Growth regulation of human B lymphocyte progenitor cells

by

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Synopsis

This thesis describes an investigation into the growth characteristics of a human pre-B cell acute lymphocytic leukaemia cell line, SMS-SB. Most lymphocytic tumours are difficult to adapt to tissue culture and enter a crisis after a few rounds of cell division where the majority of cells die. To sustain proliferation of the remaining cells, addition of exogenous mitogens is usually required. SMS-SB was an unusual leukaemia because the cells did not go through a crisis phase and grew indefinitely, in the absence of exogenous mitogens. This sustained proliferation in tissue culture appears to reflect the synthesis and secretion of an autocrine growth factor(s); the cells are density-dependent for growth, and proliferation can occur in media completely devoid of protein.

The original aim of this work was to identify and characterise the autocrine growth factor, termed SB-AF. During investigations to identify cytokines with the ability to substitute for the autocrine growth factor activity, platelet-derived growth factor (PDGF) was shown to stimulate the growth of SMS-SB cells under low cell density conditions; SMS-SB cells are known to secrete PDGF and express PDGF receptors. However, antibody inhibition experiments suggest that PDGF cannot account for all the autocrine activity of SB-AF, thus other cytokine components of SB-AF were sought.

CD23 is a 45kDa type-II transmembrane glycoprotein and a member of the C-type lectin superfamily. There is a soluble form of CD23 (sCD23) which is released by cleavage from the surface of cells into the extracellular fluid, and this form has been attributed multiple cytokine activities. It was discovered that sCD23 dramatically promotes thymidine incorporation by SMS-SB cells.

The work of this thesis has shown that SMS-SB cells undergo apoptosis when cultured at low cell density; sCD23 is the only cytokine tested with the ability to prevent SMS-SB cell apoptosis. Apoptotic SMS-SB cells have low levels of the proto-oncogene *bcl-2* but sCD23 can sustain *bcl-2* levels in the cells.

The investigations have shown that SMS-SB cells do not express CD23, negating the hypothesis that CD23 is acting in an autocrine fashion. The most interesting discovery made during these investigations was that SMS-SB cells bind CD23-containing liposomes

specifically but they do not express the known receptors for CD23, namely CD21, CD11a and CD11b; SMS-SB cells express a novel CD23 receptor.

Thus, SMS-SB cells express a novel receptor for CD23 and signalling via this receptor prevent apoptosis of the cells. Preliminary data is presented from CD23 affinity columns used to isolate and characterise the novel CD23 receptor. A protein of 85kDa has been identified as a candidate receptor, but further characterisation is required.

SMS-SB cells will provide a good model to examine the role of autocrine growth factors in early B cell development, moreover, the discovery of a novel CD23 receptor on pre-B cells, implies a role for sCD23 in early B cell development. Since sCD23 has previously been shown to promote the growth and maturation of early T cells and myeloid progenitors, it will be interesting to investigate the role of CD23, and the novel receptor, in all aspects of haematopoiesis.

for my family, and Iain; thanks for everything

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Declaration

The work described in this thesis was performed personally unless otherwise acknowledged.

Abbreviations

ALL	acute lymphocytic leukaemia
BCGF	B cell growth factor
BSA	bovine serum albumin
CLL	chronic lymphocytic leukaemia
CM	conditioned medium
CSF	colony stimulating factor
DEPC	diethyl pyrocarbonate
DiOC18	3, 3' - Dioctadecyloxacarbocyanine percholate
EBNA2	Epstein-Barr virus nuclear antigen 2
EBV	Epstein-Barr virus
ECL	enhanced chemilluminescence
EDTA	ethylenediaminetetra-acetic acid
FACS	fluorescence-activated cell sorter
FcR	receptor for the Fc region of the Ig molecule
FCS	foetal calf serum
FDC	follicular dendritic cell
FITC	fluorescein-isothiocyanate
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
HSC	haematopoietic stem cell
IFN	interferon
Ig	immunoglobulin
IL	interleukin
kDa	kilodalton
LTBMC	long-term bone marrow culture
MAb	monoclonal antibody
MOPS	4-morpholinpropanesulfonic acid
OGP	n-Octyl-β-D-glucopyranoside
PBS	phosphate buffered saline
PDGF	platelet-derived growth factor
PE	phycoerythrin
PFHMII	Protein Free Hybridoma Medium-II
POPC	1-Palmitoyl-2-oleoyl-phosphatidyl choline
RT-PCR	reverse transcriptase - polymerase chain reaction
SB-AF	SMS-SB cell autocrine growth factor
sCD23	soluble CD23 (25kDa form)
TdT	terminal deoxynucleotidyl transferase
[^{3}H]-TdR	tritiated thymidine
TGF-LD	transforming growth factor - leukaemia derived

TH	T helper cell
TLCK	Nα-*p*-tosyl-L-lysine chloromethyl ketone
TNF	Tumour necrosis factor
U	Unit of specific activity
v/v	volume by volume
w/v	weight by volume

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Chapter 1

Introduction

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Introduction

1.1 *The Immune System*

The homeostatic basis of every biological system is the absolute necessity for tightly regulated cell growth, differentiation and death. For organs, tissues and systems it is implicit that the growth of individual cells is tightly controlled and balanced by levels of cell death. If the control becomes deregulated, or an individual cell escapes from the regulation, there may be abnormal growth leading to the formation of a tumour.

The immune system is a highly complex, integrated network of cells and soluble factors which cooperate to protect the body against invasion by pathogens. Aquired immunity is induced or stimulated by exposure to foreign antigen; it is highly specific, and is able to discriminate between self and non-self. Since intercellular communication and growth regulation in the immune system are so complex, the results of inappropriate cell activation or proliferation can be disasterous, leading to the development of allergy, autoimmunity or malignancies such as lymphomas and leukaemias. Studying the mechanisms of malignant transformation *in vitro* may provide insight into the processes which regulate normal cell proliferation and differentiation, and possibly lead to rationale for cancer therapy.

Of the many cell types involved in mediating specific immune responses, T cells are recognised by many as being the most important since they can play both a cytotoxic and a helper role in destruction of pathogen-infected cells. However, the B lymphocyte may have a more important role in development of an immune response than many wish to believe. B lymphocytes express membrane immunoglobulin (Ig) on their surface and are activated when the Ig binds its specific antigen. However, to develop into plasma cells which secrete antibody, the B cells usually require T cell help, in the form of secreted interleukins and cell-cell surface interactions.

Antibodies are mediators of the humoral immune response since they bind to specific antigens and aid in their elimination. The protective biologic effects of antibodies are numerous and include: neutralisation of soluble antigen (viruses and bacteria); activation of the classical complement pathway; opsonization of particulate matter to allow enhanced phagocytosis; and antibody-dependent cell-mediated cytotoxicity (ADCC). However, the B cell itself can play another important role in an immune response - that of an antigen-presenting cell which acts to activate T-helper cells. Antigen-specific B cells are highly efficient at presenting their cognate antigen because membrane Ig molecules function as

high-affinity receptors which effectively internalise and present antigen, even at very low concentrations. This interaction, as well as activating a T cell response, ensures that the antigen-specific B cell is the preferential recipient of T cell-derived cytokines and the direct cell-cell contacts which provide additional signals to the B cell allowing a humoral response to develop.

Thus, the B lymphocyte plays a central role in the immune system, and its development is a subject of extensive investigation since there are numerous pathological conditions known to occur if this development becomes uncontrolled.

1.2 *B Cell Development*

Lymphoid, as well as erythroid and myeloid, lineages of haematopoietic cells are descendants of pluripotential, self-renewable $CD34^+$ haematopoietic stem cells (HSC) in the bone marrow (Sutherland *et al.*, 1993). From the HSC it appears that the next stage of B cell development is generation of a common, bipotential, lymphoid-committed precursor which can give rise to T and B lymphocytes (Ikuta *et al.*, 1992). The exact point at which commitment to the lymphoid lineage occurs is, at present, unknown, however, discovery of genes, for example, Ikaros, which can prevent lymphoid development if inactivated, will allow clarification of this early developmental stage (Georgopoulos *et al.*, 1994).

Later stages of development have been resolved based on phenotypic analyses, demonstrating that B lymphocyte growth and differentiation is a multi-stage process which involves the collaboration of numerous cell types and cytokines. B cell differentiation can be divided into two main phases: the antigen-independent and the antigen-dependent phase (figure 1.1). The first phase involves development of the haematopoietic stem cell into an immature surface IgM-positive B cell - it is independent of antigen involvement and occurs in the bone marrow. The second phase involves negative selection of B cells which express a receptor with specificity for self antigen and maturation of cells which are specific for non-self (Tarlinton, 1994). In the periphery and secondary lymphoid tissue, such as lymph node, the cells mature to become surface IgM^+/IgD^+ and, if they contact specific antigen, may become activated to Ab-producing plasma cells or memory B cells (reviewed by Rolink & Melchers, 1991). This antigen-dependent phase of development is also regulated by T cell-derived cytokines, for example, IL-2, IL-4, IL-6, IL-10, IL-13, IL-15 etc.

The focus of this thesis will be the antigen-independent phase of B cell differentiation.

