In Vitro *Regulation of Growth, Differentiation and Survival of Leukemic CD5+ B Cells*

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A thesis submitted for the Degree of

DOCTOR OF PHILOSOPHY

University of Technology, Sydney

1995.

Declaration

I certify that this thesis has not already been submitted for any degree and is not being submitted as part of candidature for any degree.

I also certify that this thesis has been written by me and that any help that I received in preparing this thesis, and all sources used, have been acknowledged.

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Signature removed prior to publication.

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Acknowledgements

My first thank you goes to my supervisor, Professor Bob Raison. Bob not only allowed me to undertake my studies in his laboratory but also provided me with a great deal of his time, assistance and encouragement. Something that I am particularly grateful for was the amount of latitude Bob gave me with the direction that this project took. He always gave me enough rope, but never let me hang myself. So, many thanks Bob - it has been a pleasure and a privilege to work under your supervision.

I would also like to thank Dr. Kath Weston for being very helpful during various stages of this project, and for meticulously proof-reading this thesis. Thanks, Kath, also for the many stimulating discussions about B cells, cell culture and flow cytometry. Your support, enthusiasm and friendship have been invaluable and have contributed immensely to this work.

The Immunobiology Unit, UTS, has been a great place to work and many of its members, past and present, have been of assistance throughout the last 4 years. I would particularly like to acknowledge Grant Shoebridge who, despite being a molecular biologist, always took an interest in my project, was never afraid to ask me what was I doing and why, and was always willing to take a crash course in B cellology in order to understand my work. I would also like to thank Associate Professor Kevin Broady, Carolyn Woodlands, Nick dos Remedios and Jeanette Taverner for assistance at various times during the course of this project.

The N.S.W Cancer Council, Australasian Society for Immunology, UTS Vice-Chancellors Postgraduate travel fund and Bob all deserve thanks for

providing me with travel grants that enabled me to attend international conferences.

Outside of UTS, warm thanks go to my parents, family and friends for their continued support and interest over the years. Finally, none of this would have been possible without Karen who, for many years, has continued to be an eternal and invaluable source of patience, love, support and encouragement.

Abstract

B cell chronic lymphocytic leukemia (B-CLL) is a hematologic neoplasm characterised by the proliferation and accumulation of sIgM$^+$/D$^+$ B cells that fail to progress to the final stages of B cell development. The malignant cells in B-CLL also express the pan-T cell antigen CD5, suggesting that CLL is a malignancy of the CD5$^+$ subset of B cells. Additional characteristics of the malignant clone include a low proliferative index, enhanced *in vivo* survival and constitutive expression of the anti-apoptosis oncoprotein bcl-2. The behaviour of leukemic CD5$^+$ B cells *in vitro* contrasts their arrested *in vivo* state. That is, despite the majority of cells being arrested in the G_o phase of the cell cycle, the leukemic B cells are not irreversibly frozen as they can be induced to differentiate to Ig-secreting cells under appropriate *in vitro* conditions. Furthermore, leukemic CD5$^+$ B cells rapidly undergo death by apoptosis following *in vitro* culture. This thesis describes the requirements for *in vitro* activation of leukemic CD5$^+$ B cells, the characterisation of the events involved in apoptosis of these cells as well as the identification of various growth factors capable of modulating these events.

Stimulation of unfractionated peripheral blood lymphocytes (PBLs) from three patients with B-CLL with the phorbol ester PMA and the mitogens PHA and PWM resulted in significant increases in cell proliferation, RNA synthesis and IgM secretion when compared to unstimulated cell populations. PMA was the most potent inducer of IgM secretion and this occurred irrespective of the presence of residual T cells. PMA-induced proliferation and RNA synthesis were also independent of T cells. However, in the presence of T cells, these parameters of cellular activation were enhanced during *in vitro* culture. Thus, the inductive ability of PMA on leukemic CD5$^+$ B cells was independent of T cells. In

contrast, activation and differentiation of the leukemic CD5+ B cells into IgM-secreting cells following culture with mitogens did not occur in the absence of T cells. Interestingly, co-stimulation of leukemic CD5+ B cells with PMA and anti-Ig induced cellular responses that exceeded those induced by either activator alone. Thus, leukemic CD5+ B cells from patients with B-CLL can be activated *in vitro* and differentiate in response to stimulation via both T cell-dependent and T cell-independent mechanisms.

Apoptotic cell death was characterised in purified leukemic CD5+ B cells obtained from six B-CLL patients. All leukemic CD5+ B cell populations entered an apoptotic pathway *in vitro* as evidenced by a reduction in cell size, loss of cell viability and fragmentation of DNA into multimers of ~180 base pairs. Following 24 hours of *in vitro* culture 24.0±16% of DNA was fragmented. After 8 days, the majority of DNA was fragmented, and fewer than 10% of cultured cells were viable. Examination of bcl-2 expression in the malignant B cells by flow cytometry revealed a unimodal pattern of expression in >85% of cells from each B-CLL patient prior to culture. During *in vitro* culture, bcl-2 expression became bimodal such that the B cells displayed a bcl-2_{high} and bcl-2_{low} phenotype. The level of expression by the bcl-2_{high} cells was similar to that observed prior to *in vitro* culture, indicating that bcl-2 is down-regulated in apoptosing cells. Interestingly, despite this down-regulation, the overall number of cells positive for bcl-2 remained constant. This suggests that the enhanced survival of leukemic CD5+ B cells *in vivo* is mediated by the sustained expression of bcl-2 and that additional mechanisms exist capable of overriding the protective effect of bcl-2 when bcl-2 is present at reduced levels.

Leukemic B cell apoptosis has previously been reported to be delayed or prevented by IL-4, IFN-γ and IFN-α. These results were confirmed in this study where it was found that culture of leukemic $CD5^+$ B cells with IL-4 or IFN-γ enhanced cell viability and delayed apoptosis in 6/6 and 5/6 populations of leukemic B cells, respectively. This function was also found to be shared by IL-2, IL-6, IL-13 and TNF-α as these cytokines enhanced cell viability and delayed apoptosis in some of the cell populations examined at a level similar to that observed for IL-4 and IFN-γ. These cytokines may mediate their effect via the expression of bcl-2 as culture in the presence of IL-2, IL-4, IL-6, IL-13, IFN-γ or TNF-α resulted in a higher percentage of cells displaying the $bcl\text{-}2_{high}$ phenotype, compared to unstimulated cells. Taken together, these results suggest that autocrine and/or paracrine growth loops may play a role in the pathogenesis of B-CLL and that cytokines that prevent apoptosis *in vitro* may be targets for treatment of this B cell malignancy.

Publications arising from work presented in this thesis

Tangye SG, Weston KM and Raison RL (1995). Phorbol ester activates $CD5^+$ leukemic B cells via a T cell-independent mechanism. **Immunol. Cell Biol.** 73: 44-51.

Tangye SG and Raison RL (1995). Leukemic $CD5^+$ B cell apoptosis: co-incidence of cell death and DNA fragmentation with reduced bcl-2 expression. **Br. J. Haem.** 92: 950-953.

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Abbreviations

AET	2-aminoethyisothiouronium bromide
Ag	antigen
AML	acute myeloid leukemia
BCIP	bromochloroindolphenyl
B-CLL	B cell chronic lymphocytic leukemia
bp	base pairs
BSA	bovine serum albumin
BSF-MP6	B cell stimulatory factor derived from a T cell hybridoma
C	constant
cIg	cytoplasmic Ig
°C	degrees Cecius
CD	cluster of differentiation
CD40L	ligand for CD40
CLL	chronic lymphocytic leukemia
CD	cluster of differentiation
CIAA	chloroform isoamylalcohol
CPM	counts per minute
ELISA	enzyme-linked immunosorbant assay
EBV	Epstein Barr virus
FACS	fluorescence activated cell sorter
$F(ab')_2$ anti-μ	$F(ab')_2$ fragments of rabbit polyclonal Ig specific for human μ chains of IgM
FBS	fetal bovine serum
FcR	Fc receptor
FITC	fluorescein isothyocyanate
FSC	forward angle light scatter
H	Ig heavy chain

HCL	hairy cell leukemia
HIGM-1	hyper IgM immunodeficiency syndrome
IFN	interferon
Ig	immunoglobulin
IgA	immunoglobulin A
IgD	immunoglobulin D
IgE	immunoglobulin E
IgG	immunoglobulin G
IgM	immunoglobulin M
IL	interleukin
kDa	kilodalton
L	Ig light chain
LMW-BCGF	low molecular weight B cell growth factor
LPS	lipopolysaccharide
mAb	monoclonal antibody
MFI	mean fluorescence intensity
mins	minutes
µL	microlitre
mL	millilitre
NBT	nitroblue tetrazolium
NK	natural killer
nm	nanometre
pNPP	p-nitrophenyl phosphate
PBL	peripheral blood lymphocytes
PBS	phosphate buffered saline
PBS-BSA	0.1% BSA prepared in PBS, containing 0.1% sodium azide
PE	phycoerythrin
PHA	phytohemagglutinin

PKC	protein kinase C
PLL	prolymphocytic leukemia
PMA	phorbol myristic acetate
PMA_{low}	a suboptimal, submitogenic concentration of PMA
PMA_{opt}	optimal concentration of PMA
PWM	pokeweed mitogen
rpm	revolutions per minute
SAC	*Staphylococcus aureus* Cowan I
SCID	severe combined immunodeficiency
s.d.	standard deviation
SDS	sodium dodecyl sulfate electrophoresis
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
s.e.m.	standard error of the mean
sIg	surface immunoglobulin
SRBC	sheep red blood cells
SSC	side (90°) light scatter
ssDNA	single stranded DNA
TBS	tris buffered saline
TBS-T	tris buffered saline containing 0.05% Tween
TGF	transforming growth factor
TNF	tumor necrosis factor
TNF-R	tumor necrosis factor receptor
TPA	tetradodecanoyl phorbol acetate
U	units
V	variable
XID	x-linked immunodeficiency

Chapter 1:

Introduction

B cell development involves the differentiation of progenitor cells into Ig-secreting B cells. During this procedure, developing B cells receive signals from the surrounding microenvironment which dictate whether or not the B cells survive to undergo further development. Receipt of a death signal can initiate a cascade of events termed programmed cell death, or apoptosis, resulting in the selective loss of unwanted or potentially harmful cells. Cytokines and growth factors have been shown to be important in the regulation of B cell development, apoptosis, growth and differentiation. One of the molecules involved in regulating the survival of B cells is the product of the bcl-2 gene, an oncoprotein capable of suppressing the induction of apoptosis in different cells and cell lines.

The failure of cells to undergo apoptosis, or the deregulated expression/production of a particular cytokine, may disrupt the homeostasis of B cell development and thus give rise to B cell malignancies. Malignant B cells from patients with B cell chronic lymphocytic leukemia represent cells arrested at a fixed point of the B cell developmental pathway. Therefore, they are a convenient source to study the effect of cytokines and growth factors on the growth, survival and further differentiation of these leukemic B cells. Futhermore, interaction of cytokines with leukemic B cells may yield information pertaining to the underlying causes of these malignancies and may also offer insight into possible therapeutic regimes. This chapter describes the development, function, differentiation and survival of normal and malignant B cells.

B CELL DEVELOPMENT, FUNCTION AND DIFFERENTIATION

B Cells as Immunoglobulin-Secreting Cells

The primary role of B cells is to secrete immunoglobulin (Ig) specific for a given antigen (Ag). Each Ig molecule is a tetrameric protein, consisting of two heavy and two light chains covalently linked via disulfide bonds. Each chain contains a variable (V) and constant (C) region and the domains arising from the V and C region genes constitute the idiotypic and isotypic determinants, respectively, of the Ig molecule. Association of the heavy and light chain V domains forms the antigen-binding site of the Ig molecule while the C region bestows the Ig with specific effector functions, such as the capacity to fix complement, initiate an allergic response or neutralise viruses. The V region of the heavy chain (V_H) is the product of three distinct genetic elements - variable (V_H), diversity (D_H) and joining (J_H) - while the V_L is encoded by V_L and J_L genes. There are 50-250 V_H, 30 D_H and 6 J_H genes that are discontiguously arranged on the same chromosome (14 in humans; 12 in mice). The exon encoding the V_H results from a series of gene rearrangements where one or more D_H genes is initially juxtaposed to a single J_H gene. The DJ_H element is then joined to one V_H gene, forming a unique V_H exon (VDJ). A complete Ig heavy chain is produced following the alignment of the VDJ element with a constant region (C) gene. Following the successful rearrangement of heavy chain genes, the resulting μ-chain molecule binds to a surrogate light chain (ψ), a protein exhibiting significant homology with human *lambda* light chains. Once assembled, the μ-ψ complex is transported to the cell surface where it noncovalently associates with a heterodimer comprised of the Igα and Igβ molecules. This complex, consisting of μ heavy chain, surrogate light chain and the Igα and Igβ molecules, represents the B cell antigen receptor. Light chain assembly

involves the rearrangement of one of 70-80 *kappa* or *lambda* V genes with one of 5 or 4 J*k* or Jλ genes, respectively, thus producing the genetic element that encodes the $V_{L.}$. Assembly of the light chain molecule is greatly facilitated by the successful rearrangement of heavy chain genes (Mulligan, 1990; Banchereau and Rousset, 1992; Schatz *et al.*, 1992; Kipps and Carson, 1993; Stewart and Schwartz, 1994).

The C region of the Ig molecule expressed in a developing B cell is of the μ isotype. Located downstream of the V, D and J genes are nine different C region genes (μ, δ, γ_1, γ_2, γ_3, γ_4, α_1, α_2, ε) that encode the Ig isotypes (IgM, IgD, IgG_1, IgG_2, IgG_3, IgG_4, IgA_1, IgA_2, IgE). During a secondary immune response, an Ig molecule can undergo isotype switching where the V region gene is ligated to a gene specific for an alternate isotype. This process is regulated by T cells and cytokines (see ***T Cell-Dependent B Cell Activation, Activation via CD40***) and involves deletion of intervening DNA sequences between the rearranged VDJ gene and the C region gene of interest. Isotype switching alters the effector function of the Ig molecule while preserving its specificity (Purkerson and Isakson, 1992; de Vries *et al.*, 1993).

B Cell Development

Pluripotent stem cells differentiate into mature Ig-secreting plasma cells in two distinct stages, known as lymphopoiesis and immunopoiesis, in different anatomical locations (reviewed in Mulligan, 1990; Banchereau and Rousset, 1992; Tarlinton, 1994). Lymphopoiesis is the sequential differentiation of pro-B cells into pre-B cells and pre-B cells into immature/virgin B cells. This occurs in the fetal liver and adult bone marrow and is independent of antigen and accessory cells such as T cells, follicular dendritic cells and monocytes/macrophages. The end result of lymphopoiesis is a mature immunocompetent B cell expressing a functionally rearranged Ig molecule. Immunopoiesis occurs in secondary lymphoid organs such as the lymph nodes, tonsils, Peyer's patches and spleen. Immunopoiesis is the antigen-driven differentiation of mature B cells into activated blast cells which further develop into antigen-specific plasma and memory B cells (Figure 1.1). Throughout both lymphopoiesis and immunopoiesis, the distinct stages of B cell differentiation can be discerned by the expression of specific cell surface and cytoplasmic molecules. Thus, pro-B, pre-B, immature B, mature B, activated B or plasma cells are detectable according to the presence or absence of cell surface molecules that can be classified as pan-B cell antigens or antigens specific for the different stages of B cell development (Table 1.1). The different stages of B cell development can also be identified by the degree of Ig gene rearrangement. Pro-B cells have Ig V genes in a germline configuration; D-to-J_H and V_H-to-DJ_H recombination occurs at the early pre-B cell stage while late stage pre-B cells express cytoplasmic Ig μ chain. Immature B cells are defined by the expression of surface IgM while the co-expression of IgM and IgD is representative of mature B cells.

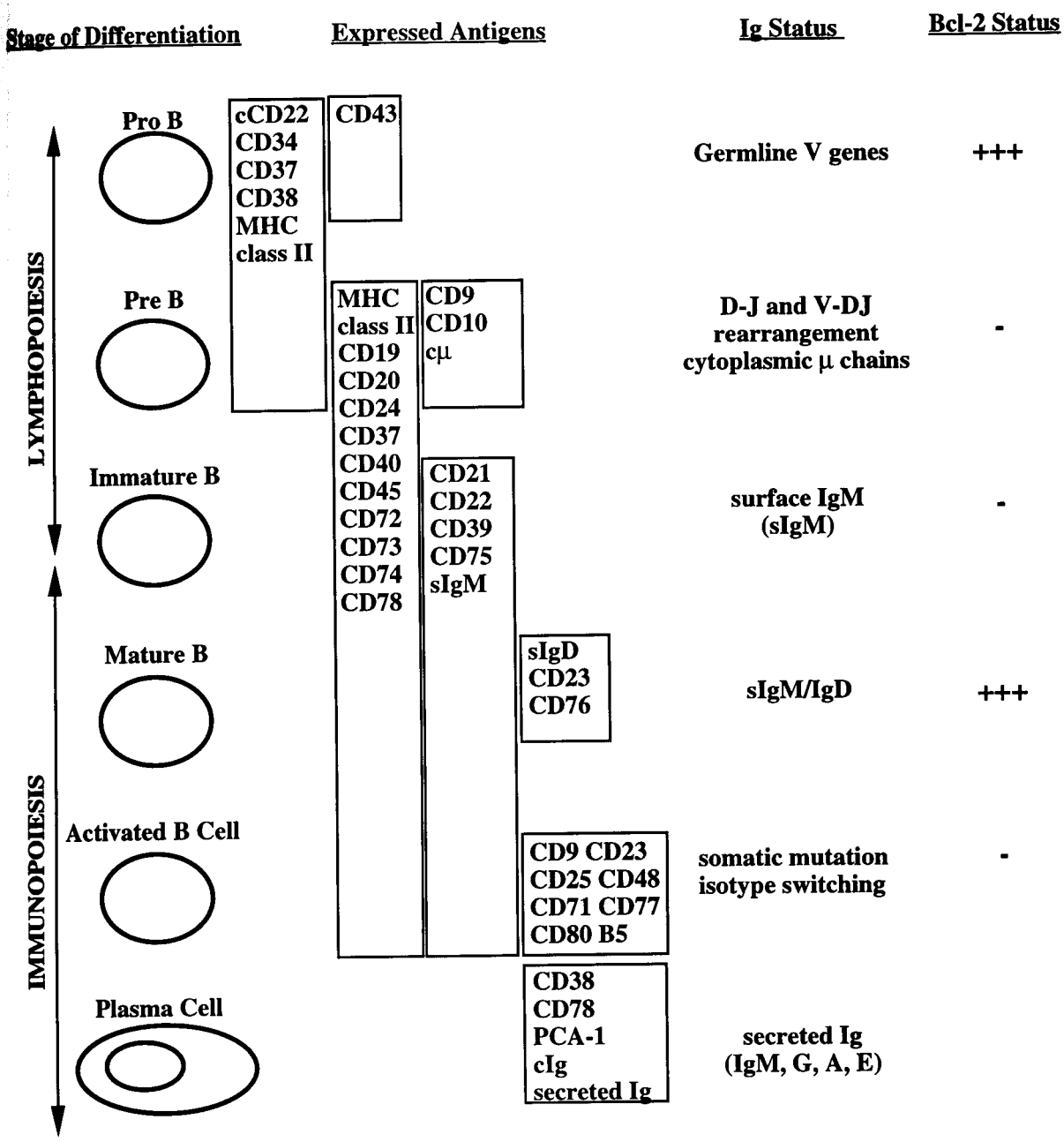


Figure 1.1: Characteristics of Developing B Cells.

(adapted from Mulligan, 1990; Uckun, 1990; Clark and Lane, 1991; Banchereau and Rousset, 1992; Freedman and Nadler, 1992).

Table 1.1: *Expression of Antigens by Developing B Cells*

Cell	*Cellular Phenotype*
Pro-B	MHC class II, CD19, cCD22, CD34, CD37, CD38, CD40, CD72
Pre-B	MHC class II, CD9*, CD10*, CD19, CD20, cCD22, CD24, CD34*, CD37, CD38*, CD40, CD45, CD72, CD73, CD74, CD78, cμ
Immature B cell	MHC class II, CD19, CD20, CD21, CD22, CD24, CD37, CD39, CD40, CD45, CD72, CD73, CD74, CD75, CD78, sIgM
Mature B cell	MHC class II, CD19, CD20, CD21, CD22*, CD23, CD24, CD37, CD39, CD40, CD45, CD72, CD73, CD74, CD75, CD76, CD78, sIgM, sIgD*
Activated B cell	MHC class II, CD9*, CD19, CD20, CD21*, CD23*, CD24, CD25, CD37, CD39*, CD40, CD45, CD48, CD69, CD71, CD72, CD73, CD74, CD75, CD76, CD77, CD78, CD80 (B-7)*, sIgM, CD48, B5, Bac-1, 4F2
Blast	MHC class II*, CD10*, CD19*, CD20*, CD24*, CD25*, CD37*, CD38, CD40*, CD45*, CD48*, CD71*, CD72*, CD73*, CD74*, CD75*, CD76*, CD78, sIgM*, CD48*, B5*, Bac-1*, 4F2*
Plasma	cIgM, CD38, CD78, PCA-1

* : this antigen is lost at the next stage of B cell development; underlines: indicates the first stage at which this antigen is expressed by developing B cells.

(adapted from Mulligan, 1990; Uckun, 1990; Clark and Lane, 1991; Banchereau and Rousset, 1992; Freedman and Nadler, 1992).

B Cell Activation, Proliferation and Differentiation *In vitro*

For a B cell to undergo differentiation, it must receive appropriate signals from the surrounding microenvironment. These include surface Ig (sIg)-mediated signals, signals delivered by co-stimulatory molecules expressed on accessory cells and signals delivered by cytokines secreted by reciprocally activated T cells.

Activation via Surface Ig

B cells undergo activation or deletion in response to ligation of sIg by specific antigen. Deletion usually occurs in immature B cells following exposure to self antigens (Goodnow, 1992). In contrast, mature B cells are activated subsequent to antigen exposure. There are two main categories of antigens that can elicit B cell activation. Thymus-dependent antigens are monovalent protein antigens that usually cannot induce sufficient crosslinking of sIg for efficient B cell responses. Thus, T cell help is required. Thymus independent antigens are usually multivalent polysaccharide antigens capable of inducing extensive crosslinking of sIg. This mode of activation is mimicked *in vitro* by the use of high-affinity anti-Ig antibodies (Noelle and Snow, 1991; Hodgkin and Kehry, 1993; Parker, 1993; Mond *et al.*, 1995). *Staphylococcus aureus* Cowan I (SAC) can also activate B cells independently of T cells by binding to both sIg, via protein A, and MHC class II molecules, via superantigens expressed by the bacteria (Banchereau and Rousset, 1992). Ligation of sIg with either anti-μ or SAC induces B cell activation and proliferation, but not Ig secretion (reviewed in Banchereau and Rousset, 1992; Hodgkin and Kehry, 1993). The induced proliferation could be enhanced by co-culturing in the presence of phorbol esters (Suzuki *et al.*, 1985) or cytokines such as TNF-α (Boussiotis *et al.*, 1994), IL-4 (Defrance *et al.*, 1987, 1988; Hodgkin *et al.*, 1991a; Rousset *et al.*, 1992), IL-2, IL-10

(Rousset *et al.*, 1992) and IL-13 (Defrance *et al.*, 1994). *In vitro* studies showed that differentiation to IgM, IgG, IgE and IgA secretion occurred following the addition of IL-2, IL-4 or IL-10 to Ig-activated B cells (Defrance *et al.*, 1988; Splawski *et al.*, 1989; Rousset *et al.*, 1992), and the amount of Ig secreted in the presence of these cytokines was potentiated by TNF-α (Jelinek and Lipsky, 1987), IL-6 (Splawski *et al.*, 1990), IL-9 (Dugas *et al.*, 1993) or IL-12 (Jelinek and Braaten, 1995). Although multiple Ig isotypes were detected, activation of sorted $sIgD^+$ (naive) and $sIgD^-$ (memory) B cells with SAC revealed that the naive B cells predominantly secreted IgM while the B cells that had undergone isotype switching *in vivo* secreted IgM, IgG and IgA. This indicated that the cytokines used in these studies to stimulate sIg-activated B cells do not induce isotype switching *in vitro* (Banchereau and Rousset, 1992). However, recent evidence has been presented demonstrating that IL-3, GM-CSF and IFN-γ can induce the secretion of downstream Ig H chain isotypes by B cells activated with a multimeric form of anti-IgD (reviewed in Mond *et al.*, 1995). Although both IL-2 and IL-4 were capable of inducing proliferation and differentiation of B cells activated with either insoluble anti-IgM or SAC, the state of cellular activation appears to dictate the intensity of the response. Co-activation of B cells with SAC and IL-2 resulted in significant proliferation and Ig secretion. For IL-4 to be effective, the B cells required a pre-incubation period of 1 day when insoluble anti-IgM was used as the co-activator, or 2-3 days when SAC was used (Defrance *et al.*, 1987; Jelinek and Lipsky, 1988). Furthermore, IL-4 inhibited IL-2-induced activation, proliferation and differentiation of SAC-activated B cells (Jelinek and Lipsky, 1988; Splawski *et al.*, 1989; Maher *et al.*, 1991). The mechanism by which IL-4 exerts an inhibitory effect is unknown. However, it appears to be unique for SAC activation because IL-4 enhances differentiation of

lipopolysaccharide (LPS)-activated B cells and also synergises with IL-2 resulting in increased levels of Ig (Splawski *et al.*, 1989).

Thus, consistent with sIg acting as a receptor for antigen, activation of B cells through their Ag-receptor gives rise to activated/cycling B cells capable of further differentiation in the presence of appropriate soluble factors. Although this mechanism of signalling represents a T-cell independent response, the finding that T-cell derived growth factors can modulate Ig-mediated B cell activation suggests an accessory cell component is required for optimal responses to such thymus-independent antigens.

T Cell-Dependent B Cell Activation

The B cell response to thymus-dependent antigens requires T cell help. This occurs once antigen has been recognised by membrane Ig and subsequently internalised. The antigen-specific B cell then processes and presents fragments of the antigen in association with MHC class II molecules to T cells. Recognition of the antigen-MHC complex by the T cell antigen receptor results in T cell activation. Once activated, T cells deliver appropriate signals to the B cell in the form of expressed membrane proteins and secreted cytokines (Abbas, 1989; Noelle and Snow, 1991; Hodgkin and Kehry, 1993; Clark and Ledbetter, 1994). Several systems have been established that are capable of mimicking the T cell-dependent differentiation of both murine and human mature B cells (i.e. immunopoiesis). Murine and human sIgM$^+$ B cells have been shown to undergo activation, proliferation and Ig secretion in an antigen-nonspecific, MHC-unrestricted manner. This occurred when the B cells were cultured in the presence of activated T cells, an activated T cell line or activated T_H cell clones (Noelle *et al.*, 1989; Hodgkin *et al.*, 1990;

Lipsky, 1990; Tohma and Lipsky, 1991; Yellin *et al.*, 1991; van Vlasselaer *et al.*, 1992a; Blanchard *et al.*, 1994). These results were not obtained when resting T cells were used as a source of help, nor when the B and T cells were physically separated. The specific roles that T cell membrane molecules and cytokines have in this mode of B cell activation were delineated by culturing B cells with either paraformaldehyde-fixed T cells/clones or T cell membranes, in the presence and absence of exogenous cytokines. Activated, but not resting, T cells or their membranes induced B cell RNA synthesis, expression of CD23 and CD25 and cell proliferation, but not Ig secretion (Hodgkin *et al.*, 1990; Noelle *et al.*, 1991; Tohma and Lipsky, 1991). Induction of IgM, IgG_1, IgG_2, IgG_3, IgG_4, IgA and IgE secretion occurred following the addition of supernatants derived from activated Th2 clones, IL-4 plus IL-5, IL-2 or IL-13 (Hodgkin *et al.*, 1990, 1991b; Noelle *et al.*, 1991; Gascan *et al.*, 1991a, 1992; Tohma and Lipsky, 1991; van Vlasselaer *et al.*, 1992a; de Vries *et al.*, 1993; Punnonen *et al.*, 1993; Blanchard *et al.*, 1994). Because the initial B cell population was comprised essentially of $sIgM^+$ cells, and the appearance of Ig molecules of different isotype occured in an ordered fashion, it was concluded that the secretion of multiple heavy chain isotypes was a result of isotype switching and not the preferential outgrowth of isotype-committed B cells (Gascan *et al.*, 1991a; Hodgkin *et al.*, 1994).

This stimulatory activity was expressed by activated $CD4^+$ T cell clones belonging to the Th0, Th1 or Th2 subsets as well as $\alpha\beta$ and $\gamma\delta$ TcR^+ T cell clones (Hodgkin *et al.*, 1991b; Noelle *et al.*, 1991; Gascan *et al.*, 1992; van Vlasselaer *et al.*, 1992a). However, thymomas, mastocytomas, B cell hybridomas (Hodgkin *et al.*, 1990), activated $CD8^+$ and $CD4^-8^-$ T cells were incapable of inducing B cell activation (Gascan *et al.*, 1992;

van Vlasselaer *et al.*, 1992a). Kinetic studies revealed that this stimulatory activity was maximal 8-12 hr after T cell activation (Bartlett *et al.*, 1990; Tohma and Lipsky, 1991; Hodgkin and Kehry, 1993) and that *de novo* protein and RNA synthesis was required for its expression (Bartlett *et al.*, 1990; Hodgkin *et al.*, 1990). Membrane activity was also lost following heat denaturation or treatment with proteases (Hodgkin *et al.*, 1990). Blocking studies using mAbs to cell surface molecules yielded conflicting data regarding the molecules responsible for the effector function of activated T cells. Thus, while some investigators found that mAbs to CD4, MHC class II (van Vlasselaer *et al.*, 1992a), LFA-1 or ICAM-1 (Tohma and Lipsky, 1991; Tohma *et al.*, 1991) inhibited B cell activation, others reported that these mAbs, as well as Abs to CD2, CD3, B220 and CD20, had no effect (Hodgkin *et al.*, 1990; Gascan *et al.*, 1992; Lederman *et al.*, 1992a and b; van Vlasselaer *et al.*, 1992a). Despite this contradiction, none of these Abs were capable of completely abrogating the B cell response. Taken together, the results indicated that a novel cell surface molecule expressed on activated T cells was responsible for the induction of B cell activation (discussed further under *Activation via CD40*).

In addition to the above described systems, B cells can secrete multiple Ig isotypes under different culture conditions. Peripheral blood mononuclear cells secrete IgE in the presence of IL-4 (Pene *et al.*, 1988) or IL-13 (de Vries *et al.*, 1993; Punnonen *et al.*, 1993). Highly purified B cells can be induced to secrete IgE by culture with IL-4 in combination with secondary signals delivered via hydrocortisone (Jabara *et al.*, 1991) or CD58 (Diaz-Sanchez *et al.*, 1994). Therefore, successful IgE secretion appears to require IL-4 or IL-13 and a second signal that can be provided by T cells or hydrocortisone. Similarly, sIgA$^-$ B cells co-stimulated with

pokeweed (PWM) and activated CD4+ T cell clones secrete IgA when supplemented with TGF-β (van Vlasselaer *et al.*, 1992b).

Activation via CD40

Although sIg plays a significant role in B cell activation, other B cell surface molecules are also involved in this process. This is evidenced by the capacity of B cells to be activated independently of sIg crosslinking, and also by T cells in the absence of antigen. One of the key molecules capable of transmitting activation signals to B cells is CD40. CD40 is a 45-50 kDa glycoprotein expressed on normal and malignant B cells, follicular dendritic cells, thymic epithelium, monocytes and some carcinomas (Clark and Lane, 1991; Armitage, 1994).

Early studies indicated that anti-CD40 mAbs failed to induce B cell proliferation when used alone. Despite this, these mAbs enhanced B cell proliferation induced by anti-CD20 or insoluble anti-Ig mAbs or that of B cells preactivated with insoluble anti-Ig and recultured with IL-4 (Clark and Ledbetter, 1986; Valle *et al.*, 1989). In addition, crosslinking CD40 resulted in increased cell size (Valle *et al.*, 1989) and homotypic adhesion mediated by upregulating the expression of CD54 (ICAM-1) (Barrett *et al.*, 1991). Furthermore, B cells activated via CD40 also differentiate to Ig-secreting cells in the presence of appropriate cytokines. Thus, human B cells secrete IgM, total IgG, IgA and large amounts of IgE when cultured with anti-CD40 mAb and IL-4 or IL-10 (Jabara *et al.*, 1990; Gascan *et al.*, 1991b; Zhang *et al.*, 1991; de Vries *et al.*, 1993; Nonoyama *et al.*, 1994). The levels of secreted IgM, IgG and IgA induced by stimulation with anti-CD40 and IL-10 could be further increased in the presence of IL-2 (Nonoyama *et al.*, 1994).

Recently, Banchereau and colleagues found that anti-CD40 mAb could induce B cell proliferation, in the absence of secondary stimuli, when the mAb was presented to B cells on murine fibroblasts stably expressing FcRγII/CD32. Furthermore, IL-4, IL-10 or the combination of these cytokines could enhance this proliferation (Banchereau *et al.*, 1991; Rousset *et al.*, 1991 and 1992). Using this system ("the CD40 system") it was further demonstrated that purified B cells secreted large amounts of IgM, IgG, IgE and IgA in the presence of IL-4, IL-10 (Rousset *et al.*, 1991 and 1992; Briere *et al.*, 1994) or IL-13 (Defrance *et al.*, 1994). Furthermore, naive sIgD$^+$ B cells underwent isotype switching to IgG_1 and IgG_3 in the presence of IL-10 (Briere *et al.*, 1994), and to IgA when TGF-β and IL-10 were included in the activation protocol (Defrance *et al.*, 1992a). In addition to inducing Ig secretion, various cytokines can regulate the pattern of isotypes secreted by activated B cells. For example, concomitant with inducing a switch to IgA secretion, TGF-β suppressed the secretion of IgM and IgG by the same B cell population (Defrance *et al.*, 1992a). Similarly, IL-4-dependent IgE secretion is sensitive to TGF-β, IFN-γ, IFN-α and IL-2 (Pene *et al.*, 1988; Gauchat *et al.*, 1992; Nonoyama *et al.*, 1994) while IgA production is inhibited by IL-4 (Rousset *et al.*, 1992; van Vlasselar *et al.*, 1992a).

It is clear from these experiments that the characteristics of B cells stimulated via CD40 were similar to characteristics of B cells stimulated by activated T cells or their membranes. The ligand for CD40 was identified as being a 30-39 kDa glycoprotein transiently expressed on activated CD4$^+$ T helper cells (Hollenbaugh *et al.*, 1992; Lederman *et al.*, 1992a; Noelle *et al.*, 1992; Lane *et al.*, 1992, 1993, Hermann *et al.*, 1993). While unstimulated T cells are negative for CD40 ligand (CD40L), expression of this molecule on T cells could be achieved by

activation with anti-CD3 mAb, the phorbol ester phorbol myristate acetate (PMA) or the mitogen conconavalin A (Con A), with maximal expression being attained after 3-16 hours. Moreover, depending on the activation regime, CD40L could still be detected 36-48 hours later (Lane *et al.*, 1992; Spriggs *et al.*, 1992; Castle *et al.*, 1993; Grabstein *et al.*, 1993). Analysis by Northern blotting and immunofluorescence indicated that $CD4^+$ and $CD8^+$ TcR $\alpha\beta$ and $\gamma\delta$ T cells, natural killer (NK) cells, monocytes, mast cells and basophils expressed CD40L transcripts and/or protein following activation (Lane *et al.*, 1992; Spriggs *et al.*, 1992; Cocks *et al.*, 1993; Gauchat *et al.*, 1993; Hermann *et al.*, 1993) and that cells present in the T cell-rich zones of germinal centres were also positive for CD40L (Lederman *et al.*, 1992b). The CD40L displays homology to other members of the TNF gene family (Hollenbaugh *et al.*, 1992). This is consistent with CD40 being a member of the tumor necrosis factor receptor (TNF-R) superfamily of molecules (Armitage, 1994).

Investigation of the functional activity of CD40L revealed similarities between the activating properties of soluble or insoluble anti-CD40 mAbs and activated T cells or their membranes. Thus, CD40L expressed on COS or CV1/EBNA cells induced vigorous B cell proliferation (Armitage *et al.* 1992, 1993; Spriggs *et al.*, 1992; Cocks *et al.*, 1993) while soluble CD40L was less effective. However, co-activation with anti-IgD, anti-CD20 or PMA enhanced by 10-20 fold the proliferation induced by soluble CD40L (Hollenbaugh *et al.*, 1992; Lane *et al.*, 1993). IL-2, IL-4, IL-10, IL-13 and IL-15 augmented B cell proliferation in response to CD40L-expressing CV1/EBNA or COS cells (Spriggs *et al.*, 1992; Armitage *et al.*, 1993, 1995; Cocks *et al.*, 1993). Furthermore, enhanced levels of secreted IgM, IgG_1 and IgA were observed in the presence of

cell surface CD40L and IL-2, IL-10 or IL-15, while IL-4 or IL-13 directed secretion of IgG_4 (IgG_1 in mice) and IgE (Spriggs *et al.*, 1992; Armitage *et al.*, 1993, 1995; Cocks *et al.*, 1993; Grabstein *et al.*, 1993). Analogous to its role in the CD40 system (Defrance *et al.*, 1992a), TGF-β inhibited IgM, IgG_1, IgG_4 and IgE secretion whilst promoting production of IgA. Additionally, IgA synthesis in the presence of IL-2 or IL-10 was reduced by IL-4 (Armitage *et al.*, 1993). To demonstrate the specificity of these responses, activation, proliferation and differentiation of B cells could be inhibited by anti-CD40 or anti-CD40L mAbs, as well as soluble forms of CD40 or CD40L (Hollenbaugh *et al.*, 1992; Lane *et al.*, 1992; Lederman *et al.*, 1992a; Noelle *et al.*, 1992; Cocks *et al.*, 1993; Grabstein *et al.*, 1993; Nishioka and Lipsky, 1994).

The significance of the CD40-CD40L interaction *in vivo* was recently realised when the CD40L gene was mapped to a region of the X chromosome believed to be responsible for the X-linked immunodeficiency hyper-IgM syndrome (HIGM-1). This immune defect is characterised by normal or increased levels of serum IgM and IgD, low or absent serum IgG, IgA and IgE, a lack of germinal centres and susceptibility to infection with opportunistic pathogens (Callard *et al.*, 1993). *In vitro* activation of peripheral blood lymphocytes (PBLs) from HIGM-1 patients failed to induce T cell-dependent-B cell activation (Fuleihan *et al.*, 1993; Korthauer *et al.*, 1993). However, purified B cells from HIGM-1 patients could undergo Ig isotype switching when provided with signals via CD40 and appropriate cytokines (Durandy *et al.*, 1993; Fuleihan *et al.*, 1993; Korthauer *et al.*, 1993). It was subsequently discovered that *in vitro* activated T cells failed to bind soluble CD40. This was due to a point mutation in the transmembrane or extracellular domains of the CD40L molecule that caused either no expression of the

CD40L molecule or it was expressed as a mutant protein (DiSanto *et al.*, 1993; Korthauer *et al.*, 1993). These findings also indicated that molecules other than CD40 participate in Ig secretion, as evidenced by the normal or elevated levels of serum IgM in these patients. This is further supported by the finding that CD40L-deficient T cell clones supported Ig secretion by B cells, albeit at reduced levels compared to normal T cell clones (Nishioka and Lipsky, 1994). Furthermore, the finding that membranes from activated T cells in conjunction with anti-CD40 mAb induced a B cell response that exceeded the response in the presence of either activator alone suggests that molecules other than CD40L are present on the surface of activated T cells and play an active role in B cell differentiation (Gascan *et al.*, 1991b). One molecule that may play a role in IgM secretion by B cells from HIGM-1 patients *in vivo* is the 26 kDa form of TNF-α. TNF-α is expressed on activated T cells with similar kinetics to CD40L. Neutralising Abs to membrane TNF-α or the 55 kDa TNF-R inhibited secretion of all Ig isotypes by human B cells in the presence of activated T cells and IL-4 (Aversa *et al.*, 1993a).

$CD4^+$ T cells clearly provide help for the induction of B cell activation and differentiation. However, B cells can mature into Ig-secreting cells in the presence of accessory cells other than T cells. Thus, IgE secretion occurred following culture of B cells in the presence of IL-4 and mast cell or basophil cell lines (Gauchat *et al.*, 1993). Similarly, NK cells stimulate B cells to secrete Ig (Yuan *et al.*, 1992; Mond *et al.*, 1995). The effects of mast cells, basophils and NK cells have been attributed to the expression by these different cell types of CD40L (Cocks *et al.*, 1993; Gauchat *et al.*, 1993). Interestingly, activated U937 monocytic cells have also been found to cause B cell proliferation and differentiation, yet this occured in a CD40L-independent manner (Aversa *et al.*, 1994).

The effect of the CD40-CD40L interaction is not restricted to B cell proliferation and Ig secretion. Cross-linking CD40 on B cells induces the expression of CD80 (B-7) and endows the B cells with enhanced allostimulatory activity (Ranheim and Kipps, 1993; Yellin *et al.*, 1994). CD80 is the counter-receptor for CD28, the ligation of which induces IL-2 synthesis by T cells. IL-2 acts on both T and B cells, heightening the proliferative responses and inducing or augmenting Ig secretion. Furthermore, co-stimulating T cells through CD3 and CD28 enhances the subsequent B cell response by initiating secretion of IL-2 and IL-4 as well as by increasing the expression of CD40L (Klaus *et al.*, 1994; Kwekkeboom *et al.*, 1994). This was confirmed by a study which demonstrated that anti-CD40L mAb inhibited the B cell augmentation of IL-2 secretion by anti-CD3 activated helper T cells (Nishioka and Lipsky, 1994). Thus, reciprocal activation events occur which mediate the specificity of a CD40-mediated immune response.

Given the current data on B cell activation, CD40 has emerged as a major modulator of B cell function. Following the discovery of the ligand for CD40, and its pattern of expression, it is clear that the CD40-CD40L interaction is a necessary event for the induction of B cell activation, clonal expansion, differentiation to Ig-secretion and isotype switching following encounter with Ag-specific T cells. The finding that this interaction induces the expression of CD80 on B cells indicates that the responding B cells can also reciprocally activate T cells, further confirming the importance that CD40 and its ligand have in antigen-specific immune responses.

The Role of CD5 and its Ligand, CD72, in Cellular Activation

CD5 is a 67kDa glycoprotein expressed by T cells during all stages of development, as well as by a subset of B cells ("CD5$^+$ B cells"). The precise role of CD5 is yet to be elucidated. However, based on the finding that CD5 is associated with the T cell receptor (Osman *et al.*, 1993) and that anti-CD5 mAbs induce T cell activation and proliferation (Ceuppens and Baroja, 1986; Spertini *et al.*, 1991), it appears to be involved in signal transduction. The ligand for CD5 has been identified in both humans and mice as being CD72, an antigen that is expressed on B cells during all stages of development except plasma cells (van de Velde *et al.*, 1991; Luo *et al.*, 1992). Interestingly, activation of B cells with anti-CD40 mAb plus IL-4, insoluble anti-IgM or PMA is enhanced by cross-linking CD72 (Kamel *et al.*, 1991). The finding that CD5 co-precipitates with sIg on CD5$^+$ B cells suggests that these B cells may have distinct activation requirements to those of conventional (CD5$^-$) B cells (Lankester *et al.*, 1994). Interestingly, CD5 expressed on T cell clones was found to reverse the growth arrest of B-lymphoma cells induced by anti-μ or ionomycin. When combined with signals delivered via CD40, the induced growth arrest was abrogated (Muthukkumar and Bondada, 1995). Presumably, CD5$^+$ B cells could also rescue B cells from growth arrest. Thus, this ligand-receptor pair may be involved in B-T cell contact and communication by inducing T cell activation and preventing Ag-induced growth arrest of B cells.

CD5+ B CELLS

In 1980, Royston *et al.* reported that sIg+ B cells obtained from fifteen different patients with B cell chronic lymphocytic leukemia (B-CLL) co-expressed the pan-T cell Ag CD5, albeit at levels lower than T cells. Cells obtained from patients with hairy cell leukemia (HCL) or lymphosarcoma cell leukemia were negative for CD5. This initial finding led to the discovery of normal CD5+ B cells in healthy individuals. CD5+ B cells were located in the mantle zone of secondary follicles, such as lymph nodes (Caligaris-Cappio *et al.*, 1982; Abe *et al.*, 1994). Activation of normal peripheral blood B cells with PMA induced the expression of CD5, in addition to B5, CD23 and CD25. Alternative inducers of B cell activation, such as crosslinking sIg, infection with EBV, culture with cytokines and PWM, resulted in B cells expressing activation antigens. However, these cells remained negative for CD5 (Miller and Gralow, 1984; Freedman *et al.*, 1987, 1989). The discovery of normal CD5+ B cells led to an extensive characterisation of this B cell subset and the realisation that CD5+ and CD5- B cells were quite distinct.

Characteristics of CD5+ B Cells

Appearance during Ontogeny and Anatomical Location

CD5+ B cells comprise 15-35% of B cells in peripheral blood and spleen and 1-6% of total mononuclear cells of normal adults (Gadol and Ault, 1986; Casali *et al.*, 1987; Hardy *et al.*, 1987; Burastero *et al.*, 1988; Bhat *et al.*, 1992; Kasaian *et al.*, 1992). Adult bone marrow is essentially devoid of these cells (Lydyard *et al.*, 1993). Although CD5+ B cells represent only a minor population of peripheral blood lymphocytes, they have been found to be the predominant cell population during fetal development and early ontogeny. The majority (60 to >90%) of B cells in fetal spleen (17-23 weeks of gestation) and cord blood express CD5

(Bofill *et al.*, 1985; Antin *et al.*, 1986; Hardy *et al.*, 1987; Caligaris-Cappio *et al.*, 1989; Durandy *et al.*, 1990; Bhat *et al.*, 1992). However, these cells are virtually absent from human fetal liver (Antin *et al.*, 1986). The proportion of $CD5^+$ B cells in peripheral blood rapidly decreases during infancy and childhood and reaches a steady level at late adolescence. Concomitantly, the number of conventional $CD5^-$ B cells increases (Bhat *et al.*, 1992). Elevated numbers of $CD5^+$ B cells are also present in a variety of autoimmune disesases such as rheumatoid arthritis and Sjogrens syndrome (Hardy *et al.*, 1987; Burastero *et al.*, 1988; Kipps, 1989; Raveche, 1990). However, it is unclear whether the presence of $CD5^+$ B cells contributes to these autoimmune conditions. This is because, unlike the pathogenic autoAbs, Ig molecules secreted by $CD5^+$ B cells are usually of the μ isotype and bind to self-antigens with a low affinity (see ***Secreted Immunoglobulin***).

An homologous population of $CD5^+$ B cells has been identified in mice and found to exist in comparable proportions. The earliest detectable population of splenic B cells is $CD5^+$ and the predominance of these cells diminishes with age such that <2% of B cells in adult spleen and lymph nodes are $CD5^+$ (Herzenberg *et al.*, 1986). In neonatal and adult mice, the peritoneal cavity is a rich source of $CD5^+$ B cells. Essentially all peritoneal B cells are $CD5^+$ at birth, and 20-40% of peritoneal B cells are $CD5^+$ in adult mice (Hardy and Hawakawa, 1986; Herzenberg *et al.*, 1986; Kipps, 1989; Lydyard *et al.*, 1993). The status of $CD5^+$ B cells in the human peritoneal cavity is unknown, although one study found low but significant numbers of $CD5^+$ B cells in fetal peritoneal and pleural cavities (Bofill *et al.*, 1985). When peritoneal washouts obtained from adult patients were used, no $CD5^+$ B cells were detected (Kipps, 1989).

These results suggest that the human peritoneum may be a source of $CD5^+$ B cells in the neonate only.

Phenotype and Morphology

Human $CD5^+$ B cells co-express sIgM and sIgD, with the expression of alternative Ig isotypes being rare (Caligaris-Cappio *et al.*, 1989). Molecules present on $CD5^+$ B cells that are absent from conventional B cells include the the mouse RBC receptor and CD11b and CD14 (Kipps, 1989; Lydyard *et al.*, 1993). A proportion of cord blood $CD5^+$ B cells expresses CD23, CD25 and CD71 (Caligaris-Cappio *et al.*, 1989; Durandy *et al.*, 1990), yet $CD5^+$ B cells purified from adult tonsil, peripheral blood and fetal spleen do not express these activation antigens (Hardy and Hawakawa, 1986; Hardy *et al.*, 1987; Nawata *et al.*, 1990; Bhat *et al.*, 1992). Tonsillar, peripheral blood and fetal splenic $CD5^+$ B cells are also of similar size to resting lymphocytes (Hardy and Hawakawa, 1986; Nawata *et al.*, 1990; Bhat *et al.*, 1992). This suggests that different subsets of $CD5^+$ B cells may exist or that the anatomical location of normal $CD5^+$ B cells may dictate their *in vivo* activation status. In mice, the phenotype of typical conventional B cells is IgM^+IgD^{++}, $CD23^+$, $CD44^-$, $B220^{++}$. $CD5^+$ B cells, on the other hand, are $IgM^{++}IgD^{\pm}$, $CD23^-$, $CD44^+$, $IL5\text{-}R^+$, CD11 $(Mac\text{-}1)^+$, $B220^+$ (Hardy and Hawakawa, 1986, 1993; Herzenberg *et al.*, 1986; Kantor and Herzenberg, 1993; Tarlinton, 1994). Murine $CD5^+$ B cells are also slightly larger than resting B cells, yet smaller than mitogen-activated B cells and express low levels of antigens found on activated B cells (Hardy and Hawakawa, 1986, 1993; Herzenberg *et al.*, 1986).

Origin of CD5+ B cells

Because CD5+ and CD5- B cells displayed unique phenotypes, it was suggested that these two B cell subsets may in fact represent distinct B cell lineages. Support for this hypothesis came from experiments where irradiated or severe combined immunodeficient (SCID) mice were reconstituted with cells of different anatomical origins. Following transfer of adult bone marrow, the peripheral blood, spleen and lymph nodes of irradiated or SCID mice were reconstituted with conventional CD5- B cells. Surprisingly, peritoneal CD5+ B cells were absent from these animals. Reconstitution with fetal omentum, neonatal or fetal liver grafts, neonatal spleen or bone marrow or adult peritoneal cells gave rise to CD5+ B cells (Herzenberg *et al.*, 1986; Solvason *et al.*, 1991; Kantor and Herzenberg, 1993). This suggested that, unlike conventional B cells, progenitors for CD5+ B cells were absent from adult bone marrow, yet existed in other tissues.

A CD5- B cell population was also detectable in the peritoneal cavity of mice when neonatal spleen, neonatal bone marrow, fetal omentum or adult peritoneal cells were used as the source of progenitor cells (Herzenberg *et al.*, 1986; Solvason *et al.*, 1991). Interestingly, these CD5- B cells were phenotypically similar to CD5+ B cells, except for the expression of CD5. Consequently, this population of cells was termed the CD5- "sister" population. Despite the phenotypic similarities, peritoneal CD5+ and the CD5- sister population appear distinct because each population could only be reconstituted by CD5+ or CD5- peritoneal cells (Herzenberg *et al.*, 1986; Kantor and Herzenberg, 1993). CD5+ B and the CD5- sister B cells may, therefore, represent distinct lineages of cells that are long-lived and capable of self-replenishment, independently of a progenitor source. The demonstration that CD5+ B cells displayed

prolonged survival *in vitro* supports this suggestion (Hardy and Hawakawa, 1986). In contrast, conventional B cells are continually replenished from undifferentiated progenitors present in adult bone marrow (Kantor and Herzenberg, 1993). Accordingly, a new nomenclature has been proposed for murine B cells: $CD5^+$ and the $CD5^-$ sister B cell population have been renamed B-1a and B-1b cells, respectively, while $CD5^-$ B cells are B-2 cells (Kantor, 1991). The presence or absence of separate B cell lineages in humans is yet to be fully investigated. However, human adult bone marrow contains $CD5^+$ B cell precursors because they are the predominant B cell population in the peripheral blood of patients recovering from bone marrow transplantation (Antin *et al.*, 1987).

Secreted Immunoglobulin

Due to the association of $CD5^+$ B cells with autoimmune diseases, the antigen-specificity of Ig secreted by these cells was examined. Surprisingly, the IgM secreted by cord blood and peripheral blood $CD5^+$ B cells recognised IgG Fc, thus displaying rheumatoid-factor activity, and ssDNA (Casali *et al.*, 1987; Hardy *et al.*, 1987; Burastero *et al.*, 1988). Subsequent studies utilising malignant and normal $CD5^+$ B cells confirmed and extended these initial findings by demonstrating that the IgM recognised antigens of both exogenous and endogenous origin. Thus, IgM secreted by $CD5^+$ B cells binds bacterial polysaccharides, lipopolysaccharide, tetanus toxin, thyroglobulin, insulin, myosin, tubulin and histones, in addition to ssDNA and IgG Fc (Broker *et al.*, 1988; Nakamura *et al.*, 1988; Sthoeger *et al.*, 1989; Borche *et al.*, 1990; Lydyard *et al.*, 1990; MacKenzie *et al.*, 1991; Bhat *et al.*, 1992). Similar studies in mice demonstrated that $CD5^+$ B cells spontaneously secreted IgM which displayed reactivity with ssDNA, bromelain-treated mouse red

blood cells, phosphatidyl choline and determinants present on thymocytes (Hawakawa *et al.*, 1984; Herzenberg *et al.*, 1986; Hardy and Hawakawa, 1993). Due to their capacity to bind multiple, structurally distinct antigens, these IgM molecules have been termed polyreactive and are believed to comprise the "natural autoantibodies" found in the serum of healthy individuals (Herzenberg *et al.*, 1986; Nakamura *et al.*, 1988). Polyreactive IgM molecules displayed low binding affinities, ranging from 10^{-3}-10^{-7}M, for the different antigens examined (Burastero *et al.*, 1988; Nakamura *et al.*, 1988; Casali and Notkins, 1989). Furthermore, the binding of polyreactive IgM to antigen could be inhibited not only by homologous soluble antigen but also by heterologous antigens (Burastero *et al.*, 1988; Nakamura *et al.*, 1988; Casali and Notkins, 1989). Consistent with the identification of the B1b population, some cell lines derived from $CD5^-$ B cells were capable of secreting polyreactive IgM (Nakamura *et al.*, 1988; Lydyard *et al.*, 1990; Bhat *et al.*, 1992). Kaisian *et al.* (1992) further characterised this population of human $CD5^-$ B cells and found that they could be distinguished from conventional $CD5^-$ B cells by the expression of CD45RA. Thus, B2 cells are $CD5^-CD45RA^{hi}$, $CD5^+$ B/B1a cells display intermediate expression of CD45RA, and the novel population of B cells (B1b) are $CD5^-CD45RA^{lo}$. Polyreactive IgM was present in supernatants of $CD5^-CD45RA^{lo}$ and $CD5^+CD45^{int}$ B cells only. $CD5^-CD45RA^{hi}$ B cells secreted IgM and IgG, however these Ig molecules displayed monoreactivity. Although the $CD45RA^{lo}$ B cells did not express surface CD5, they contained CD5 mRNA, the level of which was comparable to that found in B1a cells (Kaisian *et al.*, 1992). These findings suggest that $CD5^-CD45RA^{lo}$ B cells represent the human B-1b population previously described in mice. This is compatible with the reduced expression by these cells of B220, the murine homolog of CD45.

Mechanisms by which polyreactive Ig molecules bind multiple antigens have been investigated. A frequent usage of V_H and V_L genes that have not undergone significant somatic mutations has been found to occur. Twenty five percent of *kappa*-positive CD5+ CLL B cell populations utilise the VkIII gene HumKv 325. This gene was in essentially germline configuration with no or minimal somatic mutations (Kipps *et al.*, 1987, 1988). Similarly, an overrepresentation of selected V_H genes belonging to the V_H1, 4, 5 and 6 gene families that were homologous with unmutated germline genes was demonstrated for Ig molecules that were polyreactive and/or derived from normal and malignant CD5+ B cells (Kipps *et al.*, 1989; Sanz *et al.*, 1989; Kipps *et al.*, 1990; Lydyard *et al.*, 1990; Mayer *et al.*, 1990; Mageed *et al.*, 1991; Schutte *et al.*, 1991; Rassenti and Kipps, 1992; Shokri *et al.*, 1993; Ikematsu *et al.*, 1994). Thus, the restricted use of a small number of unmutated V genes that do not differ significantly from germline configuration may result in an Ig binding site capable of recognising multiple structurally distinct antigens. However, there have been some reports that CLL B cell populations display V_H genes that have undergone somatic mutation. In particular, a proportion of CLL B cell populations express V_H5 genes that differ significantly from known germline sequences (Cai *et al.*, 1992). Interestingly, these Ig V genes exhibited mutations in the complementarity determining regions, suggesting an antigen-driven selection event had occurred. These results clearly contrast those of Rassenti and Kipps (1992). Consequently, cells with highly mutated V_H5 genes may represent a unique subset of CLL. However, further work examining the expression of V_H genes by leukemic B cells is necessary in order to clarify this discrepancy.

In vitro *Responses*

Because of their distinct phenotypes, it has been suggested that human $CD5^+$ and $CD5^-$ B cells may have different activation requirements. Although several investigators have attempted to demonstrate this, few consistent findings have been reported. Activation of tonsillar $CD5^+$ and $CD5^-$ B cells with insoluble anti-μ, SAC or anti-CD40 caused similar levels of proliferation and IgM, IgG and IgA secretion by both cell populations (Zupo *et al.*, 1991; Defrance *et al.*, 1992b). However, cord blood B cells displayed reduced responses to anti-μ, compared to adult peripheral blood, and secreted only IgM following activation with SAC (Durandy *et al.*, 1990). Because the $CD5^+$ B cells used in these studies were only 50-90% pure, it is difficult to interpret the influence that contaminating $CD5^-$ B cells had on the measured responses. In fact, the amount of IgG secreted by tonsillar $CD5^+$ B cells was approximately 10% of that secreted by $CD5^-$ B cells that had been activated by the same stimuli (Defrance *et al.*, 1992b). Importantly, by using peripheral blood $CD5^+$ and $CD5^-$ B cells that had been purified to >98%, Nawata and colleagues (1990) observed unique responses following activation via ligation of sIg. SAC induced the proliferation of both B cell populations while only $CD5^-$ B cells were sensitive to signals delivered by anti-μ. Although IL-2 enhanced the proliferative responses of $CD5^-$ B cells, it had no effect on SAC-induced $CD5^+$ B cell proliferation. Following preactivation with SAC, IL-2 induced $CD5^-$ B cells to secrete IgM, IgG and IgA, while IgM was the predominant Ig isotype secreted by $CD5^+$ B cells (Nawata *et al.*, 1990). According to this report, $CD5^+$ and $CD5^-$ B cells have distinct activation requirements and differ in their responsiveness with regard to stimulating agent, the intensity of the response and the capacity to undergo isotype switching. However, Punnonen *et al.* (1992) found that stimulation of human fetal splenic

$CD5^+$ B cells with anti-CD40 mAbs, in conjunction with a T cell clone and IL-4, resulted in the secretion of several Ig isotypes, including IgE. The response of these cells was sensitive to factors previously reported to inhibit the IgE secretion of mature $CD5^-$ cells. The mechanism of isotype switching in $CD5^+$ B cells *in vitro* thus appears to be dependent on T cell derived signals, rather than the T cell-independent system described by Nawata *et al.* (1990). Consistent with this, Tarlinton (1994) recently reported that, in the absence of T cells, the number of murine peritoneal B-1a cells which switch to the IgG_1 or IgG_3 isotype was <1% of the number of cells committed to IgM production. However, the serum obtained from irradiated mice reconstituted with peritoneal cells contained IgM as well as IgG_{2a} and other Ig isotypes. The observation that serum of mice reconstituted with cells that give rise to $CD5^+$ B cells contained significant levels of IgM, IgG_3 and IgA is evidence that these cells undergo isotype switching *in vivo* (Solvason *et al.*, 1991).

Function of Normal $CD5^+$ B Cells

$CD5^+$ B cells have been suggested to play an active role in the responses of immunologically immature hosts. Due to their predominance during fetal ontogeny and neonatal life, it is thought that $CD5^+$ B cells may act as a first line of defense against invading pathogens that can be neutralised by the polyreactive IgM secreted by these cells (Kipps, 1989; Raveche, 1990; Bhat *et al.*, 1992). They could also play a role in antigen presentation, as well as the clearance of autoantigens, immune complexes or cellular debris from the circulation following interaction with the expressed or secreted polyreactive IgM (Raveche, 1990; Lydyard *et al.*, 1993). $CD5^+$ B cells may also regulate the development of the B cell repertoire via both the establishment of idiotypic networks or by the interaction between CD5 and CD72 expressed on $CD5^+$ and $CD5^-$ B cells

(Raveche, 1990; Kearney, 1993; Lydyard *et al.*, 1993; Muthukkumar and Bondada, 1995). Although the expression of polyreactive IgM contributes to many of the functions ascribed to $CD5^+$ B cells, this property may also be integral to the development of $CD5^+$ B cell malignancies. Continual stimulation with endogenous antigen may result in the expansion of a population of $CD5^+$ B cells that accumulate in the periphery. This mode of pathogenesis may also explain why some $CD5^+$ B cell populations express certain activation antigens.

CHRONIC LYMPHOCYTIC LEUKEMIA

Characteristics of the Malignant Clone

B cell chronic lymphocytic leukemia (B-CLL) is a hematologic neoplasm charaterised by the proliferation and accumulation of mature-appearing B cells expressing sIgM, and often sIgD, that fail to progress to the final stages of B cell development (Gale and Foon, 1987; Freedman and Nadler, 1992). The malignant cells in B-CLL display a low proliferative index, with >99% of cells being in the G_0 phase of the cell cycle (Andreeff *et al.*, 1980; Carlsson *et al.*, 1988a; Stephenson *et al.*, 1991). Thus, CLL B cells are quiescent cells that, similar to normal resting B cells, express MHC class II, CD19, CD20, CD24, CD40 and CD45RA. However, they are negative for antigens characteristic of pre-B or plasma cells. Unlike normal B cells, CLL B cells also express the mouse erythrocyte rosetting receptor (MER), the myelomonocytoid-associated antigens CD11b/c, CD14 and CD15, as well as CD5. The intensity of expression of sIg and CD5 is approximately 10-fold less than that expressed on normal B cells and T cells, respectively. Furthermore, a number of cases have been reported to express the activation antigens B5, CD23, CD25, CD48 (Blast-1) and CD71 (Gale and Foon, 1987; Freedman *et al.*, 1987; Morabito *et al.*, 1987; Dighiero *et al.*, 1991; Freedman and

Nadler, 1992). According to the data identifying normal CD5+ B cells, the normal cellular counterpart of the malignant cell in B-CLL appears to be a B cell activated *in vivo* via protein kinase C and/or a component of secondary follicles. This latter suggestion is supported by findings that the profile of cytokines secreted by CD5+ B cells is similar to that of resting B cells located within the mantle zone of germinal centres (Schena *et al.*, 1992a). Furthermore, the pattern of expression of the anti-apoptotic oncoprotein bcl-2 (see **APOPTOSIS**) by B-CLL cells is similar to resting mantle zone B cells (Schena *et al.*, 1992b; Abe *et al.*, 1994). Consequently, CLL is not a malignancy of a normal peripheral blood B cell, but rather of CD5+ B cells.

In vitro Activation of Leukemic CD5+ B Cells

The reasons for investigating *in vitro* responsiveness of leukemic B cells are two-fold. Firstly, because B cell malignancies represent a rich source of monoclonal cells arrested at a fixed point of B cell development, many investigators have used such cells as a model for normal B cell development. Secondly, the contribution of particular growth factors to B cell malignancies may be revealed. Consequently, leukemic CD5+ B cells have been stimulated with phorbol esters, mitogens, antibodies to cell surface antigens, cytokines and combinations thereof, and the responses of these cells monitored. Despite their state of developmental arrest *in vivo*, leukemic CD5+ B cells obtained from patients with B-CLL can undergo activation, proliferation and differentation to Ig-secreting cells *in vitro*. However, there has been a great deal of heterogeneity reported for the response of different leukemic B cell populations to the same growth signals.

Phorbol Esters

PMA, or tetradodecanoyl phorbol acetate (TPA), is a tumor promotor derived from croton oil. PMA achieves cellular transformation by binding to protein kinase C (PKC) via a diacylglycerol moiety within its structure (Polliack, 1990). Since its first description as an inducer of CLL B cell differentiation (Totterman *et al.*, 1980), PMA has been used extensively as an agent to investigate the *in vitro* activation of malignant B cells. Following activation with PMA, $CD5^+$ B cells from CLL patients have been reported to undergo lymphoblastoid/plasmacytoid transformation. This was evidenced by an increase in cell size and cellular RNA content, entry into G_1 and an increase in the amount of cytoplasmic Ig (Totterman *et al.*, 1980; Carlsson *et al.*, 1988a, 1988b). These morphological changes were accompanied by a reduction in sIg (predominantly sIgD) expression, increased MHC class II and CD54 expression, the appearance of the activation antigens 4F2, Bac-1, CD23, CD25, CD71 and CD80, and enhanced allostimulatory activity (Okamura *et al.*, 1982a, 1982b; Gordon *et al.*, 1984; Carlsson *et al.*, 1988a, 1988b, 1993). Furthermore, PMA increased transcription of the *c-myc* and *c-fos* oncogenes (Larsson *et al.*, 1987), as well as the secreted form of Ig μ chain (Forsbeck *et al.*, 1987), occassionally resulting in the secretion of IgM by the activated CLL B cells (Okamura *et al.*, 1982a, 1982b; Gordon *et al.*, 1984). Leukemic B cell differentiation occurred in the absence of proliferation (Carlsson *et al.*, 1988b; Nilsson, 1992) indicating that, although these cells can mature into IgM-secreting cells, they were incapable of entering the cell cycle. However, proliferation could be induced by supplementing cultures of leukemic B cells with a B cell stimulatory factor (BSF-MP6) derived from a T cell hybridoma, the calcium ionophore A23187, or various cytokines such IL-4 and TNF-α (Drexler *et al.*, 1987; Pfeffer *et al.*, 1987; Carlsson *et al.*, 1988a, 1989a,

van Kooten *et al.*, 1993a, 1993b). Concomitantly, there was increased monoclonal IgM secreted by the leukemic B cells, and the appearance of plasmablasts, in response to activation with PMA and these co-stimuli (Pfeffer *et al.*, 1987; Carlsson *et al.*, 1989b; van Kooten *et al.*, 1993b, 1993c). The combination of PMA, BSF-MP6 and/or IL-4 was found to be the most potent inducer of proliferation and differentiation for leukemic $CD5^+$ B cells. In fact, this protocol induced IgM secretion in populations of leukemic B cells that were unresponsive to PMA alone. Two-colour immunofluorescence, utilising antibodies specific for the light chain expressed by the leukemic B cells and bromodeoxyuridine that had been incorporated into cycling cells, demonstrated proliferation of cells from the malignant clone (Stevenson *et al.*, 1991). Thus, proliferation and differentiation can occur simultaneously in selected leukemic B cell populations in the presence of appropriate/additional stimuli. However, controversy exists regarding the role that T cells and their soluble products play in PMA-induced B cell differentiation, because this event has been found to be both mediated by residual normal T cells (Danersund *et al.*, 1985; Nilsson, 1992) and to also occur in T cell-depleted preparations of leukemic B cells (Drexler *et al.*, 1987).

Thus, it is clear that PMA is a potent inducer of leukemic B cell activation. This is evidenced by altered morphology, expression of activation antigens, cell proliferation, and differentiation into Ig-secreting cells. These parameters are indicative of maturation toward a terminal stage of B cell development. Furthermore, particular messenger pathways are intact within these cells despite their maturational arrest *in vivo*. Consequently, the precise activation stage of the leukemic B cells will probably predict the degree of responsiveness *in vitro*.

Mitogens

Stimulation with various mitogens has also been found to cause CLL B cell activation. Proliferation, lymphoblastoid transformation and/or Ig secretion has been observed in cultures of leukemic B cells activated with staphylococcal enterotoxin, PWM, phytohemagglutinin (PHA) and LPS (Fu *et al.*, 1978; Robert *et al.*, 1979; Nowell *et al.*, 1981; Johnstone *et al.*, 1982; Juliusson *et al.*, 1983; Bloem *et al.*, 1984; Pfeffer *et al.*, 1987; Stephenson *et al.*, 1991; Duan *et al.*, 1992). Staphylococcal enterotoxins were particularly potent cellular activators, as noted by responses of cells that failed to undergo activation in the presence of other polyclonal B cell stimuli (Duan *et al.*, 1992). In several of these cases proliferation and IgM secretion occurred independently (Nowell *et al.*, 1981; Duan *et al.*, 1992). Mitogen-induced leukemic B cell differentiation was strictly dependent on the presence of autologous or allogeneic T cells (Fu *et al.*, 1978; Robert *et al.*, 1979; Nowell *et al.*, 1981; Duan *et al.*, 1992). Although the responses of the malignant clone of cells were less than those of normal B cells (Robert *et al.*, 1979; Johnstone *et al.*, 1982), leukemic B cell differentiation was confirmed by the detection of secreted IgM bearing the same class of light chain as that expressed on the surface of the CLL B cells (Robert *et al.*, 1979; Johnstone *et al.*, 1982; Juliusson *et al.*, 1983; Duan *et al.*, 1992).

Activation Via Cell Surface Antigens

As described previously, normal human and murine B cells can be activated by cross-linking sIg or CD40. In the following section, studies describing the effects of anti-Ig or anti-CD40, when used alone, on leukemic B cells will be discussed. Subsequent sections will detail the role that these cell surface molecules play in leukemic B cell differentiation when combined with additional stimuli.

Activation via CD40

Although CLL B cells express some activation antigens, the majority of samples tested were negative for surface expression of CD80. Following culture with mitomycin C-treated activated T cells, a $CD40L^+$ T cell line or with anti-CD40 mAb, leukemic B cells were induced to express CD80 (Ranheim and Kipps, 1993; Yellin *et al.*, 1994). This response was specific to the CD40 pathway of activation, as antibodies to CD40 or CD40L inhibited CD80 expression (Yellin *et al.*, 1994). In addition to inducing CD80, ligation of CD40 on leukemic B cells led to the expression of CD23 (Yellin *et al.*, 1994) and increased the expression of the adhesion molecules CD11a, CD54 and CD58 (Ranheim and Kipps, 1993). CD40-activated B cells displayed enhanced allostimulatory activity compared to resting cells. The proliferation of allogeneic T cells was not induced by B cells incubated with a CD40L-negative T cell line and could be inhibited by soluble CD80-ligand or anti-CD40L mAb, indicating that the allostimulatory activity of the B cells is attributable to activation via CD40 (Ranheim and Kipps, 1993; Yellin *et al.*, 1994). Taken together, these findings suggest that the enhanced allostimulatory activity conferred on the B cells by PMA resulted from the induced expression of CD80.

Similar to normal B cells, leukemic B cells failed to enter the cell cycle following culture with soluble antibodies to CD40 (Beiske *et al.*, 1988; Garcia *et al.*, 1993). However, proliferation and differentiation of CD40-activated leukemic $CD5^+$ B cells occurred in the presence of appropriate growth factors (see ***Cytokines***).

