establish whether a significant increase in specific antibody secretion was being observed. Both these experiments showed clearly that the inhibition is dependent on the sCD40L signal being delivered early (in the first 24 hours) of culture.

Fig.3.3

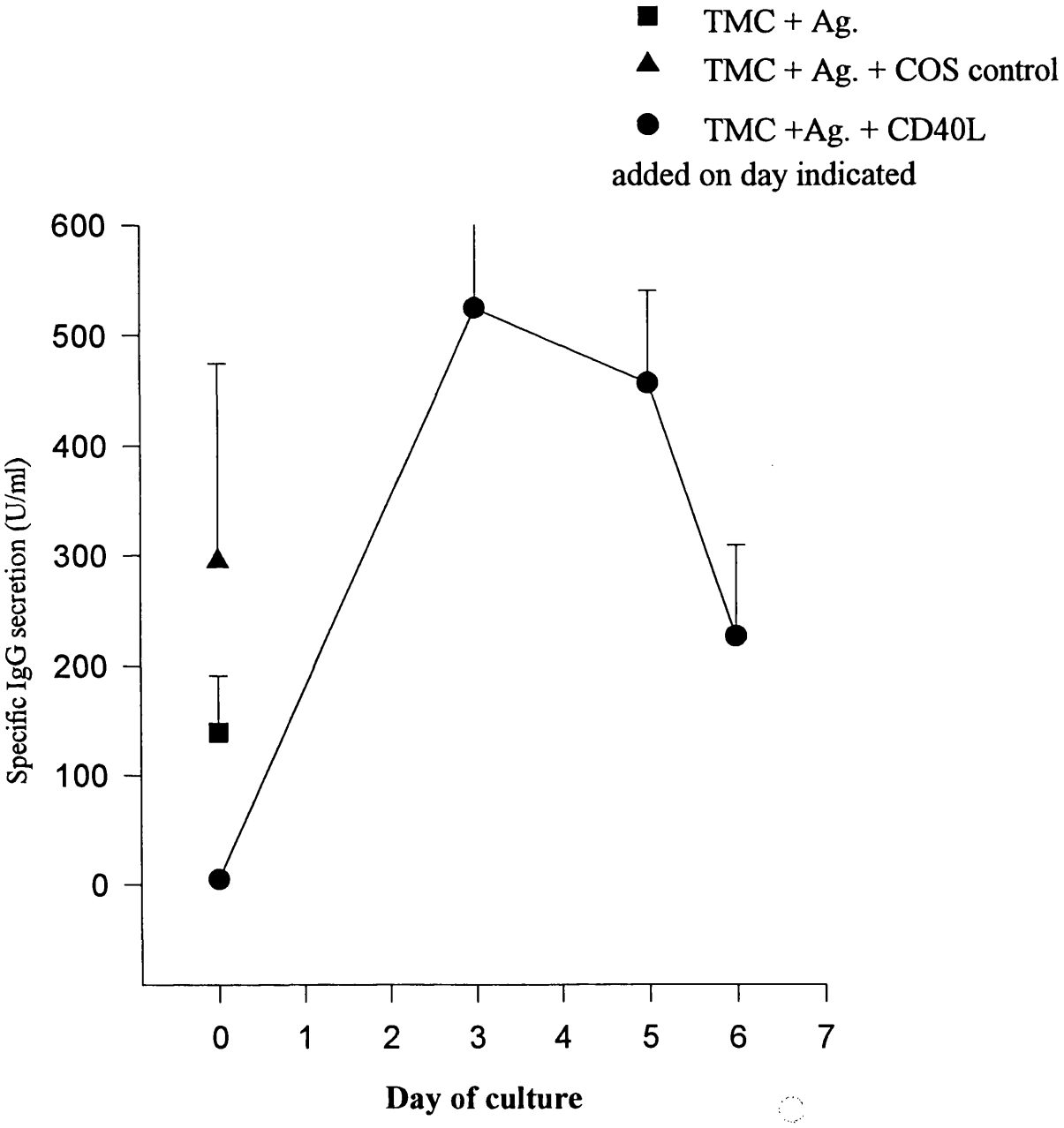


Fig. 3.3. Timecourse of action of CD40L in the specific antibody response by TMC

TMC were stimulated with antigen and sCD40L added to the cultures on the days indicated. On Day 6 the CD40L was added post-wash as a control. Results are expressed as mean antibody secretion from triplicate cultures +/- 1 s.e.m.

Fig. 3.4

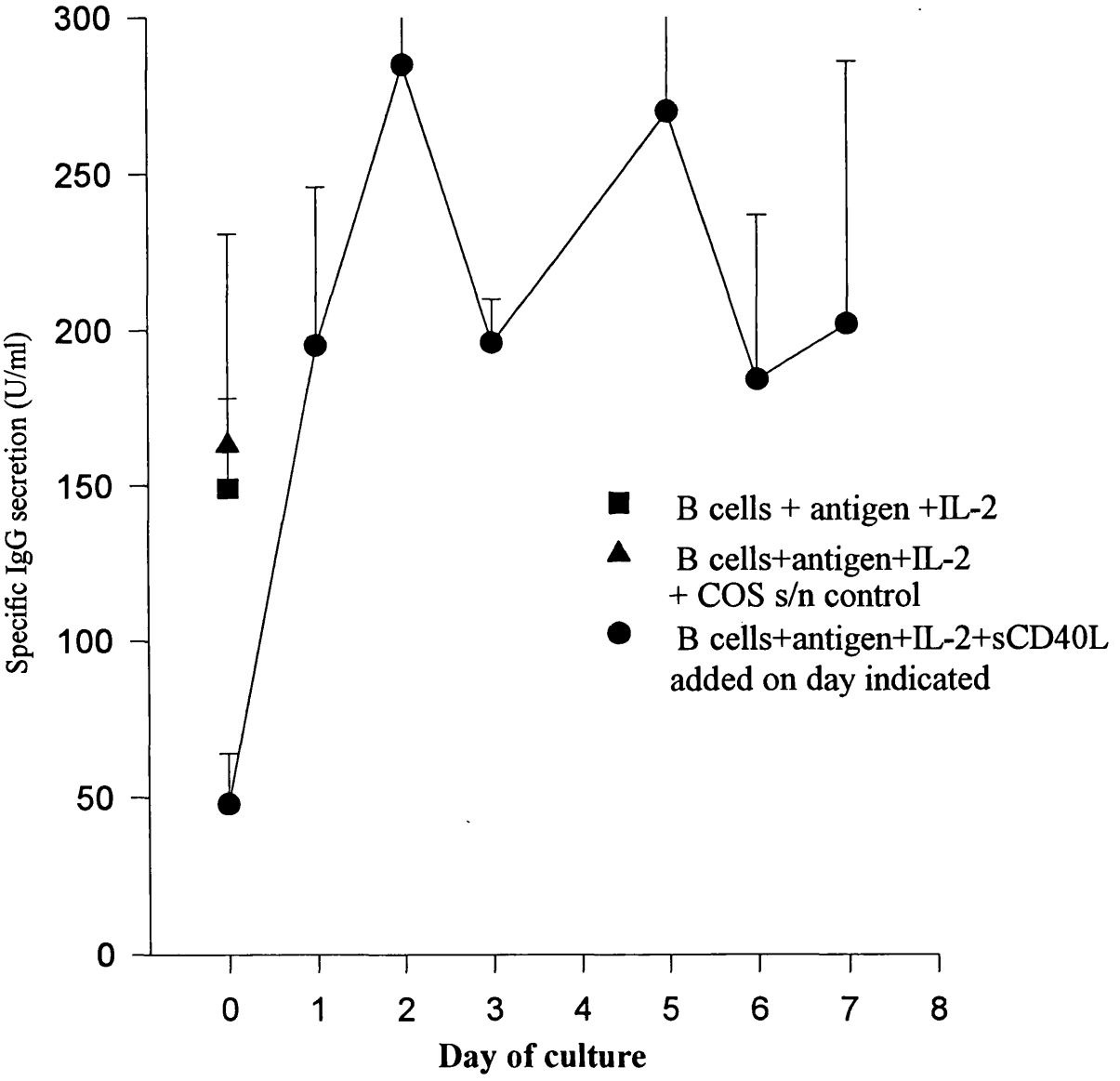


Fig. 3.4 Timecourse of action of CD40L inhibition of the specific antibody responses by purified B cells cultured with antigen and TRF

Purified B cells and IL-2 were stimulated with antigen and sCD40L added to the cultures on the days indicated. On Day 7, CD40L was added post-wash as a control. Results are expressed as mean antibody secretion from triplicate cultures +/- 1 s.e.m.

3.8. Comparison of mAbs to CD40 with different epitope specificities on specific antibody responses and B cell proliferation

We obtained the panel of eight mAbs to CD40 (clones EA-5, G28.5, BL-0GY/C4, 5C3, HB14, 14G7, M2-CD40 and M3-CD40) submitted to the 5th International Workshop on
Human Leucocyte Differentiation Antigens. These mAbs in addition to mAb 89 and sCD40L were compared for their ability to inhibit specific antibody production and to co-stimulate B cell proliferation with anti-IgM.

The results of two experiments showing the effect of the panel of mAbs on specific antibody response are shown in Fig.3.5. In both experiments, CD40L completely abrogated the response whereas the control COSs/n slightly increased specific antibody secretion. The panel of mAbs had variable effects. One mAb, BL-OGY/C4 inhibited antibody secretion in both experiments to background levels. Some mAbs e.g. G28.5, EA5, M2-CD40, M3-CD40 showed full inhibition in one experiment but only partial inhibition in the other. Finally, other mAbs gave only partial inhibition or had no effect.

We next compared the different CD40 mAbs and CD40L for their ability to co-stimulate with anti-IgM to induce B cell proliferation. Firstly, purified B cells were cultured either alone or with 100U/ml IL-4 or 10μg/ml anti-μ beads and in the presence of 0.5 μg/ml G28.5 (mAb to CD40), or 10^5 fixed CV1/EBNA cells expressing CD40L on their surface (CD40L). CV1/EBNA cells containing the empty

Fig.3.5

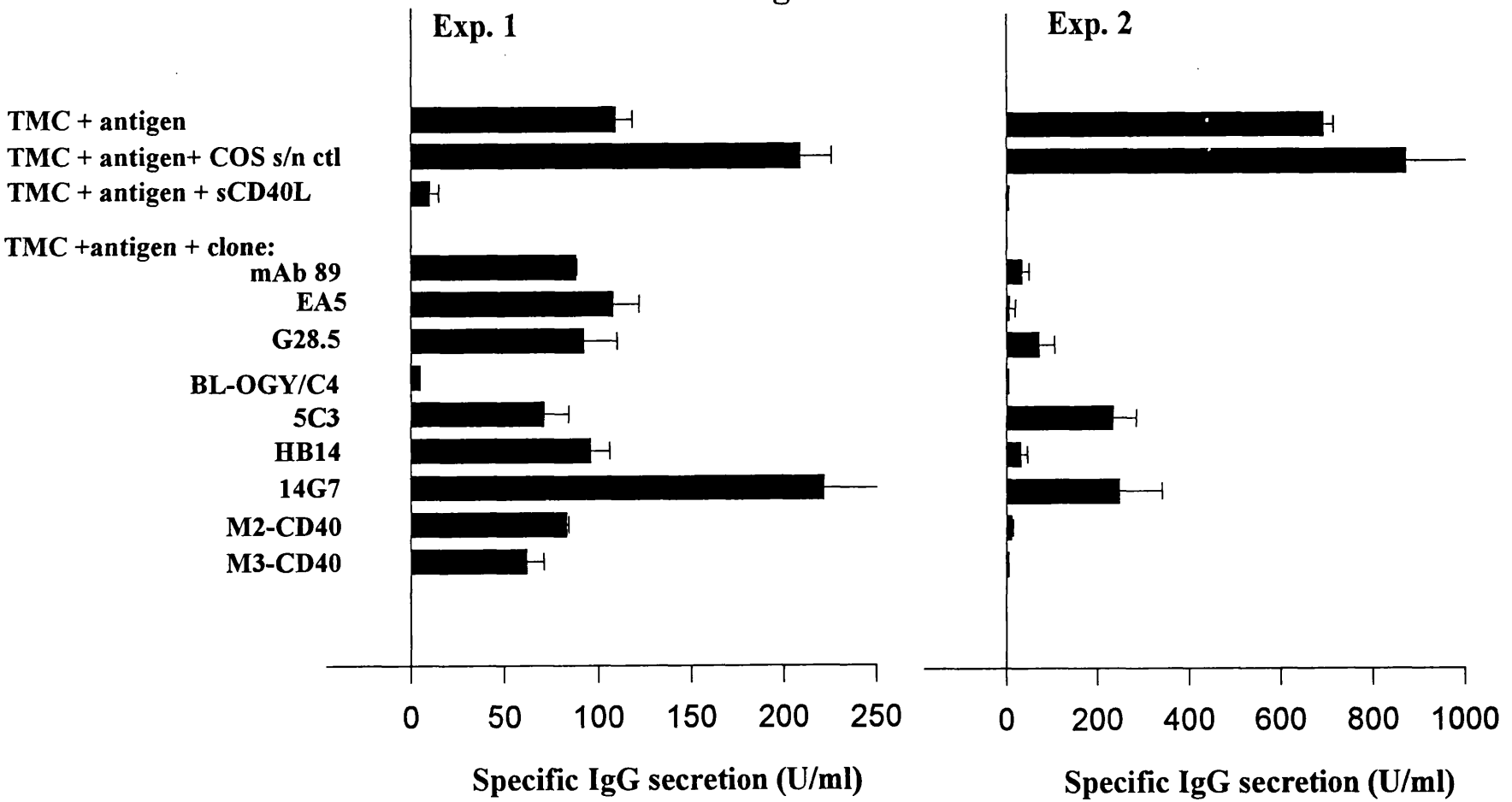


Fig. 3.5 Effect of CD40 mAbs on specific antibody production by human TMC

TMC were stimulated with antigen in the presence of CD40 mAbs from the HLDA workshop. Each mAb was added at a concentration of 10μg/ml. Day 7 supernatants were analysed for secretion of specific IgG.

vector were used as a control (Table 3.5). In another experiment, B cells were co-stimulated with sCD40L and either anti-IgM beads(10μg/ml) or IL-4 (100U/ml). Supernatant from COS cells containing the empty vector (COS s/n) was used as a control. (Table 3.6.)

CD40L alone (present as either CV1/EBNA$_{CD40L}$ or sCD40L) and to a lesser extent G28.5 both had a direct mitogenic effect on B cells compared with control cultures. In addition CD40L or G28.5 co-stimulated with both IL-4 and anti-IgM to induce proliferation. Anti-IgM was a stronger co-stimulatory signal than IL-4 and CD40L plus IL-4 were more effective at inducing proliferation than IL-4 and G28.5.

The panel of CD40 mAbs were then compared to sCD40L for their ability to co-stimulate with anti-IgM to induce proliferation. Results from a typical experiment are shown in Fig. 3.6. In this experiment CD40L co-stimulated well with anti-IgM. The workshop mAbs EA-5, G28.5, 5C3 and HB14 were good co-stimulators but not as powerful as the sCD40L. mAb 89, 14G7 and BL-OGY were poor stimulators of proliferation and the values for ^{3}HTdR uptake barely exceeded the control value for E^{-} cultured with anti-IgM alone. MAbs M2-CD40 and M3-CD40, however, were extremely efficient co-stimulatory agents and were to sCD40L.

The results from the experiments showing inhibition of the specific antibody responses and co-stimulation of proliferation by sCD40L and the panel of CD40 mAbs are summarised in Table 3.7. These results show there is no clear cut relationship between the ability of the mAbs to stimulate proliferation and inhibit specific antibody secretion.

Table 3.5. Proliferation of human B cells in response to CD40 mAbs or CD40L expressed on CV1/EBNA cells

	^{3}H TdR uptake (dpm) (x10^{-3})[a]					
	Exp.1			Exp.2		
	medium	IL-4	anti-IgM	medium	IL-4	anti-IgM
medium	1.9	4.7	16.1	1.5	2.9	4.9
CV1/EBNA$_{(empty\ vector)}$	2.8	5.3	15.6	2.1	4.0	22.6
CV1/EBNA$_{CD40L}$	29.0	84.0	93.0	44.0	75.6	115.8
G 28.5	11.6	21.5	185	5.1	12.6	55.0

[a] mean dpm from triplicate cultures. Standard errors were all <10%.

Table 3.6 Proliferation of human B cells in response to soluble trimeric CD40L

	^{3}H TdR uptake (dpm x 10^{-3})[a]					
	Exp. 1			Exp. 2		
	medium	IL-4	anti-IgM	medium	IL-4	anti-IgM
medium	0.2	22.3	1.7	0.7	2.1	5.7
COS s/n	1.5	3.2	4.5	1.2	3.8	5.3
sCD40L	40.0	128.6	95.5	31.0	28.0	72.1

[a] mean dpm from triplicate cultures. Standard errors were all <10%.

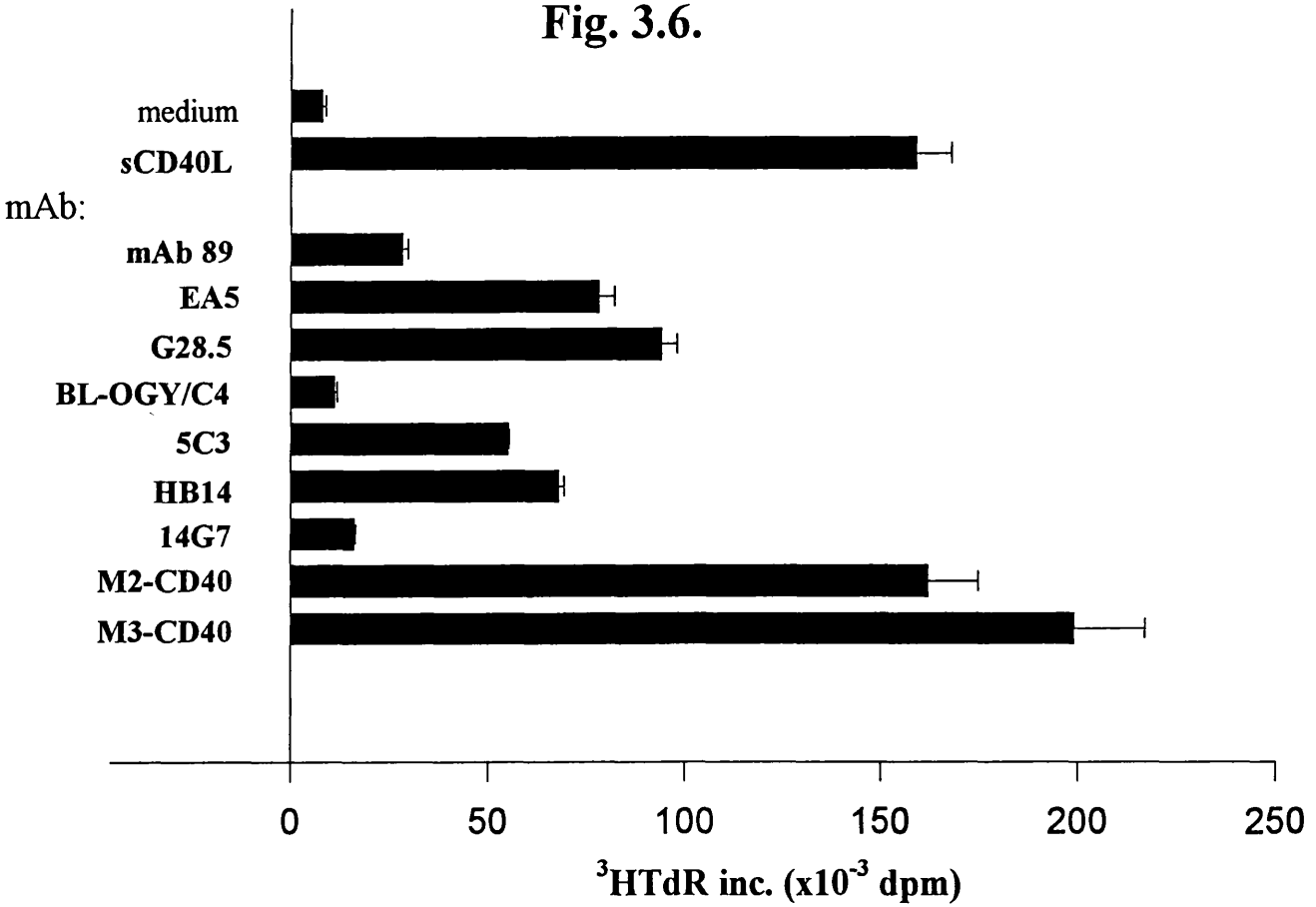


Fig. 3.6. Effect of a panel of mAbs to CD40 on B cell proliferation

B cells were co-stimulated with 10μg/ml of anti-IgM beads and CD40mAbs from the HLDA workshop at a concentration of 10μg/ml. B cell proliferation was measured by the uptake of ^{3}H-TdR after 72 hours. Results are expressed as mean dpm from triplicate cultures +/- 1 s.e.m..

Table 3.7. Summary of the effects of a panel of mAbs to CD40 on proliferation and specific antibody production by B cells.

CD40 mAb	Effect on proliferation	Degree of inhibition of specific antibody response	
		Exp.1	Exp.2
mAb 89	↑[a]	+[b]	+++
EA5	↑↑	-	+++
G28.5	↑↑	+	++
BL-OGY/C4	-	+++	+++
5C3	↑↑	+	+
HB14	↑↑	-	+++
14G7	↑	↑↑(*)	+
M2-CD40	↑↑↑	+	+++
M3- CD40	↑↑↑↑	++	+++
sCD40L	↑↑↑	+++	+++

[a] Results for proliferation are expressed according to whether the mAb was a strong (↑↑↑↑ or ↑↑↑), moderate (↑↑), or poor (↑) co-stimulator of proliferation or had no stimulatory activity (-).

[b] In describing the results for specific antibody production:
+ + + = potent inhibition, + + = moderate inhibition + = poor inhibition
- = no inhibition, (*) ↑↑ = stimulation of Ab. secretion

3.9 Discussion

The data described in this chapter clearly show that CD40-ligation of antigen-stimulated human tonsil B cells results in the inhibition of specific antibody production. This is a novel and perhaps a surprising outcome given that most other experimental systems have demonstrated a stimulatory role for CD40-CD40L. The only other study to date that reports any inhibitory activity by CD40L is by Funakoshi *et al* who showed that the addition of CD40L to B-cell lymphoma cells inhibits their proliferation (146). In the search to elucidate the role of CD40-CD40L interactions in B cell differentiation, most early studies relied on observations made in experimental systems which employed polyclonal activators. The availability of the well-characterised assay for *in vitro* specific antibody production by human B cells to influenza virus provided an alternative approach for studying the mechanisms involved in regulation.

In attempting to explain why CD40 ligation should inhibit specific antibody production by B cells stimulated with antigen, it is useful to consider the role of CD40-CD40L interactions at different stages of B cell differentiation. The B cells which secrete specific antibodies to the influenza virus are memory cells shown by the fact that all people have serum antibodies to the virus. In addition, IgG is the predominant isotype secreted in response to a challenge with influenza virus and it has been shown that the response is made by μ- (δ+) cells (61). Thus the B cells in question have previously been selected and have survived events in the germinal centre required for secondary high affinity responses including class switching, somatic hypermutation, and subsequent rescue from apoptosis during affinity

maturation. *In vivo*, the affinity maturation process for B cells residing in 2^{O} lymphoid tissue of the tonsil is likely to continue beyond the initial exposure of antigen via interactions with unprocessed antigen complexed on the FDC.

The observation that CD40L can inhibit some aspect of T-dependent B cell responses has not been reported elsewhere. These results are unique but also potentially controversial. The only other similar study examining the role of CD40 in specific antibody responses is by Nonoyama *et al* (294). In this study, peripheral blood B cells were collected before and after intravenous immunisation with the antigen, ϕx 174 and stimulated *in vitro* with antigen and either CD40 mAb (G28.5) plus IL-10 or autologous T cells. Day 12 supernatants were assayed for phage-specific immunoglobulin by ELISA and a phage-neutralising assay. The response was shown to be antigen and T cell- dependent however T cell help was replaced by addition of anti-CD40 and IL-10. Peripheral blood B cells isolated after the first immunisation secreted mainly IgM whereas after the second immunisation, B cells secreted predominantly IgG consistent with a memory response. In these experiments, antibody production could be inhibited by a blocking antibody to CD40L and no secondary response was elicited from XHIGM patients. They concluded that *in vitro* antigen-specific antibody production requires the presence of antigen, IL-10 and activation via CD40 ligation.

The reason for the discrepancy between the observations of ourselves and Nonoyama *et al* are not obvious. Although antigen-dependence was demonstrated, the possibility that the ϕx 174 antibodies were a component of a polyclonal response rather than a specific antibody response was not formally excluded. CD40 mAb and IL-10 are

known to stimulate polyclonal antibody production and it is possible that the requirement for CD40 was a component of the polyclonal response. (58,64). In contrast, specificity of the influenza antibody response used here has been confirmed by crossover experiments with two non cross-reacting strains of virus. In addition, Nonoyama *et al* measured specific antibody production after 12 days. We have found specific antibody secretion to influenza to be maximal after 7 days. In contrast, polyclonal antibody secretion by B cells stimulated with CD40L and cytokines or with EBV and cytokines is maximal after 12 days.