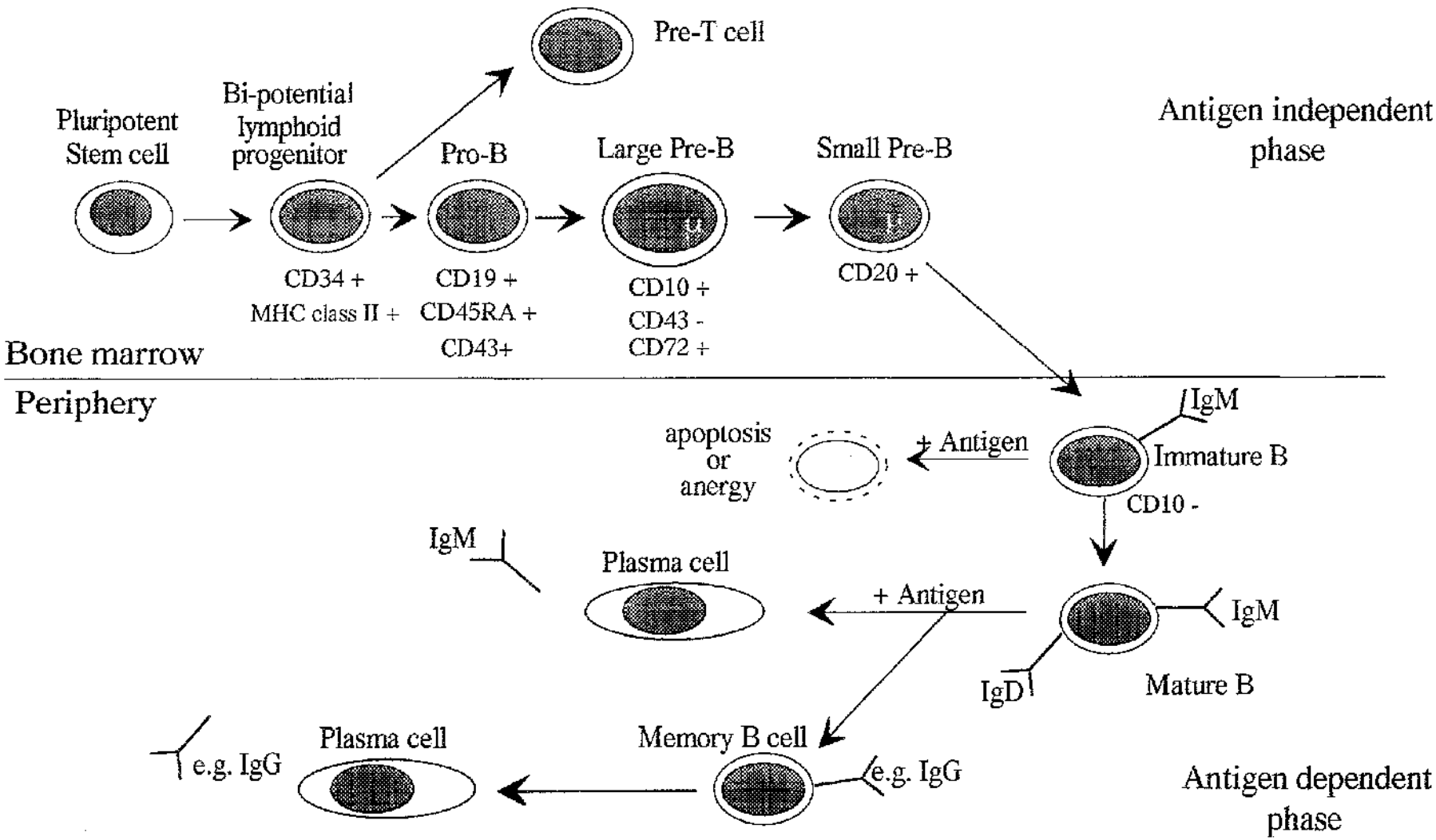


Fig.1.1 Describes B lymphocyte development; the antigen independent and dependent phases. +/- represents major antigens gained or lost at each stage; μ represents Ig μ heavy chains.

1.3.1 *Antigen - independent B cell differentiation*

The initial phase of B cell development occurs in the bone marrow - an environment which consists of various types of stromal cells including fibroblasts, macrophages, adipocytes, reticulocytes, adventitial and endothelial cells (Dorshkind & Landreth., 1992). The influence of stromal cells is mandatory for B cell development both via production of cytokines and through cell-cell contacts. The stages of early B cell differentiation are carefully ordered and can be defined by phenotypic changes, including the status of Ig gene rearrangement.

1.3.2 *Phenotypic changes in B cell development.*

Hardy and colleagues, 1991, developed a scheme to compartmentalise murine B cell precursors into five distinct fractions based on their expression of various surface molecules; these categories are also used to describe human B cell precursors (Hardy *et al.*, 1991; Tarlinton, 1994). The first committed B cell precursors are referred to as pro-B cells. They express cell surface CD45RA (B220) and leukosialin (CD43) but have no other B cell specific markers and have not yet begun immunoglobulin (Ig) gene rearrangement. Pre-B cells express cytoplasmic μ chains and also B cell specific markers such as CD10, CD24, CD19, CD20, CD45, CD72 (Schwarting *et al.*, 1992; Duperray *et al.*, 1990), BP-1 and Heat stable antigen (HSA) but have lost expression of CD43 (Hardy, 1991). They also begin to express major histocompatibility complex (MHC) class II (Ia) antigens (Tarlinton, 1994). Both pro-B and large pre-B cells are highly proliferative and, by definition, lack surface immunoglobulin. Expression of CD20 at the small pre-B stage coincides with cessation of cell division, exit from the bone marrow, and maturation towards a mature B-cell phenotype (Salvaris *et al.*, 1992), with a concomitant decrease in expression of surface antigens such as CD10 and CD24 (Duperray *et al.*, 1990).

1.3.3 *Ig gene rearrangement determines the stage of B cell differentiation*

The early stages of B cell development involve production of Ig heavy and light chains which can be assembled as the B cell antigen receptor - surface Ig.

The Ig heavy and light chains are composed of multiple gene segments which undergo a precise sequence of somatic recombination to create the variable and constant regions of the molecule. The loci which compose heavy chains include a variable (V_H) region as well as diversity (D_H) and joining (J_H) regions, all of which encode the antigen binding site, and constant (C) regions. Light chains are encoded by a combination of V_L, J_L and C_L loci and so have less variability than heavy chains. The first recombination events occur in the heavy chain genes by joining of a D_H to a J_H locus. At this stage the precursors express the nuclear enzyme terminal deoxynucleotidyl transferase (TdT) which is involved in N-region addition and hence generation of additional diversity during gene rearrangement. Subsequently, one of the many V_H region genes is juxtaposed to the rearranged D_HJ_H, the

rearranged heavy chain gene is transcribed and the remaining non-coding sequences between the VDJ_H and the Cμ exons removed by post-transcriptional processing. The result is formation of a V_H-D_H-J_H-Cμ transcript which is translated into heavy chain protein (Rolink & Melchers, 1993).

B cells appear to be able to detect if the Ig gene locus is productively rearranged by displaying the Ig heavy chain on the cell surface. If productive, the heavy chain is postulated to bind to a ligand in the stromal cell environment and signal the cell to proceed to the next stage of rearrangement. At first, this explanation appeared unlikely since heavy chains were known to require assembly with light chains before they could be expressed on the cell surface (McCubrey *et al.*, 1985). The problem was solved after the discovery of two genes, V_{preB} and λ_5. These encode proteins able to associate non-covalently to form, what is now known as, a surrogate light chain (Sakaguchi & Melchers, 1986; Kudo & Melchers, 1987). These genes appear to be expressed exclusively in B cells, from the pro-B cell stage through to immature mIg^+ B cells in the bone marrow (Melchers *et al.*, 1993), with the λ_5 protein able to bind covalently to $D_HJ_HC\mu$ proteins and to fully rearranged heavy chains, allowing their expression on the cell surface (Karasuyama *et al.*, 1990). It is still not clear exactly what the μ-V_{preB}-λ_5 complex binds to in the bone marrow, but it functions in the selection and amplification of pre-B cells with functionally rearranged Ig heavy chain genes and its expression is obligatory for conventional (B-2) B cell differentiation to occur (Ehlich *et al.*, 1993). After successful heavy chain rearrangement, cytoplasmic μ chains are found in the cell and it is said to have differentiated to the pre-B cell stage. Successful expression of heavy chain protein with V_{preB} - λ_5 prevents further heavy chain rearrangement (allelic exclusion) and is thought to signal the initiation of light chain gene rearrangements (Tsubata, 1992). However, Ehlich *et al.*, 1993, have evidence that light chain rearrangment can occur at the pre-B cell stage and without prior rearrangement of heavy chain genes. There are also data to suggest that light chain rearrangement can occur before that of heavy chains in human EBV-transformed pre-B cells (Kubagawa *et al.*, 1989).