Activation via sIg

As is the case for immature and mature $CD5^-$ B cells, signals delivered through sIg have been shown to exhibit a stimulatory or inhibitory effect

on CLL $CD5^+$ B cells. Anti-μ or anti-idiotypic antibodies have been found to abrogate spontaneous and induced proliferation of 7 CLL B cell populations (Baeker and Rothstein, 1986; Steinberg *et al.*, 1987a; Mongini *et al.*, 1989; van Endert and Moldenhauer, 1992; van Endert *et al.*, 1992). This phenomenon is not restricted to B cells obtained from CLL patients as a similar degree of inhibition has been observed for cells obtained from other B cell malignancies, including prolymphocytic leukemia (Chan *et al.*, 1990), HCL (Mongini *et al.*, 1989), lymphoma (van Endert and Moldenhauer, 1992), centrocytic lymphoma and immunocytoma (van Endert *et al.*, 1992). Interestingly, inhibition of HCL proliferation was associated with cell death (Chan *et al.*, 1990). Consequently, immunotherapy with anti-IgM antibodies may be a mechanism for the treatment of a variety of B cell neoplasias. In contrast, monoclonal and polyclonal anti-IgD, when used at the same concentration as anti-μ, failed to inhibit B cell proliferation (Mongini *et al.*, 1989). The difference in effectiveness of the antibodies was not due to a difference in expression of sIgD nor a significant difference in antibody affinity (Mongini *et al.*, 1989). Furthermore, when conjugated to Sepharose, the same preparations of antibodies induced cell proliferation (van Endert and Moldenhauer, 1992).

Populations of malignant B cells have also been identified that proliferate in response to anti-IgM antibodies in the absence (Baeker and Rothstein, 1985; Steinberg *et al.*, 1987b) and presence of B cell growth factors (Yoshizaki *et al.*, 1982; Steinberg *et al.*, 1987a ,1987b; see below). Similarly, Carlsson *et al.* (1989a) reported that IL-2 enhanced the rate of IgM secretion by one clone of leukemic B cells following pre-activation with SAC. Interestingly, the combination of IL-2 and the BSF-MP6 supernatant synergised to induce a high-rate of proliferation and

differentiation of SAC-activated leukemic B cells independently of residual T cells and monocytes. Thus, the capacity of sIg ligation to result in a proliferative or inhibitory signal appears to be dependent on the specificity and form of the antibody. Because CLL is a heterogenous disease and not all malignant B cells respond similarly, the stage of differentiation of the cell may also influence the outcome of sIg cross-linking.

Cytokines

The expression by leukemic $CD5^+$ B cells of receptors for particular cytokines (Kabelitz *et al.*, 1985; Digel *et al.*, 1989; Trentin *et al.*, 1993) indicates that these cells may be capable of receiving signals from such growth factors. IL-2, IL-7 and TNF-α alone can induce proliferation in a proportion of CLL B cell populations (Lantz *et al.*, 1985; Digel *et al.*, 1989 and 1991; Aderka *et al.*, 1993). In contrast, other cytokines failed to initiate cell division in the majority of cases tested (Ghaderi *et al.*, 1988; Digel *et al.*, 1991; van Kooten *et al.*, 1992). However, in the presence of a co-stimulus, such as PMA, anti-Ig, SAC or anti-CD40/$CD32^+$ L cells, IL-2, IL-4, IL-6, IL-7, IL-10, IL-13, TNF-α and IFN-α and γ have all been found to induce *in vitro* proliferation of leukemic $CD5^+$ B cells (Lantz *et al.*, 1985; Ostlund *et al.*, 1986; Malkovska *et al.*, 1987; Murphy *et al.*, 1987; Ghaderi *et al.*, 1988; Karray *et al.*, 1988a, 1988b; Totterman *et al.*, 1988; Carlsson *et al.*, 1989a, 1989b; Digel *et al.*, 1989, 1991; Galanaud *et al.*, 1990; Defrance *et al.*, 1991; Fluckiger *et al.*, 1992, 1993, 1994a; van Kooten *et al.*, 1992, 1993a, 1993b; Aderka *et al.*, 1993, McManus *et al.*, 1993, Trentin *et al.*, 1993). Some of these modes of activation also resulted in increased cell size, as well as the up-regulation of expression of activation and adhesion molecules. SAC, IL-2, IL-4, IL-7, IL-10, IL-13, IFN and TNF-α led to the appearance or increase in expression of

CD23, CD25, CD71 or CD11c/18 (Robert *et al.*, 1985; Malkovska *et al.*, 1987; Murphy *et al.*, 1987; Totterman *et al.*, 1987; Carlsson *et al.*, 1989a; Digel *et al.*, 1991; Fluckiger *et al.*, 1992, 1994a; Fournier *et al.*, 1992a; Carlsson *et al.*, 1993; van Kooten *et al.*, 1993d). This suggests that, despite their developmental arrest *in vivo*, leukemic B cells are capable of undergoing activation and proliferation following *in vitro* culture.

The effects of IL-4 are similar to anti-Ig in that they appear to be dependent on the activation status of the cell. IL-4 synergised with anti-CD40 or PMA and BSF-MP6 to induce significant leukemic B cell proliferation (Carlsson *et al.*, 1989b; Fluckiger *et al.*, 1992). In contrast, spontaneous proliferation or proliferation induced by IL-2, TNF-α, IFN-γ or LMW-BCGF was reduced or abrogated by IL-4 (Karray *et al.*, 1988a; Carlsson *et al.*, 1989b; Galanaud *et al.*, 1990; Defrance *et al.*, 1991; Luo *et al.*, 1991; van Kooten *et al.*, 1992, 1993b; Rettie and Hoffbrand, 1994). IL-4 inhibits proliferation by retaining the cells in the G_o,phase of the cell cycle, thus preventing their entry into G_1. The exact mechanism by which IL-4 inhibits leukemic B cell proliferation induced by IL-2 and TNF-α is unknown. However it is not due simply to a reduction in expression of cell surface receptors for these cytokines (Karray *et al.*, 1988a; van Kooten *et al.*, 1992, 1993a). Several hypotheses have been proposed to explain the inhibition. The first is based on two independent observations : (1) IL-4 increases intracellular cAMP levels in normal B cells, and (2) TNF-α-induced leukemic B cell proliferation is inhibited by an analog of cAMP or by an inhibitor of cAMP degradation. Accordingly, van Kooten *et al.* (1993a) proposed that the inhibitory action of IL-4 is due to the resultant increase in intracellular levels of cAMP. Secondly, because IL-4 inhibits the secretion of TNF-α by CLL B cells (Rettie and Hoffbrand, 1994), IL-4 may inhibit proliferation by

preventing autocrine TNF-α production. Further support for this suggestion was the finding that IL-2-induced leukemic B cell proliferation could be reduced both by anti-TNF-α Abs or pentoxifylline, a drug that disrupts TNF-α synthesis (van Kooten *et al.*, 1993a). This would implicate an autocrine role for endogenously produced TNF-α, a notion previously put forward by Hoffbrand *et al.* (1993). However, this suggestion runs counter to a previous study that found the inhibition by IL-4 could not be overcome by exogenous TNF-α (Luo *et al.*, 1991). It must be pointed out that the addition of TNF-α may not overcome the intrinsic effects that IL-4 exerts on the leukemic B cells. An additional mechanism by which IL-4 may inhibit IL-2-induced CLL B cell activation comes from the discovery that the 64 kDa γ-chain subunit of the IL-2 receptor (IL-2Rγ) is also a functional component of the IL-4 receptor (Russell *et al.*, 1993). Thus, in the presence of IL-2, IL-4 may effectively compete for receptor binding and consequently reduce the amount of cell surface-bound IL-2. However, because the IL-2Rγ-chain is also present in the IL-7 receptor (Noguchi *et al.*, 1993), and proliferation induced in resting CLL B cells by IL-7 was not affected by IL-4 (Digel *et al.*, 1991), this proposed mechanism for inhibition requires further examination.

A definitive role for IL-6 in the disease process of B-CLL is yet to be demonstrated. Previously, Ghaderi *et al.* (1988) found that the combination of PMA and IL-6 (±IL-1) induced proliferation of 2/2 B-CLL populations. However, others have reported that when used alone or in combination with PMA or other activators and cytokines, IL-6 failed to induce significant proliferation in any of the different CLL samples tested (Carlsson *et al.*, 1989a; Defrance *et al.*, 1991; Nilsson *et al.*, 1992; van Kooten *et al.*, 1992, 1993b; Fluckiger *et al.*, 1992). Furthermore, IL-

6 can inhibit spontaneous and TNF-α induced proliferation of purified leukemic B cells, although to a lesser extent than IL-4 (Aderka *et al.*, 1993; van Kooten *et al.*, 1993b). Proliferation in response to TNF-α could be enhanced in the presence of antibodies to IL-6 or IL-6R (Aderka *et al.*, 1993), indicating that endogenously produced IL-6 may regulate leukemic B cell growth. Another study, however, failed to confirm this result (van Kooten *et al.*, 1992). The results for IL-6 are intriguing as this cytokine has previously been described as being a growth factor for the mature B cell malignancies, myeloma and plasma cell leukemia (Kawano *et al.*, 1988; Freeman *et al.*, 1989; Klein *et al.*, 1995). Clarification of the activity of IL-6 in CLL thus appears necessary.

In the presence of co-stimuli, cytokines can also induce differentiation of leukemic $CD5^+$ B cells into Ig-secreting cells independently of concomitant proliferation. Thus, IFN-α and γ, IL-2 plus SAC, or anti-CD40 plus IL-2 and/or IL-10 induces or enhances IgM secretion and the appearance of plasmacytoid cells (Robert *et al.*, 1985; Ostlund *et al.*, 1986; Malkovska *et al.*, 1987; Totterman *et al.*, 1988; Carlsson *et al.*, 1989a; Defrance *et al.*, 1991; Fluckiger *et al.*, 1992, 1993). IFN-induced differentiation was found to be independent of other stimulatory factors (Ostlund *et al.*, 1986; Totterman *et al.*, 1988) while the effect of IL-10 was mediated through the up-regulation of the high affinity IL-2 receptor on the leukemic B cells (Fluckiger *et al.*, 1993). The combination of IL-2 and IL-10 on CD40-activated leukemic B cell was the most efficient means of inducing differentiation (Fluckiger *et al.*, 1993), a finding similar to that described for the effects of these cytokines on normal human B cells (Rousset *et al.*, 1992). When used alone, IL-4 does not initiate differentiation of leukemic B cells (Karray *et al.*, 1988a; Luo *et al.*, 1991; van Kooten *et al.*, 1993b). However, similar to proliferation,

IL-4 can antagonise differentiation induced by certain growth stimuli or synergise with other activating agents to modulate the amount of IgM secreted by these cells (Carlsson *et al.*, 1989b; Fluckiger *et al.*, 1992; van Kooten *et al.*, 1993b). The presence of a single class of light chain in the majority of secreted IgM indicated that it is highly likely to be derived from the malignant clone of B cells (Malkovska *et al.*, 1987; Robert *et al.*, 1985; van Kooten *et al.*, 1993b). Despite the ability of IL-6, IL-7 and TNF-α to induce cell division, these cytokines failed to stimulate Ig secretion when used as the sole activator or in the presence of SAC or anti-CD40 (Digel *et al.*, 1989; Fluckiger *et al.*, 1992; Aderka *et al.*, 1993).

Isotype Switching in CLL B Cells

B-CLL cells usually express surface IgM and/or IgD. The expression of surface IgG, or other Ig isotypes, rarely occurs. The issue of isotype switching by leukemic $CD5^+$ B cells is controversial and ambiguous. Several groups have described isotype switching in B-CLL cells following *in vitro* stimulation (Saiki *et al.*, 1980; Yoshizaki *et al.*, 1982; Malkovska *et al.*, 1987). However, in these cases, the cultured cells either expressed surface IgG, as well as IgM and IgD (Saiki *et al.*, 1980; Yoshizaki *et al.*, 1982), or the cell surface Ig phenotype prior to culture was not reported (Malkovska *et al.*, 1987). Because these cells already expressed IgG on their surface, this could not be classified as isotype switching. Several other studies have confirmed either the absence of the switched phenotype on the B-CLL cell surface prior to stimulation and/or the detection of monoclonal cytoplasmic and secreted Ig of the same light chain isotype as that expressed on the cell surface. SAC was shown to induce the production of IgG from the cells of 1/4 B-CLL patients (Juliusson *et al.*, 1983); conditioned T cell culture supernatant induced the cells from 3/10

B-CLL patients to secrete IgG (Steinberg *et al.*, 1987b) while IL-4, in conjunction with hydrocortisone, induced the malignant cells from 11/15 B-CLL patients to synthesise IgE (Sarfati *et al.*, 1989). However, culturing leukemic B cells in the CD40 system supplemented with IL-4 or TGF-β failed to initiate isotype class switching (Defrance *et al.*, 1991; Fluckiger *et al.*, 1992). In normal B cells, this combination gives rise to IgE and IgA, respectively (Defrance *et al.*, 1992a). Although PMA is a potent inducer of IgM secretion by activated CLL B cells, there is no evidence that PMA is capable of inducing a switch in the isotype of the secreted Ig (Okamura *et al.*, 1982; Gordon *et al.*, 1984; van Kooten *et al.*, 1993c).

***Heterogeneity of Leukemic B Cell Responses*, In vitro**

Although there is compelling evidence that leukemic B cells from patients with B-CLL are not irreversibly frozen at a fixed point of B cell development and can in fact be induced to undergo proliferation and differentiation, significant heterogeneity exists regarding the responses of different populations of leukemic B cells to the same growth stimuli. Results reported by some authors were not confirmed in subsequent studies. In addition, significant heterogeneity has been observed to occur when cells are treated with one activator or with a combination of progression signals. These include activation with mitogens, cytokines, anti-IgM and phorbol esters (Okamura *et al.*, 1982; Hivroz *et al.*, 1986; Karray *et al.*, 1987; Pfeffer *et al.*, 1987; Nerl *et al.*, 1988; Carlsson *et al.*, 1989a; Kawano *et al.*, 1989; Duan *et al.*, 1992; Fluckiger *et al.*, 1992). Furthermore, the outcome of responses was not always correlated with the expression of growth factor receptors (Kabelitz *et al.*, 1985; Hivroz *et al.*, 1986; Mitsui *et al.*, 1991). This heterogeneity may arise from subtle differences in the stage of development at which the B cells are arrested.

Also, the purity of the leukemic B cell preparations may influence the degree of heterogeneity observed.

Cytokines Secreted By CLL B Cells

$CD5^+$ B cells from B-CLL have been reported to express and secrete various cytokines. IL-1β, IL-6, IL-7, IL-8, IL-10, TNF-α, TGF-β, LMW-BCGF, thioredoxin and IFN-γ have been found to be constitutively expressed by the majority of leukemic B cell populations assayed or detected in the supernatants of unstimulated or *in vitro* activated purified CLL B cells (Pistoia *et al.*, 1986; Kawamura *et al.*, 1986; Morabito *et al.*, 1987; Uggla *et al.*, 1987; Cordingley *et al.*, 1988; Biondi *et al.*, 1989; Hahn *et al.*, 1991; Foa *et al.*, 1990; Ericsson *et al.*, 1992; Fournier *et al.*, 1992b; Kremer *et al.*, 1992; Nilsson *et al.*, 1992; Schena *et al.*, 1992a; Buschle *et al.*, 1993; Finke *et al.*, 1993; Larsson *et al.*, 1993; Plate *et al.*, 1993; di Celle *et al.*, 1994; Lotz *et al.*, 1994; Reittie and Hoffbrand, 1994; Long *et al.*, 1995). Despite the findings of IL-6 mRNA and protein in CLL B cells, Freeman *et al.* (1989) failed to detect expression of IL-6 mRNA in 8 samples of purified leukemic B cells. Similarly, the results for IL-7 require verification because another study failed to detect any evidence of IL-7 mRNA in the CLL samples studied (di Celle *et al.*, 1994). Further analysis revealed that secretion of some of these cytokines is regulated by other soluble factors. Thus, the production of TNF-α by resting or activated CLL B cells could be enhanced by IL-2, IFN-γ and exogenous TNF-α (Cordingley *et al.*, 1988; Foa *et al.*, 1990; Heslop *et al.*, 1990; van Kooten *et al.*, 1992; Larsson *et al.*, 1993). The amount of IL-6 secreted was increased in the presence of TNF-α (Heslop *et al.*, 1990) or by activation with SAC and IL-2 or PMA and IL-4 (Nilsson *et al.*, 1992). Interestingly, secretion of TNF-α and IL-6 by CLL B cells was inhibited by IL-4 and IFN-α (Cordingley *et al.*, 1988; Reittie and

Hoffbrand, 1994). The conflicting data regarding the effect of IL-4 on IL-6 production by CLL cells may be due to the use of only one population of leukemic cells by Nilsson *et al.* (1992). Thus, the results reported by these authors may not necessarily be representative of the majority of CLL cells. Alternatively, activation of the CLL B cells with PMA may result in IL-4 having a positive effect on IL-6 secretion. This would be consistent with the induction of IgM secretion by the combination of PMA and IL-4 in leukemic $CD5^+$ B cells (van Kooten *et al.*, 1993c). Interestingly, murine B cells and lymphomas that display a CD5 phenotype have also been found to produce IL-6 and TNF-α and β (O'Garra *et al.*, 1990). Furthermore, the secretion of cytokines by human leukemic $CD5^+$ B cells appears to be similar to that observed for normal human B cells. TNF-α protein and mRNA, IL-6 and IL-10 were detected in or secreted by human B cells activated with cytokines (IL-4, TNF-α), phorbol esters, or antibodies CD40 or sIg (Smeland *et al.*, 1989; Rieckmann *et al.*, 1991; Boussiotis *et al.*, 1994; Burdin *et al.*, 1995)

Because a number of cytokines found to be secreted by leukemic $CD5^+$ B cells also induce their proliferation, it is important to determine the contribution that each cytokine may play in the disease process of B-CLL.

APOPTOSIS

Apoptosis is a series of biochemical events that ultimately gives rise to cell death. Because it occurs in a relatively ordered fashion, apoptosis has also been termed programmed cell death . Apoptosis usually occurs at a specific stage of cell development and is a mechanism by which the body maintains cellular homeostasis because cell death is usually of benefit to the organism. Several of the events involved in apoptosis have been identified. These include changes occurring at both the cellular and

nuclear level. One of the earliest observations is the rapid reduction in cell size and volume followed by perturbations to the cell membrane such that the plasma membrane becomes ruffled and undergoes blebbing (zeiosis). Despite these gross changes, the cell membrane remains intact, thus preventing a potentially damaging inflammatory response. However, the nucleus is the main target during apoptosis and a characteristic hallmark of apoptosing cells is the fragmentation of genomic DNA into multimers of 180-200 base pairs in length. This is due to the activation of a divalent cation-dependent endogenous endonuclease and results from the regular cleavage of DNA at internucleosomal linker regions. Cell lysis appears to occur relatively late in this sequence of events. Apoptosis can be distinguished from necrosis which is representative of cell death occurring due to trauma, shock or sudden injury. Necrosis is characterised by osmotic swelling and cell lysis, release of intracellular contents and the breakdown of DNA into randomly sized units, and an associated inflammatory response (reviewed in Cohen 1991, Cohen and Duke, 1993).

Apoptosis in the Immune System

Apoptosis occurs in many systems of the body. It is an active process in the development of the immune repertoire and is thought to be responsible for the deletion of autoreactive B and T cells. As a result, the immune system has served as an excellent model for the study of apoptosis and the mechanisms which regulate the molecular events involved. Immature B cell lymphomas, as well as bone marrow-derived immature B cells, have been reported to undergo apoptosis following exposure to glucocorticoids or in response to cross-linking of their sIg (Benhamou *et al.*, 1990; Hasbold and Klaus, 1990; Cuende *et al.*, 1993; Merino *et al.*, 1994; Norvell *et al.*, 1995) while mature resting B cells

enter an apoptotic pathway either spontaneously (Illera *et al.*, 1993; Mower *et al.*, 1994; Lomo *et al.*, 1995) or following hypercrosslinking of their sIg (Parry *et al.*, 1994a, 1994b). These systems are believed to represent various mechanisms by which autoreactive B cells are removed from the immune system and tolerance is maintained. Firstly, immature B cells may undergo clonal deletion following interaction with self-antigen, while mature B cells may be similarly deleted following hyperstimulation of the Ag receptor by thymus-independent Ags. Germinal center B cells also spontaneously apoptose under *in vitro* culture conditions (Liu *et al.*, 1989). This is believed to represent a means by which developing germinal centre B cells (centrocytes), that display reduced affinity for Ag following somatic hypermutation of their Ig genes, are eliminated.

The use of such models of apoptosis has also increased the understanding of the requirements for the occurrence and prevention of apoptosis of developing and activated B cells. Mature B cell apoptosis occurs at an increased rate in the presence of TGF-β, the cAMP-inducing agent forskolin (Lomo *et al.*, 1995), and the inhibitor of protein synthesis cycloheximide (Illera *et al.*, 1993; Mower *et al.*, 1994). PMA and two inhibitors of PKC, staurosporine and H7, could respectively reduce and accelerate the appearance of fragmented DNA in cultures of unstimulated B cells, implicating a primary role for PKC in B cell survival and differentiation (Illera *et al.*, 1993; Lomo *et al.*, 1995). Apoptosis occurring in immature B cells, mature B cells and B-lymphoma cell lines either spontaneously or in response to cross-linking of sIg could be delayed by IL-4 or ligation of CD40 (Illera *et al.*, 1993; Tsubata *et al.*, 1993; Komada *et al.*, 1994; Parry *et al.*, 1994a, 1994b; Santos-Argumedo *et al.*, 1994; Sumimoto *et al.*, 1994; Lomo *et al.*, 1995; Norvell *et al.*, 1995). An enhanced degree of cell survival occurred in the presence of

both anti-CD40 Ab and IL-4 (Parry *et al.*, 1994a, 1994b). Consistent with the upregulation of adhesion molecules on CD40-activated B cells, the prevention of anti-Ig-induced B cell apoptosis appeared to involve a CD11a/CD18a-dependent pathway (Sumimoto *et al.*, 1994). Interestingly, centrocytes could be prevented from entering an apoptotic pathway, and continue to differentiate, following ligation of sIg or CD40 (Liu *et al.*, 1989). This effect was enhanced by the simultaneous cross-linking of sIg and CD40. This situation is believed to mimick the interaction of centrocytes with Ag, which is on the surface of Ag-presenting cells, and CD40L-expressing activated $CD4^+$ T cells. Germinal centre B cells could also be prevented from entering apoptosis *in vitro* by culturing in the presence of IL-10 (Levy and Brouet, 1994), following ligation of CD38 (Zupo *et al.*, 1994) or by co-culture with soluble recombinant CD23 and IL-1α (Liu *et al.*, 1991a). These latter signals are likely to be derived from the $CD23^+$ follicular dendritic cells present in the outer zone of the germinal centre. Additional evidence that dendritic cells are involved in the selection of Ag-specific B cells *in vivo* came from a recent report which found that ligation of the adhesion molecules CD11a and CD49a on the surface of germinal centre B cells with their natural ligands CD54 and CD106 (VCAM-1), respectively, prevented apoptosis and that this effect was enhanced by culture with anti-IgM (Koopman *et al.*, 1994). This suggests that these adhesion molecules co-operate with the Ig-delivered signal in giving rise to Ag-specific B cells. Based on these results, the death or survival of developing B cells following cross-linking of their Ag receptor appears to be mediated by the presence or absence of dendritic cells expressing particular adhesion molecules or activated T cells, which would express CD40L and secrete cytokines such as IL-4 and IL-10, in the microenvironment where selection of Ag-specific B cells occurs.

Genetic Regulation of Apoptosis

A number of gene products have been implicated in either the induction or prevention of apoptosis. These include *bcl-2*, APO-1/Fas (CD95), p53, and *c-myc*. Of these, bcl-2 and Fas have been most thoroughly investigated.

Bcl-2

The *bcl-2* gene is located on chromosome 18. In ~85% of human follicular lymphomas, it is translocated to chromosome 14. Consequently, the *bcl-2* gene becomes juxtaposed to the Ig heavy chain gene locus, causing the deregulated expression of the chimeric (bcl-2-Ig) gene product, and aberrantly elevated levels of bcl-2-Ig mRNA and protein (reviewed in Korsmeyer, 1992a, 1992b). Immunolocalisation studies revealed that bcl-2 is a 25 kDa protein present in the inner and outer mitochondrial membranes, as well as the endoplasmic reticulum and nuclear envelope (Hockenberry *et al.*, 1990; Lithgow *et al.*, 1994; Reed, 1994). Transfection of growth-factor dependent hemopoietic cell lines with the bcl-2 gene resulted in their enhanced *in vitro* survival following the removal of the growth factor (Vaux *et al.*, 1988, 1992; Hockenberry *et al.*, 1990; Nunez *et al.*, 1990). Unlike other oncogenes, bcl-2 did not promote the proliferation of bcl-2-transfected cells. Thus, following rescue from apoptosis, the bcl-2 positive cell lines remained in the G_0 phase of the cell cycle for up to 30 days in the absence of the necessary growth factors (Vaux *et al.*, 1988; Hockenberry *et al.*, 1990; Nunez *et al.*, 1990). However, cell proliferation and enhanced survival (characteristics of tumorigenesis) occurred in cell lines expressing both *c-myc* and *bcl-2* (Vaux *et al.*, 1988). *Bcl-2*, therefore, appears to be a unique oncogene in that it is a regulator of cell death, rather than an inducer of proliferation, such as *c-myc*, or a tumor suppressor gene, such as p53 and Rb

(Korsmeyer, 1992a). The oncogenic potential of *bcl-2* may lie in its capacity to rescue cells from programmed cell death without inducing cell division, thus allowing for the relentless accumulation of non-dividing cells. In addition, bcl-2 may allow particular cells to persist *in vivo* in a resting state. These cells may subsequently become malignant following the activation of other oncogenes, eg *c-myc* (Vaux *et al.*, 1988; Korsmeyer, 1992a). Additional evidence that bcl-2 affects apoptosis came from studies which demonstrated that the expression of bcl-2 protein was restricted to tissues where apoptosis is an active process (Hockenberry *et al.*, 1991; Liu *et al.*, 1991b). Hence, bcl-2 was found in areas of the germinal centre and thymus where selection of B and T cells occurs. An extension of this was the finding that the expression of bcl-2 by B cells is developmentally regulated (Merino *et al.*, 1994). Thus, pre-B and immature B cells lacked bcl-2 while pro-B and mature B cells expressed high levels of this protein (see Figure 1.1). This co-incided/correlated with the sensitivity of pre-B and immature B cells to glucocorticoid-induced apoptosis and also with the window period during which time developing B cells undergo apoptosis physiologically; that is, either as a consequence of failing to produce a functional Ig receptor (pre-B cells) or following exposure to self antigen (immature B cells) (Merino *et al.*, 1994). Finally, mechanisms which rescue germinal centre B cells from apoptosis result in the upregulation of bcl-2 protein (Liu *et al.*, 1991b; Levy and Brouet, 1994; Zupo *et al.*, 1994).

In addition to preventing apoptosis, bcl-2 may be involved in immunological memory. Compared to control littermates, transgenic mice expressing a deregulated *bcl-2* gene displayed greatly increased numbers of B cells, antigen-specific B cells and increased levels of serum IgM, IgG and IgA after a secondary immune response. These effects of

bcl-2 expression on B cells were evident for protracted periods of time and were attributed to enhanced B cell longevity (McDonnell *et al.*, 1989; Nunez *et al.*, 1991; Strasser *et al.*, 1991a; Smith *et al.*, 1994). Furthermore, clonal deletion of B cells recognising a membrane-bound self Ag was delayed in mice expressing the bcl-2 transgene (Hartley *et al.*, 1993). In mice lacking this transgene, cell death occurred after 1-3 days of exposure to self Ag. In contrast, mice expressing bcl-2 possessed relatively large numbers of self-Ag specific immature B cells in the peripheral blood, bone marrow, spleen and lymph nodes following 6 weeks of exposure (Hartley *et al.*, 1993). Collectively, these results suggest that bcl-2 can override the normal mechanisms controlling the size of the B cell pool during B cell development and differentiation.

However, not all mechanisms that induce apoptosis are reversed by the expression of bcl-2. While the expression in thymocytes of bcl-2 in transgenic mice could prevent apoptosis induced by glucocorticoids, ionizing radiation and anti-CD3, clonal deletion of superantigen-specific thymocytes was not affected (Sentman *et al.*, 1991; Strasser *et al.*, 1991b). Thus, the process of negative selection of T cells may operate independently of bcl-2. In addition, WEHI-231 B-lymphoma cells transfected with bcl-2 were resistant to apoptosis induced by heat-shock, yet the pathway of cell death initiated by sIg crosslinking remained intact (Cuende *et al.*, 1993). Similarly, the high level of expression of bcl-2 by mature peripheral blood B cells failed to rescue these cells from apoptosis induced by TGF-β1 or forskolin (Lomo *et al.*, 1995). Although effective at sustaining the viability of certain growth factor-dependent hemopoietic cell lines (IL-3), the expression of bcl-2 did not prolong the *in vitro* survival of an IL-2-dependent T or an IL-6-dependent myeloma cell line in the absence of IL-2 or IL-6 (Nunez *et al.*, 1990). In addition, apoptosis

was induced in targets of cytotoxic T cell irrespective of bcl-2 expression (Vaux *et al.*, 1992). Thus, although bcl-2 has an active role in delaying or preventing programmed cell death in a variety of cell types, there appear to be alternative mechanisms of apoptosis that operate independently of bcl-2. These mechanisms appear to involve the recently discovered molecules bax, the two isoforms of bcl-x (bcl-x_L and bcl-x_S), BAG-1 and Bad. Interestingly, these molecules have been found to be either homologues of bcl-2 (bcl-x_L, bcl-x_S, Bad) or act as bcl-2-binding proteins (bax, BAG-1), and can duplicate or antagonise the action of bcl-2 (Boise *et al.*, 1993; Oltvai *et al.*, 1993; Nunez *et al.*, 1994; Yin *et al.*, 1994; Takayama *et al.*, 1995; Yang *et al.*, 1995). Transfection of an IL-3-dependent cell line with bcl-x_L prevented apoptosis induced following withdrawal of IL-3 to an extent that was indistinguishable from bcl-2 (Boise *et al.*, 1993). Similarly, a T-lymphoma cell line co-expressing BAG-1 and bcl-2 displayed greater resistance to induction of apoptosis by various means than cells expressing only bcl-2 (Takayama *et al.*, 1995). In contrast, the protective effect of bcl-2 or bcl-x_L was negated when the same cells were co-transfected with bcl-x_S, bax or Bad (Boise *et al.*, 1993; Oltvai *et al.*, 1993; Yang *et al.*, 1995). Interestingly, bcl-x_S has been found to be predominantly expressed in immature thymocytes (Boise *et al.*, 1993), while bax transgenic mice have significantly reduced numbers of thymocytes *in vivo*, compared to nontransgenic littermates. Taken together, these findings suggest that the inability of bcl-2 to prevent clonal deletion in bcl-2-expressing transgenic mice may be due to the dominant effect of bcl-x_S. Additionally, bax and bad form heterodimers with bcl-2 and bcl-x_L when these latter molecules are overexpressed, and the inherent ratios of bax, bad, bcl-2 and bcl-x_L appear to determine the susceptibility of cells to death following receipt

of an apoptotic signal (Oltvai *et al.*, 1993; Nunez *et al.*, 1994; Yang *et al.*, 1995).

Fas/APO-1/CD95

Fas is a 50 kDa cell surface molecule differentially expressed on resting and activated neutrophils, monocytes, lymphocytes and fibroblasts. Like CD40, Fas belongs to the TNF-R superfamily (Itoh *et al.*, 1991; Daniel and Krammer, 1994; Iwai *et al.*, 1994). Cells expressing Fas constitutively, or following *in vitro* activation, undergo apoptosis in the presence of an anti-Fas mAb (Itoh *et al.*, 1991; Daniel and Krammer, 1994). This mechanism of inducing apoptosis appears to play an integral role in limiting an immune response by eliminating specific cells following neutralisation of the Ag. Interestingly, the action of Fas appears to be influenced by the co-expression of bcl-2. Thus, cells such as neutrophils that are negative for bcl-2 ($bcl\text{-}2^{-}/Fas^{++}$) rapidly enter an apoptotic pathway following exposure to anti-Fas. In contrast, lymphocytes strongly expressing bcl-2 ($bcl\text{-}2^{++}/Fas^{+/-}$) were resistant to the effects of this Ab, while monocytes which express less bcl-2 than lymphocytes ($bcl\text{-}2^{+}/Fas^{+}$) display a rate of cell death intermediate to that of lymphocytes and neutrophils (Iwai *et al.*, 1994). These results are supported by the findings that an IL-3-dependent cell line transfected with Fas cDNA was susceptible to anti-Fas mediated apoptosis and that co-transfection of bcl-2 partially inhibited this form of cell death (Itoh *et al.*, 1993).

Apoptosis and Leukemic $CD5^{+}$ B Cells

In contrast to their enhanced longevity *in vivo*, leukemic $CD5^{+}$ B cells obtained from patients with B-CLL rapidly die by apoptosis *in vitro* (Collins *et al.*, 1989; Buschle *et al.*, 1993; Panayiotidis *et al.*, 1993, 1994).

This phenomoenon was independent of autologous T cells, cell density or serum components (Collins *et al.*, 1989; Robertson *et al.*, 1993). The rate of apoptosis could be accelerated by actinomycin D, cycloheximide, glucocorticoids, calcium ionophore A23187, inhibitors of PKC, IL-5, IL-10, purine nucleoside analogues or crosslinking sIgM (McConkey *et al.*, 1991; Dancescu *et al.*, 1992; Forbes *et al.*, 1992; Panayiotidis *et al.*, 1993; Robertson *et al.*, 1993; Fluckiger *et al.*, 1994b; Mainou-Fowler *et al.*, 1994). The ability of anti-IgM mAb to induce DNA fragmentation may be the means by which this reagent inhibits spontaneous and induced proliferation of some leukemic B cells (see *Activation via sIg*). The results of experiments examining the effect of IL-10 on leukemic B cell survival strongly contrast those obtained from both the murine and human systems where it has been reported that IL-10 sustains the viability of small dense splenic B cells (Go *et al.*, 1990) and germinal centre B cells (Levy and Brouet, 1994). Furthermore, the sensitivity of leukemic $CD5^+$ B cells to IL-10 is specific for this malignancy as B cells obtained from patients with non Hodgkins lymphoma or HCL did not display an apoptotic morphology in the presence of this cytokine (Fluckiger *et al.*, 1994b). Interestingly, however, Itoh and Hirohata (1995) recently reported that the effect of IL-10 on human B cell survival was highly dependent upon the stage of activation of the cells. This suggests that the discrepancy in the findings for the effect of IL-10 on CLL B cells and germinal centre B cells may lie in the activation status of these cells.

Identification of mediators of leukemic B cell survival may shed light on the possible pathogenesis of this malignancy and also give rise to targets for potential treatment/therapy. Several factors have been identified which prevent or delay apoptosis of CLL B cells. PMA, IL-4 and IFN-α and γ were found to prevent spontaneous apoptosis or apoptosis of

leukemic CD5+ B cells induced by various reagents. These reagents include colchicine, methylprednisolone, etoposide, anti-IgM mAb, A23187 and IL-10 (McConkey *et al.*, 1991; Forbes *et al.*, 1992; Dancescu *et al.*, 1992; Buschle *et al.*, 1993; Panayiotidis *et al.*, 1993, 1994; Fluckiger *et al.*, 1994b). Thus, similar to splenic B cells (Illera *et al.*, 1993), programmed cell death of leukemic B cells can be prevented by PMA and IL-4. Interestingly, apoptosis could also be prevented when leukemic B cells were cultured on a monolayer of human umbilical cord endothelial cells, suggesting a role for adhesion molecules in the survival of leukemic B cells *in vivo* (Long *et al.*, 1995). The prevention of apoptosis in CLL B cells appears to be restricted to IL-4 and the IFNs because similar experiments investigating the effects of other soluble factors failed to reveal a role for IL-1, 2, 3, 5, 6, 7, TNF-α or TNF-β (Dancescu *et al.*, 1992; Buschle *et al.*, 1993; Sarfati, 1993; Panayiotidis *et al.*, 1994). However, although unable to prevent spontaneous apoptosis, IL-2 reduced the amount of fragmented DNA in cultures of IL-10-stimulated leukemic B cells (Fluckiger *et al.*, 1994b). This may be due to the upregulation of the IL-2 receptor on IL-10-treated CLL B cells (Fluckiger *et al.*, 1993). Analogous to normal B cells, anti-CD40 mAb presented on CD32+ L cells could overcome both spontaneous and IL-10 induced leukemic B cell apoptosis (Fluckiger *et al.*, 1994b). This contrasts the finding by Dancescu *et al.* (1992) who reported that anti-CD40 had no effect on leukemic B cell apoptosis. However, the latter report utilised soluble anti-CD40, suggesting that the form of the antibody dictates its ability to rescue cells from spontaneous apoptosis. This is also consistent with observations made in normal B cell systems. Thus, autocrine (CLL B cell-derived IFN-γ) and/or paracrine (T cell-derived IL-4 and IFN) factors may play an active role in the survival and progressive accumulation of leukemic B cells in B-CLL. Additional evidence for an

autocrine role of cytokines in B-CLL came from the finding by Buschle *et al.* (1993) that the leukemic B cells used in their study were capable of secreting IFN-γ *in vitro* and that relatively high amounts of IFN-γ (60 - >2200pg/ml) were found in the sera of 10 CLL patients. This contrasts the virtual absence of IFN-γ from the sera of normal donors.

Expression of bcl-2 and Fas in CLL B Cells

Bcl-2 mRNA and protein have been detected in all CLL B cells examined (Schena *et al.*, 1992b; Mapara *et al.*, 1993). In >95% of cases, this has been found to occur in the absence of any gene rearrangements (Hanada *et al.*, 1993). The level of bcl-2 in leukemic B cells is at least equal to or up to 25 times greater than that found in B cell lines carrying a translocated bcl-2 gene (Schena *et al.*, 1992b; Hanada *et al.*, 1993). Despite its constitutive expression, bcl-2 can be down-regulated *in vitro* in response to signals which induce cell proliferation (Schena *et al.*, 1992b; Mapara *et al.*, 1993). Thus, activation signals may also initiate premature death of leukemic B cells or render these cells susceptible to induction of apoptosis. Interestingly, in the presence of SAC and IL-2, leukemic B cells were found not only to down-regulate bcl-2 expression, but also to up-regulate the expression of Fas (Mapara *et al.*, 1993). Proliferation induced by this combination of activators was inhibited by anti-Fas mAb due to the activation of apoptosis. This is consistent with the regulation of apoptosis in normal cells by bcl-2 and Fas.

However, conflicting results have been found for the expression of bcl-2 by unstimulated leukemic B cells during *in vitro* culture. Using Western blotting techniques it was found that expression of bcl-2 was lost after 1-4 days (Dancescu *et al.*, 1992; Panayiotidis *et al.*, 1993, 1994). In contrast, analysis by immunofluorescence and flow cytometry revealed CLL cells

continued to express bcl-2 at levels that were similar to that observed prior to culture following 1-2 days in culture (Kobayashi *et al.*, 1993; Mainou-Fowler *et al.*, 1994). A further study (Fluckiger *et al.*, 1994b), using seven different leukemic B cell samples, reported that after 3 days, bcl-2 expression had become bimodal, with some cells displaying reduced bcl-2 while the remainder exhibited bcl-2 at levels comparable to that of uncultured cells. Similarly, inconsistent findings have been reported relating to the expression of bcl-2 by CLL B cells in the presence of cytokines that prevent apoptosis. Thus, expression of bcl-2 by CLL B cells rescued from apoptosis by IL-4 and IFN-α has been found to be sustained (Panayiotidis *et al.*, 1993, 1994), increased (Dancescu *et al.*, 1992; Jewell *et al.*, 1994), decreased (Chaouchi *et al.*, 1994) or unaltered (Fluckiger *et al.*, 1994b; Mainou-Fowler *et al.*, 1994). Consequently, the issue of bcl-2 expression, and its subsequent function in leukemic $CD5^+$ B cells requires addressing.

The enhanced rate of apoptosis in the presence of IL-10 was also associated with the accelerated reduction of bcl-2 expression in CLL B cells (Fluckiger *et al.*, 1994b). Consistent with the prevention of IL-10-induced apoptosis, anti-CD40, IL-2 and IL-4 maintained levels of bcl-2 comparable to freshly obtained cells. (Fluckiger *et al.*, 1994b). Thus, enhanced leukemic B cell survival *in vivo* may result from the constitutive expression of this anti-apoptosis oncoprotein, the expression of which is regulated by mechanisms independent of chromosomal rearrangements. This is supported by two recent findings. Firstly, cells expressing lower levels of bcl-2 die *in vitro* with increased kinetics compared to cells that have a high level of bcl-2 (Hanada *et al.*, 1993). Secondly, bcl-2 expression is maintained for up to 30 days in populations of leukemic B cells that do not spontaneously enter an apoptotic pathway

in vitro (Panayiotidis *et al.*, 1994). However, the role that molecules such as bcl-x and bax have in the regulation of leukemic B cell apoptosis can not be discounted.

AIMS OF THIS STUDY

Investigations of the *in vitro* activation of leukemic CD5+ B cells is necessary to enhance our understanding of the requirements for the further growth and differentiation of these clonally arrested B cells. Requirements for *in vitro* activation may reveal intrinsic differences between normal B cells and malignant B cells. Furthermore, because leukemic B cells undergo spontaneous apoptosis following *in vitro* culture, signals involved in promoting the enhanced survival of these cells *in vivo* may be elucidated. An understanding of the factors involved in *in vitro* activation of leukemic B cells may be crucial to the development of treatment strategies for these malignancies.

The aims of this project involved investigating the *in vitro* activation, differentiation and survival of leukemic CD5+ B cells, obtained from different patients with B-CLL. This was achieved by culturing cells in the presence of different stimulators including phorbol esters, mitogens, and antibodies to sIgM. The contribution of T cells to these responses was also determined by comparing the responses of unfractionated peripheral blood lymphocytes, comprised of leukemic B cells and normal residual T cells, to those of purified leukemic B cell preparations depleted of T cells. Furthermore, the phenomenon of spontaneous apoptosis of leukemic CD5+ B cells, as well as its modulation by different cytokines, was characterised with respect to the kinetics of cell death, DNA fragmentation and expression of the anti-apoptotic oncoprotein bcl-2.

Chapter 2:

Materials and Methods

Antibodies for Immunofluorescence

MAb specific for the human peripheral blood mononuclear cell markers CD3, CD4, CD5, CD19, CD25, CD80, LeuM3 as well as isotype-matched control mAb, were purchased from Becton Dickinson, Mountain View, CA, U.S.A. Anti-CD40 mAb was purchased from Sanbio (The Netherlands). Anti-CD8 mAb was purchased from Boehringer-Mannheim (Mannheim, Germany). Anti-bcl-2 mAb was purchased from Dako Corporation, Real Carpinteria, CA, U.S.A. FITC-conjugated $F(ab')_2$ fragments of goat antiserum specific for total human Ig, μ, γ, δ and α Ig heavy chain isotypes, as well as *kappa* and *lambda* light chains, were purchased from Kallestad, Austin, TX, U.S.A. FITC-conjugated polyclonal sheep anti-mouse Ig antiserum was purchased from Silenus Laboratories, Hawthorn, Vic., Australia.

Reagents/Antibodies for Immunoassays

$F(ab')_2$ fragments of polyclonal rabbit anti-human IgM (μ-chain specific) were purchased from Dako Corporation, Real Carpinteria, CA, U.S.A. Standard human IgG and IgM were purchased from Behringwerke (Marburg, West Germany) and Silenus Laboratories (Hawthorn, Vic., Australia), respectively. The mouse mAb K121, specific for human free *kappa* light chains and the *kappa* myeloma antigen (KMA) (Boux *et al.*, 1983, 1984), was purified from ascites fluid and spent tissue culture media by affinity chromatography over a column comprised of human *kappa* light chains immobilised on CNBr-activated Sepharose CL4B. The following reagents were purchased from the Sigma Chemical Company, St. Louis, U.S.A: soluble Protein A; alkaline phosphatase-conjugated polyclonal antibodies specific for human μ, γ, *kappa* and *lambda* chains, as well as mouse Ig (whole molecule); the phosphatase substrates p-

nitrophenyl phosphate (pNPP) and nitroblue tetrazolium /bromochloroindolphenyl (NBT/BCIP)

Mitogens

PMA, PHA and PWM were purchased from the Sigma Chemical Company, St. Louis, U.S.A.

Cytokines and Related Antibodies

IL-1β was purchased from Collaborative Biomedical Products (Massachusetts, U.S.A). IL-2, IL-4 and IL-6 were purchased from Boehringer-Mannheim (Mannheim, Germany). IL-5 and TGF-β were purchased from the Sigma Chemical Company (St. Louis, U.S.A). IL-10, IL-13 and TNF-α were purchased from R&D Systems (Minneapolis, U.S.A). IFN-γ was a generous gift from Dr. Vic Danis (Kolling Institute, Royal North Shore Hospital, Sydney). Polyclonal goat antisera specific for human IL-2, IL-4 and IL-10 were also purchased from R&D Systems (Minneapolis, U.S.A).

B-CLL Cells

Throughout the course of this study, cells from six different patients with B-CLL were used. Peripheral blood lymphocytes (PBLs) were obtained from B-CLL patients following leukapheresis at either the Royal Prince Alfred Hospital, Camperdown, NSW, Australia or the Royal North Shore Hospital, NSW, Australia. Red blood cells were lysed by incubation with 10 mL Tris/NH_4Cl (17mM Tris, 0.14M NH_4Cl, pH 7.2) at 37°C for 5 minutes, followed by washing with RPMI 1640 and centrifugation and then a further Tris/NH_4Cl treatment for 1 minute at 37°C. The lymphocytes were washed with RPMI 1640 and stored in liquid nitrogen in RPMI-1640 containing 40% FCS and 10% DMSO. On thawing, excess

dead cells were removed from the preparations by centrifugation in a Beckman GPR refrigerated centrifuge over Ficoll-Hypaque (Pharmacia LKB, Uppsula, Sweden) for 20 minutes at 1800 rpm (600 *g*). Viability of cells recovered from above the interface was >90%, as determined by exclusion of trypan blue or ethidium bromide (see below).

Depletion of T Cells from CLL PBLs

Residual T cells were depleted from the B-CLL cell preparations by rosetting with 2-aminoethyisothiouronium bromide (AET)-treated sheep red blood cells (SRBC), as described by Kaplan and Clarke (1975).

Preparation of AET-treated SRBCs

SRBC were obtained from the Wellcome Research Laboratories (Royal North Shore Hospital, Australia) and stored in an equal volume of Alsever's solution (20.5 g/L dextrose, 8 g/L sodium citrate, 4.2 g/L NaCl, 0.55 g/L citric acid; pH 6.2). Prior to use, the SRBC were washed 5 times with sterile PBS by centrifuging in a Beckman GPR refrigerated centrifuge at 1400 rpm (360*g*) for 10 minutes at 4°C. A 0.18 M solution of AET was prepared by dissolving 1.6 g of AET in 40 mL of dH_2O and adjusting the pH to 9.0 with concentrated (10M) NaOH. Following the final wash, the SRBC were resuspended in sterile PBS and 4 volumes of the AET solution were added. The cells were incubated at 37°C for 15 minutes, with mixing by inversion occurring every 5 minutes. Following the AET treatment, the cells were washed a further 5 times with sterile PBS, resuspended in RPMI-1640 at a density of 10%, and then stored at 4°C.

Rosetting

Prior to rosetting, the thawed CLL PBLs were centrifuged for 20 minutes at 1800 rpm over Ficoll-Hypaque to remove excess dead cells. The CLL PBLs were resuspended in tissue culture media composed of RPMI-1640 supplemented with fetal bovine serum (FBS; 10%), HEPES (4.76 g/L), sodium bicarbonate (8.5 mg/L), penicillin (100 IU/mL), streptomycin (0.1 mg/mL) and L-glutamine (2 mM), hereafter referred to as complete media. AET-treated SRBC were added to the cells and incubated at 37°C for 15 minutes, with mixing by inversion occurring every 5 minutes. The cells were pelletted by centrifugation at 1400 rpm for 10 minutes and then incubated on ice for 1 hour. Rosetting (T cell enriched population) and non-rosetting (T cell depleted/B cell enriched population) cells were separated by centrifugation over Ficoll-Hypaque with the former population passing through the density gradient. The B cells were recovered from the interface, treated with SRBC lysis buffer (8.3 g/L NH_4Cl, 37 mg/L EDTA, 1.0 g/L $KHCO_3$, pH 7.3) for 10 minutes at 37°C, centrifuged and then resuspended in complete media. The efficiency of the rosetting procedure was determined by staining the resultant cell population with mAb specific for normal T cells and leukemic $CD5^+$ B cells. One cycle of rosetting was usually sufficient to yield cell populations comprised of 95-99% sIg/CD19/$CD5^+$ B cells and 0.01-1.7% $CD3^+$ T cells (see Figure 3.1 and Table 4.1).

Purification of T Cells from Normal Peripheral Blood

Normal T cells were purified from peripheral blood obtained from healthy donors. Whole blood was diluted with an equal volume of sterile PBS and then centrifuged over Ficoll-Hypaque (1800 rpm, 20 minutes). Mononuclear cells were collected from the interface and washed once with RPMI-1640. T cells were enriched following incubation with AET-

treated SRBC, as described above, and subsequent centrifugation over Ficoll-Hypaque. The SRBC present in the rosetting (T cell) fraction were lysed by incubation with SRBC lysis buffer for 10 minutes at 37°C. The T cells were recovered by centrifugation at 1200 rpm for 10 minutes at room temperature. The phenotype of the purified T cell preparations was determined by surface immunofluorescence and analysis by flow cytometry, as described below. Typically, the population enriched for T cells following one round of rosetting was found to be comprised of 85-90% CD3/CD5$^+$ T cells, 5-10% CD19/sIg$^+$ B cells and 2-5% LeuM3$^+$ monocytes.

Surface Immunofluorescence

Aliquots of cells were placed in 1.5 mL Eppendorf centrifuge tubes (10^6 cells/tube), washed once with 0.5 mL PBS containing 0.1% BSA and 0.1% NaN_3 (0.1% BSA-PBS) and then centrifuged for 4-6 seconds at 14000 rpm in an Eppendorf 5415C centrifuge. To determine the phenotype of CLL PBLs, T cell depleted CLL B cells (i.e. purified leukemic B cells) and T cells purified from peripheral blood of normal healthy donors, the cells were incubated with 25 µL of unconjugated anti-CD40, or FITC-conjugated anti-humanIg, anti-CD19 or anti-CD3 and PE-conjugated anti-CD5 for 30 minutes at 4°C. The frequency of CD4$^+$ cells within the T cell populations was determined by incubating the cells with FITC-conjugated anti-CD4 and PE-conjugated anti-CD5 for 30 minutes at 4°C. The light chain isotype of the malignant B cells was determined by incubating the cells with FITC-conjugated goat anti-human *kappa* or burro anti-human *lambda* antiserum for 30 minutes at 4°C. Expression of the activation antigens CD25 and CD80 was determined by incubating the cells with PE-conjugated anti-CD25, unconjugated anti-CD80 or the appropriate isotype matched control mAb. For indirect

immunofluorescence, bound unconjugated mAbs (anti-CD40, anti-CD80) were detected following a second incubation period of 30 minutes at 4°C with FITC-conjugated sheep anti-mouse Ig. The cells were then washed three times with 0.5 mL or twice with 1.0 mL 0.1% BSA-PBS, resuspended in 250-500 µL 0.1% BSA-PBS or 1% paraformaldehyde (in PBS, pH 7.2) and analysed by flow cytometry.

Stained single cell suspensions were analysed on a single-laser FACScan flow cytometer (Becton Dickinson, Mountain View, CA, USA). Surface staining was measured on a logarithmic scale. FITC and PE were excited at a wavelength of 488 nm and fluorescent emissions were selectively collected at 511 and 543 nm respectively. The compensation was set on the instrument such that single labelled cells appeared in different quadrants of the resulting profile. During each experiment the laser power, photomultiplier tube voltage, scatter and fluorescence gains were kept constant. The collected data were analysed using Lysis II and CellQuest software programmes.

B Cell Cultures

Cell Proliferation and RNA Synthesis

For assessment of cellular proliferation and RNA synthesis, cells were cultured in flat-bottomed 96-well microtitre plates (Costar, Massachusetts, U.S.A; Falcon, Becton Dickinson, New Jersey, U.S.A), in a final volume of 200 µL. Eighteen hours prior to harvest, triplicate cultures were pulsed with 1 µCi of either ^{3}H thymidine or ^{3}H-uridine (Amersham Australia Pty. Ltd., Castle Hill, N.S.W). Cells were harvested using a PHD Cell Harvester (Cambridge Technologies Inc., MA, U.S.A) and the amount of incorporated radioactivity was determined by liquid scintillation counting.

Optimisation of Mitogen Concentrations

Unfractionated PBLs were cultured at a density of $1.5x10^6$ cells/mL in complete medium at 37°C in an atmosphere of 5% CO_2. Cell density was optimised by culturing cells from 1 patient (MIL) at $0.5x10^6$, $1.0x10^6$ and $1.5x10^6$/mL. *In vitro* cell responses were negligible at a density of $0.5x10^6$ cells/mL and were maximal at $1.5x10^6$ cells/mL. The CLL PBLs were cultured in the presence of varying concentrations of the tumor PMA (0-100 ng/mL), or the mitogens PHA (0-10 μg/mL) or PWM (0-5 μg/mL). The optimal concentration of each activator was determined by measuring the amount of ^{3}H-thymidine incorporated into the cells during the last 18 hours of a 3-4 day incubation period. Subsequently, unfractionated PBLs or T-depleted purified B cell preparations were either unstimulated or cultured for 8 days in the presence of PMA, PHA or PWM at concentrations determined to evoke maximal proliferative responses. DNA and RNA synthesis, as well as IgM secretion were determined after 2, 4, 6 and 8 days of culture (see below).

Activation via sIgM

Unfractionated PBLs or T cell depleted preparations ($1.5x10^6$/mL) were cultured in the presence of increasing concentrations of a polyclonal rabbit anti-human IgM ($F(ab')_2$ fragment; μ-chain specific) antiserum ($F(ab')_2$ anti-μ; 0-100 μg/mL), with or without varying concentrations of PMA (1.6-100 ng/mL). After 4 days, the rates of cell division (proliferation) and RNA synthesis were assessed by determining the incorporation of ^{3}H-thymidine and ^{3}H-uridine, respectively. For kinetic studies, the T-depleted cell preparations were cultured for up to 10 days with $F(ab')_2$ anti-μ at a concentration found to be optimally synergistic with PMA. DNA and RNA synthesis were determined at various times after the initiation of culture. For investigation of IgM secretion using

this mode of activation, a two-step culture procedure was used. The leukemic CD5+ B cells were preactivated for 48 hours with $F(ab')_2$ anti-μ in the presence and absence of PMA. The cells were then washed twice with PBS and once with RPMI-1640, and then recultured for an additional 7 days in complete media, after which time the amount of IgM secreted was measured.

Investigation of Leukemic CD5+ B Cell Survival, In vitro

Purified leukemic B cells were cultured in 24-well plates (Falcon, Becton Dickinson, New Jersey, U.S.A) for up to 8 days at a density of 1.5 x 10^6/mL in a total volume of 1.0-2.0 mL. All cultures were performed in complete medium, at 37°C in an atmosphere of 5% CO_2. Cell viability, DNA fragmentation, DNA extractions and bcl-2 expression were determined after various times of *in vitro* culture.

Co-culture with Allogeneic T Cells

Leukemic CD5+ B cells and normal T cells were co-cultured. However, the cell populations were physically separated by a 0.45μm Millicell culture plate insert (Millipore, Bedford, MA, U.S.A). Purified leukemic B cells (600 μL) were added to the wells of a 24-well plate (Falcon, Becton Dickinson, New Jersey, U.S.A) at an initial density of $3.0x10^6$ cells/mL and allowed to settle for 1 hour. The culture plate insert was soaked in complete media for 1 hour and then placed over the B cells. Increasing numbers of purified allogeneic normal T cells (0, $2x10^5$, $4x10^5$, $6x10^5$, in 300 μL) were then added to the upper chamber. The total volume in the upper chamber was made to 600 μL with either complete medium or PHA, prepared at 4-times the optimal concentration. Consequently, the final density of the leukemic CD5+ B cells was

$1.5x10^6$/mL. The cultures were incubated for 4-5 days, after which time the viability of the B cells was determined (see below).

Cytokine Cultures

To optimise culture conditions, leukemic $CD5^+$ B cells were cultured for 4-8 days, depending on the cell population, in the presence of different cytokines at a range of concentrations [IL-1β (1-50 U/mL), IL-2 (5-100 U/mL), IL-4 (1-5 U/mL), IL-5 (1-25 ng/mL), IL-6 (50-1000 U/mL), IL-10 (0.5-20 ng/mL), IL-13 (1-20 ng/mL), IFN-γ (1-50 ng/mL), TGF-β (0.5-10 ng/mL) and TNF-α (0.5-20 ng/mL)]. After the appropriate incubation period, cell viability was determined. The kinetics of cell death and apoptosis were further investigated by culturing the leukemic B cells for 8 days in the absence or presence of appropriate cytokines at their optimal concentration. Cell viability, DNA fragmentation and bcl-2 expression were determined at various times following initiation of the culture period.

Neutralisation of or Delayed Addition of Cytokines

Leukemic $CD5^+$ B cells were cultured at a density of $1.5x10^6$/mL in a total volume of 1.0mL of complete media alone or media supplemented with either IL-2 or IL-4. At various times following the initiation of culture, 250 μL of each culture supernatant was removed and replaced with an equal volume of goat anti-human IL-2, goat anti-human IL-4 or a goat antiserum of irrelevant specificity, prepared at four-times the optimal concentration. Alternatively, leukemic $CD5^+$ B cells were cultured in 1.0 mL of complete media. At various times following the initiation of culture, 250 μL of complete media was removed and replaced with an equal volume of the appropriate cytokine prepared at four-times the optimal concentration. The effects of adding or removing

the cytokine on the leukemic $CD5^+$ B cells was assessed by determining cell viability after 7 days of *in vitro* culture.

Detection of Secreted Immunoglobulin

Cultures designed for IgM and IgG quantitation usually consisted of cells grown at a density of 1.5 x 10^6 cells/mL in a volume of 1.5 or 2 mL in the wells of a 24-well tissue culture plate (Costar, Massachusetts, U.S.A). If reagents were limiting, cells were grown at the same density, in 48-well plates (Costar, Massachusetts, U.S.A), in a volume of 500 µL. For the detection of IgM and IgG, ninety six-well microtitre plates (Costar, Massachusetts, U.S.A) were coated with 50 µL of either affinity purified anti-IgM ($F(ab')_2$ fragment) antibody or Protein A prepared in PBS containing 0.02% sodium azide (PBS-azide), respectively. Following a 1 hour incubation at 37°C, or overnight at 4°C, the plates were washed 3 times with PBS-azide and the non-specific binding sites were blocked by addition of 3% BSA (200 µL), prepared in PBS-azide. Culture supernatants and IgM or IgG standard proteins were added to the wells in duplicate, serially diluted in 1% BSA-PBS and then incubated for at least 1 hour at 37°C. The plates were washed 3 times with PBS-azide and bound IgM and IgG were detected by the addition of alkaline phosphatase-conjugated affinity purified goat anti-human µ or γ chain antibodies (1:2500 dilution), respectively, and incubating for 1 hour at 37°C. The plates were washed twice with PBS-azide, once with Milli Q water and once with sodium carbonate buffer (0.015 M Na_2CO_3, 0.035 M $NaHCO_3$, pH 9.6) prior to the addition of p-nitrophenyl phosphate (pNPP; 1.0 mg/mL, prepared in sodium carbonate buffer, pH 9.6) to each well. Color development was measured on an Organon Teknika Microelisa system reader at a wavelength of 405 nm. The concentrations

of secreted IgM and IgG were determined by comparison with a standard curve generated for each of the different human Ig heavy chain isotypes.

The homogeneity of the secreted IgM was determined by light chain specific immunoassays. Briefly, 96-well microtitre plate wells were coated with $F(ab')_2$ anti-μ and blocked with 3% BSA as described above. Culture supernatants or monoclonal human IgM*kappa* and IgM*lambda* paraproteins were added to the wells in duplicate, serially diluted in 1% BSA-PBS and incubated for 1 hour at 37°C. The plates were washed 3 times with PBS-azide and bound IgM*kappa* and IgM*lambda* detected by the addition of alkaline phosphatase-conjugated affinity purified goat anti-human *kappa* or *lambda* light chain antibodies, respectively, and pNPP. The detecting polyclonal antisera specific for human *kappa* and *lambda* light chains displayed no cross-reactivity with monoclonal IgM paraproteins expressing the alternate light chain isotype.

Detection of Binding of Secreted IgM to Mouse IgG

An immunoassay was developed to demonstrate the binding of human IgM, secreted by *in vitro* activated CLL B cells, to mouse Ig. This phenomenon has been previously described to occur at the surface of CLL B cells via a low affinity mechanism not involving the Ag-binding site of the mouse Ig and is believed to be due to the polyreactive nature of the human Ig expressed by these leukemic $CD5^+$ B cells (Weston and Raison, 1991). Ninety six-well microtitre plates (Costar, Massachusetts, U.S.A) were coated with 50 μL of a 100 μg/mL solution of mouse Ig (K121) and blocked with 3% BSA as described above. IgM secreted by activated CLL B cells and normal human IgM, at known concentrations, were added to duplicate wells and serially diluted in 1% BSA-PBS. Following a 2 hour incubation period at 37°C, the plate was washed twice

with PBS-azide. Bound IgM was detected by the addition of alkaline phosphatase-conjugated affinity purified goat anti-human μ chain antibodies and visualised by the addition of pNPP.

Determination of Cellular Viability

Cultured leukemic CD5+ B cells were collected, centrifuged briefly in an Eppendorf 5415C centrifuge and resuspended in PBS containing 0.1% BSA and 0.1% sodium azide (0.1% BSA-PBS). To determine the viability of the cell population, ethidium bromide (Sigma Chemical Company, St. Louis, U.S.A), prepared in PBS-azide, was added to the cells at a final concentration of 5 μg/mL. Cells were analysed immediately by flow cytometry. Cells incorporating the ethidium bromide were deemed to be non-viable and were detected by fluorescence of the ethidium bromide at 595nm when excited with a wavelength of 488 nm. The forward and 90° angle light scattering characteristics of the cells were determined simultaneously. All assays were performed in triplicate.

Determination of Apoptosis

Agarose Gel Electrophoresis

Cultured leukemic CD5+ B cells (~3-4 x 10^6 cells) were aliquotted into Eppendorf tubes and briefly centrifuged. Following aspiration of the supernatants, the cell pellets were resuspended in 300-500 μL of lysis buffer (10 mM Tris-HCl, 1 mM EDTA, 1% SDS, pH 8.0, containing 100 μg/mL of proteinase K) and incubated overnight at 37°C. Cell lysates were extracted with equal volumes of phenol and chloroform:isoamylalcohol (CIAA; 24:1). The resultant aqueous phase was digested with DNAse-free RNAse (40 U/mL) for 3 hours at 37°C. The lysates were sequentially extracted with an equal volume of phenol and then with CIAA. DNA was precipitated overnight at -20°C in the

presence of 0.3M sodium acetate and 2 volumes of 100% ethanol. The following day, the DNA was pelleted by centrifugation for 30 minutes at 14000 rpm in an Eppendorf 5415C centrifuge at 4°C. The pellet was washed once with 70% ethanol, dried in a vacuum dessicator and resuspended in 40 µL of buffer containing 10 mM Tris and 1 mM EDTA (TE, pH 8.0). Samples were electrophoresed for 15-18 hours at 25V through a 1.8% agarose gel in buffer comprised of 40 mM Tris, 1.0 mM EDTA and 20 mM acetic acid (pH 8.0; TAE buffer). DNA was visualised by UV light following staining of the gel with ethidium bromide.

Apoptotic Nuclei

The amount of fragmented DNA in cells cultured *in vitro* was quantitated according to the protocol described by Nicoletti *et al.* (1991). Leukemic CD5+ B cells were collected, pelleted in an Eppendorf centrifuge, resuspended in 100 µL of hypotonic lysis buffer (0.1% sodium citrate, 1.0 mM Tris, 0.1 mM EDTA, 0.1% Triton-X, 50 µg/mL propidium iodide [Sigma Chemical Company, St. Louis, U.S.A]; pH 8.0) and incubated overnight in the dark at 4°C. The following day, 100 µL of the lysis buffer lacking propidium iodide was added to each sample prior to analysis by flow cytometry. Fluorescence of propidium iodide was measured on a logarithmic scale using a single-laser FACScan flow cytometer. Using this method, diploid DNA of uncultured non-apoptotic B cells appears as a tight unimodal peak of relatively high fluorescence. During apoptosis, the percentage of cells with diploid DNA is diminished and a peak with reduced fluorescence appears which represents hypodiploid/apoptotic nuclei. The results obtained by quantitating apoptosis by this method were found to correlate with those obtained using the classical colorimetric assays (Nicoletti *et al.*, 1991; Illera *et al.*, 1993). All assays were performed in triplicate.

Expression of bcl-2 Protein

SDS-PAGE and Western Blot Analysis

Cultured leukemic $CD5^+$ B cells ($1.5\text{-}3x10^6$) were centrifuged and resuspended in 20-40 µL of SDS-PAGE reducing sample buffer (0.25M Tris, pH 8.6 containing 10% (w/v) glycerol, 2% SDS and 0.15% dithiothreitol). The samples were boiled for 10 minutes and then stored at -20°C. Cell lysates were subsequently electrophoresed through a 12% polyacrylamide gel, in the presence of 0.1% SDS, according to the discontinuous gel system of Laemmli (1970), using a Bio-Rad Mini-Protean II apparatus (Bio-Rad Laboratories, Richmond, CA, U.S.A). Proteins of known molecular weight (lysozyme 14.3 kDa, trypsin inhibitor 21.5kDa, carbonic anhydrase 30kDa, ovalbumin 46kDa, bovine serum albumin 67kDa, phosphorylase B 97kDa, myosin 200kDa; Amersham, USA) were concomitantly electrophoresed. The proteins were then electrotransferred to a nitrocellulose membrane (0.5 µm; Bio-Rad Laboratories, Richmond, CA, U.S.A) for 60 minutes at 4°C, using a Biorad Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad Laboratories, Richmond, CA, U.S.A), according to the manufacturers instructions. Nitrocellulose membranes were blocked with 3% BSA, prepared in PBS containing 0.02% azide, for 30-45 mins. Following 3 washes with Tris-buffered saline containing 0.05% Tween (TBS-T), mouse anti-human bcl-2 mAb was added to the NC membrane and incubated for 1 hour. Bound mouse Ig was detected by incubation with an alkaline phosphatase-conjugated sheep polyclonal anti-mouse Ig, and subsequently visualised by the addition of the phosphatase substrate, comprised of 330 µg/mL of NBT and 165 µg/mL BCIP, prepared in alkaline phosphatase buffer (100 mM Tris, 100mM NaCl, 5 mM $MgCl_2.6H_2O$; pH 9.6). The nitrocellulose membranes were washed 3 times for 5 minutes with TBS-T between each incubation step. All

incubations were performed at room temperature on a platform rocker. The proteins in an identically prepared gel were concomitantly transferred to another nitrocellulose membrane. This membrane was probed with the secondary detecting antiserum (alkaline phosphatase conjugated sheep anti-mouse Ig antiserum) only, to determine the extent of cross-reactivity between this antiserum and proteins present in the cell lysates.

Immunofluorescence and Flow cytometry

The expression of bcl-2 protein was also examined flow cytometrically, based on the method described by Aiello *et al.* (1992), with minor modifications. This procedure involved fixation of the cells with paraformaldehyde, followed by permeabilisation with Triton X-100. This method was optimised for leukemic B cells by altering the concentration of paraformaldehyde. Thus, leukemic B cells ($2.5\text{-}5.0 \times 10^6$) were centrifuged and resuspended in 250 µL of 0.5%, 1.0%, 1.5% or 2.0% paraformaldehyde (prepared in PBS-azide, pH 7.4). The cells were incubated on ice for 10 minutes, after which time 30 µL of 0.5% Triton-X 100 (final concentration 0.05%) was added. After 10 minutes on ice, the cells were washed once with 1.0 mL 0.1% BSA/PBS-Az and resuspended in 250 µL of 4% human serum (Gilford, Irvine, CA, U.S.A), prepared in 0.1% BSA/PBS-Az, and incubated for a further 10 minutes at room temperature. The cells were centrifuged and resuspended in 30 µL PBS-azide containing either mouse anti-human bcl-2 antibody or an isotype matched control mAb (5 µg/mL). Following a 30 minute incubation period at 4°C, the cells were washed twice with 1.0 mL of 0.1% BSA/PBS-Az and bound mAb was detected by the addition of 25 µL FITC-conjugated sheep anti-mouse Ig. Following a further 30 minute incubation at 4°C, the cells were washed twice and resuspended in 250 µL

of 0.1% BSA/PBS-Az. Samples were analysed on a FACScan flow cytometer, as described above.

Treatment with Bcl-2 Antisense Oligonucleotides

Phosphorothioate-treated sense and antisense oligonucleotides corresponding to the translation initiation site of the bcl-2 gene (Reed *et al.*, 1990) were purchased from Auspep Pty Ltd (Parkville, Vic., Australia). The sequence of the antisense oligonucleotide was 5' CAGCGTGCGCCATCCTTCCC- 3', while the sequence of the sense oligonucleotide was 5' -GGGAAGGATGGCGCACGCTG- 3' (Reed *et al.*, 1990). Leukemic B cells from 3 B-CLL patients (MIL, MCK and MOT) were cultured at a density of 1.5 x 10^6/mL in the absence or presence of each oligonucleotide at a final concentration of 20 μM. Unlike previous cultures, the amount of FBS in the complete medium was reduced to 5% so as to minimise the DNAse activity of the medium. After 4 or 5 days, light scattering characteristics, viability and bcl-2 expression of oligonucleotide-treated CLL B cells were determined and compared to that of untreated CLL B cells.

Chapter 3:

In vitro *Activation of Leukemic CD5+ B Cells.*

Introduction

B cell malignancies represent monoclonal populations of cells arrested at a fixed point of development. These malignancies are therefore a convenient source of large numbers of clonally arrested B cells. Consequently, B cell malignancies have often been used as models to investigate the activation and differentiation requirements of normal B cells. Furthermore, *in vitro* examination of the responsiveness of malignant B cells may provide insight into the underlying mechanism(s) that result(s) in the block in cellular development. The majority of B-CLL cells resemble small resting $CD5^+$ B cells arrested in the G_0 phase of the cell cycle (Andreef *et al.*, 1980). However, based on many published reports, these cells are not irreversibly frozen, as they can be driven to differentiate into Ig-secreting plasma cells under appropriate *in vitro* culture conditions. PMA, used alone or in combination, will usually induce activation of CLL B cells (Totterman *et al.*, 1980; Gordon *et al.*, 1984; Carlsson *et al.*, 1988b). However, controversy exists as to the role that T cells and their secreted products play in the PMA-induced activation of CLL B cells. Thus, PMA-mediated differentiation of CLL B cells has been found to occur in both the absence (Drexler *et al.*, 1987, 1988) and presence (Danersund *et al.*, 1985; Nilsson, 1992) of residual normal T cells. CLL B cells have also been found to respond to various cytokine-mediated signals. TNF-α, IL-2 and IFN-α and γ induce B-CLL cell proliferation and differentiation in the presence and absence of pre- or co-activation (Lantz *et al.*, 1982; Digel *et al.*, 1989; Ostlund *et al.*, 1986; Totterman *et al.*, 1988; Carlsson *et al.*, 1989a). Interestingly, the effects of IL-4 and anti-Ig reagents on leukemic $CD5^+$ B cells *in vitro* were found to be particularly dependent on the pre- or co-activating signal received by these cells (Baeker and Rothstein, 1986; Carlsson *et al.*, 1989b; Defrance *et al.*, 1991; Luo *et al.*, 1991; Fluckiger *et al.*, 1992).