There may be further differences between the two experimental systems, for example with regard to the precise stage of B cell development at which antigen stimulation occurs. In the case of the Nonoyama study, donors were immunised twice with the antigen and peripheral blood was taken 6 weeks after the last immunisation. The B cells used in our study were derived from tonsil tissue and the time of the exposure to influenza was not known. It is possible, that B cells which have been residing in the tonsil tissue have been in contact with the FDC network. They may therefore have undergone more stages of development than recently primed cells circulating in the peripheral blood and thus may react differently to stimulation with antigen. Finally, we have not fully investigated the effect of adding IL-10 to our assays. Early studies showed that alone it does not replace T cell help in the same way as IL-2. However it is possible that it could have some major effect in the response to influenza which remains to be defined.

CD40 ligation consistently inhibits specific antibody production

Ligation of CD40 on B cells with mAb 89, G28.5, CD40L expressed on fixed CV1/EBNA cells or sCD40L consistently resulted in the inhibition of specific antibody responses. It was possible that cross-linking of CD40 with mAbs was simply resulting in blocking the B cell interaction with Th cells and thereby ablating the antibody response. It has been shown that CD40L expression on T cells is diminished upon culture in the presence of B cells (412,427) which could result in reduced T cell help and exert a regulatory effect on B cell activation and differentiation. It was therefore possible that the continued presence of CD40L in T cell-containing cultures was affecting the B cell response via feedback mechanisms involving other T-B cell interactions.

In order to test this, the experiments were repeated using purified B cells cultured in the presence of a TRF. The results confirmed the initial observations using unseparated TMC that cross-linking CD40 inhibited specific antibody production and further that the effect was due to the transduction of a negative signal directly to the B cell and is not mediated via T cells.

In experiments in which B cells were cultured with antigen and IL-2, we observed inhibition of specific antibody production by the control CV1/EBNA cells containing the empty vector as well as $CV1/EBNA_{CD40L}$. However, this inhibition was not observed if $BCGF_{lmw}$ was used as a TRF or in responses using whole TMC. Thus, the inhibitory effect was not generally caused by the CV1/EBNA cells but some unexpected interaction between IL-2 and CV1/EBNA cells. Given that the

CV1/EBNA cells are fixed, it is unlikely that an inhibitory factor is being secreted by the cells. However, the CV1/EBNA cell line is derived from a monkey kidney epithelial line and could conceivably express other cell surface molecules which might induce interactions with B cells and interfere with specific antibody production. It is also possible that the CV1/EBNA cells have other receptors which can bind IL-2 thus effectively depleting the cultures of a TRF and causing a lack of responsiveness in the B cells. If this is a competitive inhibitory effect, it may have been possible to overcome the inhibition by increasing the dose of IL-2 used as a TRF. However we did not follow this up as in future experiments we used the soluble, trimeric CD40L.

sCD40L was obtained from COS cells transfected with modified CD40L cDNA with a leucine zipper to produce the trimeric form (12). The soluble trimer had a dramatic inhibitory effect on specific antibody production. However, we also noticed that the control supernatants (COS cells transfected with the empty vector) consistently enhanced specific antibody production. Similar observations were made by our collaborators at Immunex and characterisation of the stimulatory factor revealed the novel cytokine, IL-15, which has functional homology with IL-2 as well as sharing some of its receptor components. We therefore attribute the stimulatory activity in specific antibody production by the control COS cell supernatants largely to its intrinsic IL-15 activity although the presence of further factors cannot be ruled out. It would have been preferable to use purified sCD40L, free from COS supernatant, but unfortunately such a preparation was not available as purification has proved difficult. However, these observations do not affect the conclusions regarding the inhibitory effect of CD40L.

Addition of CD40L to antigen-stimulated B cells decreases both polyclonal and specific antibody production.

The specific antibody response *in vitro* to influenza virus is a typical amamnestic response by B cells to a protein antigen, secreting mainly IgG with little, if any, IgM. On the other hand, CD40L stimulates polyclonal antibody secretion in normal B cells which can be augmented by the addition of cytokines such as IL-2. We therefore measured both the total immunoglobulin and specific antibody secretion by B cells stimulated with influenza virus in order to investigate the possibility that CD40L was, for some reason, enhancing polyclonal immunoglobulin secretion in preference to specific antibody secretion, perhaps by over-riding the signal delivered by antigen.

Although these experiments gave complex results, it was apparent that addition of CD40L to antigen-stimulated cultures resulted in a decrease of both polyclonal and specific IgG secretion. There was no significant specific IgM antibody production and the total IgM was not altered significantly by the presence of either CD40L or cytokines. It is possible that the presence of specific antigen in cultures restricts the CD40L action to a specific subset of the total B cells present for example, B cells activated by antigen binding to specific surface IgG. CD40L may act selectively on a population of antigen-activated B cells in order to reduce secretion of non-specific antibody.

Is inhibition is mediated via monocyte interactions?

Alderson *et al* have shown that the presence of GM-CSF, IL-3 or IFN-γ enhanced expression of CD40 on human monocytes (2). Ligation of CD40 on monocytes with

CD40L-transfected cells enhanced the secretion of TNF-α and IL-6 in the presence of GM-CSF, IL-3 or IFN-γ and the production of IL-8 in the presence of GM-CSF or IL-3. Some of these cytokines may decrease B cell function and we therefore considered the possibility that CD40L was mediating an inhibitory effect via an interaction with monocytes. TMC or B cell preparations typically consist of only about 2% monocytes which can be easily reduced to <0.2% by adherence to tissue culture plates or through negative selection of CD14+ cells. Preliminary experiments showed that even after monocyte depletion potent inhibition of specific antibody secretion by CD40L still occurred (data not shown). Some monocytes are necessary for efficient specific antibody secretion - possibly in their capacity as antigen-presenting cells - and it is possible that this residual population of CD14+ cells could still be involved in CD40L-mediated inhibition. This seems unlikely though because our results did not show significant differences in antibody secretion in cultures after monocyte depletion and inhibition with CD40L was unaffected.

CD40L-mediated inhibition of specific antibody production is an early event.

The results from the two timecourse experiments show that inhibition of specific antibody production by CD40L is an early event. Inhibition was lost if CD40L was added beyond the first 24 hours of culture. In addition, it is possible that addition of sCD40L after day 0 or day 1 enhances specific antibody production. The increases observed were not always significantly higher than the control values however and further experiments are needed to evaluate this. There may be a direct effect on the B cells through CD40 ligation resulting in increased B cell proliferation in the early stages of culture. The increased antibody secretion observed by addition of CD40L

after Day 1 may be due solely to increased B cell proliferation or may reflect the transduction of a further signal via CD40-CD40L interaction which drives antibody secretion. It is also possible that the IL-15 that may be present in the CD40L/COS s/n is mediating some effect. Finally there seems to be a difference in the results obtained depending on whether whole TMC or purified B cells were used. It is now known that CD40L can activate T cells resulting in proliferation and increased secretion of IL-2, TNFα and IFNγ (12).The apparent increase in specific antibody production may be due to a positive effect of the sCD40L on T cells, which in turn enhances the B cell response via increased T cell help, however this has not been investigated further.

Effects of different mAbs to CD40 on B cell responses - proliferation vs differentiation?

Early observations of cross-linking CD40 on B cells with mAbs indicated that the effects of CD40 ligation (as measured by ^{3}HTdR uptake) could be enhanced by a secondary cross-linking antibody (44). In addition, it was reported elsewhere that some CD40 mAbs had an additive effect on proliferation. This suggested that there was more than one functional epitope present within the CD40 molecule and that the degree of cross-linking may be important in the regulation of B cell responses. Continuing investigations have confirmed and extended these observations (35,214) and through functional studies with the panel of mAbs to CD40 submitted to the Boston workshop, Pound *et al* (321) suggested that there is a minimum of four functional epitopes on CD40.

Our own study, in which the same panel of mAbs were added to the specific antibody assay supports these observations. It was found that some of the mAbs were able to inhibit specific antibody secretion completely whereas others acted only as partial inhibitors and some had little effect. We have not yet taken the opportunity to investigate any possible co-operative effects between the mAbs. However, the major observation was that the mAbs with the best inhibitory effect on specific antibody production were not necessarily the most potent stimulators of proliferation. In contrast, CD40L was always profoundly inhibitory with respect to specific antibody production and was a powerful stimulator of proliferation. Only mAb BL-OGY/C4 consistently inhibited specific antibody production and yet had little proliferative activity. A possible explanation is that it engages part of the CD40 receptor involved in antibody secretion but not necessary for proliferation. In our study, it was found that the different mAbs have different effects on cells from the same donor, and also that some of the mAbs exhibited different degrees of inhibition of specific antibody secretion between donors. This suggests that engaging different epitopes on CD40 can elicit different B cell responses. The different effects may be mediated via different signalling mechanisms which in turn may depend upon several factors, for example, whether CD40 and CD40L occur as dimers (CD40) or trimers (CD40L). Also, the expression, affinity and aggregation of the various epitopes of CD40 may affect the extent to which cross-linking is achieved (35).

CD40, germinal centres, and memory B cells.

A recent study by Arpin *et al* has demonstrated *in vitro*, the signals which drive human germinal centre B cells to become plasma cells or memory B cells (13). Briefly, germinal centre B cells continuously stimulated by CD40L expressed on L-

cells, IL-2 and IL-10, developed a memory cell phenotype. These cells were CD38-CD20+, secreted virtually no immunoglobulin and showed very weak intracytoplasmic staining with anti-Ig, but were responsive to further proliferative signals from CD40L, IL-2 and IL-10. In contrast, cells which received CD40L-stimulation for only the first three days in a seven day culture displayed a plasma cell phenotype. These cells were CD38+ CD20-, showed strong intracytoplasmic and surface immunoglobulin staining, and secreted IgG, IgM and IgA, but were unresponsive to proliferation signals. Taken together, these results indicate that CD40 ligation directs the formation of memory B cells from germinal centre cells.

This study seems to be in agreement with our own findings that the presence of CD40L in culture inhibits antibody forming cell (AFC) development. There are differences between the two experimental systems, however, the greatest being that we are studying the response of memory B cells to CD40L stimulation whereas Arpin *et al* studied the differentiation of germinal centre B cells. It is possible that, in our assay, the stimulation of memory B cells with CD40L during the whole culture period of the specific antibody response maintains the memory phenotype. Arpin *et al* showed that after the first three days of culture with CD40L the germinal centre B cells were proliferating and were in the exponential growth phase. This observation fits with our model that the CD40L stimulates initial and rapid clonal expansion. We have yet to investigate whether removal of the CD40L after, for example, three days would then promote the generation of specific antibody-secreting plasma cells. It is not known at this stage whether pre-switched memory B cells would respond in the same way as germinal centre cells. There is a certain amount of debate as to whether memory B cells can give rise to germinal centres or undergo further somatic mutation

following antigen recognition, but it is not an impossible scenario. Finally, the study by Funakoshi *et al,* who found that ligation of CD40 expressed by B lymphoma cells induced the death of the malignant cells (146), provides a further example of the fact that CD40-CD40L interactions are not always stimulatory with respect to increased proliferation or immunoglobulin secretion.

Initial observations with CD40 and CD40L were concerned with their role in events giving rise to a primary response including germinal centre formation and less attention was focused on the response by memory B cells. The results presented here suggest that CD40L can elicit different effects following B cell encounter with antigen. Taking all this into account, I propose that upon re-challenge with antigen, ligation of CD40L serves to induce clonal expansion of the B cell in preference to terminal differentiation into an antibody-secreting cell in order to mount the most effective T-B response possible. This would ultimately ensure the secretion of much greater quantities of specific antibody. Obviously it would be necessary for such a proliferative step to be rapid as immunoglobulin secretion in memory responses increases significantly after only a few days. It is possible that the CD40L can induce levels of proliferation in the memory B cell similar to those observed in the initial stages of germinal centre formation. The exact mechanisms involved remain to be elucidated, but will be considered in the final chapter.

CHAPTER 4

CD40L AND THE HYPER-IGM SYNDROME

4.1 Introduction

Hyper-IgM syndrome is a severe immunodeficiency characterised clinically by low levels of serum IgG, IgA and IgE with normal or elevated IgM and susceptibility to opportunistic infections, autoimmune disorders and tumours (301). Patients have normal numbers of circulating B cells but no germinal centres. They are unable to mount effective responses to T-dependent antigens and vaccination is ineffective. The disease can occur as a primary or acquired disorder. The primary form includes an X-linked pattern of inheritance as well as both autosomal dominant and recessive forms.

The X-linked form of the disease (XHIGM) was mapped to chromosome Xq24-27 (307,308). When the ligand for CD40 was cloned it was mapped to Xq26.3-27.1 (165) and proposed as the candidate gene for XHIGM. Studies by several groups demonstrated a variety of mutations in the CD40L gene of patients with XHIGM and transfection experiments showed that mutated CD40L was unable to stimulate B cells (14,96,116,144,227,330). As yet, much less is known about the non-X linked forms of HIM.

The immunology department at Great Ormond Street Hospital is the major tertiary referral centre for childhood immunodeficiencies in the UK. Therefore, despite the low occurrence of this disease a significant number of patients attend the hospital for diagnosis and treatment. We embarked on a major study of X-linked and non X-linked HIM patients from 1992 - 1994 including genetic analysis, CD40L expression and

functional studies of B cells from patients and obligate carriers. My work was concerned with B cell function in XHIGM and is described in this chapter.

4.2 Patients, carriers and parents involved in the clinical study

The patients, carriers and parents who donated blood for the study are listed in Table 4.1. In addition, four healthy volunteers, FB, JH, LH and SS were used as controls.

Table 4.1. Patients, carriers and parents involved in the study

Subject	DOB	Sex	Diagnosis	Mode of inheritance
CS	23-03-88	M	XHIGM	X-linked[a] maternal uncle died at 6/12
BW	03-09-88	M	XHIGM	X-linked 6 male deaths over two generations
AT	17-03-63	M	XHIGM	X-linked affected males over several generations
GP	13-04-82	M	XHIGM	sporadic[b]
JP	17-09-88	M	XHIGM	sporadic
LAS	22-11-75	F	HIM	non X-linked[c]
VE	07-04-85	F	HIM	non X-linked
HG	15-03-89	F	HIM	non X linked
FZ	16-10-82	M	HIM	autosomal recessive,[d] parents 1st cousins, sister dead
CS (c)	ns	F	carrier[e]	mother of CS
BW(c)	ns	F	carrier	mother of BW
GP (p)	ns	F	parent	mother of GP
VE (p)	ns	F	parent	mother of VE
FZ (p)	ns	M	parent	father of FZ
BS (p)	ns	F	parent	mother of BS

[a] X-linked patients that had a well-documented family history of the disease and the gene defect has been identified.

[b] Patients termed 'sporadic' had clinical features of HIM but no family history. These patients have subsequently been confirmed as X-linked through identification of the CD40L gene mutation.

[c] Patients had clinical features of HIM but are female and clearly not X-linked, possibly an autosomal defect.

[d] Patient FZ subsequently found to be MHC class II deficient

[e] In patients whose X-linked status was confirmed by a family history of the disease, the mothers were termed obligate carriers

ns, information not supplied

4.3 CD40L expression on activated T cells

As part of this laboratory's investigation of HIM, CD40L expression on activated T cells from HIM patients and normal controls was carried out routinely by Sue Smith in the immunobiology unit. Briefly, peripheral blood was taken from patients and volunteer donors and separated into B and T cell populations by one round of rosetting with AET-SRBC followed by density centrifugation over Percoll (1.080g/L). T cells were stimulated overnight with PMA(20ng/ml) plus calcium ionophore A23187 (100ng/ml). CD40L expression on activated T cells was detected by FACS analysis after staining with a fusion protein, CD40-Fc (11), a monoclonal antibody, mAb 5c8 (249) or a polyclonal anti-sera, anti-TRAP (165). (CD40.Fc and 5c8 are thought to bind to the CD40 binding domain on CD40L whereas anti-TRAP recognises different epitopes on the molecule (60). The CD40L expression for a normal donor two X-linked and one non-X-linked HIM patient are shown in Fig. 4.1.

CD40L was readily detected on T cells from normal donors after 16h stimulation with PMA and calcium ionophore by both antibodies and the CD40.Fc. No significant staining was observed on non-activated T cells. Two patterns of CD40L expression were observed on T cells from X-linked patients. Type I showed no staining with CD40.Fc., anti-TRAP or 5c8 and suggested a complete inability to express CD40L. Activated T cells from some patients were able to bind anti-TRAP but not CD40.Fc

Fig. 4.1

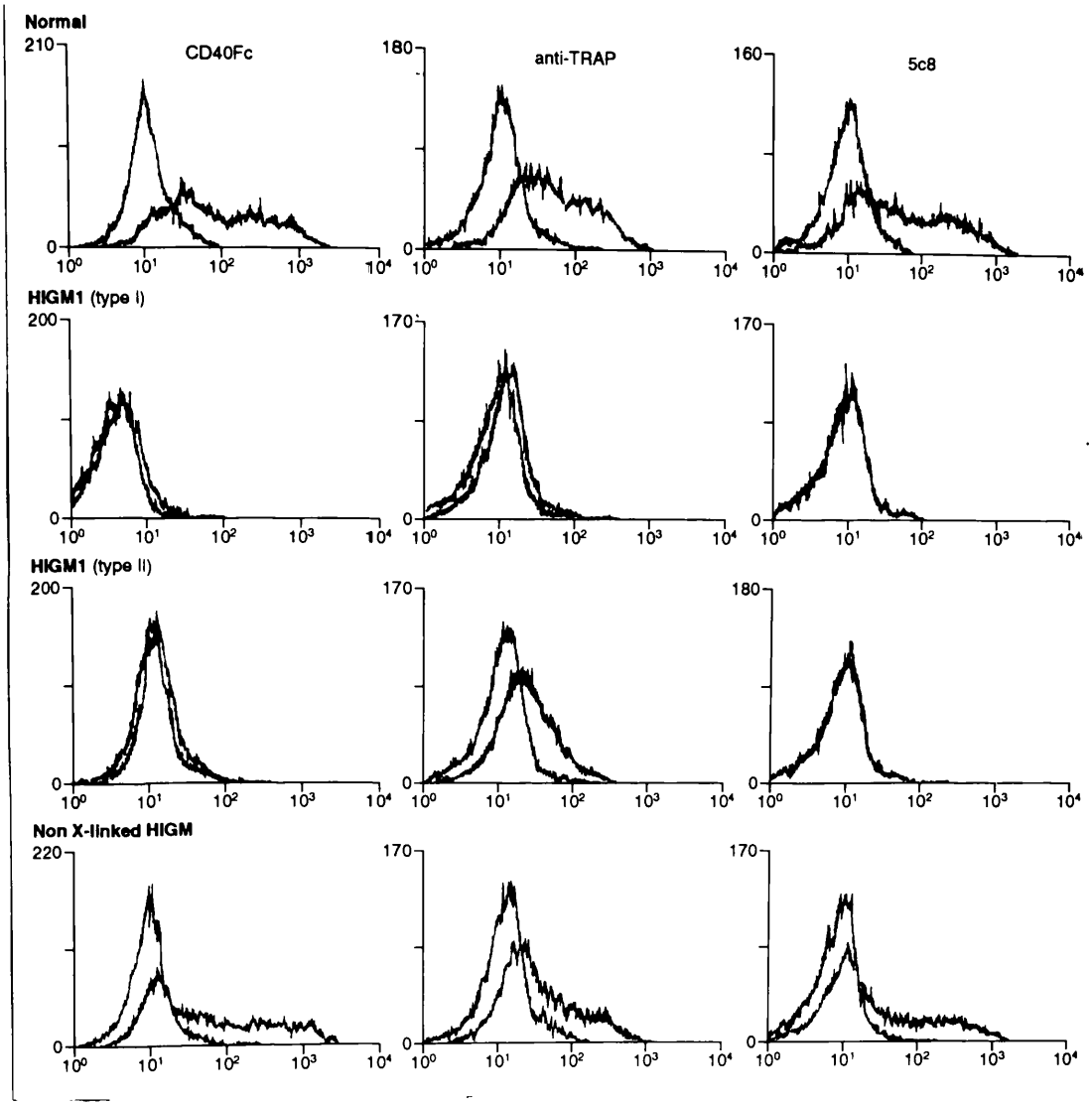


Fig. 4.1. Expression of CD40L on activated T cells

Two types of CD40L expression were identified on activated T cells from XHIGM patients. In one group (Type I) there was no binding of CD40Fc , 5c8 or anti-TRAP. In a second group, (Type 2) there was no binding of either CD40Fc or 5c8, but normal expression of CD40L as detected with anti-TRAP. Expression of CD40L was normal on activated T cells from a female (non-X-linked) HIM patient. (These studies were carried out by Sue Smith.)

or 5c8 (typeII staining) suggesting that a non-functional CD40L was being expressed. Activated T cells from all our sporadic patients tested for expression of CD40L displayed either type I or type II patterns of staining defined by the X-linked patients. They have subsequently all been shown to have XHIGM by DNA sequencing of the CD40L gene. Activated T cells from obligate carriers exhibited variable levels of expression of CD40L compared with the controls. Activated T cells from three non X-linked, LAS, VE and HG and one male autosomal recessive patient, FZ were also analysed. The three females all expressed CD40L although levels were reduced in one. Patient FZ consistently displayed weak expression of CD40L compared to the normal staining pattern of his father. This patient has subsequently been found to be HLA class II deficient and has a reversed CD4/CD8 ratio, i.e. the numbers of CD4+ cells are very low. Given that only a small subset of CD8+ cells express CD40L this fact may explain patient FZ's abnormal CD40L profile.

4.4. Expression of CD19 and CD40

In order to exclude an obvious B cell defect in the non-X-linked HIM patients, B cells from all patients, carriers and controls were analysed by flow cytometry for the expression of CD19 and CD40. Patients and carriers all displayed normal expression of these two cell surface antigens. (Data not shown).

4.5 Proliferation and antibody secretion by B cells from HIM patients

Early studies showed that B cells from patients with XHIGM were able to proliferate in response to CD40 ligation and make IgE when co-stimulated with IL-4. No studies had been undertaken with non-X-linked HIM. As these patients expressed normal levels of CD40L, there was a possibility that they had an intrinsic B cell defect. In order to investigate this possibility, I carried out a series of proliferation and differentiation assays utilising CD40L expressed on CV1/EBNA cells and various cytokines. Results with X-linked and non X-linked patients and normal donors. were compared.

4.5.1 XHIGM

Proliferation

B cells from XHIGM were cultured in 96-well plates at 2 x 10^5 per well either alone or in the presence of $CV1/EBNA_{CD40L}$ cells at 2 x 10^4 /well, with or without 5U/ml of human recombinant IL-2 (hrIL-2). Control wells contained B cells cultured in medium alone, B cells cultured with $CV1/EBNA_{empty\ vector}$ with or without IL-2, and B cells cultured with 10μg/ml anti-μ beads plus 100U/ml IL-4 as a positive control. The results are shown in Table 4.2.

B cells from the control donors (FB, JH) and parents (GP, JP) proliferated when cultured with $CV1/EBNA_{CD40L}$ alone or 5U/ml IL-2 + $CV1/EBNA_{CD40L}$ although the levels of ^{3}HTdR uptake induced was variable between donors. Culture of the B cells with IL-2 stimulated only low levels of proliferation. Similarly, B cells cultured with $CV1/EBNA_{empty\ vector}$ with or without IL-2 had no appreciable effect on proliferation above the small amount seen with IL-2 alone. B cells from the XHIGM patients

Table 4.2 Proliferation of XHIGM B cells in response to CD40 ligation

Patient	^{3}HTdR uptake (dpm)						
	medium	empty vector[a]	CD40L[b]	IL-2 (5U/ml)	IL-2 + empty vector	IL-2+ CD40L	IL-4 + anti-IgM
Controls							
FB	2,166	2,332	16,462	4,935	3,737	19,275	6,191
JH	214	285	2,332	4,094	4,019	18,653	36,282
GP(p)	666	570	8,919	6,968	7,442	24,230	2,711
JP(p)	741	738	5,681	1,688	2,286	7,890	4,992
X-linked patients							
CS	3,094	3568	71,781	16,162	21,422	74,295	9,116
BW	1,086	1063	16,089	1,422	10,432	22,430	12,418
GP	826	732	2,750	2,550	2,955	4,253	9,024
JP	643	874	5,144	1,551	1,735	6,799	12,649

[a] Control CV1/EBNA cell containing empty vector.
[b] CD40L expressed on surface of CV1/EBNA cells.

exhibited similar patterns of proliferation as B cells from normal donors in response to exogenously added CD40L or CD40L+IL-2 confirming that patients with X-linked hyper-IgM syndrome have normal B cell proliferative responses following ligation of CD40.

Antibody Secretion

We next tested the ability of B cells from patients with XHIGM to secrete antibody in response to exogenously added CD40L and cytokines (IL-2 or IL-10). B cells from control donors and XHIGM patients were cultured with 5U/ml IL-2 or 100ng/ml IL-10 and 10^5cells/ml CV1/EBNA$_{CD40L}$ or CV1/EBNA$_{empty\ vector}$ as a control. Culture supernatants were analysed for secretion of IgM, IgG and IgA (Table 4.3)

B cells from normal donors, cultured in medium alone secreted low and variable amounts of IgM, IgG and IgA. Addition of either CD40L or IL-2 to the cultures generally enhanced immunoglobulin secretion to a variable degree but the presence of CD40L and IL-2 together gave a significant increase in immunoglobulin production of all three classes. In the X-linked patients, the levels of IgM secreted by B cells alone were quite low compared to some of the normal controls suggesting that a deficiency in CD40L expression is not always accompanied by an increase in IgM secretion. In patients CS, BW and GP, addition of either IL-2 or CD40 alone had no appreciable effect on IgM secretion. However, the addition of IL-2 and CD40L together enhanced IgM secretion a little in CS and BW but still failed to have a significant effect on GP. In contrast, B cells from patient AT secreted very low levels of IgM in the absence of any stimulation, but were responsive to the presence of either CD40L

Table 4.3. Immunoglobulin secretion by XHIGM B cell stimulated with CD40L and IL-2.