The kappa light chain is the most common light chain found in humans, with rearrangement at the λ locus occuring only if κ rearrangements fail to generate a functional light chain (Tarlinton, 1994). After formation of a V_L-J_L-Cκ/λ transcript and its translation to light chain protein, rearrangement of the remaining alleles is halted and the IgM molecule is assembled on the cell surface marking the transition from a non-dividing small pre-B cell to an immature B lymphocyte. The immature B lymphocyte, with its clonotypic receptor (Burnet, 1959), then leaves the bone marrow for the periphery where, as a $mIgM^+/IgD^+$ B cell, antigen is essential for further differentiation.

1.3.4 CD5-positive B cells

CD5 is a 67kD glycoprotein whose murine homologue, Ly-1, was originally discovered on T cells and thought to be expressed exclusively by $CD4^+$ T helper cells (Cantor & Boyse, 1977). However, later studies in humans reported CD5 expression on all normal T cells (Ledbetter *et al.*, 1981), on some B cell tumours (especially chronic lymphocytic leukaemia (B-CLL) (Martin *et al.*, 1981) and, as in the mouse, on a small proportion of normal B cells (Calligaris-Cappio *et al.*, 1982). It has now been established that CD5 is expressed by a sub-population of B cells, termed B-1a cells, that express normal B cell markers such as CD19, CD20, CD21 and class II MHC, but are CD23-, have high levels of mIgM and dull to moderate levels of mIgD (Herzenberg *et al.*, 1986; Gadol & Ault, 1986). These cells may represent the normal cellular counterparts of B-CLL cells (Calligaris-Cappio *et al.*, 1982). In the mouse, Ly-1 cells can be divided into two sister populations - B-1a ($CD5^+$) and B-1b ($CD5^-$) compared to conventional (or B-2) B cells (Kantor, 1991), and a similar $CD5^-$ population, which has all the functional characteristics of $CD5^+$ cells, has been described in human peripheral blood B cells (Kasaian *et al.*, 1992).

B-1 cells can be distinguished from conventional B cells by patterns of gene expression, anatomical localisation and functional characteristics. The phenotype of these cells has already been mentioned but, in addition, B-1 cells tend to use a restricted set of V_H genes (Pennell *et al.*, 1988). Several of these V_H families, including V_H5, appear to be overexpressed in B-CLL (Humphries *et al*, 1988). Another distinguishing characteristic of B-1 cells is their ability to self-replenish, unlike conventional B cells (Kantor, 1991). $CD5^+$ cells are responsive to numerous cytokines including IL-2 and especially IL-5, and may themselves produce IL-10 (Calligaris-Cappio *et al.*, 1989; Tominaga *et al.*, 1991; O'Garra & Howard, 1992). IL-10 has been implicated in the development of $CD5^+$ B cells and is thought to have an autocrine growth factor function. In mice, administration of anti-IL-10 antibody depletes the $CD5^+$ B cell population but not the conventional B cell population (Ishida *et al.*, 1992).

Early experiments showed that CD5+ B cells produce auto- and polyreactive antibodies (Broker *et al.*, 1988; Sthoeger *et al.*, 1989). Indeed, this population in man appears to be responsible for production of the majority of anti-Fcγ and anti-Fcμ antibodies, (Hardy *et al.*, 1987) and for production of IgM which reacts with low avidity with a variety of self- and non-self antigens (MacKenzie *et al.*, 1991).

The $CD5^+$ population is prominent early in ontogeny and this is thought to mimic phylogeny since $CD5^+$ cells produce these 'natural antibodies' - polyreactive, and often autoreactive, antibodies of the IgM isotype (Bofill *et al.*, 1985).

LOCALISATION: B-1 cells represent approximately 1% of the total B cell population in mice and they are mainly localised in the peritoneal cavity with some in the marginal zones of the spleen (Herzenberg *et al.*, 1986). In humans, the first B cells to appear in the developing lymph nodes are virtually 100% $CD5^+$ (Bofill *et al.*, 1985) but the numbers decrease with age until, in the adult, only 5-30% of the circulating B cells are $CD5^+$. Less than 30% of lymph node B cells are $CD5^+$ and these are largely found at the periphery of germinal centres (Lydyard *et al.*, 1993).

There are contrasting views as to whether B-1 cells actually represent a distinct lineage of B cells or whether CD5 is in fact an activation marker. That the B-1 cells represent a separate lineage of cells is supported by the finding that hematopoetic stem cells (HSC) from foetal or neonatal liver will repopulate the B-1 and conventional B-2 cell compartments of irradiated SCID mice, but HSC transferred from adult bone marrow will only repopulate the B-2 compartment (Hardy *et al.*, 1986). Thus, progenitors of B-1 cells are abundant in foetal liver but are rare in adult bone marrow. Also, if mice are irradiated, only those repopulated with peritoneal cells in addition to bone marrow have B-1 cells (Herzenberg *et al.*, 1986). Further evidence for a separate B-1 lineage comes from the λ_5 knockout mouse which has perturbation of B-2 cell development but apparently normal B-1 cell development (Kitamura *et al.*, 1992), and from PAX-5 knockout mice which lack CD5+ B cells (Urbanek et al., 1994). It is possible that this observation can be explained by the ability of 'surviving' B-1 cells to self-replenish but it may also be argued that SCID and knockout mice provide an alien environment for B cell development.

The argument that $CD5^+$ B cells represent an activated subpopulation is supported by reports that human $CD5^-$ B cells can be induced to become $CD5^+$ *in vitro* by activation with phorbol esters, and several cytokines modulate expression of the molecule (Paavonen *et al.*, 1990). In the mouse, stimulation of splenic B cells with anti-IgM and IL-6 induces expression of CD5 and down-regulates CD23 (Kantor, 1991; Ying-zi *et al.*, 1991). Activation of human B cells by EBV has the opposite effect and causes down-regulation of surface CD5 (Van de Velde *et al.*, 1991). IL-4 also reduces the expression of CD5 on normal and malignant B cells (Freedman *et al.*, 1989) which suggests that CD23 and CD5 undergo reciprocal regulation. However, co-expression of CD5 and CD23 can occur in humans and, indeed, is used to diagnose B-CLL cells (Delespesse, 1992). This is of interest since the work of Van de Velde and colleagues, 1991 demonstrated that CD72, a member of the same superfamily as CD23, is the receptor for CD5. The CD5/CD72 interaction is thought to aid B/T cell interaction although $CD5^+$ B cells may also interact with other B cells. It is important to note that $CD5^+$ B cells themselves express very high levels of CD72 (Lydyard *et al.*, 1993).

1.3.4-2 CD72 structure and proposed functions

CD72 is a B cell marker found to be expressed at the very earliest (pro-B) stages of B cell development and lost after terminal differentiation to plasma cells (Gordon, 1994). It is highly B cell restricted but there are reports of expression on some macrophages, FDCs and some epithelial cells. In germinal centres and the follicular mantle, B cells are strongly positive for CD72. CD72 is a type II integral membrane protein and, in common with CD23, is a member of the C-type lectin family (described in section - CD23). Like CD23, it has a leucine zipper motif between the membrane and lectin-like domains which cause an α-helical coiled-coil structure and thus there is oligomerisation of CD72 to create a stable homodimer of 86kD (Gould *et al*, 1991). Unlike CD23, there is no evidence that CD72 has a soluble form. Transformation with EBV upregulates expression of CD23 but appears to downregulate CD72 expression, as well as that of CD5 (Gordon, 1994).

The function of CD72 remains elusive but, as mentioned above, its interaction with CD5 has been implicated in B-T cell contacts since T cells express CD5. This may be the case in murine B cells where antibody to murine CD72 blocks the *in vitro* response to T-dependent antigens (Yakura *et al.*, 1981), but it has not been demonstrated for human CD72. In resting human B cells, anti-CD72 mAbs deliver a co-mitogenic signal with immobilised anti-IgM (mimics T cell-independent antigens) to stimulate DNA synthesis. However, soluble anti-μ, which mimics T cell-dependent antigen, was unable to interact with the CD72 signal (Kamal *et al.*, 1991). Therefore, it has been suggested that CD72 may play a role during T-cell-independent B cell responses, possibly by interaction with $CD5^+$ B-1 cells. As previously noted, human $CD5^+$ B cells have a tendency to express low-affinity polyspecific antigen receptors and it has been suggested that these cells could serve as antigen-presenting cells (APC) for conventional B (B-2) cells which express more specific receptors. It is proposed that CD5 on the B-1-APC would interact with CD72 on the B-2 cell to augment the Ag-delivered signal and activate the cell. This hypothesis may be supported by studies which show that CD5 deficient mice are unable to mount responses to certain T-independent antigens (Gordon, 1994).

It has been shown that CD5 can exist as a soluble molecule, extending the possiblity that CD72 may also be triggered on the B cell surface by a soluble ligand (Jamin *et al.*, 1991). However, the role of CD72 remains obscure and the real function of CD5+ cells in the immune system is not yet clear. It is possible that CD5+ cells develop early in life due to selection by endogenous antigens, including anti-idiotypic B cells, which are present in the foetus and not in the adult. In this role they may parallel the $\gamma\delta$ T cells, progenitors of which are only present in foetal HSC (Kantor, 1991), and so represent the primitive layer of the immune system, with B-2 cells and $\alpha\beta$ T cells making up the most developed layer. B-1 cells may have evolved to continually produce autoantibodies with a regulatory role and possibly 'natural' antibodies which offer a first line of defence against pathogens. They

do not seem to become permanently committed to antibody production and appear to retain their self-renewal capacity (Herzenberg *et al.*, 1986).