While there is considerable information regarding the *in vitro* activation of B-CLL cells, heterogeneity has been reported to exist in the responses of cells from different patients to the same stimuli (Hivroz *et al.*, 1986; Pfeffer *et al.*, 1987). It has been suggested that this may be due to the malignant clones being arrested at different stages of activation or development. Furthermore, several published reports detailing the *in vitro* responses of CLL B cells employed cell preparations that contained significant numbers of residual contaminating T cells. Thus, the frequency of contaminating T cells may also contribute to the heterogeneity of responses of leukemic B cells. In this chapter, the activation and differentiation of $CD5^+$ CLL B cells using phorbol esters, mitogens, exogenous human cytokines and antibodies to sIgM is described. It was found that malignant B cells can be induced to undergo lymphoblastoid transformation, proliferation, RNA synthesis and differentiation to IgM secretion *in vitro* at levels significantly higher than unstimulated controls. These responses were found to occur via both T cell-dependent and T cell independent mechanisms.

Results

3.1. B-CLL Cell Phenotype

The cells used in the experiments described below were obtained from CLL patients MIL, KAR and MOT. The phenotype of these cell populations was determined by immunofluorescence staining and flow cytometry. Analysis was performed on cells having forward and 90° angle light scatter characteristics of lymphocytes. Cells falling within this gate typically represented 96.5-99% of the viable population. PBL populations from CLL patients were usually comprised of >90% leukemic B cells, as determined by the co-expression of the B cell restricted antigens CD40 (not shown), sIg and CD19, as well as CD5 (Figure 3.1). The large majority of the remaining cells were $CD3^+$ T cells (Figure 3.1). Unfractionated PBLs were depleted of T cells by rosetting with AET-treated SRBCs. Based on flow cytometric analysis, the rosetting procedure yielded preparations comprising 98-99.5% sIg^+CD5^+ (leukemic B) cells with <0.2% $CD3^+$ (T) cells (Figure 3.2). The histograms in Figures 3.1 and 3.2 are representative of the phenotypic distribution of leukemic $CD5^+$ B and residual normal T cells present in the CLL PBLs and T-depleted preparations, respectively, used in experiments described in this chapter. The monoclonality of the leukemic $CD5^+$ B cells was confirmed by demonstrating that a single class of Ig light chain was present on their surface (Figure 3.3). That is, MIL and KAR CLL $CD5^+$ B cells expressed *kappa* light chains while MOT B cells were positive for *lambda* light chains. All 3 cell populations co-expressed sIgM and sIgD, yet failed to express IgG (Figure 3.3). Thus, the Ig phenotype expressed by the malignant B cells from patients MIL and KAR was μδκ, while MOT expressed the μδλ isotypes (Figure 3.3). The *in vivo* activation status of the leukemic $CD5^+$ B cells was investigated by incubating the cells with mAb specific for the activation

Figure 3.1: Phenotype of Unfractionated PBLs from CLL Patients.

PBLs from CLL patients MIL (top), KAR (middle) and MOT (lower) were incubated with FITC-anti CD19, FITC-anti Ig, FITC-anti CD3, PE-anti CD5 (solid histograms) or an appropriate isotype control mAb (outline histogram) for 30 minutes at 4°C. Cells were washed 3 times prior to analysis by flow cytometry. The *x*-axis represents relative fluorescence intensity and the *y*-axis represents cell number. The values in each histogram represent the percentage of cells positive for the indicated cell surface Ag. Although only ~75% of MOT B cells express CD5, a unimodal shift in fluorescence intensity was apparent.

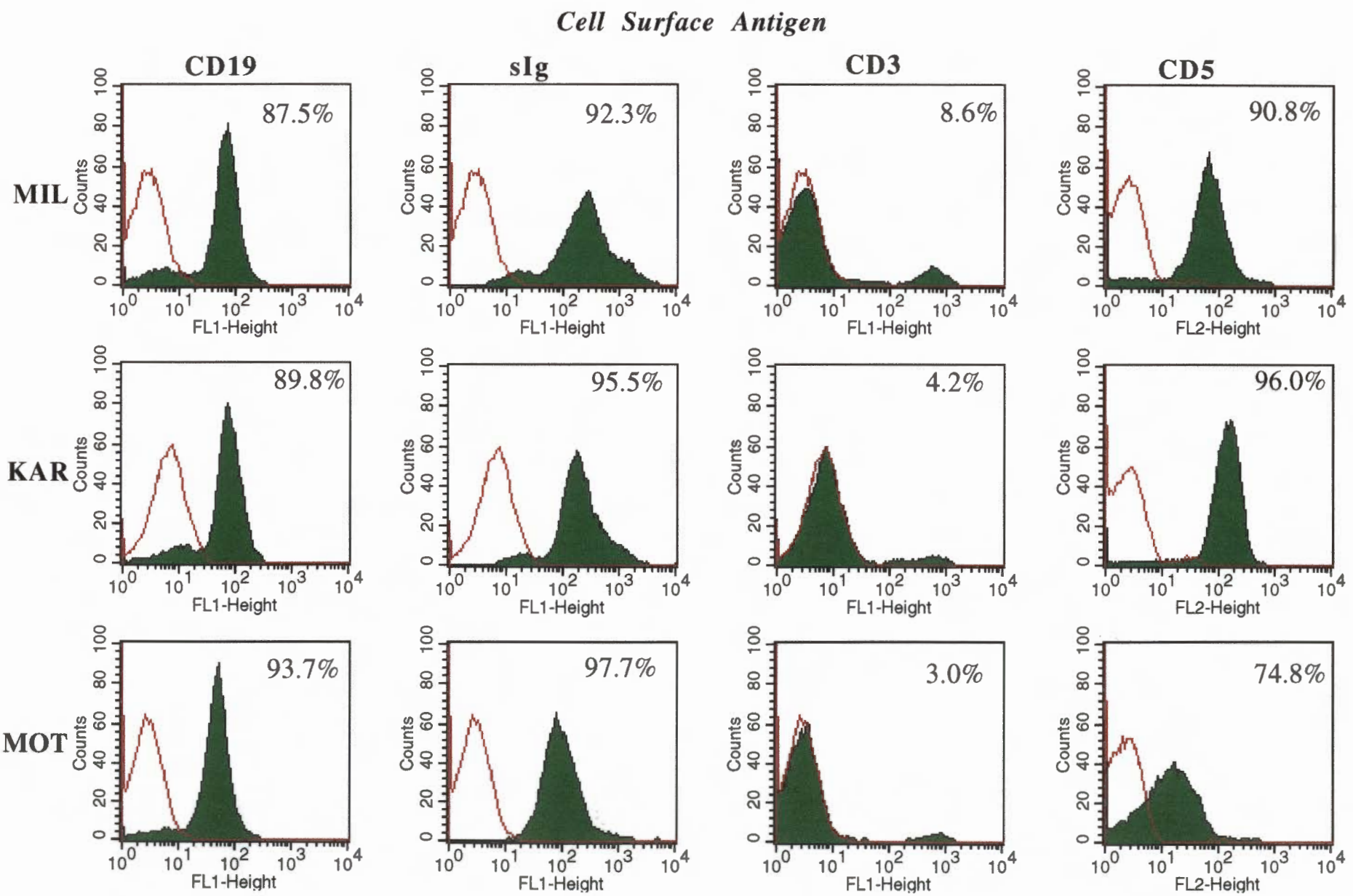
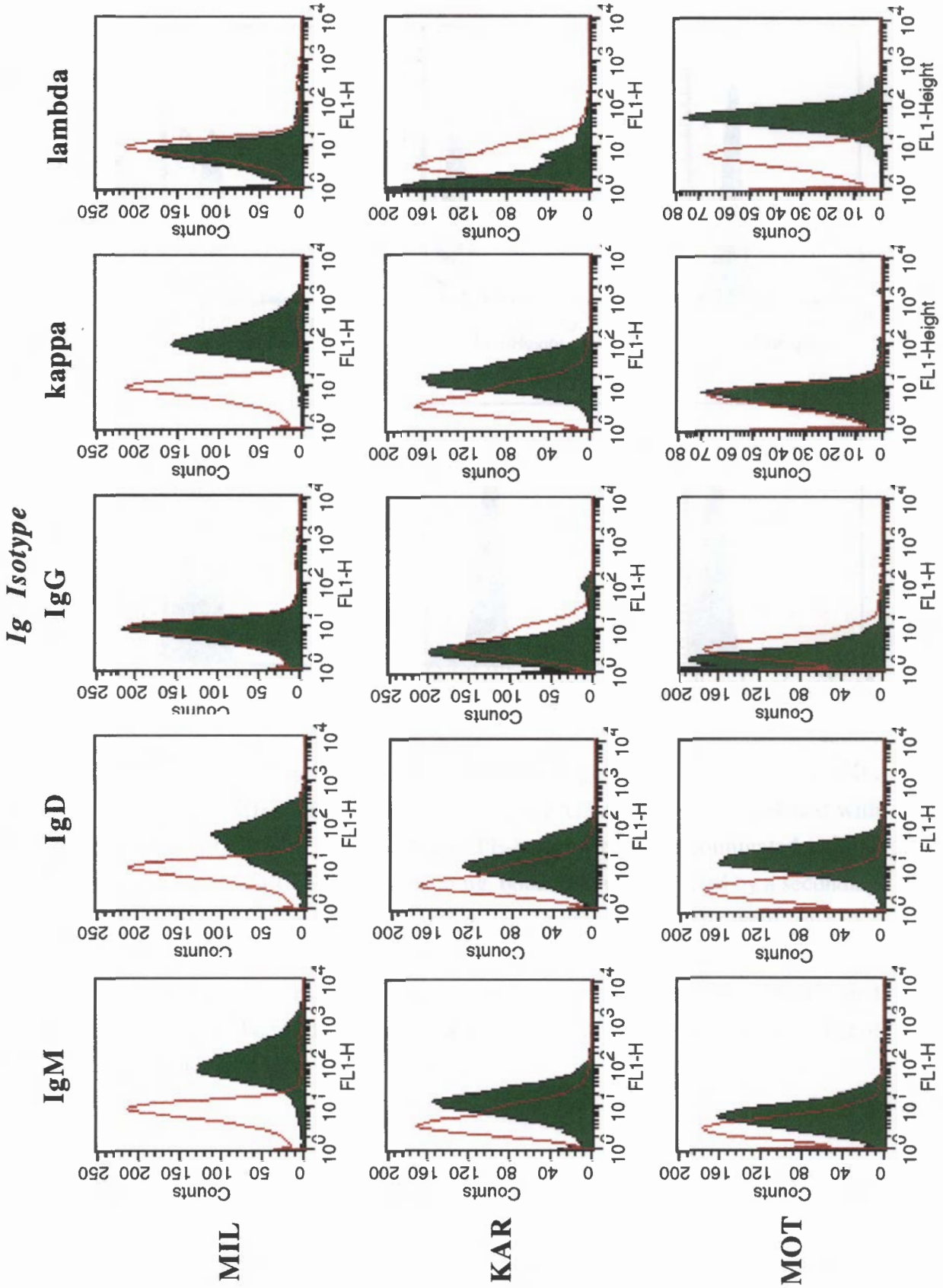

Cell Surface Antigen
CD19
sIg
CD3
CD5
MIL
KAR
MOT
87.5%
92.3%
8.6%
90.8%
89.8%
95.5%
4.2%
96.0%
93.7%
97.7%
3.0%
74.8%
Counts
FL1-Height
FL2-Height

Figure 3.2: Phenotype of T-Depleted CLL B Cell Preparations. CLL B cell preparations were depleted of residual T cells by rosetting with AET-treated SRBCs. Resultant T-depleted B cell preparations from patients MIL (top), KAR (middle) and MOT (lower) were incubated with FITC-anti CD19, FITC-anti Ig, FITC-anti CD3, PE-anti CD5 (solid histograms) or an appropriate isotype control mAb (outline histogram) for 30 minutes at 4°C. Cells were washed 3 times prior to analysis by flow cytometry. The values in each histogram represent the percentage of cells positive for the indicated cell surface Ag. The *x*-axis represents relative fluorescence intensity and the *y*-axis represents cell number.

Cell Surface Antigen
CD19
sIg
CD3
CD5
MIL
KAR
MOT
98.4%
99.8%
0.2%
99.2%
96.0%
98.1%
0.2%
98.0%
97.3%
99.3%
0.1%
76.4%
Counts
FL1-Height
FL2-H
FL2-Height

Figure 3.3: Expression of sIg by CLL B Cells.

Purified B cells from MIL, (top), KAR (middle) and MOT (lower) were incubated with FITC-conjugated $F(ab')_2$ fragments of polyclonal goat Ig specific for human IgM, IgD, IgG, or *kappa* or *lambda* light chains (solid histograms) for 30 minutes at 4°C. The fluorescence intensity of cells incubated with a FITC-conjugated goat antiserum of irrelevant specificity is indicated by the outline histogram. Cells were washed 3 times prior to analysis by flow cytometry. The *x*-axis represents relative fluorescence intensity and the *y*-axis represents cell number.


Ig Isotype
IgM
IgD
IgG
kappa
lambda
MIL
KAR
MOT
Counts
FL1-H
FL1-Height

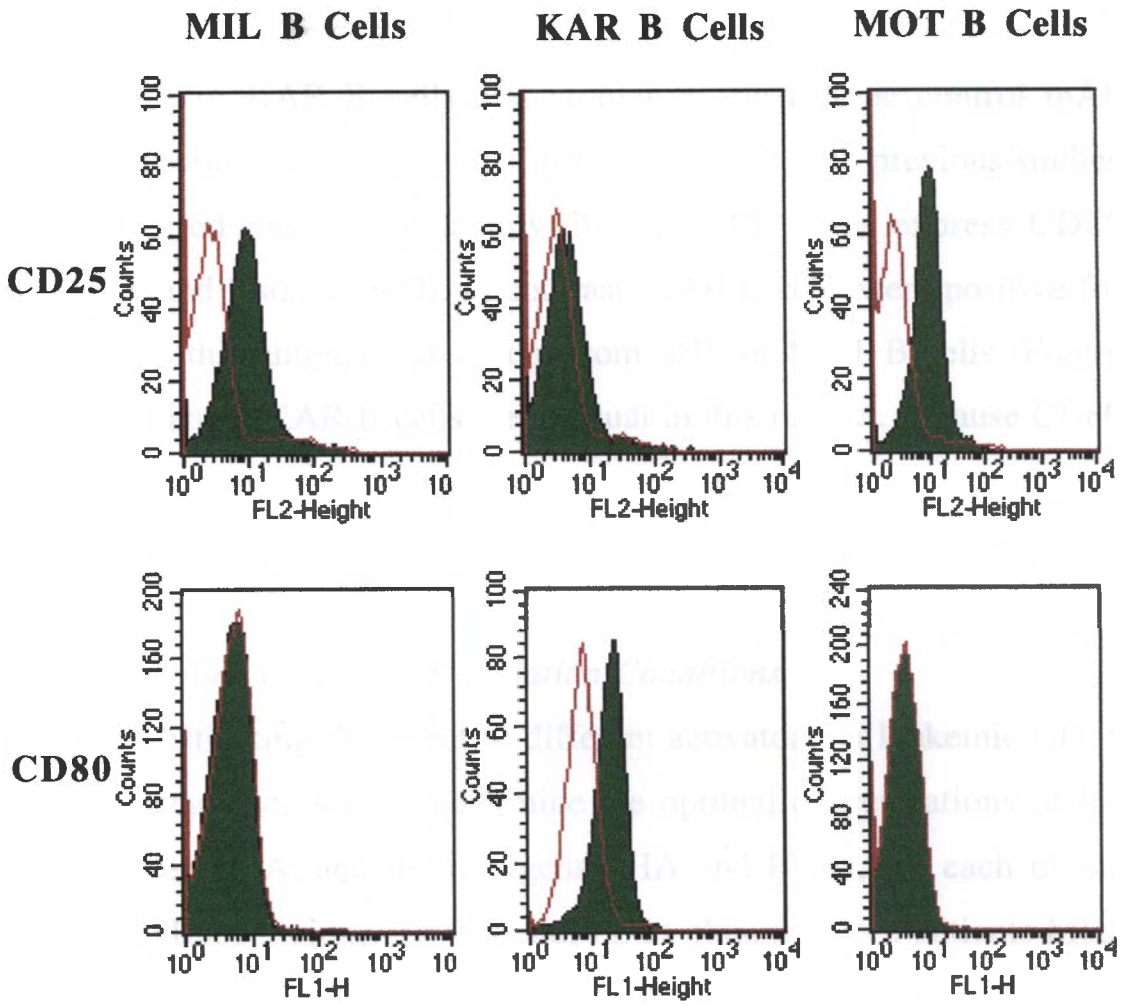


Figure 3.4: Expression of Activation Antigens by CLL B Cells. Purified B cells from MIL (left), KAR (middle) and MOT (right) were incubated with PE-anti-CD25, anti-CD80, or an appropriate PE-conjugated or unconjugated isotype control for 30 minutes at 4°C. For CD80 staining, bound Ab was detected by a secondary incubation with FITC-conjugated sheep-anti mouse Ig antiserum. Cells were washed 3 times prior to analysis by flow cytometry. The *x*-axis represents relative fluorescence intensity and the *y*-axis represents cell number. The expression patterns of CD25 and CD80 are indicated by the solid histograms, while the outline histogram represents that of the isotype control mAb.

antigens CD25 (IL-2 receptor) and CD80 (B-7). MIL and MOT B cells were found to express significant amounts of CD25 while the fluorescence of KAR B cells was similar to the isotype control mAb (Figure 3.4, upper panel). This finding is consistent with previous studies which reported that approximately 50% of CLL cases express CD25 (Freedman and Nadler, 1992). In contrast, KAR B cells were positive for CD80 while this antigen was absent from MIL or MOT B cells (Figure 3.4, lower panel). KAR B cells are unusual in this respect, because CD80 has been reported to be infrequently expressed on leukemic $CD5^+$ B cells obtained from B-CLL patients (Freedman *et al.*, 1987).

3.2. Optimisation of In vitro *Stimulation Conditions*

Prior to investigating the effect of different activators on leukemic $CD5^+$ B cells, it was necessary to determine the optimal concentrations of the phorbol ester PMA, and the mitogens PHA and PWM, for each of the different cell populations. To this end, PBLs obtained from patients MIL, KAR and MOT were cultured in the presence of increasing concentrations of activator for 3-4 days, after which time incorporation of ^{3}H-thymidine was determined. The PBLs responded to PMA, PHA and PWM. However the extent of the response and the optimal concentration of each activator was specific for the different leukemic cell populations assayed. For PHA and PWM, a clear optimal concentration was realised as concentrations exceeding or less than this resulted in reduced responses. Proliferation of the PBLs to PMA, PHA and PWM is illustrated in Figures 3.5 a, b and c, respectively, and the optimal concentration of each activator is listed in Table 3.1.

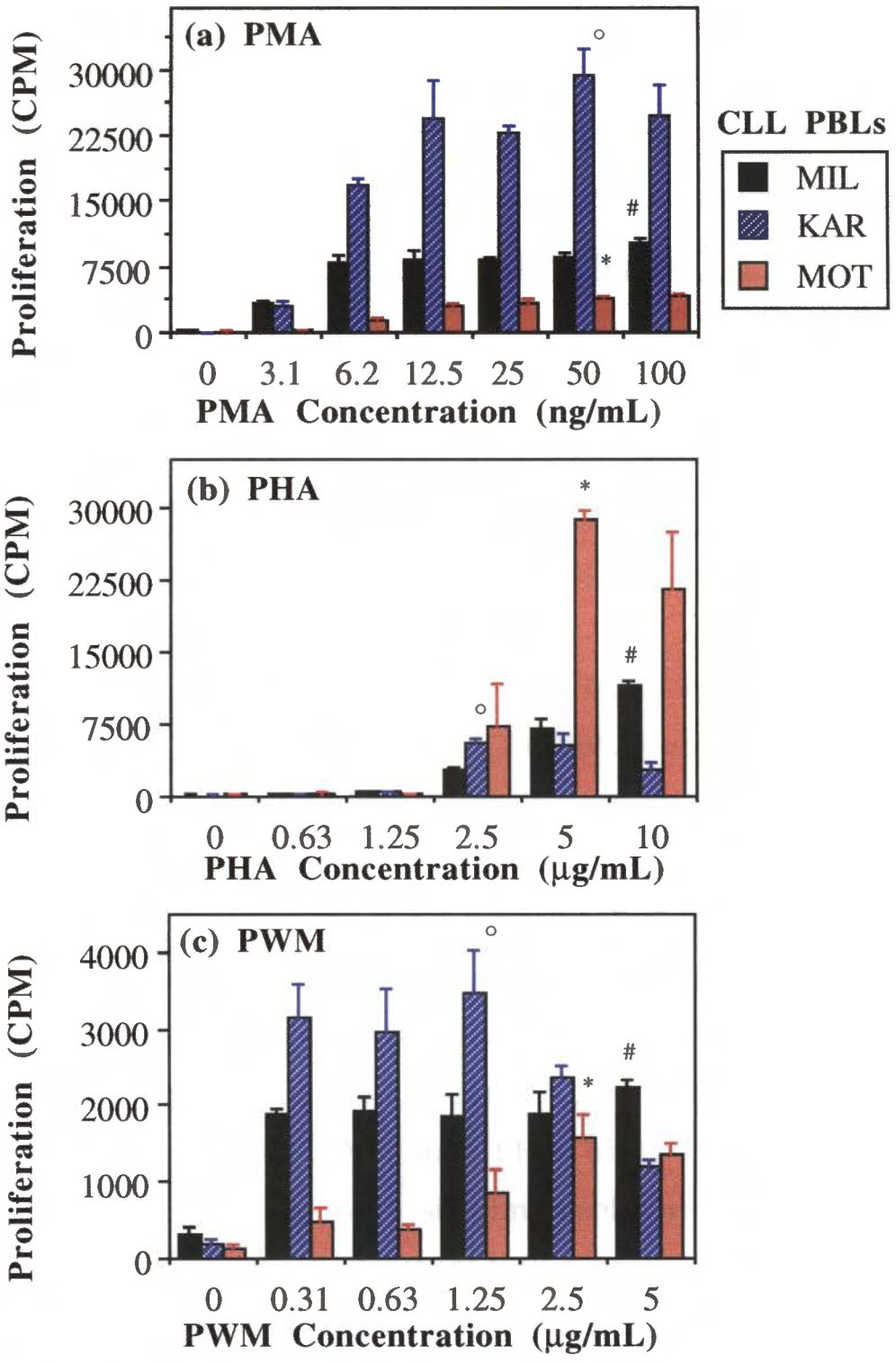


Figure 3.5: Optimisation of PMA, PHA and PWM.

Unfractionated CLL PBLs from patients MIL, KAR and MOT were cultured for 3-4 days in the presence of increasing concentrations of (a) PMA, (b) PHA or (c) PWM. Proliferation was determined by harvesting the cells after an 18 hour pulse with 1 µCi ^{3}H-thymidine. Each column represents the mean counts per minute (cpm) ± 1 s.d. of triplicate samples. Symbols indicate the optimal concentration of each activator for the different CLL PBLs (# MIL; ° KAR; * MOT).

Table 3.1: *Optimal Concentrations of Activators for* In vitro *Stimulation of CLL B Cells*

	Activator		
CLL Patient	PMA (ng/mL)	PHA (μg/mL)	PWM (μg/mL)
MIL	100	10	5
KAR	50	2.5	1.25
MOT	50	5	2.5

Values represent the concentration of each activator that induced maximal uptake of ^{3}H-thymidine during the last 18 hours of a 3-4 day incubation period.

3.3. Kinetics of CLL PBLs Responses Following In vitro *Stimulation*

Unfractionated MIL, KAR and MOT CLL PBLs were cultured for 8 days in the absence or presence of optimal concentrations of PMA, PHA or PWM. These cell preparations were found to undergo proliferation (Figures 3.6 a, c and e) and RNA synthesis (Figures 3.6 b, d and f) at rates significantly greater than those of unstimulated cells. Maximal proliferation and RNA synthesis varied for each patient and also with the nature of the stimulating agent. Generally, this occurred following 4-8 days of culture, with PMA and PHA being potent inducers of proliferation and RNA synthesis. Immunofluorescence analysis of mitogen-activated PBLs from patient MIL confirmed proliferation of the leukemic $CD5^+$ B cells. This was indicated by the relative proportions of leukemic B cells and normal T cells remaining constant during *in vitro* culture. Prior to *in vitro* culture, the preparation of MIL CLL PBLs was comprised of 93% $sIg^+/CD5^+$ B cells and 7.8% $CD3^+CD5^+$ T cells. After 4 days, there were 93±1% and 8.6±1.4% of leukemic $CD5^+$ B and normal T cells, respectively, in the PHA-stimulated preparations, while

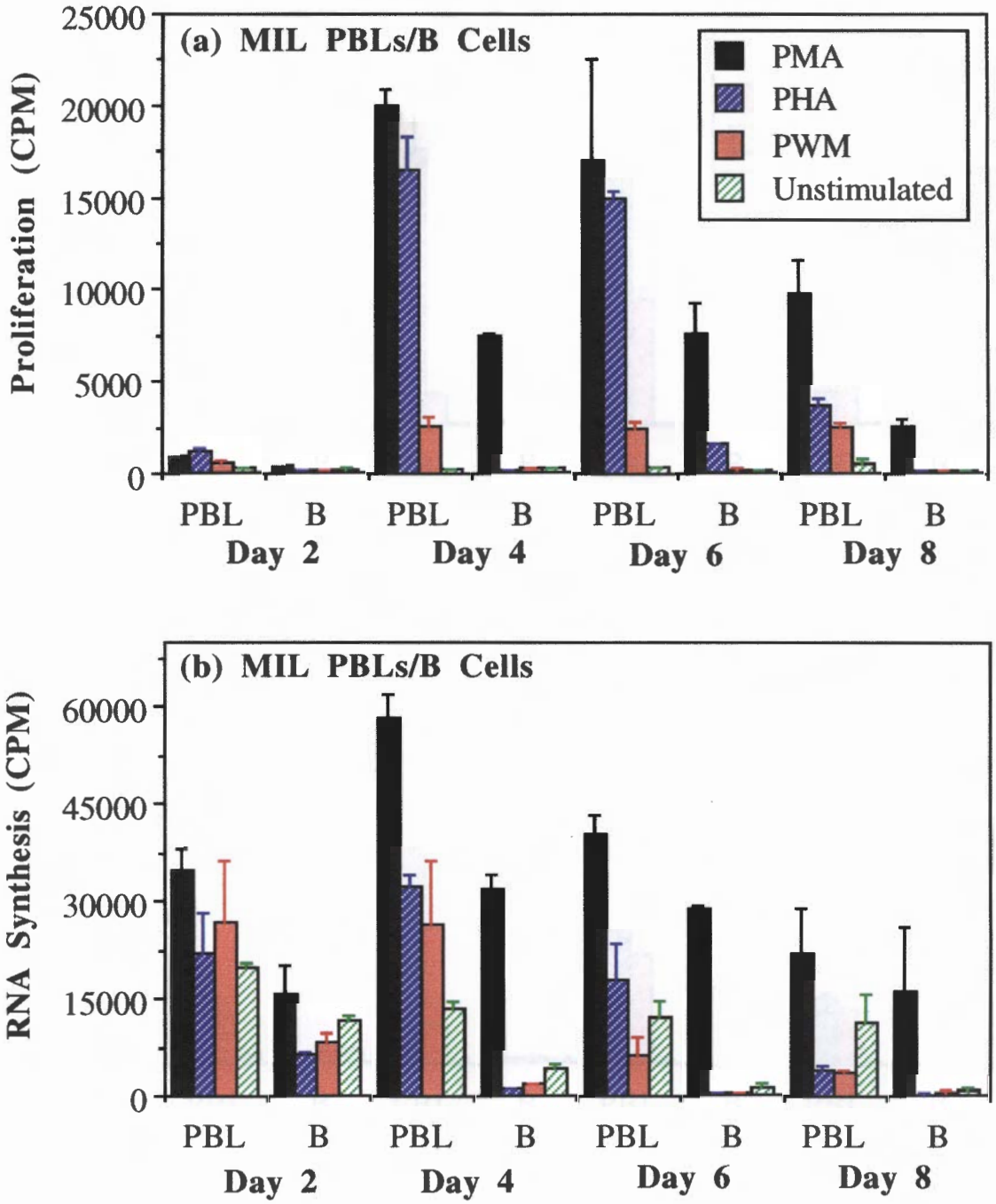


Figure 3.6: PMA-induced Activation of Unfractionated PBLs and Purified B cells from B-CLL Patients.

Unfractionated PBLs (PBL) and purified B cells (B) from patients MIL (a, b), KAR (c, d) and MOT (e, f) were cultured for 8 days in the absence or presence of PMA, PHA or PWM. Proliferation (a, c, e) and RNA synthesis (b, d, f) were determined on days 2, 4, 6 and 8 by pulsing the cultured cells with 1 μCi of ^{3}H-thymidine and ^{3}H-uridine, respectively, and harvesting 18 hours later. Each column represents the mean cpm ± 1 s.d. of triplicate samples, and is representative of at least two independent experiments.

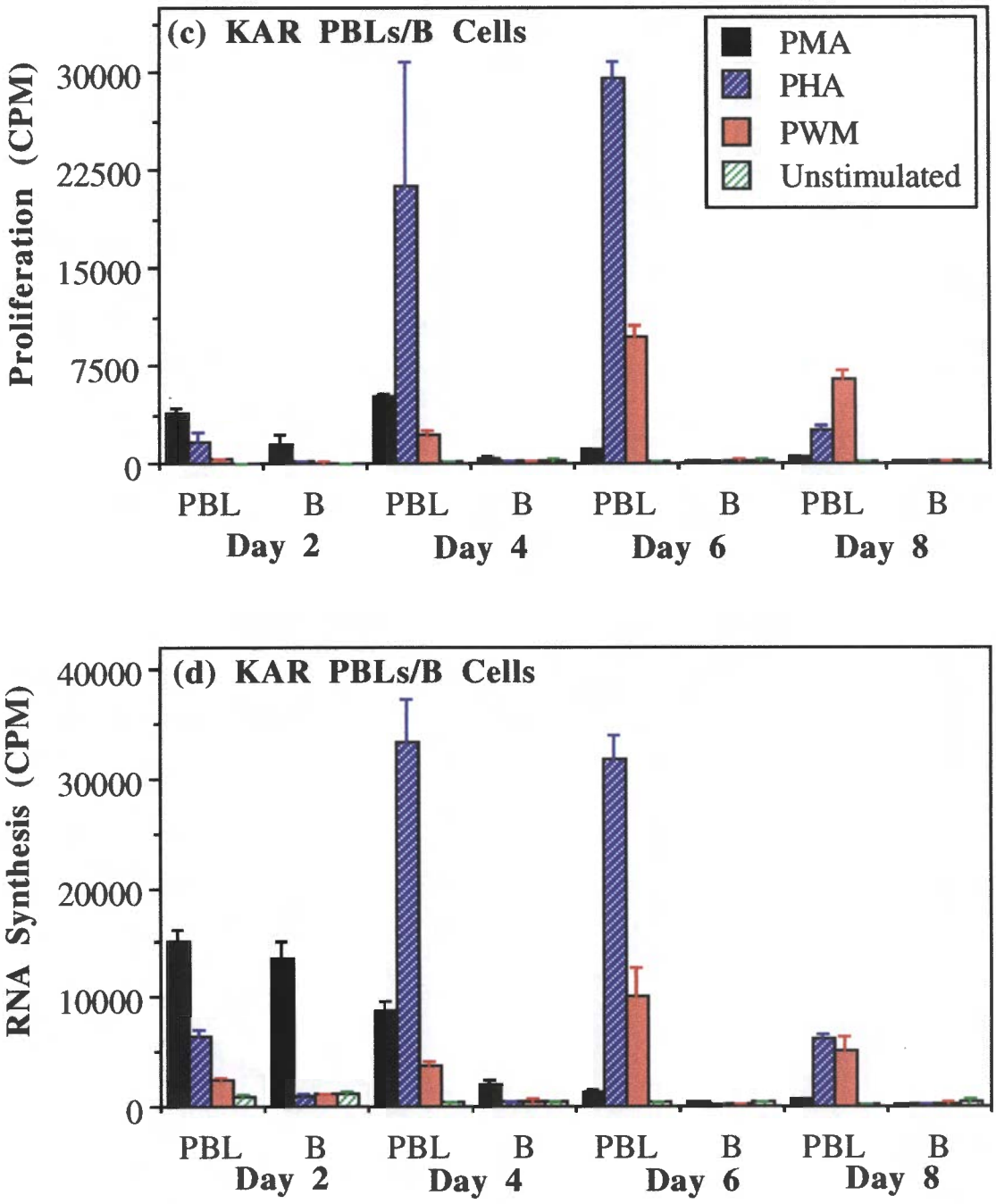


Figure 3.6: PMA-induced Activation of Unfractionated PBLs and Purified B cells from B-CLL Patients (continued).

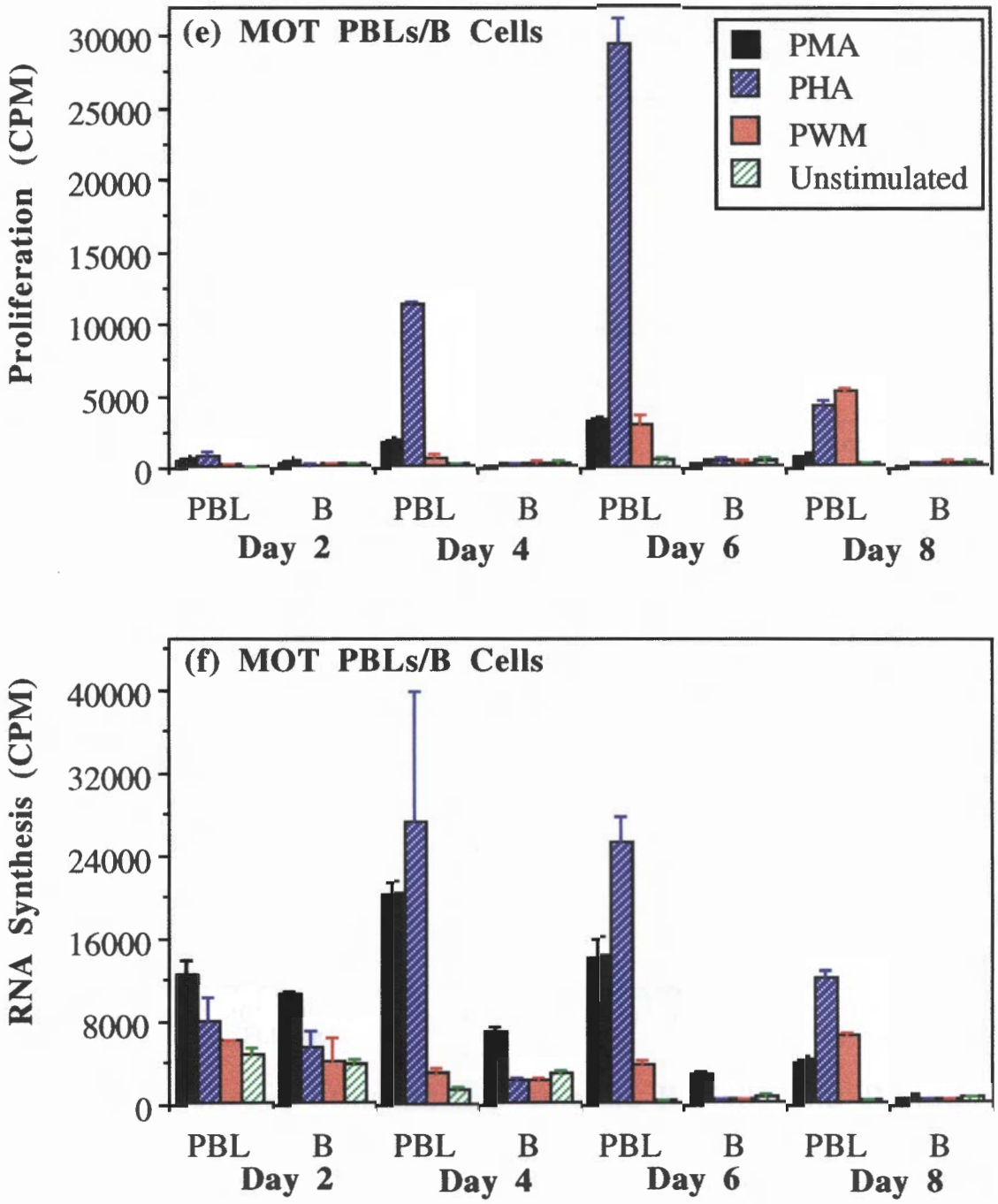


Figure 3.6: PMA-induced Activation of Unfractionated PBLs and Purified B cells from B-CLL Patients (continued).

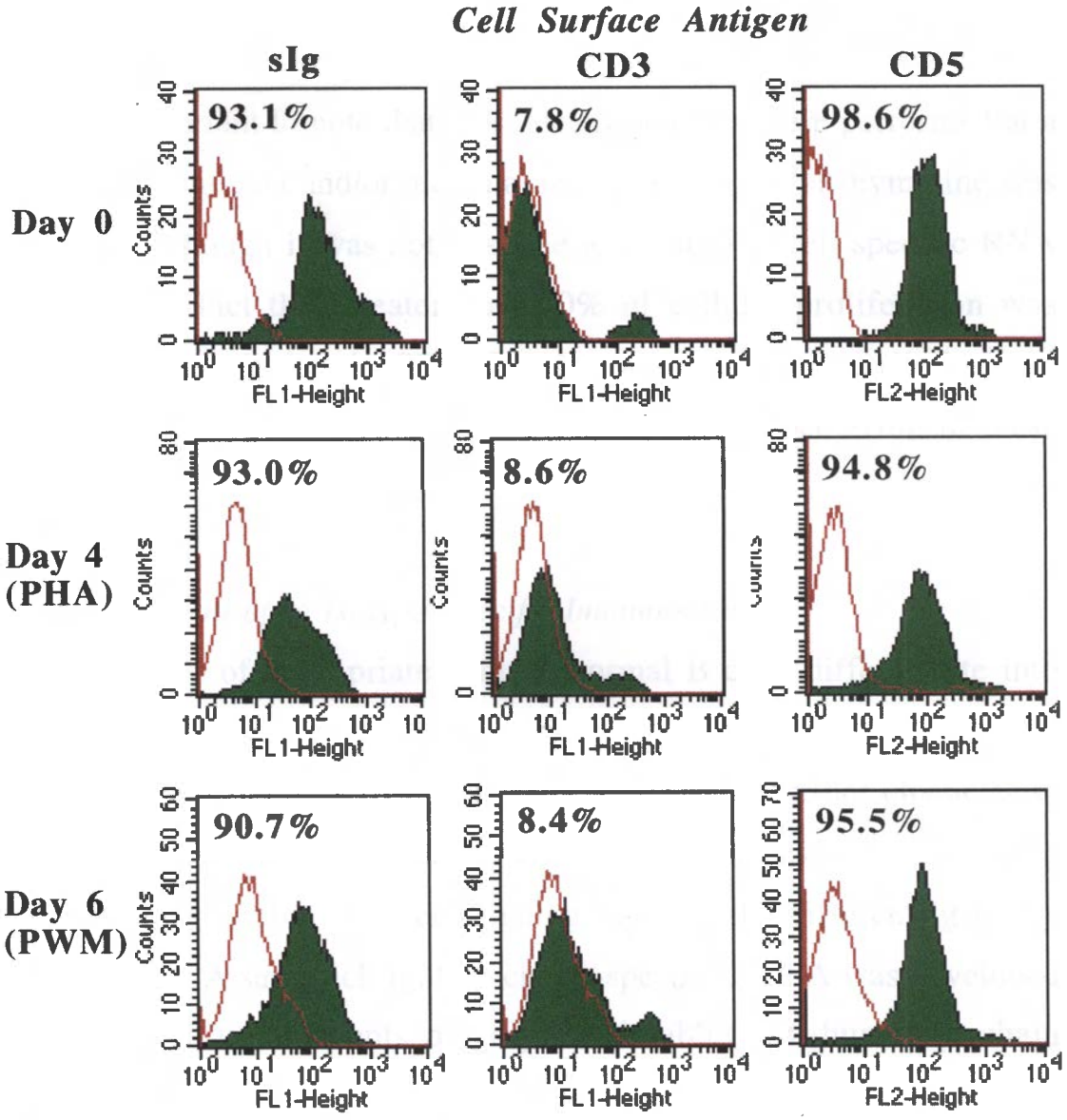


Figure 3.7: Mitogen-Stimulated Leukemic B Cells and Residual T Cells Proliferate at a Similar Rate.

PBLs from CLL patient MIL were cultured for 4 or 6 days with PHA (middle panel) and PWM (lower panel), respectively. After these times, the percentage of leukemic B and normal T cells was determined by incubation with FITC-anti-Ig, FITC-anti-CD3 or PE-anti-CD5 (solid histogram). The outline histogram represents the fluorescence of cells incubated with an isotype-matched control Ab. The percentage of sIg^+, $CD3^+$ and $CD5^+$ cells present in the starting population (Day 0) is indicated by the histograms in the upper panel. The value in each histogram plot represents the % of cells expressing the Ag of interest.

after 6 days, the percentages of leukemic CD5+ B and normal T cells in the PWM-stimulated preparations were 90.7±1% and 8.4±0.5% (Figure 3.7). It is important to note that these determinations were performed at a time when significant and/or maximal incorporation of ^{3}H-thymidine was occurring. Although it was not possible to identify B cell specific RNA synthesis, the fact that greater than 90% of cellular proliferation was derived from the leukemic CD5+ B cells suggests that a similarly large proportion of the RNA synthesis observed was also occurring in these cells.

3.4. Development of Ig Isotype-Specific Immunoassays

In the presence of appropriate stimuli, normal B cells differentiate into plasma cells, as evidenced by the appearance of Ig molecules in the culture supernatants of activated B cells. In order to further characterise the *in vitro* activation of CLL B cells, it was necessary to develop immunoassays capable of discriminating between the different Ig heavy chain isotypes. A sandwich IgM (μ-chain)-specific ELISA was developed using the $F(ab')_2$ fragments of polyclonal rabbit anti-human μ chain ($F(ab')_2$ anti-μ) as the capture Ab. For the detection of secreted IgG molecules, soluble Protein A was used to capture IgG. Bound IgM and IgG were detected by alkaline phosphatase-conjugated goat antiserum specific for human μ and γ chains, respectively, and subsequently visualised by the addition of pNPP. As detailed in Figure 3.8, when the capture reagents were titrated, it was found that human IgM and IgG were optimally detected by 20 μg/mL $F(ab')_2$ anti-μ and 100 μg/mL Protein A, respectively. IgM was not detected when assayed in the γ-chain-specific ELISA (Figure 3.9a). In contrast, levels of IgG >0.2 μg/mL displayed significant cross-reactivity in the μ-chain specific ELISA (Figure 3.9b). However, because the linear section of the IgM

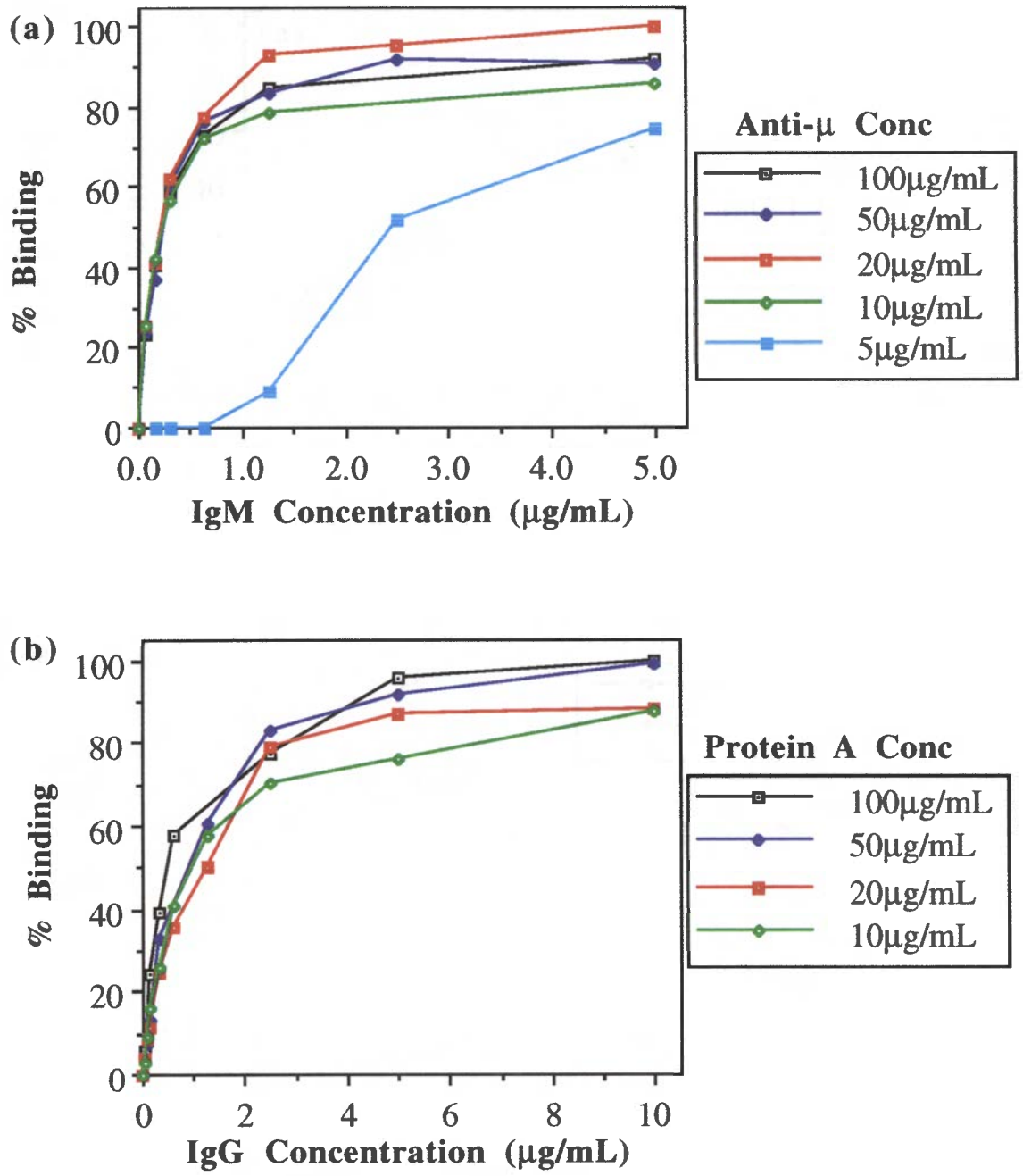


Figure 3.8: Development of Isotype-Specific Immunoassays.
Ninety-six well microtitre plates were coated with different concentrations of (a) $F(ab')_2$ fragments of rabbit polyclonal Ig specific for human IgM (µ-chain specific) or (b) soluble Protein A. Dilutions of human IgM or IgG standards were added to the wells and subsequently detected with alkaline phosphatase-conjugated anti-human µ or γ chain specific goat antiserum, respectively. Results are expressed as the percentage of the maximum absorbance value obtained for the highest concentration of human IgM or IgG standard.

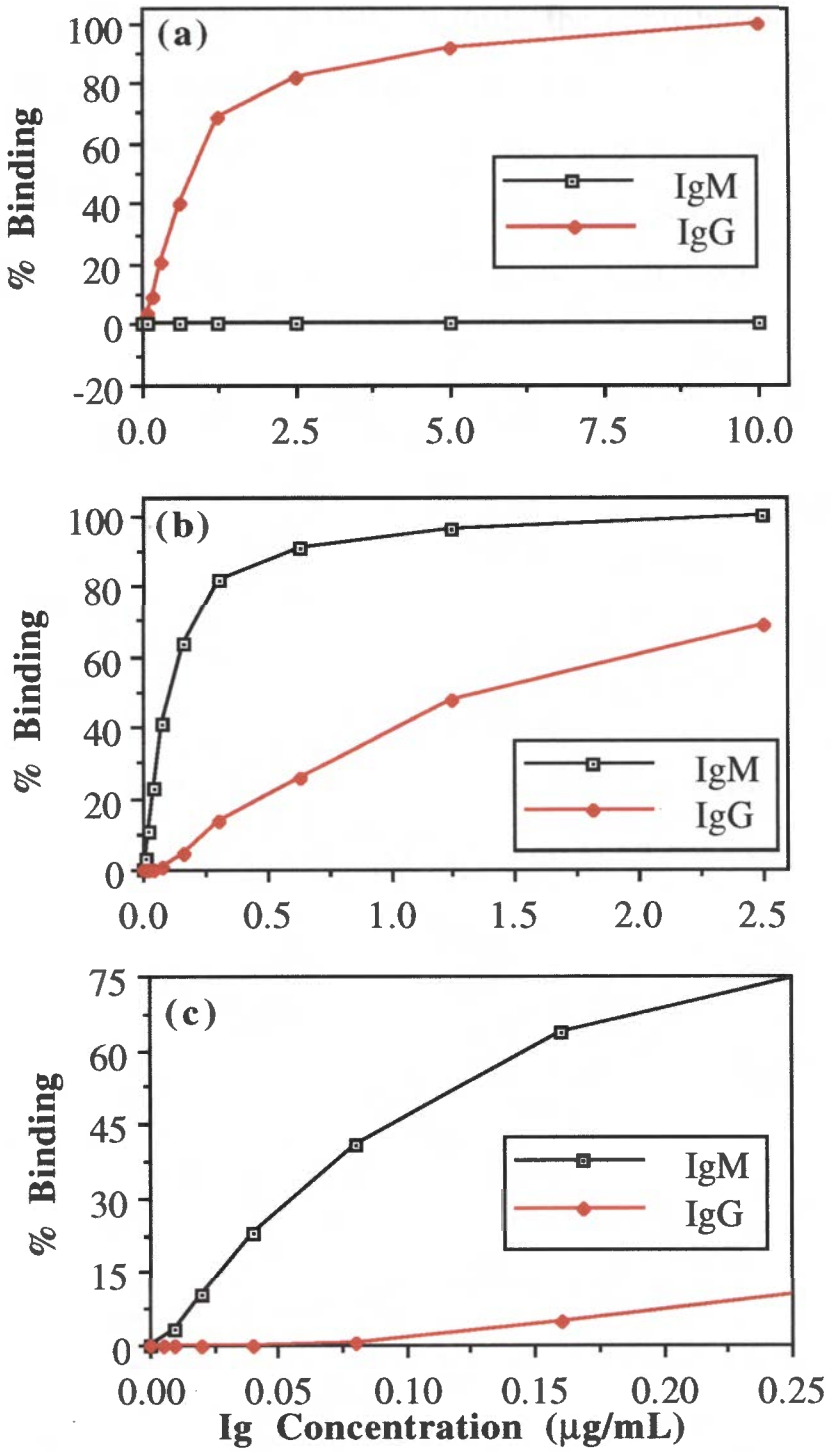


Figure 3.9: Cross-Reactivity of IgM and IgG in the Isotype-Specific Immunoassays.

Two 96-well microtitre plates were coated with either (a) Protein A or (b) $F(ab')_2$ anti-µ. Human IgG and IgM standards were added to both plates and the degree of cross-reactivity assessed by detecting bound Ig with alkaline phosphatase-conjugated γ-chain specific or µ-chain specific antiserum. Results are expressed as the percentage of the binding of the highest concentration of the human Ig standard. (c) represents the cross-reactivity of IgG in the µ-chain specific ELISA over the linear range of this curve.

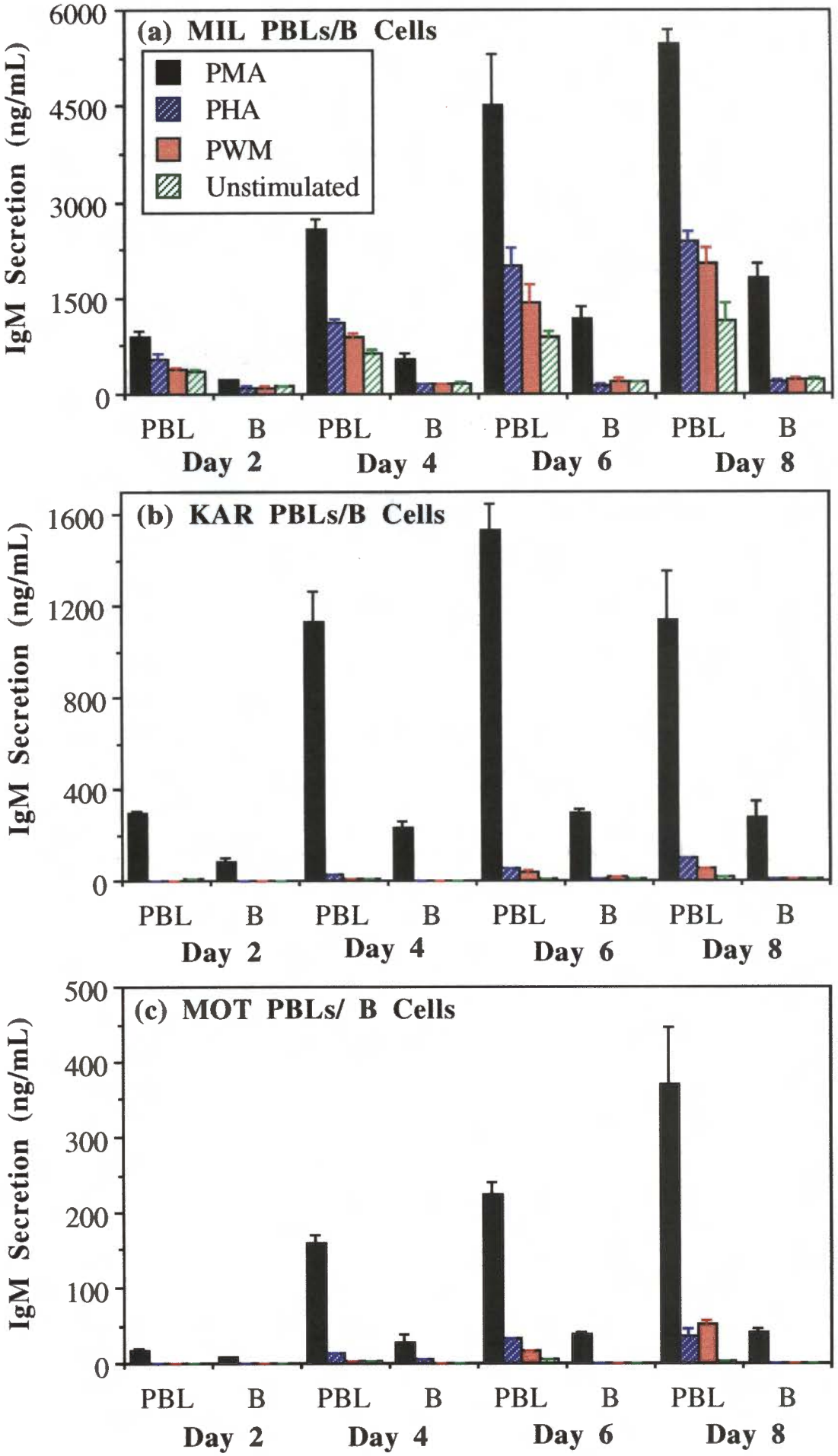
standard curve ranged from 0-0.2 μg/mL, the contribution of IgG to the total absorbance value would be insignificant because the absorbance due to the corresponding concentrations of IgG was minimal (Figure 3.9c). The lower limit of detection of each of these assays was 5 ng/mL of IgM and 10ng/mL of IgG. Variations on these assays allowed for the detection of Ig expressing either *kappa* or *lambda* light chains. Here, the detecting secondary Ab was a light chain specific antiserum. The sensitivity of these assays was similar to that for the μ-chain-specific ELISA (data not shown).

3.5. Stimulation of CLL PBLs with Cellular Activators Induces IgM Secretion

In addition to proliferation and RNA synthesis, secretion of IgM by the unfractionated PBLs from the 3 patients with B-CLL was either enhanced or induced following *in vitro* stimulation with PMA, PHA and PWM (Figure 3.10a-c). Unstimulated PBLs from the patient MIL spontaneously secreted IgM. However, spontaneous IgM secretion by unfractionated PBLs from the other 2 patients was minimal (<15 ng/mL). After 2 days, IgM was detectable in most supernatants obtained from activated cells and the amount secreted continued to increase even after maximal proliferation had occurred (Figure 3.10). Consistent with its effect on proliferation and RNA synthesis, PMA was the most effective inducer of IgM secretion, yielding concentrations 5-100 times greater than that of the unstimulated cells. Although not as effective as PMA at inducing IgM secretion, both PHA and PWM were capable of inducing secretion of IgM at concentrations ~2-20 times greater than that of unstimulated PBLs. Light chain specific immunoassays indicated that the secreted IgM bore the same light chain isotype as that expressed by the malignant $CD5^+$ B cells. As shown in Figure 3.11, IgM of the specific light chain isotype

Figure 3.10: *In vitro* Activation of Leukemic CD5+ B Cells Induces IgM Secretion.

PBLs (PBL) or T-depleted preparations (B) from patients MIL (a), KAR (b) and MOT (c) were cultured for 8 days in the absence or presence of PMA, PHA or PWM. IgM secretion was determined by assaying culture supernatants after 2, 4, 6 and 8 days of culture. Each point represents the mean ng IgM/mL ± 1 s.d. of at least duplicate determinations, and is representative of at least two independent experiments.


(a) MIL PBLs/B Cells
PMA
PHA
PWM
Unstimulated
IgM Secretion (ng/mL)
6000
4500
3000
1500
0
PBL B PBL B PBL B PBL B
Day 2 Day 4 Day 6 Day 8
(b) KAR PBLs/B Cells
IgM Secretion (ng/mL)
1600
1200
800
400
0
PBL B PBL B PBL B PBL B
Day 2 Day 4 Day 6 Day 8
(c) MOT PBLs/ B Cells
IgM Secretion (ng/mL)
500
400
300
200
100
0
PBL B PBL B PBL B PBL B
Day 2 Day 4 Day 6 Day 8

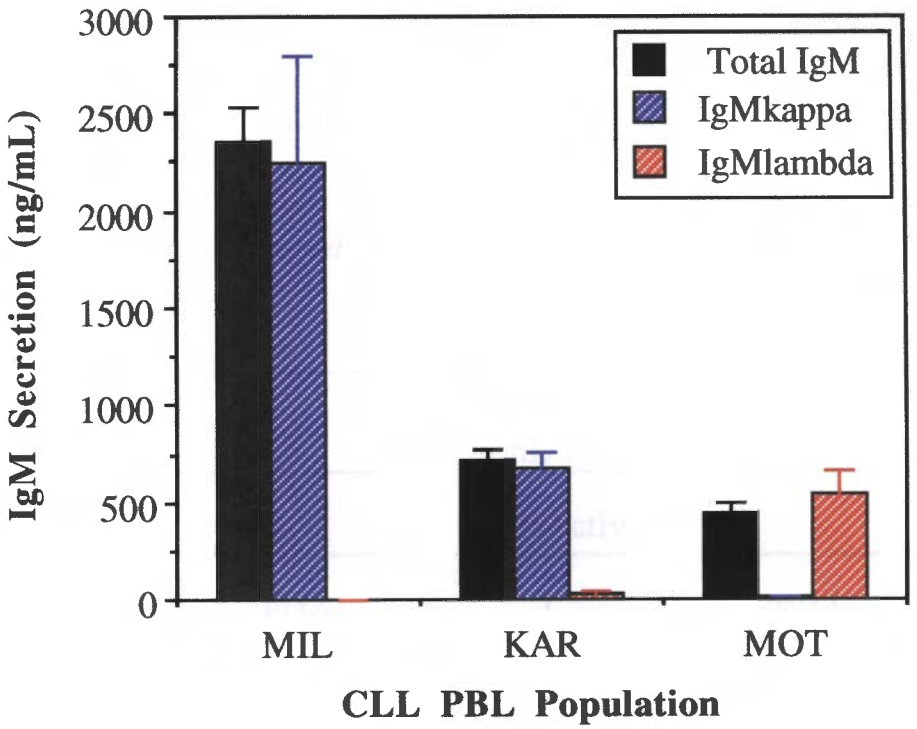


Figure 3.11: Light-chain isotype of IgM secreted by PMA-stimulated CLL PBLs.

Supernatants from PMA-stimulated PBLs were collected after 4 (MIL) or 8 days (KAR, MOT) of culture. The amounts of total IgM, IgM*kappa* and IgM*lambda* were determined by μ-chain specific and light-chain specific immunoassays. Data represent mean ng/mL ± 1 s.d. of samples assayed at 1 or more dilutions.

represented >93% of the total amount of IgM secreted by the CLL PBLs following stimulation with PMA. Less than 5% of the secreted IgM expressed the alternate light chain isotype. Immunoassays performed on supernatants from mitogen-stimulated unfractionated PBL preparations

Table 3.2: *IgM Secreted by Mitogen-Stimulated CLL PBLs is Monoclonal*

	IgM Secretion ng/mL (s.d.)					
	Cellular Activator					
	PHA			PWM		
Cells	Total	IgM*k*	IgMλ	Total	IgM*k*	IgMλ
MIL	1800 (250)	1520 (211)	120 (0)	1280 (80)	1317 (280)	<5
KAR	24 (11)	30 (8)	<5	14 (3)	19 (4)	<5
MOT	NT	<5	213 (38)	32 (11)	<5	35 (1)

Unfractionated CLL PBLs from the 3 different patients were cultured with the optimal concentration of PHA or PWM. The amounts of total IgM, IgM*kappa* and IgM*lambda* secreted by the activated cells were determined by μ-chain specific and light chain specific immunoassays. Similar results were obtained in two or three independent experiments. NT - not tested.

Additional evidence that the secreted IgM was derived from the malignant clone of cells came from experiments demonstrating that the IgM bound mouse Ig. It has been previously found that mouse Ig binds to leukemic CD5+ B cells via a low-affinity mechanism mediated by the polyreactive Ig expressed on the surface of these cells (Weston and Raison, 1991).

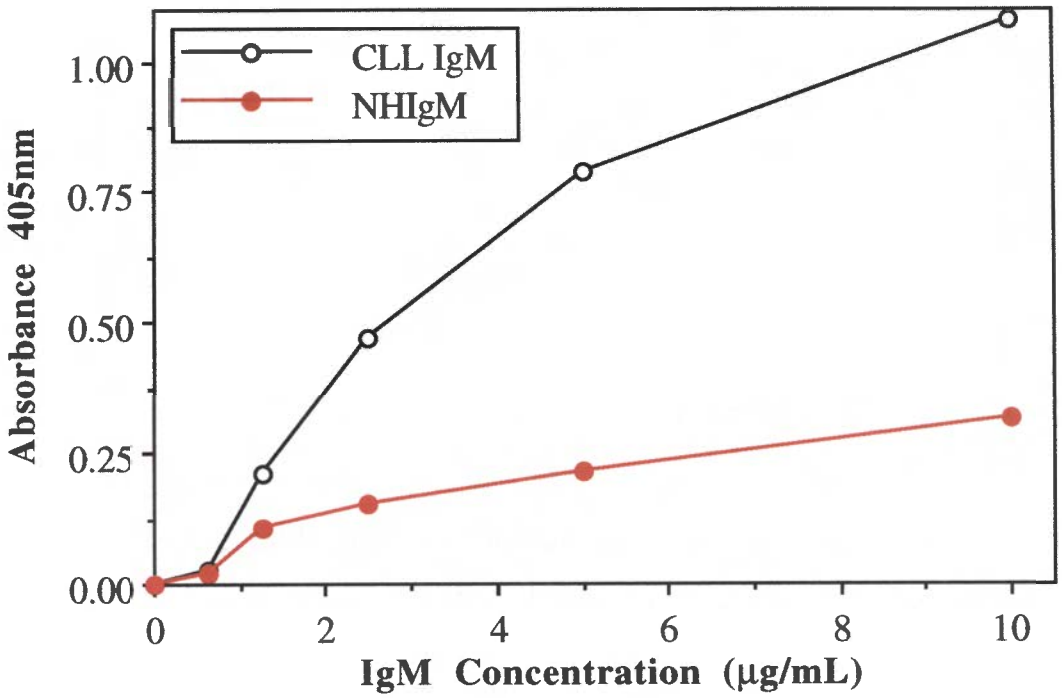


Figure 3.12: Binding of IgM Secreted by CLL B Cells to Mouse Ig.

Mouse IgG (K121; 100 µg/mL) was immobilised to the wells of a 96-well microtitre plate and non-specific binding sites blocked with 3% BSA. IgM present in supernatants of activated MIL CLL B cells (CLL IgM) or normal human serum (containing a known amount of IgM; NHIgM) were added to the wells and serially diluted. Bound human IgM was detected by alkaline phosphatase-conjugated anti µ-chain specific antiserum and visualised following the addition of pNPP. The resultant absorbance was measured at 405nm. This result is representative of multiple independent experiments which used IgM obtained from MIL B cells that were unstimulated or activated *in vitro* with PMA, PHA or PWM.

Figure 3.12 shows the binding of IgM contained in supernatants of activated MIL CLL PBLs to mouse IgG (K121), compared to normal human IgM present in a control serum sample (NHIgM). At the highest concentration of IgM assayed, CLL IgM bound to mouse IgG at a level that was 5 times greater than that exhibited by the NHIgM. The observation that there is a low degree of binding by the NHIgM possibly reflects the presence of polyreactive antibodies that have been found to exist in the peripheral blood of normal healthy individuals (Casali and Notkins, 1989). Taken with the results of the light chain-specific immunoassays, these data demonstrate that the secreted IgM is likely to be homogeneous and derived from the malignant B cells.

IgG was not detectable in the supernatants of KAR or MOT CLL PBLs following activation with PMA, PHA or PWM (data not shown). Similarly, PMA did not result in the production of IgG by MIL PBLs (Figure 3.13). However, the mitogens did give rise to secreted IgG in cultures of MIL PBLs. Compared to IgM, the amounts of secreted IgG were relatively low and were not evident until day 4, after which time there was increased secretion of IgG (Figure 3.13). The delayed appearance of IgG is suggestive of isotype switching, yet further characterisation of the IgG molecules revealed the presence of both classes of light chains, indicating a polyclonal response (data not shown). That is, unlike the vast majority of secreted IgM, the IgG appeared to be produced by the residual population of normal B cells present at a frequency of ~1%. Thus, the detection of IgG at a later stage of the culture period could be explained by the production of IgG by only a small proportion of normal B cells.

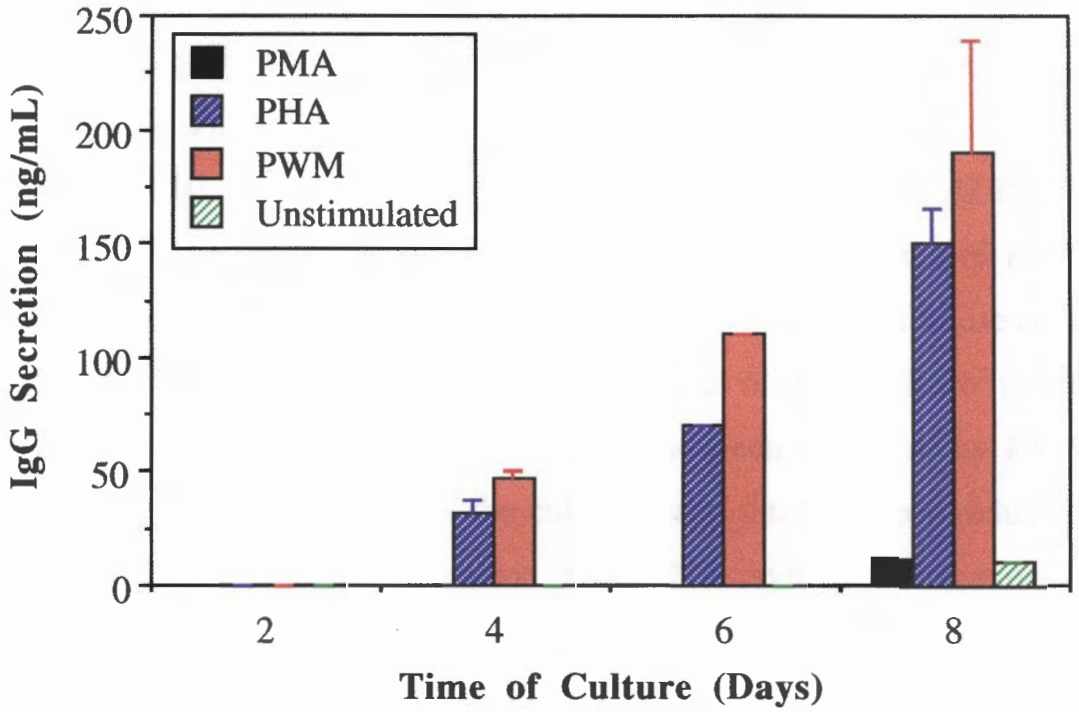


Figure 3.13: Mitogenic-stimulation of MIL CLL PBLs Results in the Secretion of IgG.

MIL CLL PBLs were cultured for 8 days in the absence or presence of PMA, PHA or PWM. IgG secretion was determined by assaying culture supernatants 2, 4, 6 and 8 days after the initiation of the culture. Each point represents the mean ng IgG/mL ± 1 s.d. of triplicate determinations and is representative of two independent experiments.

3.6. PMA Induces Cellular Activation In the Absence of T Cells

T-depleted B cell preparations purified from the peripheral blood of the 3 B-CLL patients were cultured under the same conditions as described above. These purified B cell preparations continued to respond to PMA (Figures 3.6a-f), as indicated by either proliferation (2/3 B cell populations; MIL and KAR) and/or enhanced RNA synthesis (3/3 B cell populations). The proliferation of KAR B cells in the presence of PMA may have been greater than that detected because PMA caused these cells to become extremely adherent to the surface of the culture plate. Following harvest, many KAR B cells that had been activated by PMA remained attached to the wells of the culture plate, thus possibly reducing the actual amount of proliferation and RNA synthesis measured. However, the rate of proliferation and/or RNA synthesis of the T cell-depleted preparations was less (30-80%) than that of the unfractionated PBLs. Furthermore, in the absence of T cells, both proliferation and RNA synthesis by the purified B cells from patients MOT and KAR peaked earlier and were less sustained in response to PMA than proliferation and RNA synthesis of the unfractionated PBL preparations (Figure 3.6).

PMA-stimulated purified leukemic $CD5^+$ B cells continued to secrete significant levels of IgM (Figure 3.10). However, as with proliferation and RNA synthesis, the amount of IgM secreted by the purified B cells from the 3 patients in response to PMA was only ~20-40% of that secreted by the PBLs from these patients. The amount of IgM spontaneously secreted by unstimulated MIL purified B cells was also reduced to a similar degree, compared to the unstimulated PBL preparation. Despite this reduction the levels of IgM secreted by the

PMA-stimulated purified B cells remained significantly greater than that secreted by the unstimulated B cells.

PMA-stimulated B cells also displayed an increase in forward and 90° angle light scattering properties, compared to unstimulated cells. This indicates an increase in both size and internal complexity (granularity) of these cells since forward and 90° angle light scatter are associated with cell size and granularity. Figure 3.14 shows the histograms of forward and 90° angle light scatter of PMA-stimulated leukemic $CD5^+$ B cells, compared to uncultured cells. The viable cells in unstimulated cultures had the light scattering characteristics of freshly prepared uncultured lymphocytes, whereas in the presence of PMA both the forward and 90° angle light scatter of leukemic $CD5^+$ B cells were significantly increased. It is clear from these results that leukemic $CD5^+$ B cells undergo lymphoblastoid transformation following *in vitro* stimulation with PMA.