Donor	isotype	Immunoglobulin secretion (ng/ml) medium	CV1/EBNA/ CD40L	IL-2	CV1/EBNA/ CD40L+IL-2
control					
FB	IgM	185 ± 73	678 ± 297	107 ± 10	456 ± 169
	IgG	71 ± 30	67 ± 128	41 ± 10	120 ± 63
	IgA	1134 ± 373	1544 ± 182	856 ± 221	1037 ± 28
JH	IgM	81 ± 9	116 ± 11	228 ± 75	1428 ± 231
	IgG	205 ± 50	192 ± 27	240 ± 13	1055 ± 79
	IgA	561 ± 96	1168 ± 515	730 ± 166	2012 ±689
GP(p)	IgM	310 ± 54	956 ± 38	1390 ± 1089	3584 ± 648
	IgG	157 ± 66	706 ± 251	1630 ± 910	19,233 ± 2879
	IgA	26 ± 11	318 ± 76	263 ±255	2182 ± 526
XHIGM					
CS	IgM	29 ± 2	45 ± 3	27 ± 3	56 ± 6
	IgG	7 ± 3	11 ± 9	<5	<5
	IgA	61 ± 27	81 ± 26	39 ± 24	<5
BW	IgM	55 ± 7	61 ± 7	28 ± 9	148 ± 6
	IgG	25 ± 8	30 ± 24	34 ± 17	<5
	IgA	5 ± 2	7 ± 3	27 ± 23	<5
AT	IgM	<5	63 ± 5	149 ± 13	1779 ±135
	IgG	<5	<5	<5	<5
	IgA	68 ± 42	60 ± 30	62 ± 17	38 ± 11
GP	IgM	10 ± 2	11 ± 2	24 ± 10	35 ± 9
	IgG	29 ± 5	11 ± 3	<5	10 ± 2
	IgA	<5	<5	<5	<5

or IL-2 and the presence of the two together stimulated a response comparable to that from normal donors. B cells from a few patients spontaneously secreted a small amount of IgG and IgA whereas in the others (CS, AT) it was virtually undetectable. Addition of CD40L and IL-2 either alone or in combination did not increase IgG or IgA secretion.

4.5.2 Non X-linked patients

Proliferation

Proliferation assays were performed as described with B cells from non-X-linked patients and controls. The results are shown in Table 4.4.

Table 4.4 Proliferation of B cells from patients with non-X-linked HIGM in response to CD40L and IL-2

Patient	**^{3}HTdR inc. (dpm:- x10^{-3})**						
	medium	**empty[a] vector**	**CD40L[b]**	**IL-2 (5U/ml)**	**IL-2 +empty vector**	**IL-2 + CD40L**	**hrIL-4 + anti-IgM**
Controls							
LH	1,415	638	20.107	5,459	4,713	25,301	10,671
CJ	1,331	1,229	9,784	5,742	5,492	18,575	23,786
SS	414	480		9,304	11,662	15,550	9,281
VE(p)	788	1071	882	15,880	907	13,003	29,711
Non-X-linked HIM patients							
LAS	135	293	38,352	732	715	41,901	25,648
VE	580	699	1,083	20,116	849	13,110	31,572
FZ	191	300	6,242	2,496	3,872	13,458	51,392

[a] Control CV1/EBNA cell containing empty vector.
[b] CD40L expressed on surface of CV1/EBNA cells.

As expected, B cell from normal donors, gave a strong proliferative response to CD40L which was further enhanced in the presence of 5U/ml IL-2. Similarly, B cells from all three non X-linked patients were responsive to CD40L and IL-2 and showed no obvious abnormalities with respect to CD40-induced proliferation.

Antibody secretion

B cells from patients with non-X-linked HIGM and control donors were cultured with 5U/ml IL-2 and 10^5cells/ml CV1/EBNA$_{CD40L}$ or CV1/EBNA$_{empty\ vector.}$ Culture supernatants were analysed for secretion of IgM, IgG and IgA (Table 4.5).

Two types of response were observed. B cells from LAS spontaneously secreted a large amount of IgM. This was enhanced slightly by the addition of CD40L alone but not by IL-2. Addition of CD40L and IL-2 together induced a huge response - seventeen to forty-three times greater than the response by B cells from the two normal donors. The B cells from the other patients, VE and FZ secreted very little IgM when cultured alone or with the addition of CD40L or IL-2. However, CD40L and IL-2 together were able to stimulate some IgM production. In the non-X-linked patients, IgG secretion was undetectable by B cells from LAS - the patient with extremely high IgM levels. In patient VE, addition of IL-2 and CD40L together slightly enhanced the low level of IgG secretion, but B cells of patient FZ were unresponsive. No significant IgA secretion was obtained from any of the non-X-linked patients.

Table 4.5 Immunoglobulin secretion by non X-linked HIM B cells stimulated with CD40L and IL-2.

		Immunoglobulin secretion (ng/ml)			
Donor	**Ig Isotype**	**medium**	**CD40L[a]**	**IL-2**	**CD40L+IL-2**
control					
SS	IgM	47 ± 5	105 ± 20	98 ± 6	330 ± 81
	IgG	243 ± 3	443 ± 38	278 ± 3	713 ± 244
	IgA	25 ± 2	56 ± 20	20 ± 4	75 ± 24
LH	IgM	52 ± 2	95 ± 47	97 ± 8	127 ± 31
	IgG	238 ± 47	298 ± 51	125 ± 20	803 ± 47
	IgA	1327 ± 141	1929 ± 114	887 ± 75	1647 ± 420
Non X-linked patients					
LAS	IgM	1299 ± 41	1861 ± 35	1190 ± 82	5476 ± 302
	IgG	<5	<5	<5	<5
	IgA	5 ± 0.7	7 ± 0.3	6 ± 1	5 ± 0.6
VE	IgM	8 ± 1	23 ± 2	15 ± 6	86 ± 20
	IgG	20 ± 3	26 ± 0.8	15 ± 3	73 ±16
	IgA	<5	<5	<5	<5
FZ	IgM	<5	7 ± 1	4 ± 1	156 ± 114
	IgG	20 ± 3	22 ± 2	26 ± 2	32 ± 0.1
	IgA	<5	<5	<5	<5

[a]CD40L expressed on surface of CV1/EBNA cells.

4.6 Effect of IL-10 and CD40L on antibody secretion

Early studies with IL-10 had demonstrated its properties as a potent differentiation factor to B cells and a potential switch factor. IL-2 only showed BCDF activity and the low responses observed by the patient's B cells to IL-2 and CD40L prompted me to try IL-10. A concentration of 100ng/ml IL-10 was chosen which was added to culture with or without CD40L as described for IL-2. Table 4.6 shows the results for five patients, one parent and one normal donor. The results are expressed as the mean immunoglobulin secretion from triplicate cultures ± 1 s.e.m. No IgE was detected.

The experiments with IL-10 gave much higher levels of immunoglobulin secretion than with IL-2. B cells from normal donors and obligate carriers responded in a similar way. The addition of IL-10 alone enhanced IgM, IgG and IgA secretion as did the addition of CD40L alone to cultures. Addition of CD40L and IL-10 together resulted in a huge increase in immunoglobulin secretion. Interestingly, the IgA secretion was apparently extremely susceptible to the action of CD40L and IL-10 and it was not uncommon to measure amounts of IgA greater than 10,000ng/ml.

In the XHIGM patients, addition of IL-10 and CD40L enhanced IgM secretion, but the ability of this combination to induce IgG or IgA secretion was variable. In one patient (AT), there was a big increase in IgG secretion from almost undetectable levels when B cells were incubated with either CD40L or IL-10 alone, to a large amount on the addition of CD40L and IL-10 together. The large standard deviation

Table 4.6 Effect of IL-10 and CD40L on immunoglobulin secretion by B cells from HIM and normal controls

Donor		Ig. secretion(ng/ml)			
	Ig Isotype	CV1/EBNA empty vector	CV1/EBNA/ CD40L	IL-10	IL-10 + CV1/EBNA/ CD40L
Normal					
SS	IgM	47 ± 8	105 ± 35	520 ± 64	>10,000
	IgG	140 ± 14	443 ± 66	1416 ± 606	3,324 ± 104
	IgA	28 ± 3	56 ± 34	205	>10,000
TK	IgM	53 ± 13	1067 ± 257	680 ± 122	3,393 ± 581
	IgG	55 ± 15	968 ± 64	360 ± 67	1291 ± 73
	IgA	60 ± 9	1430 ± 462	133 ± 23	>10,000
XHIGM					
AT	IgM	20 ± 13	63 ± 8	113 ± 65	>10,000
	IgG	<5	<5	<5	496 ± 392
	IgA	<5	<5	<5	<5
XHIGM					
BW	IgM	80 ± 25	61 ± 7	163 ± 46	268 ± 8
	IgG	27 ± 8	30 ± 41	5 ± 1	6 ± 3
	IgA	19 ± 15	7 ± 2	<5	5 ± 1
XHIGM					
GP	IgM	9 ± 2	11 ± 2	28 ± 10	467 ± 172
	IgG	<5	11 ± 5	9 ± 4	14 ± 10
	IgA	<5	<5	16	53
Non-X-linked					
HG	IgM	15 ± 6	10 ± 6	13 ± 7	48 ± 3
	IgG	19 ± 5	20 ± 4	19 ± 10	14 ± 2
	IgA	7 ± 4	<5	<5	<5
VE	IgM	13 ± 4	23 ± 4	52 ±11	665 ± 35
	IgG	22 ± 1	26 ± 2	61 ± 37	63 ± 28
	IgA	<5	<5	<5	<5

may be caused by switching of only a few B cells - possibly even in just one culture from the triplicate. No IgA secretion was observed from AT's B cells. B cells from the other X-linked patient, (BW), were unable to secrete either IgG or IgA in response to IL-10 and CD40L, whereas patient GP was found to secrete a small amount of IgA but no IgG.

Of the two female, non-X-linked patients, only one, (VE), appeared to show an increase in anything other than IgM secretion upon stimulation with IL-10 and CD40L. In this patient a small increase in the secretion of IgG was observed. However this was obtained by the addition of IL-10 alone and not significantly increased by the further addition of CD40L and is unlikely to be due to a switching event. No IgA was detected from this patient. The lack of response to CD40L observed in these patients is consistent with the opinion that the non-X-linked form of HIM is due to an intrinsic B cell defect.

4.7 IgE secretion by non-X-linked patients in response to IL-4

It has been demonstrated by several groups that B cells from XHIGM patients were able to secrete IgE through cross-linking CD40 and the addition of IL-4 thus confirming that the B cell switching mechanism is intact. On the other hand, if the non-X-linked patients have an intrinsic B cell defect, it is likely that IL-4 and CD40L would be unable to induce IgE secretion. To clarify this, we obtained more blood from patients VE and LAS and cultured the B cells with 100U/ml of IL-4 and CD40L and measured IgE secretion. The results (including a normal donor) are shown in Table 4.7 and are expressed as mean IgE secretion from triplicate cultures ± 1 s.e.m.

Table 4.7 IgE secretion by B cells from Non-X-linked hyper-IgM patients stimulated with IL-4 and CD40L.

	IgE secretion (ng/ml)		
Stimulus	Control	Patient LAS	Patient VE
medium	<5	<1	<1
CV1/EBNA/CD40L	<5	<1	<1
CV1/EBNA(control)	<5	<1	<1
IL-4	<5	<1	<1
IL-4 + CV1/EBNA/CD40L	20 ± 6	<1	9 ± 1
IL-4+ CV1/EBNA/control	<5	<1	<1

As expected, B cells from the normal donor did not secrete IgE unless stimulated by CD40L and IL-4. B cells from LAS were unresponsive to the signal and were unable to switch. Cells from VE secreted a small amount of IgE in response to addition of CD40L and IL-4 suggesting that at least some normal B cell function could be reconstituted.

4.8 Discussion

'Hyper-IgM' - a disease of considerable diversity

The hyper-IgM syndrome is defined by recurrent infections and hypogammaglobulinaemia with normal or elevated levels of IgM. It occurs as an acquired and primary disorder and the latter exists in both X-linked and non-X-linked forms. HIM thus embraces a number of disorders.

The X-linked form of hyper-IgM Syndrome (XHIGM) is caused by a mutation in the gene encoding CD40L on T cells. Two types of CD40L defect have been identified by flow cytometry. In Type 1, the mutation results in a complete lack of expression of CD40L on the surface of activated T cells and in Type 2 a ligand is expressed but is apparently non-functional. The B cells from these patients express CD40 and are able to proliferate normally when co-stimulated with cytokines and exogenous CD40L. Several studies have demonstrated that B cell function in these patients is also normal with respect to their ability to secrete IgE or IgA in response to stimulation with IL-4 and CD40L or IL-10, CD40L and SAC respectively. In my experiments however, the ability of XHIGM B cells to secrete other isotypes (IgG, IgA) in response to cytokines and CD40L was variable. IL-10 was found to be a more potent differentiation/switch factor than IL-2.

IL-10 stimulates immunoglobulin secretion more effectively than IL-2

The ability of patient's B cells to secrete immunoglobulin other than IgM when stimulated by CD40L expressed on CV1/EBNA cells and cytokines was assessed in this study. IL-2 was consistently found to be ineffective in stimulating antibody secretion, other than IgM, by XHIGM B cells (tables 4.3 - 4.4). This was consistent with the role of IL-2 as a B cell differentiation factor but not a switch factor where IgM secretion but not IgG, IgA or IgE would be expected due to the lack of switched cells in the patients. In contrast, the addition of CD40L plus IL-10 to B cells from some patients was able to induce IgG and IgA secretion (Table 4.5). This was in accordance with the findings of Korthauer *et al* (227) although they seemed more successful at stimulating IgG and IgA and detected both isotypes in each of three

patients whereas in our studies only 1 patient out of 3 secreted IgG or IgA. One possible explanation for the difference between my results and those of Korthauer *et al* is the use of *Staphylococcus aureus* Cowan (SAC) in the Korthauer study as a stimulator of antibody secretion (19,103). The SAC may have amplified the signals from anti-CD40 plus cytokines to enhance antibody secretion. SAC has also been shown to increase the expression of CD40 on B cells and this too may have been a factor in the increased switching and differentiation observed. Secondly, in the Korthauer study, CD40 was cross-linked with mAb 89 - a mAb to CD40 rather than CD40L. Although this might not be expected to make a difference, in a study by Allen *et al* (96), mAb G28.5 was found to be better able to induce IgE secretion from patient B cells than CD40L expressed on CV1/EBNA cells - in some cases increasing IgE secretion by 50%. In fact most of the studies that have attempted to stimulate antibody production from the B cells of HIM patients have relied on mAbs to CD40. It is also worth noting that most reported studies have concentrated on IgE secretion induced by IL-4 and anti-CD40. Only ourselves and two others have also studied IgG and IgA secretion (14,227). It is possible that IgE switching occurs via a different mechanism to IgG and IgA switching and that it may be easier to achieve *in vitro*. IL-4 is also known to increase CD40 expression which, like the SAC, may have a secondary effect of amplifying the response.

It is not clear then, whether the infrequent responses observed in this study from the patients with respect to IgG and IgA secretion were simply due to the chosen assay system not providing optimal stimulation or if it shows that B cells from some XHIGM patients do not switch in response to stimulation with IL-10 and CD40L.

IL-10 - switch factor or differentiation factor?

IL-10 has been shown to induce B cells to produce IgA and IgG. Further, it has been shown to stimulate IgD+ cells to make IgG1 and IgG3. These findings are consistent with heavy chain switching. It was thought likely therefore that IL-10 was inducing an isotype switch in IgM+ B cells from some of our XHIGM patients resulting in secretion of IgG and IgA. However, not all the patients were completely devoid of serum IgG and IgA which suggested that a low level of switching had occurred *in vivo*. To address this question, colleagues at Schering Plough, Dardilly, used PCR to amplify the mRNA in several XHIGM patients to look for evidence of switching (331). Patient AT - our Type 1 XHIGM patient - expressed mRNA for IgG and showed evidence of somatic hypermutation. No ε mRNA was detected and the presence of α mRNA was not certain. Two of the female patients were also analysed, HG was found to express γ and α mRNA and LAS expressed only α. All were positive for μ and δ. The data were not quantitative and therefore it is not possible to state the physiological relevance of these findings. However, it does suggest a role for IL-10 as a potent differentiation factor which stimulates secretion from switched cells - albeit present at a low frequency. Results from control donors showed that stimulation with IL-10 alone or together with CD40L significantly enhanced the secretion of IgM, IgG and IgA and it is more probable that IL-10 is acting on pre-existing switched cells.

Some patients may produce a soluble CD40L

Sequencing the CD40L gene in patient AT revealed a point mutation at nucleotide 163 which resulted in the exchange of a neutral methionine residue for a positively charged arginine (227). The mutation occurred in the transmembrane domain of the

molecule and was responsible for preventing the expression of CD40L on the cell surface. This may be compared to a study by Bonifacino *et al* which demonstrated that the insertion of a positively charged amino acid into the transmembrane domain of Tac results in a signal for endoplasmic degradation (37).

Alternatively, the mutation in the transmembrane domain may result in secretion of a soluble form of the CD40L rather than degradation. This might help to explain the presence of switched cells found in this patient (AT). In the initial studies of expression of CD40L, T cells were activated overnight and expression was detected at 12 -18 hrs. In a further study, F. Brière *et al* investigated the possibility that expression was occurring earlier. She found that a T cell clone from AT showed a transient expression of CD40L which was maximal at 2 hrs and was lost again by 6 hours (331). This is different to the kinetics of normal expression which showed maximal expression between 10-16 hours following activation and which was lost by 24 hours. The existence of a soluble form of CD40L is considered possible and is supported by the presence of soluble forms of other members of the TNF family. Further, these molecules can often be detected in serum or urine and thus one might also expect to be able to detect CD40L in this way. Initial attempts to identify such a molecule in our laboratory have thus far proved unsuccessful. Even if patient AT does secrete sCD40L, which might account for the presence of switched cells, there is evidently still a defect as shown by his lack of serum IgG, IgA or IgE.

CD40L may not be the sole factor involved in isotype switching

During the early studies into CD40L expression, the existence of a second ligand for CD40 was thought possible although to date no firm evidence for this hypothesis has

been published. A study from Life *et al* (251) showed that T cell clones from a patient with XHIGM were able to induce IgE synthesis *in vitro* despite lacking surface expression of CD40L. Furthermore, Sanchez *et al* found that binding of CD58 to CD2 provides an alternate IL-4-dependent, CD40-independent mechanism for the secretion of IgE (112). Together, these data suggest that molecules other than CD40L may be involved in isotype switching.

The nature of the defect causing a lack of immunoglobulin production in non-X-linked HIM

This study included three female patients with primary HIM and a male patient (FZ) with an affected sister suggesting an autosomal dominant mode of inheritance. His T cells expressed CD40L although the expression was lower than normal. This patient has subsequently been found to be HLA Class II deficient with low numbers of T cells and an inverted CD4/CD8 ratio which is likely to be the cause of his immunodeficiency. CD8+ve T cells express lower levels of CD40L than CD4+ cells (235) which would explain the low CD40L expression observed. T cells from the female patients were all positive for CD40L expression although one showed reduced expression. B cells from one female (LAS), consistently failed to secrete any isotype other than IgM in response to CD40L and cytokines (331). Her B cells secreted large amounts of IgM even when unstimulated in culture which were further increased by the addition of CD40 with either IL-2 or IL-10. Analysis of B cell mRNA by PCR showed evidence of switched cells, but the SAC assay also failed to induce the secretion of any isotype other than IgM. The results obtained from LAS are consistent with an intrinsic B cell defect. In contrast, the other two patients VE and HG secreted lower levels of IgM and responded similarly to the addition of CD40L and cytokines as the XHIGM patients.

One patient, VE, also secreted IgG and IgE when stimulated with CD40L in the presence of IL-10 and IL-4 respectively.

We have considered the possible B cell defects that could result in non-X-linked HIM. The two early activation molecules, B7-1 and B7-2, are important in T cell-B cell interactions and were possible candidates. Using mAbs, activated B cells from these three patients were analysed for the expression of B7-1, but the results so far have been largely inconclusive. Expression of B7-2 has not been analysed yet but it too might be an important molecule as it is expressed before B7-1.

CD40 was another possible candidate which could be responsible for the defect. A CD40-deficient mouse was found to elevated serum IgM and low levels of the other isotypes (73). However, CD40 expression on B cells of all patients had been analysed and was normal (data not shown). After studying the panel of mAbs to CD40 (Chapter 3) which suggested that CD40 was composed of more than one functional epitope, we considered the possibility that an altered epitope expression in CD40 in the patients may be involved in the immunodeficiency. B cells from LAS, several normal donors and two HIM patients were analysed by flow cytometry using the panel of eight mAbs from the workshop (data not shown). Early data suggests that there may be some differences in the expression of different epitopes although more controls need to be included.

Is there a link between phenotype and genotype?

Identification of the two different patterns of CD40L expression in XHIGM led us to question whether a particular pattern may be related to a specific mutation. On a more simplistic level we also considered the possibility that the severity of the disease was

also linked to type of staining observed. This was partly through observation of one of the patients, AT who showed Type 1 expression (i.e. no CD40L expression) but was apparently quite healthy. In comparison, some of the other patients with Type 2 staining (expression of a defective CD40L) were thriving less well. Interpretation was difficult however as a lack of clinical symptoms was thought to be more likely related to the age at which the diagnosis was made and therapy commenced. A review of AT's case and family history support the latter explanation as his therapy started early following a number of deaths of siblings and close relatives. Any comparison between the phenotype and genotype of the disease requires information on the mutation in the CD40L. The mutations described so far have nearly all been unique and it has not been possible to identify a link between the two.

CHAPTER 5

EFFECT OF IL-4 ON HUMAN SPECIFIC ANTIBODY RESPONSES

5.1. Introduction

In addition to its activation and growth factor activity, IL-4 has a potent effect on immunoglobulin production by human B cells. It displays activity both as a B cell differentiation factor enhancing IgM and IgG secretion (105) and as a switch factor involved in the regulation of IgG4 and IgE secretion (150,151,318,413). One study from this laboratory addressed the role of IL-4 in the regulation of immunoglobulin isotypes secreted by human B cells stimulated with EBV (228). Briefly, low concentrations of IL-4 (<10U/ml) increased production of IgM, IgG1, IgG2, IgG3 and IgA with little or no IgG4 or IgE. At higher doses (100U/ml) however, there was a dramatic increase in IgE secretion with some IgG4 also produced whereas secretion of all the other isotypes was unaffected or, more often, was decreased. This suggested that IL-4 had a dual role in antibody secretion acting as a BCDF at low concentrations and a switch factor at high concentrations.

These effects of IL-4 on polyclonal immunoglobulin secretion raised important questions about IL-4 regulation of the specific antibody response to influenza virus. In a preliminary study from this laboratory, it was found that IL-4, at doses as low as 10U/ml, had a profound inhibitory effect on the specific antibody responses by human B cells to influenza virus (63). Moreover, addition of anti-IL-4 either had no effect, or in some cases only slightly enhanced, specific antibody production suggesting that IL-4 was not required for the specific antibody response. These early studies did not exclude the preferential effect on one or more IgG subclasses produced in the specific antibody response or on IgE. In this chapter I have extended the original observation

and investigated the effect of IL-4 on specific IgG subclass responses to influenza virus antigen.

5.2. Characterisation of the isotypes secreted by human TMC in the specific antibody response to influenza virus.

To investigate the possible selective effect of IL-4 on the specific antibody isotypes to influenza, I first determined the immunoglobulin class and IgG subclasses produced under normal conditions. In these experiments, whole TMC were cultured with influenza virus and supernatants were assayed on Day 7 for secretion of specific IgM, IgG, IgA and IgE and also IgG1, IgG2, IgG3 and IgG4 antibody. Table 5.1 shows the results of three such experiments using TMC from different donors.

IgG and IgA were detected in each experiment but no IgM or IgE secretion was obtained from any of these donors which is typical of a secondary, amamnestic response to a viral antigen. Some donors do secrete some IgM (see later experiments) which may reflect the donor's history of exposure to influenza. IgG1 was always found to be the predominant IgG subclass produced with variable amounts of IgG3. No IgG2 or IgG4 was detected from these donors although IgG4 has also been observed in other experiments. The considerable variation between donors in both the amount of antibody secreted and the isotype distribution is a consistent feature of the specific antibody response and may

Table 5.1. Antibody isotypes produced in the specific antibody response to influenza virus by human TMC.

Isotype	Specific antibody secretion (U/ml) [a]		
	Exp. 1	**Exp. 2**	**Exp. 3**
IgM	<5	<5	<5
IgG [b]	>2000	336 ± 111	90 ± 12
IgG1	>2000	429 ± 115	78 ± 14
IgG2	6	<5	<5
IgG3	48 ± 16	62 ± 13	nd
IgG4	<5	<5	<5
IgA	>2000	432 ± 69	18 ± 6
IgE	<5	<5	<5

[a] Mean specific antibody secretion from triplicate cultures of TMC stimulated with antigen in Units/ml ± 1 s.e.m. Secretion in control cultures without antigen was always <5U/ml.
[b] Total specific IgG

reflect the time since the last exposure to influenza and/or recirculation of influenza-specific T and B lymphocytes. The lack of any IgE or IgG4 secretion makes this a good assay for testing the role of IL-4 which may be expected to increase or stimulate production of these isotypes.