The ability of $CD5^+$ cells to produce IL-10 may also be important in immune regulation since IL-10 can inhibit cytokine synthesis by macrophages and inhibit TH_1 cell function, but favour the function of TH_2 cells. IL-10 is also produced by macrophages and T cells, so its regulation and effects will be complex. However, in diseases, for example CLL, elevated levels of $CD5^+$ B cells, leading to overproduction of IL-10, may explain the dysregulation of immune function (O'Garra & Howard, 1992).

It is not yet clear which, if any, of these roles of B-1 cells is most important in maintenance of an effective normal immune system. Both B-1 and conventional, B-2, B cells must derive from a common progenitor but the exact point in B cell development at which the two lineages branch is unknown.

1.4 *Regulation of B cell development by stromal cells*

Progenitor B cells proliferate and differentiate in close contact with bone marrow stromal cells, within the interstices of a network of cytoplasmic processes (Jacobsen & Osmond, 1990). Cell-cell contacts and the cytokines that stromal cells secrete are both essential for B lymphopoiesis, but relatively little is known about the exact interactions which occur. The bone marrow is a difficult environment to study *in vivo* so most information has come from *in vitro* long-term bone marrow culture techniques (Whitlock & Witte, 1982). Several stromal cell lines have been developed and appear to exhibit heterogeneity of function with respect to the stage of B cell development they can support (Dorshkind & Landreth, 1992). Some cell lines support the maturation of pro-B cells to pre-B cells but others will support only proliferation, not maturation, of these cells (Nishikawa *et al.*, 1988). It is not clear whether these cell lines represent distinct populations of stromal cells or that, as cell lines, they are deficient in certain functions and so unable to provide complete support for B lymphopoiesis. However, the discovery of these cell lines has demonstrated the existence of multiple signals which act at defined points in B cell development.

1.4.2 *Cell-cell contacts*

As mentioned previously, developing human and murine lymphoid cells can be observed in direct, intimate contact with stromal cells in bone marrow cultures (Dorshkind & Landreth, 1992) and *in vivo* they have been shown to make extensive contact with stromal reticular cell processes (Jacobsen & Osmond, 1990). This contact appears to be essential for the long-term survival of $B220^-$ cells (Kearney *et al.*, 1987) and understanding how B cells modulate and are affected by this adhesion has relevance to both normal B cell differentiation and to the spread of leukaemic cells outside the bone marrow environment.

Several adhesion molecules have been implicated in these cell to cell contacts. Developing B lymphocytes express VLA-4 integrin which may bind to VCAM-1 found on stromal cells; during murine B lymphopoiesis, the interaction between VLA-4 on progenitor B cells and the murine homologue of VCAM-1, has been shown to be essential for maturation (Miyake *et al.*, 1991). The adhesion molecules VLA-4 and VLA-5 are also expressed by progenitor B cells, and are able to bind to fibronectin. Antibodies to CD9, a member of the newly defined tetrahelical family of proteins which are proposed to modulate adhesive processes, have been shown to induce pre-B cell adhesion to bone marrow stromal cells by promoting reognition of fibronectin by VLA-4 and VLA-5 (Masellis-Smith & Shaw, 1994). Since CD9 is present on pre-B cells but not on circulating B cells, it could serve to retain B cell progenitors within the bone marrow microenvironment.

A stromal cell surface molecule termed BST-1, which has 33% homology with CD38, also facilitates pre-B cell growth but the ligand for BST-1 is, at present, unknown. Stromal cell lines from rheumatoid arthritis patients have enhanced BST-1 expression and were previously shown to have an enhanced ability to support the growth of a pre-B cell line when compared to stromal cell lines from healthy donors (Kaisho *et al.*, 1994).

In addition to facilitating B cell development, there is another role for cell-cell contact in the bone marrow. In the mouse, many B lineage cells are observed in such close contact with macrophages that their cell membranes are indistinguishable. Apoptotic cells are phagocytosed after recognition and binding by macrophages and, during B cell development, apoptosis probably occurs after a cell has undergone non-functional Ig gene rearrangement to 'abort' their development (Jacobsen & Osmond, 1990).

The exact role of cell-cell contact in B lymphopoiesis is not clearly understood but the existence of progenitors whose survival is absolutely dependent upon contact with stromal cells suggests that it plays a crucial role. It is possible that a function of adhesion is to expose B cell progenitors to regulatory cytokines produced by stromal cells, however, there is evidence that the maturation (as opposed to growth) of pro-B cells, although dependent upon stromal cells, does not require direct contact but can be potentiated by soluble mediators (Landreth & Dorshkind, 1988).

1.4.3 *Cytokines*

Normal stromal cells, from primary cultures, have been shown to produce numerous cytokines including IL-3, IL-7 and IL-11, macrophage (M)-colony-stimulating factor (CSF), stem cell growth factor (SCGF) and occasionally Insulin-like growth factor 1 (IGF-1) (Witte *et al.*, 1993). However, in contrast to some observations with stromal cell

lines and clones, there was no evidence for constitutive expression of IL-4, IL-6 or GM-CSF. Witte *et al.*, 1993 suggest that this discrepancy may have been caused, in the case of IL-6, by the cells becoming more fibroblast-like after extended culture. Most of the stromal cells examined were shown to produce IL-7 but about 20% had no detectable IL-7 protein. Thus, primary stromal cells may include functional subsets and these, at least in regard to IL-7 production, mirror the differences in cytokine profiles seen in stromal cell lines. However, it is possible that IL-7 is produced by all stromal cells but constitutive levels are maintaind by highly localised inductive signals. Indeed, Sudo and colleagues (Sudo *et al.*, 1989), showed that transcription of IL-7 was actually induced in a cloned stromal cell line after contact with pre-B cells, although other reports dispute this observation (Witte *et al.*, 1993). It has been suggested that, in addition to cytokines, stromal cells may secrete extracellular matrix proteins which act to stabilise and provide a high local concentration of the secreted growth factors .

There are numerous cytokines, for example IL-1, IL-3 and IL-11, which have a general involvement in haematopoiesis, including stimulation of stem cells. IL-11 appears to act in synergy with IL-3, stem cell factor and also G-CSF to expand and sustain long-term culture-initiating cells (Brandt *et al.*, 1993). However, there are cytokines which act more specifically on lymphopoiesis; those involved in B cell development will be described below.

PRO-B CELLS: The stromal cell cytokines involved in maturation and proliferation of pro-B cells are poorly defined. Long-term survival of these cells apparently requires direct stromal cell contact (Nagasawa, 1994) and this makes it difficult to examine which soluble factors are actually involved. Maturation of $B220^-$ cells to pre-B cells is not affected by IL-7 or *kit*-ligand but conditioned medium from stromal cells can mediate this effect (Landreth & Dorshkind, 1988), suggesting that cytokines are involved. As yet, these additional cytokines have not been identified, however, the stromal cell line PA6, which can maintain pro-B cell growth, has been shown to produce a novel cytokine called pre-B cell growth stimulating factor (PBSF) which is a member of the intercrine α family. Although the observed effects of PBSF were to promote the growth of pre-B cells, the authors suggest that it may also act to increase the numbers of pro-B cells able to mature to pre-B cells (Nagasawa, 1994).

PRE-B CELLS: Most information about cytokine effects on early B lymphopoiesis relates to the pre-B cell stage. IL-7 is known to play a key role in B lymphopoiesis but, although necessary, it is clearly not sufficient for pre-B cell development and most of its effects are observed in synergy with other stromal cell derived cytokines. The primary action of IL-7 is to promote proliferation, with this responsivness only developing after B cells have begun to express B220 (Lee *et al.*, 1989). Administration of IL-7 to normal mice

increases the number of $B220^{+}/sIgM^{-}$ pre-B cells, which subsequently mature to $sIgM^{+}/sIgD^{+}$ B cells (Morrissey *et al.*, 1991), and administration of monoclonal antibodies to IL-7 virtually eliminates the pre-B cell population in mice (Grabstein *et al.*, 1993).

Another cytokine involved in B lymphopoiesis is *kit*-ligand (KL), the gene product of the *Steel* locus and also known as stem cell factor (SCF), whose effects appear only in synergy with IL-7 (McNeice *et al.*, 1991). Most stromal cells secrete KL and this may explain the synergy observed between IL-7 and stromal cell supernatants (Billips *et al.*, 1992). However, injection of anti-SCF mAb does not significantly affect murine B lymphopoiesis (Ogawa *et al.*, 1991) so other molecules may be able to compensate for KL functions. As mentioned previously, pre-B cell growth stimulating factor (PBSF) can stimulate proliferation of a pre-B cell line by itself and, furthermore, synergistically augment the growth of bone marrow B cell progenitors in the presence of IL-7. Samal and colleagues, 1994, cloned a 52kDa human pre-B cell colony enhancing factor produced by stromal cells and showed that it acts in synergy with IL-7 and SCF to enhance murine pre-B colony formation (Samal *et al*, 1994). Insulin-like growth factor-1 (IGF-1) is also able to promote proliferation of IL-7-dependent pre-B cells (Landreth *et al*, 1992).