3.7. T Cell Depletion of CLL PBLs Abrogates Mitogen-Induced Activation and Differentiation

In contrast to PMA, the mitogens PHA and PWM failed to induce significant activation or differentiation of the purified B cell preparations. The rates of proliferation (Figure 3.6a, c, e), RNA synthesis (Figure 3.6b, d, f) and IgM secretion (Figure 3.10) by the purified leukemic $CD5^+$ B cell preparations were reduced by greater than 90% following depletion of the residual T cells.

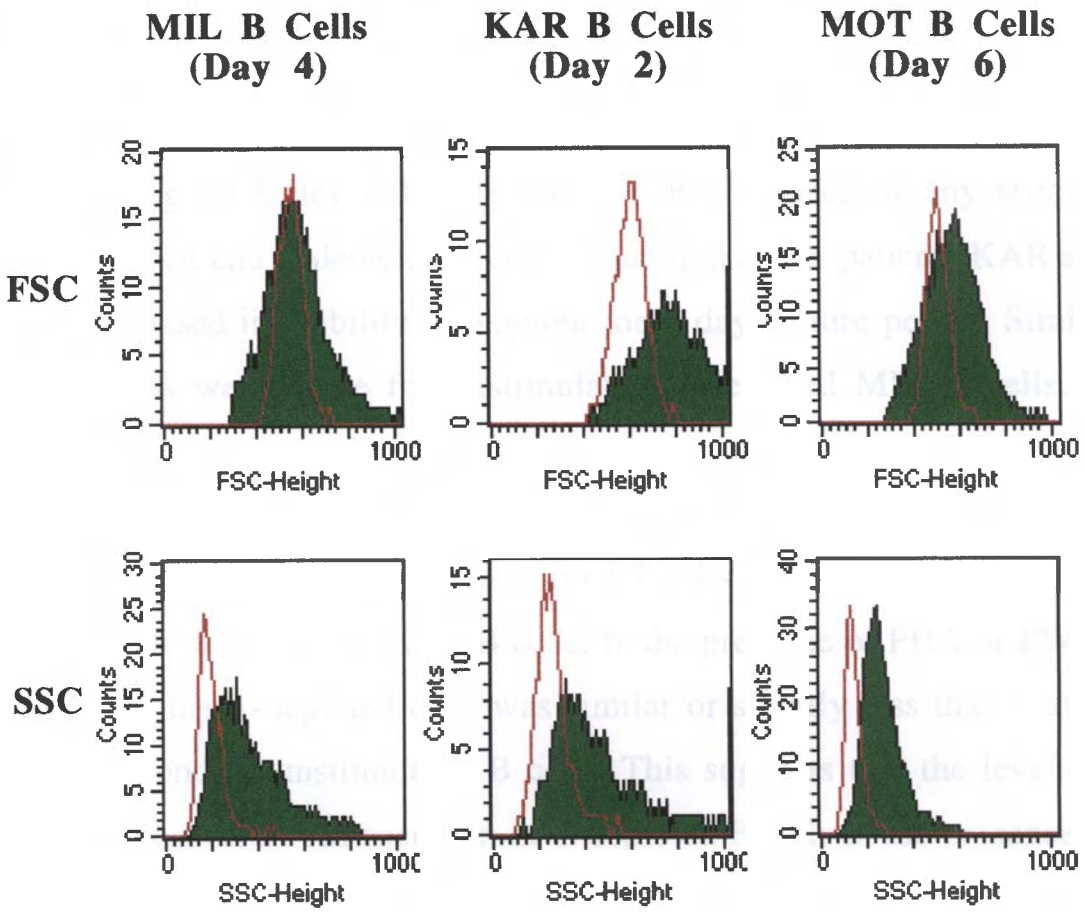


Figure 3.14: PMA Activation Induces Lymphoblastoid Transformation.

Purified leukemic CD5+ B cells from patients MIL, KAR and MOT were cultured *in vitro* with and without PMA for 4, 2 and 6 days, respectively. The light scattering characteristics of viable cultured cells were then determined by flow cytometric analysis. The solid histogram represents the forward (FSC; upper panel) and 90° angle (SSC; lower panel) light scatter of PMA-stimulated leukemic B cells while the outline histogram represents that of unstimulated cells.

3.8. Effect of Residual T Cells on Leukemic B Cell Viability

The viability of unstimulated, PMA-stimulated or mitogen-stimulated CLL PBLs and purified leukemic CD5+ B cells was assessed at various times during an 8 day culture period. In the absence of any stimuli, unfractionated and T-depleted B cell preparations from patients KAR and MOT decreased in viability throughout the 8 day culture period. Similar observations were made for unstimulated T-depleted MIL B cells. In contrast, at the completion of the culture period, the viability of unstimulated MIL CLL PBLs remained at ~80% (Figure 3.15). Thus, in 2/3 cases, the presence of residual normal T cells had no effect on the rate of cell death of leukemic CD5+ B cells. In the presence of PHA or PWM, viability of the T-depleted cells was similar or slightly less than that of the corresponding unstimulated B cells. This suggests that the levels of RNA synthesis and IgM secretion of T-depleted B cells in the presence of mitogens may have been less than unstimulated cells because there were fewer viable cells following activation with PHA or PWM. However, activation of CLL PBLs with these mitogens resulted in there being significantly more viable cells compared to unstimulated CLL PBLs and/or mitogen stimulated T-depleted cells (Figure 3.15). It is worth noting that, based on the data presented in Figure 3.7, this increase in viability is not due to the preferential survival of the normal T cells because leukemic CD5+ B cells comprised a significant proportion of cells present in the mitogen-stimulated CLL PBL preparations. This suggests that mitogen-activated T cells may deliver survival signals to the co-cultured leukemic CD5+ B cells via either cell-contact or cytokine mediated mechanisms. In contrast to the effects of mitogens on purified leukemic CD5+ B cells, PMA preserved cell viability independently of the presence of residual T cells (Figure 3.15; see *Section 3.12*).

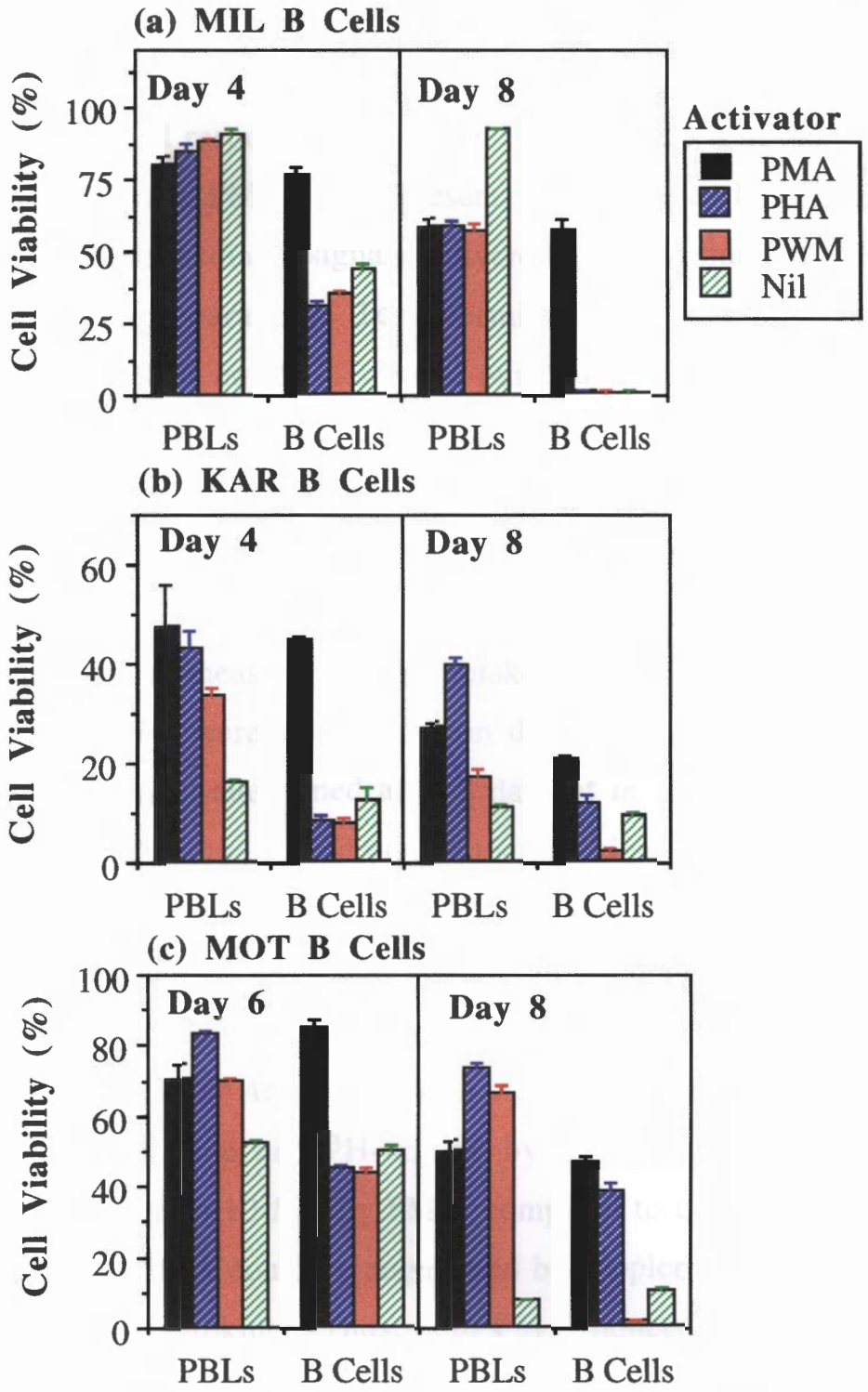


Figure 3.15: Mitogen Stimulation Enhances the Viability of CLL PBLs, but not T-depleted B Cells.

PBLs and T-depleted cell preparations (B cells) obtained from patients MIL (a), KAR (b) and MOT (c) were cultured for 8 days with or without PMA, PHA and PWM. Cell viability was determined at various times following the initiation of the culture period by the ability of the cells to exclude ethidium bromide and analysis by flow cytometry. Each column represents the mean % of viable cells ± 1 s.d. of triplicate samples.

3.9. Exogenous Cytokines Augment PMA-Induced Leukemic CD5+ B Cell Activation

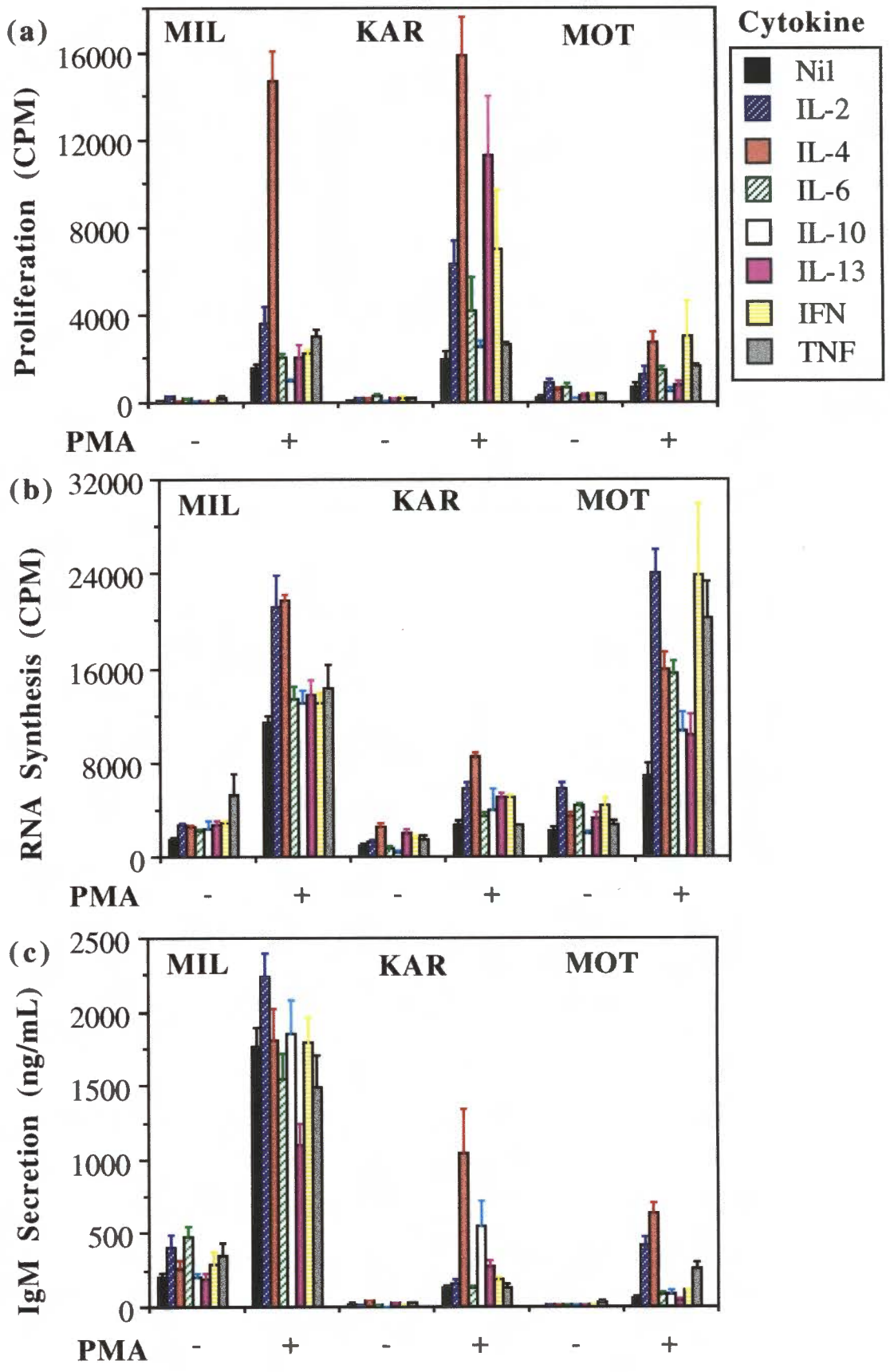
The reduced responses of purified leukemic CD5+ B cells, compared to unfractionated PBLs, in the presence of PMA may be due to cell contact or cytokine-mediated signals delivered by the residual T cells present in the PBL preparations. To determine the effect that T cell-derived cytokines had on B-CLL cells, purified leukemic CD5+ B cells were cultured in the presence or absence of IL-2, IL-4, IL-6, IL-10, IL-13, IFN-γ and TNF-α, with or without PMA. The cytokines were used at concentrations found to maximally enhance the viability of unstimulated leukemic CD5+ B cells (see Chapter 5). Cell proliferation and RNA synthesis, as measured by the uptake of ^{3}H-thymidine and ^{3}H-uridine, respectively, were determined on day 4, while differentiation to IgM secretion was determined after 8 days of *in vitro* culture. As shown in Figures 3.16a and b, addition of the cytokines alone had minimal effect on B cell proliferation and RNA synthesis. At best, several of these cytokines caused a 2-3 fold increase in the uptake of ^{3}H-thymidine and ^{3}H-uridine. Consistent with the results described in section 3.6 (*PMA Induces Cellular Activation In the Absence of T Cells*), the rate of uptake of ^{3}H-thymidine and ^{3}H-uridine by the leukemic CD5+ B cells was enhanced up to 10-fold by PMA, compared to unstimulated B cells. The effects of PMA could be augmented by supplementing the cultures with exogenous cytokines. Thus, both PMA-induced proliferation and RNA synthesis of KAR B cells were significantly increased by IL-2, IL-4, IL-13 and IFN-γ; MIL B by IL-2, IL-4 and TNF-α; and MOT B cells by IL-2, IL-4, IL-6, IFN-γ and TNF-α (Figure 3.16a, b). Similarly, the level of IgM secreted by KAR B cells in response to stimulation with PMA was enhanced by IL-4, IL-10 and IL-13, and MOT B cells by IL-2, IL-4 and TNF-α. Interestingly, none of the exogenous cytokines could

reproducibly enhance the amount of IgM secreted by PMA-activated MIL B cells (Figure 3.16c). In contrast to its effect on KAR B cells, IL-13 had no effect on the proliferation and RNA synthesis of MIL and MOT B cells (Figure 3.16a, b). Furthermore, IL-13 was found to inhibit the level of IgM secreted by MIL and MOT B cells following activation with PMA ($p<0.05$; Figure 3.16c). The level of inhibition by IL-13 was ~30-50% for both populations of leukemic $CD5^+$ B cells. This effect was observed in two or three independent experiments performed on both populations of cells. Although several of the assayed cytokines displayed a positive effect on PMA-induced leukemic $CD5^+$ B cell activation, the degree of enhancement was variable. Thus, while IL-2, IL-6, IFN-γ and TNF-α increased cell proliferation 2-3 fold, IL-4 (and IL-13 in the case of KAR B cells) was the most potent co-stimulant, causing a 7-10 fold increase in the proliferation induced by PMA alone. In addition, IL-4 caused the maximal amount of IgM secretion by PMA-stimulated cells, resulting in levels that were also 7-10 times greater than that induced by PMA alone.

Figure 3.16: Exogenous Cytokines Enhance PMA-Induced Activation of Leukemic CD5+ B Cells.

Purifed leukemic CD5+ B cells from patients MIL, KAR and MOT were cultured in the absence and presence of PMA, with or without IL-2, IL-4, IL-6, IL-10, IL-13, IFN-γ and TNF-α at the concentrations listed below. Proliferation (a) and RNA synthesis (b) were determined on day 4 following an 18 hour pulse with ^{3}H-thymidine or ^{3}H-uridine. IgM secretion (c) was determined after 8 days of culture by a μ-chain specific immunoassay. Each point represents the mean cpm ± 1 s.d. or mean ng of IgM/mL ± 1 s.d. of at least triplicate determinations, and is representative of two or three independent experiments.

	Leukemic CD5+ B Cell Population		
Cytokine	MIL	KAR	MOT
IL-2 (U/mL)	100	100	50
IL-4 (U/mL)	2	2	2
IL-6 (U/mL)	1000	1000	1000
IL-10 (ng/mL)	10	10	10
IL-13 (ng/mL)	2.5	20	1
IFN-γ (ng/mL)	50	50	20
TNF-α (ng/mL)	10	10	10


(a)
MIL
KAR
MOT
Proliferation (CPM)
16000
12000
8000
4000
0
PMA - + - + - +
Cytokine
Nil
IL-2
IL-4
IL-6
IL-10
IL-13
IFN
TNF
(b)
MIL
KAR
MOT
RNA Synthesis (CPM)
32000
24000
16000
8000
0
PMA - + - + - +
(c)
MIL
KAR
MOT
IgM Secretion (ng/mL)
2500
2000
1500
1000
500
0
PMA - + - + - +

3.10. Induction of Leukemic CD5+ B Cell Activation via Ligation of Surface IgM

Additional evidence that leukemic CD5+ B cells were responsive to exogenous stimuli came from experiments that utilised $F(ab')_2$ fragments of polyclonal rabbit anti-human IgM (μ-chain specific; $F(ab')_2$ anti-μ) to deliver signals to the B cells via cross-linking of surface IgM (sIgM). Preliminary experiments were carried out using unfractionated CLL PBLs. Ligation of sIgM with a range of $F(ab')_2$ anti-μ concentrations (0.8-100 μg/mL) was insufficient to induce cell proliferation (data not shown). However, co-stimulation with a concentration of PMA that was 10-25 fold lower than optimal (4-6 ng/mL; PMA_{low}) yielded a proliferative response that was 5-20 fold greater that the response to PMA_{low} alone (Figure 3.17). Consistent with the finding that each CLL PBL population responded maximally to different concentrations of PMA and mitogens, the concentration of $F(ab')_2$ anti-μ at which significant proliferation was first detectable was also cell source specific. Thus, proliferation of MIL CLL B cells was significantly greater than PMA_{low} alone when $F(ab')_2$ anti-μ was present at 6.3 μg/mL and the concentration of $F(ab')_2$ anti-μ required for maximal proliferation was 50 μg/mL. Proliferation of KAR and MOT B cell was significantly increased by 1.6 and 6.3 μg/mL of $F(ab')_2$ anti-μ, respectively, while the optimum $F(ab')_2$ anti-μ concentration for both of these populations was 25 μg/mL (Figure 3.17). These experiments were repeated using T-depleted preparations of leukemic CD5+ B cells so as to eliminate the response due to the residual T cells following activation with PMA. Under these conditions, results similar to those obtained for unfractionated CLL PBLs were observed for MIL and KAR (Figure 3.18). However, consistent with the finding that MOT B cells failed to proliferate in response to PMA, there was minimal incorporation of ^3H-thymidine by these cells in the presence of the co-

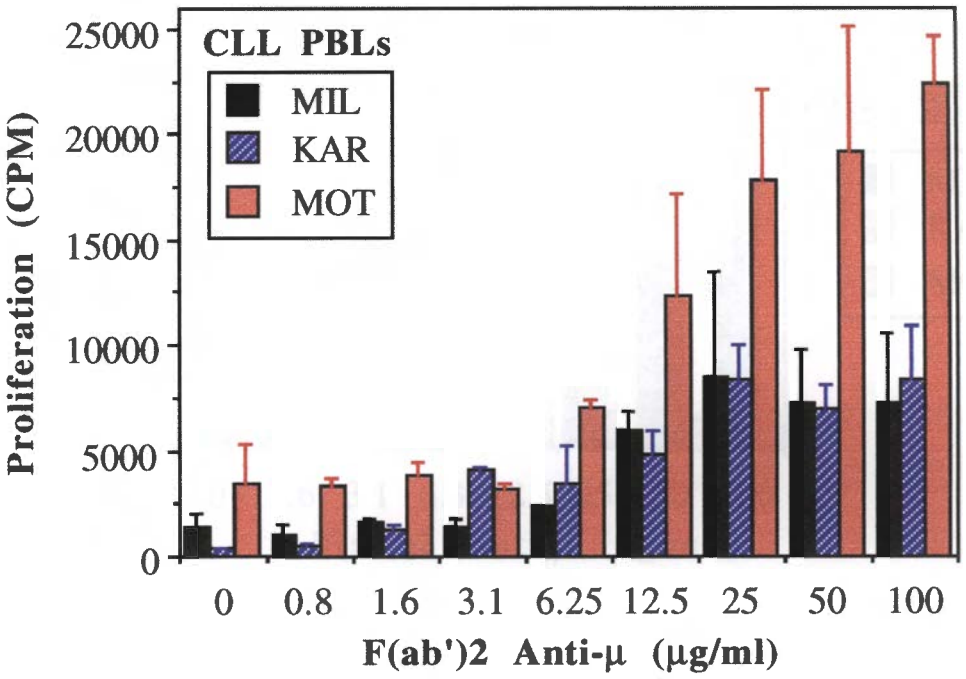


Figure 3.17: Anti-μ Synergises with PMA to Induce Proliferation of CLL PBLs.

Unfractionated PBLs from patients MIL, KAR and MOT were cultured for 4 days in the presence of a suboptimal dose of PMA (5 ng/mL) with or without varying concentrations of $F(ab)'_2$ anti-μ. Proliferation was determined after an 18 hour pulse with ^3H-thymidine. Each point represents the mean cpm ± 1 s.d. of triplicate samples.

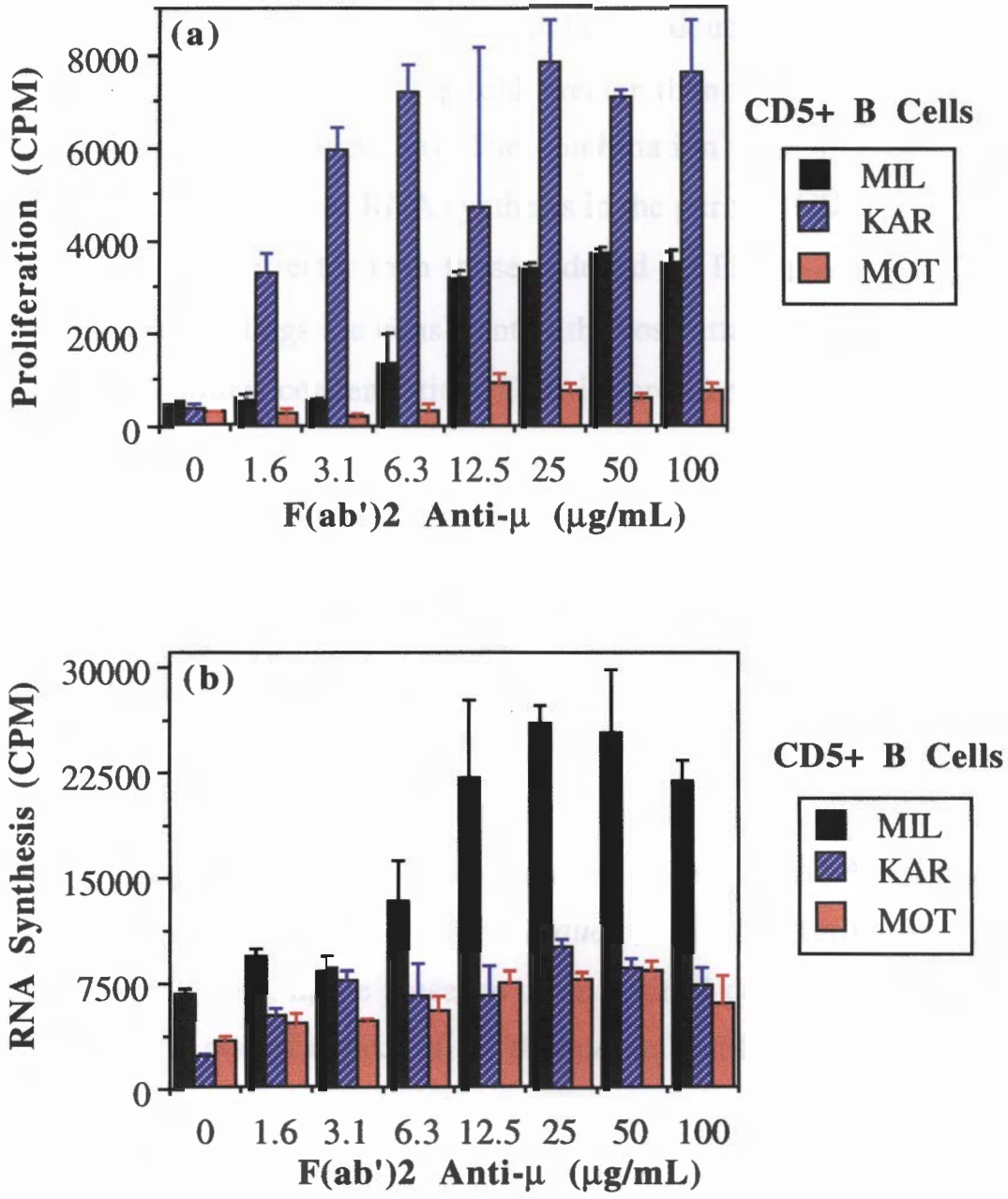


Figure 3.18: Responses of Purified CD5+ Leukemic B Cells to Anti-μ and PMA.

T-depleted B cell preparations from MIL, KAR and MOT were cultured for 4 days in the presence of PMA (5 ng/mL) with or without varying concentrations of $F(ab')_2$ anti-μ. Proliferation (a) and RNA synthesis (b) were determined after an 18 hour pulse with ^{3}H-thymidine or ^{3}H-uridine, respectively. Each point represents the mean cpm ± 1 s.d. of triplicate samples.

stimulus (Figure 3.18a). Thus, while MIL and KAR B cells displayed a 10-25 fold increase in proliferation following activation with $F(ab')_2$ anti-μ and PMA_{low}, the rate of proliferation of doubly stimulated MOT B cells was equal to or only 2-3 fold greater than cells stimulated with PMA_{low} alone (Figure 3.18a). The combination of $F(ab')_2$ anti-μ and PMA_{low} yielded levels of RNA synthesis in the purified $CD5^+$ B cells that were 2.5-5.0 fold greater than those induced by PMA_{low} alone (Figure 3.18b). These findings are consistent with those attained when PMA was used at an optimal concentration. That is, proliferation was induced in purified leukemic $CD5^+$ B cells from patients MIL and KAR, while the rate of RNA synthesis was elevated in all 3 cell populations. Having established that $F(ab')_2$ anti-μ+PMA_{low} induced significant responses, it was of interest to determine whether or not proliferation and/or RNA synthesis could be augmented by higher (i.e. optimal) concentrations of PMA. To this end, purified leukemic $CD5^+$ B cells from patients MIL, KAR and MOT were cultured with a fixed concentration of $F(ab')_2$ anti-μ (25 or 50 μg/mL) and varying concentrations of PMA. As previously demonstrated in section 3.6 (*PMA Induces Cellular Activation In the Absence of T Cells*), in the presence of an optimal concentration of PMA (PMA_{opt}), the rate of uptake of ^{3}H-thymidine by MIL and KAR B cells was ~50 times greater than unstimulated cells (Figure 3.19a-c). Similarly, the rate of RNA synthesis by the 3 different cell populations was elevated 5-6 fold by PMA alone, compared to the basal level of unstimulated cells (Figure 3.19d-f). These parameters of cellular activation were significantly augmented in cells receiving a co-stimulus of PMA and $F(ab')_2$ anti-μ. Maximal activation, as indicated by the highest CPM, occurred in the presence of $F(ab')_2$ anti-μ and 50-100 ng/mL of PMA, i.e. PMA_{opt}. Under these conditions, proliferation of MIL and KAR B cells was enhanced a further 3-fold, while RNA synthesis was heightened

by a factor of 1.5-3.0 (Figure 3.19a-f). Most interesting was the observation that co-stimulation with $F(ab')_2$ anti-μ and 10-100 ng/mL of PMA induced proliferation in MOT B cells (Figure 3.19c). These cells were refractory to the proliferation-inducing activity of PMA alone. The level of ^{3}H-thymidine incorporation by doubly-stimulated MOT B cells was 40-50 times greater than that in the presence of anti-μ alone (5123±1290 CPM vs 96±35 CPM). This induced proliferation was similar to that displayed by MIL and KAR B cells in response to PMA_{opt} alone (Figure 3.19). It is unclear why MOT B cells showed little proliferation in response to $F(ab')_2$ anti-μ+PMA_{low}. It can not be attributed to the reduced expression by these cells of sIgM because, according to flow cytometric analysis, the level of sIgM expressed on MOT B cells was similar to that on KAR B cells (Figure 3.3), and this latter population of leukemic $CD5^+$ B cells incorporate a significant amount of ^{3}H-thymidine following activation with these reagents. As expected, the maximum stimulation index for $F(ab')_2$ anti-μ+PMA stimulated MIL and KAR B cells, compared to PMA stimulated cells, occurred at concentrations of PMA between 1.6 and 6.3 ng/mL. Although the total uptake of radio-label was lower than when PMA_{opt} was employed, in the presence of such sub-mitogenic concentrations the stimulation indices for MIL and KAR B cells were 20.0 and 9.7, respectively. At these lower concentrations of PMA, co-stimulation with $F(ab')_2$ anti-μ resulted in cellular responses that were comparable to those observed with PMA alone. At higher concentrations of PMA, the stimulation indices were reduced due to the increased B cell proliferation induced in the presence of PMA_{opt} alone (Figure 3.19). Thus, ligation of sIgM significantly reduced the activation threshold of MIL and KAR B cells and prepared these cells for entry into the cell cycle. Importantly, this mode of activation also initiated

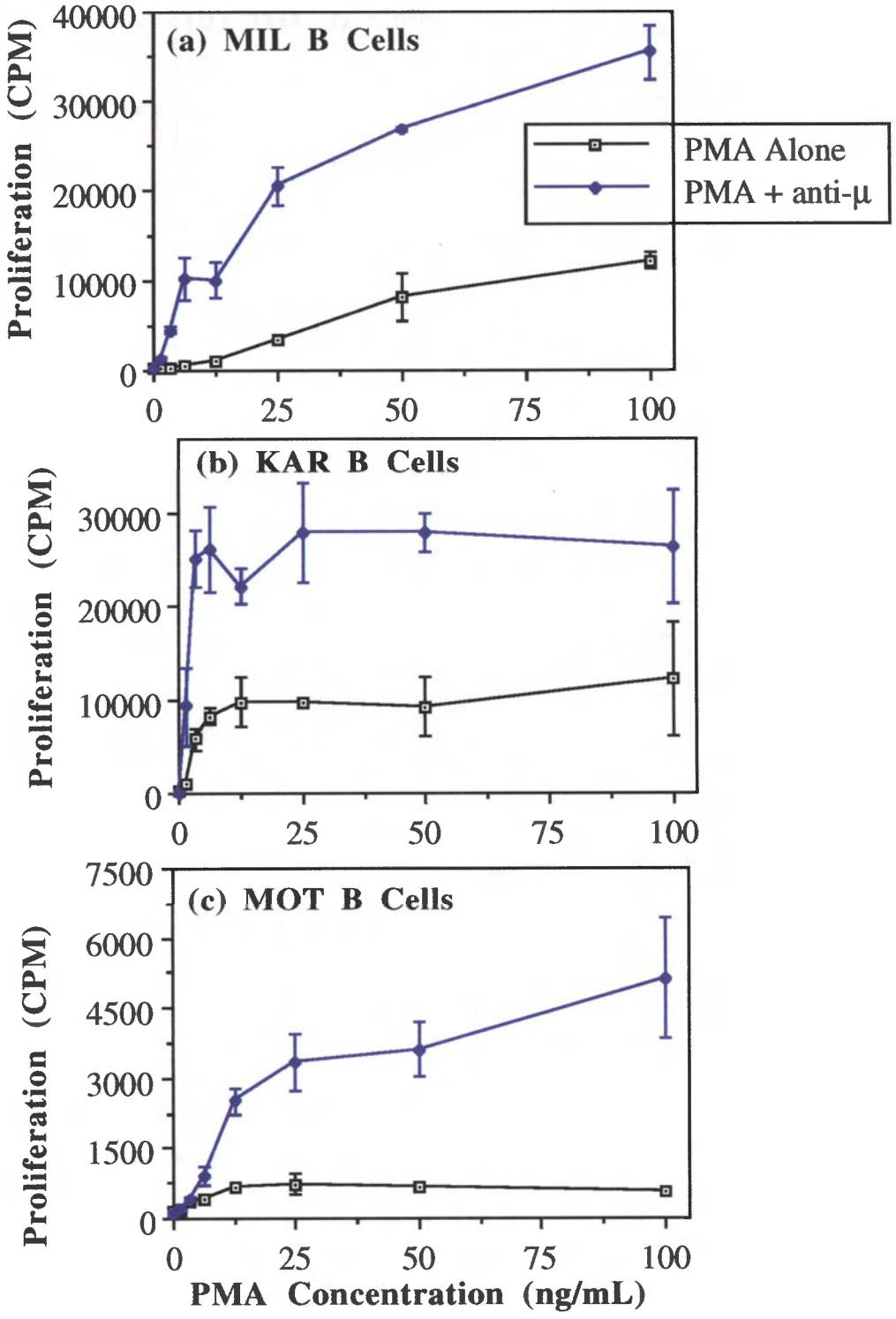


Figure 3.19: Activation via sIgM is Enhanced with Increasing Concentrations of PMA.

T-depleted preparations from MIL (a, d), KAR (b, e) and MOT (c, f) were cultured for 4 days with 50 μg/mL (MIL) or 25 μg/mL (KAR, MOT) of $F(ab')_2$ anti-μ in the presence of increasing concentrations of PMA (0-100 ng/mL). Proliferation (a-c) and RNA synthesis (d-f) were determined after an 18 hour pulse with ^{3}H-thymidine or ^{3}H-uridine, respectively. Each point represents the mean cpm ± 1 s.d. of triplicate samples.

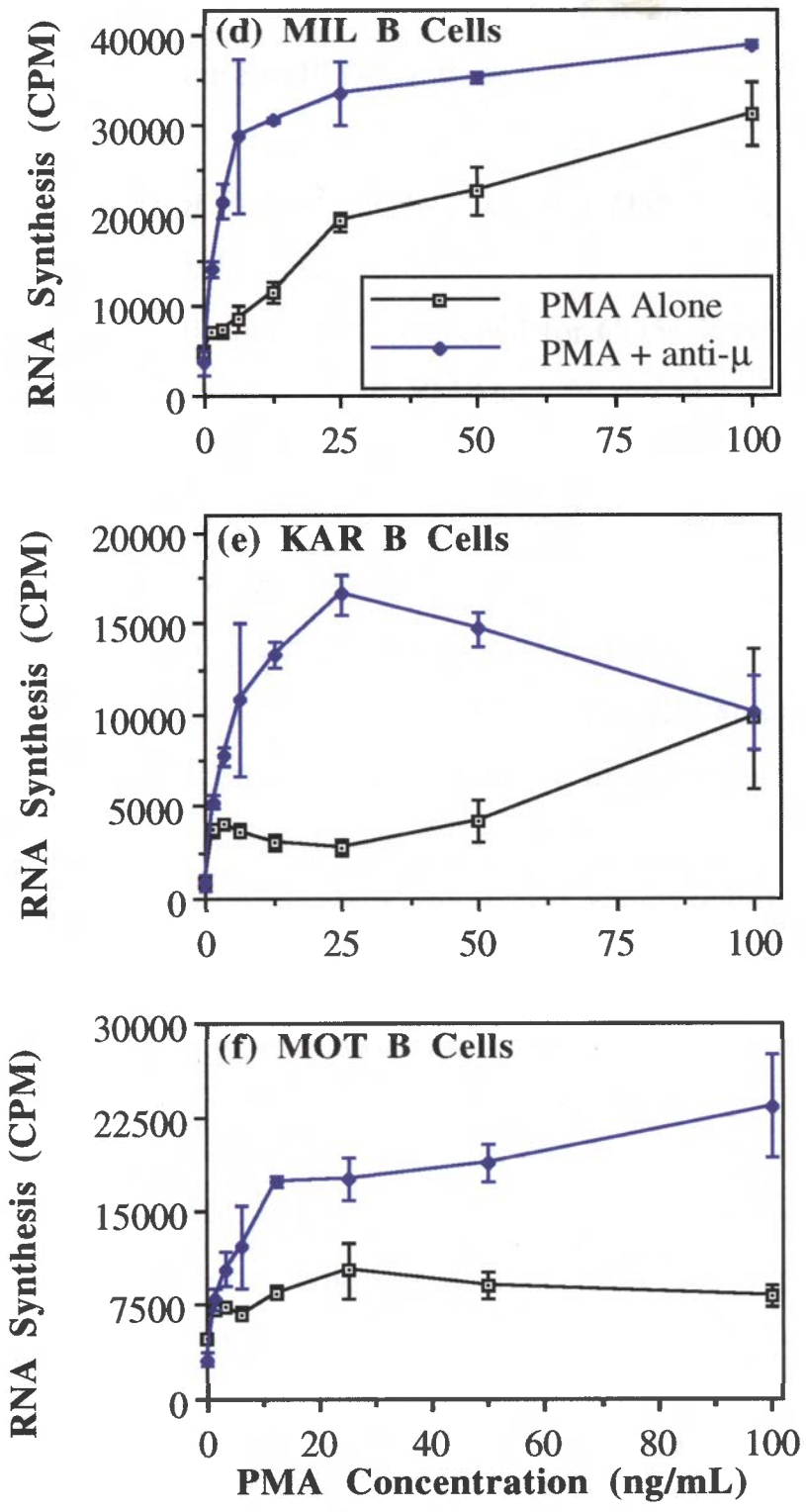


Figure 3.19: Activation via sIgM is Enhanced with Increasing Concentrations of PMA (continued).

proliferation in purified leukemic CD5+ B cells that failed to undergo division in response to PMA_{opt} alone.

3.11. Kinetics of Responses of Leukemic CD5+ B Cells to Anti-μ + PMA are Similar to PMA_{opt} Alone

Leukemic B cell populations enriched for CD5+ B cells were cultured for 10 days in the presence of PMA_{low} (4 ng/mL), PMA_{opt} (50 or 100 ng/mL) and $F(ab')_2$ anti-μ at the concentration determined above to elicit maximal proliferation (25 or 50 μg/mL). Cell proliferation and RNA synthesis were determined every second day following an 18 hour pulse with ^{3}H-thymidine and ^{3}H-uridine, respectively. To compare responses, the cells were also cultured with PMA_{opt} or PMA_{low}, as well as $F(ab')_2$ anti-μ alone. Figure 3.20 illustrates the incorporation of ^{3}H-thymidine and ^{3}H-uridine as determined after various times of *in vitro* culture. It is clear that the 3 different leukemic CD5+ B cell populations responded to PMA_{opt}, as previously demonstrated. MIL and KAR B cells displayed maximal proliferation on days 6 and 4, respectively, while proliferation of MOT B cells was minimal. The enhancement of RNA synthesis peaked after 2-6 days, depending on the cell population. These data are similar to those presented in section 3.6 (*PMA Induces Cellular Activation In the Absence of T Cells*). When used alone, PMA_{low} caused no or minimal proliferation of the leukemic CD5+ B cells. Interestingly, the rates of RNA synthesis could be enhanced to a comparable degree in KAR B cells by both the optimal and low concentration of PMA, suggesting that the concentration determined to be optimal for cell proliferation may not necessarily elicit the maximal rate of transcription. Anti-μ alone also had no effect on cell proliferation or RNA synthesis at any time point throughout the culture period. The combination of anti-μ and PMA_{low} induced a proliferative response in MIL and KAR B cells and enhanced

RNA synthesis in all 3 CLL populations to a degree that was significantly greater than that induced by either activator alone (Figure 3.20). In fact, the CPM induced were comparable to or greater than those of PMA_{opt}, a mode of activation previously shown to be the most efficient inducer of proliferation and RNA synthesis by purified leukemic $CD5^+$ B cells. Furthermore, the combination of anti-μ and PMA_{opt} induced a proliferative response in all 3 CLL populations that was approximately 10 times greater than that induced by PMA_{opt} alone at the time of maximal proliferation (Figure 3.20). Thus, culture with these two activators resulted in a synergistic response. However, the kinetics resulting from the single and double stimuli were similar, with maximal activation occurring at the same times. Thus, despite inducing similar or greater rates of proliferation and RNA synthesis than PMA_{opt}, co-stimulation of CLL B cells with PMA_{low}/PMA_{opt} + $F(ab')_2$ anti-μ does not appear to cause extended cycles of cell division that would be evidenced by a sustained or prolonged response. Despite this, co-stimulation of the leukemic $CD5^+$ B cells with PMA + $F(ab')_2$ anti-μ prevented the rapid decline in uptake of ^{3}H-thymidine and ^{3}H-uridine that is usually observed by these cells once PMA-induced proliferation and RNA synthesis have peaked. This resulted in significant uptake of ^{3}H-thymidine or ^{3}H-uridine ocurring after 8-10 days of culture in the presence of anti-μ + PMA_{low} while in the presence of PMA_{opt} the rate of uptake approaches background levels after 6 days (Figure 3.20, and data not shown).

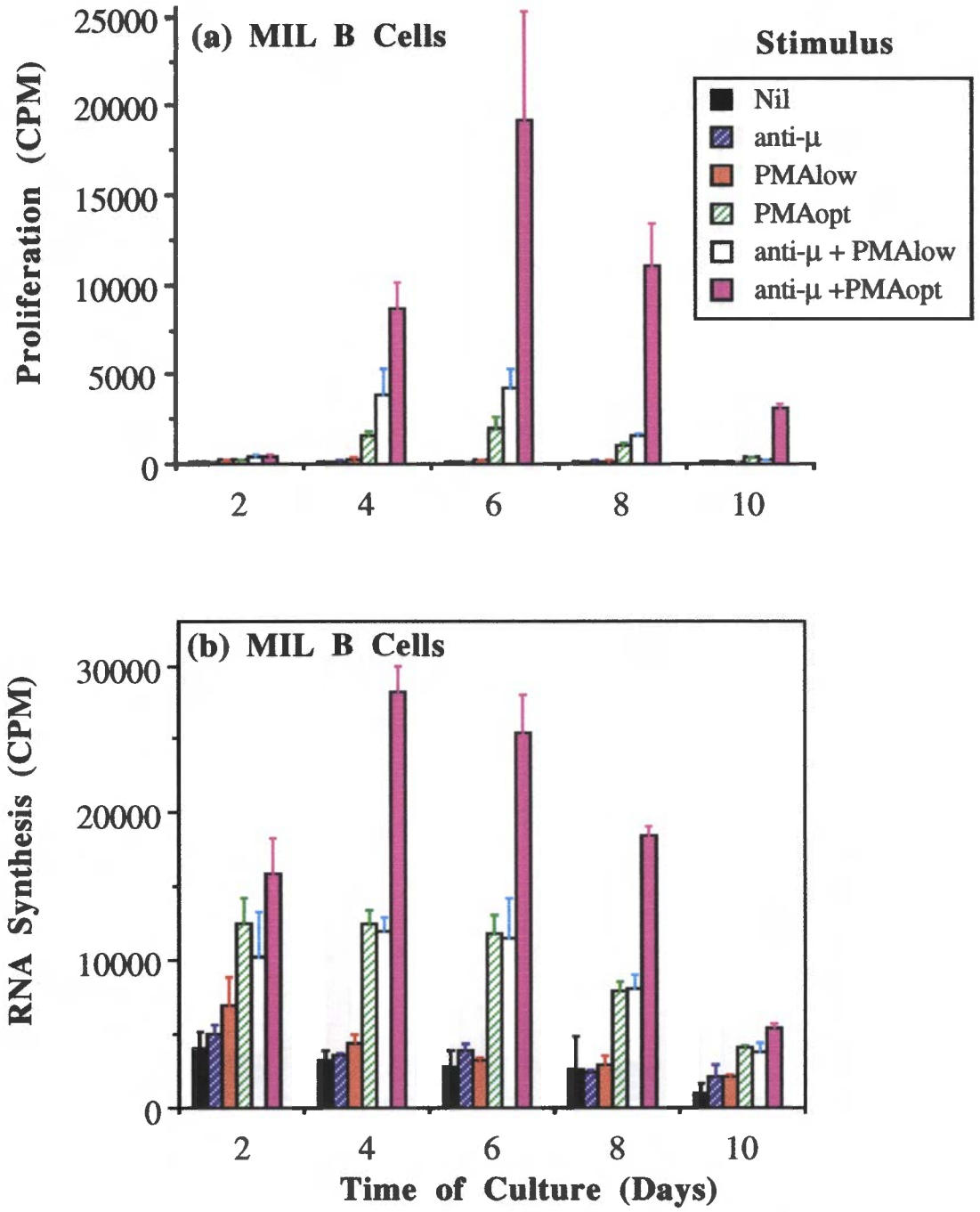


Figure 3.20: Kinetics of Proliferation and RNA Synthesis Induced by PMA and Anti-μ in Leukemic CD5+ B Cells.

Purified B cells from patients MIL (a, b), KAR (c, d) and MOT (e, f) were cultured for 10 days in the absence or presence of PMA_{low} (4 ng/mL), PMA_{opt} (50 or 100 ng/mL), $F(ab')_2$ anti-μ (25 or 50 μg/mL), $F(ab')_2$ anti-μ + PMA_{low} or $F(ab')_2$ anti-μ + PMA_{opt}. Proliferation (a-c) and RNA synthesis (d-f) were determined on days 2, 4, 6, 8 and 10 by pulsing the cultured cells with 1 μCi of ^{3}H-thymidine and ^{3}H-uridine, respectively, and harvesting 18 hours later. Each point represents the mean cpm ± 1 s.d. of triplicate samples.

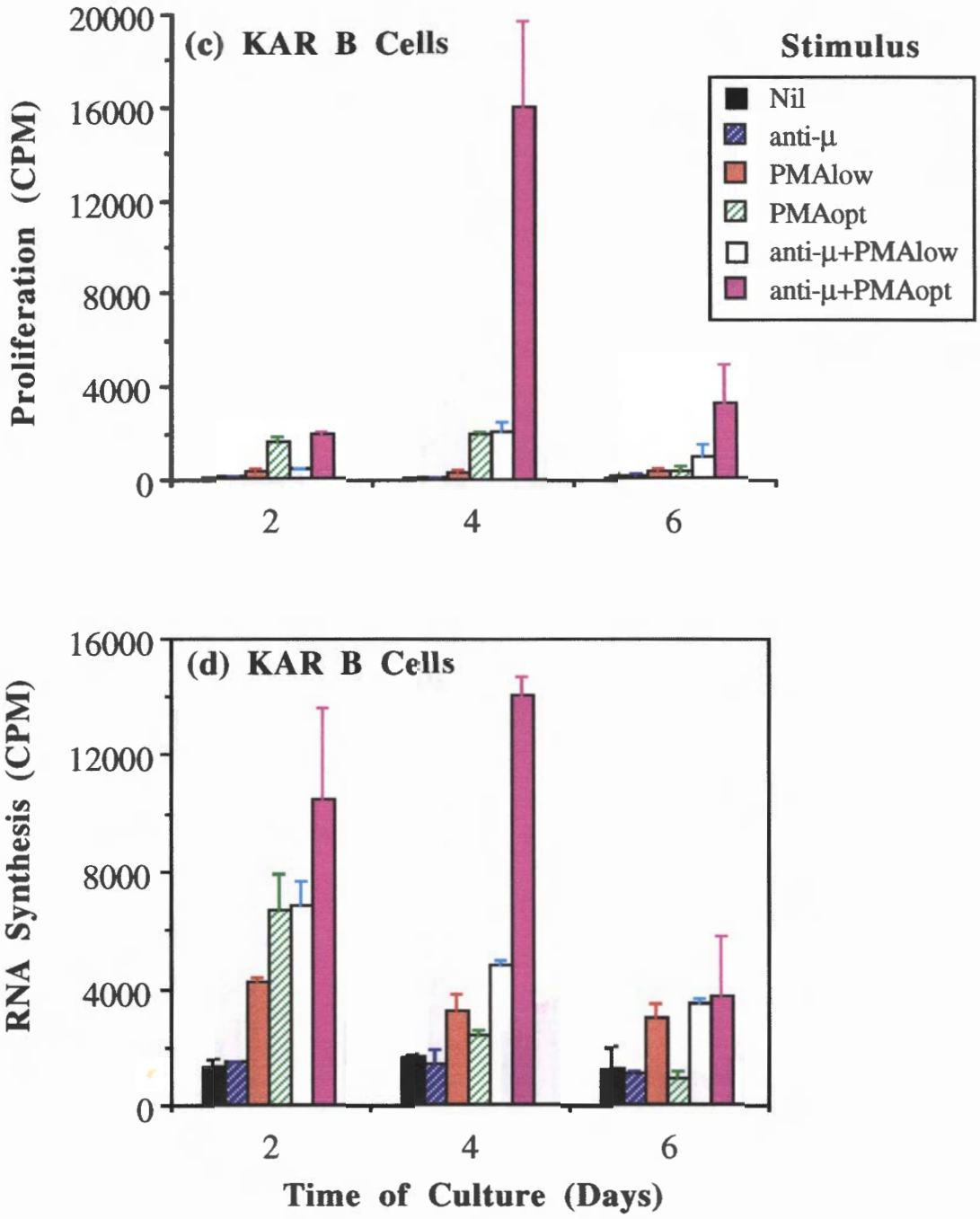


Figure 3.20: Kinetics of Proliferation and RNA Synthesis Induced by PMA and Anti-μ in Leukemic CD5+ B Cells (continued).

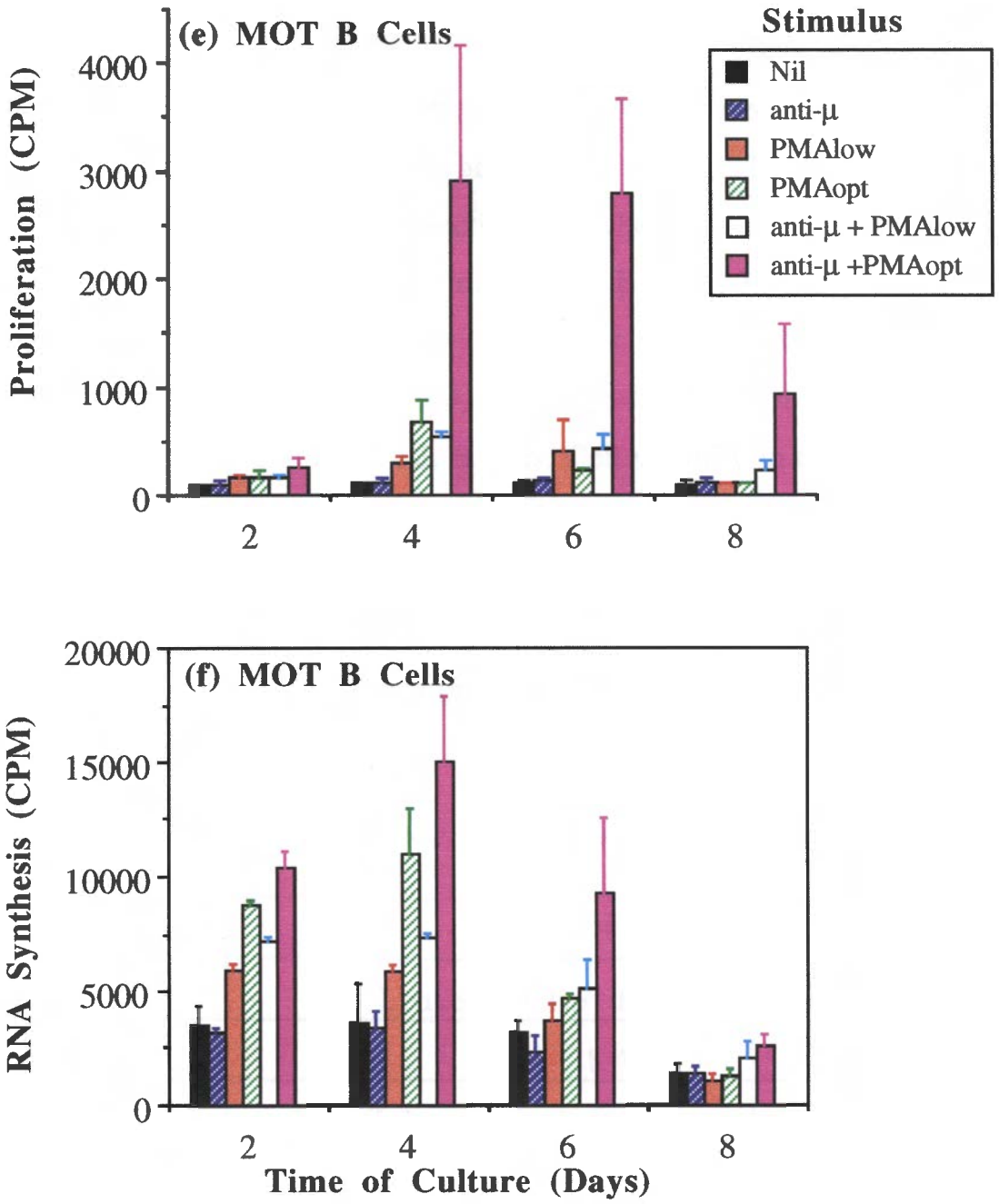


Figure 3.20: Kinetics of Proliferation and RNA Synthesis Induced by PMA and Anti-μ in Leukemic CD5+ B Cells (continued).

3.12. Anti-μ has no Effect on the Enhancement of Viability of Leukemic CD5+ B Cells Caused by PMA.

It has been previously demonstrated that PMA prevents leukemic B cells from undergoing apoptosis (Forbes *et al.*, 1992). These results were confirmed by data presented in Section 3.8, where it was found that PMA enhanced the viability of leukemic CD5+ B cells. In the presence of PMA_{low} or PMA_{opt}, there were ~30% more viable cells, compared to unstimulated controls (Table 3.3). $F(ab')_2$ anti-μ had no effect on the viability of *in vitro* cultured CLL B cells. Cell viability in the presence of anti-μ and either concentration of PMA was similar to that with PMA alone (Table 3.3). This suggests that crosslinking sIg in the presence of PMA resulted in an increased rate of DNA and RNA synthesis by the leukemic CD5+ B cells as opposed to causing an increase in the proportion of viable cells.

Table 3.3: *Anti-μ Does Not Synergise with PMA to Enhance Cell Viability*

	Cell Viability % (s.d.)		
	Leukemic CD5+ B Cell Population		
Activator	MIL	KAR	MOT
Nil	32.9 (1.3)	9.4 (1.6)	9.6 (0.4)
anti-μ	32.1 (1.3)	9.1 (1.4)	11.4 (1.3)
PMA_{low}	58.6 (1.1)	40.4 (2.7)	42.2 (1.6)
PMA_{opt}	62.3 (1.8)	NT	45.1 (1.4)
anti-μ/PMA_{low}	65.0 (1.2)	41.9 (2.0)	44.9 (1.5)
anti-μ/PMA_{opt}	60.2 (2.3)	NT	NT

Purified leukemic CD5+ B cells from the 3 different B-CLL patients were cultured in the absence or presence of PMA without or with $F(ab')_2$ fragments of rabbit anti-human μ chain antibody (25 or 50 μg/mL). Cell viability was determined after 4 or 5 days of *in vitro* culture. These results are representative of 2 independent experiments.

NT - not tested.

3.13. *Crosslinking sIgM Does not Enhance Differentiation to IgM Secretion*

To investigate whether the dual stimulus of sIgM crosslinking and PKC activation potentiated differentiation of leukemic CD5+ B cells, a two-step culture system was employed. The CLL B cells were cultured for 2 days in the presence of the appropriate stimulus, after which time the cells were thoroughly washed and then recultured for 7 days in complete medium. This allowed for the detection of secreted IgM, which was otherwise unable to be detected in the single-step culture system as the activating $F(ab')_2$ anti-μ was also used as the capture Ab in the μ-chain specific immunoassay. To monitor activation, proliferation and RNA synthesis of the preactivated cells were determined after 4 days of secondary culture. Following removal of the stimulus, the proliferative activity of all 3 populations and the RNA synthesis of KAR and MOT B cells were reduced to background levels (data not shown). This suggested that efficient induction of cell proliferation required the continual presence of the stimuli. Despite the absence of a proliferative response, MIL B cells preactivated with either the optimal or low dose of PMA, or a combination of anti-μ plus PMA, displayed RNA synthesis that was 3-7.5 fold greater than cells cultured initially in complete medium. Consistent with the findings for proliferation and RNA synthesis, KAR and MOT B cells failed to secrete IgM at detectable levels (< 5ng/mL) during the secondary culture (data not shown). This is further evidence for the requirement of continual stimulation for the induction of activation/differentiation. However, IgM was detected in supernatants of MIL B cells (Table 3.4). This was not surprising as these cells were previously found to spontaneously secrete relatively large amounts of IgM (see section 3.6, *PMA Induces Cellular Activation In the Absence of T Cells*). Interestingly, the level of secreted IgM was often greater in

wells containing cells that had been preactivated with an optimal or a low concentration of PMA, with the amount being highest following preactivation with PMA_{opt} (Table 3.4). This suggests, for MIL B cells at least, that although the rates of RNA synthesis in the presence of an optimal and sub-mitogenic concentration of PMA are similar, maximal cell proliferation and differentiation to IgM secretion is only achieved by one PMA concentration that is optimal for both of these parameters of cellular activation. Cells preactivated with $F(ab')_2$ anti-μ in concert with PMA_{low} or PMA_{opt} secreted IgM at a level that was only 1.5-2.0 fold greater than that of cells preactivated with PMA_{low} or PMA_{opt} alone (Table 3.4). These results can be interpreted as indicating that co-stimulation of leukemic $CD5^+$ B cells via ligation of sIg and activation of PKC does not cause a large increase in the extent of cellular differentiation, compared to either activator alone, even though this protocol is an efficient inducer of cell division and RNA synthesis.

Table 3.4: *Secretion of IgM by MIL Leukemic $CD5^+$ B cells Following Preactivation with PMA and/or anti-μ*

	IgM Secretion ng/mL (s.d.)		
Activator	Expt 1	Expt 2	Expt 3
Nil	34 (12)	28 (5)	141 (12)
anti-μ	NT	46 (4)	195 (44)
PMA_{low}	266 (55)	NT	140 (27)
PMA_{opt}	702 (92)	379 (64)	495 (60)
anti-μ/PMA_{low}	380 (101)	185 (24)	329 (21)
anti-μ/PMA_{opt}	NT	NT	665 (91)

Purified leukemic $CD5^+$ B cells from patient MIL were cultured in the absence or presence of PMA without or with $F(ab')_2$ anti-μ chain antibody. After 48 hours, the cells were washed thoroughly and recultured for an additional 7 days in complete medium. IgM secretion was then determined by a μ-chain specific ELISA. NT - not tested.

Discussion

This chapter describes the activation and differentiation of $CD5^+$ B cells from 3 B-CLL patients following *in vitro* stimulation with the phorbol ester, PMA in the presence and absence of antibodies to sIgM. In addition, the effects of culturing leukemic $CD5^+$ B cells with the mitogens PHA and PWM are described. Furthermore, the contribution of T cells and T cell-derived cytokines to these responses was assessed by comparing the responses of CLL PBLs and purified B cell preparations to the same activators.

The tumor promoter PMA has been used extensively as an agent to investigate the *in vitro* activation of B-CLL cells. Early studies showed that stimulation with PMA resulted in 80-90% of malignant B-CLL cells undergoing lymphoblastoid transformation (Totterman *et al.*, 1980). This was reflected by a reduction in the expression of sIg, an increase in the amount of IgM present in the cytoplasm (cIg) (Totterman *et al.*, 1980) and an accompanying increase in cell size. These induced cellular changes were dependent on the presence of residual T cells and occurred in the absence of proliferation (Totterman *et al.*, 1980, 1987; Danersund *et al.*, 1985; Carlsson *et al.*, 1988b; Nilsson, 1992). Additional experiments indicated that the uptake of ^{3}H-thymidine by cultured B-CLL cells could be induced when PMA was used in combination with the BSF-MP6 T cell supernatant (Carlsson *et al.*, 1988b). This demonstrated that the malignant B cells were not refractory to progressive signals. In contrast to the induction of IgM secretion by PMA, it was found that B-CLL cell proliferation induced by PMA/BSF-MP6 was not affected by the depletion of T cells and monocytes (Carlsson *et al.*, 1988b). PMA has also been reported to induce the appearance of B cell activation antigens (Gordon *et al.*, 1984; Totterman *et al.*, 1987) and enhance the stimulatory

activity of B-CLL cells in mixed lymphocyte cultures (Okamura *et al.*, 1982b).

Consistent with these results, the data presented here indicate that PMA induced activation of leukemic CD5+ B cells obtained from 3 different patients with B-CLL. This was evidenced by the induction or enhancement of cellular proliferation, RNA synthesis and IgM secretion to levels that significantly exceeded those of unstimulated leukemic CD5+ B cells. PMA-induced cellular activation was also accompanied by alterations to cellular morphology, suggestive of lymphoblastoid transformation (Figures 3.6, 3.10, 3.14). These morphological and differentiation-associated changes induced by PMA occurred in the absence of both T cells and exogenous cytokines, albeit at levels considerably less than those observed for unfractionated PBLs. This suggests that PMA is capable of activating these leukemic CD5+ B cells directly and not via an accessory cell dependent mechanism. However, although PMA acted independently of T cells with respect to leukemic CD5+ B cell activation, the presence of the residual T cells enhanced the rates of both proliferation and RNA synthesis and also the time of peak activation. In addition, the magnitude of the PMA-induced response was increased in the presence of residual T cells (Figure 3.6).

These findings are similar to those described recently for normal murine B cells, stimulated with activated T cell membranes in the presence and absence of a supernatant containing T cell derived B cell growth factors (Hodgkin *et al.*, 1994). These authors found that murine resting splenic B cells underwent proliferation in the presence of activated T cell membranes alone, and maximal proliferation was observed 3 days after the initiation of culture. In the presence of growth factors, the degree of

proliferation was not only enhanced but also maintained such that it peaked on day 4 (Hodgkin *et al.*, 1994). As one of the active components of the T cell membrane is known to be the ligand for CD40, it is likely that this mode of B cell stimulation results following the ligation of CD40 expressed on the B cell surface. Regarding the responses of the leukemic $CD5^+$ B cells described in this chapter, the enhanced proliferation in the presence of the residual T cells could be explained by two possible scenarios. Firstly, because PMA, in combination with ionomycin, is capable of inducing expression of CD40L on the surface of T cells (Fuleihan *et al.*, 1993; Korthauer *et al.*, 1993) the leukemic $CD5^+$ B cells may subsequently receive signals via activation of protein kinase C (PMA) and ligation of CD40. Although this possibility was not investigated, it is consistent with the finding that mAbs to human CD40 significantly enhanced the proliferation of normal human B cells to PMA alone (Ledbetter *et al.*, 1987). Secondly, cytokines secreted by the PMA-stimulated normal residual T cells may also increase the rate of uptake of ^{3}H-thymidine by the leukemic $CD5^+$ B cells in the presence of PMA. This was found to be the case as numerous cytokines, such as IL-2, IL-4, IL-6, IL-13, IFN-γ and TNF-α, were capable of significantly enhancing the level of proliferation induced in the purified leukemic $CD5^+$ B cell populations by PMA alone (Figure 3.16). These findings are consistent with those previously described for some CLL B cell populations that were activated with PMA and either IL-2 (Kabelitz *et al.*, 1985), IL-4 in combination with BSF-MP6 (Carlsson *et al.*, 1989b), or TNF-α (van Kooten *et al.*, 1992, 1993a, 1993b). The enhanced proliferation by the leukemic $CD5^+$ B cells in response to the co-stimulus of PMA and IL-2 was not unexpected because it was found that 2 of the B cell populations examined (MIL, MOT) expressed CD25 prior to culture (Figure 3.4). Furthermore, although unstimulated KAR B cells were negative for this

receptor, it has previously been reported that activation with PMA induced the expression of CD25 (Carlsson *et al.*, 1988a, 1988b; Mitsui *et al.*, 1991), possibly rendering these cells sensitive to signals mediated by IL-2. Taken together, it appears that the enhanced proliferation of unfractionated CLL PBLs, over purified leukemic $CD5^+$ B cells, may be attributable to the action of IL-2, IL-4, IL-6, IL-13, IFN-γ and/or TNF-α. However, as mentioned above, the influence that cell contact mediated signals play in this model of leukemic B cell activation cannot be discounted.

The results regarding the effect of IL-13 on leukemic $CD5^+$ B cell activation are intriguing for several reasons. The activities of IL-13 very closely resemble those of IL-4. Thus, both cytokines have been found to upregulate CD23 expression on B cells (McKenzie *et al.*, 1993; Punnonen *et al.*, 1993), induce B cell proliferation and Ig isotype switching to IgG_4 and IgE when used in conjunction with antibodies to CD40 (Cocks *et al.*, 1993; Punnonen *et al.*, 1993), inhibit the proliferation of human B cell precursors (Pandrau *et al.*, 1992; Renard *et al.*, 1994) and induce similar phenotypic and functional changes to human monocytes (de Waal Malefyt *et al.*, 1993a). In addition, proliferation of leukemic B cells activated by anti-CD40 antibodies could be similarly enhanced by addition of either IL-4 or IL-13 (Fluckiger *et al.*, 1994a). Furthermore, it appears that the receptors for IL-4 and IL-13 share common components (Aversa *et al.*, 1993b; Zurawski *et al.*, 1993). In light of these findings, it is not clear why IL-13 could enhance the PMA-induced proliferation of KAR B cells only, while in the presence of IL-4 the level of proliferation of all 3 B cell populations was significantly augmented. An immediate explanation could be the lack of expression by MIL and MOT B cells of components unique to the IL-13 receptor, or the necessary signalling molecules.

Alternatively, the results may be attributable to the constitutive expression of the activation antigen CD80 on the surface of KAR B cells. This molecule was absent from MIL and MOT B cells (Figure 3.4). Because activation of leukemic B cells with antibodies to CD40 induces the expression of CD80 (Ranheim and Kipps, 1993; Yellin *et al.*, 1994), this mode of activation may also confer on the cells the ability to respond to IL-13; i.e. to express the necessary components of the IL-13 receptor. Although culture with PMA has also been reported to induce expression of CD80 (Gordon *et al.*, 1984), it does not appear to be as efficient as culture with anti-CD40 antibodies. This is based on comparisons of the fluorescence histograms presented by the authors of these previous reports (Gordon *et al.*, 1984; Ranheim and Kipps, 1993; Yellin *et al.*, 1994). Furthermore, PMA failed to induce cell surface expression of CD80 on MIL B cells or augment the level of CD80 present on KAR B cells over a 72 hour incubation period (data not shown). This may explain why CLL B cells proliferated in the presence of anti-CD40 and IL-13 (Fluckiger *et al.*, 1994a) yet CLL B cells lacking CD80 (i.e. MIL and MOT B cells; Figure 3.16), as described in this chapter, failed to enter the cell cycle following activation with PMA and IL-13. The suggestion that incubation with PMA or anti-CD40 antibodies differentially activates leukemic $CD5^+$ B cells is supported by previous studies which found that PMA, in conjunction with IL-4, induced IgM secretion by leukemic B cells (van Kooten *et al.*, 1993c), while no IgM was detected in cultures of leukemic B cells following cross-linking of CD40 in the presence of IL-4 (Defrance *et al.*, 1991). This is not the first report of unresponsiveness of abnormal B cells to the effects of a cytokine known to stimulate normal B cells. Go *et al.* (1990) found that the level of expression of class II MHC antigens on the surface of B cells obtained from male mice suffering from an X-linked immunodeficiency (XID) were unaffected following *in*

vitro culture in the presence of IL-10. This contrasts normal resting murine B cells, where it was observed that IL-10 caused a significant upregulation in expression of MHC class II antigens. These results were interpreted as possibly reflecting a lack of IL-10 receptor or signal-transducing molecules from the surface of the abnormal murine B cells (Go *et al.*, 1990). Interestingly, the ability of IL-4 to upregulate MHC class II expression occurred when normal or XID B cells were used (Go *et al.*, 1990).

Interpretations of conclusions reached by other authors regarding PMA-induced differentiation of CLL B cells are complicated by the omission in these reports of the exact level of residual T cells (Totterman *et al.*, 1981; Okamura *et al.*, 1982a, 1982b; Gordon *et al.*, 1984). Furthermore, comparisons between the effects of PMA on unfractionated CLL PBLs and preparations enriched for B cells are not often performed (Pfeffer *et al.*, 1987; Beiske *et al.*, 1988; Carlsson *et al.*, 1988b), leaving unresolved the influence that accessory cells may have on the differentiation of leukemic B cells. Despite these complications, reports detailing the responses of highly purified preparations of leukemic B cells suggest that B-CLL cells differentiate to IgM-secreting cells in the presence of PMA by mechanisms that are both dependent and independent of the presence of residual normal T cells. Drexler *et al.* (1987, 1988) found that PMA induced a 7 fold increase in IgM secretion by cell preparations comprised of >98% leukemic B cells and <1% T cells, compared to unstimulated controls. This response could be enhanced nearly 2-fold by the co-stimulus of PMA plus the calcium ionophore A23187, while IL-2, IL-6 and autologous T cells had no effect. In contrast, the vast majority (8/10) of CLL cell populations investigated by van Kooten *et al.* (1993c) failed to secrete detectable IgM following activation with PMA. However, in

combination with IL-4, 70% of samples matured into IgM-secreting cells. Consistent with Drexler *et al.* (1988), this study also failed to demonstrate a role for IL-2 in the PMA-mediated differentiation of leukemic B cells (van Kooten *et al.*, 1993c).