5.3 Effect of IL-4 on specific antibody isotype production by whole TMC

Previous work from this laboratory demonstrated inhibition of specific antibody responses by IL-4 (63). However, only IgG, IgM and IgA were measured in the earlier experiments and the study did not address the possibility that IL-4 was initiating a switch to IgG4 or IgE secretion. Having analysed the normal range of isotypes secreted by B cells stimulated with antigen alone, I then proceeded to investigate the effect of IL-4 on IgG subclass production. Whole TMC were cultured with antigen in the presence of IL-4 over a dose range of 0.05-100 U/ml, Day 7 supernatants from the cultures were analysed for all isotypes (IgM, IgG, IgA and IgE) and IgG subclasses as before. The results for IgG and IgA from one representative experiment are shown in Fig.5.1. Specific IgG and IgA production was inhibited in a dose dependent fashion to a minimum response (maximum inhibition) at the highest dose of IL-4 (100 U/ml). At all other doses of IL-4, the degree of inhibition obtained was variable. In some cases inhibition occurred immediately with the lowest dose of IL-4 as demonstrated by IgA secretion in Fig. 5.1 whilst in others antibody secretion was unaffected by the lower doses of IL-4 (as seen

Fig. 5.1

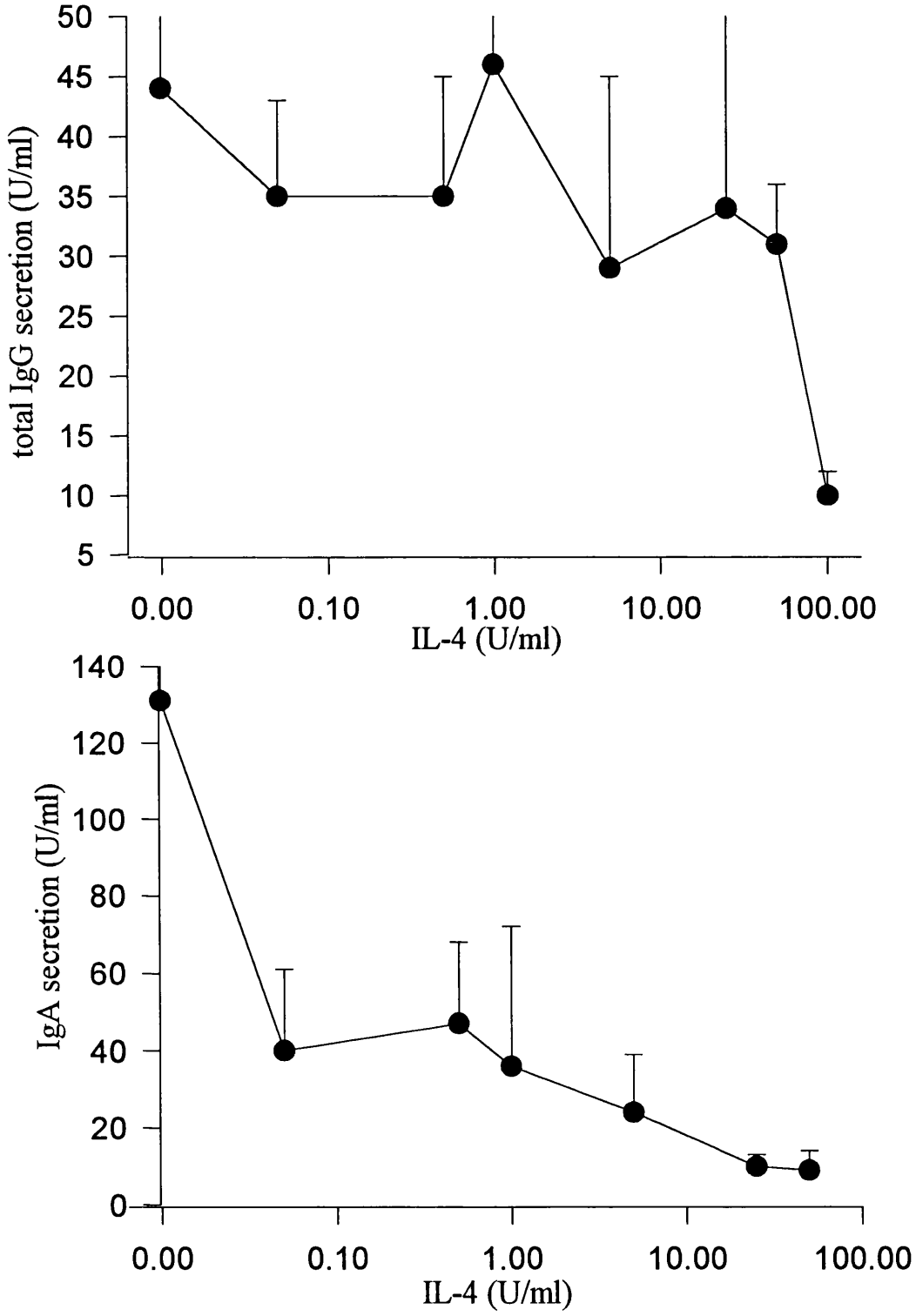


Fig. 5.1 Effect of IL-4 on the secretion of specific total IgG and IgA by TMC.

TMC were cultured with antigen and a range of IL-4 concentrations from 0.05 - 100 U/ml. Culture supernatants were analysed for secretion of specific IgG and IgA. Results from two representative experiments are shown.

with IgG secretion). Similar patterns of inhibition were noted when the IgG subclasses were measured.

5.4 Comparison of high and low doses of IL-4 on specific immunoglobulin isotype and IgG subclass antibody production by TMC.

In all the measurements of specific antibody production there was significant variability between triplicate cultures as indicated by the high standard deviations (evident in Fig. 5.1). This variability sometimes made the results difficult to interpret, particularly at the intermediate concentrations of IL-4, even though a distinct trend was often visible. To reduce this variation, the experiments were repeated using an increased number of replicate cultures (n = 20) to allow statistical analysis of the results. Two concentrations of IL-4 were chosen - (2U/ml and 150 U/ml) in order to assess any differential effect due to high and low doses of IL-4.

In these experiments, 2 x 10^6 whole TMC were cultured with influenza virus as previously described and IL-4 was added at either 2 U/ml or 150 U/ml. Cultures were performed in replicates of twenty and incubated as before. For statistical analysis, antibody production in the control cultures containing only TMC and influenza virus was compared with that from cultures containing IL-4 at either 2 U/ml or 150 U/ml. A paired t-test was performed on the data (Table 5.2).

Table 5.2. Effect of high and low doses of IL-4 on specific antibody secretion by TMC.

Exp. 1

Isotype [b]	Specific Antibody Secretion (U/ml) [a]		
	IL-4 (U/ml)		
	0	2	150
IgM	<5	<5	<5
IgG	503 ± 69	347 ± 20 $p < 0.05$ [c]	244 ± 26 $p < 0.002$ [d]
IgG1	244 ± 20	290 ± 23 ns	265 ± 29 ns
IgG2	<5	<5	<5
IgG3	113 ± 53	68 ± 27 ns	89 ± 38 ns
IgG4	<5	<5	<5
IgA	139 ± 32	91 ± 41 $p < 0.02$	79 ± 18 $p < 0.002$
IgE	<5	<5	<5

continued.....

Table 5.2 continued

Exp. 2

Isotype [b]	Specific Antibody Secretion (U/ml) [a]		
	IL-4 (U/ml)		
	0	2	150
IgM	10 ± 1	7 ± 0.4 p < 0.02 [c]	7 ± 0.6 p < 0.01 [d]
IgG	45 ± 4	29 ± 2 p < 0.002	31 ± 5 p < 0.002
IgG1	50 ± 3	37 ± 2 p < 0.002	35 ± 3 p < 0.002
IgG2	<5	<5	<5
IgG3	15 ± 4	10 ± 4 ns	5 ± 1 p < 0.02
IgG4	13 ± 2	13 ± 2 ns	22 ± 2 p < 0.002
IgA	46 ± 8	33 ± 5 p < 0.05	12 ± 1 p < 0.002
IgE	<5	<5	<5

[a] Specific antibody production by TMC stimulated with antigen and IL-4 expressed as mean specific antibody secretion from 20 cultures ± 1 s.e.m.
[b] Total IgG (all subclasses), IgM, IgA, IgE and IgG1-4 were assessed
[c] p-value calculated from the standard error of difference of the means between control cultures and cultures containing 2U/ml IL-4
[d] p-value was calculated from the standard error of difference of the means between the control cultures and cultures containing 150U/ml IL-4

In both experiments, IgG and IgA secretion were inhibited at both high and low doses of IL-4 (p-values ranged from 0.002 to 0.05). In addition, a small amount of IgM was detected in Exp.2 which was also inhibited at both doses. The effect of IL-4 on IgG subclasses was not consistent as the standard deviations were still high.

These experiments were both undertaken with unseparated TMC and the effect of IL-4 may be obscured by the presence of T cells. The experiments were therefore repeated with purified B cells cultured in the presence of TRF. This produced a more precisely controlled culture system and reduces any effects - inhibitory or stimulatory - from other cytokines which may be produced by T cells or through T-B cell interactions.

5.5 Effect of high and low doses of IL-4 on the specific immunoglobulin isotype and IgG subclass antibody responses by purified B cells stimulated with antigen and TRF.

To eliminate the possibility that IL-4 was acting indirectly through T cells, E^- cells were separated from whole TMC by rosetting with AET-SRBC and cultured at 0.75 x 10^6 E^- cells/ml (in multiples of 20) with antigen in the presence of TRF (5U/ml IL-2). IL-4 at 2U/ml or 150U/ml was added at the start of culture. The results are shown in Fig. 5.2.

Fig. 5.2

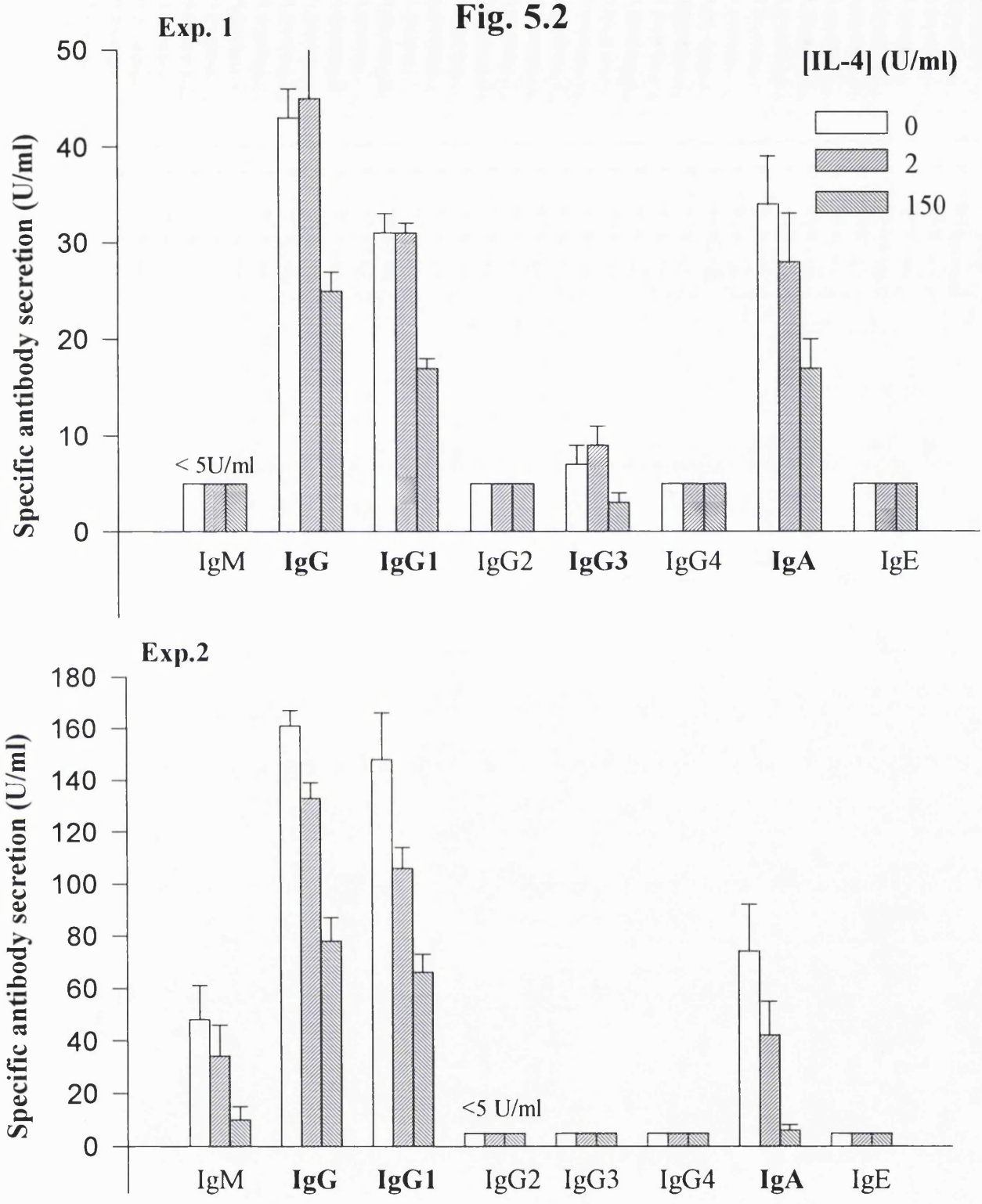


Fig 5.2. Effect of high and low doses of IL-4 on specific antibody secretion by B cells

Tonsil (E^-) cells were cultured either with antigen alone or in the presence of hrIL-4 at 2U/ml or 150 U/ml. Culture supernatants were analysed for immunoglobulin secretion on day 7. Results are expressed as mean specific antibody secretion from 20 replicates +/- 1 s.e.m. Unstimulated cultures all gave background values <5U/ml

By using E^- cells cultured in replicates of 20, the standard errors have been kept low. IgG, IgG1, IgA production in both experiments and IgM in Exp.2 were inhibited reduced maximally at 150U/ml IL-4. In several cultures, specific antibody secretion was also inhibited significantly at the lowest concentration of IL-4. Notably, there was no selective effect on any isotype at either concentration, and no induction of IgE or IgG4 at the highest doses. These experiments with purified B cells also confirm that IL-4 is acting directly on the B cells.

5.6 Comparison of the effects of IL-13 and IL-4 on specific antibody production.

The effects of IL-13 on B cell function have been found to be very similar to IL-4 and it may also be involved in the regulation of IgE and IgG4 production. This section compares the effects of IL-4 and IL-13 on specific antibody responses.

Whole TMC were stimulated with antigen as described with the addition of either IL-13 at concentrations 0.1 - 200ng/ml or IL-4 at concentrations of 0.1 - 200U/ml. (In B cell proliferation experiments, comparable stimulation was obtained with 20U/ml of IL-4 and 20ng/ml of IL-13.) Culture supernatants were analysed for the production of specific IgG only. Results for two experiments from different donors are shown in Fig.5.3.

In both experiments, IL-4 inhibited specific antibody production at the highest dose (200U/ml) with a variable response at lower concentrations consistent with previous

Fig. 5.3

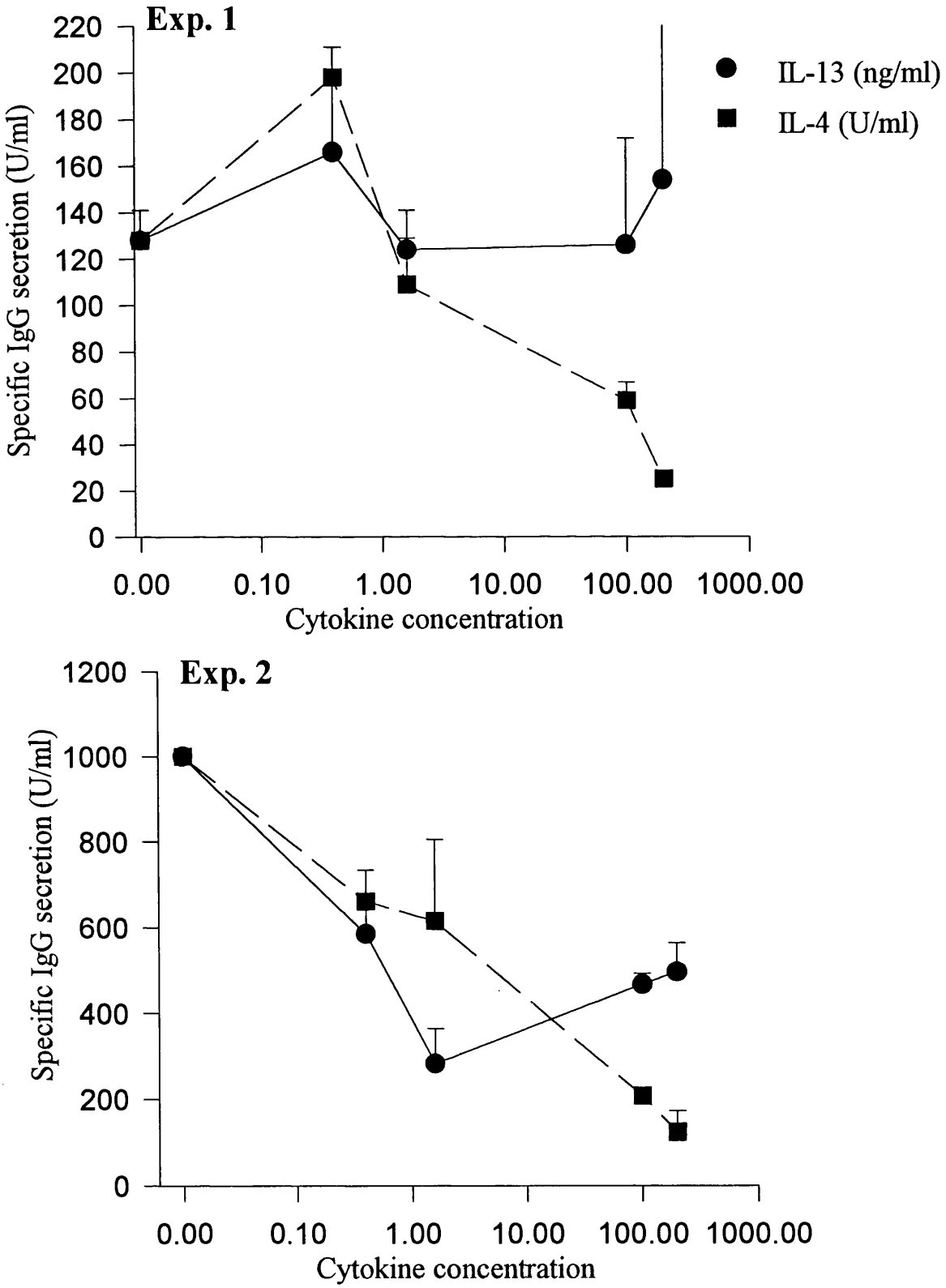


Fig. 5.3. Comparison of the effects of IL-13 and IL-4 on specific antibody production by TMC

TMC were stimulated with antigen and either IL-4 or IL-13 over a range of concentrations between 0.01 -200 U/ml or ng/ml respectively. Results are shown for two separate experiments. Only positive error bars have been added for clarity.

observations. In one experiment, IL-13 had no marked inhibitory effect on specific antibody secretion even at the highest concentration. In the second experiment, the response to IL-13 was much more erratic but at the highest concentration (200ng/ml) it inhibited specific antibody production although not as much as IL-4.

5.7 Discussion

These results show clearly that addition of IL-4 to antigen-stimulated cultures inhibits specific antibody secretion. Moreover, IL-4 had no selective effect on the secretion of any isotype or subclass and was not able to induce switching to IgE or IgG4 production even at high concentrations. Inhibition was due to a direct effect on the B cells as shown by experiments which used IL-2 as a TRF.

Inhibition was dose-dependent with no qualitative difference between high and low concentrations of IL-4. The effect of high and low doses of IL-4 on differentiation of EBV-activated B cells has already been described (228) where it was shown that low doses of IL-4 acted as a BCDF and increased secretion of IgM, IgG1, IgG2, IgG3 and IgA whereas high doses of IL-4 acted as a switch factor inducing IgE and IgG4 secretion. No such effect was observed on specific antibody responses.

Possible mechanisms for IL-4-mediated inhibition of specific antibody production .

The exact mechanism of the inhibition remains unknown and literature regarding this dilemma is conflicting. One study suggested that IL-4 inhibited IL-2 mediated proliferation of SAC-activated B cells but did not affect differentiation (101). Another study showed that IL-4 inhibited IL-2 mediated help in specific IgM secretion in a primary response (258) and further, that IL-4 inhibited by counteracting the effect of IL-2. In the specific antibody response to influenza virus, IL-2 is important as both a BCDF and a TRF. In addition, the inhibitory effect of IL-4 was more pronounced in experiments with E^- where IL-2 was present as a TRF. These observations are consistent with IL-4 counteracting IL-2 activity.

One possible explanation is competition between IL-2 and IL-4 for receptor-binding. The receptors for IL-2 and IL-4 both utilise the γ_c (355). IL-2 requires all three receptor components (α,β,γ_c) to bind with maximal affinity (276,392). Competition with IL-4 for utilisation of the γ_c may result in IL-2 binding to the lower affinity components ($\alpha\beta$) resulting in reduced helper activity. If this was a competitive effect, it may be possible to overcome it by increasing the concentration of IL-2.

In cultures of unseparated TMC it was possible that IL-4 could mediate an effect through the T cells, for example, through stimulation of T cell subsets. In humans, Th1 cells predominantly secrete IL-2, IFN-γ and TGF-β and are involved in both humoral and cell-mediated immune responses (285,347). The majority of Th1 clones studied are cytolytic and can provide help for secretion of IgG, IgM and IgA but not

IgE (108) and are appropriate for responses to viral antigens. This view is supported by several studies including the isolation of Th1-type clones specific for influenza virus (348). In contrast, Th2 cells are associated with humoral responses particularly IgE and IgG4, but have low cytolytic potential. Furthermore, IL-4 appears to be required for the development of Th2 clones (369,389). Thus, in specific antibody responses, addition of IL-4 to cultures of TMC stimulated with influenza virus may promote the development of Th2 cells which secrete cytokines (IL-4, IL-13 etc.) that are not effective in anti-viral antibody responses.

Alternatively, IL-4 may have a more positive role. Horohov *et al* (195) suggested that IL-4 can suppress antibody production in favour of enhancing cytotoxic T cell activity (195). IL-4 was demonstrated to increase the proliferation of CD8+ve T cells which were specific for the influenza virus when added at the onset of culture whilst addition 5 or 6 days into an 8-day culture increased the cytolytic activity. In our experimental system, addition of IL-4 at the onset of culture may induce proliferation of any residual CD8+ cells resulting in an increase in cytolytic activity and accompanied reduction in antibody secretion.

Inhibition by IL-4 was also obtained in cultures containing E^- cells plus TRF where T cell involvement should be minimal or absent. In these experiments, B cells were purified by one round of rosetting with AET-SRBC and T cell (CD3+) contamination was routinely <3%. Without sorting the cells to reduce T cells even further, the possibility cannot be completely excluded that IL-4 could still exert an effect on the residual T cells. Despite this possibility, the observation that IL-4 inhibited specific

antibody responses by E^- cells plus TRF more than responses by whole TMC would suggest that IL-4 inhibits the TRF activity of IL-2 through altered receptor binding.

Does IL-4 play a role in specific antibody responses?

The role of IL-4, if any, in specific antibody production remains unclear. One of the effects of IL-4 is to increase expression of cell surface molecules necessary for T-B contact in specific antibody responses. In fact, IL-4 appears not to be required for specific antibody production since an IL-4 blocking antibody does not affect specific antibody secretion (63). However its inhibitory effect *in vitro* suggests that it does perform some regulatory function. IL-6, on the other hand, does not elicit any effect in the response (95).

The inhibition of specific antibody secretion observed in the presence of IL-4 is similar to that obtained with addition of CD40L to the assay. Inhibitory action by both molecules is an early event and is lost after two or three days in culture. (61,63,258). Furthermore, IL-4 enhances the expression of CD40 on B cells (161) and the cross-linking of CD40 in the presence of IL-4 provides a potent mitogenic signal to B cells (160,162). In chapter 3, it was proposed that cross-linking of CD40 expressed on memory B cells inhibited the immediate terminal differentiation in favour of clonal expansion of that specific clone. It is possible that IL-4 exerts a similar proliferative effect on B cells and that IL-4 and CD40 together could act as co-factors to optimise memory responses.

I have not determined the extent to which B cells proliferate in response to stimulation with influenza virus or if the response is modulated by the addition of IL-4

and/or CD40 but this may prove informative. A study by Taieb et al showed that IL-4 could inhibit B cell proliferation induced by soluble anti-μ antibodies (391). General protein synthesis, particularly CD23 however, was increased. In addition, the inhibitory effect could be overcome by cross-linking sIg., MHC II or CD40 suggesting a strong requirement for contact-mediated signals. They concluded that IL-4 acted to prime B cells to receive further contact-mediated mitogenic signals, and that in the absence of the correct stimuli, IL-4 could block DNA synthesis thus reducing the risk of uncontrolled proliferation. If this hypothesis is applied to the observations from the specific antibody response, it may be that IL-4 primes the B cells in response to antigen but perhaps does not receive the correct progression signal and thus the response is abrogated. This might be true in experiments using IL-2 as a TRF, but should not be a factor in experiments using whole TMC where T cell help is available. Of course, in that case, the interaction of IL-4 with the T cells may be the limiting factor.