As well as its ability to promote pre-B cell proliferation, IL-7 has been implicated in the maturation of pre-B cells to immature B cells, with responsiveness to IL-7 lost once the cells have surface Ig (Dorshkind & Landreth, 1992). A study involving a human acute lymphocytic leukaemia (ALL) showed limited maturation of the pre-B cells to CD20 expressing B cells which were sIg-negative, on addition of IL-7 (Ohyashiki *et al.*, 1993). However, another report demonstrated that *c-kit*, the receptor for *kit*-ligand, and the IL-7R are no longer expressed once cells have undergone kappa rearrangements and thus, they suggest a minimal role for IL-7 and KL in mediating the pre-B to B cell transition (Henderson *et al.*, 1992). Another cytokine involved in this transition is an IL-4-like molecule produced by the stromal cell line SCL 160 (Dorshkind & Landreth, 1992). IL-4 itself has been shown to inhibit growth of stromal cell-dependent pre-B cell lines (Rennick, 1987; Ohyashiki *et al.*, 1993) consistent with it acting to induce pre-B cell differentiation. Another group failed to see an effect of IL-4 on the differentiation of leukaemic pre-B cells but it is possible that these cells had a differentiation block and so were unable to respond (Consolini *et al.*, 1993).

A second model of pre-B cell maturation is proposed by Rolink *et al.*, 1991, who suggest that, in the mouse, it is removal of IL-7 from cultures which releases cells to differentiate to surface Ig-positive cells. Their hypothesis is that proliferation of precursors pushes them away from the centre of the bone marrow and thus away from stromal cell contacts and the appropriate cytokine supply, forcing or allowing the cells to differentiate.

NEGATIVE REGULATION: There is emerging evidence that B cell development is sensitive to negative regulation in culture and *in vivo*. Various cytokines, including transforming growth factor-β (TGF-β), IL-1, IL-4 and granulocyte-macrophage colony-stimulating factor (GM-CSF) appear to inhibit early B lymphopoiesis. This inhibition may be elicited through action on the stromal cells since treatment of stromal cells with GM-CSF abrogates their ability to support pre-B cell differentiation (Lee *et al.*, 1993; Dorshkind & Landreth, 1992). In addition, pre-incubation of stromal cells with either IL-1α or IL-4 completely abrogates their ability to support pre-B cell generation in subsequent culture with freshly isolated bone marrow cells, apparently via induction of an inhibitory cytokine (Billips *et al.*, 1990).

Thus, the regulation of B lymphopoiesis appears to be highly complex, involving positive and negative acting cytokines and direct B cell-stromal cell contact. It is likely that other, as yet unidentified, cell surface antigens and cytokines are also involved in early B cell development.

The cytokines mentioned previously are all produced by bone marrow cells other than B cells themselves. There is, however, evidence that B cells produce cytokines to control their own growth and this autocrine control may have a role in B lymphopoiesis. Dysregulation of such growth control could lead to development of malignancies.

1.5 *Autocrine control of B cell development.*

There are multiple regulatory controls operating during B lymphopoiesis and it has become apparent that B cells are able to produce factors which affect their own growth. These factors have been termed autocrine growth factors. The original observation of the existence of autocrine factor production by B cells came from work involving Epstein-Barr virus (EBV)-transformed B cells (Blazar *et al.*, 1983). EBV is a herpesvirus which enters B cells via binding to CD21 (Fingeroth *et al.*, 1984). Once EBV has circularised, the B cells express increased levels of the cellular gene CD23 (Hurley & Thorley-Lawson, 1988) and are immortalised *in vitro*. Blazar and colleagues demonstrated that EBV-transformed B cells produce an autocrine growth factor after they observed that conditioned media from human B cell lines contained a stimulatory activity which, when added back to the same cells cultured at low cell density, reduced the lag phase and subsequently enhanced growth of the cells (Blazar *et al.*, 1983). Subsequently, Gordon *et al.*, 1984a, reported that EBV-transformed cells, if starved of their autocrine growth factor by culture at low cell density, arrested in the G1 phase of the cell cycle. This was in contrast to cells starved of supplements such as transferrin where the cells arrested at the G1/S transition stage. The authors went on to demonstrate that the autocrine activity was due to a protein of between 25 and 30 kDa in size. This autostimulatory activity, termed B

cell-derived B cell growth factor (B-BCGF) also acted in synergy with anti-Ig to stimulate proliferation of normal tonsil B cells (Gordon *et al.*, 1984b).

It has been proposed that the association between EBV and malignancies such as Burkitt's lymphoma is due to the induction of autocrine growth factor production by the B cell and subsequent removal of normal growth regulation. However, the picture may not be so simple because many EBV-negative cell lines, and active cycling normal B cells, produce a 'BCGF' arguing that it is not a property specifically induced by EBV transformation (Gordon, 1985; Jurgensen *et al*, 1986). Thus, it appears that autocrine growth factor production may be a property of continuously growing cells and, after activation of normal cells, it may result in clonal expansion (Muraguchi *et al.*, 1986).

Since these initial observations of autocrine factor production, numerous 'B-BCGF' have been recognised and now several cytokines have been implicated as autocrine factors:

IL-1: In 1984, Scala *et al* reported that an EBV-transformed cell line could produce IL-1α and that it had an autostimulatory effect on the cells. There have been several other reports of IL-1or IL-1-like activity acting as an autocrine growth factor and there are several demonstrations of B cell chronic lymphocytic leukaemia (B-CLL) clones producing IL-1 (Gordon & Cairns, 1992). IL-1 is most prominently produced by monocytes and, indeed, IL-1 production appears to be restricted to B-CLL cases expressing the monocyte-associated antigen, CD14 (Morabito *et al.*, 1987). In B-CLL, the leukaemic cells do not divide in the blood but presumably are produced by rapidly dividing progenitor cells in the bone marrow. In most cases the leukaemic cells are refractory to the effects of autologous IL-1 but it is possible that the progenitor cells use IL-1 to sustain their own growth.

TNF-α: Tumour-necrosis factor has been implicated as an autocrine factor for B-CLL cells (Cordingley *et al.*, 1988), and recently, for normal human splenic B cells (Boussiotis *et al.*, 1994). When the B cells are activated with either anti-CD40 antibodies and IL-4, i.e. mimicking activation by T-dependent antigen, or with anti-Ig i.e. mimicking T cell-independent antigen, there is induction of TNF-α gene transcription and production of TNF-α (Boussiotis *et al.*, 1994). That antibodies to TNF-α inhibit the B cell proliferation confirms the autocrine effect and suggests that this regulation might have a role in infections where polyclonal B cell expansion occurs.

TNF-β: More commonly referred to as lymphotoxin, TNF-β also acts as an autocrine factor but only in human EBV- transformed cell lines (Estrov *et al.*, 1993). No production of TNF-α by these cells was evident .

IL-6: Interleukin-6, although normally produced by fibroblasts, endothelial cells or monocytes (Biondi *et al.*, 1989), has been identified as an autocrine growth factor for both EBV-immortalised B cells and multiple myeloma cell lines (Tosato *et al.*, 1990: Kawano *et al.*, 1988). The involvement of IL-6 in multiple myeloma, where neutralising antibodies to IL-6 inhibits the *in vitro* proliferation of the cells, demonstrates that autocrine growth regulation can contribute to pathogenesis of a B cell malignancy (Kawano, 1988).

Lactic acid: In the EBV-immortalised cell lines reported by Tosato and colleagues, 1990, IL-6 did not appear to be the sole autocrine growth factor since a low-molecular weight fraction (<5000 Da) contained a large proportion of the stimulatory activity. This activity has surprisingly been identified as lactic acid, a metabolic product of glycolysis (Pike *et al.*, 1991). Lactic acid alone was found to account for greater than 70% of the autocrine activity in serum-free supernatants from EBV-immortalised B cells. That the majority of the stimulatory activity is due to lactic acid may explain why previous studies using anti-IL-6 neutralising antibodies failed to inhibit autocrine activity of unfractionated supernatants from lymphoblastoid cell lines (Tosato *et al.*, 1990).

Thioredoxin: A structurally novel 12 kDa protein with IL-1-like activities was reported to be an autocrine factor for EBV-transformed B cells. The factor was termed 3B6 IL-1 (Wakasugi *et al.*, 1987) but is now known to belong to the family of reducing enzymes called thioredoxins (Bertoglio *et al.*, 1989). Thioredoxins are found in almost all cell types and are attributed with numerous properties ranging from hydrogen donor for ribonucleotide reductase in DNA synthesis, to degradation of insulin. Recently, thioredoxin has been shown to be involved in autocrine growth stimulation of EBV-transformed cells after it was discovered that it can increase cell proliferation by triggering protein kinase C translocation (Biget *et al.*, 1994). It is possible that, by amplifying intracellular signals, thioredoxin can make EBV-positive cells more sensitive to sub-optimal concentrations of growth factors to which normal cells would not respond.