As described in the results section, it was demonstrated that PMA enhanced or induced the secretion of IgM by unfractionated PBLs from three patients with B-CLL (Figure 3.10). The secreted IgM was of the same light chain isotype as that expressed on the leukemic $CD5^+$ B cells of each patient, indicating that the secreted material was the product of these cells (Figure 3.11). This was further confirmed by demonstrating that IgM secreted by mitogen-activated leukemic CD5+ MIL B cells bound to mouse Ig in a solid-phase ELISA (Figure 3.12), a phenomenon previously documented to occur at the cell surface level and believed to be mediated by the polyreactive IgM and IgD expressed by the leukemic $CD5^+$ B cells (Weston and Raison, 1991). The profiles of IgM secretion by PMA-stimulated unfractionated PBLs and those of purified B cells obtained from patient MIL were similar, with the amount of IgM secreted being 5-8 fold greater than unstimulated cultures. For the other 2 patients, the secretion of IgM by the PMA-stimulated purified B cells was also significantly greater than that produced by the unstimulated B cells, although the actual quantity of IgM secreted by the purified B cell preparations was never as great as that of the unfractionated PBLs (Figure 3.10). Thus, these results display both similarities and differences to previous reports. First, PMA induced or enhanced the secretion of IgM by purified B cells obtained from 3 different B-CLL patients. Secondly, this response was greater when unfractionated cell preparations were used. Similar to the enhanced proliferation and RNA synthesis, the increased quantities of secreted IgM observed in the presence of residual

T cells (<10% of total cells) could result from cell contact or soluble signals delivered by the PMA-activated T cells. When the purified leukemic CD5+ B cells were co-cultured with PMA and a panel of different cytokines, IL-4 was found to increase by 5-10 times the amount of IgM secreted by 2 of the different populations, compared to that secreted in the presence of PMA alone (Figure 3.16). These data are consistent with those of van Kooten *et al.* (1993c) who reported that IL-4, in combination with PMA, induced IgM secretion by 7/10 populations of purified leukemic B cells. However, the ability to augment PMA-mediated leukemic CD5+ B cell differentiation was not restricted to IL-4. In fact, the amount of IgM secreted by PMA-stimulated KAR B cells was substantially increased by IL-10 or IL-13 and that secreted by MOT B cells was increased when cultured with IL-2 or TNF-α (Figure 3.16). The majority of these cytokines have previously been found to be involved in the differentiation of normal B cells (Banchereau and Rousset, 1992; Rousset *et al.*, 1992). In contrast, exogenous cytokines had very little effect on the amount of IgM secreted by PMA-activated MIL B cells. This suggests that contact mediated signals delivered to leukemic CD5+ B cells by the PMA-activated T cells, or combinations of cytokines, are required for maximal IgM secretion by MIL B cells. It therefore appears that leukemic CD5+ B cells can be classified into one of at least 3 groups, according to their ability to differentiate in the presence of PMA. The first group would comprise cells that are initially resistant to PMA-induced differentiation but are rendered susceptible in the presence of a co-stimulus. A second group would represent cells that secrete IgM in the presence of PMA, and the amount of Ig secreted is not influenced by additional signals. The last group of cells would be those capable of differentiating into IgM secreting cells under the influence of PMA and the rate of differentiation is potentiated by secondary signals. The three

cell populations examined in this study would fall into this last category as the purified leukemic CD5+ B cells all responded to PMA, yet the amount of IgM secreted could be augmented in the presence of autologous normal T cells and/or exogenous cytokines. Interestingly, PMA has a minimal effect on Ig secretion by normal human B cells. However, this response could be enhanced 2-4 fold in the presence of autologous T cells (Ralph and Kishimoto, 1982; Drexler *et al.*, 1988). This suggests that the activation requirements of normal human B cells are similar to those of particular leukemic CD5+ B cell clones. Thus, PMA was capable of inducing activation and IgM secretion of leukemic CD5+ B cells in the presence and absence of T cells. This contrasts the previous finding that PMA-induced B-CLL cell differentiation is mediated by residual T cells, and occurs in the absence of DNA synthesis (Danersund *et al.*, 1985; Totterman *et al.*, 1988; Nilsson, 1992). Moreover, it highlights intrinsic differences between the activation requirements of particular clones of leukemic B cells and normal B cells.

In the presence of autologous T cells, the mitogens tested in this study were also capable of inducing IgM secretion (Figure 3.10). This is in agreement with previously published results which describe mitogen-induced B-CLL cell activation (Bloem *et al.*, 1984; Duan *et al.*, 1992). Bloem *et al.* (1984) reported that PBL preparations from 2 patients with B-CLL could be induced to incorporate ^{3}H-thymidine and secrete monoclonal IgM in the presence of PWM and *Staphylococcus aureus.* Similarly, Duan *et al.* (1992) recently demonstrated that differentiation of leukemic B cells to Ig-secreting cells was achievable following stimulation with Staphylococcal enterotoxins as well as conventional mitogens such as PWM. The results presented in this chapter are consistent with these two studies and confirm the finding that mitogen-induced leukemic B cell

differentiation is dependent on the presence of autologous or allogeneic T cells. The amount of IgM detected in the supernatants of stimulated KAR and MOT PBLs were relatively low compared to the levels observed for MIL PBLs. It could be argued that these low levels were due to the secretion of IgM by normal B cells present in the cell preparations. However, this appears unlikely because the IgM secreted by mitogen-stimulated PBLs from patients KAR and MOT was demonstrated to be homogeneous with respect to light chain isotype, and consistent with the phenotype of the leukemic CD5+ B cells (Figure 3.3, Table 3.2). In addition to inducing IgM secretion, mitogen-activated autologous T cells appeared to deliver a survival signal to the leukemic CD5+ B cells during *in vitro* culture. This could also be mediated via T cell contact or soluble signals from the T cells to the B cells. It has previously been reported that triggering normal human B cells through CD40 with anti-CD40 mAb or recombinant CD40L prevents their death (Liu *et al.*, 1991b; Holder *et al.*, 1993). Because mitogen-stimulation of T cells induces expression of CD40L (Castle *et al.*, 1993), it is possible that the subsequent interaction of CD40L with CD40 resulted in the enhanced viability of the leukemic CD5+ B cells. The role that cytokines play in leukemic CD5+ B cell survival is described in Chapter 5.

The concentrations of PMA required for activation of normal B cells are 10-20 fold less than those found to be optimal for the leukemic CD5+ B cells used in this study (Suzuki *et al.*, 1985; Beiske *et al.*, 1988). Furthermore, the subsequent activation of leukemic B cells achieved by PMA is considerably less than that of normal B cells (Beiske *et al.*, 1988). These findings are consistent with the suggestion that malignant B cells are more resistant to *in vitro* activation and that stimulation with one agent, which induces significant activation of normal B cells, is often

insufficient for the successful activation of leukemic B cells. Earlier studies indicated that antibodies to sIg either have no effect on CLL B cells, or deliver stimulatory or inhibitory signals resulting in an enhancement or reduction in cell proliferation. Studies that failed to find an effect of anti-μ on CLL B cells attributed this to the low levels of Ig expressed on the B cell surface or abnormal capping of sIg, resulting in impaired signalling via sIg (Beiske *et al.*, 1988). When used alone, it was also found that $F(ab')_2$ anti-μ, at a range of concentrations, did not induce leukemic $CD5^+$ B cell activation as evidenced by lack of cell enlargement, cell division or RNA synthesis (Figures 3.19, 3.20, data not shown). This provides additional data that CLL B cells are resistant to even the earliest stages of cellular activation as anti-Ig has been found to cause an increase in the volume/size of both human and murine normal B cells (Beiske *et al.*, 1988; Hodgkin *et al.*, 1991a). However, Ig expressed by CLL B cells could clearly deliver progression signals. This was indicated by the ability of $F(ab')_2$ anti-μ to enhance by 2-20-fold the uptake of ^{3}H-thymidine and ^{3}H-uridine induced by a suboptimal concentration of PMA in PBLs obtained from the 3 CLL patients. This effect was underscored by the demonstration that preparations depleted of T cells responded similarly (Figures 3.17, 3.18). Co-stimulation of leukemic $CD5^+$ B cells with $F(ab')_2$ anti-μ + PMA_{low} induced a level of proliferation and RNA synthesis that was comparable to that induced by an optimal concentration of PMA. Interestingly, although the stimulation indices where greatest when the cells were co-stimulated with $F(ab')_2$ anti-μ and PMA_{low}, maximal uptake of ^{3}H-thymidine and ^{3}H-uridine occurred following activation with $F(ab')_2$ anti-μ plus PMA_{opt}. Importantly, this protocol induced proliferation in a population of leukemic $CD5^+$ B cells that were refractory to proliferative signals delivered by PMA alone (MOT; Figure 3.19c). The kinetics of the responses of leukemic $CD5^+$ B cells were

comparable to normal human B cells which proliferated maximally after 3 days of stimulation with PMA and anti-μ when used at concentrations of 0.1-1 ng/mL and 75 μg/mL, respectively (Suzuki *et al.*, 1985). It has been previously demonstrated that PMA prevents leukemic B cells from undergoing apoptosis (Forbes *et al.*, 1992). These findings were reproduced in this study where 30-40% more viable cells were recovered from cultures of cells stimulated with PMA_{opt} and PMA_{low}, compared to cultures of unstimulated CLL B cells (Figure 3.15, Table 3.3). The enhancement of cell viability caused by PMA was not further increased when combined with $F(ab')_2$ anti-μ. Consequently, the higher level of proliferation and RNA synthesis induced by $F(ab')_2$ anti-μ in concert with PMA does not appear to be due to uptake of ^{3}H-thymidine or ^{3}H-thymidine by a larger proportion of viable cells, but is more likely to result from a higher level of incorporation per cell. Thus, although previous reports have found that antibodies to sIg can result in the death of leukemic B cells, the results presented here indicate that sIg can act as a receptor for signals which result in increased activation of CLL B cells co-stimulated with PMA.

Following preactivation with $F(ab')_2$ anti-μ and PMA for 48 hours and reculture in complete media for 7 days, 2 of the leukemic $CD5^+$ B cell populations failed to secrete detectable IgM, while the third population (MIL) did. However, the amount secreted by the cells preactivated with anti-μ in concert with PMA was only 1.5-2 fold greater than that secreted by cells preactivated with PMA alone (Table 3.3). Thus, although proliferation and RNA synthesis of leukemic $CD5^+$ B cell in the presence of anti-μ/PMA_{low} is similar to that induced by PMA_{opt}, co-stimulation of the cells does not induce as great a degree of differentiation. These results can be interpreted as indicating that co-stimulation of leukemic $CD5^+$ B

cells via ligation of sIg and activation of PKC does not cause a large increase in the extent of cellular differentiation, compared to either activator alone, even though this protocol is a more potent inducer of cell division and RNA synthesis. That is, activation signals delivered to the leukemic CD5+ B cells by anti-μ primarily result in enhanced cell proliferation and RNA synthesis, but have minimal effect on cell viability or differentiation to IgM secretion.

Similar to the results described here, B cells obtained from patients with non-Hodgkin's lymphoma have also been reported to proliferate in the presence of antibodies to sIg and PMA (Godal *et al.*, 1982; Beiske *et al.* 1984a). Under these conditions, proliferation in response to anti-sIg/PMA was 2-5 times greater than that induced by PMA alone. Differentiation of such lymphoma B cells also occurred in the presence of anti-sIg and PMA. This was demonstrated by plasmacytoid transformation and the accumulation of cIg, although the level of secreted Ig was not determined in these studies (Beiske *et al.*, 1984a, 1984b). However, because the cell preparations contained up to 20% T cells, the lymphoma B cells were likely to have been activated not only by anti-sIg and PMA but also with cytokines secreted by and cell surface molecules expressed on PMA-activated T cells. In addition, although there is clearly a response by the B cells, the full extent of this may be masked by T cell proliferation induced by PMA. Interestingly, some CLL B cells have also been found to increase the amount of cIg following PMA stimulation without concomitant secretion of intact Ig molecules (Totterman *et al.*, 1981). Based on these observations, it is possible that the combination of anti-μ plus PMA did induce the leukemic CD5+ B cells described in this chapter to undergo partial differentiation, as defined as an increase in cIg content, yet maturation into Ig-secreting cells may have required an additional

stimulus. This is supported by the finding that plasmacytoid transformation of some non-Hodgkin's lymphoma B cells stimulated with anti-μ plus PMA occurred independently of cIg synthesis and accumulation (Beiske *et al.*, 1984a), suggesting that partial, but not terminal, differentiation of the B cells can result from this mode of stimulation.

Despite previous reports describing the heterogeneity of responses of B-CLL cells from different patients to the same stimuli, several consistencies were observed in the results reported here with respect to the *in vitro* activation of the leukemic CD5+ B cells obtained from 3 patients with B-CLL. Firstly, mitogen-induced activation and differentiation of leukemic CD5+ B cells was abolished following the depletion of the residual normal T cells. This was not surprising, given the data of previous studies which demonstrate the critical role played by activated T cells in B cell differentiation (reviewed in Banchereau and Rousset, 1992). Secondly, PMA was capable of inducing lymphoblastoid transformation, proliferation, RNA synthesis and IgM secretion in leukemic CD5+ B cells purified from the peripheral blood of these 3 patients. While PMA-induced activation was achievable in the absence of T cells, the extent of these cellular responses was enhanced and prolonged in the presence of the T cells. This is likely to be mediated by the secretion of cytokines by the PMA-activated T cells. However the contribution of signals delivered by cell-cell contact to the enhanced responses of leukemic CD5+ B cells in the unfractionated CLL PBLs cannot be discounted. Interestingly, *in vitro* activation and differentiation of leukemic CD5+ B cells, achieved either by a T cell-dependent or T cell-independent mechanism, was associated with the recovery of significant numbers of viable cells. This suggests that efficient *in vitro*

activation of leukemic CD5+ B cells only occurs following receipt of a survival signal. Finally, the combination of $F(ab')_2$ fragments of polyclonal rabbit anti human μ-chain antiserum and varying concentrations of PMA induced proliferation and/or RNA synthesis in the leukemic CD5+ B cells to levels similar to or exceeding those induced by the optimal PMA concentration. These results indicate that leukemic CD5+ B cells can be stimulated to undergo proliferation, enhanced RNA synthesis and differentiation to IgM secretion in the absence of T cell generated signals and that the signalling pathway mediated via sIg ligation remains intact in these clonally arrested B cells.

Chapter 4:

Characterisation of In Vitro *Apoptosis of Leukemic CD5+ B Cells*

Introduction

Apoptosis, or programmed cell death, is a series of biochemical events that ultimately give rise to cell death. This mechanism of cell death is distinct from necrosis and is characterised by a rapid reduction in cell size and volume, perturbations to the cell membrane (zeiosis) and the fragmentation of genomic DNA into multimers of 180-200 base pairs in length (Cohen, 1991, Cohen and Duke, 1992). One of the regulators involved in the prevention of apoptosis is the product of the *bcl-2* gene, a 25 kDa protein present in the inner and outer mitochondrial membranes, as well as the endoplasmic reticulum and nuclear envelope (Hockenberry *et al.*, 1990; Lithgow *et al.*, 1994; Reed, 1994). Transfection of growth-factor dependent hemopoietic cell lines with the *bcl-2* gene resulted in their enhanced *in vitro* survival following removal of the growth factor (Vaux *et al.*, 1988; Hockenberry *et al.*, 1990; Nunez *et al.*, 1990). Mice expressing the *bcl-2* transgene possess increased numbers of B cells and serum Ig as a result of *bcl-2* overriding the normal mechanisms controlling the size of the B cell pool (Nunez *et al.*, 1990; Strasser *et al.*, 1991a; Smith *et al.*, 1994). Furthermore, when cultured *in vitro*, B cells from bcl-2-transgenic mice survived for significantly longer periods of time than littermate controls (McDonnell *et al.*, 1989). Interestingly, the majority of human follicular lymphomas harbour the t(14;18) chromosome translocation which places the *bcl-2* gene adjacent to the Ig heavy chain gene. This translocation causes both the deregulated expression of the chimeric (*bcl-2-Ig*) gene product and aberrantly elevated levels of bcl-2-Ig mRNA and protein in the affected cells (reviewed in Korsmeyer, 1992a, 1992b; Reed, 1994). Consequently, bcl-2 appears to contribute to B cell lymphomas by preventing apoptosis. Interestingly, a number of homologs of bcl-2 have been recently identified that either duplicate (bcl-x_L and BAG-1) or antagonise (bcl-x_S,

bax and Bad) the function of bcl-2 indicating that this family of molecules may play a significant role in the induction and prevention of programmed cell death (Boise *et al.*, 1993; Oltvai *et al.*, 1993; Yin *et al.*, 1994; Takayama *et al.*, 1995; Yang *et al.*, 1995).

Unique characteristics of the malignant cells in B-CLL include (i) a low proliferative index, with >99% of cells in the G_0 phase of the cell cycle (Andreeff *et al.*, 1980); (ii) enhanced *in vivo* survival (Hoffbrand *et al.*, 1993), and (iii) constitutive expression of *bcl-2* protein (Schena *et al.*, 1992b). Unlike the majority of B cell lymphomas, the expression of bcl-2 is independent of chromosomal translocation and is therefore regulated by an alternative mechanism(s) (Zutter *et al.*, 1991; Hanada *et al.*, 1993). Interestingly, and in contrast to their enhanced survival *in vivo*, leukemic $CD5^+$ B cells from patients with B-CLL have been reported to undergo spontaneous apoptosis *in vitro* (Collins *et al.*, 1989). This chapter describes the characterisation of apoptosis in six different CLL samples. All cell populations examined expressed high levels of the bcl-2 protein, as determined by flow cytometry. Despite this, the cells underwent spontaneous apoptosis *in vitro*, as demonstrated by DNA fragmentation and alterations in cell morphology typical of apoptosing cells. The *in vitro* half-lives of the six different leukemic $CD5^+$ B cell populations ranged from 36 hours to >120 hours. Irrespective of the half-life, cell death and apoptosis were accompanied by a 2-3 fold reduction in expression of bcl-2 by the apoptosing cells. However, the percentage of cells expressing bcl-2 remained constant throughout the culture period. This suggests that the enhanced survival of leukemic $CD5^+$ B cells *in vivo* may be mediated by the sustained expression of bcl-2 and that additional mechanisms exist that are capable of overriding the protective effect of bcl-2 when this anti-apoptotic oncoprotein is present at reduced levels.

Results

4.1. Phenotype of CLL B Cells

In addition to the three leukemic B cell populations described in Chapter 3, a further three populations of leukemic CD5+ B cells were examined. All samples were obtained from patients suffering from B-CLL. The proportion of leukemic CD5+ B cells was enriched following depletion of residual normal T cells by rosetting with AET-treated SRBCs. The purity of the resultant cell preparation was determined by immunofluorescence staining and flow cytometric analysis. Prior to rosetting, 0.5-8.0% of cells present in the the unpurified CLL PBL preparations were CD3+ T cells. As listed in Table 4.1, one round of rosetting was sufficient to reduce the level of T cells present in the B cell-enriched population to 0-1.5%, for the six different populations examined. The values listed in Table 4.1 are representative of the purity of the different leukemic CD5+ B cell preparations used at different times throughout the course of this study, and also apply to the results described in this and the following Chapter.

4.2. Kinetics of Leukemic CD5+ B Cell Death, In vitro

The kinetics of cell death were investigated by culturing B cells from six different B-CLL patients for 8 days and determining cell viability at various time points by ethidium bromide exclusion and quantitation by flow cytometry. The viability of the different leukemic CD5+ B cell populations was >90% prior to culture. As can be seen in Figure 4.1a, the viability of RAN and KAR CLL cells after 48 and 96 hours of *in vitro* culture was ~50% and ~25%, respectively. The viability of MUR and MCK cells, on the other hand, was >70% for the first 4 days of *in vitro* culture. MOT and MIL cells also followed a similar course during *in vitro* culture, with cell viability being ~70% and ~50% after 48 and 96

hours, respectively. Despite the differences observed during the first half of the culture, at the completion of the culture period only 5-15% of cells in all populations examined were viable. Table 4.2 lists the half-lives for the different leukemic CD5+ B cell populations and highlights the significant differences in kinetics of cell death.

Table 4.1: *Phenotype of Purified Leukemic CD5+ B Cells*

	Cell Surface Antigen			
CLL Patient	CD3	CD5	CD19	sIg
MIL	0.66±0.28	97.9±1.35	97.5±1.1	99.2±0.58
KAR	0.42±0.25	98.0±1.8	95.4±1.2	99.1±0.7
MOT	0.068±.0.1	73.5±1.0	97.7±1.1	99.2±0.8
RAN	0.35±0.4	98.8±0.7	97.8±0.35	99.4±0.3
MUR	1.55±0.15	99.5±1.0	97.5±1.0	99.0±1.0
MCK	1.0±0.5	99.1±0.8	97.5±0.55	98.8±0.2

Purified leukemic B cell preparations were prepared by rosetting PBLs from CLL patients. Cells were then incubated with FITC- or PE-conjugated mouse mAb specific for CD3, CD5 or CD19, or polyclonal FITC-conjugated goat anti-sIg. Each value represents the percentage of cells expressing the antigen of interest.

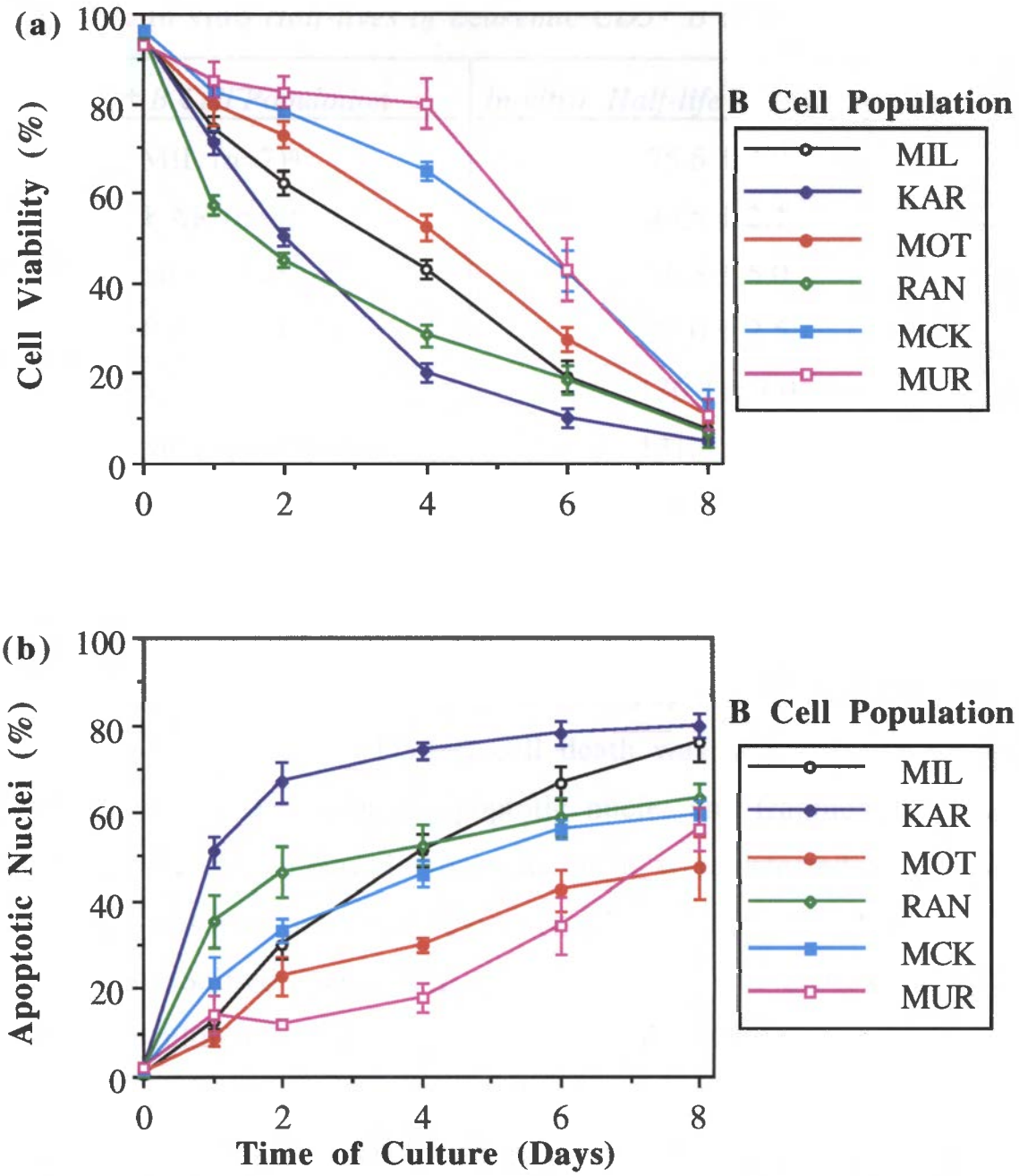


Figure 4.1: Kinetics of Leukemic CD5+ B Cell Death and Apoptosis.

Purified leukemic B cells from 6 different B-CLL patients were cultured for 8 days in the absence of exogenous stimuli. At various time points, the percentage of viable cells (a) and apoptotic nuclei (b) were measured by flow cytometry, as described in *Materials and Methods*. Each point represents the mean percentage of viable cells (a) or the mean percentage of apoptotic nuclei (b) ± s.e.m. of at least 3 independent experiments (see Table 4.2).

Table 4.2: In vitro *Half-lives of Leukemic CD5+ B Cells*

CD5+ B Cell Population	*In vitro Half-life* [b] (hours ± sem)
MIL (n=7)[a]	75.6 ± 4.9
KAR (n=5)	49.5 ± 2.1
MOT (n=5)	96.5 ± 5.0
RAN (n=4)	39.0 ± 2.5
MUR (n=3)	136.4 ± 7.0
MCK (n=7)	131.0 ± 6.1

a: n is the number of independent experiments performed using a particular population of leukemic CD5+ B cells; b: half-life was defined as being the time at which 50% of cells remained viable.

4.3. Leukemic B Cells Undergo Spontaneous Apoptosis, In vitro

The cause of leukemic CD5+ B cell death was demonstrated to be apoptosis by the detection of apoptotic nuclei and fragmented DNA throughout the culture period. Apoptotic nuclei were detected by a flow-cytometric based procedure previously described by Nicoletti *et al.* (1991) and modified by Illera *et al.* (1993). Using this method, diploid DNA of uncultured cells appears as a tight unimodal peak of relatively high fluorescence. During apoptosis, the proportion of cells with diploid DNA diminishes, and a peak with reduced fluorescence apears which represents hypodiploid or apoptotic nuclei. Prior to the initiation of culture, the level of apoptotic nuclei in the different cell populations was minimal (<2%). After 24 hours of *in vitro* culture, at least 10% of unstimulated leukemic CD5+ B cells displayed apoptotic nuclei. The rate of appearance of apoptotic nuclei in the different cell populations was similar to the kinetics of cell death. That is, the cell populations exhibiting the shortest *in vitro* half-lives (RAN and KAR) also contained the highest amount of apoptotic nuclei after 24 hours of culture (40-50%). Similarly,

accumulation of apoptotic nuclei in cultures of MCK B cells, which had an *in vitro* half-life of >120 hours, was significantly less than that of cells which underwent *in vitro* death at faster rates. Cells from patients MOT and MUR displayed <20% apoptotic nuclei after 48 hours and the gradual increase with time was considerably slower. This is consistent with their prolonged cell survival relative to the other leukemic CD5+ B cell populations (Figure 4.1b). Despite this variability, the percentage of apoptotic nuclei continued to increase throughout the culture period or until a plateau was reached for all cell populations examined (Figure 4.1b). After 8 days of *in vitro* culture, 50-80% of cells in the 6 different populations exhibited apoptotic nuclei (Figure 4.1b).

Apoptosis was also demonstrated by electrophoretic analysis of DNA extracted from unstimulated leukemic CD5+ B cells following different times of *in vitro* culture. Figure 4.2 illustrates the time course of DNA fragmentation for two populations of cells. From this result, it is clear that the starting cell preparation (time 0) contained little or no fragmented DNA. This is consistent with the detection of apoptotic nuclei by the flow cytometric based method, as illustrated in Figure 4.1b. However, 24 hours after the initiation of the culture, fragmented DNA was detectable for the two leukemic CD5+ B cells populations. The fragments appeared to correspond to multimers of 180-200 base pairs, consistent with the description of apoptosis occurring in other cells and cell lines. The relative amount of fragmented DNA appeared to increase with time and this was accompanied by a reduction in the amount of high molecular weight, intact genomic DNA. Similar results were observed when cells from CLL patient RAN were subjected to the same experiment (result not shown). These results are consistent with the findings shown in Figure

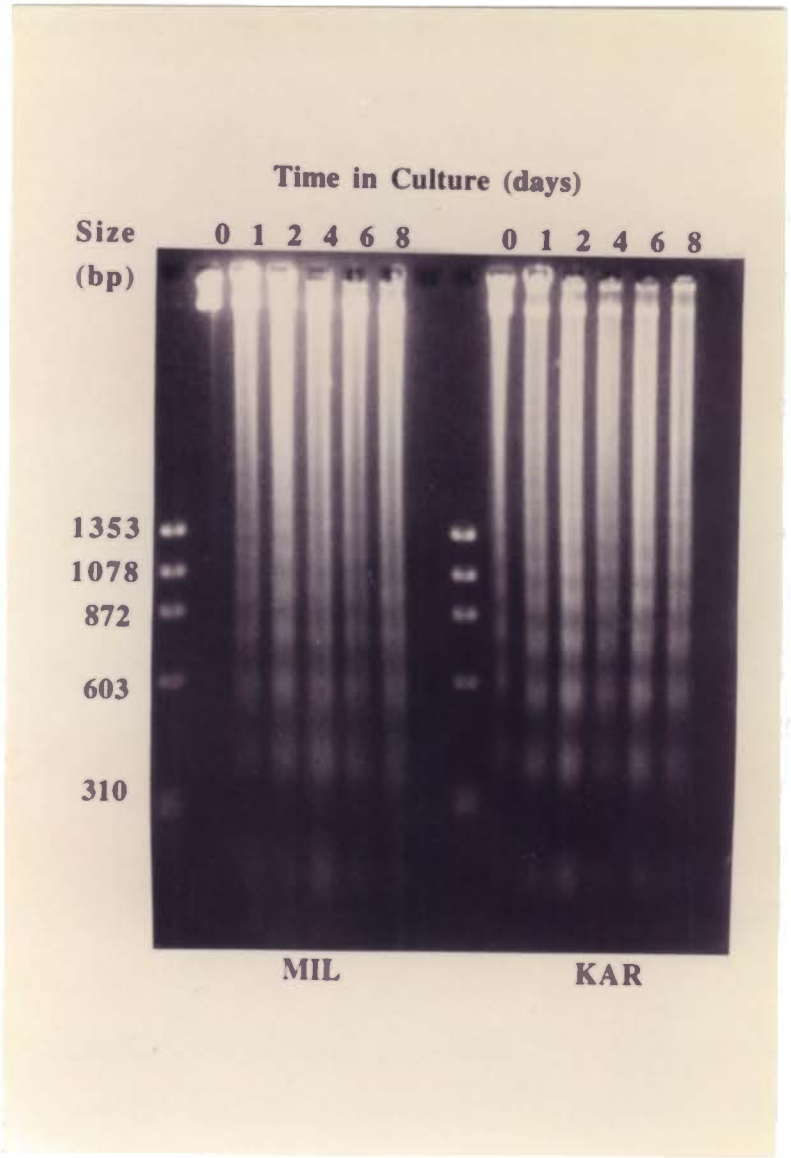


Figure 4.2: Leukemic CD5+ B cells Undergo DNA Fragmentation *in vitro.*

Purified leukemic B cells from 2 different B-CLL patients were cultured for 8 days in the absence of additional stimuli. At various time points, DNA was extracted from the cells and electrophoresed through a 1.8% agarose gel. The gel was stained with ethidium bromide and the DNA visualised under UV light.

4.1b. Thus, it is clear from Figures 4.1 and 4.2 that leukemic $CD5^+$ B cells from a number of CLL patients undergo apoptosis following *in vitro* culture in the absence of any additional stimuli.

4.4. Characterisation of Apoptotic Cell Populations

Throughout the culture period, cell death and DNA fragmentation were accompanied by the appearance of a population of cells with reduced forward light scattering characteristics compared to the starting cell population. This is likely to represent a reduction in cell size because the parameter of forward light scatter equates with cell size. The kinetics of appearance of cells with reduced size from patients MIL, KAR and MOT over an 8 day culture period are shown in Figure 4.3. Cells with light scatter properties of uncultured cells appear in region 1 (R1), while cells with reduced size are in region 2 (R2). Prior to *in vitro* culture (day 0), the majority of cells (>90%) appeared in R1. Following 48 hours of *in vitro* culture, a population of cells with reduced forward scatter (i.e. size) was present in cultures of cells obtained from the three different B-CLL patients. Interestingly, the cells from patient KAR that displayed reduced forward scatter also demonstrated increased 90° angle light scatter. As the culture period progressed, the number of cells appearing in R2 continued to increase, at the expense of cells in R1. At the completion of the culture period (day 8), >90% of cells resided in R2 (Figure 4.3). These two differently sized cell populations could be separated by density gradient centrifugation. Figure 4.4 portrays the light scatter profiles, the ability to exclude ethidium bromide, the presence of apoptotic nuclei and the expression of bcl-2 (see section 4.6 for a detailed description of bcl-2 expression by *in vitro* cultured leukemic $CD5^+$ B cells) of cultured MCK B cells that had been enriched for or depleted of viable cells following density gradient centrifugation. The unfractionated population was

Figure 4.3: Apoptosis is Accompanied By Reduced Forward Light Scatter.

The light scatter characteristics of MIL (top), KAR (middle) and MOT (lower) B cells were determined by flow cytometry after 0, 2, 4 and 8 days of culture. The value indicates the percentage of cells in region 1. The x and y axes represent forward and 90° angle light scatter, respectively.

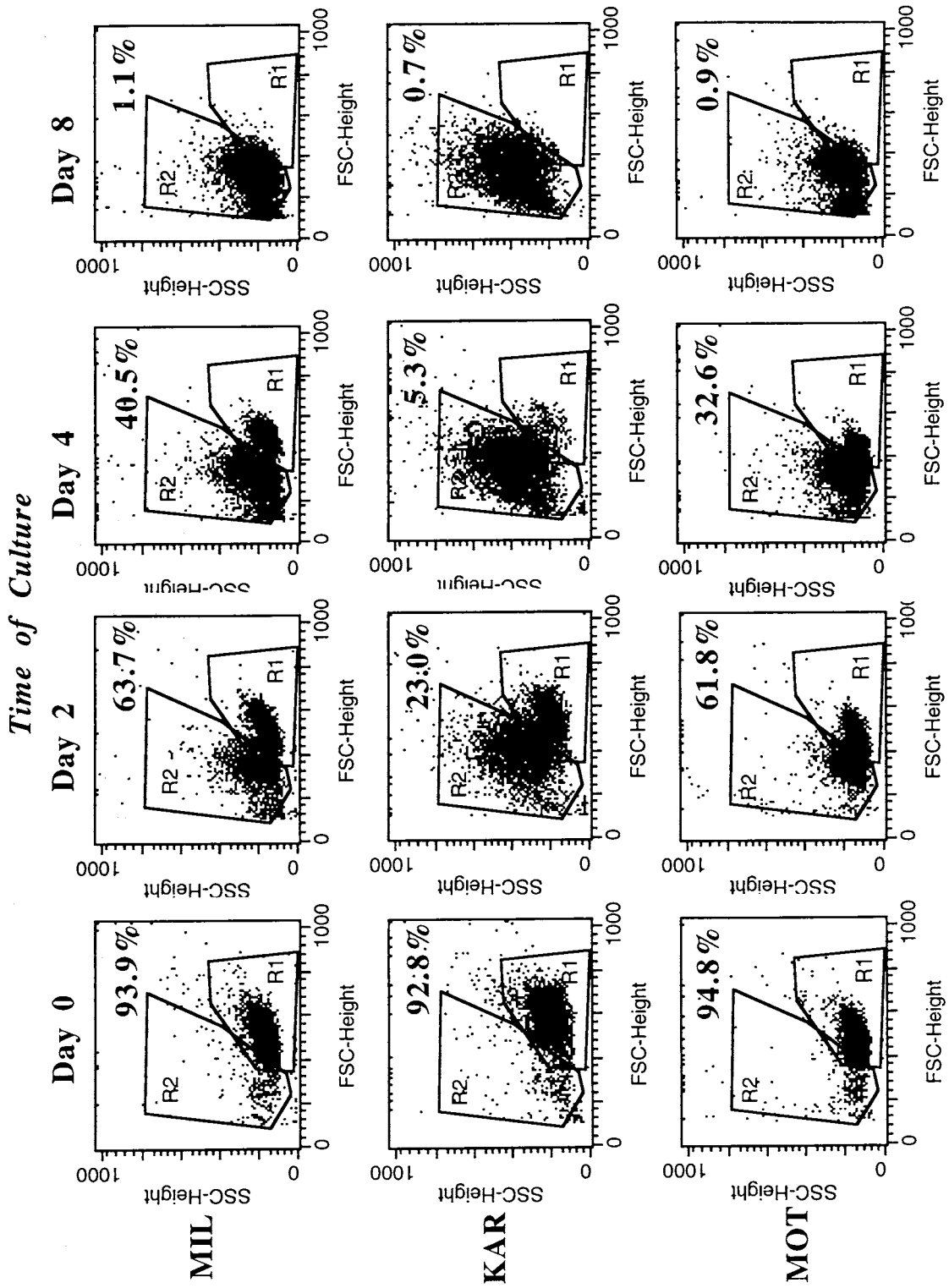
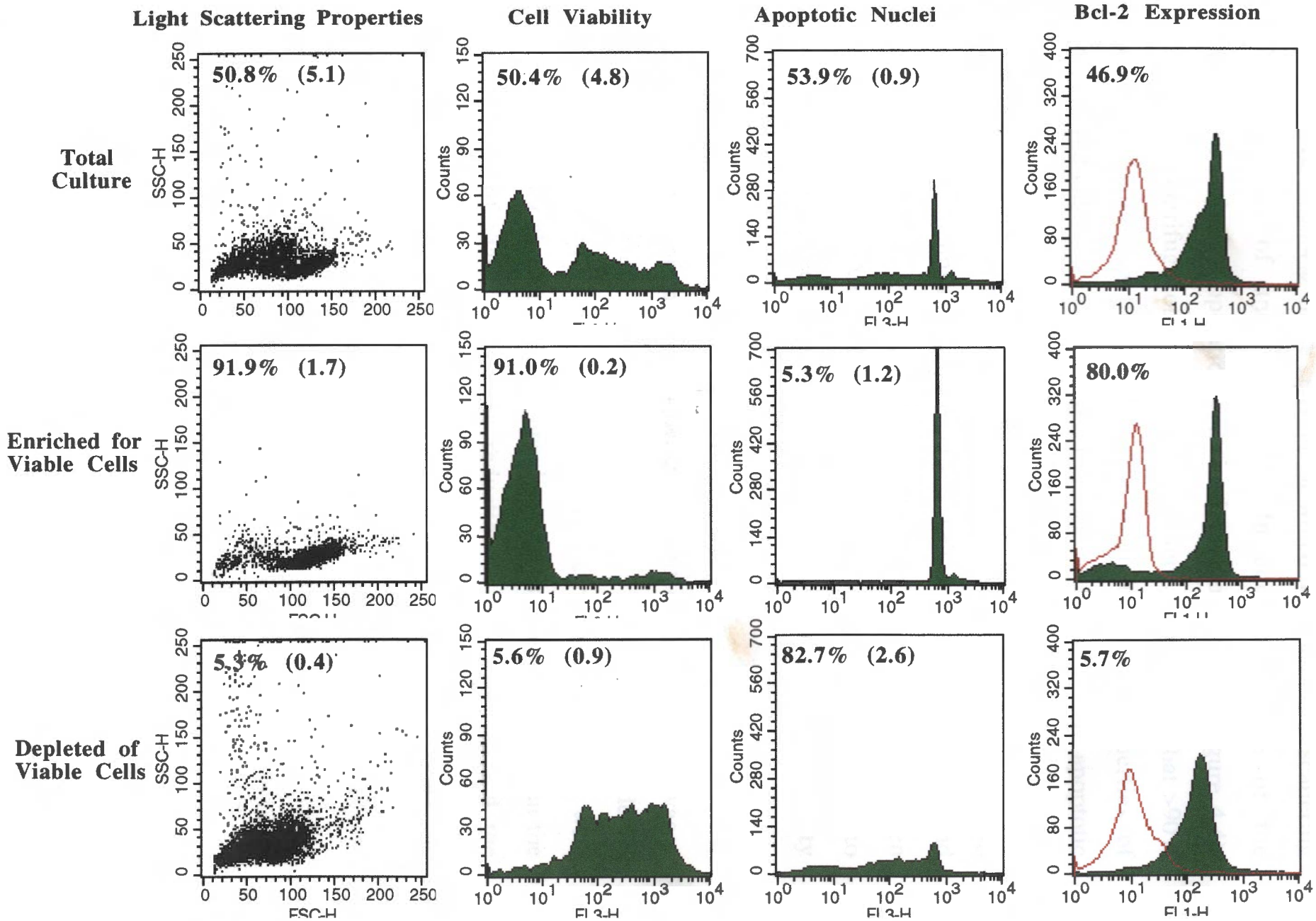

Time of Culture
Day 0
Day 2
Day 4
Day 8
MIL
KAR
MOT
93.9%
63.7%
40.5%
1.1%
92.8%
23.0%
5.3%
0.7%
94.8%
61.8%
32.6%
0.9%
R1
R2
SSC-Height
FSC-Height
0
1000

Figure 4.4: Characterisation of Apoptotic Cell Populations.

MCK B cells were cultured in the absence of any stimuli for 5 days, after which time the cells were enriched for or depleted of viable cells by centrifugation over Ficoll-Hypaque. Viable cells were recovered from the interface of the density gradient while non-viable cells were located beneath the density gradient. Light scatter profiles, cell viability, apoptotic nuclei and bcl-2 expression of the total culture and the fractionated populations were then determined, as described in *Materials and Methods*. The percentage of cells with light scatter properties of uncultured cells, the percentage of viable cells, percentage of cells exhibiting apoptotic nuclei and the percentage of cells displaying a bcl-2_{high} phenotype are indicated on each panel. The values in parentheses indicate the standard deviation of the mean of triplicate samples. For the histograms of bcl-2 expression, the solid histogram represents the fluorescence of cells incubated with anti-bcl-2 mAb while the outline histogram represents the fluorescence of cells incubated with an isotype-matched control mAb.


Light Scattering Properties
Cell Viability
Apoptotic Nuclei
Bcl-2 Expression
Total Culture
Enriched for Viable Cells
Depleted of Viable Cells
50.8% (5.1)
50.4% (4.8)
53.9% (0.9)
46.9%
91.9% (1.7)
91.0% (0.2)
5.3% (1.2)
80.0%
5.3% (0.4)
5.6% (0.9)
82.7% (2.6)
5.7%
SSC-H
FSC-H
Counts
FL3-H
FL1-H

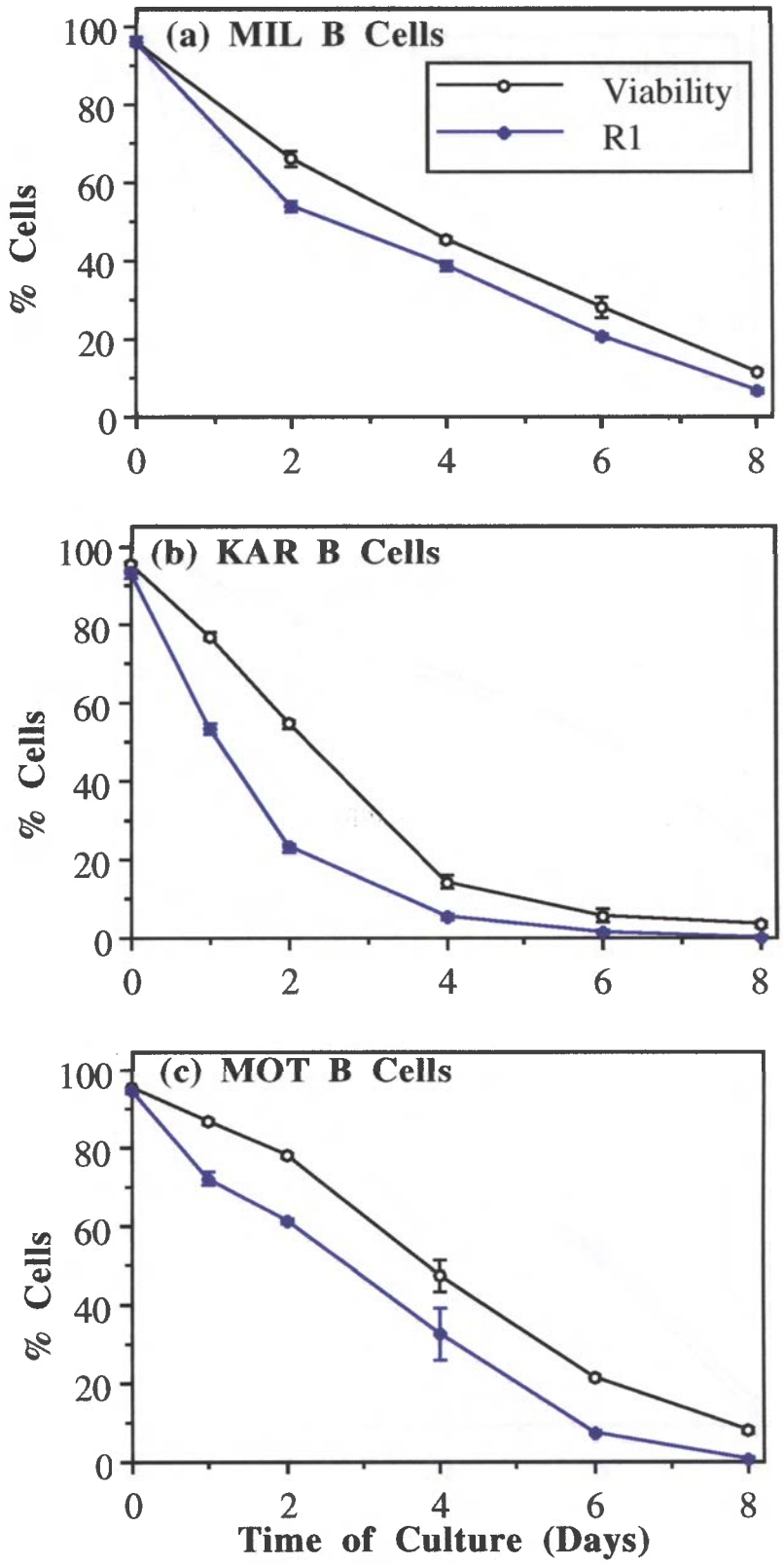
comprised of 50.4% viable cells, all of which displayed light scattering characteristics of uncultured cells. In addition, 53.9% of the unfractionated cell population contained apoptotic nuclei (Figure 4.4). Following centrifugation over a density gradient, it was found that >90% of cells comprising the population with light scatter properties of uncultured cells were viable and only 5.3% of the cells were apoptotic (Figure 4.4). Thus, this cell population was reasonably homogeneous with regard to the stage of apoptosis. The population of cells displaying reduced size contained few viable cells as well as cells with apoptotic and diploid nuclei (Figure 4.4). Similar results to these were obtained when cultured MIL and MOT leukemic $CD5^+$ B cells were fractionated into populations enriched for or depleted of viable cells following density gradient centrifugation.

The appearance of the population comprised of smaller cells was a relatively early event in apoptosis and appeared to precede cell death. This was reflected by the observation that the percentage of cells with light scattering characteristics of uncultured cells was often less than the percentage of viable cells. The reduction in cell viability and the reduction in the proportion of cells with light scattering characteristics of uncultured cells (i.e. cells in R1) for the six different populations of leukemic $CD5^+$ B cells over an 8 day culture period are shown in Figure 4.5. These results demonstrate that a reduction in cell size often precedes the loss of membrane integrity. These findings also reinforce the finding that significant numbers of viable cells are present in the population with reduced size, although the proportion of viable cells in this population varied from patient to patient. For instance, ~80-90% of viable MIL, MCK and MUR B cells resided in the population displaying light scattering properties of uncultured lymphocytes. This contrasts KAR,

MOT and RAN B cells, where viable cells were located in populations of both sizes, with 30-50% of cells with reduced size remaining viable (Figures 4.3 and 4.5).

Figure 4.5: Reduction in Cell Size Precedes Cell Death.

Leukemic $CD5^+$ B cells from patients MIL (a), KAR (b), MOT (c), RAN (d), MCK (e) and MUR (f) were cultured for 8 days in the absence of any exogenous stimuli. After various times, the percentage of viable cells and the percentage of cells displaying light scattering characteristics of uncultured cells (i.e. those in R1) were determined. Each point represents the mean ± s.d. of triplicate samples and is representative of at least 3 independent experiments (see Table 4.2).


(a) MIL B Cells
Viability
R1
% Cells
100
80
60
40
20
0
0
2
4
6
8
(b) KAR B Cells
% Cells
(c) MOT B Cells
% Cells
Time of Culture (Days)

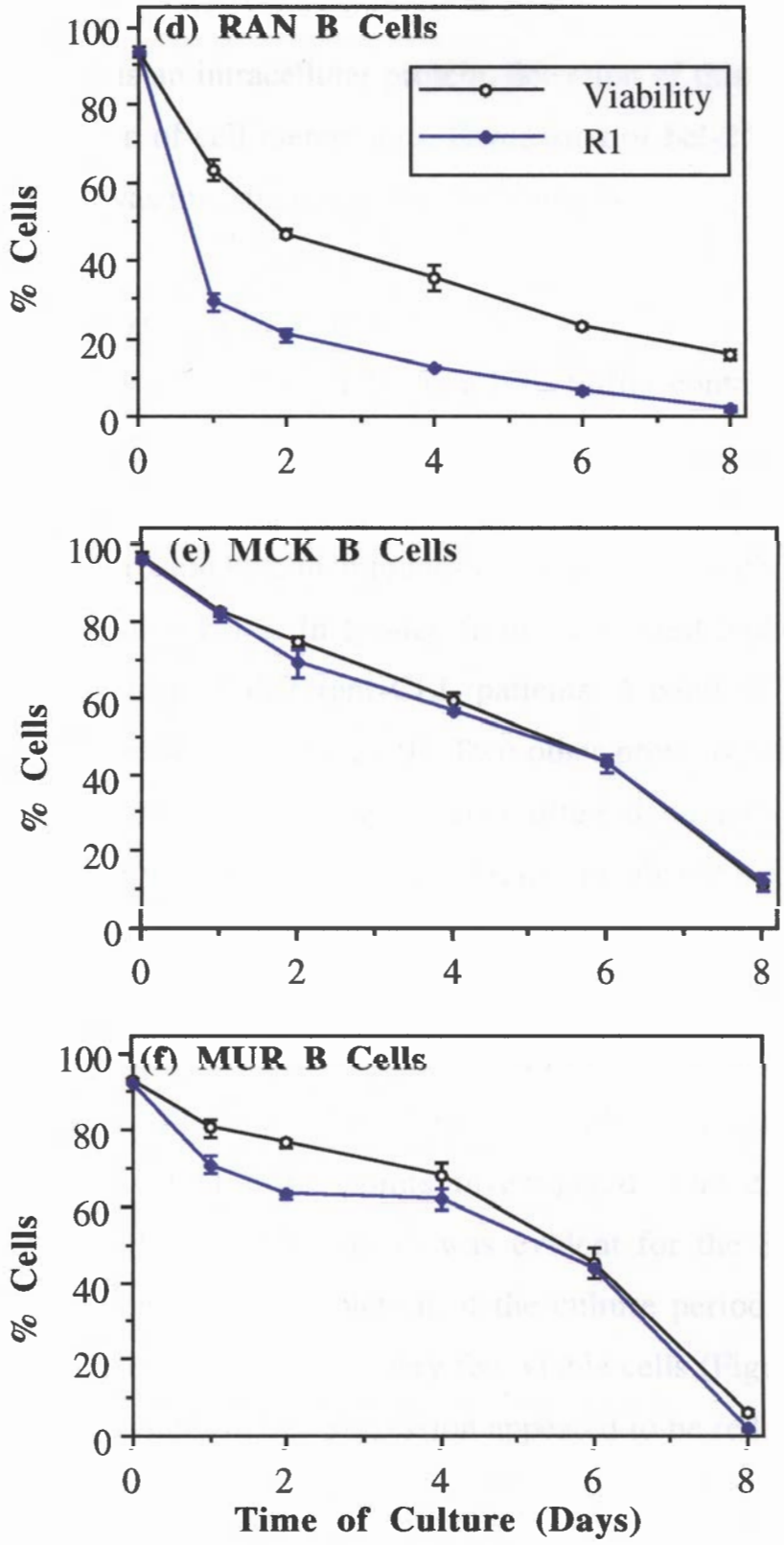


Figure 4.5: Reduction in Cell Size Precedes Cell Death (continued).

4.5. Expression of Bcl-2 by Leukemic B Cells

Because bcl-2 is an intracellular protein, detection of this protein requires permeabilisation of cell membranes. Expression of bcl-2 by the leukemic CD5+ B cells was investigated by two procedures.

i. SDS-PAGE/Western Blotting

Firstly, the cells were resuspended in a lysis buffer containing 0.1% SDS and then subjected to electrophoresis through 12.5% acrylamide gels. Bcl-2 was detected following electrotransfer of proteins present in the cell lysates onto nitrocellulose membranes and incubation with a mouse mAb specific for human bcl-2. In lysates from uncultured leukemic CD5+ B cells obtained from 3 different CLL patients, a band of ~25 kDa was detected (Figures 4.6a, b, c; Day 0). Two other proteins, of ~15 kDa and ~35 kDa molecular weight, were also often detected (Figure 4.6d). However, these were present on membranes incubated with the alkaline phosphatase-conjugated anti-mouse Ig secondary Ab only (Figures 4.6d). This indicates that these proteins were not specifically detected by the anti-bcl-2 Ab, but were cross-reacting with the secondary detecting Ab. During *in vitro* culture, bcl-2 remained detectable in lysates of leukemic CD5+ B cells at all time points investigated. The 25 kDa protein recognised by the anti-bcl-2 mAb was evident for the 3 different cell populations even at the completion of the culture period, a time which corresponded to the presence of very few viable cells (Figures 4.6a, b, c). At this time, the intensity of expression appeared to be reduced, compared to the starting cell population. However bcl-2 was clearly evident in these cell preparations. This suggested that spontaneous apoptosis of CLL B cells was a bcl-2-independent event.

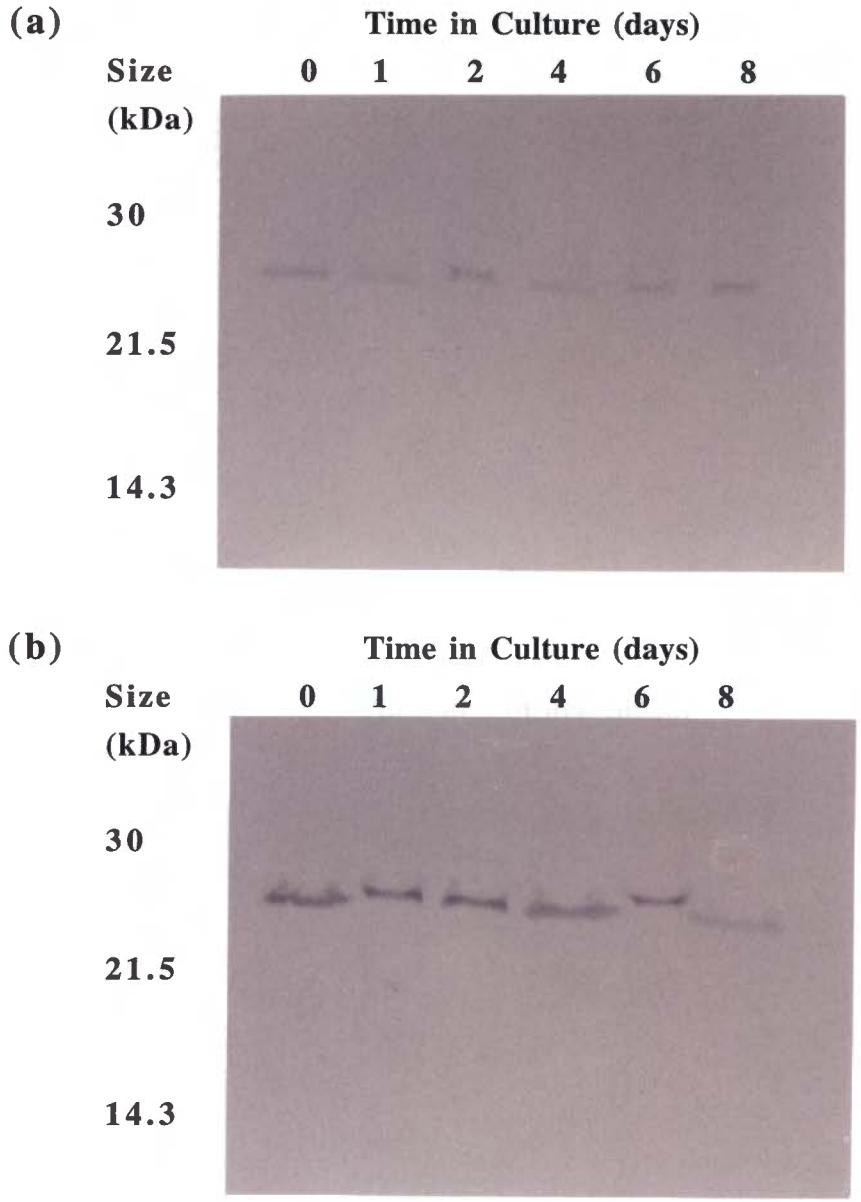


Figure 4.6: Detection of bcl-2 in Leukemic CD5+ B Cells by SDS-PAGE and Western Blotting.

Leukemic CD5+ B cells from patients MIL (a), MCK (b) and KAR (c, d) were cultured for 8 days in the absence of exogenous stimuli. Bcl-2 expression was determined in uncultured cells (day 0) and in cells cultured for 1, 2, 4, 6 and 8 days. The cells were resuspended in lysis buffer containing 0.1% SDS and electrophoresed through a 12.5% polyacrylamide gel. Proteins present in the cell lysates were transferred to a nitrocellulose membrane which was probed with an anti-bcl-2 mAb, followed by an alkaline phosphatase-conjugated anti-mouse antiserum (a, b, c) or with the alkaline phosphatase-conjugated anti-mouse antiserum alone (d).

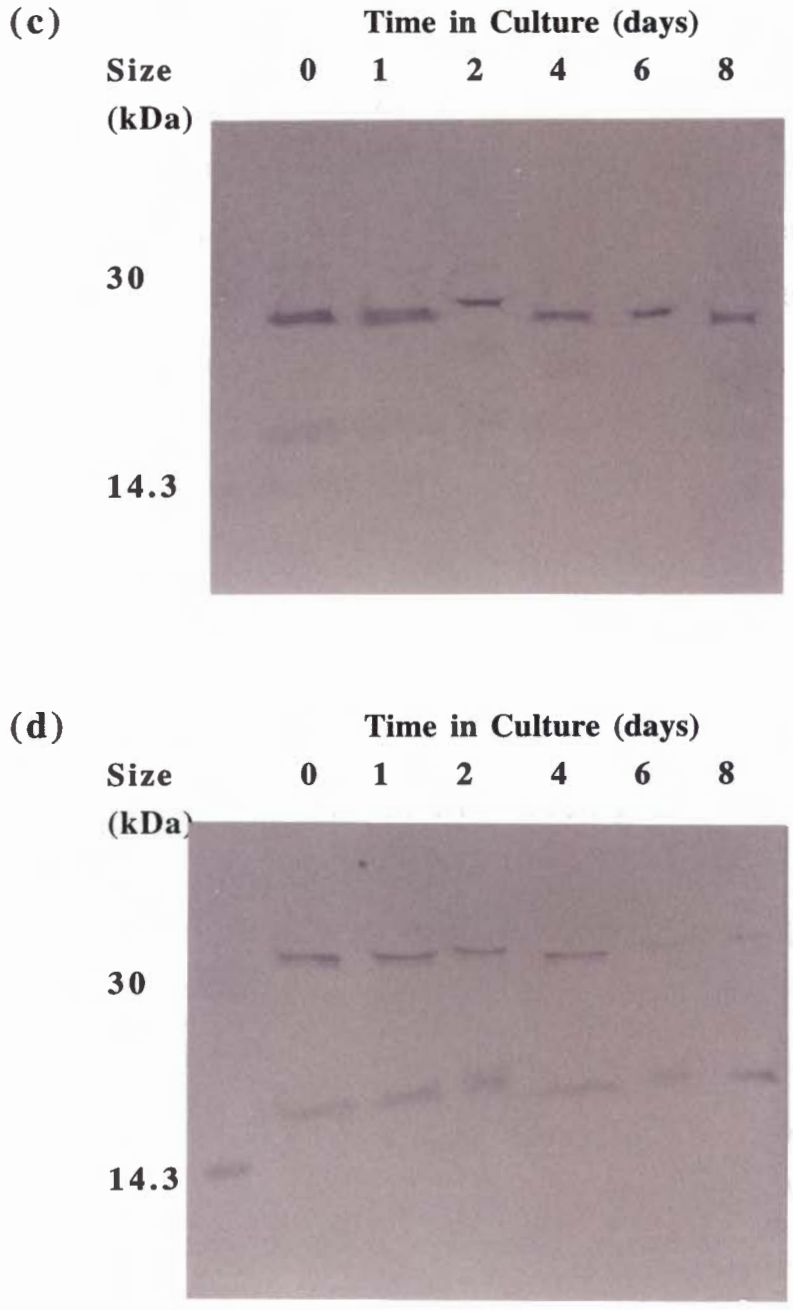


Figure 4.6: Detection of bcl-2 in Leukemic CD5+ B Cells by SDS-PAGE and Western Blotting (continued).

ii. Immuno-flow Cytometry

Optimisation of Detection of Bcl-2

A method for the detection and quantitation of intracellular bcl-2 expression by immunofluorescence and flow cytometric analysis was recently described by Aiello *et al.* (1992). This involves fixing the cells with 2% paraformaldehyde prior to permeabilisation with Triton X-100. The method was optimised for leukemic CD5+ B cells by determining the concentration of paraformaldehyde that resulted in maximal fluorescence intensity of bcl-2 following incubation of fixed B cells with an anti-bcl-2 mAb. Optimisation was achieved by fixing the leukemic CD5+ B cells with 2, 1.5, 1.0 or 0.5% paraformaldehyde, followed by permeabilisation with 0.05% Triton X-100, as described (Aiello *et al.*, 1992). As can be seen in Figure 4.7 the mean fluorescence intensity (MFI) of bcl-2 expression by MIL leukemic CD5+ B cells was greatest when a concentration of 0.5-1.0% paraformaldehyde was used. Under these conditions, the fluorescence intensity of bcl-2 staining was 30-35 times greater than the MFI of the isotype matched control mAb. This contrasted the higher concentrations (1.5% and 2.0%) of paraformaldehyde, where the relative increase over the isotype control was only 8-12 fold. Similar results were obtained when MCK B cells were used to optimise paraformaldehyde concentration (not shown). Thus, paraformaldehyde at concentrations exceeding 1.0% significantly reduced the MFI of bcl-2 expression. Consequently, all subsequent experiments aimed at investigating bcl-2 expression utilised paraformaldehyde at a concentration of 0.5-1.0%. Furthermore, each new stock solution of paraformaldehyde was prepared at 2.0% and the optimal concentration was re-assessed, as described above.

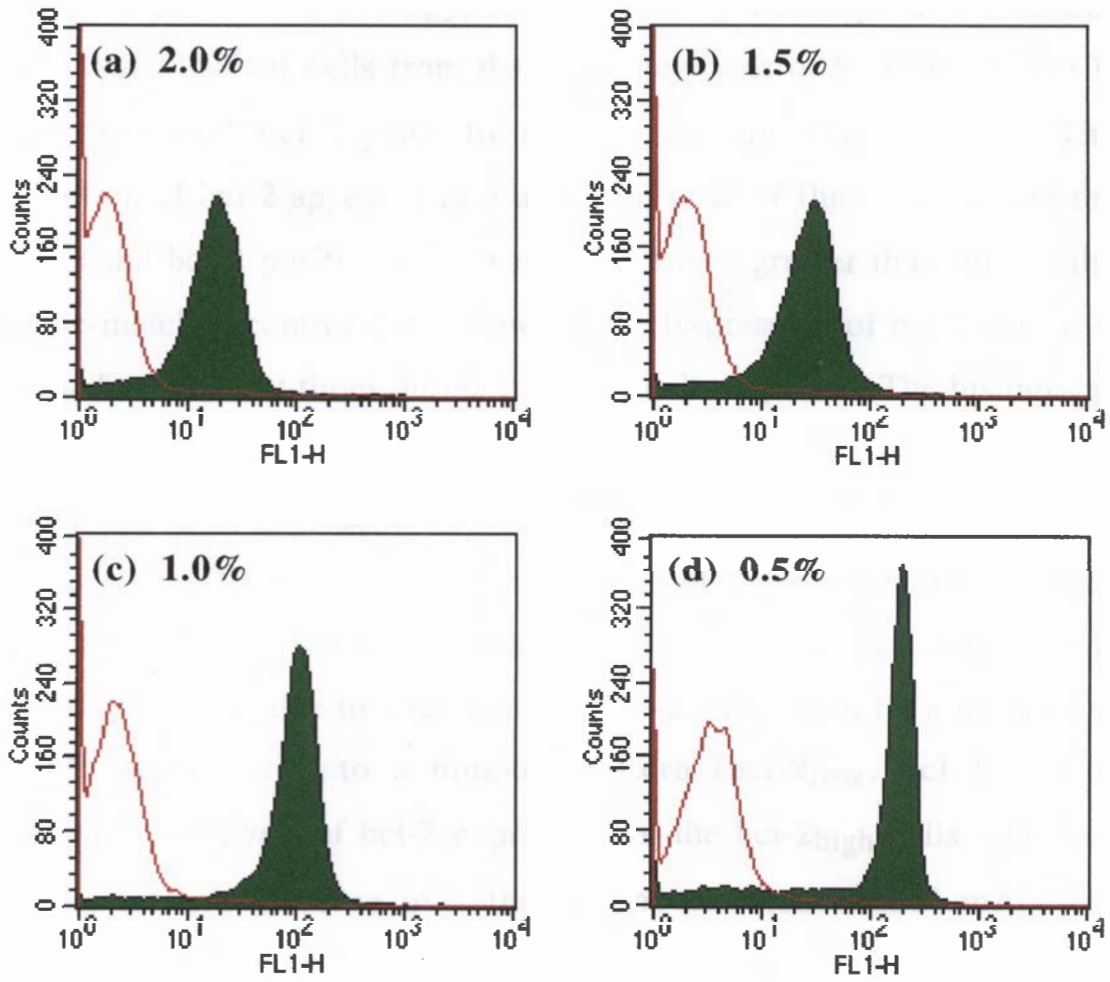


Figure 4.7: Optimisation of Detection of Bcl-2 by Immuno-flow Cytometry.

Leukemic CD5+ B cells from patient MIL were fixed with paraformaldehyde at concentrations of 2.0% (a), 1.5% (b), 1.0% (c) or 0.5% (d) for 10 minutes on ice. Following permeabilisation with 0.05% Triton-X, the cells were incubated with either anti-bcl-2 mAb (solid histogram) or an isotype-matched control mAb (outline histogram). Bound Ab was detected with FITC-conjugated anti-mouse Ig antiserum. Fluorescence was measured on a logarithmic scale.