Finally, the observations with IL-13 were not conclusive. Early evidence suggested that that IL-13 could bind the γ_c (433) but experiments with X-SCID patients showed that γ_c was not essential for responses to IL-4 or IL-13 (267) and recently a distinct receptor component for IL-13 has been described (61). If both IL-4 and IL-13 were binding γ_c, they might have a similar inhibitory effect on specific antibody secretion by competition with IL-2 for γ_c (Fig. 5.3 Exp.2). Conversely, if IL-4 or IL-13 bound independently of the γ_c , and was not competing with IL-2, then specific antibody secretion may not be affected (Fig 5.3, Exp.1). This is a highly speculative

interpretation of limited data as the responses of only two donors have been examined to date. However, if a pattern of responses to IL-13 did emerge that was different to that obtained with IL-4, it may provide a useful clue to the question of the relationship between these two cytokines.

CHAPTER 6

COMPARISON OF IL-2 AND IL-15 IN SPECIFIC ANTIBODY PRODUCTION

6.1 Introduction

It is well established that the response of B cells to influenza virus has a requirement for IL-2 and further that IL-2 can replace T cell help in the specific antibody response (64). The only other soluble factor with TRF activity identified to date is low molecular weight BCGF; a semi-purified preparation from the supernatants of PHA-activated T cells (62,380).

Culture supernatants from the simian kidney epithelial cell line CV1/EBNA (272) were found to support growth of an IL-2-dependent line CTLL (153). From these supernatants, a novel cytokine was isolated by Grabstein *et al* in 1994 and designated IL-15 (163). In addition, IL-15 induces proliferation of activated B cells, and stimulates immunoglobulin secretion (IgM, IgG, IgA) upon cross-linking CD40. IL-15 has also been found to replace T cells *in vitro* in primary specific responses to SRBC; a property hitherto only ascribed to IL-2 and $BCGF_{lmw}$ (9). Although the functional properties of IL-15 and IL-2 are very similar, there is little sequence identity between the two cytokines. They do however have structural homology, and IL-15 belongs to the helical cytokine family whose other members include IL-2, IL-4 and GM-CSF (25). IL-15 was subsequently found to share the β and γ (but not α) components of the IL-2R (154,163).

This chapter describes the experiments performed in the first stages of characterising the role of IL-15 in the specific antibody response by comparing its activity as a TRF with IL-2.

6.2 Effect of IL-15 on B cell proliferation

IL-15 was first identified by its ability to support the proliferation of CTLL and normal peripheral blood T lymphocytes (163). The similar activities of IL-15 and IL-2 suggested that IL-15 might also stimulate B cell proliferation. This was subsequently confirmed by Armitage *et al* (9). On obtaining a supply of IL-15, the cytokine was initially titrated in a proliferation assay for comparison with IL-2 and to obtain a suitable concentration for use in other experiments. Results from two proliferation assays are shown in Fig. 6.1. Proliferation to a single (optimal) dose of IL-2 is included for comparison.

In both experiments, IL-15, in the presence of 10μg/ml anti-IgM beads, induced proliferation in a dose-dependent manner. In control cultures, IL-2 also co-stimulated with anti-IgM to induce proliferation. IL-15 alone was unable to stimulate B cell proliferation In Exp. 1, 5U/ml of IL-2 stimulated moderate proliferation and was equivalent to 30 ng/ml of IL-15. In Exp. 2, IL-2 and anti-IgM were more effective co-stimuli and a higher concentration of IL-15 was required to induce comparable levels of proliferation.

Fig.6.1.

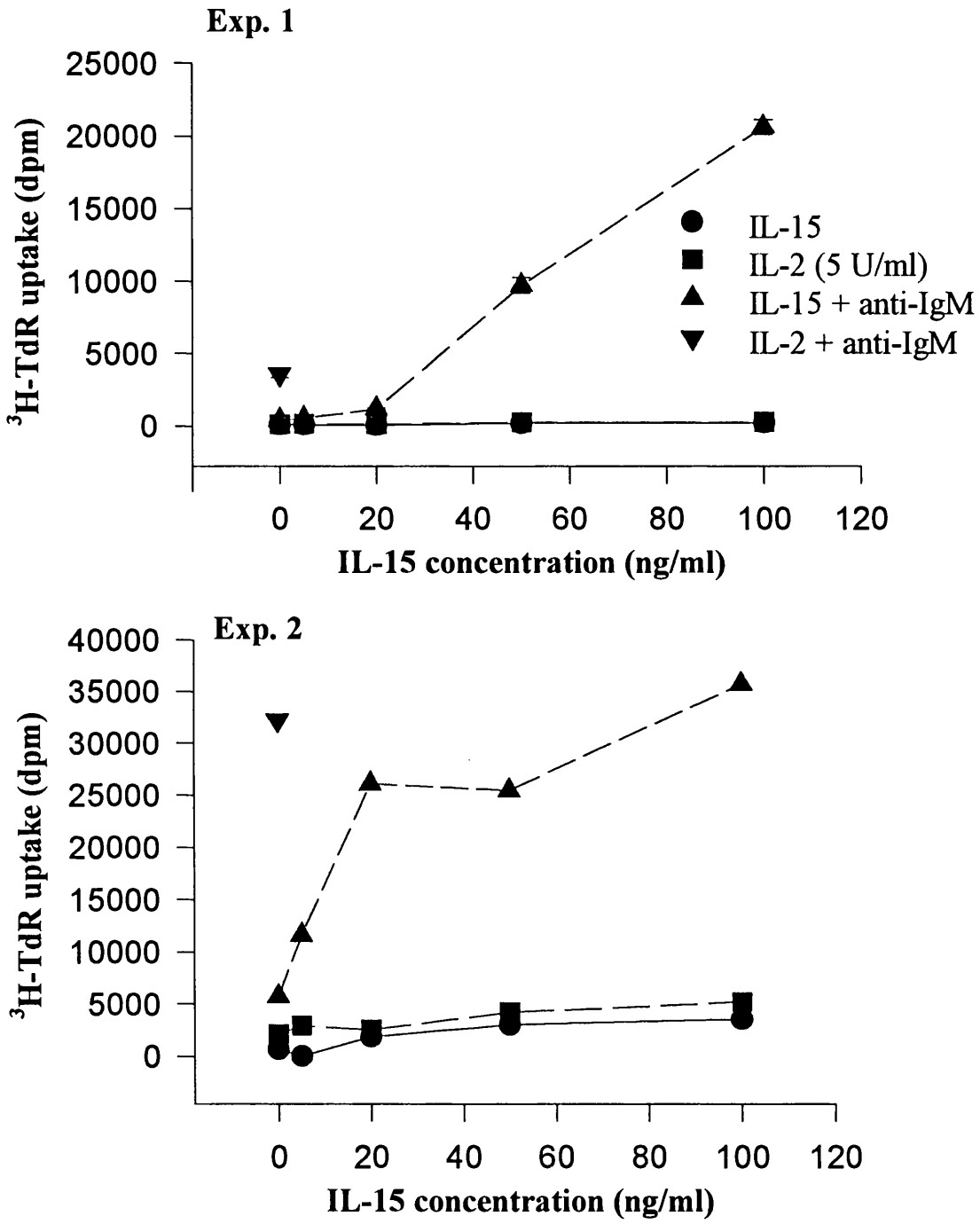


Fig.6.1. IL-15 is a B cell growth factor

B cells were prepared from frozen (Exp.1) or fresh (Exp.2) tonsil tissue. and co-stimulated with 10ug/ml anti-IgM, 5U/ml IL-2 and IL-15 at concentrations shown. Results are expressed as mean dpm from triplicate cultures . Standard errors were all <10%.

6.3. T cell replacing factor-activity of IL-15

I was specifically interested to see if IL-15, like IL-2 and $BCGF_{lmw}$, had TRF activity in the specific antibody response. In initial, experiments, B cells from frozen TMC which had previously been found to respond to IL-2 in TRF assays were stimulated with antigen and either IL-2 or IL-15. The results from two experiments are shown in Table 6.1.

Table 6.1 IL-15 is a T cell replacing factor

Cells[a]	Antigen	TRF[b]	IgG secretion (U/ml)[c]	
			Exp.1	Exp.2
E^-	-	-	<5	5 ± 1
E^-	+	-	<5	220 ± 73
E^- + E+	+	-	>2000	> 2000
E^-	+	IL-2 (5U/ml)	72 ± 20	1415 ± 111
E^-	+	IL-15 (20ng/ml)	104 ± 11	1117 ± 299

[a] E^- cells from TMC purified by one round of E-rosetting cultured with either E+ cells or TRF as indicated.
[b] IL-2 (5U/ml) or IL-15 (20ng/ml) were added as TRF
[c] Mean specific IgG secretion from triplicate cultures ± 1 s.e.m.

The results from these two experiments demonstrate the ability of IL-15 to replace T cell help. Its effect was very similar to IL-2 and the magnitude of the response to both cytokines was approximately the same. IL-15 was used at a concentration of 20ng/ml. At this dose it was calculated to have almost equal specific activity to IL-2 in B cell proliferation experiments. Over these and other experiments there was no clear distinction between the two cytokines as to which was the best TRF and any differences are likely to be due to variations between donors.

Although the IL-15 receptor has its own unique α chain (155), it has been shown that binding to both the β and γ chains of the IL-2R is necessary for signalling. In the above experiments it is not clear whether IL-15 mediates its activity exclusively via the β and γ chains or through an additional unique receptor component. The next experiments investigated the effects of adding IL-2 and IL-15 together in the cultures to determine whether they could co-stimulate or if they were competing for receptor binding which might reduce the TRF activity in the assay. The results for two experiments are shown in Table 6.2.

In Exp.1, the batch of IL-15 used had optimal activity in a B cell proliferation assay at a concentration of 200ng/ml. In this experiment, background levels of antibody secretion (from cultures containing E^- + antigen) were very high and it is difficult to draw conclusions. Nevertheless IL-2 appears to act as a TRF and enhances antibody secretion from 386U/ml (control value) to 719U/ml. There also appears to be an

Table 6.2 Effect of presence of both IL-2 and IL-15 on TRF activity in the specific antibody response

Cells[a]	Antigen	TRF[b]	Specific IgG (U/ml)[c]
Exp. 1			
E^-	-	-	<5
E^-	+	-	386 ± 27
E- + E+	+	-	1042 ± 78
E^-	+	IL-2	719 ± 153
E^-	+	IL-15 (200ng/ml)	496 ± 68
E^-	+	IL-2 + IL-15 (200ng/ml)	922 ± 132
Exp. 2			
E^-	-	-	<5
E^-	+	-	<5
E^- + E+	+	-	246 ± 8
E^-	+	IL-2	45 ± 5
E^-	+	IL-15 (5ng/ml)	7 ± 2
E^-	+	IL-15 (20ng/ml)	10 ± 3
E^-	+	IL-15 (50ng/ml)	23 ± 9
E^-	+	IL-15 (100ng/ml)	126 ± 36
E^-	+	IL-2 + IL-15 (5ng/ml)	105 ± 65
E^-	+	IL-2 + IL-15 (20ng/ml)	65 ± 1
E^-	+	IL-2 + IL-15 (50ng/ml)	188 ± 59
E^-	+	IL-2 + IL-15 (100ng/ml)	409 ± 239

[a] E^- cells from TMC purified by one round of E-rosetting, cultured with either E+ cells or TRF as indicated.
[b] IL-2 was present at 5U/ml throughout, IL-15 was added at the concentration shown
[c] Mean specific IgG secretion from triplicate cultures ± 1 s.e.m.

additional increase when IL-2 was in combination with IL-15. The effect was additive rather than synergistic. In Exp.2, the TRF activity of IL-15 increased in a dose dependent manner with a maximal response at 100ng/ml. This was comparable to the concentration required to co-stimulate with anti-IgM for B cell proliferation. In the presence of 5U/ml of IL-2 , the response was increased synergistically even at the lowest concentration of IL-15. The ability of IL-15 to increase IL-2 responses suggests that IL-15 is not competing with IL-2 for binding to the β and γ chains of the IL-2R, and can enhance IL-2 mediated reactions and is consistent with the presence of a separate receptor component for IL-15.

6.4 Effect of IL-2 on different sub-populations of B cells

Until now, IL-2 and $BCGF_{lmw}$ were the only two cytokines found to have TRF activity in the specific antibody response by human B cells (380). The precise nature of the activity $BCGF_{lmw}$ has never been elucidated as it was a commercial preparation derived from the culture supernatants of activated T cells that is no longer available. In a previous study, IL-2 was found only to function as a TRF for low density ("activated") B cells whereas $BCGF_{lmw}$ could also stimulate specific antibody secretion from high density ("resting") populations of B cells (62). The following experiments were performed to define the sub-population of B cells for which IL-15 had stimulatory activity. Purified B cells were separated into heavy (resting) and light (activated) populations by discontinuous density centrifugation. Each of the populations were stimulated with antigen and either IL-2, IL-15 or both. Table 6.3 shows the results from two experiments.

Table 6.3 Comparison of TRF activities of IL-2, IL-15 and $BCGF_{lmw}$ on different B cell populations.

Cells[a]	Antigen	TRF [b]	IgG secretion (U/ml) [c] B cell population:		
			Unseparated	'Light'	'Heavy'
Exp. 1					
E^-	-	-	<5	<5	<5
E^-	+	-	<5	<5	<5
$E^- + E+$	+	-	78 ± 27	145 ± 79	36 ± 12
E^-	+	IL-2	68 ± 10	<5	<5
E^-	+	IL-15	125 ± 23	58 ± 2	13
E^-	+	$BCGF_{lmw}$	135 ± 47	180 ± 42	16 ± 8
Exp. 2					
E^-	-	-	<5	<5	<5
E^-	+	-	5 ± 2	<5	<5
$E^- + E+$	+	-	16 ± 5	29 ± 3	39 ± 8
E^-	+	IL-2	3 ± 2	7 ± 2	<5
E^-	+	IL-15	3 ± 1	<5	<5
E^-	+	IL-2 + IL-15	24 ± 6	47 ± 3	26 ± 8

[a] E^- cells from TMC purified by one round of E-rosetting, cultured with either E+ cells or TRF as indicated.
[b] IL-2 (5U/ml), IL-15 (100ng/ml) or 10%v/v $BCGF_{lmw}$.
[c] Mean specific IgG secretion from triplicate cultures ± 1 s.e.m.

In Exp.1, IL-2, IL-15 and $BCGF_{lmw}$ all had TRF activity in cultures containing unseparated B cells. Cultures containing 'light' (activated) B cells also secreted specific antibody in the presence of IL-15 and $BCGF_{lmw}$, but, unexpectedly, did not in the presence of IL-2. In cultures that contained the high density population of B cells, IL-2 was unable to replace T cell help as expected. In this experiment, IL-15 showed similar activity to $BCGF_{lmw}$ in its ability to act as a TRF for heavy B cells although the response was rather low.

In Exp. 2, $BCGF_{lmw}$ was no longer commercially available and the effect of addition of IL-2 and IL-15 together was investigated. In this experiment, all antibody responses were very low. Even in the unseparated population, IL-2 or IL-15 alone were not able to stimulate antibody secretion. However IL-2 and IL-15 together stimulated a response which was comparable to reconstituting the assay with E+ cells. Neither the heavy nor the light populations of B cells responded to IL-2 or IL-15 alone but both populations secreted IgG when IL-2 and IL-15 were added together. This experiment has been repeated several times but no clear pattern has emerged except that the combination of IL-2 and IL-15 does seem to replace T cell help even if either cytokine alone is ineffective.

6.5 Discussion

This study demonstrated that IL-15 can support B cell proliferation with anti-IgM-coated beads as a co-stimulatory agent. However, some differences between batches of IL-15 the two experiments in were noted (Fig.6.1). In Exp. 1, the levels of ^{3}HTdR uptake did not reach a plateau, but in Exp. 2, peak proliferation was obtained at 20 -

50 ng/ml IL-15. B cells from frozen tissue were used in Exp.1 and from fresh tissue in Exp.2 which may partly explain the differences between these observations. Alternatively the differences may have been due to the use of different batches of IL-15. We consistently experienced some variation in activity between different batches of IL-15 which highlights the importance of titrating each new batch of the cytokine.

IL-15 was found to replace T cell help in the specific antibody response; a property hitherto only attributed to IL-2 and $BCGF_{lmw}$. In addition, there was evidence of synergistic activity when IL-2 and IL-15 were added to the assay together (Tables 6.2 and 6.3) IL-2 and $BCGF_{lmw}$ have previously been shown to have TRF activity on different populations of B cells in response to influenza virus(62). In these experiments, IL-2 was unable to activate resting B cells and therefore was only a TRF for activated B cells. In contrast, $BCGF_{lmw}$ had TRF activity for both resting and activated T cells and was assumed to be able to activate resting B cells.

In similar experiments, I attempted to establish which population of B cells responded to TRF activity of IL-15. The initial results suggested that IL-15 had similar activity to $BCGF_{lmw}$ (Table 6.2, Exp.1), and was able to activate resting B cells. However, subsequent experiments failed to clarify this. In some cases, IL-2 and IL-15 together were able to stimulate specific antibody secretion by resting B cells where IL-2 or IL-15 alone had no effect, thus suggesting some degree of cooperativity between the two cytokines. It is attractive to speculate that the original $BCGF_{lmw}$ preparations contained both cytokines but further experiments are required to answer this question satisfactorily.

As research into cytokines has progressed, a pattern has emerged of cytokine pairs which have very similar activities. For example IL-4 and IL-13 (which were discussed in the previous chapter), TNFα and β, and IL-1α and IL-1β. IL-2 and IL-15 seem to be another cytokine pair which have very similar functional properties. The reason for the existence of such 'pairs' of cytokines, with similar functions, is intriguing. Do they represent an extraordinarily high degree of redundancy which serves to protect from the occurrence of lethal mutations, or do each have more distinct functions which may not be exclusive to the immune system? A case in point is the observation that mice deficient for the IL-2 gene can still maintain immune responses *in vivo* and have no serious developmental abnormalities, although *in vitro* memory responses are impaired (232). However IL-15 and IL-2 are expressed in mutually exclusive cell types which suggests that they may have distinct immune functions (163). IL-2 is only secreted by activated T cells whereas IL-15 is secreted by a number of cell types and tissues. The most abundant sources of IL-15 are adherent PBMC (monocyte enriched), epithelial and fibroblast lines, and placental and muscle tissue. The secretion of IL-15 by many cell types but not T cells suggests that its function may not be restricted to T-B cell responses. Furthermore, it may mediate an effect via some other principle target cell, for example within the endothelium. In order to help clarify this aspect, it has been the strategy of this group and others to try and identify differences as well as similarities in function between the two cytokines in question.

CHAPTER 7

GENERAL DISCUSSION

If it is possible to elucidate the mechanisms involved in the production of specific antibodies to antigens such as influenza the possibility exists of manipulating the response for therapeutic purposes. For example, it may be desirable to enhance immune responses in immunocompromised individuals or for optimising vaccines. Alternatively, suppression of the immune response may be useful, for example in the treatment of autoimmune disorders or for preventing rejection following organ transplantation. An experimental system such as the assay for specific antibody production allows the study of the role of cytokines and cell surface antigens in T-B interactions following re-stimulation of memory cells by antigen.

CD40-CD40L interactions in Th-B responses

CD40-CD40L interactions have been shown to have key regulatory functions affecting a number of different aspects of T-B responses. This is illustrated by the XHIGM patients who lack germinal centres and are unable to undergo isotype switching or mount memory responses and is supported by a number of *in vitro* and *in vivo* studies. The burning question remains: how does one receptor-ligand pair induce so many effects?

Early studies predicted the existence of more than one CD40L molecule but as yet there is no evidence for this. A number of more recent models propose a sequence of events based on a progression of co-stimulatory interactions between CD40L on T cells and CD40 on accessory cells and help delivered to B cells through a CD40-

CD40L interaction between the B and Th cells. These interactions would be optimised through other adhesion molecules whose expression in turn is likely to be regulated by the release of cytokines from other accessory cells within a particular microenvironment e.g. the germinal centre. The nature of CD40L expression on T cells i.e. rapid transient expression following activation, confers another level of regulation and allows the delivery of a quick and precise signal. In addition, CD40L can directly stimulate T cells (presumably through binding to low levels of CD40 expressed on the surface) and may have a role in optimal T cell activation. CD40 expression is constitutive on B cells and engagement is therefore primarily dependent upon the activation state of the T cell. However CD40 expression on B cells is enhanced by IL-4 and also following B7/CD28/CTLA-4 interactions which may increase the chances of a successful CD40-CD40L interaction. CD40 is also expressed on dendritic cells, FDCs monocytes (enhanced in the presence of GM-CSF, IL-3 and IFNγ) and endothelial cells all of which may have a regulatory function.

A possible scenario is as follows: antigen/pathogen is captured by dendritic cells e.g. in skin or mucosa which migrate into T cell rich areas of lymph nodes. Activation of the dendritic cell induces/enhances CD40 expression. IDC processes antigen and presents it to a T cell in MHC-restricted manner. The T cell, once activated, expresses CD40L and can bind CD40 on IDCs enhancing cytokine expression for further T cell activation, proliferation and differentiation. IDC may present antigen to B cells initiating B-T cell interaction via CD40-CD40L binding. CD40 cross-linking on the B cell at this stage may be a key event in the recruitment of B cells to primary follicles and germinal centre formation. In the fully developed germinal centre, centroblasts occupy the dark zone and are thought to undergo somatic mutation within their Ig-

gene V regions. Centroblasts mature into centrocytes and migrate to the light zone where they encounter a large number of FDCs that present unprocessed antigen to the B cells. Here the processes of selection on the basis of affinity for antigen, isotype switching and rescue from apoptosis occur.

The latter processes require CD40-CD40L interactions. Close examination reveals a potential problem in the source of CD40L. This was highlighted in a study by Grammer et al (166) who point out that isotype switching and rescue from apoptosis in the germinal centre occur not before 72 hours after initial T cell activation by which time CD40L is no longer expressed by Th cells. In addition, although germinal centre T cells were found to express CD40L, there is a considerable degree of compartmentalisation of B and T cells in the lymph nodes. Thus early activation events largely occur in the T-cell-rich areas in the paracortex of the lymph nodes and also the junction between the follicular mantle and the germinal centre whereas the majority of the B cell activity occurs in the dark zone of the germinal centre where there are virtually no T cells. The finding that B cells can be induced to express CD40L identical to that expressed by T cells offers an explanation for this. It is possible that CD40L expressed on T cells is required for initial activation and formation of germinal centres but CD40L expressed on B cells provides the necessary signals for rescue and isotype switching later in the response.

Another possible mechanism of control is via differential expression of functional epitopes of CD40. Collective data from the panel of mAbs to CD40 that were submitted to the B cell section of the 5th international workshop and conference on HLDA antigens concluded that several distinct functional epitopes are present on

CD40, the engagement of which can elicit different responses with respect to their ability to perform functions such as CD40L binding, rescue from apoptosis or induce proliferation. These were consistent with our observations that the different mAbs could inhibit specific antibody production to varying degrees (described in Chapter 4). We used the panel of mAbs to examine the expression of CD40 on B cells from normal donors and both X-linked and one non-X-linked HyperIgM patient and found variable patterns of expression (data not shown) which again seems consistent with the existence of several different epitopes. It can be envisaged that the regulation of expression of a particular epitope that may be restricted to a particular stage of activation would provide an efficient regulatory mechanism for CD40 mediated functions. It would be interesting to extend this study to assess what effect the stage of differentiation, e.g. germinal centre cell or memory cell or mode of activation of the B cell, has on CD40 epitope expression.

Perspectives for studying the specific antibody response

From our studies with CD40 we propose that signalling via CD40 expressed on an isotype-switched, high affinity memory B cell following encounter with antigen, leads to expansion of the memory clone rather than immediate terminal differentiation into a plasma cell. One way of studying this would be to stimulate B cells with CD40L and influenza virus and then transform the cells with Epstein Barr Virus. The cells are cultured in a limiting dilution assay based on original cell numbers and incubated until signs of transformation are visible. Supernatants are then analysed by ELISA for the presence of specific antibody. The rationale is that EBV stimulates antibody production from transformed clones. If CD40L induced proliferation in antigen

stimulated cells this would be reflected in greater frequency of antibody secreting cells in the limiting dilution assay compared to control cultures that received no CD40L stimulation. This method of limiting dilution following EBV transformation has been applied previously to the specific antibody response for the determination of the frequency of antigen-specific helper T cells (378). Initial attempts to apply this technique to the 'CD40 question' have proved unsuccessful although this has probably been due largely to technical difficulties.

Identifying B cell subsets with properties of memory cells

In attempting to elucidate the mechanisms involved in the formation and activation of memory cells, we may need to try and isolate more accurately a specific memory B cell population. No one marker specifying a mature memory cell has been identified, rather researchers rely on the presence or absence of a number of cell surface molecules. Pascual *et al* describe a Bm5 B cell subset in humans, which is IgD-CD38- (312) that is considered to represent a memory B cell population. Secondly, Defrance *et al* showed that CD5+ cells largely secreted IgM when stimulated with cross-linked CD40mabs and IL-2 whereas CD5- cells secreted high levels of IgG (104).

Researchers studying memory cell formation in the mouse have also addressed this problem with some successful results. In the mouse, B cells secreting specific antibodies have been isolated following immunisation with phycoerythrin (PE) or 4-hydroxy-3-nitrophenylacetyl (NP) (177,269) where they comprise only 0.02% of a whole spleen. Their memory cell status was confirmed by analysis of the V region

heavy chain genes which were found to have undergone somatic hypermutation (269). McHetzer-Williams *et al* found that isolated memory B cells could undergo extensive proliferation *in vitro* (stimulated by lipopolysaccharide) but did not undergo further somatic mutation during this proliferative phase.