Other cytokines: Several other cytokines are thought to act as autocrine factors for B cells, including IL-2, IL-5 and IL-10. Leukaemic cell lines of pre-B cell origin produce IL-2 and also synthesise IL-2 receptors; but there has been no direct demonstration of autocrine activity (Holan *et al.*, 1993).

In 1990, Paul and colleagues discovered that EBV-transformed B cells produce IL-5 (Paul *et al.*, 1990) and in 1992 they went on to show that IL-5 was, indeed, acting as an autocrine factor (Baumann & Paul, 1992). As yet, there is no evidence of IL-5 acting as an autocrine factor for non-EBV transformed human B cells but it is possible that the response is inducible if the cells are appropriately activated.

With respect to IL-10, it has been shown that the IL-10 coding sequence is highly homologous to an EBV open reading frame gene, BCRF1 (Moore *et al.*, 1990). Human IL-10 enhances the viability of resting B cells and the proliferation of activated B cells and the EBV-encoded product has the same biological activities, so was termed viral IL-10 (vIL-10) (Stewart *et al.*, 1994). It is possible that EBV uses the vIL-10 to keep cells proliferating while it is actively replicating and spreading during the early stages of infection. IL-10 is produced by activated T cells, activated monocytes and some B cells. Some Burkitt's lymphoma cell lines produce IL-10 (Stewart *et al.*, 1994) and CD5-positive B cells, capable of self-renewal, produce high levels of IL-10, as detected by PCR (Raveche *et al.*, 1992). In fact, murine B-1 cells are known to constitutively produce elevated levels of IL-10 and mice treated with a neutralising anti-IL-10 antibody from birth lack B-1 cells (O'Garra and Howard, 1992). This appears to be due to elevated endogenous IFN-gamma but does demonstrate a role for IL-10 in B-1 cell development. IL-10 appears to be expressed at a discrete stage of B cell differentiation corresponding to mature and/or activated B cells. It is not clear whether IL-10 acts as an autocrine factor for normal cells but it may be important in B cell malignancies.

IL-7: Expression of IL-7 has not been detected in normal B cells but malignant B cell phenotypes, including lymphoblastoid cell lines and Burkitt's lymphoma cell lines, have been shown to constitutively secrete IL-7 and some mature B cell lines express IL-7 receptors. Numerous human B cell lines express IL-7 transcripts but only EBV-positive cell lines constitutively secrete IL-7; the cell lines which express IL-7 receptors do not secrete IL-7, arguing against an autocrine effect (Benjamin *et al.*, 1994). At present there are no reports of IL-7 acting as an autocrine growth factor for B cells except a report where IL-7-bearing retroviruses were used to transform IL-7 dependent cell lines. Subsequently these cell lines became independent of exogenous IL-7, their proliferation was inhibited by anti-IL-7 antibodies and they were acutely tumorigenic in syngeneic hosts (Overell *et al.*, 1991).

CD23: CD23 is a 45kD type-II transmembrane glycoprotein and a member of the C-type lectin superfamily (see section 1.5 for more details). It was first described as the EBV cell surface antigen (EBVCS) by Kitner and Sugden (1981), and expression of CD23 was shown to be a requirement for transformation of B cells suggesting that it was involved in growth stimulation. It is now apparent that normal cells also express CD23 when activated (Walker *et al.*, 1986) but, in EBV-transformed B cells, CD23 appears to be superinduced and constitutively expressed (Thorley-Lawson *et al.*, 1985). Swendeman and Thorley-Lawson, 1987, observed that CD23 is released into the supernatant as a soluble molecule of 25kD molecular weight (sCD23) which is involved in autocrine proliferation of EBV-infected B lymphoblasts and is a growth factor for normal, receptor-

stimulated, B cell blasts. However, production of CD23 by EBV-transformed cells does not correlate with their tumourigenicity and, in fact, there may be an inverse relationship between these two factors (Gordon *et al.*, 1985; Klein & Klein, 1985).

Synergistic effects: As mentioned with respect to IL-6 and lactic acid, it is possible that more than one autocrine factor is present in the supernatant of autonomously growing cells. This can make identification of factors very difficult. A paper by Abken *et al*, 1992, actually indicates involvement of four secreted cytokines which act synergistically to maintain long-term proliferation of EBV-negative human B cell lines. IL-1α, IL-6, TNF-α and TNF-β are all necessary for the continued growth of some established human B cell clones. These cytokines appear to regulate and balance production of one another since the IL-6 promotor is activated by TNF-α and IL-1α and, furthermore, IL-1α induces transcription of TNF-α and TNF-β mRNA (Abken *et al*, 1992). Each of these cytokines has independently been shown to modulate neoplastic cell growth in various cell systems but the human B cell clones tested by Abken and colleagues are non-tumorigenic.

It is possible that normal B cells actively control their own growth, since mitogenically stimulated B cells from peripheral blood secrete very high levels of IL-1α, IL-6, TNF-α and TNF-β (higher than the cell lines). However, the normal B cells do not proliferate continuously in the presence of these cytokines. It is not clear whether the cells are refractory to high concentrations of the cytokines or they may also produce growth inhibitory cytokines such as TGF-β.

Negative autocrine factors: It is likely that the establishment of an immortalized cell line will depend partly on the balance of autocrine stimulatory and inhibitory factors that the cells produce. In the first description of B-BCGF production, Vesole *et al.*, 1979, reported an inhibitory activity which could be separated from the 30kDa stimulatory activity. Kerhl *et al.*, 1986 showed that activated B cells release TGF-β into the supernatant and that it is capable of inhibiting B cell growth stimulation, depending on the concentration of stimulatory cytokine present. Examples of mechanisms which could circumvent negative factors include: loss of receptors for the cytokine, for example, an EBV-transformed B cell line which lacks TGF-β receptors and fails to be inhibited by TGF-β; and the production of soluble cytokine receptors, for example, IL-1 receptors released by cells to downregulate the stimulatory effects of autocrine growth factors (Gordon and Cairns, 1991). Thus, the deregulation of negative control factors may play an important role in the development of leukaemias and lymphomas.

Autocrine factors in tumorigenesis: Several investigators have demonstrated that, for B cells, the production of and dependence upon autocrine stimulation is inversely proportional to the degree of tumourigenicity (Gordon *et al.*, 1985; Klein & Klein, 1985;

Abken *et al.*, 1992). In agreement with this, Swendeman and Thorley-Lawson, 1987, showed that EBV-transformed cell lines, although expressing the highest CD23 levels, are less tumourigenic than the EBV-negative Burkitt's lymphoma cells which have low levels of CD23. Thus, autocrine growth factor production is apparently not the sole requirement for transformation of B cells; an idea supported by Young *et al*, 1991, who overexpressed IL-7 in pre-B lymphoid cell lines which subsequently attain autocrine growth and secrete biologically active IL-7 but rarely acquire a tumorigenic phenotype. They concluded that, at least with regard to IL-7, autocrine production may be one of several synergistic events which can lead to malignant conversion. Indeed, Overell and colleagues found that transformation of murine pre-B cell lines with an IL-7-bearing retrovirus occurred at a low level, indicating that secondary events were required. However, the transformation involved an autocrine mechanism and the "IL-7-independent" cells were tumourigenic, in contrast to their parental IL-7-dependent cells (Overell *et al.*, 1991).

Internal autocrine factors: Another hypothesis for autocrine growth control is the existence of internal autocrine regulation (Goldwasser *et al.*, 1991). There is not much information about this potential mechanism, but it may exist with respect to erythropoietin (EPO) in haematopoietic stem cells where antisense to EPO suppresses both colony number and cell growth but neutralising antibodies to EPO have no effect. This effect is also seen with GM-CSF antisense in normal mouse bone marrow (Goldwasser *et al.*, 1991). The authors hypothesise that this mechanism is used for proliferation of the multipotent cells but, as differentiation occurs, the cells express specific external receptors for the various ligands, and the internal autocrine mechanism ceases to function with the receptor expressed on the cell membrane and the cell dependent on external, possibly still autocrine, signals.