4.6. Detection of bcl-2 Expression

Using the above methodology, and analysis by flow cytometry, it was found that >85% of cells from the six populations of leukemic $CD5^+$ B cells expressed bcl-2 prior to *in vitro* culture (Figure 4.8). The expression of bcl-2 appeared as a unimodal peak of fluorescence and the MFI of the bcl-2 positive cells was 15-45 times greater than that of the isotype-matched control mAb (Figure 4.8). Expression of bcl-2 was also assessed at different times during an 8 day culture period. The histograms in Figure 4.9 represent the expression of bcl-2 by cells obtained from patients MIL, MOT and MCK as determined at various times during *in vitro* culture. It is clear from these data that the overall fluorescence intensity of bcl-2 was reduced in the three different cell populations assayed. This was due to a reduction in bcl-2 expression by a proportion of cells, giving rise to a bimodal pattern ($bcl\text{-}2_{low}$, $bcl\text{-}2_{high}$) of expression. Analysis of bcl-2 expression in the $bcl\text{-}2_{high}$ cells indicated that the relative increase over the isotype-matched control mAb was similar to that observed prior to the initiation of culture. Cells displaying the $bcl\text{-}2_{low}$ phenotype were evident as early as 48 hours after the initiation of culture. Furthermore, the percentage of cells down-regulating the expression of bcl-2 continued to increase, at the expense of $bcl\text{-}2_{high}$ cells, such that after 8 days the majority of cells displayed the $bcl\text{-}2_{low}$ phenotype (Figure 4.9). Despite this reduction, the percentage of cells positive for bcl-2 remained constant (ie >85%). Figure 4.10 illustrates the results of experiments where different leukemic $CD5^+$ B cell populations were cultured for 8 days and cell viability and the percentage of cells displaying the $bcl\text{-}2_{high}$ phenotype were concomitantly determined. The loss of bcl-2 protein was found to correlate with the rate of cell death. Thus, while ~75% of MCK cells displayed a $bcl\text{-}2_{high}$ phenotype after 2 days, the percentage of KAR and RAN B cells

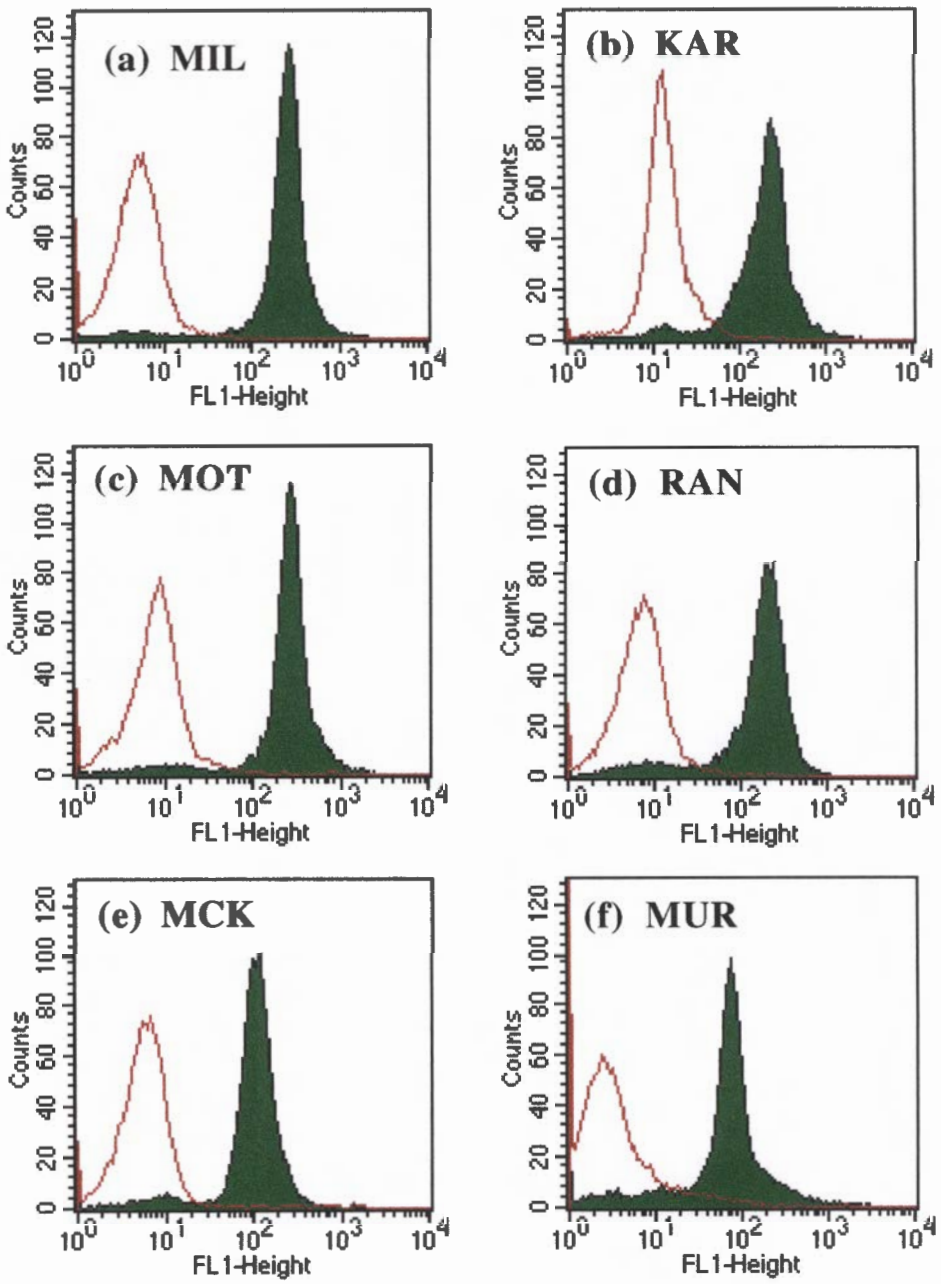
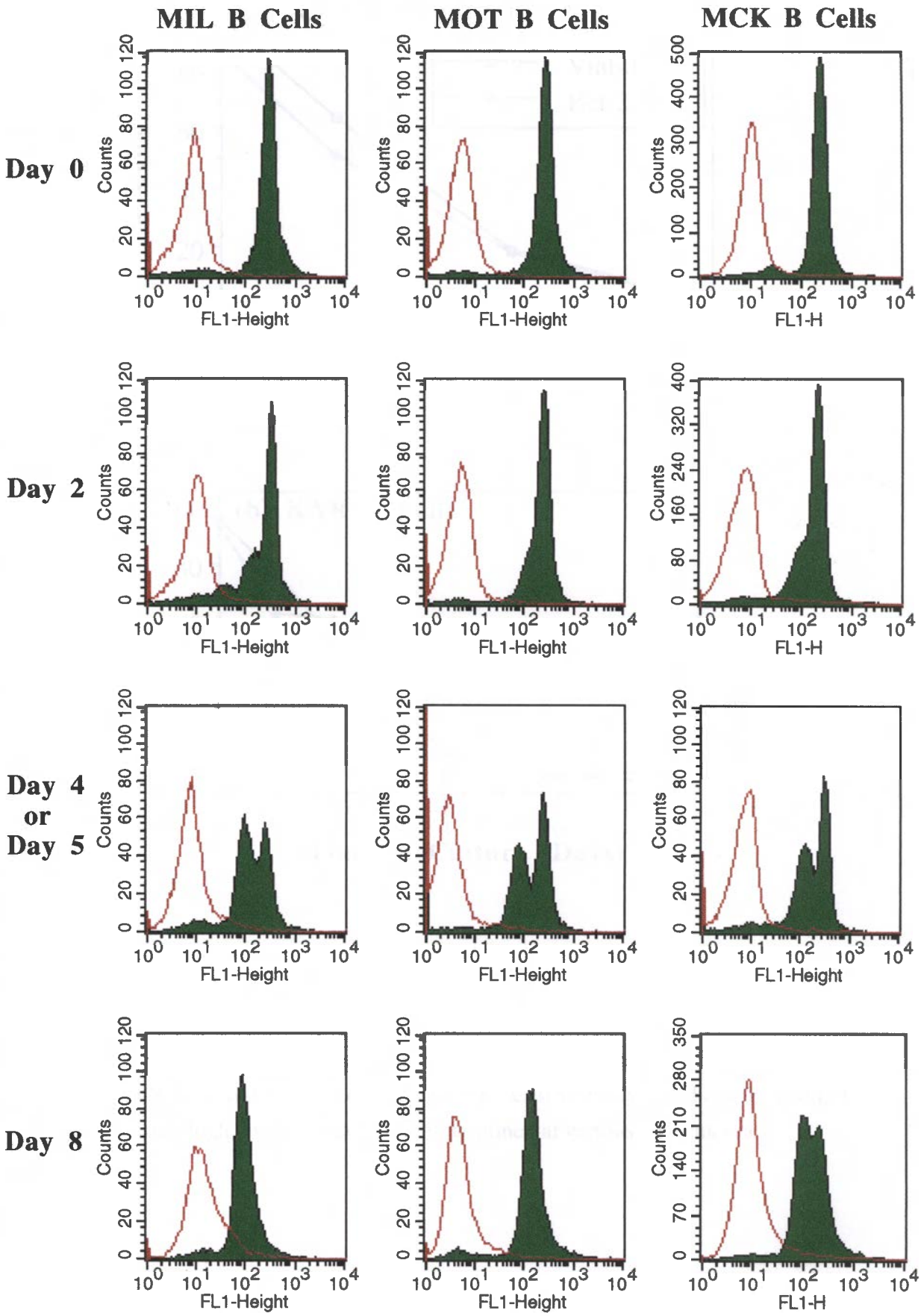


Figure 4.8: Leukemic CD5+ B Cells Express High Levels of bcl-2.

Leukemic CD5+ B cells from patients MIL (a), KAR (b), MOT (c), RAN (d), MCK (e) and MUR (f) were stained for bcl-2 (solid histogram) prior to culture, as described in *Materials and Methods*. The outline histogram indicates the fluorescence of an isotype-matched control antibody. Fluorescence was measured on a logarithmic scale.

Figure 4.9: Kinetics of Reduction of Bcl-2 Expression by *In vitro* Cultured Leukemic CD5$^+$ B cells.

Purified CD5$^+$ B cells from patients MIL, MOT and MCK were cultured for 8 days. Bcl-2 expression (solid histogram) was determined on days 0, 2, 5 and 8 (MIL, MCK) or days 0, 2, 4, and 8 (MOT), as described in *Materials and Methods*. Fluorescence was measured on a logarithmic scale. The outline histogram indicates the fluorescence of the isotype-matched control antibody.


MIL B Cells
MOT B Cells
MCK B Cells
Day 0
Day 2
Day 4
or
Day 5
Day 8
Counts
FL1-Height
FL1-H

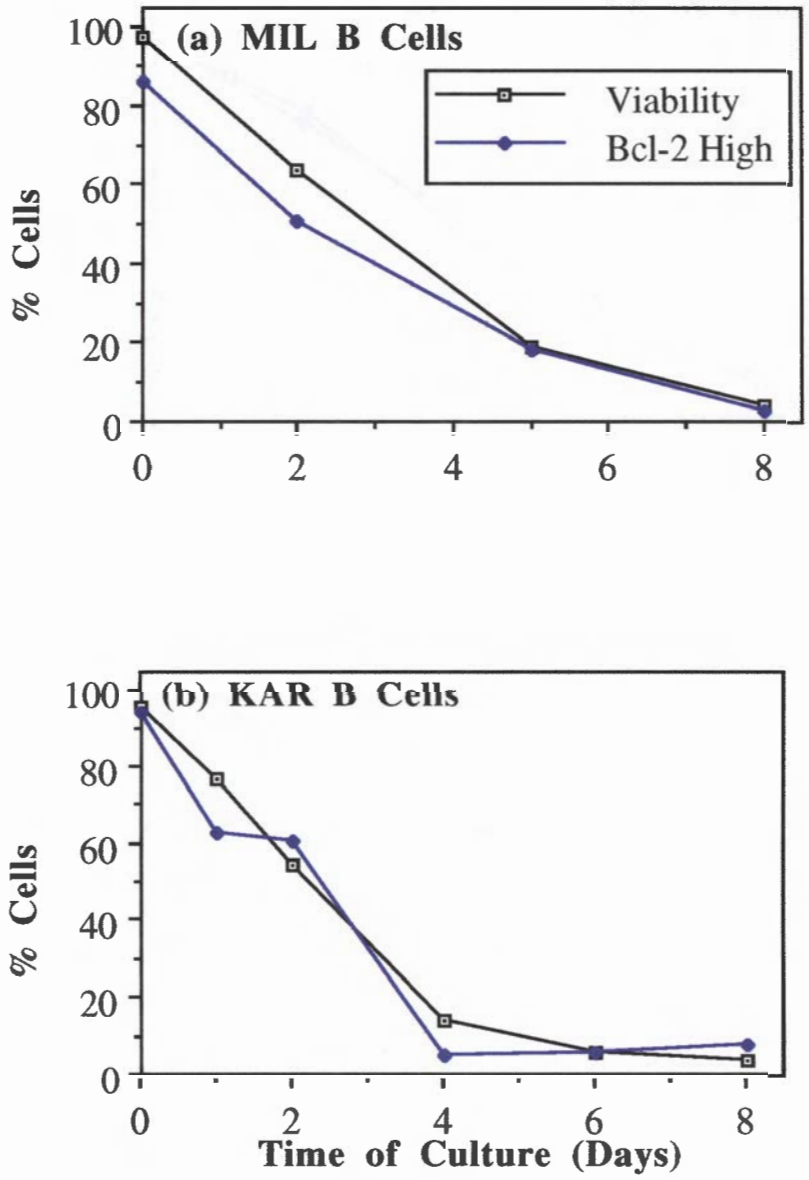


Figure 4.10: Reduced bcl-2 Expression Correlates with Cell Death.

MIL (a), KAR (b), MOT (c), RAN (d) and MCK (e) leukemic CD5+ B cells were cultured for 8 days in the absence of any stimuli. Cell viability and the proportion of cells expressing high levels of bcl-2 were determined at various time intervals.

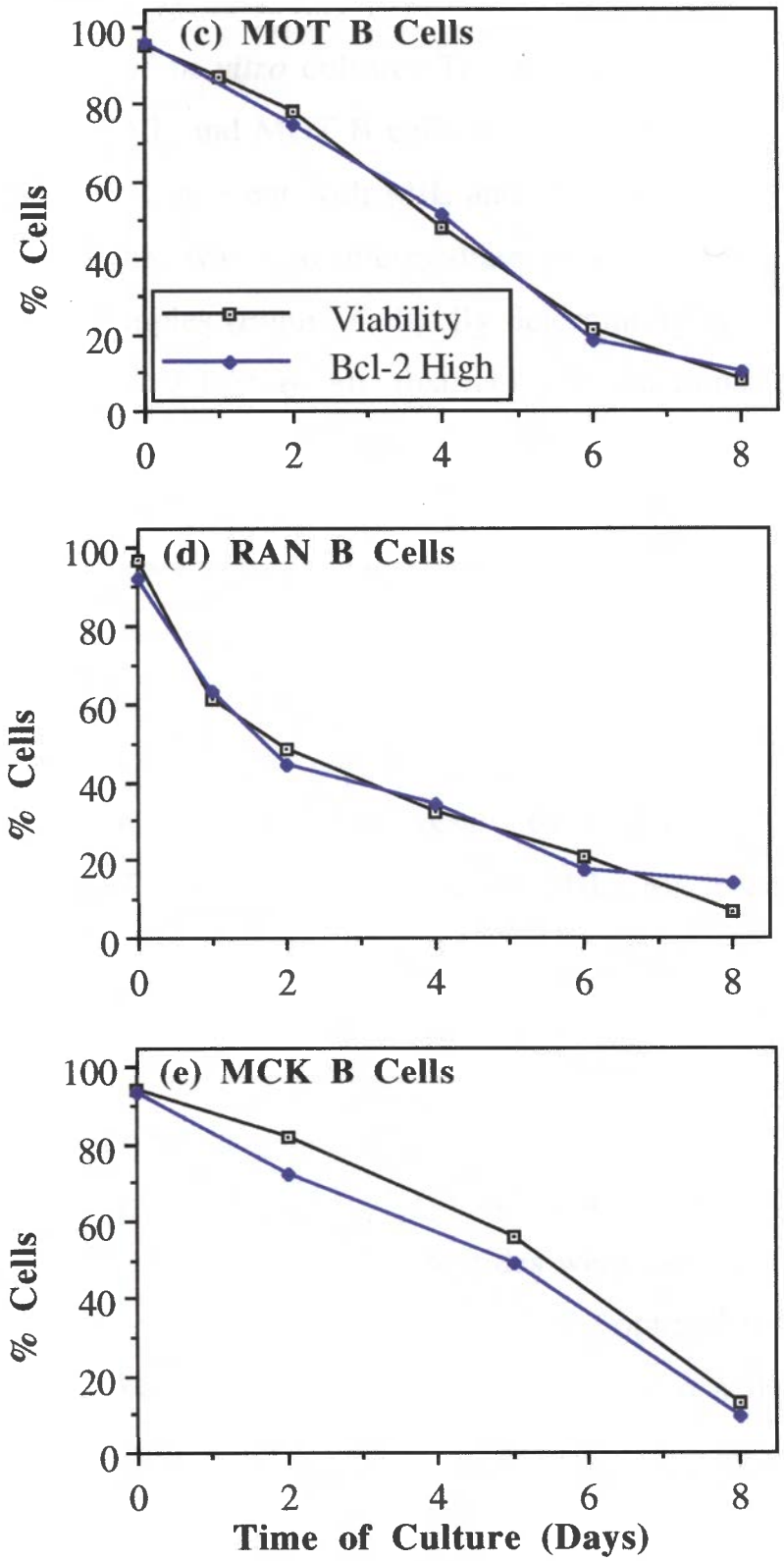


Figure 4.10: Reduced bcl-2 Expression Correlates with Cell Death. (continued).

displaying this phenotype was significantly decreased to 40-50% after a similar period of *in vitro* culture. The kinetics of reduction in bcl-2 expression by MIL and MOT B cells were intermediate to that of MCK and KAR/RAN, consistent with MIL and MOT B cells exhibiting an *in vitro* half life that was also intermediate to that of the other leukemic CD5+ B cell samples (Figure 4.10). By determining bcl-2 expression of cultured leukemic CD5+ B cells that had been fractionated according to cell density, it was revealed that the cells with reduced forward scatter comprised the bcl-2_{low} population. Similarly, the majority of cells expressing the bcl-2_{high} phenotype were viable and displayed light scattering characteristics of uncultured lymphocytes (Figure 4.4).

4.7. Effect of Antisense Oligonucleotides on Viability and Bcl-2 Expression by In vitro *Cultured Leukemic CD5+ B Cells*

Leukemic CD5+ B cells from patients MIL, MCK and MOT were cultured for 4-5 days in the presence or absence of antisense oligonucleotides specific for the translation initiation site of the *bcl-2* gene. The control oligonucleotide was the corresponding sense sequence. The aim of these experiments was to artificially reduce the level of bcl-2 expressed by the leukemic CD5+ B cells in an attempt to accelerate the kinetics of cell death. The oligonucleotides were used at a concentration of 20μM, similar to that previously found to be optimal for the inhibition of growth and bcl-2 expression of the human pre-B acute lymphocytic leukemic cell line, 697 (Reed *et al.*, 1990). Light scattering characteristics, viability and bcl-2 expression of oligonucleotide-treated cells were determined and compared to those characteristics of untreated cells. Irrespective of the oligonucleotide sequence, the light scattering properties of the treated cells were drastically altered after *in vitro* culture with the oligonucleotides (Figure 4.11). The population of cells

that appeared during *in vitro* culture with reduced size was most affected as these cells took on an even smaller appearance, and also displayed an increased 90° angle light scatter, indicative of increased cellular granularity/internal complexity. The light scattering properties of untreated and oligonucleotide-treated MIL, MOT and MCK B cells are presented in Figure 4.11. Interestingly, the percentage of MIL and MCK B cells with light scattering properties of uncultured cells in the presence of the sense oligonucleotide was in fact less than the corresponding cells that were cultured without either oligonucleotide or with the antisense oligonucleotide. For MOT B cells, the percentage of B cells with light scattering properties of uncultured cells was similar, regardless of the culture regime (Figure 4.11). Viability of MIL and MCK B cells was lowest in the presence of the sense oligonucleotide, while that of cells cultured with the antisense sequence was similar to untreated cells (Table 4.3). This is probably a consequence of the reduced numbers of cells with light scattering properties of uncultured cells in the presence of the sense oligonucleotide. Consistent with the light scattering profiles of oligonucleotide-treated and untreated MOT B cells, viability of these cells was also similar. Bcl-2 expression by the treated and untreated cells was also investigated. As demonstrated above, unstimulated leukemic $CD5^+$ B cells express the bcl-2 oncoprotein in a bimodal fashion during *in vitro* culture. For MIL and MCK B cells, the percentage of cells displaying the $bcl\text{-}2_{high}$ phenotype was similar with and without the oligonucleotides (Table 4.3). Thus, although cell viability was reduced in the presence of the sense oligonucleotide, this was not reflected by a decrease in the number of cells expressing high levels of bcl-2. Interestingly, in the presence of the antisense oligonucleotide, the percentage of MOT B cells with the $bcl\text{-}2_{high}$ phenotype was only 25%, compared to cultures of untreated or sense oligonucleotide-treated cells where 42% of cells

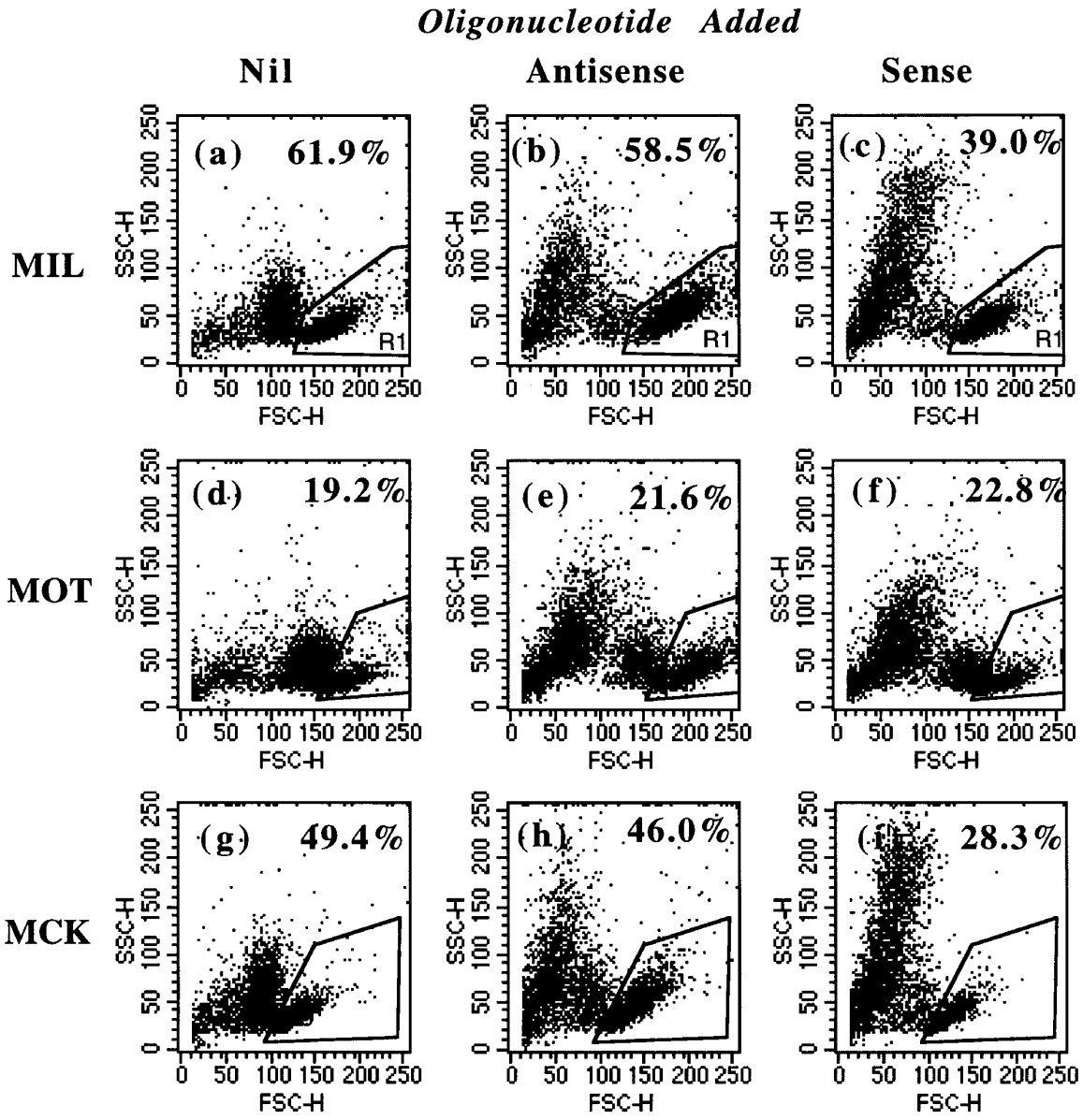


Figure 4.11: Effect of Phosphorothioate-treated Oligonucleotides on the Light Scattering Charactertistics of Leukemic CD5+ B Cells.

MIL (a-c), MOT (d-f) and MCK (g-i) CD5+ leukemic B cells were cultured without (a, d, g) or with antisense (b, e, h) or sense (c, f, i) oligonucleotides specific for the translation initiation site of bcl-2. The light scattering characteristics of leukemic B cells was determined after 4 or 5 days by flow cytometry. The value indicates the percentage of cells in region 1.

displayed this phenotype (Table 4.3). However, despite this apparent effect on bcl-2 expression, viability of the corresponding cells was no less than the control treated cells. Consequently, no correlation could be found for the effect of bcl-2 antisense oligonucleotides on cell viability and bcl-2 expression.

Table 4.3: *Effect of Oligonucleotides on Viability and Expression of Bcl-2 by Leukemic CD5+ B Cells*

CLL B cells	*Oligonucleotide*	*Cell Viability (%)*	*Bcl-2 High (%)*
MIL	Nil	61.4	60.0
	Antisense	60.2	67.3
	Sense	40.0	60.8
MOT	Nil	36.3	42.4
	Antisense	39.4	25.1
	Sense	33.9	42.8
MCK	Nil	59.9	63.3
	Antisense	56.7	59.0
	Sense	33.7	60.0

Leukemic CD5+ B cells from patients MIL, MOT and MCK were cultured in the absence (nil) or presence of oligonucleotides specific for the translation initiation site of the bcl-2 gene (antisense) or the corresponding sense oligonucleotide (sense). Cell viability and the proportion of cells displaying the bcl-2_{high} phenotype were determined after 4 or 5 days of *in vitro* culture.

Discussion

It has recently been demonstrated that B cell apoptosis occurs in three distinct stages. The first stage is characterised by the appearance of cells with decreased packing of membrane phospholipids and decreased forward angle light scatter, indicative of reduced cell size. Second stage apoptotic cells have undergone DNA fragmentation, while stage 3 cells display membrane permeability (Mower *et al.*, 1994). Thus, although cells have entered apoptosis, their cell membrane remains intact and continues to exclude vital dyes, until they enter stage 3.

In agreement with Collins *et al.* (1989), the results presented here demonstrate that $CD5^+$ B cells obtained from patients with B-CLL spontaneously undergo apoptosis following *in vitro* culture. A significant (~10-50%) proportion of leukemic $CD5^+$ B cells from all CLL samples exhibited apoptotic nuclei 24 hours after the initiation of culture. This value continued to increase such that after 48 hours 20-60% of nuclei in the leukemic $CD5^+$ cells were fragmented (Figures 4.1, 4.2). These results are similar to previous reports which found that after 16-30 hours of culture 5-59% of leukemic $CD5^+$ B cells displayed fragmented DNA (Buschle *et al.*, 1993; Panayiotidis *et al.*, 1993). Apoptosis of leukemic $CD5^+$ B cells was accompanied by cell death (as determined by loss of membrane integrity) and the appearance of cells with reduced size and, occasionally, increased granularity (Figure 4.3). By fractionating cultured cells on the basis of density, it was found that the majority of cells in the population displaying the forward and 90° angle light scatter characteristics of uncultured B cells were viable and contained diploid DNA (Figure 4.4). Depending on the origin of the leukemic $CD5^+$ B cells being investigated, the population of smaller cells often contained significant numbers of viable cells possessing diploid DNA. However, the

majority of these cells were non-viable and apoptotic. A reduction in cell size, as assessed by reduced forward light scatter, was an early event in leukemic CD5+ B cell apoptosis and often preceded cell death because a significant proportion of cells with reduced size remained capable of excluding ethidium bromide (Figures 4.3 and 4.5). Thus, these results are consistent with the events documented to occur when normal B cells undergo programmed cell death. That is, an early feature of apoptosing cells is the rapid loss of cell size and volume, concomitant with DNA fragmentation (Mower *et al.*, 1994). These results are also in agreement with the majority of reports that detail the *in vitro* apoptosis of leukemic CD5+ B cells. However, the broad range of *in vitro* half-lives observed for the different populations of leukemic CD5+ B cells in this study (Table 4.2) indicates that heterogeneity exists in the kinetics by which these cells undergo apoptosis.

The apoptotic process in leukemic CD5+ B cells was further characterised with respect to the expression of the anti-apoptotic oncoprotein bcl-2. Freshly obtained CLL B cells constitutively express high levels of bcl-2. However, in contrast to other B cell malignancies, bcl-2 expression in CLL is largely independent of chromosomal rearrangements (Schena *et al.*, 1992b; Hanada *et al.*, 1993). This study also demonstrated the constitutive expression of bcl-2 by uncultured leukemic CD5+ B cells both by Western blotting techniques and immunofluorescence (Figures 4.6, 4.7, 4.8). Further analysis of bcl-2 expression in leukemic CD5+ B cells by SDS-PAGE and Western blotting indicated that this protein remained detectable in cells that had been cultured *in vitro* for up to 8 days. Under these conditions, the vast majority of CLL B cells were non-viable and displayed fragmented DNA. These results contrasted those observed previously by Dancescu *et al.* (1992) and Panayiotidis *et al.* (1993 and

1994) who found that expression of bcl-2 by leukemic CD5+ B cells was lost after 1-4 days of *in vitro* culture. This apparent conflict was resolved by examining the expression of bcl-2 by immunofluorescence staining and analysis by flow cytometry. The method chosen to measure bcl-2 expression was optimised for leukemic CD5+ B cells. It was found that paraformaldehyde at concentrations of 0.5-1.0% produced fluorescence histogram profiles depicting maximal expression of bcl-2 by the leukemic CD5+ B cells. Concentrations of paraformaldehyde exceeding 1.0% significantly reduced the MFI of bcl-2 expression by 4-5 fold (Figure 4.7). This may arise from denaturation or alteration of the epitope recognised by the anti-bcl-2 mAb as a result of fixation. Alternatively, the higher concentration may cause excessive cross-linking of cellular proteins, resulting in reduced accessibility of the detecting mAb for the epitope. This important finding is in contrast to the published procedure (Aiello *et al.*, 1992), which used 2.0% paraformaldehyde, and suggests that although this method will result in the detection of bcl-2 expression, the apparent level of expression may be falsely reduced.

Having optimised this methodology, an exhaustive analysis of bcl-2 expression by leukemic CD5+ B cells during *in vitro* culture was undertaken. After 8 days, the mean intensity of bcl-2 expression was reduced 2-3 fold, compared to expression by uncultured cells. This was attributable to the reduced expression of bcl-2 in a proportion of cells (Figure 4.9). Although not investigated, it was likely that the mRNA for bcl-2 in these cells was also down-regulated during *in vitro* culture. This is based on the finding that in a cytotoxic T cell line (Deng and Podack, 1993) and in human leukemic cell lines (Chen *et al.*, 1995) the levels of bcl-2 mRNA rapidly declined within 3-4 hours of induction of apoptosis

by various means (deprivation of essential growth factors, ionising radiation, TNF-α or ceramide).

Consistent with the induction of apoptosis, the number of leukemic $CD5^+$ B cells with lower levels of bcl-2 continued to increase with time (Figure 4.9). The results from five cell populations examined were averaged and indicated that after 2 days 68.7 ± 9.2% (mean±sem) of the CLL cells expressed bcl-2 at a high level, while after 8 days this value decreased to 13.4 ± 2.0%. These results confirm those of Fluckiger *et al.* (1994b) who also reported a bimodal pattern of bcl-2 expression observed in seven different leukemic B cell samples after 3 days of *in vitro* culture. An extension of these previous findings is that down-regulation of bcl-2 continues during *in vitro* culture, such that by 8 days the majority of cells display the $bcl\text{-}2_{low}$ phenotype (Figure 4.9). The percentage of cells exhibiting high of levels bcl-2 was similar to the percentage of viable cells at all time points for each of the 5 populations examined suggesting that cell death may be a consequence of reduced bcl-2 expression (Figure 4.10). It is important to emphasise that the cells never became negative for bcl-2 and that even after 8 days of *in vitro* culture ~85% of cells remained positive for bcl-2, although the level of expression was reduced compared to the starting cell population. Thus, it was still possible to detect bcl-2 protein by Western blotting techniques, even though the cells were undergoing apoptosis. This is another example of the induction of apoptosis irrespective of the constitutive expression of bcl-2. This suggests that the protective effect of bcl-2 is only sustainable when this protein is present at a minimum level and that below this level, apoptotic events may be activated. If bcl-2 has a role *in vivo*, it would be assumed that this minimal level of expression would be that displayed by the cells prior to *in vitro* culture i.e. $bcl\text{-}2_{high}$. This is supported by a number of

recent findings. Enforced expression of bcl-2 in an IL-2-dependent T cell line or an IL-6-dependent myeloma cell line failed to prevent the death of these cells following withdrawal of the necessary growth factor (Nunez *et al.*, 1990). However, subsequent reports found that bcl-2 could prevent apoptosis in such cell lines if the transfected bcl-2 gene was expressed at high enough levels (Deng and Paddock, 1993; Schwarze and Hawley, 1995). These reports found that the ability of transfected bcl-2 to prevent apoptosis of an IL-2-dependent cytotoxic T cell line and an IL-6-dependent myeloma cell line, following withdrawal of these growth factors, correlated with the levels at which this ectopic gene was expressed. This is similar to the situation described here for leukemic CD5+ B cells; i.e. despite the constitutive expression of bcl-2 protein, CLL B cells continue to enter an apoptotic pathway.

Bcl-2 may be unable to prevent apoptosis in *in vitro* cultured leukemic CD5+ B cells because when bcl-2 is present at a reduced level in these cells, proteins that inhibit bcl-2 function, such as bax and bcl-xS, may have increased efficacy (Boise *et al.*, 1993; Oltvai *et al.*, 1993). In this situation, the bax protein, which associates with bcl-2 and accelerates apoptosis (Oltvai *et al.*, 1993), may exist in relatively increased amounts over bcl-2 and, by association with bcl-2, may neutralise its anti-apoptotic effect and subsequently initiate programmed cell death. This is a similar scenario to the one proposed by Oltvai *et al.* (1993) who found that the rate of apoptosis of a growth-factor dependent cell line differed according to the ratio of expression of bcl-2 to bax. Furthermore, in a recent report examining the regulation of anti-IgM-induced apoptosis of human B-lymphoma cell lines, it was found that bax mRNA and protein were rapidly induced in the cells following cross-linking of their sIg. In contrast, the expression of bcl-2 and bcl-x remained unaltered (Bargou *et*

al., 1995). In other reports, bcl-x_L was found to be down-regulated in a murine myeloma cell line and human leukemic cell lines undergoing apoptosis, yet the level of expression of bax remained similar to that of non-apoptotic cells (Chen *et al.*, 1995; Schwarze and Hawley, 1995). In these latter reports, alterations in the levels of bcl-2, bcl-x and bax could all contribute to bax existing in relatively increased amounts, compared to bcl-2 and bcl-x. Once again, this is compatible with the suggestion that the inherent ratio of bax to bcl-2/bcl-x dictates whether or not a cell undergoes programmed cell death (Yin *et al.*, 1994).

In an attempt to demonstrate a specific role for bcl-2 in the process of leukemic CD5+ B cell death and apoptosis, cells from three different patients were cultured with sense and antisense oligonucleotides specific for the translation initiation site of the bcl-2 gene. The aim of this experiment was to artificially reduce the level of bcl-2 mRNA, and consequently bcl-2 protein, which would presumably accelerate the kinetics of cell death. Unfortunately, this was not found to be the case. Bcl-2 antisense oligonucleotides did not cause a significant reduction in cell viability compared to cells cultured in complete medium or cells cultured with the control sense oligonucleotide (Table 4.3). Similarly, no consistent effect was observed for the level of bcl-2 expression in cells cultured with the specific oligonucleotide, compared to control cultures. One reproducible feature of cells cultured in the presence of oligonucleotides was an alteration in cellular morphology (Figure 4.11). In the presence of both antisense and sense oligonucleotides, a proportion of cells displayed reduced forward angle light scattering properties (i.e. size) and increased 90° angle light scatter (i.e. internal complexity/granularity). Because the oligonucleotides were derivatised with phosphorothioate, this result was interpreted as being a chemical

effect, possibly due to the sulfur molecules present in the oligonucleotides following derivatisation. The results obtained here contrast those obtained by other investigators, who employed identical oligonucleotides. In one study, it was found that the expression of bcl-2 was significantly reduced in the human pre-B cell acute lymphocytic leukemia cell line, 697, following culture with the antisense, but not the sense, oligonucleotide. This was specific as the cell surface expression of MHC class II was unaffected (Reed *et al.*, 1990). The reduction in bcl-2 expression was accompanied by an inhibition of cell proliferation as well as accelerated cell death, suggesting that maintenance of high levels of bcl-2 may contribute to the enhanced viability of 697 leukemic cells. Another report found that apoptosis was induced in 7 out of 17 different populations of blast cells from patients with acute myeloid leukemia (AML) following incubation in the presence of bcl-2-specific antisense oligonucleotides. Concomitant with these findings was the reduced expression of bcl-2 protein by the oligonucleotide-treated AML blasts (Keith *et al.*, 1995). The reason for the absence of an effect of the oligonucleotides in this study is unknown. However, it may be due to the fact that the leukemic $CD5^+$ B cells are in the G_0 phase of the cell cycle; i.e. they are non-proliferating cells. All studies describing the effect of oligonucleotides have utilised cell lines (Reed *et al.*, 1990; Ozaki *et al.*, 1994; Sumimoto *et al.*, 1994; Keller and Ershler, 1995). Although the study by Keith *et al.* (1995) examined the effect on AML blasts, these blasts, nonetheless, displayed spontaneous proliferation. Furthermore, although that study found an effect of bcl-2 antisense oligonucleotides, this was observed in less than half of the samples investigated. Antisense technology has been applied to CLL B cells previously (Fournier *et al.*, 1994). In this case, to investigate the role of CD23 in the regulation of leukemic B cell proliferation, CLL B cells were incubated with oligonucleotides specific

for CD23 cDNA. Interestingly, the surface expression of CD23 on both normal and B-CLL cells was enhanced, rather than diminished, in the presence of the antisense, but not sense, oligonucleotides. This suggests that oligonucleotides can also sustain protein expression by possibly stabilising the mRNA. Thus, based on the available literature, antisense oligonucleotides are most effective when assayed on established cells lines, rather than *in vitro* activated normal or leukemic blood cells. For antisense oligonucleotides to be useful in the culture of leukemic $CD5^+$ B cells, a more efficient system of delivery of the oligonucleotides to the nucleus of the cells will require development and optimisation.

In conclusion, this chapter describes the characterisation of apoptosis in six different populations of leukemic $CD5^+$ B cells. All populations were found to enter an apoptotic pathway during *in vitro* culture. However the kinetics of cell death revealed significant heterogeneity between the individual populations. This heterogeneity may be attributable to mechanisms responsible for maintaining the elevated expression of bcl-2 as it was observed that cells displaying delayed cell death kinetics down-regulated bcl-2 expression at a later time point. Because the cells continued to express bcl-2 at levels significantly greater than background, it is suggested that additional mechanisms may be involved in the initiation of leukemic $CD5^+$ B cell apoptosis. These mechanisms may be regulated by the expression of the bcl-x_S and bax proteins. Because B cell leukemias represent a convenient source of large numbers of B cells that are homogeneous with respect to their development, and these cells rapidly enter an apoptotic pathway on *in vitro* culture, leukemic $CD5^+$ B cells may serve as a useful model for studying the regulation, control and occurrence of apoptosis.

Chapter 5:

Regulation of Leukemic CD5+ B Cell Apoptosis In Vitro *By Cytokines*

Introduction

Despite their state of developmental arrest *in vivo*, leukemic CD5+ B cells obtained from patients with B-CLL can undergo activation, proliferation and differentiation *in vitro* following the appropriate stimulation. Because various cytokines, including IL-2, IL-4, IL-7, IL-10, IL-13, TNF-α and IFN-γ, are capable of inducing the proliferation of leukemic CD5+ B cells (Lantz *et al.*, 1985; Ostlund *et al.*, 1986; Karray *et al.*, 1988a; Totterman *et al.*, 1988; Carlsson *et al.*, 1989b; Digel *et al.*, 1989, 1990; Fluckiger *et al.*, 1993, 1994a), it has been suggested that CLL may be the manifestation of unregulated production of such growth factors. Consequently, autocrine and paracrine growth loops may be central to this malignancy (Hoffbrand *et al.*, 1993; Sarfati, 1993). Additional evidence that cytokines may play a role in B-CLL came from the finding that the serum of CLL patients contained increased levels of TNF-α (Foa *et al.*, 1990; Adami *et al.*, 1994), IL-6 (Hoffbrand *et al.*, 1993) and IFN-γ (Buschle *et al.*, 1993), compared to donor-matched normal serum, and that leukemic CD5+ B cells express and secrete various cytokines, including IL-1β, IL-6, IL-7, IFN-γ, TNF-α, TGF-β and LMW-BCGF (Uggla *et al.*, 1987; Cordingley *et al.*, 1988; Biondi *et al.*, 1989; Foa *et al.*, 1990; Fournier *et al.*, 1992b; Kremer *et al.*, 1992; Schena *et al.*, 1992a; Plate *et al.*, 1993; Reittie and Hoffbrand, 1994).

As demonstrated in the previous chapter, leukemic CD5+ B cells from patients with B-CLL undergo spontaneous apoptosis *in vitro*, despite their enhanced *in vivo* survival. Apoptosis was characterised by a reduction in cell size, DNA fragmentation and loss of membrane integrity. This suggests a role for apoptosis-inhibitory factors in the accumulation of these cells *in vivo*. These factors may be absent from the *in vitro* culture conditions employed in this and previous studies. Leukemic B cell

apoptosis can be delayed under various conditions. Spontaneous and hydrocortisone-induced apoptosis of leukemic CD5+ B cells can be delayed *in vitro* by the cytokines IL-4, IFN-α and IFN-γ (Dancescu *et al.*, 1992; Buschle *et al.*, 1993; Panayiotidis *et al.*, 1993; Chaouchi *et al.*, 1994; Jewell *et al.*, 1994). Some of these reports have also indicated that these cytokines maintain or increase the expression of bcl-2, which is otherwise diminished in the absence of any exogenous stimuli (Dancescu *et al.*, 1992; Buschle *et al.*, 1993; Panayiotidis *et al.*, 1993, 1994; Jewell *et al.*, 1994). In contrast, other investigators have reported that IL-4, IFN-α and IFN-γ prevent leukemic CD5+ B cell apoptosis by mechanisms unrelated to the modulation of bcl-2 expression (Chaouchi *et al.*, 1994; Fluckiger *et al.*, 1994b; Mainou-Fowler *et al.*, 1994). Apart from IL-4 and the IFNs, no role has been found for IL-1, IL-2, IL-3, IL-5, IL-6, IL-7, IL-13, TNF-α or TNF-β in the prevention of apoptosis in unstimulated leukemic CD5+ B cells (Dancescu *et al.*, 1992; Sarfati, 1993, Fluckiger *et al.*, 1994a, 1994b). Consequently, the correlation between leukemic CD5+ B cell apoptosis, bcl-2 expression and the effect of other cytokines on these parameters remains unresolved.

This chapter describes the effect of a variety of cytokines on the *in vitro* survival of leukemic CD5+ B cells obtained from 6 patients with B-CLL. The cytokines used in this study were those that have previously been reported to be secreted by CLL B cells and/or induce a proliferative response in leukemic CD5+ B cells, either alone or in conjunction with a co-activator. IL-13 was included due to the observation that many of its functions are identical to that of IL-4 (Zurawski and de Vries, 1994). The results presented confirm previous studies by demonstrating that IL-4 and IFN-γ can delay apoptosis in different populations of leukemic CD5+ B cells, *in vitro*. In addition, the results showed that delaying leukemic

CD5+ B cell apoptosis was accompanied by the recovery of significant numbers of viable cells, compared to unstimulated controls. Furthermore, it was found that IL-2, IL-6, IL-13 and TNF-α also delayed apoptosis and cell death in some of leukemic CD5+ B cell populations examined. Concomitant with enhancing cell viability and delaying apoptosis, these cytokines also sustained the elevated expression of bcl-2 protein by the leukemic CD5+ B cells. These results suggest that autocrine and paracrine growth factors may play a role in the pathogenesis of B cell malignancies by delaying cell death and sustaining the expression of the anti-apoptotic oncoprotein bcl-2.

Results

5.1. Soluble Factors Secreted by Activated T Cells Enhance Leukemic CD5+ B Cell Survival

Results presented in Chapter 3 indicated that an increased percentage of viable cells was recovered from cultures of mitogen activated CLL PBLs, compared to mitogen-activated T-depleted cell preparations (see section 3.8 *Effect of Residual T Cells on Leukemic CD5+ B Cell Viability*). This suggested that residual normal T cells delivered a survival signal to the leukemic CD5+ B cells. This effect could be mediated either by physical interaction of the T cells with the B cells or by secretion of soluble products capable of having a positive effect on B cell viability. To delineate the specific roles that molecules expressed on or secreted by activated T cells have on B cell viability, CD5+ B cells purified from 3 B-CLL patients were cultured with increasing numbers of resting or PHA-activated allogeneic normal human T cells. PHA was also added to wells containing leukemic B cells alone to control for the effects that this mitogen may have on cell viability. The normal T and leukemic CD5+ B cells, however, were separated by a 0.45μm Millicell culture plate insert that permitted diffusion of soluble products yet prevented physical association of the two cell types. Resting T cells slightly increased the recovery of viable B cells in a dose-dependent manner (Figure 5.1), suggesting that the T cells spontaneously secrete cytokines which act on the leukemic CD5+ B cells. However, this increase in leukemic CD5+ B cell viability was further enhanced ($p < 0.05$) in the presence of mitogen-activated T cells. This effect was dose-dependent, with the maximal effect occurring in the presence of the highest number of T cells tested.

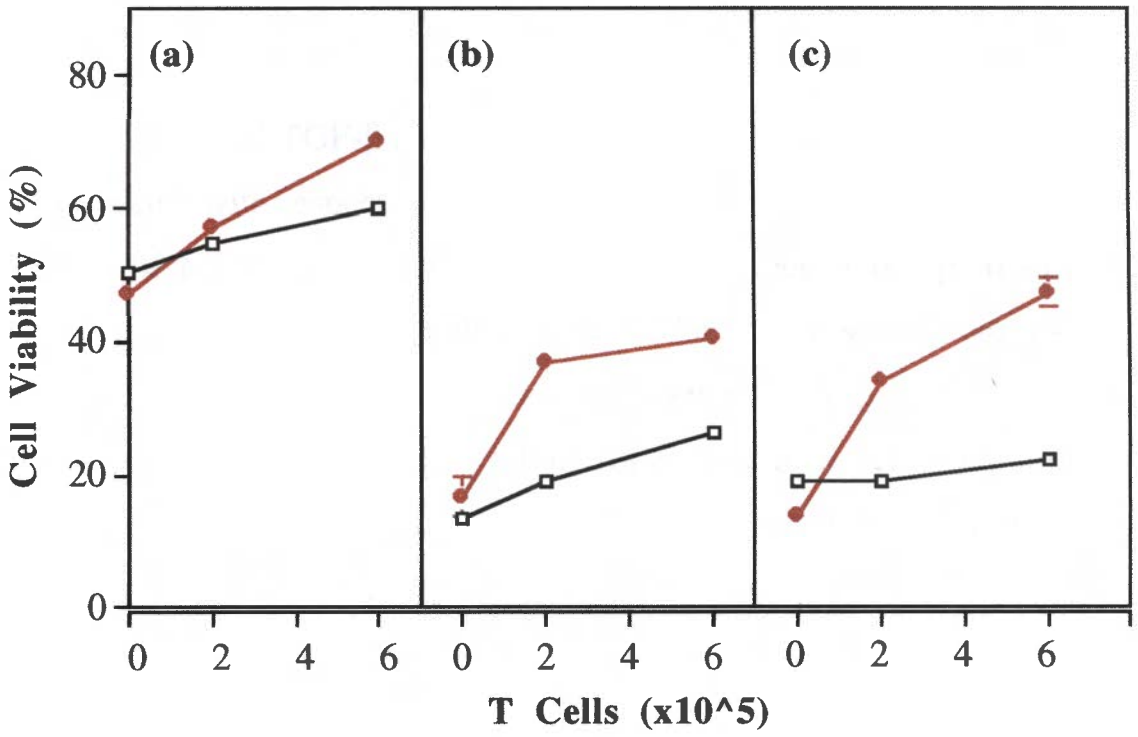


Figure 5.1: Soluble Factors Secreted by *In vitro* Activated T Cells Enhance Viability of Leukemic CD5+ B Cells.

Leukemic CD5+ B cells ($1.8x10^6$) from patients MIL (a), KAR (b) and RAN (c) were cultured in the presence of 0, $2x10^5$ or $6x10^5$ normal allogeneic T cells, in a final volume of 1.2 mL. The added T cells were either unstimulated (black line) or activated with PHA (red line). The leukemic B and normal T cells were separated from one another by a 0.45μm culture plate insert. Cell viability was determined after 4 (KAR, RAN) or 5 (MIL) days of culture. Each point represents the mean % viable cells ± 1 s.d. of triplicate samples.

5.2. Effect of Cytokines on Viability of Leukemic CD5+ B Cells, In vitro

To identify cytokines that can increase cell viability, purified leukemic CD5+ B cells from the CLL patients were cultured in the presence of varying concentrations of IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-13, IFN-γ, TNF-α and TGF-β so as to determine the optimal concentration of each cytokine with respect to enhancement of cell viability. Due to limited numbers of MUR leukemic CD5+ B cell, the effect of IL-1β, IL-5 and IL-13 on these cells was not determined. The dose response curves for each of the ten different cytokines are presented in Figure 5.2 and the optimal concentration of each cytokine for each CLL B cell population is listed in Table 5.1. Figure 5.3 indicates the mean effect that each of these cytokines had on cell viability when assessed after different periods of *in vitro* culture. From these experiments, a number of interesting observations were made. Firstly, IL-1β, IL-5, IL-10 and TGF-β failed to increase cell viability. This group of cytokines was slightly cytotoxic to the B cells at the highest concentrations tested, with TGF-β causing the greatest increase in cell death (Figures 5.2 and 5.3). Secondly, IL-4 increased the viability of leukemic CD5+ B cells from the six CLL patients by 20-40%. IFN-γ had a similar effect as IL-4, enhancing the viability of 5/6 leukemic CD5+ B cell populations by 15-25%. In contrast to IL-4, IFN-γ had no effect on leukemic CD5+ B cells from patient MIL. Thirdly, IL-2, IL-6, IL-13 and TNF-α were also found to be viability-enhancing cytokines when tested on some of the leukemic CD5+ B cell populations. IL-2 and IL-6 increased the viability of 4/6 leukemic CD5+ B cell populations by 15-30%. Interestingly, the cell populations whose viability could be enhanced by IL-2 could also be enhanced by IL-6. In the presence of TNF-α, the viability of KAR and MUR B cells was increased by 15-20%, while that of MOT and MCK B cells was enhanced by ~5-10%. The responses of the leukemic CD5+ B cells to IL-13 were

heterogenous. Surprisingly, this cytokine displayed a comparable effect to IL-4 on only one population of leukemic CD5+ B cells. When cultured *in vitro* with either IL-4 or IL-13, the viability of KAR B cells was increased by 27% and 26%, respectively, compared to unstimulated cells. The other cell populations were either unaffected by IL-13 (MIL), or cell viability was only mildly (~5-10%) increased. Overall, the viability of the different populations of leukemic CD5+ B cells could be enhanced by at least 2 individual cytokines, and in some cases 4 or 5 cytokines were effective.

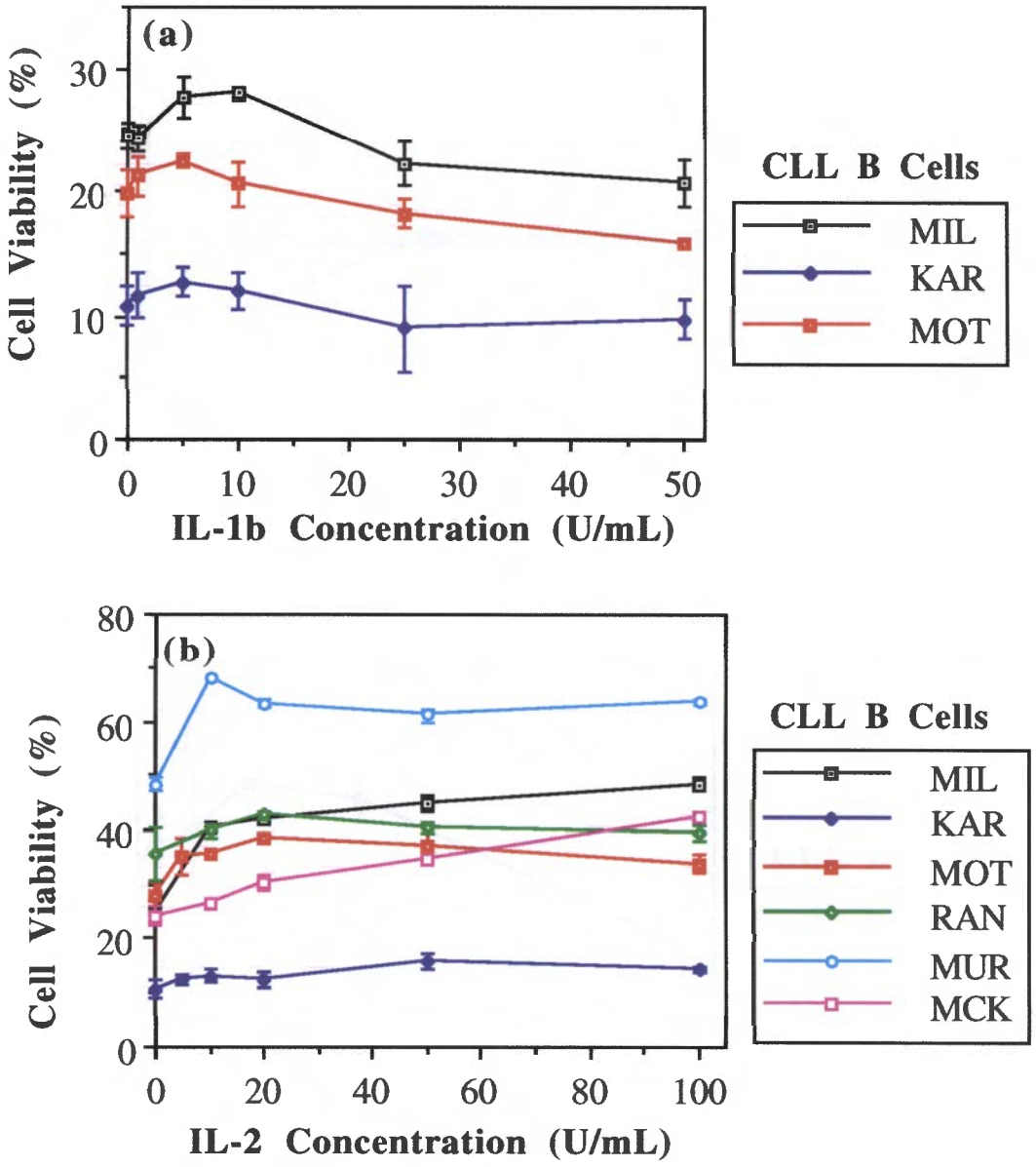


Figure 5.2: Optimisation of Cytokine Concentrations for Enhancement of Viability of Leukemic CD5+ B Cells.

Leukemic CD5+ B cells from 6 different B-CLL patients were cultured in the presence of varying concentrations of (a) IL-1β, (b) IL-2, (c) IL-4, (d) IL-5, (e) IL-6, (f) IL-10, (g) IL-13, (h) IFN-γ, (i) TNF-α or (j) TGF-β. Cell viability was determined after 4 (KAR, RAN), 5 (MIL) or 6 (MOT, MUR, MCK) days. Each point represents the mean % viable cells ± 1 s.d. of triplicate samples.

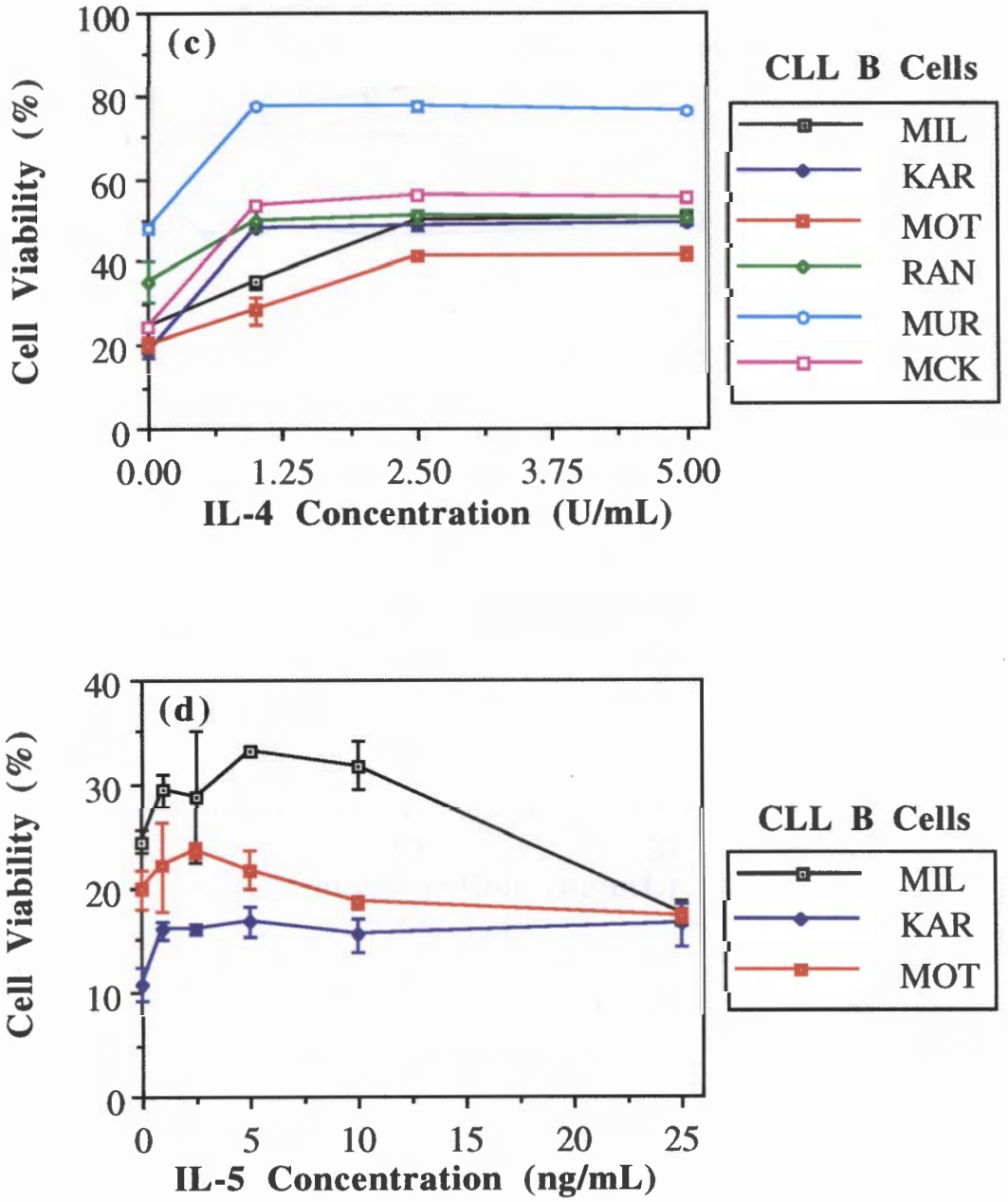


Figure 5.2: Optimisation of Cytokine Concentrations for Enhancement of Viability of Leukemic CD5+ B Cells (continued).

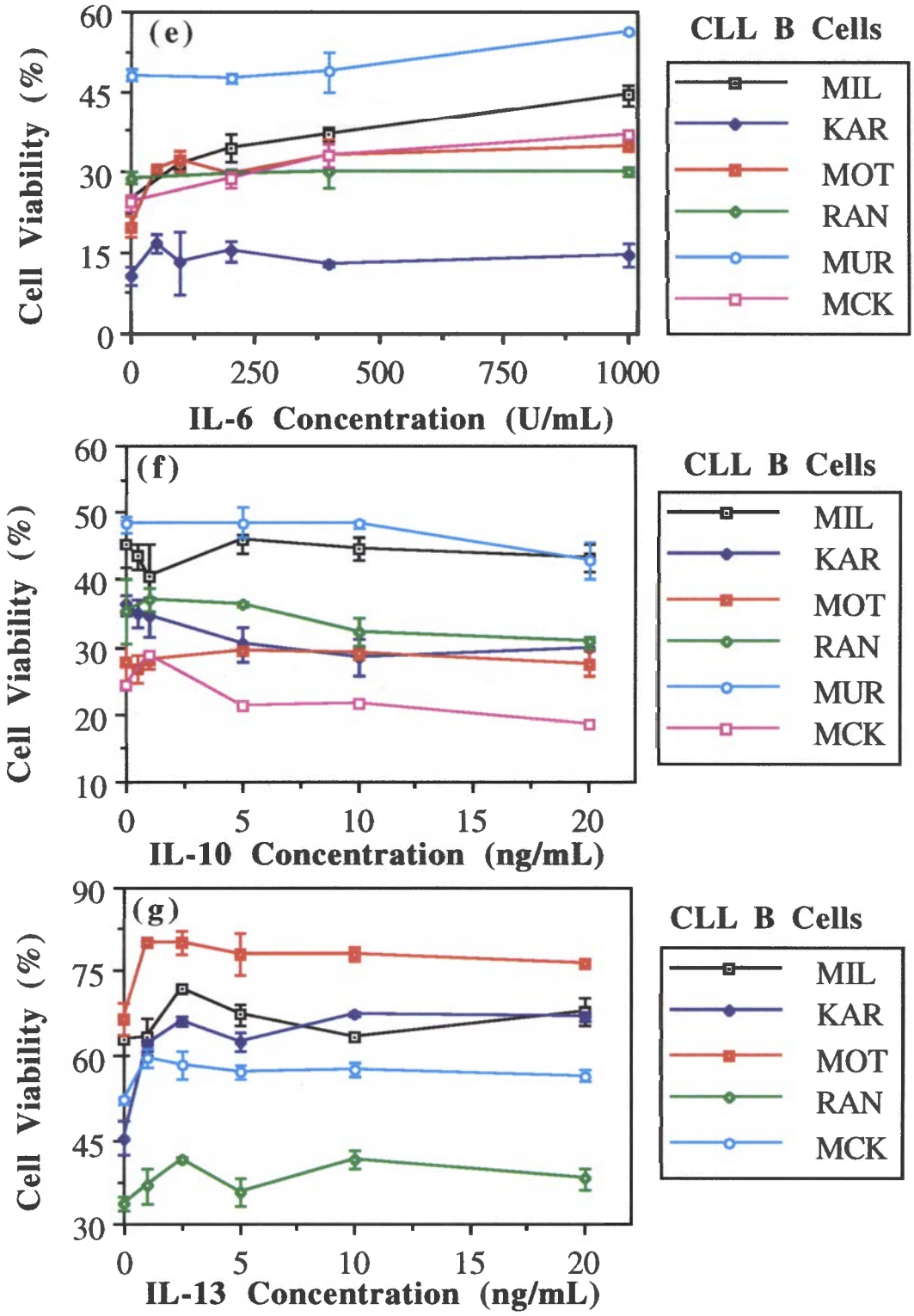


Figure 5.2: Optimisation of Cytokine Concentrations for Enhancement of Viability of Leukemic CD5+ B Cells (continued).

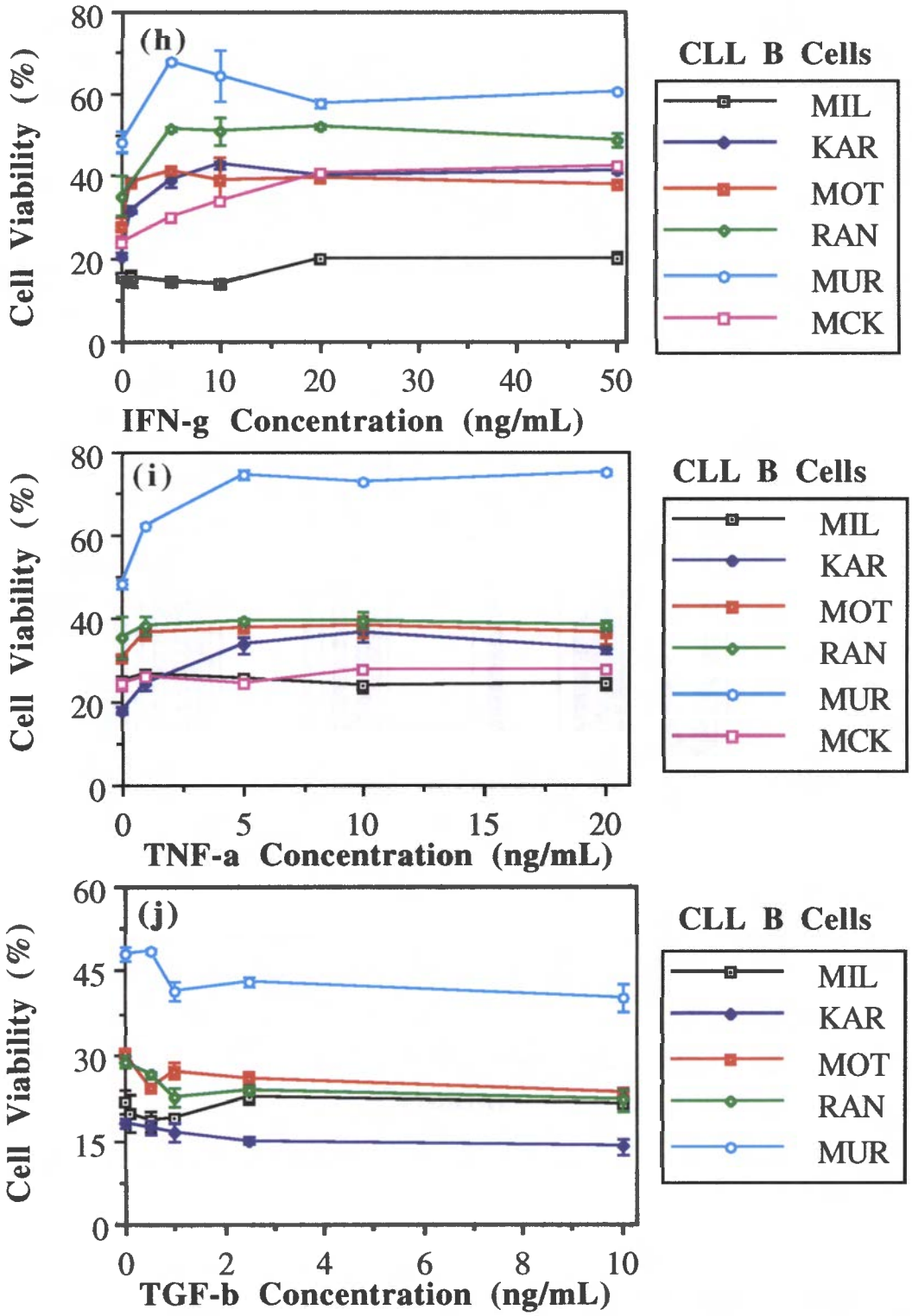


Figure 5.2: Optimisation of Cytokine Concentrations for Enhancement of Viability of Leukemic CD5+ B Cells (continued).

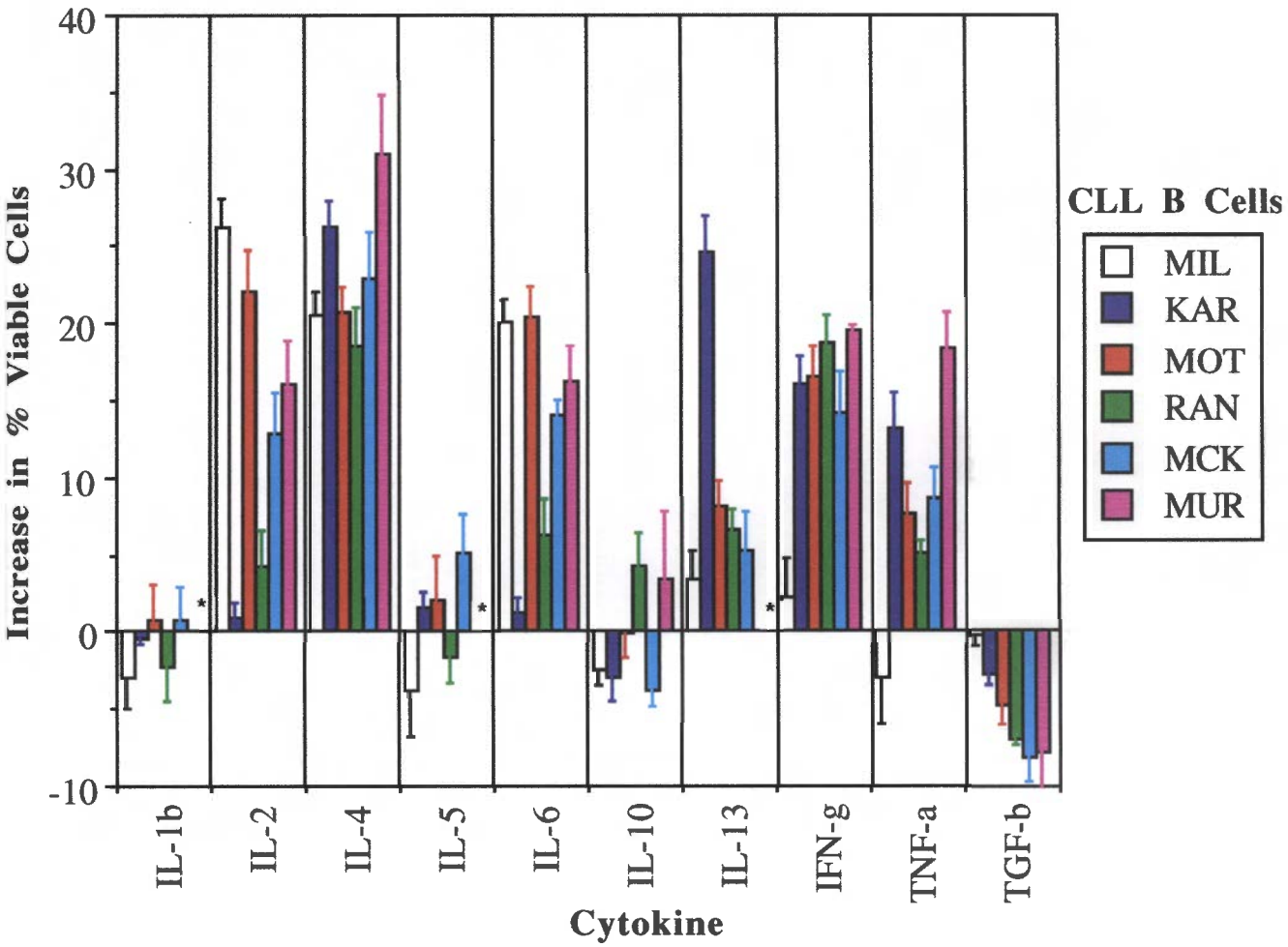


Figure 5.3: Effect of Cytokines on Leukemic B Cell Survival, *In vitro*.

Leukemic CD5+ B cells from patients MIL, KAR, MOT, RAN, MCK and MUR were cultured in the presence of optimal concentrations of IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-13, IFN-γ, TNF-α or TGF-β. Cell viability was determined after 4-7 days of culture. The *y*-axis represents the mean increase in the percentage of viable cells (±s.e.m., of 3-12 independent experiments), compared to cells cultured in complete medium without exogenous cytokines. The asterisks indicate that the effect of the cytokine on the corresponding leukemic CD5+ B cells was not examined.

Table 5.1: *Optimal Viability-enhancing Concentrations of Cytokines for* In vitro *Cultured Leukemic CD5+ B Cells.*

	Cytokine Concentration					
	Leukemic CD5+ B Cell Population					
Cytokine	MIL	KAR	MOT	RAN	MCK	MUR
IL-1 (U/mL)	50	50	50	NT	NT	NT
IL-2 (U/mL)	100	100	50	50	100	10
IL-4 (U/mL)	2	2	2	2	2	1
IL-5 (ng/mL)	50	50	50	NT	NT	NT
IL-6 (U/mL)	1000	1000	1000	1000	1000	1000
IL-10 (ng/mL)	10	10	10	20	20	20
IL-13 (ng/mL)	2.5	10	1	10	1	NT
IFN-γ (ng/mL)	50	50	20	20	50	5
TNF-α (ng/mL)	10	10	10	10	20	5
TGF-β (ng/mL)	10	10	10	10	NT	10

Purified leukemic CD5+ B cells from six different CLL patients were cultured in the presence of varying concentrations of the indicated cytokine. The concentration of each cytokine found to maximally enhance cell viability was considered to be optimal. When a cytokine decreased cell viability (i.e. IL-1β, IL-5, IL-10 or TGF-β; see Figures 5.2 and 5.3), the concentration that resulted in the greatest degree of death was used for future experiments. NT = not tested.

5.3. Responsiveness to IL-2 Correlated with the Expression of CD25

The ability of IL-2 to exert an effect on leukemic CD5+ B cells generally correlated with the presence or absence of the low affinity receptor for IL-2, CD25, on the surface of these cells. That is, of the 6 B cell populations investigated, five were found to express significant amounts of CD25 (Figure 5.4). Thus, while the fluorescence intensity of cells from patient KAR that had been incubated with anti-CD25 was similar to that of cells incubated with the isotype-matched control mAb, the remaining leukemic CD5+ B cell populations expressed this receptor at levels 3-4.5 times greater than background (Figure 5.4). The viability of four of the five CD25-expressing leukemic CD5+ B cell populations was substantially increased in the presence of IL-2 (Figure 5.3), suggesting that this cell surface cytokine receptor is functional and capable of signal transduction in the majority of cases. However, despite expressing CD25 at levels comparable to those found for the IL-2 responsive populations (Figure 5.4), the viability of leukemic CD5+ B cells from patient RAN was unaffected following culture with this cytokine *in vitro* (Figure 5.3).