A similar approach to isolating memory cells in human B cell populations is an attractive idea but would require a probe to allow sorting of antigen-specific B cells. It may be possible to tag the influenza virus with a dye to allow sorting. Molecular analysis might yield information about the levels of mutation and hence about the development of memory B cells.

More in depth signalling studies may also prove to be informative. CD40 signals through members of the *src*-family of tyrosine kinases. It has been suggested that these kinases might activate different downstream pathways as different *src*-family members appear to be able to associate with different downstream effects and have different substrate peptide preferences. In addition, changes in expression of *src*-family molecules at different points during B cell differentiation may contribute to changes in cellular responses to antigen (68) and may also be influenced by a particular cytokine or other component for example, CD40.

The idea of analysing single antigen-specific B cells is attractive and likely to be informative. The possibility of cloning such cells and culturing them *in vitro* would enable the study of the effects of single molecules such as CD40L on processes such as proliferation or further rounds of somatic mutation. It must be borne in mind however, that B cells do not act in isolation *in vivo* neither, for the most part, do

cytokines or cell surface molecules act singly. Therefore another approach to studying *in vitro* antibody responses might lead in the opposite direction to that described above i.e. by attempting to re-create an appropriate microenvironment representative of a particular stage of B cell differentiation. This should include accessory cells such as IDCs and FDCs to 'complete' the picture although a drawback to this would be the difficulty in controlling the potential multiplicity of interactions.

Reversal of inhibition of specific antibody response by CD40 or IL-4

One can gain clues to normal regulatory function involved in a response by studying its inhibition. In the specific antibody response, the reversal of CD40-mediated inhibition is a potential strategy. Possible candidates for interacting with CD40 or CD40L are other members of the TNF or TNFR superfamilies. Rodriguez *et al* showed that human tonsil B cells required TNF-α for the first 12 hours of in vitro culture for spontaneous immunoglobulin secretion (340) and the interaction between CD40 and TNFα is possible line of investigation.

There are in fact relatively few cytokines or antibodies to cell surface antigens that have a significant effect on the specific antibody response as confirmed by studies using both the B cell panel and the cytokine receptor panel of mAbs from the Fifth International Workshop (data not shown). Further we found no effect on specific antibody responses by an OX40.Fc protein or mAbs to OX40 (data not shown).

Beyond the follicle - CD40 and CD40L in other cellular interactions

A glance through the recent publications in CD40 research shows there is still much interest in the molecular mechanisms driving CD40-CD40L interactions in Th-B cell responses. Significant attention is also shifting towards the importance of CD40 in other cellular interactions. CD40L, although largely expressed on CD4+ T cells, is also detected on a small subset of CD8+ T cells, and possibly mast cells, basophils, NK cells, monocytes and also B cells. Similarly, CD40 is expressed on a variety of cells including B cells, monocytes, dendritic cells and thymic epithelium and there is great scope for CD40-CD40L interactions between a large number of different effector and accessory cells. For example, the role of CD40 expressed on endothelial cells is intriguing. The current opinion is that it is likely to be involved in 'lymphocyte homing' and is probably expressed during an inflammatory response and responsible for recruiting the appropriate effector cells.

A possible link between CD40 and cancer?

Finally, the role of CD40 expressed on malignant cells may prove to be important. The observation that a large number of transformed cells expressed CD40 whereas their normal tissue counterparts did not was made early in the CD40 story. Given that CD40 cross-linking enhances cell proliferation and further inhibits apoptosis suggests that CD40 expression might play a positive role in aspects of carcinogenesis such as the loss of growth control. The observations by Funakoshi *et al* that ligation of CD40 on B cell lymphomas by CD40L inhibits their proliferation *in vitro* and can induce the regression of tumours *in vivo* (146) offers an alternative of a potentially protective role of CD40 in neoplastic cells. The study is supported by previous observations that signals with growth-promoting effects in normal tissues can have an anti-tumour

effect on malignant cells of the same tissue (15,48,309). Our own observations that CD40 ligation on B cells has an inhibitory effect also supports the idea that CD40 suppresses some aspects of cellular activity. Expression of CD40 by malignant cells might recruit CD40L-expressing cytotoxic T cells as a line of defence or, as found with the monocytes may induce tumourigenic effects. Such a concept has vast potential in the therapeutic war against cancer especially if it was relevant to malignancies other than those of haematopoietic origin.

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CD40 Ligand (CD40L) Expression and B Cell Function in Agammaglobulinemia with Normal or Elevated Levels of IgM (HIM)

Comparison of X-Linked, Autosomal Recessive, and Non-X-Linked Forms of the Disease, and Obligate Carriers[1]

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Hyper-IgM syndrome is a rare immunodeficiency characterized by low or absent IgG, IgA, and IgE with normal or elevated levels of IgM. It can occur as an acquired or familial disorder with either X-linked or autosomal modes of inheritance. The X-linked form (HIGM1) is a result of mutations in the CD40 ligand (CD40L) gene, but the defect in non-X-linked forms of the disease (HIM) has not been determined. We show here that CD40L expression on activated T cells from non-X-linked patients can be detected by CD40Fc, 5c8 Mab, and anti-TRAP, whereas activated T cells from HIGM1 patients either had no detectable CD40L (Type I), or stained with anti-TRAP but not CD40Fc or 5c8 (Type II). Activated T cells from obligate carriers varied from low to normal expression of CD40L. B cells from HIGM1 and non-X-linked HIM patients proliferated in response to CD40L. Costimulation of B cells from HIGM1, from sporadic HIM, or from non-X-linked HIM patients with CD40L plus IL-2 resulted in some IgM production, but no significant IgG or IgA. Costimulation with CD40L plus IL-10 resulted in significant IgG and/or IgA secretion by B cells from some HIGM1 patients, but consistently failed to stimulate IgG or IgA secretion by B cells from non-X-linked patients. In addition, costimulation with CD40L and IL-4 failed to induce IgE secretion by B cells from one non-X-linked HIM patient, and induced a weak response in another. These results suggest that patients with non-X-linked forms of HIM may have an intrinsic B cell defect preventing heavy chain switching, which is not related to expression of CD40L. *The Journal of Immunology*, 1994, 153: 3295.

Immunodeficiency with hyper IgM (HIM)[3] is a rare syndrome characterized by an increased susceptibility to recurrent infections and low or absent serum IgG, IgA, and IgE, and normal or more often elevated levels of IgM (1–3). It is clinically heterogeneous and can occur either as an inherited or acquired disorder. Family studies have established an X-linked mode of inheritance mapping to Xq24–27 for some instances of HIM (3, 4), but about 22% of cases are girls indicating the existence of a non-X-linked or autosomal recessive form (5–7), and some pedigrees have been reported with autosomal dominant transmission (8, 9). The X-linked form of the disease (HIGM1) has been shown recently to be a result of a mutation in the CD40 ligand (CD40L) gene (1, 10–14), but the defect in non-X-linked forms has not yet been identified.

CD40L is a type II integral membrane protein expressed on the surface of activated T cells (15–17). It has significant sequence homology with TNF and a predicted tertiary structure very similar to the TNF trimer (18). Binding of CD40L to CD40 on B cells stimulates B cell proliferation (16) and, in combination with IL-2 or IL-10, stimulates B cells to secrete IgM, IgG, and IgA, but not IgE (16,

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[3] Abbreviations used in this paper: HIM, hyper IgM syndrome; CD40Fc, CD40 IgG1 Fc fusion protein; CD40L, CD40 ligand; E^+ cells, lymphocytes forming rosettes with AET-treated sheep red blood cells (T cells); E^-, non-rosette-forming cells; HIGM1, X-linked form of hyper-IgM syndrome; PMA, phorbol 12-myristate 13-acetate.

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Table I. *Subjects investigated in this study* [a]

Subject	DOB	Sex	Diagnosis	Family History and Inheritance Pattern
BW*	09/04/88	M	HIGM1	6 male deaths over 2 generations: X-linked
CS*	03/23/88	M	HIGM1	maternal uncle died at 6/12: X-linked
AT	03/17/63	M	HIGM1	affected males over several generations: X-linked
PO	04/17/89	M	HIGM1	brother died: X-linked
GM	08/13/85	M	HIGM1	three affected males in one generation: X-linked
KS	05/27/85	M	HIGM1	cousin of GM: X-linked
PS	05/01/92	M	HIGM1	brother of KS, cousin of GM: X-linked
NC*	07/20/73	M	HIGM1	brother died: X-linked
FM	09/09/81	M	HIGM1	brother died: X-linked
BS	10/12/76	M	HIGM1 (S)	Sporadic
JP*	09/17/88	M	HIGM1 (S)	Sporadic
GP*	04/13/82	M	HIGM1 (S)	Sporadic
CSh	05/20/76	M	HIGM1 (S)	Sporadic
AG	01/12/78	M	HIGM1 (S)	Sporadic
HG	03/15/89	F	HIM	Sporadic
VE	04/07/85	F	HIM	Sporadic
LS	11/22/75	F	HIM	Sporadic
FZ*	10/16/82	M	HIM	Parents 1st cousins, sister dead: autosomal recessive
C-1	na	F	Obligate carrier	Mother of BW
C-2	na	F	Obligate carrier	Mother of CS
C-3	na	F	Obligate carrier	Mother of PO
C-4	na	F	Obligate carrier	Mother of NC
C-5	na	F	Obligate carrier	Mother of AT
P-1	na	F	Parent	Mother of VE
P-2	na	F	Parent	Mother of JP
P-3	na	F	Parent	Mother of GP
P-4	na	F	Parent	Mother of BS
P-5	na	M	Parent	Father of FZ

[a] The patients marked with an asterisk have been described in detail elsewhere (24).
na, not applicable.

19). Switching to IgE requires CD40 ligand plus IL-4 (19–21). mAb binding to B cell CD40 has also been shown to rescue $CD38^+$ B cells from apoptosis in the germinal center after somatic mutation and expression of new IgRs during affinity maturation (22, 23). The vital role of CD40 activation for production of switched high affinity B cells is underlined by the inability of HIGM1 patients who have a mutation in the CD40L gene to make IgG, IgA, or IgE (1, 3). There is, however, no information on the role of CD40 and its ligand in the non-X-linked forms of the disease. In this study, we have compared CD40L expression and B cell responses in HIGM1 patients with obligate carriers (mothers), sporadic cases of HIM with no family history, and non-X-linked forms including girls and one boy with an autosomal inheritance pedigree. Our results show that only HIGM1 is associated with abnormal CD40L expression and indicate the existence of another abnormality responsible for the inability to switch from IgM to other isotypes in non-X-linked forms of the disease.

Materials and Methods

Patients

Patients were selected from children presenting to a tertiary clinical immunology center at the Great Ormond Street Hospital for Children in London for investigation of hypogammaglobulinemia. Diagnostic criteria for hyper-IgM syndrome consisted of severely reduced (usually undetectable) levels of IgG and IgA, but with IgM levels within or above the normal range for age. Our patient selection and diagnostic criteria have been described recently in a review of patients seen at the Great Ormond Street Hospital for Children (24). Seven patients were boys with a family history indicating X-linked recessive inheritance. The mothers of five of these patients were investigated as obligate carriers. Five other boys fitting our diagnostic criteria had no family history (sporadic HIM). Three girls and one boy of consanguineous parents whose sister had died of hypogammaglobulinemia with preserved IgM production suggesting autosomal recessive inheritance were also studied. All the subjects investigated in this study are listed in Table I.

Cell preparation

PBMCs were prepared by centrifugation of heparinized venous blood over Ficoll-sodium diatrizoate (Lymphoprep Flow Laboratories, Rickmansworth, UK) Mononuclear cells were collected from the interface and washed twice in RPMI 1640 (Life Technologies, Inc., Paisley, UK) containing 10 mM HEPES, 5% FCS (GIBCO BRL), and 50 μg/ml of gentamicin. Sheep erythrocyte rosette-forming (E^+) cells were prepared with AET (*S*-2-aminoethylisothiouroniumbromide hydrobromide; Aldrich Chemical Co., Gillingham, UK)-treated sheep red cells (SRBC) at 4°C (25) and separated from non-rosette-forming (E^-) cells by centrifugation over Percoll (1.080 kg/l) as described previously (26). The E^- (B) cells were recovered from the interface and the E^+ (T) cells were recovered from the pellet by lysis of the SRBC with Gey's hemolytic solution (26).

T cell activation and CD40L expression

T (E^+) cells (10^6/ml) were stimulated with PMA (20 ng/ml) plus calcium ionophore A23187 (100 ng/ml) (Sigma Chemical Co., Poole, Dorset, UK) for 16 h in RPMI 1640 containing 5% FCS and 25 mM HEPES. CD40L expression on activated T cells was detected by staining with CD40 human IgG1 fusion protein (CD40Fc) (27), a mAb to CD40L (5c8) (28), and a polyclonal rabbit Ab raised against a CD40L fusion protein (anti-TRAP) (14). Labeled cells were analyzed on a FACScan (Becton Dickinson, Mountain View, CA) by using LYSIS 2 software. Responses

of patients' T cells were compared with a normal control in each experiment and repeated at least twice.

B cell proliferation and Ig secretion in response to CD40L

B (E^-) cells at a concentration of 10^6/ml in RPMI 1640 plus 5% FCS were stimulated with CV1/EBNA cells (10^5/ml) that had been transfected with human CD40L (19) and IL-2 (5 U/ml) (Amersham International, Aylesbury, Bucks, UK) or IL-10 (100 ng/ml) (kindly supplied by Anne O'Garra, DNAX, Palo Alto, CA). CV1/EBNA cells transfected with empty vector were used in control cultures. In proliferation assays, B cells at a concentration of 10^6 cells/ml in 200 μl of RPMI 1640 containing 5% FCS and 25 mM HEPES were cultured for 3 days and then pulsed with 1 μCi of [^{3}H]TdR (TRA 120; Amersham International) for 18 h before harvesting and counting on a liquid scintillation counter. In assays for Ig production, B cells at a concentration of 0.5×10^6/ml in 1 ml of RPMI containing 5% FCS and 25 mM HEPES were cultured in 2054 Falcon tubes for 8 days. Supernatants were then removed and assayed by ELISA for IgM, IgG, IgA, and IgE. Responses of patients' B cells were compared with a normal control in each experiment and repeated at least twice.

ELISA assays for secreted Igs

The ELISA assays were conducted essentially as described previously (26, 29). In brief, flat-bottom 96-well Immulon II plates (Dynatech Labs, Chantilly, VA) were coated with goat anti-human IgM, IgG, IgA, or 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 ortho-phenyldiamine was determined on a Dynatech plate reader at 492 nm. The assays had lower detection limits of 0.5, 1.0, and 1.0 ng/ml for IgM, IgG, and IgA, respectively, and 300 to 600 pg/ml for IgE. IgG subclasses were assayed by coating plates with rabbit anti-human IgG or sheep anti-human IgG4 capture Abs 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 horseradish peroxidase-conjugated streptavidin. The IgG subclass assays had lower detection limits of between 1 and 10 ng/ml.

Results

Expression of CD40L on activated T cells from HIGM1 patients and obligate carriers

CD40L expression was detected on activated T cells by binding of CD40Fc, 5c8 mAb, and anti-TRAP (Fig. 1). CD40L was readily detected on T cells from normal donors activated for 16 h with PMA and calcium ionophore with all three reagents. Freshly prepared T cells or T cells cultured for 16 h in medium alone sometimes had low levels of expression compared with background staining, but this was always insignificant compared with expression on activated T cells. All normal donors gave similar results to the example shown in Figure 1. Time course experiments showed that CD40L expression was optimal after 10 to 16 h of activation.

Two quite distinct patterns of CD40L expression were observed on activated T cells from HIGM1 patients. In type I, staining with CD40Fc, 5c8, and anti-TRAP was consistently negative (e.g., CS and AT in Fig. 1). In some patients, however, apparently normal staining was obtained with anti-TRAP but not with either CD40Fc or 5c8 (e.g., BW in Fig. 1). This result shows that the CD40L mutation does not necessarily prevent insertion of the defective protein in the membrane of activated T cells. In this respect, it is worth noting that activated T cells from the only patient known to have a mutation in the transmembrane domain of CD40L (AT in Fig. 1) were negative for both 5c8 and anti-TRAP.

Activated T cells from five obligate carriers were also examined for CD40L expression. Although CD40L was detected by all three reagents on activated T cells from every carrier tested, the level of expression was variable. Of the five carriers tested, expression was low in two carriers compared with normal controls (e.g., mother of CS in Fig. 1), whereas in the other three it approached normal levels (e.g., mother of BW in Fig. 1). The low level of expression in some carriers did not simply demarcate a minor $CD40L^+$ subpopulation of T cells consistent with a skewed X-inactivation profile, but seemed to be low on all cells. These results suggest that measurement of CD40L expression is not likely to be a reliable method of carrier detection.

In all these experiments, staining with 5c8 consistently gave the same result as staining with CD40Fc, raising the possibility that 5c8 was recognizing a determinant on CD40L within or close to the CD40 binding site. This possibility was confirmed by blocking experiments in which preincubation of activated T cells with 5c8 completely inhibited subsequent binding of CD40Fc (Fig. 2).

Expression of CD40L on activated T cells from sporadic cases of boys with HIM

Boys presenting with HIM without a family history may be sporadic cases of either X-linked or autosomal forms of the disease. We have examined activated T cells from five sporadic HIM patients and their mothers for CD40L expression. Typical results from two of these patients and their mothers are shown in Figure 3. Expression of CD40L in all sporadic HIM patients determined with CD40Fc, 5c8, and anti-TRAP was either of type I (negative for all three) (e.g., BS in Fig. 3) or type II (negative for CD40Fc and 5c8 and positive for anti-TRAP) (e.g., GP in Fig. 3) described above for HIGM1. Activated T cells from the mothers of each patient displayed normal CD40L expression. Our results are consistent with the sporadic HIM boys in our series being HIGM1 and not autosomal. To date, single strand conformational polymorphism mutational analysis and sequencing of the CD40L gene has shown mutations in the open reading frame of the CD40L gene of two patients with sporadic HIM. In one (CS), a 7-bp deletion at nucleotides 172–178 gave rise to a stop codon and truncation after amino acid 124. The other (BS) had an insertion at nucleotide 455 giving rise to a stop codon and truncation after amino acid 141. A third patient

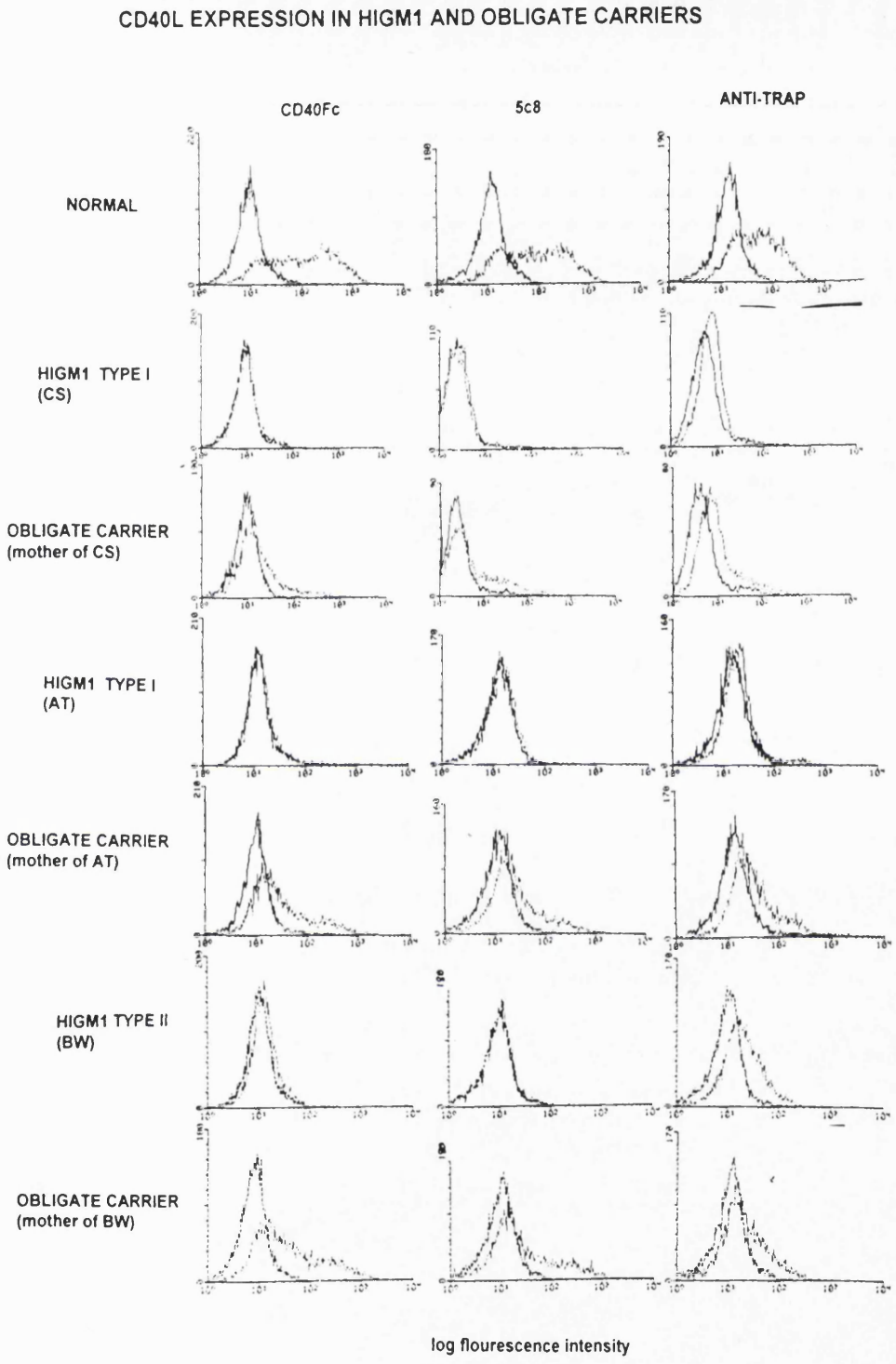

FIGURE 1. CD40L expression on activated T cells from HIGM1 patients and obligate carriers. E^+ (T) cells from patients with HIGM1 and obligate carriers were activated with PMA plus ionomycin for 16 h and then examined for CD40L expression. T cells from normal laboratory volunteers were used as controls. Examples are given for both Type I expression (CS and AT) showing no staining with CD40Fc, 5c8, or anti-TRAP, and Type II expression (BW) with no staining by CD40Fc and 5c8, but positive staining by anti-TRAP. T cells from obligate carriers (mothers of CS, AT, and BW) showed variable staining with all three reagents from low to normal.

had no detectable mutation in the ORF.[4] No CD40L was detected on activated T cells from any of these three patients, and it is not clear yet whether the third patient with no mutation in the open reading frame has a mutation in a regulatory sequence or the lack of CD40L expression is a result of some other defect. Until this question is resolved,

[4]Mutation in exon 5 has now been found in this patient.

5c8 INHIBITS BINDING OF CD40Fc TO ACTIVATED T CELLS

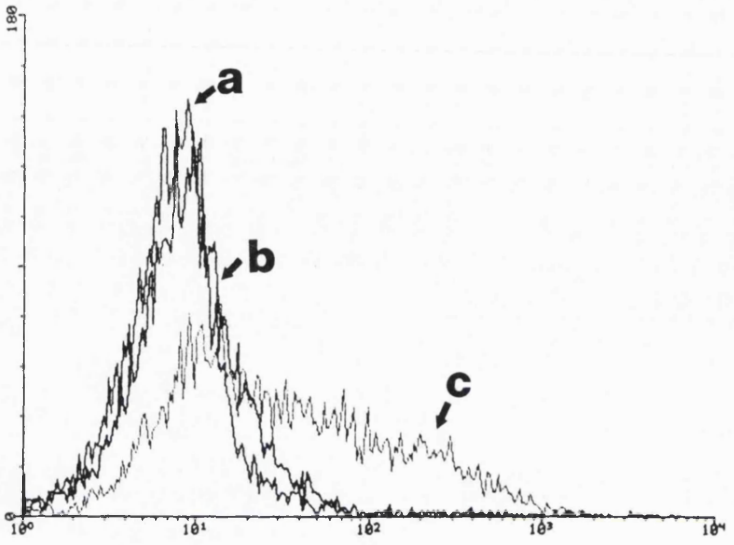

FIGURE 2. Inhibition of CD40Fc binding to CD40L on activated T cells by 5c8 mAb. E^+ (T) cells from normal volunteers were activated with PMA plus ionomycin for 16 h and then examined on the FACS after staining with CD40Fc (c) or CD40Fc after previous incubation with 5c8 mAb (b). The control (a) was obtained by staining with mouse IL-4RFc fusion protein which does not bind to human B cells.

staining of activated T cells with CD40Fc or 5c8 cannot be used unequivocally to distinguish between X-linked and other forms of HIM.

CD40L expression in non-X-linked and autosomal forms of HIM

In a recent survey of the literature on immunodeficiency with hyper-IgM (HIM), 15 of 67 patients (22%) were female and therefore not X-linked (3). An autosomal recessive inheritance pattern may account for some girls with HIM, and some sporadic presentations by boys. Moreover, pedigrees reported by Beall et al. (8) and Brahmi et al. (9) are consistent with autosomal dominant inheritance. We have investigated three girls with HIM, and one boy with an affected sister from a consanguineous marriage consistent with autosomal recessive inheritance. In each case, CD40L was detected on activated T cells with CD40Fc, 5c8, and anti-TRAP (Figs. 4 and 5). In replicate experiments, expression of CD40L on T cells from two of the girls investigated was not different from nomal controls, and was slightly low in the other one. An example of one such experiment is shown in Figure 4. Activated T cells from the boy with an autosomal form of HIM consistently showed weak expression of CD40L compared with normal expression by T cells from his father (Fig. 5). This patient was subsequently shown to be class II deficient and to have low numbers of $CD4^+$ T cells. CD40 expression on B cells from all these patients was also examined by staining with CD40 Ab (G28.5) and found to be normal (data not shown).