Platelet derived growth factors (PDGFs): These have also been implicated as having a role in internal autocrine regulation. PDGFs are a family of closely related proteins which exist as approximately 30kDa disulphide-bonded dimers. The three isoforms of PDGF - $\alpha\alpha$, $\alpha\beta$, and $\beta\beta$ are encoded by two genes termed PDGFA and PDGFB. The α and β chains are 60% homologous in amino acid sequence (Westermark and Heldin, 1991). Megakaryocytes, monocytes, and to a much lesser extent, T cells have been shown to produce PDGF. In 1983, Doolittle and colleagues demonstrated that the simian sarcoma virus oncogene, *v-sis*, is derived from the genes encoding platelet-derived growth factor (PDGF) (Doolittle *et al.*, 1983). The demonstration that antibodies against PDGF, and also treatment with suramin (a drug which prevents surface ligand/receptor interactions), can inhibit or reverse simian sarcoma virus transformation of cells and prevent their growth, has shown that transformation by the *sis* gene occurs by an autocrine mechanism, in cells which express the appropriate PDGF receptor (Betsholtz *et al.*, 1986; Tsai *et al.*, 1994). There are also two forms of PDGF receptor, PDGFR-α and PDGFR-β. The α-

receptor binds all three isoforms with high and approximately equal affinity, whereas the β-receptor preferentially binds ββ homodimers and interacts with αβ heterodimers with reduced affinity (Hart *et al.*, 1988). Both receptors have intrinsic protein-tyrosine kinase activity in their cytoplasmic domain and this is thought to be activated by conformational changes caused on dimerisation of the receptors (Westermark & Heldin, 1991). Fleming *et al.*, 1992 demonstrated that in human sarcoma tumour cells expressing PDGF ligands and receptors, the receptors were phosphorylated on tyrosine residues in the absence of exogenous ligand, suggesting chronic receptor activation. In some of these cell lines, anti-PDGF neutralising antibodies caused a reduction in DNA synthesis, suggesting that autocrine PDGF stimulation contributes to the proliferation of these tumours (Fleming *et al.*, 1992). They also found that the anti-PDGF decreased receptor phosphorylation, arguing that it was autocrine stimulation rather than genetic mutation which lead to the constitutive activation of the receptors. However, in some cell lines, anti-PDGF has no inhibitory effect on proliferation and it has been demonstrated that autocrine activation of PDGFRs can occur internally (Huang *et al.*, 1984; Fleming *et al.*, 1989), but that activated receptors must achieve a cell surface location in order to couple with intracellular signalling pathways (Johnsson *et al.*, 1985; Fleming *et al.*, 1989). This may explain why, in some situations, anti-PDGF can cause a reversion of the *v-sis*/PDGF B-transformed phenotype (Tsai *et al.*, 1994). Recent work from this laboratory also showed the expression of PDGF and its receptors by two pre-B acute lymphocytic leukaemia cell lines which suggests that PDGF may act as a lymphokine and lends the possibility that PDGF may act as an autocrine factor for B cells (Tsai *et al.*, 1994).

In conclusion, it was originally proposed that the production of autocrine growth factors is restricted to EBV-transformed and neoplastic cell lines and is the reason they grow autonomously and cause tumours such as Burkitt's lymphoma. However, autocrine factor production has been observed by EBV-negative cell lines and by normal cells, if appropriately activated. It now appears that production of autocrine factors is a property of actively cycling cells - cells which are transformed or stimulated, for example with anti-Ig and anti-CD40. The development of tumours apparently requires the simultaneous presence of several molecular perturbations but autocrine factor production is one such perturbation. Gordon *et al.*, 1985, actually showed an inverse correlation between CD23 production and tumourigenicity in EBV-transformed cells; thus, although essential for growth of the cells, production of autocrine growth factors does not correlate with tumourigenicity. Experiments where immortalised cells are transformed with growth factors and growth factor receptors show that the autocrine factors in each case increase the tumourigenicity of the cells. In contrast, if primary cells are manipulated in this way, no cellular transformation is evident.

That normal activated cells do not become malignant by production of autocrine factors may be due to production of negative regulatory molecules such as transforming growth factor-β (TGF-β). If this pathway becomes deregulated it could lead to loss of a controlling negative signal and subsequent continued growth of the cells (Gordon & Cairns, 1991). Malignancies may also develop if a mutation perturbs the differentiation potential of the cell, causing a differentiation block.

Autocrine factors may be involved in transformation of cells if there is deregulation of positive or negative growth signals, or mutation of growth factor receptors. Indeed, *in vitro* transformation of murine pre-B cells was achieved with *v-mpl*, a truncated form of a cytokine receptor related to the haematopoietin receptor superfamily and contained in myeloproliferative leukaemia virus (MPLV). This constitutively activated growth factor receptor induced high rates of proliferation of transformed cells, but did not affect their ability to differentiate (Fichelson *et al.*, 1995).

In the case of thioredoxin, an autocrine factor may increase the sensitivity of cells to low levels of growth factors by amplifying receptor signalling. The production of lactic acid could be important in solid lymphoid tumours where the centre of the mass is starved of oxygen. Anaerobic respiration may occur leading to the production and subsequent growth promoting activities of lactic acid. The deregulation of autocrine factors may be important in immunodeficiency disease, for example, in Wiskott-Aldrich syndrome (WAS), B cells from patients are transformed by EBV, shown to have defective expression of CD23 and lack of autocrine growth-stimulation (Simon *et al.*, 1992).

However, most of the information regarding autocrine factors and their role in malignant transformation, involves mature B cells with little evidence of a role for autocrine growth regulation in early B cell development.

1.5 *CD23 - adhesion molecule and cytokine*

CD23 has been mentioned previously with respect to its involvement in autocrine growth stimulation of EBV-transformed B cells. It also appears to have roles in haematopoietic cell development, antigen presentation, IgE regulation and prevention of apoptosis. The tissue distribution, structure and other properties of CD23 will now be discussed.

1.5.1 ***Distribution***: CD23 is a cell surface antigen expressed by numerous human cells including T and B lymphocytes, NK cells, macrophages, eosinophils, a subset of platelets, follicular dendritic cells, epidermal Langerhans cells, and by some epithelial cell types (Capron *et al.*, 1986; Sarfati *et al.*, 1986; Bieber *et al.*, 1989; Billaud *et al.*, 1989). It was originally identified as the low-affinity IgE receptor (FcεRII) (Gonzalez-Molina *et al.*, 1976), then independently described as a B cell activation marker on EBV-transformed

cells (Kitner & Sugden, 1981). Subsequent studies have demonstrated that the CD23 antigen has numerous roles as an adhesion molecule and cytokine (Bonnefoy *et al.*, 1987; Yukawa *et al.*, 1987) .

1.5.2 ***CD23 Structure***: The single CD23 gene is located on chromosome 19 (Suter *et al.*, 1988; Wendel-Hansen *et al.*, 1990); two transcripts (a and b), which differ only in the intracytoplasmic region where the N-terminal 7 amino acids of CD23a are replaced by 6 amino acids in CD23b, are generated by use of alternate initiation sites (Yokota *et al.*, 1988). CD23a expression is restricted primarily to B cells but the CD23b isoform is inducible by IL-4 on a variety of haematopoietic cells (Conrad, 1990). In contrast to other Fc receptors, CD23 does not belong to the Ig superfamily but is a type II transmembrane receptor and a member of the calcium-dependent (C-type) lectin family (Delespesse *et al.*, 1992). CD23 contains a conserved, cysteine-rich lectin homology domain in common with several other proteins that bind specific carbohydrates in a calcium-dependent fashion (Drickamer, 1988) (see figure 1.2). The existence of this lectin domain, the inverted membrane orientation, other conserved residues and conserved sugar motifs, including sialic acid, classify CD23 as a member of a novel superfamily of type II intergral membrane proteins, the prototype of which is the asialoglycoprotein receptor (Wong *et al.*,1991) (see table 1.1). CD23 has several distinct domains (see figure 1.2) including epitopes involved in binding IgE, which are found in the aforementioned lectin-homology domain. Near the carboxyl terminus is a triplet of amino acids "DGR" (Asp, Gly, Arg) which, in reverse configuration, i.e. "RGD", is a common recognition site of the integrin receptors but at present there are no data to suggest that this domain is involved in CD23 function (Delespesse *et al.*, 1992). Another feature of CD23 is the presence of 3 short consensus repeats of 21 amino acids and within this region are five heptadic repeats of leucines or isoleucines which form a "leucine zipper" motif.

Murine CD23 is a 49kD sialoglycoprotein which differs from human CD23 in that: 1) it has four instead of three short consensus repeats; 2) it has an extra N-glycosylation site; and 3) there is deletion of the DGR motif at the C-terminus (Bettler *et al.*, 1989a; Gollink *et al.*, 1990). Murine CD23 may also exists in two isoforms with the b isoform having an amino terminus distinct from that of human CD23b (Richards, 1991), although the existence of a murine CD23b form has recently been disputed (Conrad *et al.*, 1993).

The leucine-zipper motif, between the transmembrane and the lectin domains is now known as the "stalk region". Beavil *et al.*, 1992 found that the heptad repeats in this region are characteristic of an α-helical coiled-coil, implying that CD23 would be expressed as a dimer or trimer. This structural motif is present in other members of the C-type lectin superfamily and suggests that the proteins exist as extracellular lectin "heads" separated from the membrane by the α-helical coiled-coil "stalks" of various lengths, with these