5.4. KAR B Cells Express CD80 and Respond to IL-13

Given the overlapping functions of IL-4 and IL-13, the observation that IL-13 exerted a positive effect on the viability of only one of the cell populations studied (KAR B cells; Figure 5.3), yet IL-4 enhanced the viability of all 6 leukemic CD5+ B cell samples assayed (Figure 5.3), was unexpected. However, as reported in Chapter 3 and represented in Figure 5.5, the IL-13-responsive population of leukemic CD5+ B cells, obtained from patient KAR, constitutively expressed significant levels of CD80, while the remaining cell populations, which failed to respond to IL-13, where negative for this activation antigen. This suggests that expression

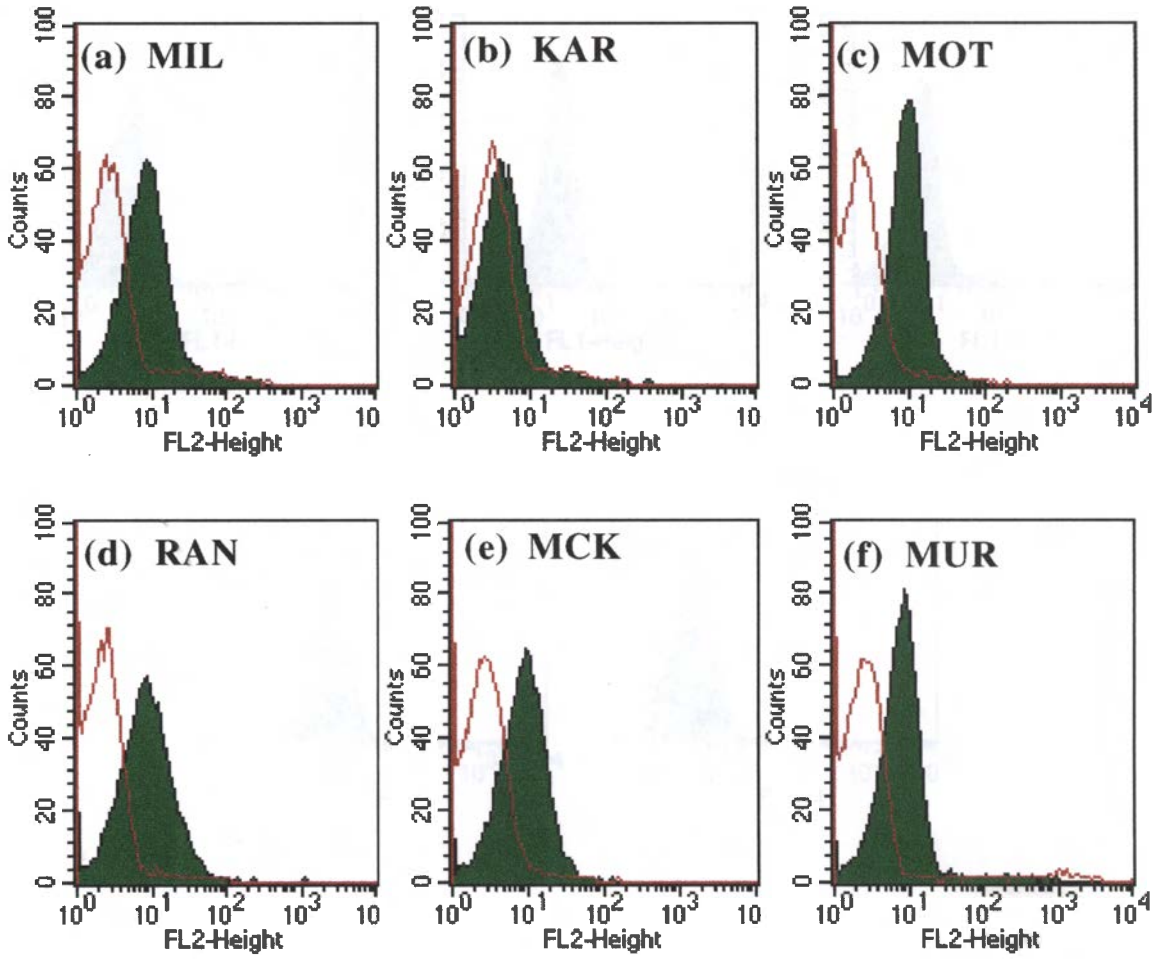


Figure 5.4: Expression of CD25 by Leukemic CD5+ B Cells.
Purified leukemic CD5+ B cells from patients (a) MIL, (b) KAR, (c) MOT, (d) RAN, (e) MCK or (f) MUR were incubated with PE-conjugated anti-CD25 (solid histogram), or an isotype-matched control mAb (outline histogram), for 30 minutes at 4°C. Cells were washed 3 times prior to analysis by flow cytometry. Fluorescence intensity was measured on a logarithmic scale. The *x*-axis represents relative fluorescence intensity and the *y*-axis represents relative cell number.

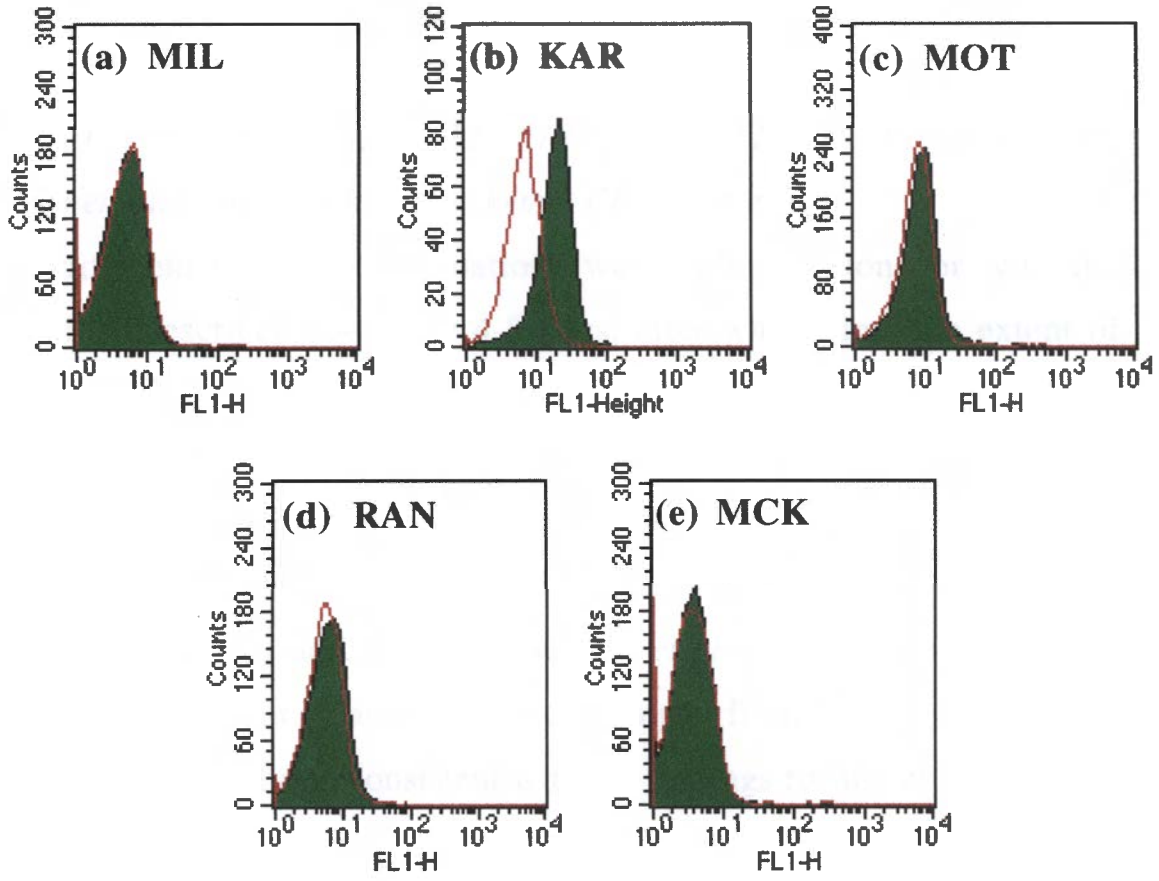


Figure 5.5: Expression of CD80 by Leukemic CD5+ B Cells.
Purified leukemic CD5+ B cells from patients (a) MIL, (b) KAR, (c) MOT, (d) RAN or (e) MCK were incubated with anti-CD80 (solid histogram), or an isotype-matched control mAb (outline histogram), for 30 minutes at 4°C. Bound mAb was detected by a second incubation step with FITC-conjugated sheep anti-mouse Ig antiserum for 30 minutes at 4°C. Cells were washed 3 times prior to analysis by flow cytometry. Fluorescence intensity was measured on a logarithmic scale. The *x*-axis represents relative fluorescence intensity and the *y*-axis represents relative cell number.

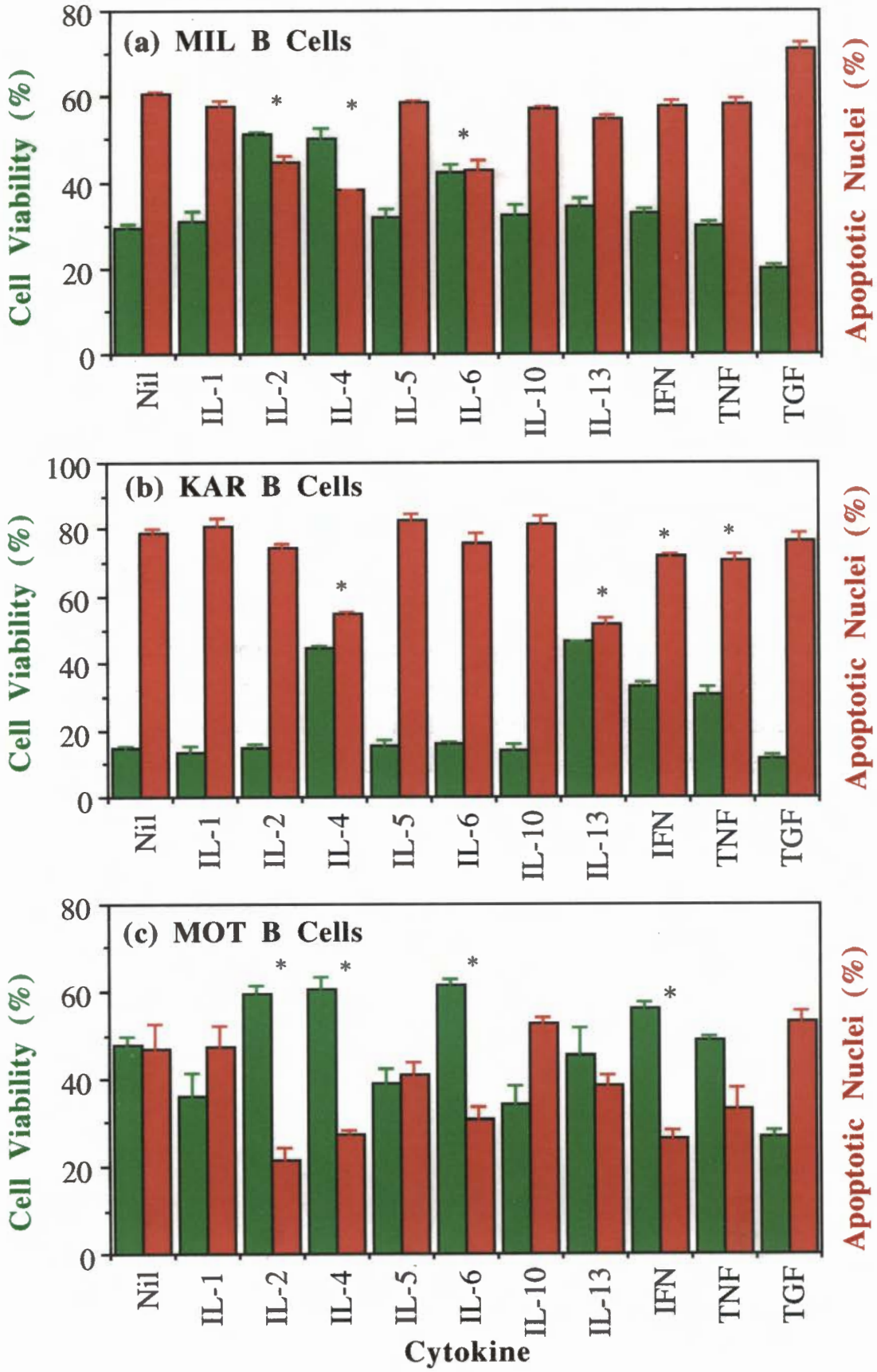
of CD80 by the leukemic CD5+ B cells may endow them with the capacity to respond to IL-13.

5.5. IL-2, IL-4, IL-6, IL-13, IFN-γ and TNF-α Prevent DNA Fragmentation in Stimulated Leukemic CD5+ B Cells

The different CLL cell populations were cultured alone or with the cytokines described above for 4-8 days, after which time the extent of apoptosis, expressed as the proportion of cells displaying apoptotic nuclei, was determined. Figures 5.6a-f illustrate the effect of these cytokines on the viability and apoptosis of the 6 different leukemic CD5+ B cell populations. The amount of apoptotic nuclei was determined by the flow cytometric method described by Nicoletti *et al.* (1991). In unstimulated cultures the amount of apoptotic nuclei ranged from 50-80%, depending on the cell population. Consistent with the findings for the effects on cell viability, IL-1β, IL-5, IL-10 and TGF-β had minimal effect on the number of apoptotic cells (Figure 5.6). In some cases, the amount of apoptotic nuclei resulting after culture with these cytokines exceeded that of unstimulated cells (e.g. IL-10, MOT and RAN; TGF-β, MIL; Figure 5.6). However, the extent of apoptosis could be significantly reduced by culture with IL-2, IL-4, IL-6, IL-13, IFN-γ and TNF-α ($p < 0.05$). Similar to the effects that these cytokines had on cell viability, the extent of this reduction varied for the different cell populations. A consistent finding was that the ability of a particular cytokine to enhance cell viability was mirrored by its capacity to also significantly reduce the proportion of cells exhibiting apoptotic nuclei. Thus, IL-4 consistently reduced apoptosis in all populations of leukemic CD5+ B cells assayed. This effect was observed in five of the six B cell populations following culture with IFN-γ and four of the six populations in the presence of IL-2 or IL-6. TNF-α reduced the extent of apoptosis in two of the six

Figure 5.6: Effect of Cytokines on Viability and Apoptosis in Different Leukemic CD5+ B Cells.

Leukemic CD5+ B cells purified from patients MIL (a), KAR (b), MOT (c), RAN (d), MCK (e) and MUR (f) were cultured in the absence or presence of the indicated cytokines for 4-7 days. Cell viability (green bars) and DNA fragmentation (red bars) were then determined as described in *Materials and Methods*. Each point represents the mean % of viable cells or the mean % of cells displaying apoptotic nuclei, ± 1 s.d., of triplicate samples. The asterisks denote a significant increase in cell viability and decrease in apoptotic nuclei following culture with a particular cytokine.


(a) MIL B Cells
(b) KAR B Cells
(c) MOT B Cells
Cell Viability (%)
Apoptotic Nuclei (%)
Nil
IL-1
IL-2
IL-4
IL-5
IL-6
IL-10
IL-13
IFN
TNF
TGF
Cytokine
*

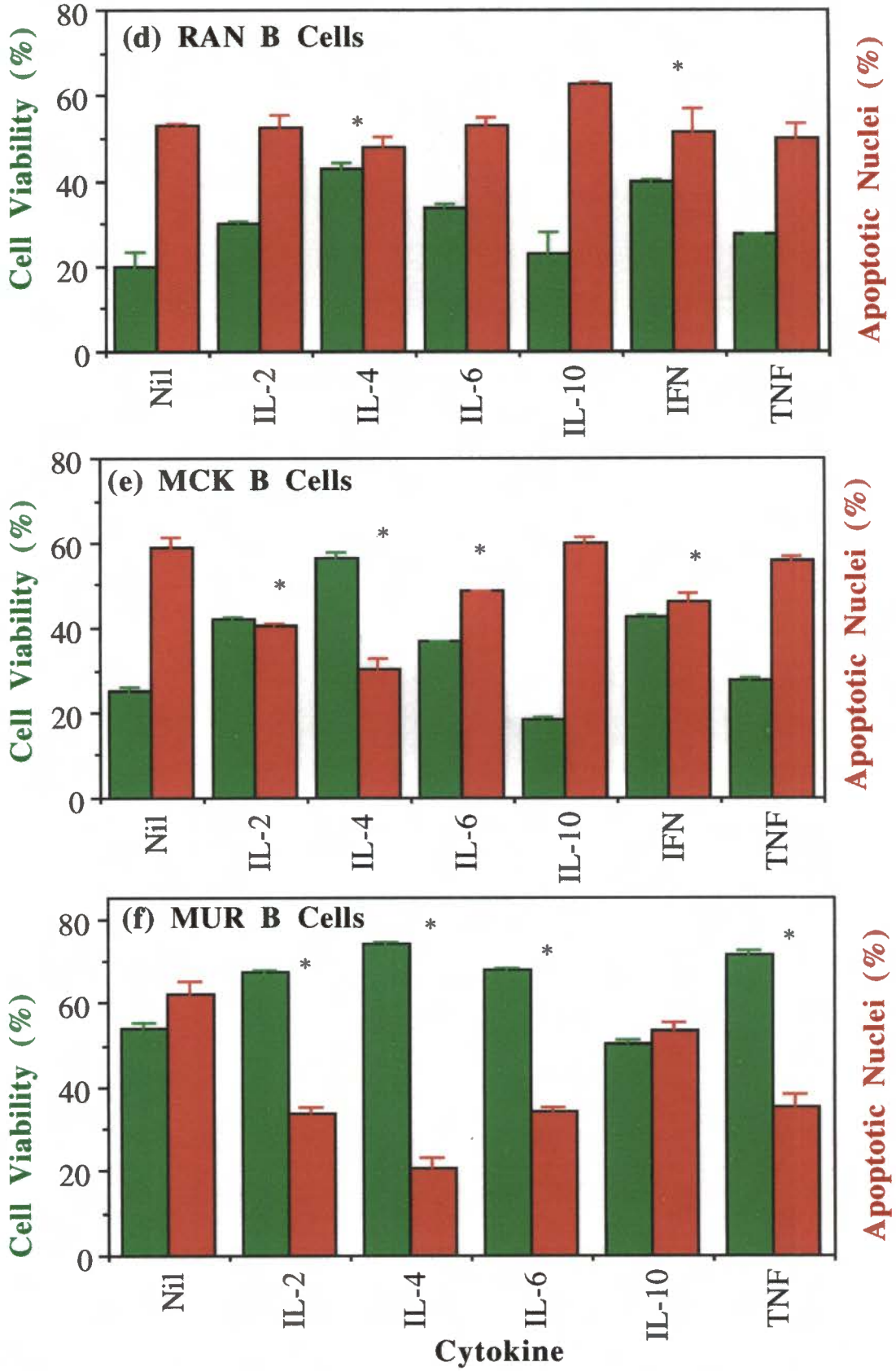


Figure 5.6: Effect of Cytokines on Viability and Apoptosis in Different Leukemic CD5+ B Cells (continued).

populations (KAR, MUR), while IL-13 suppressed apoptosis in leukemic B cells obtained from only one CLL patient (KAR). The extent of DNA fragmentation exhibited by KAR B cells was similarly reduced when these cells were cultured with IL-4 or IL-13, while the effect of IL-13 on apoptosis of the remaining cell populations was minimal. This is consistent with the effect that IL-13 had on leukemic $CD5^+$ B cell viability (Figures 5.2, 5.3 and 5.6). The reduction in the amount of apoptotic nuclei in the presence of a cytokine, compared to unstimulated cells, was often similar to the increase in the number of viable cells achieved by the same cytokine. This suggests that the cells rescued from death by the cytokines are also protected from the spontaneous induction of apoptosis.

Leukemic $CD5^+$ B cell apoptosis was also demonstrated by detecting the classical "ladder" pattern of fragmented DNA on agarose gels following electrophoresis of extracted cellular DNA. As observed in the previous chapter, this method is limited because the amount of DNA present in the fragmented form can not be easily quantitated. This is clearly demonstrated in Figure 5.7. This gel reflects the extent of DNA fragmentation in KAR B cells that were cultured in the absence or presence of IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IFN-γ and TNF-α. The amount of fragmented DNA in each lane appears to be similar. However, there are significant differences when the amount of apoptotic nuclei in similarly cultured cells was assessed by flow cytometric analysis (Figure 5.6b). Similar results were obtained when apoptosis was investigated both by agarose gel electrophoresis and flow cytometry in $CD5^+$ B cells obtained from the different CLL patients following culture with viability-enhancing cytokines. Although the results of the agarose gel experiments were not very informative, they do confirm the findings of the flow

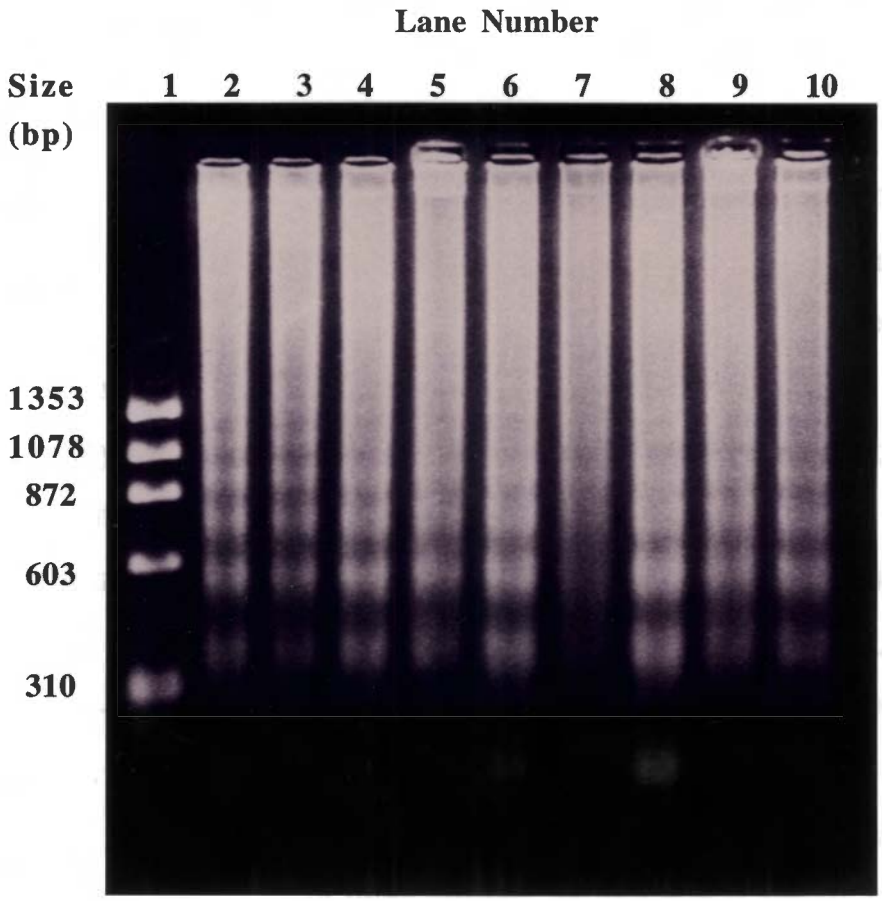


Figure 5.7: Cytokines do not Prevent Apoptosis of Leukemic CD5+ B Cells.

Leukemic CD5+ B cells from patient KAR were cultured for 4 days in the absence (lane 2) and presence of IL-1β (lane 3), IL-2 (lane 4), IL-4 (lane 5), IL-5 (lane 6), IL-6 (lane 7), IL-10 (lane 8), IFN-γ (lane 9) or TNF-α (lane 10). DNA was then extracted from the cells and electrophoresed through a 1.8% agarose gel. The gel was stained with ethidium bromide and the fragmented DNA visualised under UV light. Molecular weight standards were concomitantly electrophoresed, and are shown in lane 1.

cytometric based method for investigating apoptosis. Thus, apoptosis continued to occur in the presence of cytokines; the cytokines did not abrogate or prevent the induction of apoptosis, but merely reduced the extent to which this phenomenon occurred.

In cultures of unstimulated leukemic B cells, a population appeared displaying reduced forward light scattering properties, indicative of a reduction in cell size (see section 4.4 *Characterisation of Apoptotic Cell Populations*). In addition to reducing apoptotic nuclei and cell death, particular cytokines were capable of delaying the appearance of these cells showing reduced size. Figure 5.8 represents the forward and 90° angle light scattering properties of cells obtained from patient KAR that were either unstimulated (Figure 5.8a) or cultured with IL-4 (Figure 5.8b) or IL-13 (Figure 5.8c). Prior to *in vitro* culture, >90% of cells appeared in region 1 (R1) and cell viability was typically ~95% (see Figure 4.3 *Apoptosis is Accompanied by Reduced Forward Light Scatter*). After 4 days of *in vitro* culture, only 4.8 ± 0.3% (mean ± s.d. of triplicate samples) of cells displayed the light scattering characteristics of uncultured cells (Figure 5.8a). Stimulation with IL-4 or IL-13 resulted in 53.1 ± 0.7 or 50.4 ± 1.1% of cells, respectively, displaying the light scattering characteristics of uncultured cells and thus appeared in R1 (Figure 5.8b, c). These results were confirmed by morphological examination of cells cultured with and without these viability-enhancing cytokines (Figure 5.8d-f). In the absence of any exogenous stimuli, the majority of cells appear apoptotic, exhibiting membrane blebbing, fragmented nuclei and reduced size (Figure 5.8d). In the presence of IL-4 (Figure 5.8e) or IL-13 (Figure 5.8f) the frequency of cells displaying the morphology of apoptosis is significantly less. In contrast to unstimulated cultures, the majority of cytokine-stimulated cells retain the morphology

of uncultured cells. Similar results were obtained when the effects of viability-enhancing cytokines on the morphology of the other leukemic $CD5^+$ B cell populations were examined (results not shown).

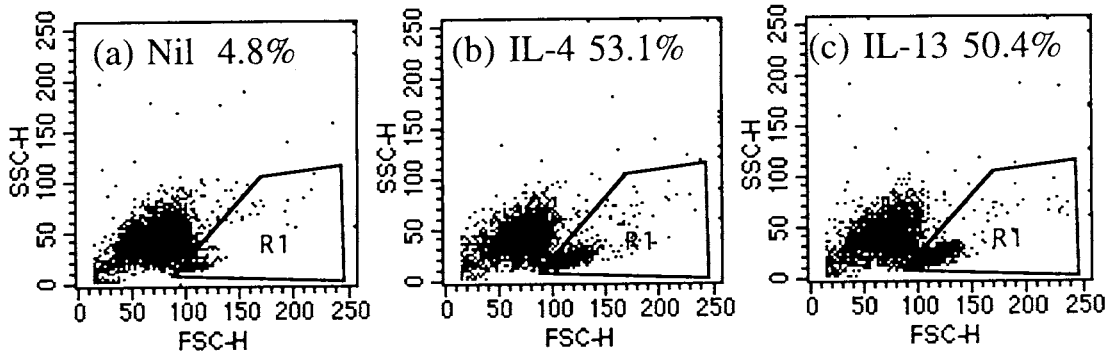
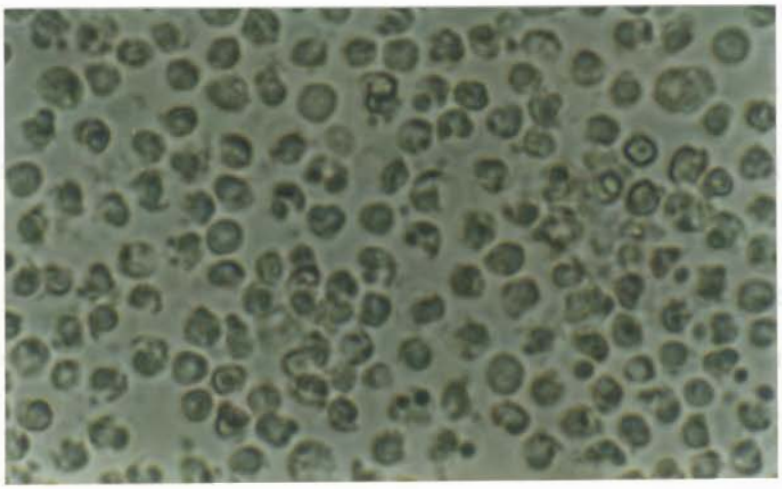


Figure 5.8: Cytokines Prevent the Reduction in Size that occurs in Apoptotic Leukemic CD5+ B Cells.

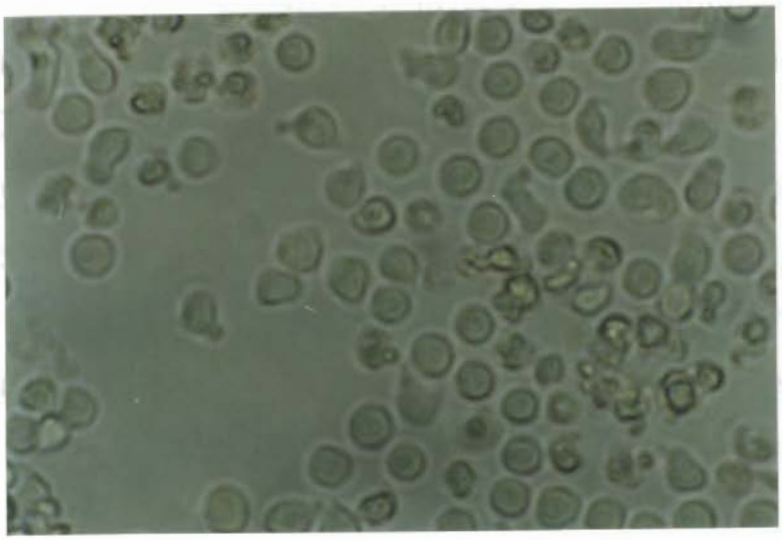
Leukemic CD5+ B cells from patient KAR were cultured for 4 days in the absence (a) or presence of IL-4 (b) or IL-13 (c). The forward (*x*-axis) and 90° angle (*y*-axis) light scattering properties were determined by flow cytometric analysis after 4 days. The value in each panel represents the percentage of cells in region 1 i.e. with the light scattering properties of uncultured cells (see Section 4.4. *Characterisation of Apoptotic Populations*).

The morphology of (d) unstimulated, (e) IL-4 or (f) IL-13-treated KAR B cells was determined after 4 days of *in vitro* culture (see over).

(d) unstimulated



(e) IL-4



(f) IL-13

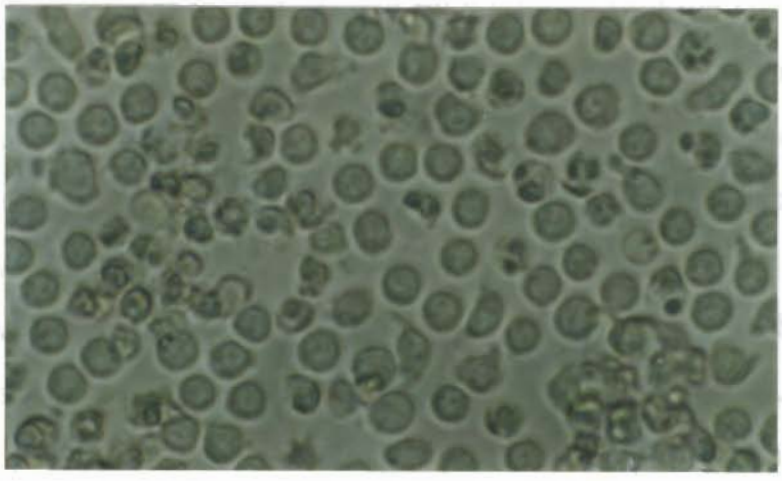


Figure 5.8: Cytokines Prevent the Reduction in Size that occurs in Apoptotic Leukemic CD5+ B Cells (continued).

5.6. The Effect of Combinations of Cytokines on Viability of Leukemic CD5+ B Cells

Because death and apoptosis of the different leukemic CD5+ B cell populations could be enhanced by at least two cytokines, the effect of combinations of cytokines on viability of leukemic CD5+ B cells was investigated. To determine if cell viability could be further increased, leukemic CD5+ B cells were cultured in the presence of one or more cytokines capable of delaying cell death in particular populations of CLL B cells. CD5+ B cells from patients MIL, MOT, MUR and MCK were cultured with IL-2, IL-4 and IL-6, as these four populations responded to each of these cytokines when assayed alone (see Figure 5.3). The leukemic CD5+ B cells were cultured for 5-7 days with these cytokines individually, or with IL-2 and IL-4, IL-2 and IL-6, or IL-4 and IL-6. To ensure a potential synergistic effect was not masked by a single cytokine alone, the efficacy of these cytokines when used at half-optimal concentrations was also determined. As expected, a single cytokine increased cell viability by 10-30%, depending on the cytokine and the cell population examined (Figure 5.9a). Combinations of IL-2 and IL-4, IL-2 and IL-6, or IL-4 and IL-6 failed to cause an increase in viability that was significantly greater than that caused by a single cytokine ($p > 0.05$). This was observed when the cytokines were present at their optimal (Figure 5.9a) or half-optimal concentration (data not shown). Thus, no synergistic or additive effect occurred in the presence of the different combinations of these cytokines. If the combination of two cytokines was to have any effect, it would likely be an inhibition of enhanced cell viability. It was not unusual for cell viability in the presence of IL-2 and IL-4, IL-2 and IL-6, or IL-4 and IL-6 to be slightly less than when the cells were cultured with a single cytokine alone (Figure 5.9a).

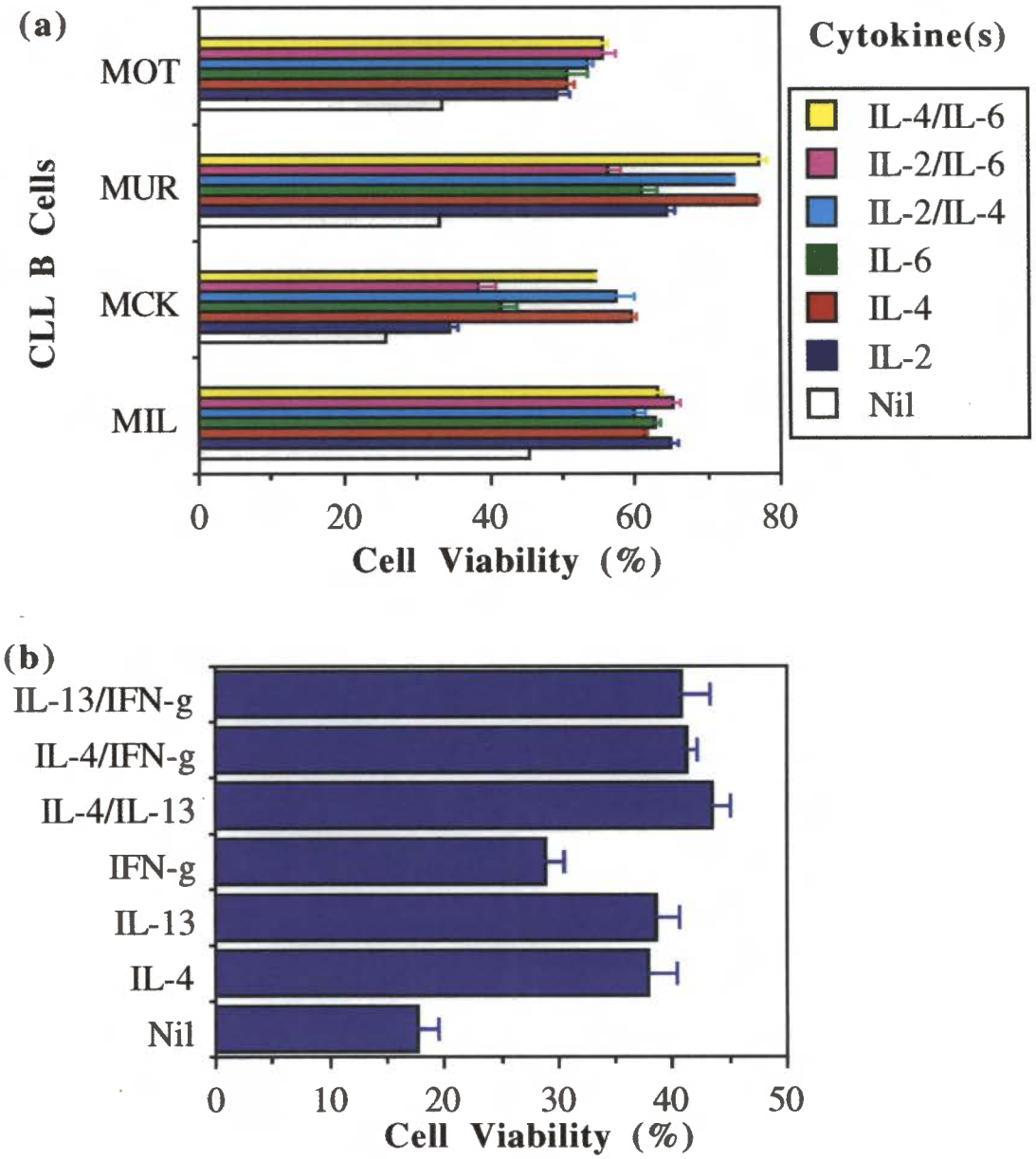


Figure 5.9: Combinations of Cytokines do not Synergise to Further Enhance Viability of Leukemic CD5⁺ B Cells.

(a) Leukemic CD5⁺ B cells from patients MIL, MOT, MCK and MOT were cultured in the absence and presence of IL-2, IL-4, IL-6 or combinations thereof. Cell viability was determined 5-7 days later.

(b) Leukemic CD5⁺ B cells from patient KAR were cultured in the absence and presence of IL-4, IL-13, IFN-γ or combinations thereof. Cell viability was determined 4 days later. Each point represents the mean % viable cells ± 1 s.d. of triplicate samples.

The viability of B cells obtained from patient KAR was unaffected following culture with IL-2 or IL-6. However, IL-4, IL-13 and IFN-γ could significantly enhance cell viability by 15-30% (Figure 5.3). Based on these observations, an experiment was performed to examine whether or not combinations of IL-4, IL-13 and IFN-γ could further increase the viability of KAR leukemic $CD5^+$ B cells caused by either of these cytokines when assayed alone. Similar to the results described above, no significant synergistic or additive effect was observed for the combinations of IL-4 and IL-13, IL-4 and IFN-γ or IL-13 and IFN-γ over that of a single cytokine (Figure 5.9b). Because MUR B cells were found to respond to both TNF-α and IL-6, and these two growth factors have been suggested to be components of an autocrine growth loop for leukemic $CD5^+$ B cells (Hoffbrand *et al.*, 1993), the combined effect of these cytokines was also examined. Viability of MUR B cells in the presence of TNF-α could be significantly enhanced by IL-6. A smaller, although still significant, increase occurred when TNF-α and IL-6 were used at half-optimal concentrations (Table 5.2). This was interpreted as being an additive effect because the increase with TNF-α plus IL-6 was less than the combined effect of the two cytokines when used alone.

Table 5.2: *TNF-α and IL-6 Combine to Further Enhance the Viability of MUR B Cells*

	Cell Viability % (s.d.)	
	Cytokine Concentration	
Cytokine	Optimal	Half-Optimal
Nil	41.6 (0.4)	41.6 (0.4)
IL-6	59.3 (1.0)	57.2 (1.6)
TNF-α	57.1 (2.3)	62.5 (2.6)
TNF-α + IL-6	68.9 (1.8)*	69.2 (0.7)*

MUR leukemic B cells were cultured in the absence or presence of IL-6 and/or TNF-α at optimal and half-optimal concentration. Cell viability was determined 7 days after the initiation of the culture period.

* $p < 0.02$; Significant increase compared to TNF-α or IL-6 when used alone.

5.7. Long Term Effects of Cytokines on Leukemic CD5+ B Cell Viability and Apoptosis

Leukemic CD5+ B cells were cultured for 8 days in the absence and presence of cytokines found to enhance the viability and prevent apoptosis of the different cell populations. Thus, MIL B cells were cultured with IL-2, IL-4 or IL-6; KAR B cells with IL-4, IL-13, IFN-γ or TNF-α; MOT B cells with IL-2, IL-4, IL-6 or IFN-γ; RAN B cells with IL-4 or IFN-γ; MCK B cells with IL-2, IL-4, IL-6 or IFN-γ; and MUR B cells with IL-2, IL-4, IL-6, IFN-γ or TNF-α. Cell viability and apoptotic nuclei were measured after various times of *in vitro* culture.

The effect of cytokines on the *in vitro* death of leukemic CD5+ B cells from patients MIL, KAR and MOT is shown in Figures 5.10a-c. The rapid reduction in viability displayed by unstimulated cells from patient

KAR during the first 1-2 days of *in vitro* culture was only marginally delayed by the cytokines previously determined to display viability-enhancing activity(Figure 5.10a). Despite this, significant numbers of viable cells were recovered following an 8 day culture period. Thus, in contrast to unstimulated cultures, where ~10% of cells remained viable at the conclusion of the culture period, culture with IL-4 or IL-13 allowed for the recovery of 25-40% viable cells (Figure 5.10a). Although not as efficient, culture with IFN-γ and TNF-α resulted in greater numbers of viable KAR B cells after 4 and 6 days of culture, compared to unstimulated cultures (Figure 5.10a). Because KAR B cells rapidly underwent cell death (*in vitro* half life = 48 hours; see Chapter 4, Table 4.2), the viability-enhancing cytokines had only a modest effect on their *in vitro* half-life. At best, this was extended by 15-30 hours (Figure 5.10a). Similar results were obtained when examining the effect of IL-4 or IFN-γ on the *in vitro* half-life of RAN B cells, which also rapidly underwent cell death (data not shown). Thus, the maximal effect of these cytokines was often observed 4-8 days after the initiation of cell culture. This suggests that the cytokines act during the latter half of the culture period and cell death is initiated early during *in vitro* culture, independently of cytokines.

However, when cell populations that underwent *in vitro* death at a rate slower than KAR and RAN B cells were cultured with particular cytokines, the effect of the viability-enhancing cytokines was two-fold. Not only were there significant numbers of viable cells (30-50% of initial cell input) recovered from the cultures at the conclusion of an 8 day culture period, but the *in vitro* half life of each of these leukemic $CD5^+$ B cell populations, as determined in Chapter 4, was significantly extended (Figure 5.10b and c). Consistent with the increases in cell viability, IL-4

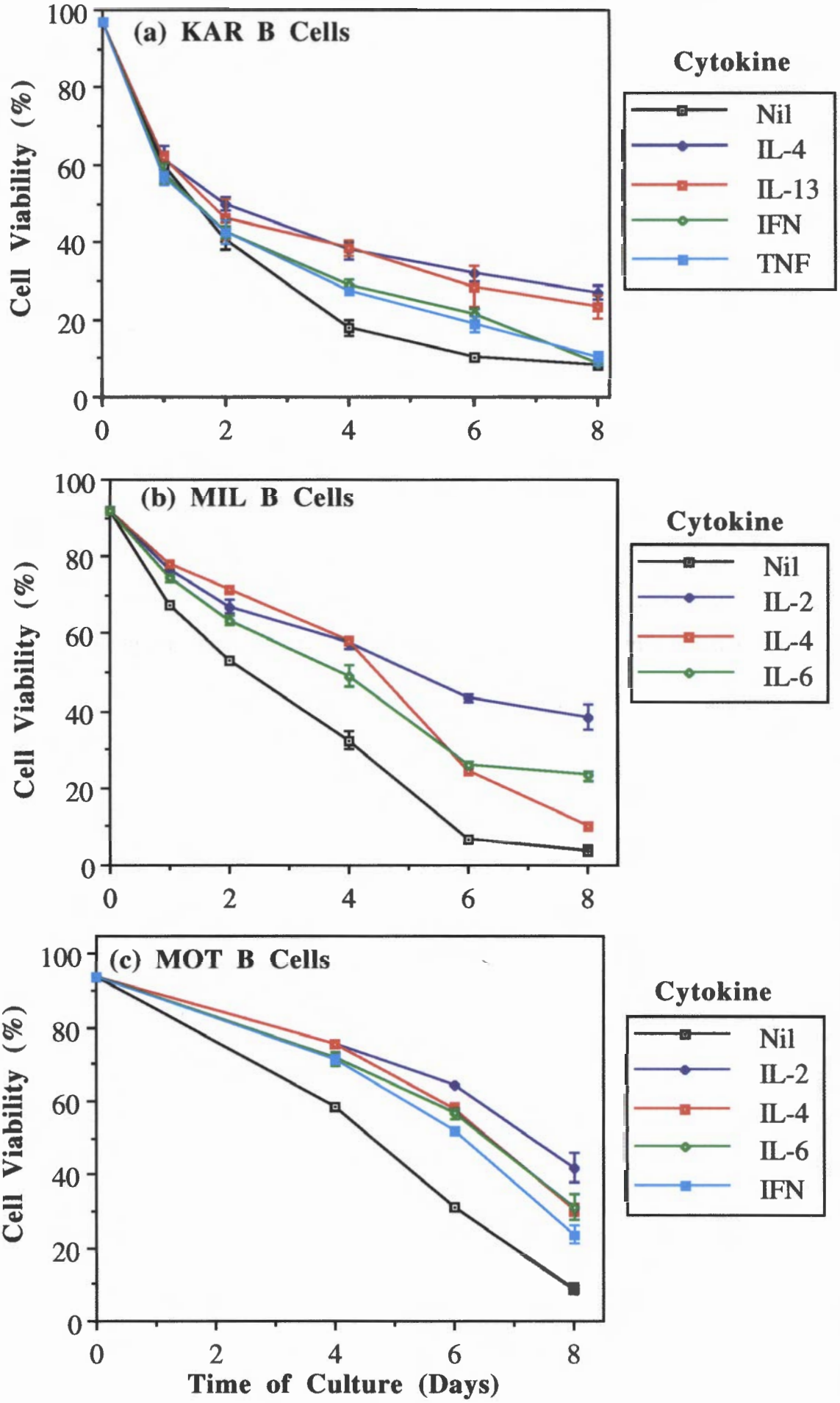

(a) KAR B Cells
Cell Viability (%)
100
80
60
40
20
0
0
2
4
6
8
Cytokine
Nil
IL-4
IL-13
IFN
TNF
(b) MIL B Cells
Cell Viability (%)
Cytokine
Nil
IL-2
IL-4
IL-6
(c) MOT B Cells
Cell Viability (%)
Cytokine
Nil
IL-2
IL-4
IL-6
IFN
Time of Culture (Days)

Figure 5.10: Long Term Effects of Cytokines on Leukemic CD5+ B Cell Viabilty.

Leukemic CD5+ B cells purified from patients KAR (a), MIL (b), and MOT (c) were unstimulated or cultured in the absence or presence of the indicated cytokines for 8 days. At various time points, cell viability was determined as described in *Materials and Methods*. Each point represents the mean % viable cells ± 1 s.d. of triplicate samples.

extended the half-life of the six CLL B cell samples by 28-72 hours (50.8 ± 11 hours; mean ± s.d.). In the presence of IL-2 and IL-6, the *in vitro* half-lives of MIL, MOT and MUR were extended by 31.2-72 hours (58.8 ± 15 hours) and 26.4-48 hours (40.8 ± 10.2 hours), respectively (Figures 5.10b, c). While not as effective as these cytokines, TNF-α and IFN-γ also significantly extended the *in vitro* half-lives of the various populations of leukemic CD5+ B cells by 26.5 ± 4.6 (n=3) and 34.3 ± 9.7 (n=4) hours, respectively (Figures 5.10b, c).

The specific cytokines that enhanced cell viability were also capable of delaying the appearance of apoptotic nuclei in cultures of leukemic CD5+ B cell populations. The kinetics of apoptosis of KAR B cells in the presence of IL-4, IL-13, IFN-γ and TNF-α, MIL B cells in the presence of IL-2, IL-4 and IL-6, MOT B cells in the presence of IL-2, IL-4, IL-6 and IFN-γ, and MUR B cells in the presence of IL-2, IL-4, IL-6 and TNF-α are illustrated in Figures 5.11a-d. As seen for the effects on cell viability, the initial rate of appearance of apoptotic nuclei exhibited by KAR B cells in the presence of IL-4 or IL-13 did not differ significantly from that of unstimulated cells (Figures 5.11a). However, the rapid accumulation of apoptotic nuclei in unstimulated KAR B cells was reduced in the presence of IL-4 or IL-13 after 48-72 hours, and significantly less apoptotic nuclei were present in cytokine stimulated cells for the remainder of the culture period (Figure 5.11a). Consistent with the effects of IL-4 and IL-13 on cell morphology and viability, the kinetics of apoptosis of KAR B cells in the presence of these two cytokines were essentially identical. When assayed over an 8 day culture period, similar results were obtained for the other leukemic CD5+ B cell populations in the presence of IL-2, IL-4, IL-6, IFN-γ or TNF-α; that is, the cytokines displaying viability-enhancing activity (Figures 5.11b-d).

Thus, after various times of *in vitro* culture, the extent of apoptosis (expressed as % apoptotic nuclei) in the presence of these cytokines was 20-40% less than that detected in cultures of unstimulated cells (Figures 5.11b-d). Consistent with the effect that these cytokines have on leukemic $CD5^+$ B cell viability, the maximal effect of these cytokines was often observed during the latter half of the *in vitro* culture period. This confirms that IL-2, IL-6, IL-13 and TNF-α, similar to IL-4 and IFN-γ, enhance viability and delay the rate of apoptosis of leukemic $CD5^+$ B cells.

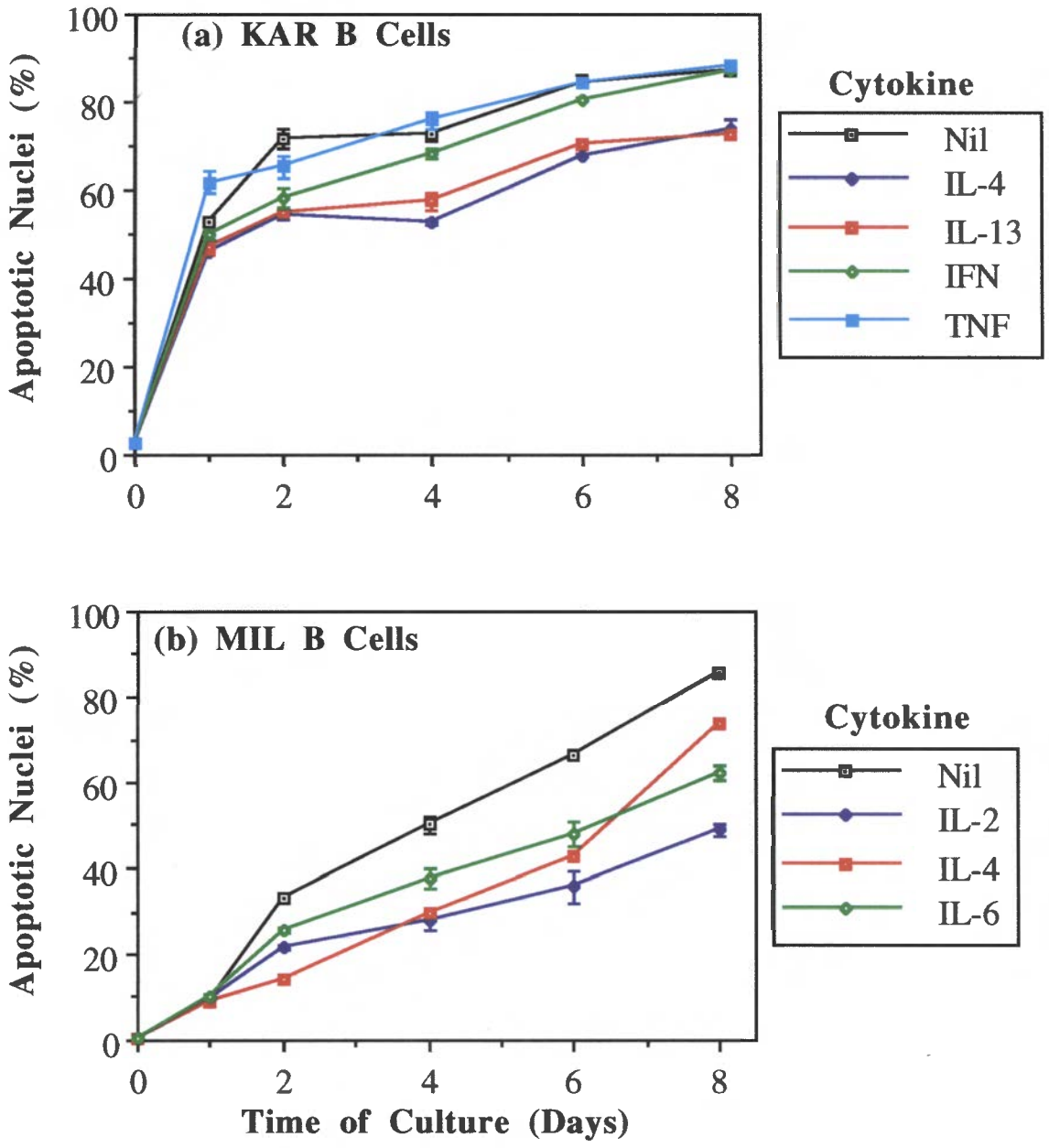


Figure 5.11: Long Term Effects of Cytokines on Apoptosis of Leukemic CD5+ B Cells.

Leukemic CD5+ B cells purified from patients KAR (a), MIL (b), MUR (c) and MOT (d) were cultured in the absence or presence of the indicated cytokines for 8 days. At various time points, DNA fragmentation was determined as described in *Materials and Methods*. Each point represents the mean % apoptotic nuclei ± 1 s.d. of triplicate samples.

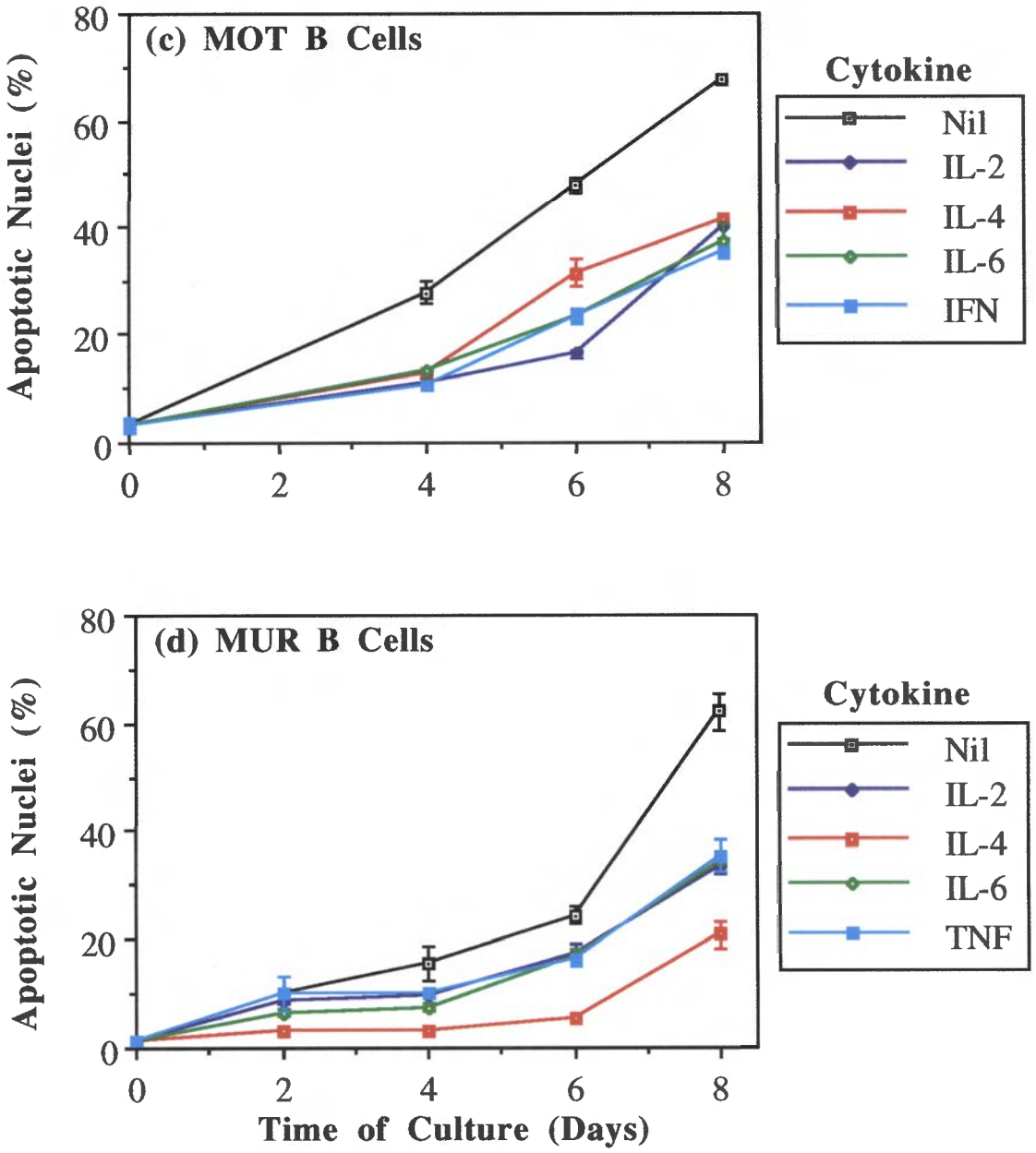


Figure 5.11: Long Term Effects of Cytokines on Apoptosis of Leukemic CD5+ B Cells (continued).

5.8. Requirements of Leukemic CD5+ B Cells for the Presence of Cytokines During In Vitro Culture

i. Delayed Addition of Cytokines

As mentioned above, the initial reduction in cell viability during the first 1-2 days of *in vitro* culture in the presence of cytokines did not greatly differ from unstimulated cells (Figures 5.10a). This suggested that the cells may enter an apoptotic process immediately on initiation of *in vitro* culture and independent of the presence of any cytokines, even though culture with exogenous cytokines resulted in the recovery of greater numbers of viable cells at the conclusion of an 8 day culture period, compared to unstimulated cultures. To determine whether or not the viability-enhancing cytokines were required by the cells during the first half of the culture, leukemic CD5+ B cells were cultured in the absence of cytokines and after various times the appropriate cytokines were added at their optimal concentrations. Cell viability was determined after 6-7 days of culture (Figure 5.12a-d). When IL-2, IL-4, IL-6, IL-13, IFN-γ or TNF-α were added to cultures of KAR, MIL, MOT or MCK B cells on day 0 (i.e at the initiation of culture), 10-30% more viable cells were recovered after 6-7 days of *in vitro* culture, compared to cells cultured without any cytokine for the entire culture period (Figure 5.12a-d). Interestingly, for cells obtained from patients MIL, KAR and MOT, the time at which the cytokines could be added, and an effect still be observed, appeared to be dependent on the *in vitro* half life of the unstimulated cell populations. Specifically, the viability enhancing effect of IL-4, IL-13 and IFN-γ was reduced by 50% when added to KAR leukemic CD5+ B cells 24-48 hours after the commencement of culture (Figure 5.12a). Similarly, the capacity of IL-2, IL-4 and IL-6 to enhance the viability of MIL B cells, and IL-2, IL-6, IFN-γ and TNF-α to enhance the viability of MOT B cells, was reduced by 50% when the addition of

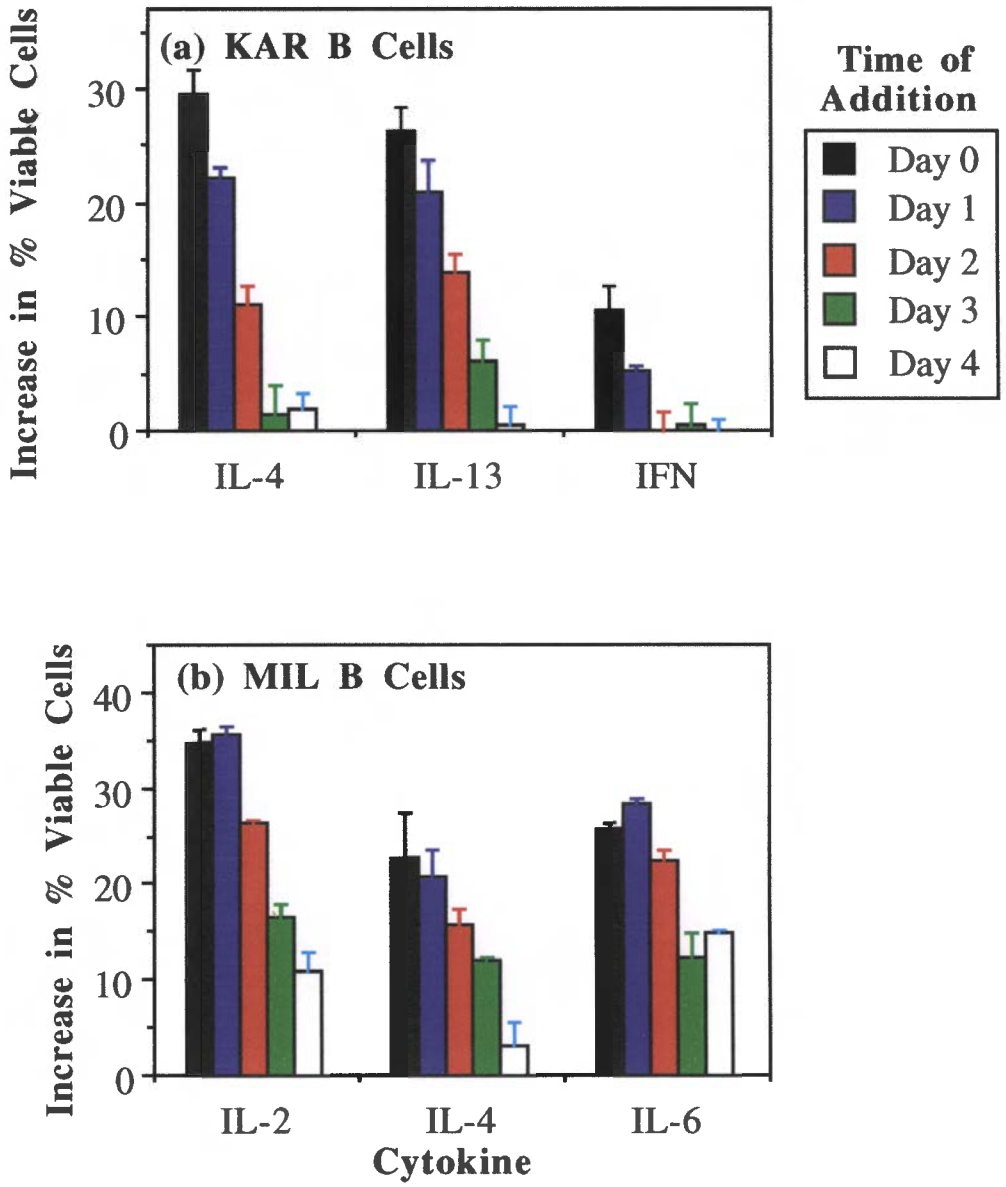


Figure 5.12: Delaying the Addition of Viability-Enhancing Cytokines by 24-72 hours Abrogates Their Effect.

Leukemic CD5+ B cells purified from patients KAR (a), MIL (b), MOT (c) and MCK (d) were cultured in complete medium. After 1, 2, 3 or 4 days the indicated cytokines were added. The cells were cultured for a total of 6 or 7 days, after which time cell viability was determined. The results are expressed as the mean increase in the percentage of viable cells, ± 1 s.d. of triplicate samples, and are compared to the results obtained when the cells were continually exposed to the cytokine (day 0). The asterisk indicates that investigation of the delayed addition of the cytokine at this time point was not determined.

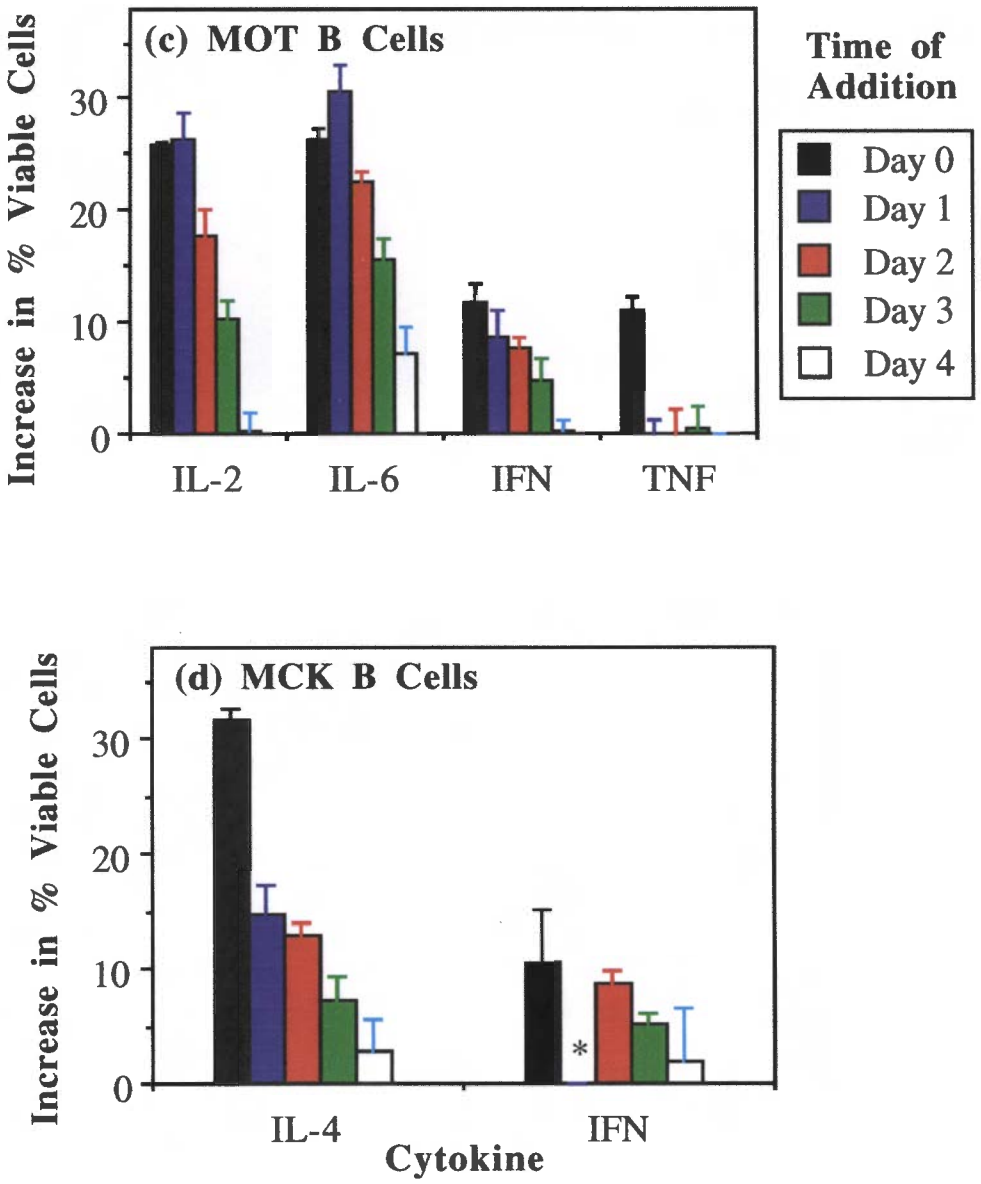


Figure 5.12: Delaying the Addition of Viability-Enhancing Cytokines by 24-72 hours Abrogates Their Effect (continued).

these cytokines to the appropriate population of leukemic CD5+ B cells was delayed for ~72 hours (Figure 5.12b and c). These times approximate to the *in vitro* half lives of these different populations of leukemic CD5+ B cells, as described in the previous chapter (Table 4.2). However, this was not the case for cells obtained from patient MCK. These cells displayed an *in vitro* half life of ~130 hours, yet delaying the addition of IL-4 by more than 24 hours reduced its effect by 50-80% (Figure 5.12d).

ii. Neutralisation of IL-2 and IL-4

The reverse experiment was performed by culturing leukemic CD5+ B cells with either IL-2 or IL-4 and then neutralising these cytokines by the addition of polyclonal goat anti-human IL-2 or anti-human IL-4 antiserum after various times. Prior to conducting these experiments, a neutralising concentration of these antibodies was determined. Leukemic CD5+ B cells from patient MIL were cultured with optimal concentrations of IL-2 or IL-4 in the presence of increasing concentrations of the goat antiserum. To control for any cytotoxic effect of the goat Ab, the cells were also cultured with polyclonal goat antiserum specific for human IL-10, with or without IL-2 or IL-4. This acted as a control for the goat antiserum due to its irrelevant specificity. Cell viability was determined after 5 days. In the absence of any goat Abs, IL-2 and IL-4 increased cell viability by 28.0% and 24.0% respectively (Table 5.3). In the presence of anti-IL-10, the relative increase in viability of IL-2 or IL-4 -stimulated cells was similar (29.3% and 22.0%, respectively), however the actual number of viable cells was slightly higher than cells cultured without anti-IL-10 (Table 5.3). This suggests that IL-10, either present in the tissue culture medium or spontaneously secreted by the leukemic CD5+ B cells, is slightly cytotoxic. This is consistent with a recent report which

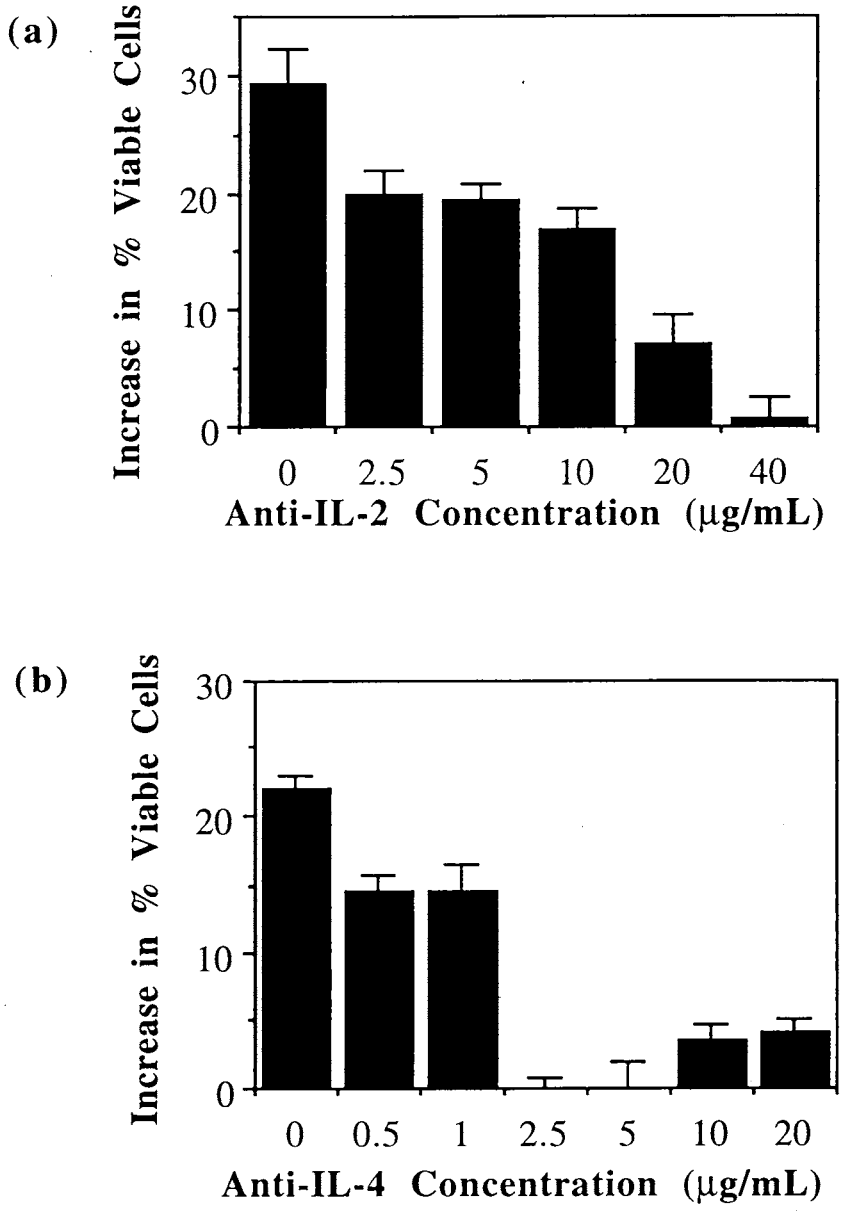


Figure 5.13: Optimisation of Anti-IL-2 and Anti-IL-4 Antiserum Concentrations.