B cell proliferation in response to stimulation by CD40L

In previous studies, HIGM1 B cells were shown to proliferate in response to CD40 Abs and CD40L in soluble form or expressed on CV1/EBNA cells, and to make IgE upon costimulation with anti-CD40 or CD40L plus IL-4 (reviewed in 1). It is not known, however, whether B cells from non-X-linked forms of the disease can also respond to CD40L. To elucidate this point, we investigated the proliferative response of T-depleted B (E^-) cells from HIM patients to CD40L with and without IL-2. In these experiments, B cells from normal donors, HIGM1 patients, and obligate carriers, as well as autosomal, non-X-linked (females), and sporadic males with HIM all proliferated when stimulated with CD40L on CV1/EBNA cells. Typical examples of these responses are shown in Table II. Addition of IL-2 did not usually stimulate significant proliferation by itself, and did not have much effect on the response to CD40L. There was no significant proliferation in control cultures with medium alone or CV1/EBNA cells transfected with empty vector (CV1/empty vector). B cells from all patients proliferated in response to anti-IgM plus IL-4 although to a lesser degree than with CD40L.

Ab secretion by HIM B cells

The ability of HIM B cells to make Ab was assessed by stimulation with CD40L plus IL-2 or IL-10. Costimulation of normal B cells with CD40L and IL-2 induced polyclonal Ig secretion of all major classes except for IgE (typical examples are shown in Tables III and IV). In other experiments with normal B cells, CD40L plus IL-2 was

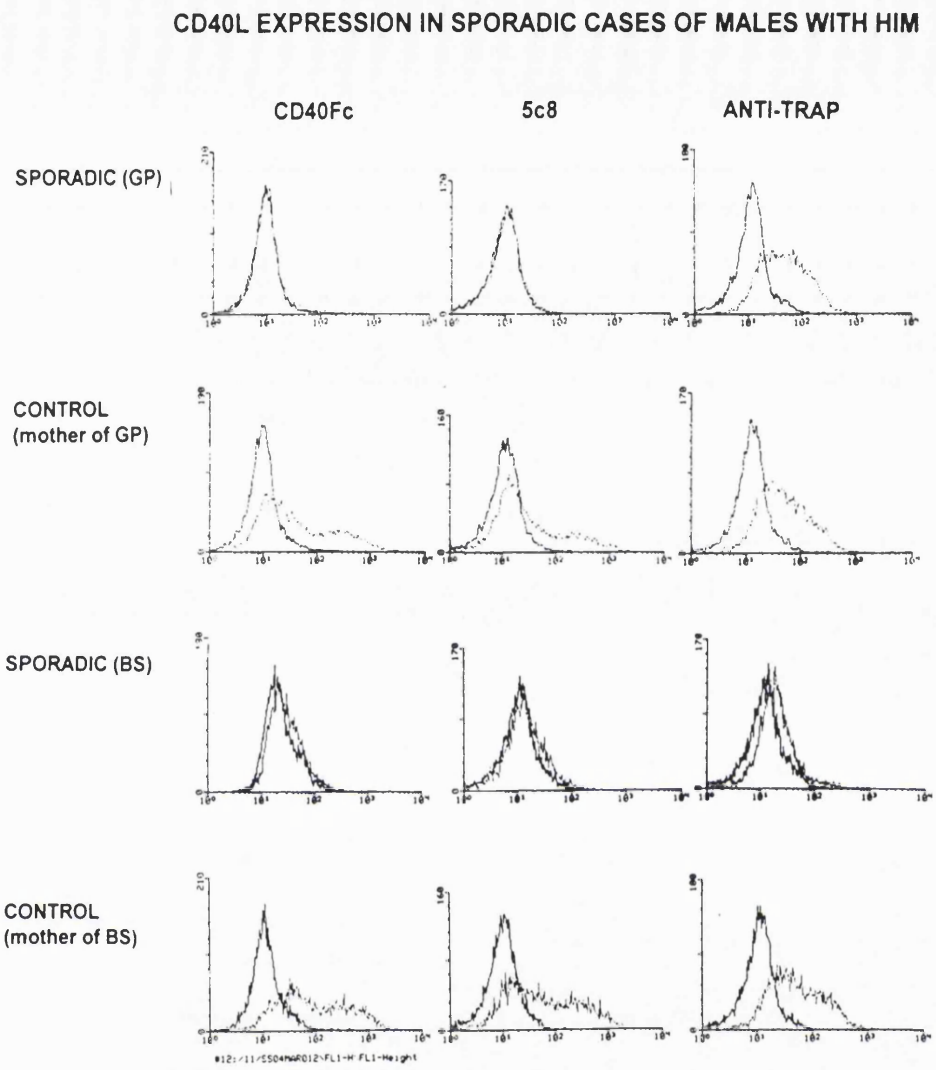

FIGURE 3. CD40L expression on activated T cells from sporadic males with HIM. E^+ (T) cells from male patients diagnosed as having hyper-IgM syndrome, but without any family history, were activated with PMA plus ionomycin for 16 h and then examined for CD40L expression. T cells from their mothers were used as controls. The examples given are for Type I expression (BS) (no staining with either CD40Fc, 5c8, or anti-TRAP) and Type II expression (GP) (negative for CD40Fc and 5c8, but positive for anti-TRAP). T cells from the mothers had normal CD40L expression

shown to stimulate production of IgG1, IgG2, IgG3, and IgG4 (data not shown). CD40L alone did not induce significant Ig secretion. Control cultures costimulated with CV1/EBNA cells transfected with empty vector plus IL-2 or IL-10 also did not produce Ab.

In contrast to the results obtained with normal B cells, little or no IgG, IgA, or IgE secretion was obtained when B cells from HIGM1, sporadic HIM, or non-X-linked HIM patients were stimulated with CD40L plus IL-2. On the other hand, B cells from obligate carriers responded similarly to normal B cells. Typical examples of each are shown in Table III. Interestingly, IgM responses (but not IgG, IgA, or IgE) were obtained with B cells from the non-X-linked (female) patients and the autosomal male patient. These results are consistent with the presence of IgM^+ (nonswitched) B cells in non-X-linked HIM which can respond to CD40L plus IL-2 to make IgM only. In contrast, only a small amount of IgM production was obtained with B cells from HIGM1 or sporadic HIM patients.

In other studies with the use of IL-4 instead of IL-2, B cells from HIGM1 patients have been shown to make IgE (10, 12–14). In addition, stimulation with CD40L and IL-10 has been shown to induce IgG and IgA secretion (14), and IgG1 and IgG3 by IgD^+ B cells (30). These results are consistent with IL-10 induced switching of IgM^+/IgD^+ B cells (30). In our hands, B cells from normal donors responded better to CD40L plus IL-10 than CD40L plus IL-2. Moreover, B cells from two of our HIGM1 patients made IgG or IgA in response to CD40L plus IL-10 but two others did not. Typical examples of a responder (AT) and a nonresponder (BW) are shown in Table IV. In contrast, no significant IgG or IgA production by B cells from non-X-linked forms of HIM (two girls and

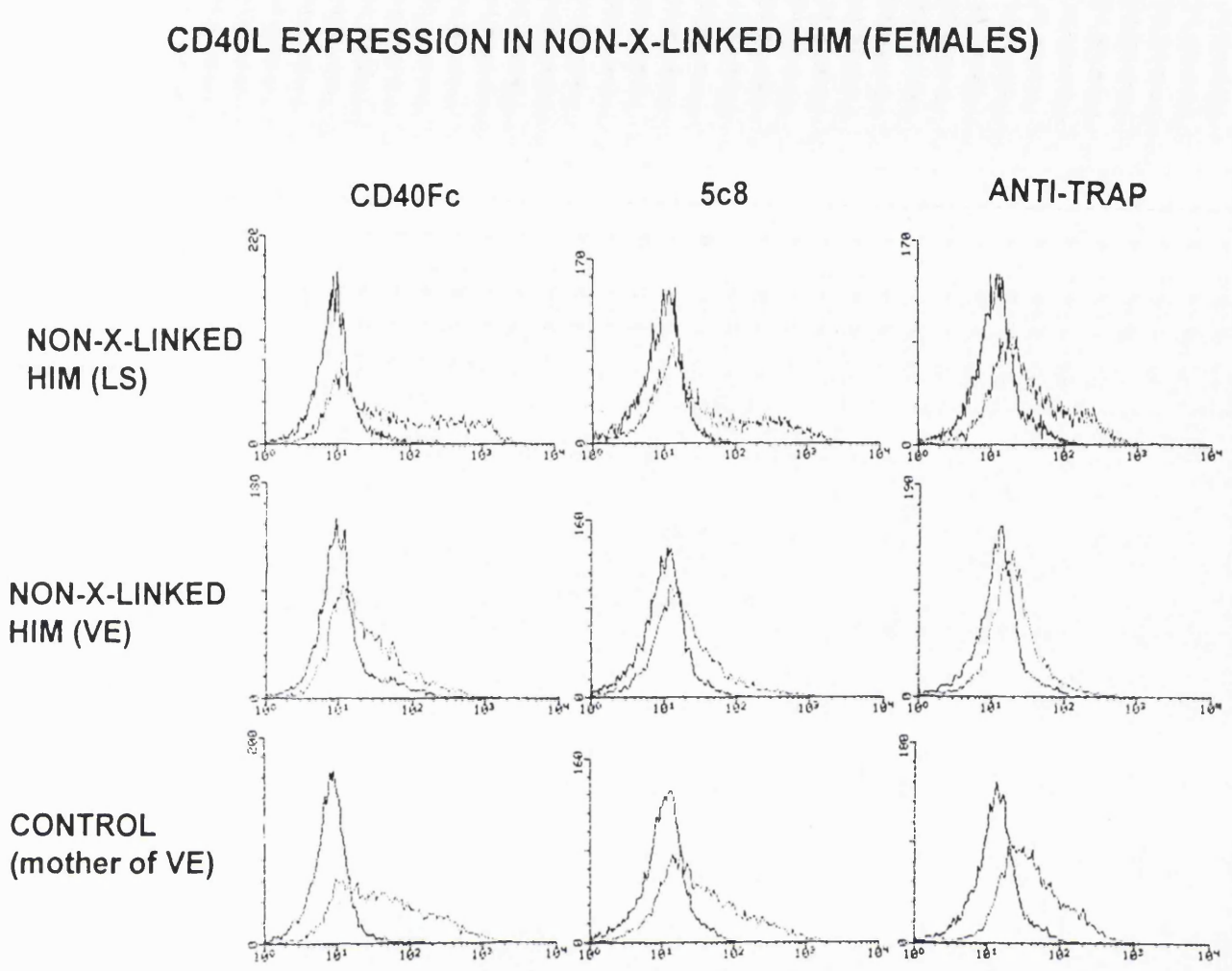

FIGURE 4. CD40L expression on activated T cells from girls with a non-X-linked form of HIM. E^+ (T) cells from girls with HIM were activated with PMA plus ionomycin for 16 h and then examined for CD40L expression. The two examples given illustrate low expression (VE) and normal expression (LS) compared with T cells from the mother of VE as a control.

a boy with an autosomal recessive form of HIM) was obtained in any experiment. We also examined IgE secretion by B cells from two non-X-linked HIM patients in response to costimulation with CD40L and IL-4. In these experiments, B cells from one patient (LS) made no detectable IgE, but a limited response was obtained with B cells from the other (VE) (Table V). Together, these results suggest that there may be an intrinsic B cell defect in these patients that prevents or inhibits heavy chain switching.

Discussion

Immunodeficiency with normal or elevated levels of IgM (HIM) is not just one disease (3). It can occur as an inherited (primary) or acquired disorder induced by environmental insults such as congenital rubella and malignancy. Primary HIM is rare among the inherited immunodeficiencies and includes a clearly defined X-linked form (3, 4), and less well defined autosomal dominant and recessive forms (5–9). Last year, five papers appeared within a few weeks of each other, each describing mutations in the gene on the X-chromosome coding for CD40L in patients with the X-linked form of the disease (HIGM1) (1, 10–14). The effect of these mutations on expression of CD40L on activated T cells was examined by staining either with a CD40 human IgG1 (or IgM) Fc fusion protein (CD40Fc) (10–14), or with a polyclonal Ab raised against a CD40L peptide fusion protein (anti-TRAP) (14). In all HIGM1 patients examined, staining with CD40Fc was extremely low or absent showing that the mutation had deleted or altered the CD40 binding site. In contrast, anti-TRAP staining was normal on T cells from two of three patients tested (14) suggesting that the mutated CD40L can be expressed on the activated T cell surface membrane.

We have confirmed that CD40L expression on activated T cells from HIGM1 patients is of two types (Fig. 1). In type I, no CD40L expression could be detected with CD40Fc, 5c8, or anti-TRAP. In some patients, however, expression of CD40L on activated T cells could be detected with anti-TRAP, but not with either CD40Fc or 5c8

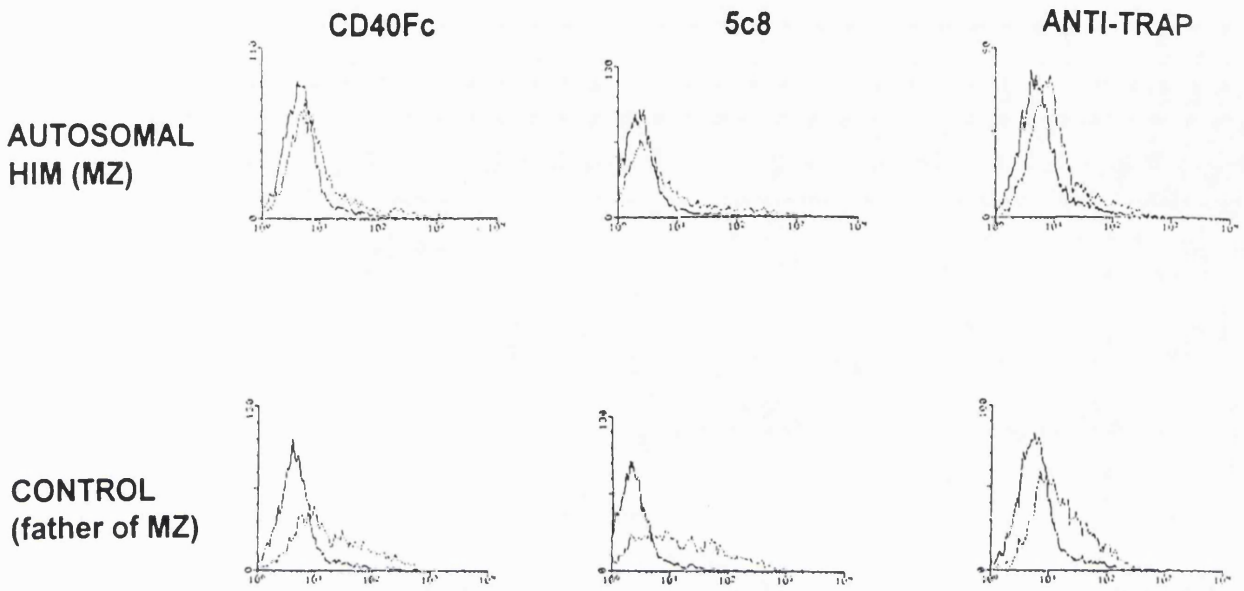

FIGURE 5. CD40L expression on activated T cells from a boy with an autosomal recessive HIM. E^+ (T) cells from a boy of consanguineous parents whose sister had died of hypogammaglobulinemia with preserved IgM production suggesting autosomal recessive inheritance were activated with PMA plus ionomycin for 16 h and then examined for CD40L expression. T cells from this patient had weak expression of CD40L compared with T cells from his father as a control.

Table II. *CD40L-induced proliferation of B cells from HIM patients*[a]

	Proliferation (dpm)					
	Normal (LH)	HIGM1 (BW)	Obligate carrier (C1)	Autosomal (FZ)	Non-X-linked (LS)	Sporadic (GP)
CV1/EBNA empty vector	638	1063	6814	300	293	874
CV1/EBNA CD40L	20107	16089	45318	6242	38352	5144
IL-2	5459	1422	10164	2496	732	1551
CV1/EBNA CD40L + IL2	25301	22430	43775	13458	41901	6799
anti-IgM + IL-4	10671	9116	6258	51392	25648	12649

[a] n.b. range of responses to CD40L by eight different normal controls was 5 to 45 × 10^3 dpm.

(type II). Activated T cells from five sporadic cases of boys with HIM also stained with one or the other of these two patterns suggesting that they were all of the X-linked form (HIGM1). Three sporadic patients have been examined so far for mutations in the CD40L gene. Two of these had mutations in the open reading frame, but no mutation has been found in the third patient. Whether a mutation in noncoding sequences of the CD40L gene is preventing expression or the defect lies elsewhere has not yet been determined.[4] Until this question is resolved, expression of CD40L on activated T cells may not be a reliable means for identifying sporadic cases of HIM as HIGM1.

One important question that has not been addressed in earlier studies of HIGM1 is whether CD40L expression on activated T cells can be used as a means of carrier detection. In one published study, polymorphic micro satellite repeat sequences in the 3′-untranslated region of the CD40L gene allowed T cells expressing the normal and mutant CD40L in an obligate carrier to be identified. Activated T cells from this carrier were separated into $CD40L^+$ and $CD40L^-$ populations by staining with CD40Fc. The normal allele was expressed only in $CD40L^+$ cells and the mutant allele in $CD40L^-$ cells (11). This finding is consistent with random X-chromosome inactivation in T cells of obligate HIGM1 carriers reported by Hendriks (31).

In our experiments, CD40Fc, 5c8, and anti-TRAP staining of activated T cells from obligate carriers was variable. In some cases, CD40L expression seemed not to be reduced at all whereas in other cases expression was much lower than expected from a pattern of random X-inactivation (Fig. 1). The variability may be a result of the statistical nature of X-chromosome inactivation or of the mutant allele affecting

Table V. *IgE secretion by B cells from non-X-linked HIM patients stimulated with CD40L and IL-4*

Stimulus	IgE Secretion (ng/ml)		
	Control	Patient LS	Patient VE
Medium	1 ± 0.2	<1	<1
CV1/EBNA/CD40L	2 ± 0.2	<1	<1
CV1/EBNA/empty vector	1 ± 0.3	<1	<1
IL-4	2 ± 2	<1	<1
IL-4 + CV1/EBNA/CD40L	20 ± 6	<1	9 ± 1
IL-4 + CV1/EBNA/empty vector	2 ± 2	<1	<1

active X-chromosome. The very low expression of CD40L on activated T cells from some carriers may be explained by this mechanism.

Expression of CD40L on activated T cells from three girls with HIM, and one boy with an affected sister from a consanguineous marriage consistent with autosomal recessive inheritance was also examined (Figs. 4 and 5). Expression of CD40L on activated T cells from the boy with autosomal recessive HIM was consistently low compared with normal controls and the patient's father. This patient was subsequently shown to be class II deficient and have low numbers of recirculating $CD4^+$ T cells. As CD40L is expressed predominantly on $CD4^+$ and not $CD8^+$ T cells (33), the low expression of CD40L can be explained by the loss of $CD4^+$ cells. In addition, his immunodeficiency is probably a result of the class II deficiency and unrelated to CD40L expression. Of the three girls with non-X-linked HIM, CD40L expression was normal in two and slightly reduced compared with normal controls in one. $CD4^+$ T cells and class II expression in these patients was normal. These results show that the immunologic defect in non-X-linked HIM cannot readily be explained by an inability to express CD40L. In this regard, our female patients seem to be different from the subset of common variable immunodeficiency patients with defective CD40L expression described by Farrington et al. (34) and is consistent with our diagnosis of HIM rather than common variable immunodeficiency. It is not known, however, whether T cells from these patients will express CD40L in response to the normal in vivo T cell activation signals. These signals have not yet been identified, but are likely to be ligation of the TCR and a second activation signal such as CTLA-4/CD28 binding to B7 on APCs. Examination of the B cells in each case of non-X-linked HIM revealed normal CD40 expression (data not shown). To date, the nature of the defect in non-X-linked HIM remains unknown. The ability to detect CD40L expression on activated T cells from all of our patients with non-X-linked HIM means that CD40L expression may be used to distinguish them from patients with the X-linked form of the disease. This is likely to be important in sporadic cases of HIM with no family history. Indeed, our analysis of five sporadic cases of boys with HIM revealed staining patterns consistent with both type I and type II HIGM1 (Fig. 1) which were quite distinct from all examples of non-X-linked HIM.

Activation with CD40 Abs or CD40L has been shown to induce proliferation in HIGM1 B cells (10, 13), indicating that B cell function in these patients may be normal. Our results confirm these earlier findings and show similar proliferative responses for B cells from HIGM1, sporadic, and non-X-linked forms of HIM (Table II). Previous studies have also shown that HIGM1 B cells activated with anti-CD40 or CD40L can be induced to secrete IgE by the addition of IL-4 (10, 12–14), or to secrete IgG and IgA by the addition of IL-10 (14, 30). In these experiments, the cytokines (IL-4 and IL-10) have been supposed to induce switching of the IgM^+ cells present in the patient's blood. We investigated the ability of B cells from our patients to make Ab by costimulation with CD40L and either IL-2 as a B cell differentiation factor without switching activity, or IL-10 to induce switching of IgM^+ cells to IgG and/or IgA secretion (30, 35). In our experiments, B cells from normal donors and B cells from obligate carriers made IgM, IgG, and IgA, but not IgE in response to CD40L plus IL-2 (Table III). These results show that pre-existing switched B cells in normal donors and in obligate carriers can be activated by CD40L plus IL-2 to secrete Ig of all classes except IgE. In contrast, costimulation of B cells from HIGM1, sporadic HIM, or non-X-linked forms of HIM with CD40L plus IL-2 failed to result in IgG, IgA, or IgE secretion. Interestingly, IgM was only marginally increased in cultures of HIGM1 B cells whereas cultures of B cells from non-X-linked forms of HIM markedly increased IgM secretion (Table III). The inability of B cells from HIGM1 patients or patients with non-X-linked forms of HIM to make Ab of any isotype except IgM is consistent with the absence of switched cells in these patients.

The situation with IL-10 was more complicated. In our experiments, B cells from all normal donors made IgM, IgG, and IgA but not IgE in response to CD40L plus IL-10. Similarly, B cells from some but not all of our HIGM1 patients secreted IgG or IgA in response to CD40L plus IL-10 (Table IV). An example of a responder (AT) and nonresponder (BW) is given in Table IV. These results are similar to the reported findings of IgG and IgA secretion by HIGM1 B cells stimulated with CD40 Ab plus staphylococcus aureus Cowan I and IL-10 (14). We have not found responses by HIGM1 B cells to IL-10 as consistently as others. The reasons for this are unclear, but could simply be a result of different patient populations and/or the use of staphylococcus aureus Cowan I plus IL-10 rather than just IL-10 in our study. IL-10 has been shown to induce B cells to make IgA (35) and IgG (14, 36). It has also been shown to stimulate IgD^+ B cells to make IgG1 and IgG3 (30) consistent with heavy chain switching, and it is likely that IL-10 is switching IgM^+ B cells from HIGM1 patients to make IgG and IgA. However, the presence of switched memory B cells in lymphoid tissues of HIGM1 patients has not yet been formally excluded, and

it is possible that IL-10 is not acting as a switch factor but is inducing Ig secretion by already committed cells. Our finding that B cells from some HIGM1 patients do not make IgG or IgA in response to CD40L plus IL-10 may be a result of the absence of already switched B cells in these particular patients. Experiments are at present being undertaken to elucidate this point.

In contrast to our results and those of others with B cells from HIGM1 patients, we were unable to show IgG or IgA production by B cells from patients with the non-X-linked form of the disease stimulated with CD40L and IL-10 (Table III and IV). Moreover, IgE production on costimulation with CD40L and IL-4 was consistently absent in one of two patients tested, and reduced compared with a normal control in the other (Table V). The nature of the defect in the non-X-linked HIM is not yet known. Activated T cells from these patients express CD40L detected with CD40Fc, 5c8, and anti-TRAP (Fig. 4), and B cells express apparently normal amounts of CD40 (data not shown). Our results are consistent with the existence of an intrinsic B cell defect in non-X-linked HIM which renders them unresponsive to another signal required for switching, although a more complex defect involving T cells cannot be formally excluded at this stage. These aspects are currently under investigation.

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CD40 cross-linking inhibits specific antibody production by human B cells

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Keywords: B cell, CD40, CD40L, differentiation, IL-2, proliferation, T cell replacing factor

Abstract

Ligation of CD40 on B cells is a co-stimulatory signal for proliferation, antibody secretion, heavy chain switching and rescue from apoptosis after somatic mutation in the germinal centre. The importance of these manifold responses to CD40 activation for humoral immunity is exemplified by the inability of boys with X-linked hyper IgM syndrome to make IgG, IgE or IgA due to a mutation in in the gene coding for CD40 ligand (CD40L). In the present study, we have investigated the effect of CD40 ligation on specific antibody production by human B cells to influenza virus. The antibody response was T cell dependent and specific for the strain of influenza virus used as antigen. Addition of either CD40 mAb or recombinant trimeric CD40L profoundly inhibited specific antibody production. Antibody production by unseparated tonsillar mononuclear cells and by T-depleted B cells stimulated with antigen in the presence of T cell replacing factor were equally inhibited with CD40 antibody showing that the effect was due to ligation of CD40 on B cells rather than blocking of T cell help. The specific antibody detected in these experiments was mostly IgG with little or no IgM and was obtained from surface IgM B cells consistent with activation of a secondary (memory) response. Co-stimulation of tonsillar B cells with CD40 antibody and anti-IgG induced proliferation of IgG^+ B cells. These results suggest that CD40 ligation can inhibit specific antibody responses and stimulate proliferation in the same IgG^+ (memory) B cell subpopulation. Addition of CD40 antibody during the first 24–48 h of the response was required for inhibition, suggesting that the effect was on early B cell activation and/or proliferation required for antibody production. There was no correlation, however, between the ability of CD40 mAb to stimulate proliferation and inhibit antibody production. We suggest that early activation of CD40 in the specific antibody response inhibits the formation of plasma cells and promotes instead the generation of memory cells.

Introduction

T cell help in antibody responses to thymus-dependent antigens is mediated by cytokines and cell surface receptor–ligand interactions. Together, these signals determine B cell responses including proliferation, antibody production and isotype switching. Of the cell surface molecules involved, CD40 ligand (CD40L) expressed on activated T cells binding to CD40 on B cells is of critical importance (1). CD40 is a 245 amino acid type I integral membrane glycoprotein belonging to the tumour necrosis factor/nerve growth factor receptor superfamily (2,3). Ligation of B cell surface CD40 with antibody or recombinant CD40L has been shown to activate B cells inducing LFA-1-dependent homotypic adhesion (4). Co-stimulation with CD40 antibody and anti-IgM or IL-4 induces proliferation (5,6), and co-stimulation with CD40 mAb or CD40L plus IL-2, IL-15 or IL-10 induces Ig secretion of all Ig classes and subclasses (7–9). CD40 signalling also plays an important role in isotype switching. Whereas IL-4 alone induces germline transcription of the C_ε heavy chain gene, recombination and secretion of IgE depends on a second signal which can be provided by CD40 (10–12). Similarly, co-stimulation with CD40L and IL-10 induces switching to IgG1 and IgG3 production by $sIgD^+$ B cells (13). Antibody cross-linking of CD40 has also been shown to rescue $CD38^+$ germinal centre B cells from apoptosis after Ig V region somatic mutation and re-expression of surface Ig during affinity maturation (14,15). Further stimulation of germinal centre B cells by CD40 in the presence of IL-2 and IL-10 promotes the generation of $CD38^-/CD20^+$ memory B cells rather than $CD38^+/CD20^-$ plasma cells (16).

The crucial importance of CD40–CD40L interactions during

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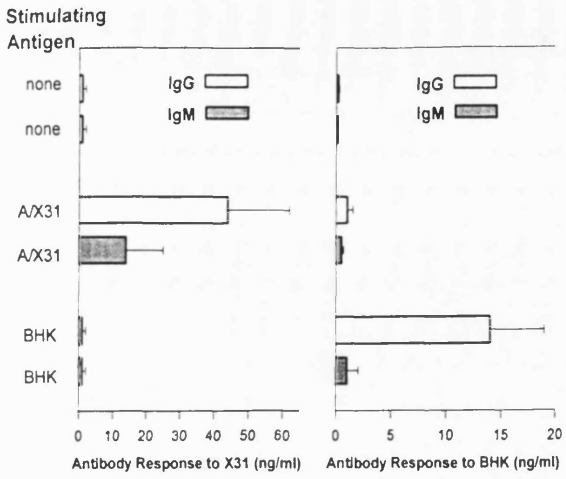

Fig. 1. Specific antibody production to influenza virus. TMC were stimulated with either influenza virus strain A/X31 or the non-cross-reacting strain BHK. Specific IgM and IgG antibody production to both strains of virus was determined by ELISA after 7 days. Specificity of the response is shown by production of antibody only to the stimulating antigen. Each point represents the mean antibody response ± SEM from triplicate cultures.

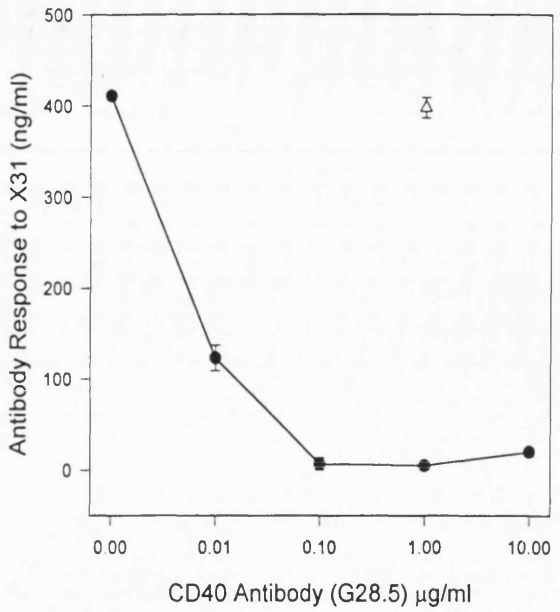

Fig. 2. Inhibition of specific antibody production to influenza virus by CD40 mAb (G28.5). TMC were stimulated with influenza virus A/X31 in the presence of G28.5 at concentrations between 0 and 10 µg/ml (●) or control IgG1 antibody at 1 µg/ml (△). Specific antibody production to A/X31 was determined by ELISA on day 7. Each point represents the mean antibody response ± SEM from triplicate cultures.

This reciprocity between two non-cross-reacting antigens is essential to establish specificity of the response.

CD40 cross-linking inhibits specific antibody production

To investigate the effect of CD40 cross-linking on the specific antibody response, TMC were stimulated with A/X31 in the presence of CD40 mAb G28.5 added at the initiation of the culture at concentrations between 0.1 and 10 µg/ml. Specific antibody was measured on day 7 and compared with antibody production by TMC cultured with A/X31 without addition of G28.5. As shown in Fig. 2, specific antibody production was significantly inhibited by G28.5 at 0.01 µg/ml and was reduced to background with concentrations of G28.5 of >0.1 µg/ml. A control IgG1 mouse mAb had no effect. In several similar experiments, optimal inhibition was always obtained at concentrations of G28.5 between 0.1 and 1 µg/ml. In all other experiments, a concentration of 1 µg/ml was used. Inhibition of the antibody response was maintained beyond the 7 days required for optimal antibody production with no recovery of the response (data not given).

CD40 antibody inhibits specific antibody production by purified B cells

In the above experiments, antibody production by unseparated TMC was inhibited by CD40 antibody. In order to establish that the effect was due to direct binding of the antibody to B cells, it was necessary to remove T cells and reconstitute $CD4^+$ T_h function with a TRF. Of all the recombinant cytokines tested to date, only IL-2 (29,30) and IL-15 (Jones *et al.*, manuscript in preparation) can restore T_h cell function in the antibody response to influenza virus. As shown in Fig. 3, depletion of T cells to <1% completely abrogated the antibody response which could be restored with IL-2. The response obtained with IL-2 used as a TRF

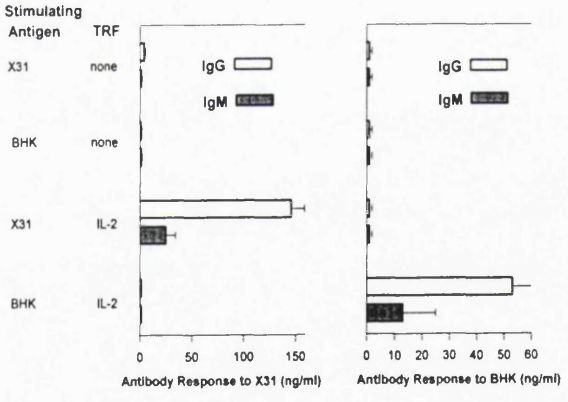

Fig. 3. T cell help in specific antibody responses to influenza virus can be replaced by IL-2. Tonsillar B lymphocytes thoroughly depleted of T cells (<1%) were stimulated with either influenza virus A/X31 or the non-cross-reacting strain BHK in medium or with 5 units/ml of IL-2 as a TRF. Specific antibody production to both strains of virus was determined by ELISA on day 7. Antibody production was restored in the presence of TRF (IL-2) and was specific for the stimulating strain of virus. Each point represents the mean antibody response ± SEM from triplicate cultures.

was also predominantly IgG and antigen specific shown by antibody production only to the stimulating antigen (A/X31 and BHK).

To examine the effect of CD40 cross-linking on specific antibody production by B cells in the absence of T cells,

Table 1. CD40 antibody (G28.5) inhibits specific antibody production by T depleted B cells cultured with antigen and TRF

T cell help	CD40 antibody	Antibody response (ng/ml)	
		Experiment 1	Experiment 2
None	none	<1	<1
T cells	none	600 ± 49	242 ± 76
TRF (IL-2)	none	494 ± 28	168 ± 24
TRF (IL-2)	G28.5 (1 μg/ml)	22 ± 3	30 ± 8

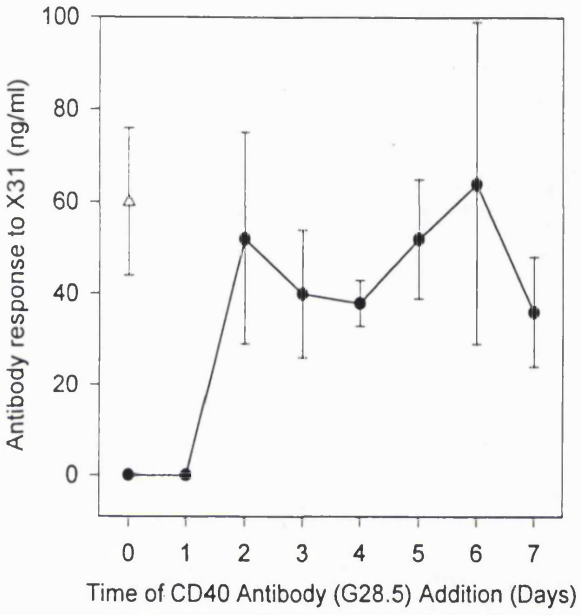

Fig. 4. Effect of time of addition of CD40 mAb (G28.5) on specific antibody production. TMC were stimulated with influenza virus strain A/X31 and G28.5 antibody at 1 μg/ml added at different times from day 0 to day 7 after the initiation of the culture (●). Response in the presence of a control IgG1 mAb (△). Specific antibody production was measured by ELISA on day 7. Each point represents the mean antibody response ± SEM from quadruplicate cultures.

purified TMC B cells were cultured with antigen (A/X31) and TRF (rhIL-2 at 5 units/ml) (Table 1). In these experiments, IL-2 at 5 units/ml fully restored specific antibody production by T-depleted B cells. Addition of G28.5 at 1 μg/ml significantly inhibited this response showing that the effect was due to direct binding of CD40 antibody to B cells.

Time-response of CD40 antibody inhibition

To determine whether inhibition by CD40 ligation was an early or late event, TMC were cultured with antigen (A/X31) and CD40 antibody (G28.5) added at 24 h intervals from the initiation of the culture until the cells were harvested at day 7. In these experiments, complete inhibition was obtained when G28.5 was added at time 0 or after 1 day in culture (Fig. 4). Antibody added at day 2 or any other time after day 2 had no effect and the antibody response was not significantly different from control cultures with no added G28.5. This result suggests that inhibition by CD40 cross-linking occurred

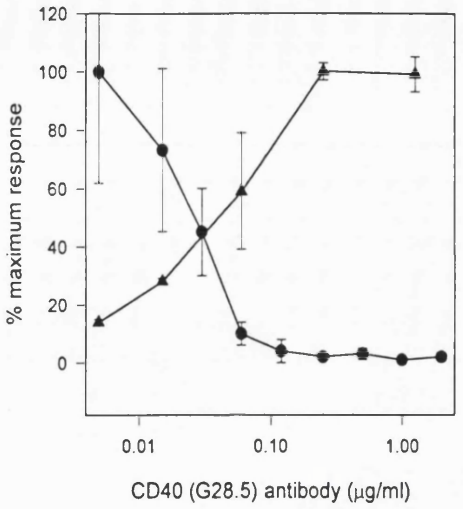

Fig. 5. Dose-response curves of CD40 mAb (G28.5) on specific antibody response and on proliferation. For the specific antibody response (●), TMC were stimulated with influenza virus in the presence of G28.5 at different concentrations from 0 and 1.5 μg/ml. Specific antibody production was determined by ELISA on day 7. For proliferation (▲), tonsillar B cells from the same TMC preparation were co-stimulated with anti-IgM and G28.5. Proliferation ([^{3}H]thymidine uptake) was determined after 4 days. Each point represents the mean ± SEM from triplicate cultures.

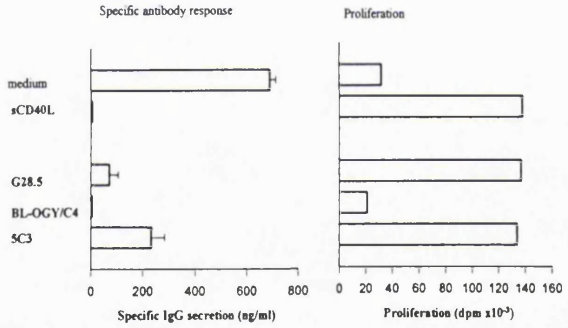

Fig. 6. Comparison of three different CD40 antibodies for their ability to induce B cell proliferation on co-stimulation with anti-IgM and inhibit specific antibody production. Inhibition of the specific antibody response was obtained by addition of 1 μg/ml of each of the CD40 antibodies to TMC cultured with influenza virus. Antibody production was measured on day 7 and expressed as the mean response of triplicate cultures ± SEM. Control cultures had no addition (medium) or sCD40L. For proliferation, B cells were cultured with anti-IgM and CD40 mAb at 1 μg/ml. Co-stimulation with sCD40L was used as a control. Proliferation was determined after 4 days and expressed as d.p.m. from triplicate cultures ± SEM.

during early B cell activation and/or proliferation rather than later in the response during B cell differentiation and antibody secretion.

Dose-response of CD40 antibody-induced proliferation and inhibition of antibody secretion

One possible explanation for the inhibition of antibody production is that proliferation induced by CD40 ligation may exclude differentiation into antibody secreting cells. In this case, the

Table 2. Effect of G28.5 on antibody responses by IgM^+ and IgM^- B cells

Antigen	CD40 mAb	Antibody response (ng/ml)					
		Experiment 1			Experiment 2		
		IgM^- B cells	IgM^+ B cells	Unseparated	IgM^- B cells	IgM^+ B cells	Unseparated
None	none	<1	<1	1 ± 1	<1	<1	<1
A/X31	none	138 ± 98	2 ± 1	36 ± 10	38 ± 11	<1	44 ± 11
A/X31	1 μg/ml	10 ± 2	5 ± 3	6 ± 1	5 ± 3	<1	4 ± 4

dose-response for G28.5-induced proliferation should be the same as the dose-response for inhibition of specific antibody production. To test this, G28.5 dose-response experiments for inhibition of antibody responses to A/X31 and co-stimulation assays with anti-IgM for proliferation were set up in parallel using B cells from the same TMC preparations. The results from these experiments (Fig. 5) show that optimal inhibition of antibody secretion is obtained with approximately the same concentration of G28.5 (0.1–1 μg/ml) that give optimal proliferation by co-stimulation with anti-IgM. Similar results were obtained on co-stimulation with anti-IgG.

Comparison of proliferation and inhibition of specific antibody production by a panel of different CD40 mAb

The possibility that inhibition of the specific antibody response was due to CD40 stimulation of B cell proliferation was explored further by comparing the activities shown by different antibodies from the CD40 panel submitted to the Fifth International Workshop of Leukocyte Differentiation Antigens. The results from one such experiment are shown in Fig. 6. In this experiment, the CD40 mAb BL-OGY/C4 profoundly inhibited the specific antibody response but was very poor at stimulating B cell proliferation. In contrast, the antibody 5C3 was good at stimulating B cell proliferation but rather poor at inhibiting the specific antibody response. Other antibodies such as G28.5 were good stimulators of B cell proliferation and inhibitors of the antibody response. The lack of correlation between the two biological activities exhibited by different antibodies from the CD40 panel suggests that inhibition of the antibody response is not simply a matter of proliferation versus differentiation.

Effect of CD40 antibody on IgM^+ and IgM^- B cells

Specific antibody responses to influenza virus are predominantly IgG suggesting that IgG^+ (IgM^-) B cells rather than IgM^+ B cells are being triggered in this response. In contrast, proliferation assays are commonly undertaken by co-stimulation with CD40 antibody and anti-IgM. This raises the possibility that CD40 cross-linking may have different effects on IgM^+ and IgM^- B cell subpopulations. Two different sets of experiments were undertaken to investigate this possibility. First, the effect of G28.5 on specific antibody production by IgM^+ and IgM^- (which includes IgG^+ B cells) B cell subpopulations cultured with T cells and antigen was determined. The results from two such experiments are shown in Table 2. As expected, specific antibody production was obtained predominantly from IgM^- B cells. In fact, antibody production by this population was generally significantly greater than from unseparated B cells which was composed mostly of unresponsive IgM^+ B cells (data not shown). Addition of G28.5 antibody inhibited antibody production by both unseparated B cells and the IgM^- subpopulation but had no observable effect on the very low response by IgM^+ B cells. Because of the very low response by IgM^+ B cells, it is not possible to say whether CD40 ligation was unable to inhibit antibody production by IgM^+ B cells or this population does not make an antibody response to be inhibited.

Table 3. Co-stimulation of B cells with G28.5 and anti-IgM or anti-IgG shows that CD40 stimulates both IgG^+ and IgM^+ B cells

CD40 antibody	Anti-Ig	Proliferation (d.p.m.)	
		Experiment 1	Experiment 2
None	none	3840 ± 1585	666 ± 150
G28.5 1 μg/ml	none	14,540 ± 2729	2471 ± 1502
None	anti-IgM	14,236 ± 788	820 ± 154
G28.5 1 μg/ml	anti-IgM	141,917 ± 10,172	24,875 ± 1013
None	anti-IgG	16,211 ± 340	2376 ± 141
G28.5 1 μg/ml	anti-IgG	59,144 ± 3923	16,215 ± 468

In a second set of experiments, proliferation of tonsillar B cells co-stimulated with G28.5 and either anti-IgM or anti-IgG was investigated. The results of two such experiments given in Table 3 show that proliferation was induced by co-stimulation of tonsillar B cells with either G28.5 plus anti-IgM or G28.5 plus anti-IgG. The response to G28.5 plus anti-IgG was always less than the response to G28.5 plus anti-IgM (about one-half to one-third) consistent with the lower numbers of IgG^+ B cells compared with IgM^+ B cells in the preparations of tonsillar B cells used in these experiments (data not shown). Together, these experiments show that CD40 cross-linking inhibits antibody production by IgM^- (IgG^+) B cells and stimulates proliferation by B cells within the same subpopulation.

Inhibition of specific antibody response by CD40L

Inhibition of antibody production in our experiments may have been due to CD40 cross-linking, or to secondary events mediated by Fc binding to monocytes or some other FcR^+ accessory cell. To distinguish between these alternatives, we compared the ability of CD40 antibody and a soluble trimeric form of recombinant human CD40L to inhibit the antibody response. In these experiments, even more potent inhibition

Table 4. Comparison of CD40 antibody and soluble CD40L as inhibitors of specific antibody responses

Antigen	CD40 cross-linker	Antibody response (ng/ml)	
		Experiment 1	Experiment 2
None	none	<5	<5
A/X31	none	120 ± 37	691 ± 23
A/X31	G28.5 mAb	28 ± 16	72 ± 34
A/X31	control SN (1:4)[a]	123 ± 16	871 ± 137
A/X31	CD40L (1:4)[b]	5 ± 2	<5
A/X31	CD40L (1:10)	<5	ND[c]
A/X31	CD40L (1:50)	125 ± 41	ND
A/X31	CD40L (1:100)	108 ± 41	ND

[a]Dilution of supernatant from COS cells transfected with empty vector.
[b]Dilution of supernatant from COS cells transfected with CD40L cDNA.
[c]Not done.

was obtained with soluble trimeric CD40L than with intact antibody (Table 4). As the trimeric CD40L does not include an Fc piece, this result shows that CD40 ligation rather than Fc activation of accessory cells is required for inhibition of specific antibody responses.

Discussion

A fundamental role for CD40–CD40L interactions in T cell help required for antibody production has been established in a number of experimental systems. In blocking experiments, anti-CD40L has been shown to inhibit T_h cell function *in vitro* (31) and to inhibit specific antibody responses to thymus-dependent antigens (red blood cells and keyhole limpet haemocyanin) but not thymus-independent type II antigens (TNP–Ficoll) *in vivo* (17). In addition, the very low or absent serum IgG, IgA and IgE in X-linked hyper IgM syndrome (HIGM-1) is due to mutations in the CD40L gene in affected boys (18,19). Our finding that CD40 ligation profoundly inhibits T cell-dependent specific antibody responses by human B cells show that CD40 has more complex functions than have hitherto been recognized.

Our findings conflict with those of Nonoyama *et al.* who showed that CD40 antibody and CD40L stimulated antibody production to the phage ϕX174 (20). The reasons for this discrepancy are unclear, but may be related to the antigen specificity of the response. In the work described by Nonoyama *et al.*, antibody to ϕX174 was detected in cultures of B cells stimulated with antigen and T cells or CD40 mAb and IL-10. Although antigen was required for optimal antibody production, no attempt was made to show that the response was specific for the stimulating antigen, and a polyclonal response particularly in cultures stimulated with CD40 mAb and IL-10 cannot be excluded. This is important because it is possible to elicit antibody to a specific antigen as a component of a polyclonal Ig response (32). In contrast, the antibody response to influenza virus *in vitro* is T cell dependent and antigen specific (21,23). Specificity of the response was firmly established with two non-cross reacting antigens (Figs 1 and 3) and by limiting dilution of antigen-specific T_h cells (23). Inhibition of specific antibody responses by CD40 mAb in our experiments was not simply due to blocking of CD40–CD40L interactions because CD40 mAb also inhibited the response by T-depleted B cells stimulated with antigen and TRF (Fig. 3 and Table 1). It is quite possible that CD40 cross-linking may have different effects in antigen-specific responses as shown for the antibody response to influenza virus, and for polyclonal responses known to occur on co-stimulation with CD40 mAb or CD40L and IL-10.

The specific antibody produced *in vitro* to influenza virus is a secondary (memory) response by B cells from immune donors who have serum antibodies to the strain of influenza virus used in this study. In addition, the specific antibody response *in vitro* was produced by surface IgM^- rather than $sIgM^+$ B cells (Table 2) and was predominantly IgG. On the other hand, cross-linking of CD40 on naive ($sIgD^+$) B cells is a signal for heavy chain switching (13) and CD40 rescue of $CD38^+$ B cells from apoptosis is in germinal centres formed by naive B cells after their first encounter with antigen (15,33). Similarly, CD4-induced B cell proliferation is often obtained by co-stimulation with anti-IgM (5,6). These considerations raise the possibility that CD40 ligation may stimulate proliferation of naive $sIgM^+$ B cells and inhibit antibody production by IgG^+ ($sIgM^-$) memory B cells as shown in Table 2. To address this question, we measured the B cell response to co-stimulation with CD40 antibody and anti-IgG. In these experiments, proliferation was obtained by co-stimulation of tonsillar B cells with either anti-IgM or anti-IgG and CD40 mAb. Proliferation in response to anti-IgG was less than obtained with anti-IgM, but not when compared to the relative numbers of $sIgM^+$ and $sIgG^+$ B cells in the tonsillar B cell preparations. From these experiments it was concluded that inhibition of antibody production could not be explained by different action of CD40 ligation on $sIgG^+$ ($sIgM^-$) and $sIgM^+$ ($sIgG^-$) B cell subpopulations.

Another question addressed in this study was whether inhibition was a direct effect of CD40 cross-linking on the B cell surface or mediated indirectly by mAb Fc binding to an accessory cell which itself delivered the inhibitory signal. Our finding that even more potent inhibition was obtained with a soluble trimeric form of recombinant CD40L than intact antibody argues against the involvement of an accessory cell. The trimeric form of CD40L is thought to be similar to CD40L expressed on activated T cells (34) and will cross-link more extensively than the bivalent antibody (24), but it does not have an Fc portion, and it is not thought to bind to B cell or monocyte cell membranes. From these results it is apparent that the extent of CD40 cross-linking rather than activation of an accessory cell by binding to Fc receptors is the important factor for optimal inhibition of specific antibody production. Evidence for a variable cross-linking effect has also been obtained with a panel of antibodies to different CD40 epitopes (35).

Ligation of CD40 within the first 24–48 h of the antibody response was required for inhibition (Fig. 4). Addition of CD40 mAb after this time had no effect. This suggests that CD40 inhibition occurs during early B cell activation and/or proliferation required for antibody production. In a recent study by Arpin *et al.*, CD40 ligation was shown to promote germinal centre B cell differentiation into memory B cells rather than

plasma cells (16). Our findings may therefore be explained by CD40 cross-linking early on in the antibody response (24–48 h) promoting the generation of memory rather than differentiation into antibody producing cells. Clonal expansion of rare antigen specific B cells after somatic mutation and rescue from apoptosis by CD40 in the germinal centre rather than terminal differentiation into antibody secreting cells would be desirable *in vivo* to optimize high-affinity antibody production.

Abbreviations

CD40L	CD40 ligand
TMC	tonsillar mononuclear cells
TRF	T cell replacing factor

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