MIL leukemic CD5$^+$ B cells were cultured with (a) IL-2 (100 U/mL) or (b) IL-4 (2 U/mL), and increasing concentrations of goat antiserum specific for IL-2 or IL-4, respectively. Cell viability was determined 5 days after the initiation of the culture period. Results are expressed as the mean increase in the percentage of viable cells, ± 1 s.d. of triplicate samples.

demonstrated that IL-10 induces apoptosis of leukemic B cells (Fluckiger *et al.*, 1994b). The concentrations at which the goat antiserum abrogated the viability-enhancing effects of IL-2 and IL-4 were 40 μg/mL and 5 μg/mL, respectively (Figure 5.13a and b). These concentrations were used for the experiments described below.

Table 5.3: *Effect of Anti-IL-10 on Leukemic CD5+ B Cell Viability*

Stimulator	*Cell Viability (% ± s.d.)*
Complete medium (CM)	10.7 ± 2.7
IL-2 (100U/mL)	38.5 ± 1.3
IL-4 (2U/mL)	35.2 ± 1.0
CM + anti-IL-10 (40μg/mL)	15.2 ± 0.5
IL-2 + anti-IL-10	44.5 ± 3.0
IL-4 + anti-IL-10	37.5 ± 1.0

MIL CD5+ B cells were cultured in complete medium (CM) alone or CM supplemented with IL-2, IL-4, goat polyclonal anti-human IL-10 antiserum, or combinations thereof. Cell viability was determined 5 days later.

The requirements for the presence of IL-2 by leukemic CD5+ B cells were investigated using cells obtained from patient MIL. Addition of goat anti-IL-2 antiserum to IL-2-stimulated MIL B cells on day 0 and day 1 resulted in viability similar to that of cells cultured in complete medium with the control goat antiserum (Figure 5.14a; compare CM/GIg with IL-2/aIL-2). Neutralising IL-2 after 2 days of *in vitro* culture resulted in >60% of the maximal activity of IL-2. This is based on a comparison with the viability of IL-2-stimulated MIL B cells in cultures where the control goat antiserum (GIg) was added to the cells after a similar time period (Figure 5.14a; compare IL-2/GIg with IL-2/aIL-2). Neutralising

IL-2 on day 4 had no effect on its viability-enhancing activity (Figure 5.14a). Thus, for IL-2 to have any effect on the viability of MIL B cells, the cells had to be continually exposed to this cytokine for the first 2 days of an *in vitro* culture period.

The requirements for the presence of IL-4 were investigated using cells obtained from patients MIL, KAR and MCK. Exposure of CLL B cells from the 3 different patients to IL-4 for only 1 day resulted in an increase in cell viability of ~15-20%. The increase in viability observed when these leukemic $CD5^+$ B cells were cultured with IL-4 for 7 days (i.e. the entire culture period) was ~25-35% (Figure 5.14b-d). Thus, a relatively short exposure period of only 1 day was sufficient for IL-4 to exert >60% of its maximal effect. Neutralising IL-4 after 2 days and later did not reduce the ability of IL-4 to maximally enhance cell viability, compared to cells cultured with IL-4 and the control goat antiserum (Figure 5.14b-d). This indicates that IL-4 need only be present during the initial phase (1-2 days) of the *in vitro* culture period. These results are similar to those obtained for leukemic $CD5^+$ B cells cultured with IL-2 and anti-IL-2 antiserum (Figure 5.14a).

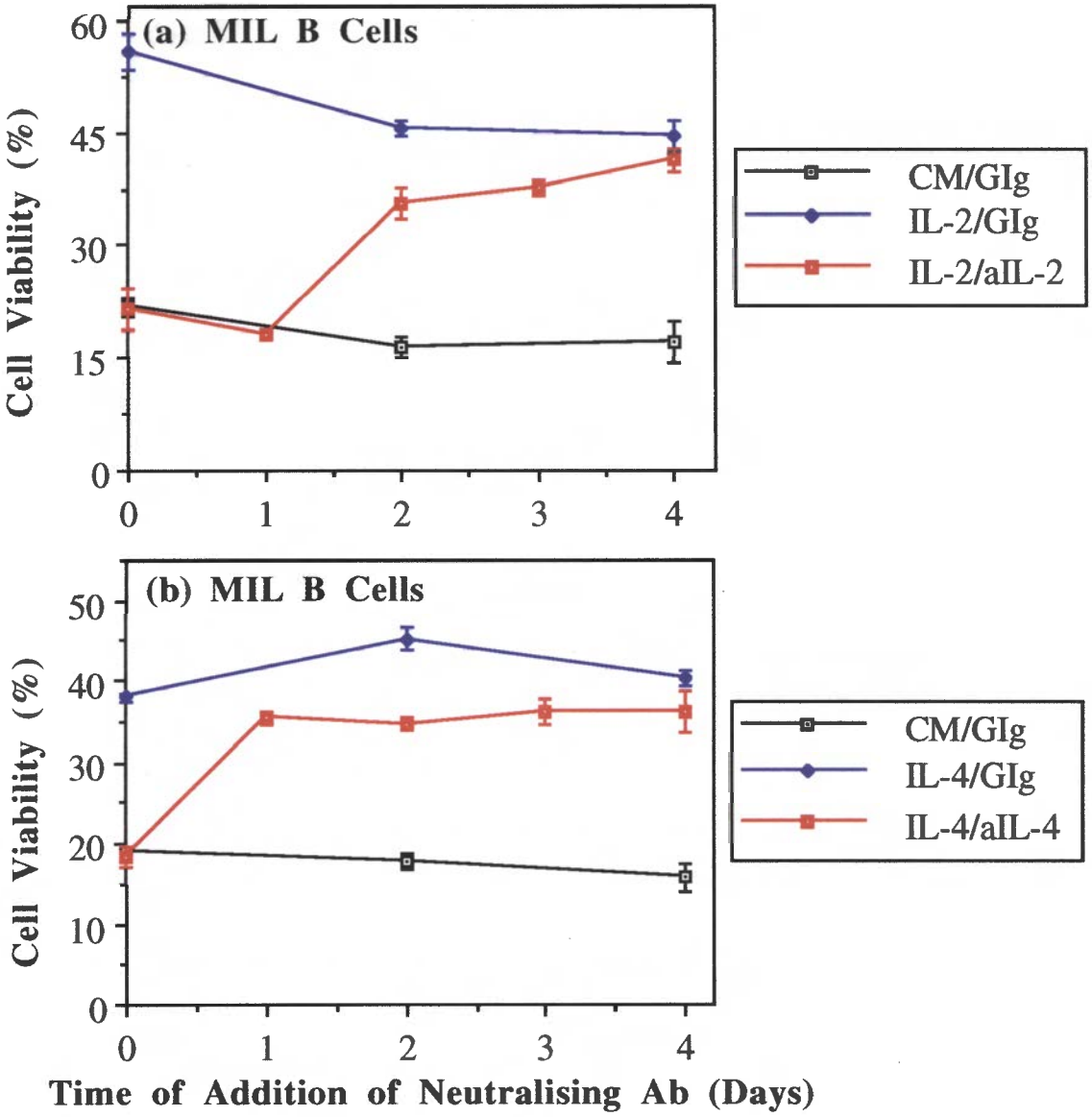


Figure 5.14: Leukemic CD5+ B Cells Must be Exposed to IL-2 or IL-4 for at Least 24 hours for these Cytokines to Have a Viability-Enhancing Effect.

(a) MIL leukemic CD5+ B cells were cultured without (complete media; CM) and with IL-2. After various times, goat antiserum specific for IL-2 (aIL-2), or a control goat antiserum (GIg), was added to the wells. Cell viability was determined after 6 days of *in vitro* culture. Leukemic CD5+ B cells from patients MIL (b), KAR (c) and MCK (d) were cultured without (complete media; CM) and with IL-4. After various times, goat antiserum specific for IL-4 (aIL-4), or a control goat antiserum (GIg), was added to the wells. Cell viability was determined after 6 (b, c) or 7 (d) days of *in vitro* culture.

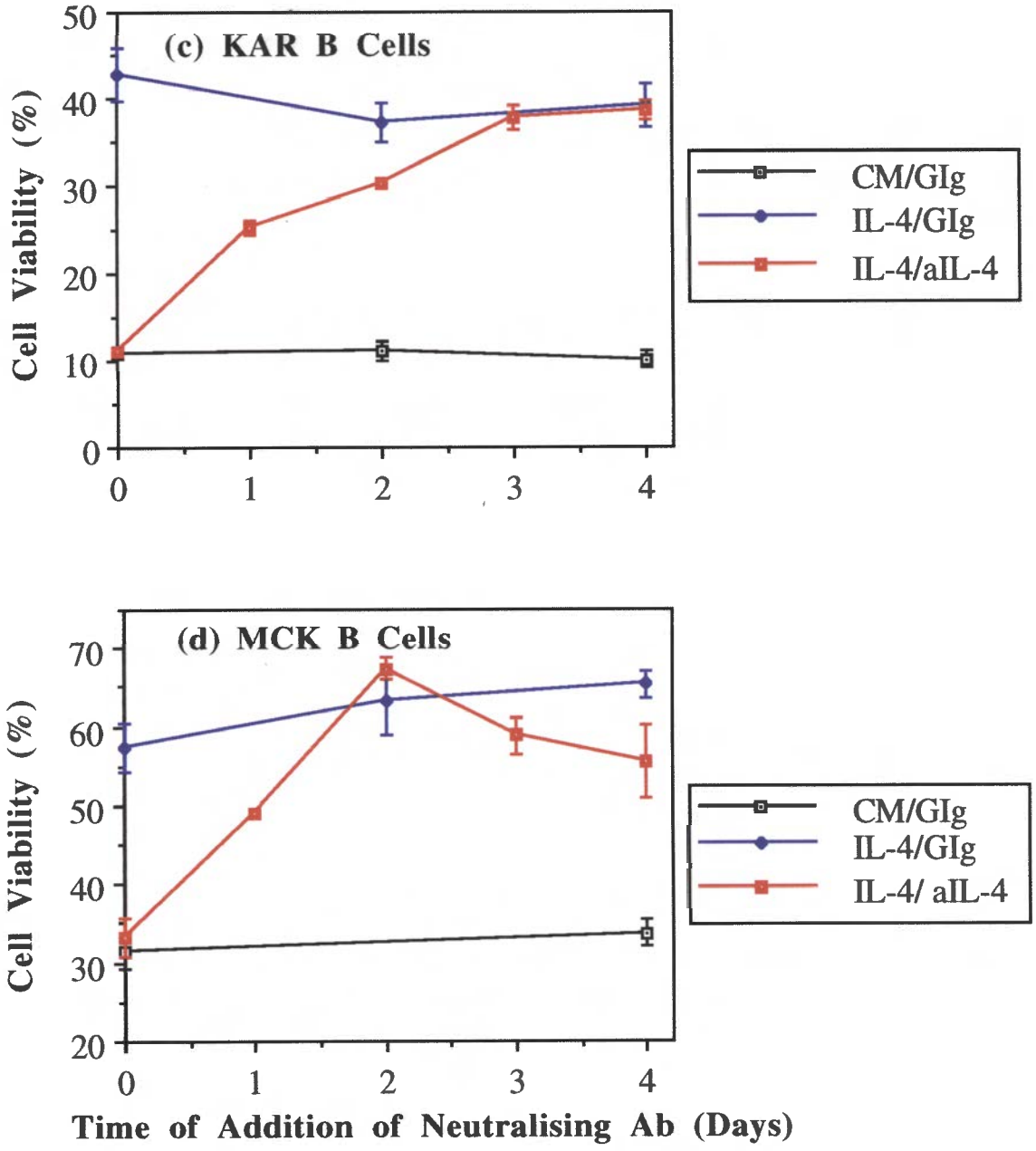


Figure 5.14: Leukemic CD5+ B Cells Must be Exposed to IL-2 or IL-4 for at Least 24 hours for these Cytokines to Have a Viability-Enhancing Effect (continued).

5.9. The Signal Delivered by Cytokines is Insufficient to Induce Cell Proliferation

In addition to determining the effect of soluble growth factors on cell viability, the ability to induce proliferation or enhance RNA synthesis of the leukemic CD5+ B cells was also assessed. IL-2, IL-4, IL-6 and TNF-α induced proliferation in some of the B cell populations. However, the amount of ^{3}H-thymidine incorporated by cytokine-stimulated cells was only 2-4 fold greater than unstimulated cells (Table 5.4). These same cytokines could also cause a slight enhancement (1.5-2.0 fold) in RNA synthesis, as indicated by the uptake of ^{3}H-uridine, by the leukemic CD5+ B cells (Table 5.5). Because unstimulated leukemic CD5+ B cells exhibit significant levels of spontaneous RNA synthesis, the enhanced rate of RNA synthesis in the presence of particular cytokines is probably due to the presence of more viable cells in these cultures. Thus, when used alone, human cytokines had minimal effect on the proliferation and RNA synthesis of the leukemic CD5+ B cells studied. However, as described in Chapter 3, co-stimulation of leukemic CD5+ B cells with F(ab')$_2$ anti-μ or PMA and various cytokines such as IL-2, IL-4, IL-6, IL-13, IFN-γ and TNF-α caused the levels of proliferation and RNA synthesis of leukemic CD5+ B cells to be 3-20 times higher than those of unstimulated cells, or cells stimulated with anti-μ, PMA or cytokine alone (see section 3.9, *Exogenous Cytokines Augment PMA-Induced Leukemic CD5+ B Cell Activation*; Figure 3.16; data not shown).

Table 5.4: *Cytokines Do Not Induce Proliferation of Leukemic CD5+ B Cells*

	Proliferation Counts per Minute (s.d.)							
Cells	Nil	IL-2	IL-4	IL-6	IL-10	IL-13	IFN-γ	TNF-α
MIL	190 (57)	320 (27)	145 (15)	187 (47)	122 (14)	193 (138)	183 (54)	230 (90)
KAR	122 (13)	190 (37)	167 (21)	316 (110)	114 (11)	163 (50)	221 (61)	210 (30)
MOT	225 (76)	896 (168)	620 (100)	686 (161)	169 (30)	309 (120)	403 (34)	372 (55)
MCK	165 (91)	220 (44)	264 (57)	253 (30)	185 (28)	171 (72)	295 (55)	522 (82)
RAN	95 (25)	400 (122)	95 (12)	157 (28)	70 (40)	NT	100 (30)	79 (19)

Leukemic CD5+ B cells from different CLL patients were cultured in the absence or presence of the indicated cytokine. Proliferation was assessed by determining the amount of ^{3}H-thymidine incorporated during the final 18 hours of a 4 day culture period.

Table 5.5: *Cytokines Do Not Enhance RNA Synthesis of Leukemic CD5+ B Cells*

	RNA Synthesis Counts per Minute (s.d.)							
Cells	Nil	IL-2	IL-4	IL-6	IL-10	IL-13	IFN-γ	TNF-α
MIL	1225 (344)	1905 (226)	2120 (418)	2051 (776)	598 (70)	649 (241)	936 (143)	870 (287)
KAR	864 (220)	1237 (160)	2621 (263)	760 (150)	417 (63)	1975 (294)	1736 (52)	1510 (312)
MOT	2187 (284)	5874 (380)	3507 (211)	4263 (330)	2014 (131)	3254 (533)	4302 (739)	2757 (252)
MCK	1996 (75)	2175 (216)	3270 (135)	2716 (190)	2253 (129)	2440 (141)	3119 (433)	2827 (140)

Leukemic CD5+ B cells from different CLL patients were cultured in the absence or presence of the indicated cytokine. RNA synthesis were assessed by determining the amount of ^{3}H-uridine incorporated during the final 18 hours of a 4 day culture period.

5.10. Expression of bcl-2 by Cytokine-treated Leukemic CD5+ B Cells

Prior to *in vitro* culture, >85% of cells from the six populations of leukemic CD5+ B cells expressed the bcl-2 protein, as determined by flow cytometry. The expression of bcl-2 appeared as a unimodal peak of fluorescence (Figure 4.8). As reported in Chapter 4, the expression of bcl-2 protein was reduced approximately 2-3 fold in unstimulated leukemic CD5+ B cells during an *in vitro* culture period of 8 days. This reduction was due to the appearance of a population of cells that had down-regulated bcl-2 expression The resultant fluorescence histogram appeared bimodal such that the cells displayed either a bcl-2_{high} or bcl-2_{low} phenotype (Figure 4.9). Despite this reduction, the percentage of cells positive for bcl-2 remained constant (ie >85%).

In addition to determining the effect that exogenous cytokines had on the death and apoptosis of leukemic CD5+ B cells, the expression of bcl-2 by the stimulated cells was concomitantly assessed and compared to that of unstimulated cells. The histograms of bcl-2 expression by leukemic CD5+ B cells obtained from patients MIL, KAR, MOT and MCK cultured in the presence and absence of different cytokines are shown in Figures 5.15a-d. Consistent with the results presented in Chapter 4, the expression of bcl-2 by unstimulated leukemic CD5+ B cells appears bimodal during *in vitro* culture. The expression by cytokine-stimulated leukemic CD5+ B cells was also bimodal. However, it is clear from the results presented in Figure 5.15 that those cytokines capable of delaying cell death and apoptosis in the different populations of leukemic CD5+ B cells, namely IL-2, IL-4, IL-6, IL-13, IFN-γ and TNF-α, also reduced the proportion of cells exhibiting the bcl-2_{low} phenotype. Thus, in the presence of particular cytokines, there was a greater percentage of cells displaying the bcl-2_{high} phenotype, suggesting that fewer cells down-regulated the

expression of bcl-2. The increase in the number of cells displaying the bcl-2_{high} phenotype in the presence of a cytokine was similar to the enhancement in cell viability induced by the same cytokine. For instance, when MIL leukemic $CD5^+$ B cells were cultured with IL-2 or IL-4, there were 26% and 22% more viable cells, respectively, compared to unstimulated cells (Figure 5.3). These cytokines also resulted in 20-25% more MIL B cells expressing the bcl-2_{high} phenotype than cells cultured with complete medium (Figure 5.15a). Similar results to these were observed for the other cell populations when cultured in the presence of viability-enhancing cytokines (compare Figures 5.3 and 5.15). This was not surprising given that earlier results indicated that down-regulation of bcl-2 expression correlated with the rate of cell death (Figure 4.10). Consistent with the similar effects of IL-4 and IL-13 on KAR B cell survival and apoptosis, the histograms of bcl-2 expression of KAR B cells stimulated with IL-4 or IL-13 were almost superimposable (Figure 5.15b). In the presence of IL-4 or IL-13, approximately 20% more KAR B cells expressed bcl-2 at a high level, compared to unstimulated cells. As described in section 5.2 (*Effect of Cytokines on Leukemic $CD5^+$ B Cell Viability* In vitro), IL-13 enhanced the viability of MOT and MCK B cells to a level that was only ~25% of that achieved by IL-4. Similarly, the capacity of IL-13 to sustain the elevated expression of bcl-2 by these cell populations was significantly less than that of IL-4 (Figure 5.15c). Not surprisingly, cytokines such as IL-10 and IL-13, which had no effect on the viability of MIL B cells, did not alter the pattern of expression of bcl-2 by these cells, compared to the corresponding unstimulated cells (Figure 5.15a). Although the proportion of cells expressing the bcl-2_{high} phenotype was greater in the presence of viability-enhancing cytokines, there was no evidence that these cytokines caused an up-regulation in the expression of this protein. This is based on analysis of the MFI of cells

with the bcl-2_{high} phenotype. The relative increase over the MFI of the isotype control was similar to that observed in uncultured cells. Thus, these cytokines appear to preserve cell viability and prevent DNA fragmentation by sustaining, rather than up-regulating, the expression of bcl-2.

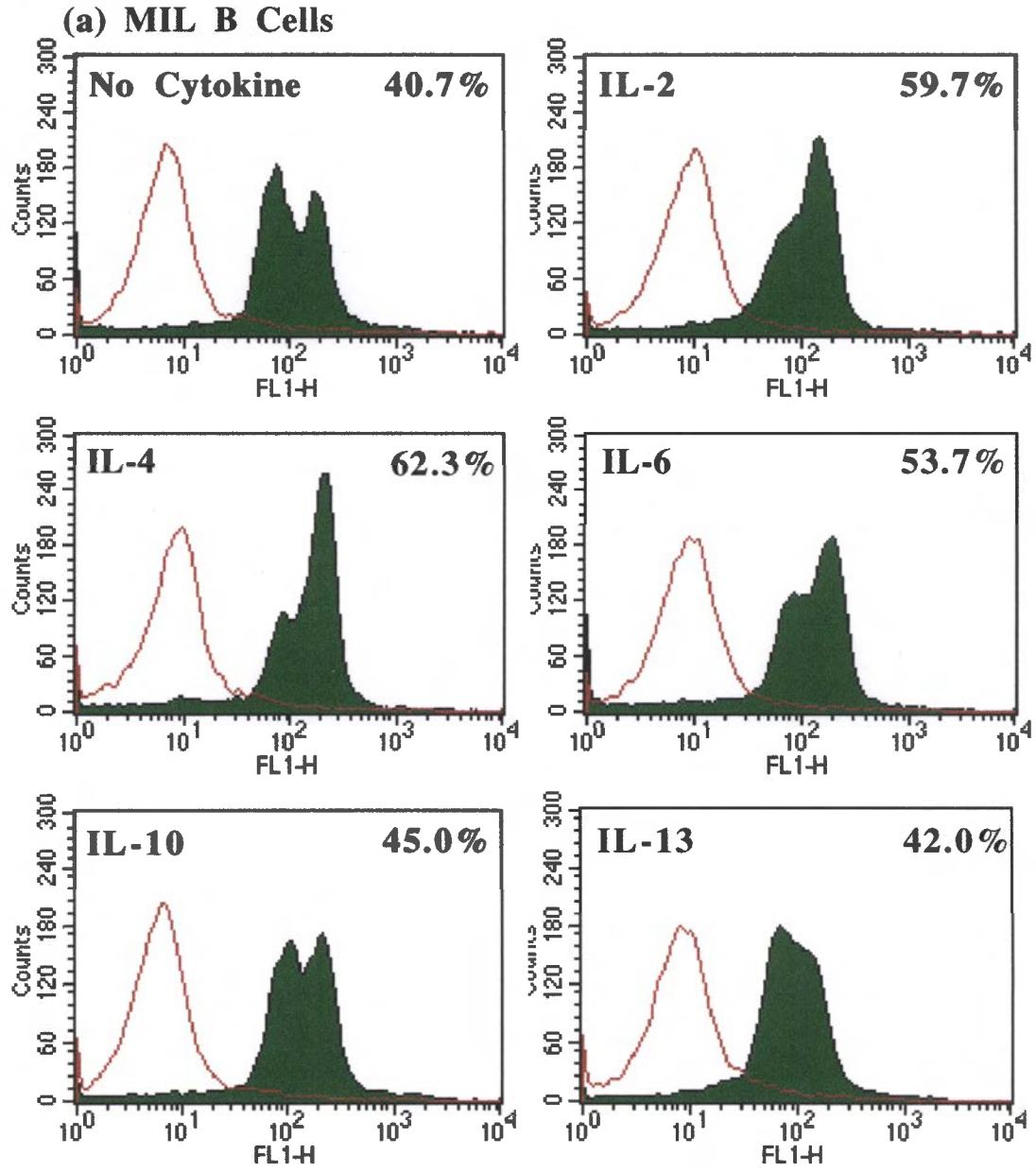


Figure 5.15: Expression of bcl-2 by Cytokine-stimulated Leukemic CD5+ B Cells.

Leukemic CD5+ B cells from patients (a) MIL, (b) KAR, (c) MOT or (d) MCK were cultured in complete medium (no cytokine) or in the presence of the indicated cytokines. Expression of bcl-2 (solid histogram) was determined after 4 (KAR), 5 (MIL), 6 (MOT) or 7 (MCK) days, as described in *Materials and Methods*. The value in each panel indicates the percentage of cells displaying the bcl-2_{high} phenotype. The outline histogram shows the fluorescence of cells incubated with an isoytpe-matched negative control mAb.

(b) KAR B Cells

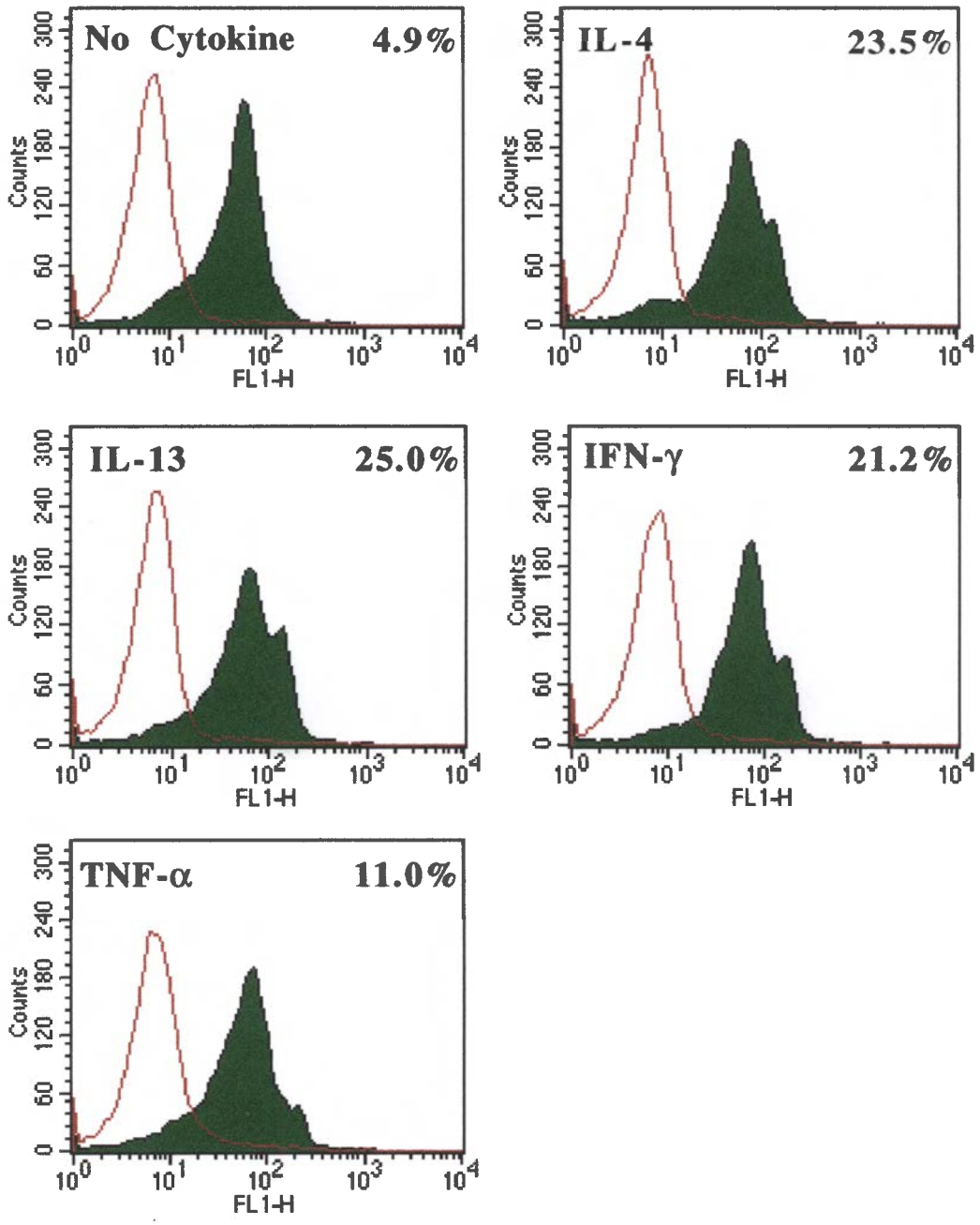


Figure 5.15: Expression of bcl-2 by Cytokine-stimulated Leukemic CD5+ B Cells (continued).

(c) MOT B Cells

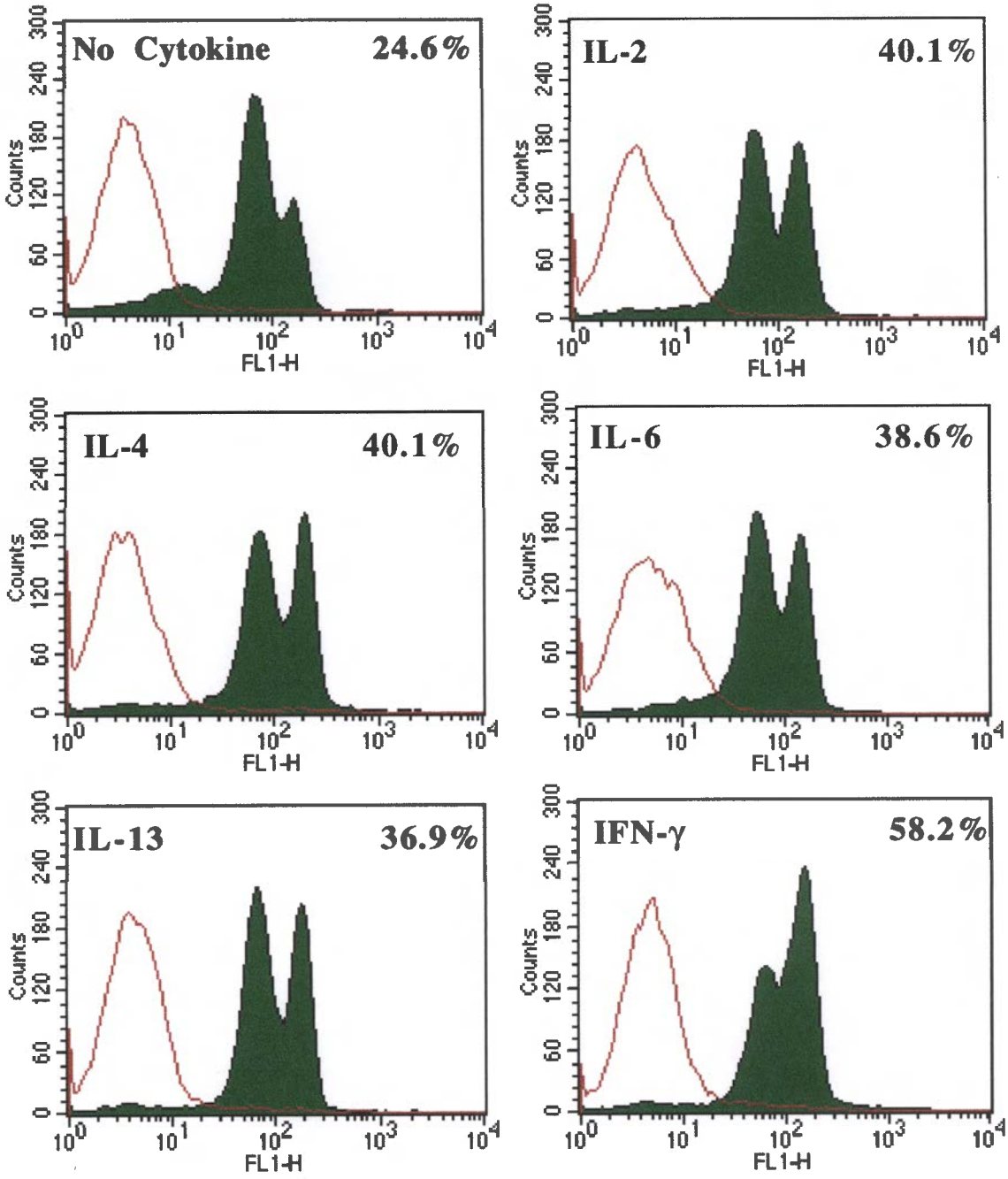


Figure 5.15: Expression of bcl-2 by Cytokine-stimulated Leukemic CD5+ B Cells (continued).

(d) MCK B Cells

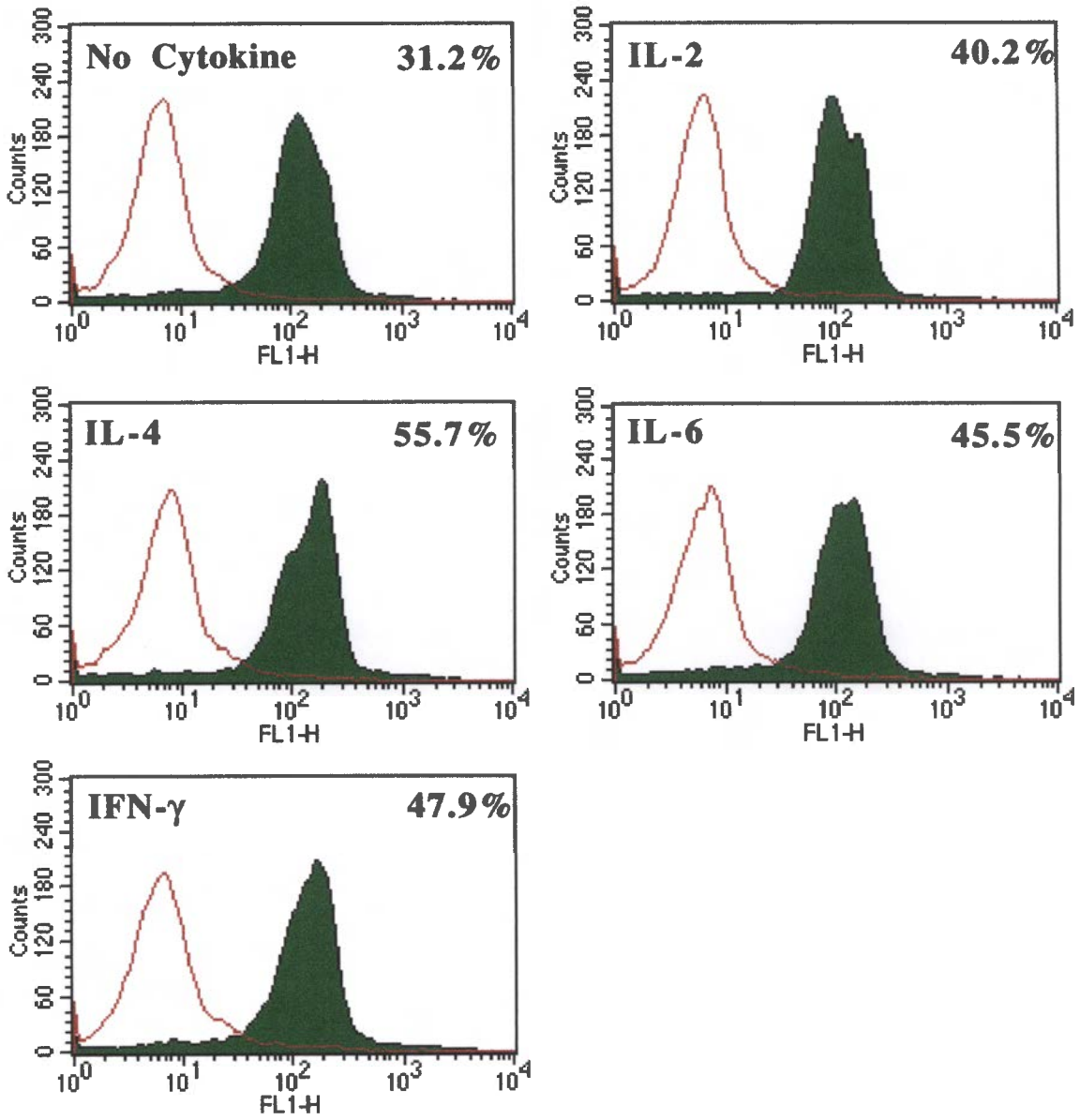


Figure 5.15: Expression of bcl-2 by Cytokine-stimulated Leukemic CD5+ B Cells (continued).

Discussion

The ability of leukemic CD5+ B cells to enter an apoptotic pathway on *in vitro* culture suggests that mediators preventing the apoptosis exist *in vivo*. Such mediators may subsequently give rise to a population of long-lived leukemic B cells. The results described in this chapter indicate that cytokines of both autocrine and paracrine origin act as anti-apoptotic factors *in vitro* and, therefore, may play a role in the pathogenesis of B-CLL. Viability of leukemic CD5+ B cells could be enhanced by co-culturing the cells with activated normal allogeneic T cells. Under these conditions, the products secreted by mitogen-activated T cells resulted in 20-35% more viable leukemic CD5+ B cells after a 4 or 5 day incubation period (Figure 5.1). However, the maximal effects of activated T cells on leukemic CD5+ B cell survival could be masked by two possible events. Firstly, the secreted products may have been utilised in an autocrine fashion to support the proliferation of the activated T cells. This may limit the amount of soluble product available to the leukemic CD5+ B cells. Secondly, there may have been exhaustion of essential nutrients in the culture media due to the rapidly proliferating T cells. To more closely investigate the effect of cytokines on cell viability, leukemic CD5+ B cells were cultured in the absence and presence of cytokines previously shown to be either produced by or activate leukemic B cells. IL-1β, IL-5, IL-10 and TGF-β displayed no viability-enhancing activity on any leukemic CD5+ B cell population tested (Figure 5.3). Thus, although it has been reported that CLL B cells possess the capacity to secrete IL-1β and TGF-β (Uggla *et al.*, 1987; Morabito *et al.*, 1987; Kremer *et al.*, 1992; Schena *et al.*, 1992a), these cytokines do not appear to be active in prolonging the survival of these cells *in vitro*. The cytotoxicity of TGF-β to CLL B cells supports a recent finding indicating that endogenously produced TGF-β can act as an inhibitor of leukemic B cell growth *in*

vitro (Lotz *et al.*, 1994). Furthermore, these findings are consistent with TGF-β being able to promote the death and apoptosis of human resting peripheral blood B cells (Lomo *et al.*, 1995). The inability of CLL B cells to respond to IL-5 (Hayes *et al.*, 1993) may be attributable to the cytotoxicity of this cytokine. A recent study also found that IL-5 increased the rate of spontaneous apoptosis exhibited by unstimulated leukemic B cells, *in vitro* (Mainou-Fowler *et al.*, 1994). Consistent with previous studies (Dancescu *et al.*, 1992; Panayiotidis *et al.*, 1993), IL-4 was found to preserve cell viability and delay the appearance of apoptotic nuclei in all of the leukemic $CD5^+$ B cell populations examined. Similar results were obtained for IFN-γ, although these effects were not as great as IL-4 (Figure 5.3). These two cytokines caused the *in vitro* half-life of the leukemic $CD5^+$ B cells to be extended by 30-72 hours, compared to the *in vitro* half-life of unstimulated B cells (Figure 5.11). Interestingly, IL-4 has also previously been reported to enhance the viability and delay apoptosis of resting murine splenic B cells (Hodgkin *et al.*, 1991; Illera *et al.*, 1993), as well as prevent anti-IgM-induced growth arrest and apoptosis of human and murine B-lymphoma cell lines (Ales-Martinez *et al.*, 1992; Komada *et al.*, 1994). Taken with the findings of this study, one of the main functions of IL-4 may be to promote the survival of B cells prior to them receiving a second signal that would initiate cell proliferation, Ig secretion and isotype switching.

According to the literature, the prevention of apoptosis in unstimulated leukemic $CD5^+$ B cells appears to be restricted to IL-4 and IFN-α and -γ because experiments investigating the effects of other soluble factors failed to reveal a significant role for IL-1, IL-2, IL-3, IL-6, IL-7, IL-13, TNF-α or TNF-β (Dancescu *et al.*, 1992; Sarfati, 1993; Fluckiger *et al.*, 1994a and b). However, in addition to the effects of IL-4 and IFN-γ, the

results described here also indicated that IL-2, IL-6, IL-13 and TNF-α were all capable of delaying the rates of cell death, and consequently apoptosis, in some of the leukemic CD5+ B cell populations examined (Figures 5.3 and 5.6). Similar to the reported effects of IL-4 and IFN-γ, these cytokines were capable of significantly increasing cell viability after various times of *in vitro* culture such that the *in vitro* half-lives of the responsive leukemic CD5+ B cell populations were prolonged by 25-50 hours. The effects of these cytokines were maximal after 6-8 days of culture, with substantial numbers of viable cells remaining at the completion of the culture period (Figure 5.11). This contrasted unstimulated cultures, where >90% of cells were dead after 8 days of *in vitro* culture. The effect of IL-2 correlated with the expression of the α-chain of the IL-2 receptor, CD25, by the responsive leukemic B cells (Figure 5.4). Thus, the viability of 4 of the 5 CD25-positive cell populations could be enhanced by IL-2, while this cytokine had no effect on cells lacking surface expression of this receptor. This suggests that IL-2 may act directly on the leukemic CD5+ B cells, and not via an indirect mechanism that could involve, for instance, IL-2-induced secretion of another cytokine (eg IL-4) by the few contaminating T cells present at very low levels (0.05-1.5%). It is unknown why leukemic CD5+ B cells from patient RAN failed to respond to IL-2, despite the expression of CD25. However, it has been previously demonstrated that CD25 expression does not always correlate with the ability to respond to IL-2. In one study, it was found that some CLL B cells expressing CD25 proliferated in response to PMA and IL-2, yet failed to secrete IgM. Other CLL B cells examined exhibited the opposite response; that is, IgM secretion in the absence of proliferation (Kabelitz *et al.*, 1985). This indicated that the ability of IL-2 to induce both cell division and differentiation does not necessarily occur in the one cell population,

despite the expression of the IL-2 receptor and the capacity of IL-2 to induce both of these B cell responses. Another study found that activation with PMA resulted in the expression of both high and low affinity receptors for IL-2 on the cells from 5 patients with B-CLL. However, 3 of the cell populations did not proliferate in response to IL-2, suggesting an inability of the expressed IL-2 receptor to transduce activation signals to the cells (Mitsui *et al.*, 1991). Thus, in a proportion of cases, expression of the IL-2 receptor on leukemic $CD5^+$ B cells does not imply that these cells will be responsive to the growth- and survival-promoting effects of this cytokine.

The results regarding the effect of IL-13 on leukemic $CD5^+$ B cell survival are intriguing for several reasons. The activities of IL-13 very closely resemble those of IL-4. Thus, both cytokines have been found to upregulate CD23 expression on resting human B cells (McKenzie *et al.*, 1993; Punnonen *et al.*, 1993), induce proliferation and Ig isotype switching to IgG_4 and IgE in anti-CD40 activated human B cells (Cocks *et al.*, 1993; Punnonen *et al.*, 1993), inhibit the proliferation of human B cell precursors (Pandrau *et al.*, 1992; Renard *et al.*, 1994) and induce similar phenotypic and functional changes in human monocytes (de Waal Malefyt *et al.*, 1993a). In addition, proliferation of leukemic B cells activated by anti-CD40 antibodies could be comparably enhanced by the addition of IL-4 or IL-13 (Fluckiger *et al.*, 1994a). Furthermore, it appears that the receptors for IL-4 and IL-13 share common components (Aversa *et al.*, 1993b; Zurawski *et al.*, 1993). Thus, it seems unusual that IL-13 could enhance the viability of only one population of cells (KAR) to a similar extent as IL-4, while IL-4 was capable of enhancing the viability of all six populations of leukemic $CD5^+$ B cells. An immediate explanation could be the lack of expression by the other CLL cells of

either the components unique to the IL-13 receptor or the necessary signalling molecules. An alternative explanation may be attributable to the constitutive expression of the B cell activation antigen CD80 on the surface of KAR B cells. This molecule is absent from MIL, MOT, RAN and MCK B cells (Figure 5.5). It was interesting to note, therefore, that the IL-13-responsive cell population expressed significant levels of CD80, with the mean fluorescence intensity being approximately 2-3 times greater than that of the isotype matched control mAb. This contrasted the other populations investigated where the fluorescence of the cells following incubation with the anti-CD80 mAb was the same as that of cells incubated with an isotype-matched control mAb. It is possible that expression of CD80 endows leukemic $CD5^+$ B cells with the ability to respond to IL-13. That is, the expression of CD80 may coincide with the expression of the necessary components of the IL-13 receptor by leukemic $CD5^+$ B cells. Two recent findings support this notion. Firstly, activation of normal human B cells or B-CLL cells via CD40 induces the expression of CD80 (Ranheim and Kipps, 1993; Yellin *et al.*, 1994). Secondly, IL-4 and IL-13 displayed comparable activities only on leukemic $CD5^+$ B cells activated with anti-CD40. Thus, while IL-4 was capable of affecting the function of unstimulated leukemic $CD5^+$ B cells or those activated through their antigen receptor with anti-IgM antibodies, IL-13 had no effect (Fluckiger *et al.*, 1994a). According to the first finding, the anti-CD40 activated cells described in the latter report would be positive for CD80, and this co-incided with the responsiveness of these cells to various functions of IL-13. This scenario is compatible with that found for KAR B cells (i.e. these cells express CD80 and respond to IL-13), and may explain why the remaining CD80-negative cell populations failed to respond to this cytokine. This is not the first time that the function a cytokine exhibits on normal B cells is absent when

the effects of the same cytokine are assessed on abnormal cells. Using splenic B cells obtained from normal mice, it was found that the level of MHC class II expressed by these cells could be significantly upregulated in the presence of IL-4 or IL-10. However, while the expression of MHC class II on B cells from immunodeficient XID mice could be increased by IL-4, IL-10 had no effect (Go *et al.*, 1990). These authors also suggested that the inability of IL-10 to exert an effect on the abnormal B cells may be a result of a lack of expression of the IL-10 receptor or associated signal transducing molecules.

IL-4 exerts numerous effects on murine and human B cells. Following activation with SAC and IL-4, Epstein Barr virus (EBV) and IL-4, or activated T cell membranes and an IL-4-containing activated T cell supernatant, B cells secreted significant levels of IgM, IgG and IgE (Defrance *et al.*, 1988; Thyphronitis *et al.*, 1991; Hodgkin *et al.*, 1994). By neutralising IL-4 or removing the supernatant, it was found that for IL-4 or the IL-4-containing supernatant to be effective, the cytokine or supernatant had to be present for the first 2-6 days of the culture period (Defrance *et al.*, 1988; Thyphronitis *et al.*, 1991; Hodgkin *et al.*, 1994). Furthermore, no effect (i.e. Ig secretion) was observed if IL-4 or the activated supernatants were added to the B cells later than 2 days following initiation of the culture period (Thyphronitis *et al.*, 1991; Hodgkin *et al.*, 1994). These results indicated that B cells must be exposed to cytokines during the early stages of *in vitro* culture, despite the observation that the maximal effects of IL-4 on Ig secretion did not occur until after 10-14 days. Similar results were obtained in this study when the requirements for the presence of cytokines by the leukemic $CD5^+$ B cells were investigated. This was pursued because the cytokines had very little effect on the kinetics of cell death observed during the first

1-3 days of culture. Depending on the *in vitro* half-life of the different leukemic CD5+ B cells, the addition of IL-2, IL-4, IL-6, IL-13, IFN-γ or TNF-α to the B cells could not be delayed by more than 1-3 days without causing a 50% reduction in the viability-enhancing activity of the particular cytokines (Figure 5.12). Although the kinetics of leukemic CD5+ B cell death proceeded at a similar rate during the first 3 days of *in vitro* culture and independently of the presence of cytokine, it is apparent from the results that these cytokines must be present early if they are to have a significant effect at a later point of the culture period. Similarly, by neutralising IL-2 and IL-4 with specific antisera, a minimum exposure time of 1-2 days was found to be necessary for these cytokines to have a significant effect on the *in vitro* survival of the leukemic CD5+ B cells (Figure 5.14). Thus, for exogenous cytokines to rescue leukemic CD5+ B cells from apoptosis and death, the cells must be exposed to the cytokines for at least 2 days, or the cytokines must be added to the B cells no later than 1-3 days after commencement of the culture period. This window of viability-enhancing activity of particular cytokines on leukemic CD5+ B cells appears to be similar to that for IL-4 in inducing Ig secretion in normal B cells.

Although IL-4 is capable of inducing CLL B cell proliferation, it can also abrogate leukemic B cell proliferation induced by a variety of growth factors, including IL-2 and TNF-α (Karray *et al.*, 1988; Carlsson *et al.*, 1989b; Galanaud *et al.*, 1990; Luo *et al.*, 1991; van Kooten *et al.*, 1992). Coupled with the finding that IL-4 can also prevent leukemic B cell apoptosis, it has been proposed that IL-4 is an important cytokine in the pathogenesis of B-CLL (Sarfati, 1993). This is based on the observation that CLL is characterised by the accumulation of long-lived B cells arrested in the G_0 phase of the cell cycle, a scenario not unlike that

induced by IL-4 *in vitro*. IL-6 can also inhibit TNF-α-induced leukemic B cell proliferation, although to a lesser extent than IL-4 (Aderka *et al.*, 1993; van Kooten *et al.*, 1993b). Based on these results, several authors have suggested that IL-4 and IL-6 may have therapeutic benefit for the treatment of B-CLL (Luo *et al.*, 1991; Aderka *et al.*, 1993). In light of the results described in this chapter, it appears that these cytokines may actually be integral to the development of the malignant clone in B-CLL. Thus, the anti-apoptotic effect of IL-6 may reflect an autocrine role for this cytokine in the development of B-CLL that may be similar to the proposed paracrine role of IL-4 in this disease. IL-4 and IL-6 may therefore mediate the accumulation of leukemic $CD5^+$ B cells *in vivo* by preventing both their proliferation and death, giving rise to a population of cells with a low mitotic index and enhanced *in vivo* longevity; i.e. features characteristic of the malignant clone in B-CLL. This is supported by the finding that serum obtained from B-CLL patients exhibit elevated levels of IL-6, compared to normal healthy controls (Hoffbrand *et al.*, 1993). However, based on the observation that IL-4 is capable of inhibiting the secretion of IL-6 by leukemic B cells (Reittie and Hoffbrand, 1994), it is unknown if these pathways occur concomitantly. Furthermore, neither a synergistic nor an additive effect was observed when IL-4 and IL-6 were combined to co-stimulate populations of leukemic $CD5^+$ B cells that were responsive to these cytokines when tested alone (Figure 5.9a). That is, the enhancement in cell viability caused by IL-4 *plus* IL-6 did not significantly exceed the enhancement caused by either of these cytokines when tested individually. Similarly, IL-2 failed to influence the increase in cell viability mediated by IL-4 and IL-6 of leukemic $CD5^+$ B cells responsive to these three cytokines. Combinations of IL-4, IL-13 and IFN-γ also failed to further enhance the viability of KAR B cells (Figure 5.9a). It remains to be established why at

least an additive effect did not occur in the presence of IL-2 and IL-4, IL-2 and IL-6, IL-4 and IL-6, or IL-4 and IL-13. It may be attributable to the inhibitory or antagonistic effect that IL-4 and IL-6 have been described to exhibit on the proliferation of leukemic $CD5^+$ B cells. This may explain why cell viability in the presence of two cytokines was slightly, yet consistently, less than that in the presence of one cytokine. In fact, the lack of co-operation between IL-4 and IL-2 is consistent with the antagonistic activity of IL-4 on IL-2-induced proliferation of leukemic B cells. Alternatively, the absence of a synergistic or additive effect may indicate that common signalling pathways are utilised by these different cytokines. This was previously suggested in a study that reported the combination of IL-4 and IL-13 did not enhance the levels of IgE and IgG_4 secreted by human peripheral blood mononuclear cells over that induced by either cytokine alone (Punnonen *et al.*, 1993). Taken together, it appears that if any of these cytokines play a role in the *in vivo* accumulation of leukemic $CD5^+$ B cells, the presence of only one cytokine would be sufficient. In contrast to these findings, when TNF-α and IL-6 were combined, the resultant viability of one cell population (MUR) was significantly greater than that achieved by either cytokine alone (Table 5.2). This indicates that an autocrine growth loop may exist which involves TNF-α and IL-6. This may involve TNF-α inducing the secretion of IL-6 by the malignant B cells, an observation previously made by Heslop *et al.* (1990). Unfortunately, because there were insufficient numbers of MUR B cells, further investigations of autocrine loops or the secretion of cytokines by these cells could not be performed. The results describing the absence of a synergistic or additive effect of IL-2, IL-4 or IL-6 on leukemic $CD5^+$ B cell viability are similar to those obtained in the murine system. The viability of resting splenic B cells can be elevated following culture with IL-4 and IL-10, yet the combination of

IL-4 and IL-10 failed to cause a further enhancement in B cell viability (Go *et al.*, 1990).

CLL B cells express relatively high levels of the anti-apoptotic oncoprotein bcl-2. Unlike other B cell malignancies, this usually occurs in CLL independently of any chromosomal rearrangements. Following *in vitro* culture, unstimulated CLL B cells have been reported to lose, down-regulate or maintain bcl-2 expression (Dancescu *et al.*, 1992; Panayiotidis *et al.*, 1993). This discrepency appears to lie in the method of detecting bcl-2 and the length of the *in vitro* culture period. The effect of cytokines on the expression of bcl-2 by *in vitro* cultured CLL B cells has also been examined, although few consistent results have been reported. IL-4 and IFN-α have been reported to have no effect (Chaouchi *et al.*, 1994; Fluckiger *et al.*, 1994b; Mainou-Fowler *et al.*, 1994), to prevent loss of bcl-2 expression (Panayiotidis *et al.*, 1993, 1994) or to upregulate its expression (Dancescu *et al.*, 1992; Jewell *et al.*, 1994). The results in Chapter 4 indicated that in the absence of any stimuli the overall expression of bcl-2 by CLL B cells was reduced, compared to the level of expression prior to culture. This was due to a reduction in bcl-2 expression by a proportion of cells ("bcl-2_{low}") such that the pattern of bcl-2 expression appeared bimodal. However, despite this reduction, the percentage of cells expressing bcl-2 remained constant. Consistent with the data describing the anti-apoptotic effect of various cytokines, it was found that the accumulation of cells with the bcl-2_{low} phenotype was substantially delayed in the presence of the appropriate growth factors (Figure 5.15). The length of time that the cytokines could cause an increase in the percentage of cells with the bcl-2_{high} phenotype, compared to unstimulated cells, was similar to the length of time that the same cytokines were capable of preserving cell viability. This is consistent with

the finding that both the increase in the percentage of cells displaying a bcl-2_{high} phenotype and the increase in cell viability observed following culture with a particular cytokine, compared to unstimulated cells, were similar. Therefore, the cytokines identified here by their ability to delay *in vitro* death and apoptosis of leukemic $CD5^+$ B cells may contribute to the enhanced survival of these cells *in vivo*, and subsequently the pathogenesis of this malignancy, by preserving the high level of bcl-2 expression by these cells. This contrasts some previous reports which proposed that cytokines such as IL-4 and IFN-γ prevent leukemic $CD5^+$ B cell apoptosis by either upregulating bcl-2 expression (Dancescu *et al.*, 1992; Chaouchi *et al.*, 1994; Jewell *et al.*, 1994) or by mechanisms independent of bcl-2 expression (Chaouchi *et al.*, 1994; Fluckiger *et al.*, 1994b). Alternatively, these cytokines may modulate the expression of other molecules involved in the regulation of apoptosis. It was recently found that IL-6 prevented apoptosis of a murine myeloma cell line by upregulating the cellular expression of bcl-x mRNA and bcl-x_L protein (Schwarze and Hawley, 1995). Thus, due to the ability of IL-6 to delay apoptosis in the majority of CLL B cell populations examined, a similar mechanism to that described for murine myeloma cells may be operative in the IL-6-mediated rescue from death and apoptosis of leukemic $CD5^+$ B cells.

The description of IL-2, IL-6, IL-13 and TNF-α as factors capable of preventing apoptosis of leukemic $CD5^+$ B cells is consistent with some of the functions that these cytokines display on various cell types. IL-2 has been found to prevent ionomycin-induced B cell apoptosis (Vasquez *et al.*, 1991) and to protect T cell lines from glucocorticoid-induced programmed cell death (Fernandez-Ruiz *et al.*, 1989; Nieto and Lopez-Rivas, 1989). Furthermore, IL-2 was found to induce transcription of

bcl-2 mRNA, and subsequently prevent apoptosis, in an IL-2-dependent cytotoxic T cell line (Deng and Podack, 1993). IL-6 has been described as being a growth factor for, or associated with, the mature B cell malignancies myeloma and plasma cell leukemia, and also to prevent apoptosis of myeloma cell lines (Kawano *et al.*, 1988; Freeman *et al.*, 1989; Hardin *et al.*, 1994; Westendorf *et al.*, 1994; Klein *et al.*, 1995, Lichtenstein *et al.*, 1995). Consequently, as proposed for IL-4, it is possible that IL-2, IL-6, IL-13 and TNF-α act primarily by delivering a survival signal to different cells prior to their subsequent activation/progression. This is supported by the finding here that although the leukemic CD5+ B cells did not undergo proliferation in the presence of cytokines alone, co-stimulation with anti-μ or PMA resulted in significant uptake of ^{3}H-thymidine. Furthermore, most of these cytokines have been reported to be capable of inducing proliferation of leukemic CD5+ B cells co-stimulated with various reagents. Thus, the pathogenesis of B-CLL, following the action of particular cytokines, may involve the appearance of a population of cells with increased longevity which may undergo expansion following subsequent co-stimulation. This may be in the form of sIg ligation or PKC activation that may occur in the presence of endogenous or exogenous antigens capable of sufficiently cross-linking the polyreactive IgM and/or IgD expressed on the surface of leukemic CD5+ B cells.

The results described here for the different leukemic CD5+ B cell populations are similar to those recently described for human myeloma cell lines. The growth and survival of several of these cell lines have been found be dependent on the presence of IL-6 (Schwabe *et al.*, 1994; Borset *et al.*, 1994; Westendorf *et al.*, 1994). Interestingly, one of these cell lines also proliferated in response to TNF-α and co-stimulation with TNF-α

and IL-6 induced a significant synergistic proliferative response (Borset *et al.*, 1994). Furthermore, IL-6 was found to prevent apoptosis of myeloma cell lines induced by dexamethasone or serum starvation (Hardin *et al.*, 1994; Lichtenstein *et al.*, 1995). Collectively, these data reinforce the notion that IL-6 is central to the development of plasma cell malignancies. Because the majority of myeloma cells or cell lines secrete IL-6 this cytokine is likely to act in an autocrine fashion (Kawano *et al.*, 1988; Freeman *et al.*, 1989; Borset *et al.*, 1994; Hardin *et al.*, 1994; Westendorf *et al.*, 1994). Taken with the results presented here, it appears that malignant B cells of B-CLL and myeloma may have similar requirements for growth and survival both *in vivo* and *in vitro.*

In addition to IL-4 and IFN-γ, IFN-α has previously been reported to prevent leukemic B cell apoptosis (Chaouchi *et al.*, 1994; Panayiotidis *et al.*, 1994). These *in vitro* findings contrast results obtained from clinical trials investigating the efficacy of IFN-α2b as a treatment for B-CLL. Infusion of B-CLL patients with IFN-α2b significantly reduced the numbers of peripheral blood leukemic B cells, as well as the serum levels of TNF-α (Morabito *et al.*, 1993; Chaouchi *et al.*, 1994). These *in vivo* data are supported by the finding that IFN-α diminished the levels of TNF-α, IL-6 and IL-1β mRNA induced in leukemic B cells following *in vitro* stimulation with exogenous TNF-α (Heslop *et al.*, 1990). Furthermore, IFN-α abrogated the TNF-α-induced proliferation of one population of leukemic B cells (Cordingley *et al.*, 1988). Based on these results, it was concluded that the clinical efficacy of IFN-α may be due to its ability to interrupt an autocrine growth loop involving TNF-α and IL-6. The results presented in this chapter are compatible with this and suggest that treatment with IFN-α may reduce the tumor load by inducing apoptosis of leukemic $CD5^+$ B cells by limiting the production, and

therefore the serum levels, of TNF-α and IL-6. These cytokines were found to enhance *in vitro* viability of, and delay apoptosis in, leukemic $CD5^+$ B cells. In keeping with the suggestion that CLL B cells and myeloma cells have similar growth requirements it is interesting to note that IFN-α is also capable of preventing growth and proliferation of U266 myeloma cells. IFN-α was found to disrupt the autocrine IL-6 growth loop for this cell line by decreasing the expression of both α- and β-chains of the IL-6 receptor (Schwabe *et al.*, 1994). Thus, in B cell malignancies, IFN-α appears capable of limiting essential components of autocrine growth loops, either by inhibiting production of the growth factor or reducing cell surface expression of the appropriate receptor.

As well as preventing apoptosis, numerous agents have been identified that are capable of increasing the rate of leukemic B cell apoptosis. One of these is IL-10 (Fluckiger *et al.*, 1994b). In this chapter, culture with IL-10 had no consistent effect on apoptosis of the six CLL samples tested. However, it was of interest to note that supplementing complete medium with goat anti-IL-10 antiserum resulted in a modest, but significant, increase in the number of viable cells recovered, compared to cells cultured with complete medium alone (Table 5.3). This suggests that IL-10, either present in the culture medium or spontaneously secreted by the leukemic $CD5^+$ B cells, may be cytotoxic to CLL B cells. Furthermore, the true effect of exogenous IL-10 on leukemic $CD5^+$ B cell viability may be masked by the effects of this endogenous source of IL-10. Further clarification of the source of this IL-10 is required. However, despite the differences in the results obtained in this chapter and those by Fluckiger *et al.* (1994b) regarding the apoptotic activity of IL-10 on leukemic $CD5^+$ B cells, these data support their proposal that IL-10 should be considered as a means of treating B-CLL. The rationale for this is based on the

capacity of IL-10 to inhibit the synthesis of IL-2, IL-4, IL-6, TNF-α and IFN-γ by human T cells, T cell clones and macrophages (de Waal Malefyt *et al.*, 1991, 1993b; Moore *et al.*, 1993). The potential therapeutic efficacy of IL-10 would, therefore, not only be due to its direct effect on leukemic CD5+ B cells, but also to its capacity to reduce the *in vivo* serum levels of particular cytokines described in this chapter to delay or prevent the *in vitro* apoptosis of leukemic CD5+ B cells. This proposal is strongly supported by two recent findings. Firstly, a correlation has been found to exist between the levels of serum TNF-α and the numbers of circulating monocytes in CLL patients, with both of these parameters continuing to increase with disease severity (Adami *et al.*, 1994). Thus, the IL-10-mediated reduction in secretion of TNF-α by monocytes may assist in the management of patients with advanced/end-stage B-CLL. Secondly, it was found that the serum levels of IFN-γ, TNF-α and IL-6 were significantly increased in mice that had been depleted of endogenous IL-10 following the administration of anti-IL-10 antibodies (Ishida *et al.*, 1992, 1993). Interestingly, these cytokines have been found to be present at elevated levels in serum from B-CLL patients (Foa *et al.*, 1990; Hoffbrand *et al.*, 1993; Buschle *et al.*, 1993; Adami *et al.*, 1994). Thus, the clinical efficacy of IL-10 as a potential therapeutic agent for human B-CLL warrants further investigation.

The apoptotic effect of IL-10, reported by Fluckiger *et al.* (1994b) is intriguing given the suggestion that IL-10 may have a role in the development of CD5+ B cells. In mice, CD5+ B cells have been identified as the major source of IL-10 (O'Garra *et al.*, 1992) and thses cells are depleted from the peritoneal cavity of mice treated with anti-IL-10 mAbs immediately after birth (Ishida *et al.*, 1992). This appeared to be an indirect effect because the treated mice displayed increased levels of

serum IFN-γ and co-administration of antibodies to IL-10 and IFN-γ resulted in normal levels of $CD5^+$ B cells (Ishida *et al.*, 1992). Furthermore, IL-10 has been found to enhance the viability of murine splenic B cells (Go *et al.*, 1990) and human germinal centre B cells (Levy and Brouet, 1994). Human $CD5^+$ and $CD5^-$ B cells secrete IL-10 following *in vitro* activation, yet expression of IL-10 mRNA and protein in resting or activated leukemic $CD5^+$ B cells was minimal (Burdin *et al.*, 1993; Finke *et al.*, 1993; Matthes *et al.*, 1993). This difference in IL-10 function in humans and mice, as well as its ability to selectively kill leukemic $CD5^+$ B cells, may reflect a perturbation in cytokine secretion due to the malignant transformation. That is, normal human $CD5^+$ B cells may be capable of secreting IL-10 and its cytotoxicity may be a mechanism which prevents the expansion of this population of B cells. However, following transformation to the malignant state, the expression of IL-10 is down-regulated, thus allowing for the clonal expansion of $CD5^+$ B cells.

The results described in this chapter support the proposal that autocrine (CLL B cell-derived TNF-α, IL-6, IFN-γ) as well as paracrine (T cell- or monocyte-derived IL-2, IL-4, IL-6, IL-13, IFN-γ, TNF-α) growth factors, and the subsequent growth loops established by interactions of such cytokines, may play an active role in the progressive accumulation and enhanced survival of leukemic $CD5^+$ B cells in B-CLL. Because of the pleiotropic effects of most cytokines, it will be necessary to assay the effects on viability of leukemic B cells of as many different cytokines as possible. With this in mind, it will be of interest to determine whether or not a role in B-CLL exists for IL-15, a recently described cytokine that displays many of the biological properties of IL-2 (Grabstein *et al.*, 1994; Armitage *et al.*, 1995). This would be particularly interesting,

considering the significant differences on the viability and apoptosis of leukemic $CD5^+$ B cells that were observed for IL-4 and IL-13, two cytokines that display comparable effects on normal resting and activated human B cells (Zurawski and de Vries, 1994).

Summary and Conclusions

The causes of the unregulated accumulation or proliferation of malignant B cells in leukemia are largely unknown. In B-CLL, the leukemic B cells are developmentally arrested in the G_0 phase of the cell cycle. Additionally, these cells display extended *in vivo* survival as well as elevated levels of the anti-apoptotic oncoprotein bcl-2. In contrast to the *in vivo* cell cycle arrest, leukemic $CD5^+$ B cells can be induced to proliferate *in vitro* following receipt of a variety of signals. As demonstrated in Chapter 3, these signals can be derived from mitogen-activated T cells, from activation of PKC following stimulation with PMA, or by the co-stimulus of crosslinking sIg molecules with $F(ab')_2$ anti-μ and PMA. The responses to PMA were significantly augmented when PMA was used in combination with several cytokines including IL-2, IL-4, IL-6, IL-13, IFN-γ and TNF-α. However, when used alone, these cytokines failed to induce a proliferative response. The T cell-derived signals are likely to be mediated via expression of the CD40L and its subsequent interaction between this and CD40 expressed on the leukemic B cell surface, as has been described for normal human B cells (Clark and Ledbetter, 1994).

The physiological relevance of leukemic B cell proliferation *in vitro* is unknown since leukemic B cells present in the peripheral blood of B-CLL patients are largely in a non-dividing state. Interestingly, *in vitro* culture with PMA or activated T cells provided leukemic B cells with a survival signal. These activation signals have also been reported to prevent apoptosis of normal resting B cells (Liu *et al.*, 1989; Illera *et al.*, 1993). Such survival signals may be important in the pathogenesis of B-CLL, resulting in the enhanced survival of the leukemic B cells, as observed *in vivo*, rather than inducing their proliferation.

It is noteworthy that leukemic B cells can be activated via their sIg because this antigen receptor is polyreactive, being capable of binding multiple structurally unrelated antigens both of endogenous and exogenous origin (Casali and Notkins, 1989). While the *in vivo* interaction with self antigens has not been described, it is tempting to speculate that chronic stimulation of $CD5^+$ B cells with sufficient auto-antigen may be involved in the pathogenesis of B-CLL. *In vivo* activation of leukemic B cells, independently of cell division, does appear to occur as evidenced by the frequent expression of activation antigens, such as CD23, CD25, CD71 and CD80.

Leukemic $CD5^+$ B cells rapidly die following *in vitro* culture. As reported in Chapter 4, cell death was mediated by apoptosis as assessed by the characteristic reduction in cell size, loss of membrane integrity and DNA fragmentation. Particularly interesting was the observation that apoptosis of leukemic $CD5^+$ B cells was associated with, and appeared to correlate with, the down-regulation of expression of bcl-2. However, while down-regulation of bcl-2 was observed, significant levels of bcl-2 continued to be detectable in non-viable, apoptotic leukemic B cells. This suggests that additional mechanisms may be involved in the regulation of leukemic B cell apoptosis which can override the protective effect of bcl-2. Alternatively, a constitutively high level of bcl-2 expression is required to protect leukemic $CD5^+$ B cells from apoptosis. This minimal level of expression would presumably be that observed prior to *in vitro* culture, i.e. the $bcl\text{-}2_{high}$ phenotype. The phenomenon of spontaneous apoptosis of leukemic B cells *in vitro* contrasts their enhanced survival *in vivo* and suggests that apoptotic-inhibitory factors may have a role in the accumulation of these cells *in vivo*. These apoptotic-inhibitory factors appear to be absent from the *in vitro* culture systems used in this and

other studies. Consequently, identification of such factors may provide an enhanced understanding of the mechanisms involved in the development of this malignancy.

In Chapter 5, IL-2, IL-4, IL-6, IL-13, TNF-α and IFN-γ were identified as factors capable of delaying death and apoptosis of leukemic $CD5^+$ B cells. In the presence of these cytokines, several *in vivo* characteristics of leukemic $CD5^+$ B cells were maintained. Firstly, because the cytokines failed to induce a significant proliferative response, the cells remained in the G_0 phase of the cell cycle. Secondly, due to the ability of the cytokines to delay apoptosis, the *in vitro* viability of cytokine-stimulated leukemic B cells was significanlty enhanced. Thirdly, as the cytokines delayed the appearance of cells displaying a bcl-2_{low} phenotype, a larger proportion of cells continued to express bcl-2 at a level similar to that observed in leukemic B cells prior to *in vitro* culture; i.e. at the level assumed to be expressed by the B cells *in vivo*. The role of these cytokines *in vivo* needs to be addressed since *in vitro* data presented in this thesis suggests a potential role for various cytokines in the accumulation of these cells *in vivo*. This is supported by the finding that the serum of CLL patients contains elevated levels of IL-6, TNF-α and IFN-γ and that these cytokines can be secreted by leukemic $CD5^+$ B cells *in vitro*. Furthermore, the cytokines identified as possessing apoptotic-inhibitory activity (IL-2, IL-4, IL-6, IL-13, TNF-α and IFN-γ) are also products of activated T cells, strengthening the suggestion for a role of residual T cells in the development of B-CLL. Curiously, these cytokines may also be involved in a proliferative event in B-CLL because the majority were capable of enhancing PMA-induced proliferation of different populations of leukemic $CD5^+$ B cells *in vitro*. Thus, B-CLL may be the manifestation of unregulated production of cytokines, linked with chronic stimulation

by auto-antigen. Because leukemic B cells and normal residual T cells can secrete various cytokines, autocrine and/or paracrine growth loops may be central to the pathogenesis of B-CLL. These growth factors may operate on several levels such as through initiation of cell proliferation in the presence of a co-stimulus (Chapter 3); through inhibition of proliferation and thereby maintaining the cells in G_0; or by preventing apoptosis and preserving expression of bcl-2 (Chapter 5). Finally, the disruption of cytokine networks *in vivo* may be used to augment existing therapies for treatment of B-CLL. For example, IL-10 and IFN-α2b may facilitate leukemic CD5+ B cell death *in vivo* by inhibiting the secretion of cytokines known to prevent apoptosis. This phenomenon may be exploited in cytokine-based therapies of B cell malignancies.

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