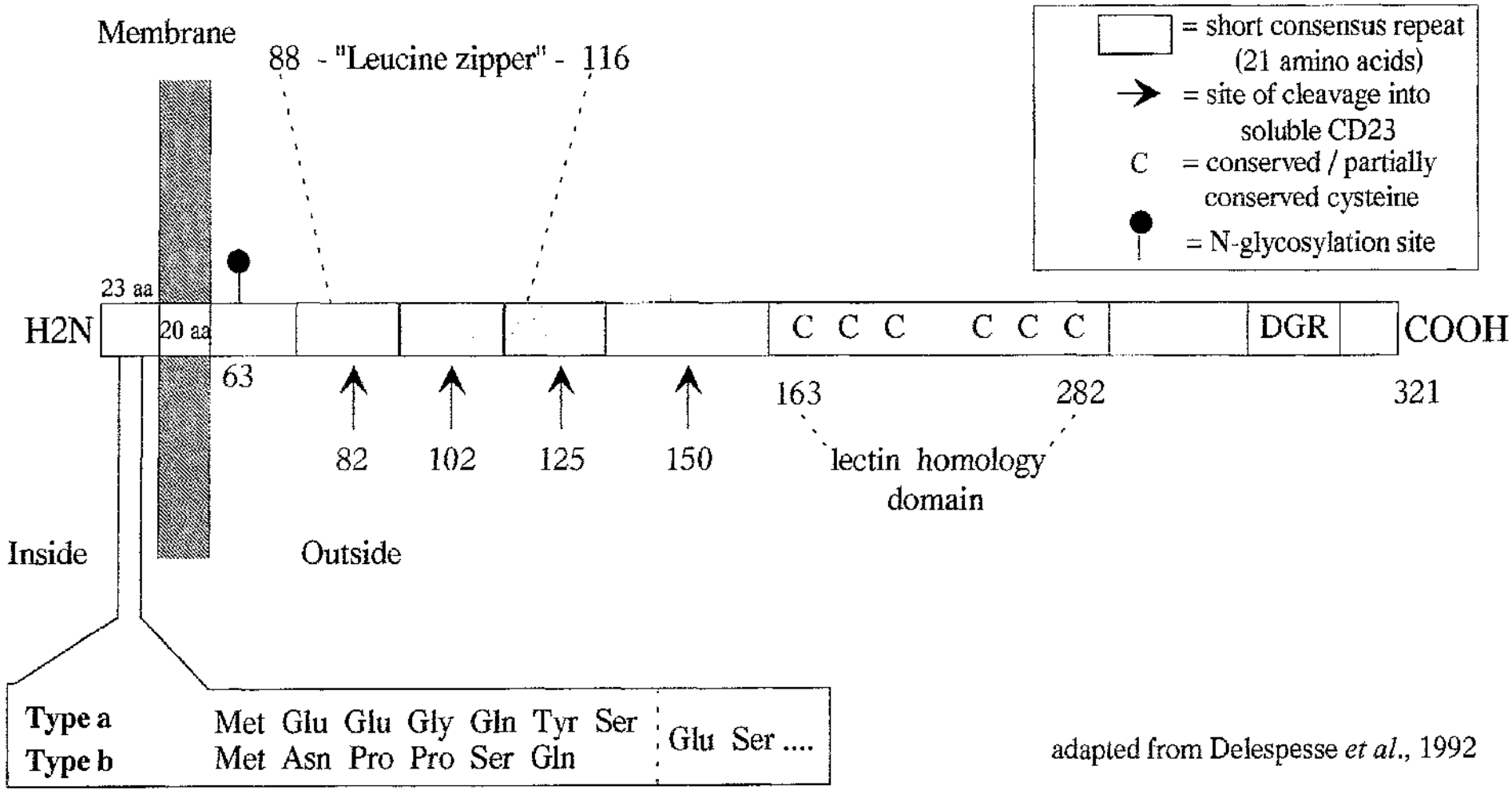


Fig. 1.2 Linear Model of the CD23 Antigen The symbols are described in a key. The region 'DGR' represents the amino acid sequence Asp, Gly, Arg. The leucine zipper and lectin-homology domains are also indicated.

stalks providing a mechanism for protein subunit dimerisation (Beavil *et al.*, 1992). A recent paper demonstrates that human CD23, if subjected to protein-protein chemical cross-linking, can form trimers on the cell surface (Beavil *et al.*, 1995). There is evidence that murine CD23 forms oligomers but whether they are dimers or trimers is not yet determined (Dierks *et al.*, 1993). Dierks and colleagues have evidence to support their theory that IgE requires at least dimers of murine CD23 to bind to cells and that the IgE probably stabilises the oligomeric structure.

Similar to most FcR, there is a soluble form of CD23 (sCD23) which is released from the surface of cells into the extracellular fluid. Numerous biological activities have been attributed to sCD23, in addition to the ability to bind IgE; CD23 is the membrane precursor of a pleiotropic cytokine. Cleavage of CD23 occurs at the residues indicated in figure 1.2, with initial release as an unstable 37 kDa fragment which then continues to be cleaved into 33, 29, 25 kDa and finally 16kDa molecules. The 25kDa form (sCD23) is the most stable (Lettelier *et al.*, 1989) and the form to which most biological activities are attributed. All cleavage products retain the ability to bind IgE (Delespesse, 1992). It has been demonstrated that CHO1-7 cells transfected with CD23 cDNA augment proteolysis of 45kDa CD23 in solution but untransfected cells do not. Highly purified 37kDa and recombinant 29kDa sCD23 also cleave the 45kDa form into 25kDa fragments. Thus, the release of sCD23 may be due to autoproteolysis (Letellier *et al.*, 1990).

After chemical cross-linking, human CD23 exists as hexamers in solution (Beavil *et al.*, 1995). It was expected that only the 37 and 33kDa sCD23 fragments would be capable of oligomerisation because they are the only fragments which possess a region of coiled coil but the 25 and 16kDa forms also form oligomers. Thus, it is apparent that the lectin heads can self-associate in solution and although the coiled-coil region is responsible for trimerization on the cell surface, additional interactions between the lectin heads result in formation of hexamers in solution.

These observations present the possibility that CD23 could bind to IgE with two lectin heads leaving a third free to interact with CD21, or indeed, it could bind to three CD21 molecules providing an avidity effect on the interaction (Beavil *et al.*, 1995). The situation with murine sCD23 oligomerisation is not clear but it appears to interact with IgE in a univalent manner. The differences in biologic activity observed in mouse and man (discussed later) may be explained by differences in ability to form oligomers in solution.

Binding of IgE to CD23 is calcium-dependent and appears to involve protein-protein interactions since deglycosylated native IgE peptides are able to bind to CD23 (Vercelli *et al.*, 1989). However, Delespesse *et al.*, 1992, showed an ability of fucose-1-phosphate to inhibit the binding of native IgE to soluble CD23. Thus, it appears to be the lectin-

homology domain which is involved in interaction of CD23 with IgE and this domain must have both carbohydrate- and protein-binding domains (Bettler *et al.*, 1989b & 1992; Bardones, 1988).

Table 1.1

Superfamily of type II membrane proteins with lectin-like domain
- Liver asialoglycoprotein receptors 1 & 2 (galactose-specific lectin)
- Macrophage asialoglycoprotein receptor (galactose-specific lectin)
- Ly-49, A,B,C. (NK cell antigen)
- NKR-P1 (signal transducing molecule on NK cells)
- CD72 (ligand for CD5)
- A1 (a T cell antigen)
- CD23 Taken from - Delespesse *et al.*, 1992

1.5.3 *Regulation of CD23 Expression*

The CD23a isoform is found exclusively on B cells and is present in small amounts on normal, peripheral B cells. This expression does not appear to be constitutive because the cells lose CD23 mRNA and protein after incubation at 37°C in the absence of stimulant (Delespesse *et al.*, 1989). However the addition of IL-4 to resting, normal human B cells induces expression of substantial amounts of CD23a and CD23b with a predominant effect on type b (Yokota *et al.*, 1988). This effect is not accompanied by activation of the cells, which remain at the G0 stage of the cell cycle (Gordon *et al.*, 1986). Expression of CD23b is also induced on other cell types including T cells and monocytes, after activation with IL-4 (Delespesse, 1992). IL-4 enhances CD23 expression on murine B cells, where an IL-4 enhancer element has recently been characterised in the CD23 gene (Richards & Katz, 1994). In humans, the expression of CD23 appears to be associated with that of IgD and even addition of IL-4 will not induce its expression on IgD-negative B cells (Kikutani *et al.*, 1986), although malignant pre-B cells from ALL patients may express CD23 after stimulation with IL-4 (Law *et al.*, 1991) and ligation of CD40 can induce expression of CD23 on normal human pre-B cells (Saeland *et al.*, 1993).

Similarly, expression of CD23 cannot be induced in normal B cells after class switching to IgG, IgA or IgE (Waldschmidt *et al.*, 1988) but some transformed or leukaemic cells, such as RPMI 8866 which secrete IgG, still express CD23 after class switching (Delespesse *et al.*, 1991). Interleukin 13 has been shown to induce CD23 expression on human B cells in an IL-4-independent fashion and there is no synergistic effect between the two cytokines (Punnonen *et al.*, 1993) .

IgE is also implicated in increasing the cellular density of CD23 on B cells but not by increasing its expression; IgE appears to protect surface CD23 from proteolytic cleavage (Lee *et al.*, 1987). The mechanism of this protection is thought to be steric hinderance of proteolytic enzyme activity (Bonnefoy *et al.*, 1990). However, a recent investigation reports that anti-IgE treatment of mice inhibits CD23 expression, along with IgE production (Haak-Fredscho *et al.*, 1994). They propose that anti-IgE may act to prevent the up-regulation of CD23 expression usually seen on immunisation and so prevent CD23/CD21 interactions and diminish the IgE response.

A recent paper by Matsui and colleagues demonstrates the existence of alternative transcripts of CD23a and -b which lack the transmembrane region and the anchoring region of the cytoplasmic tail. They suggest that these may encode secretory forms of CD23 or may function as regulatory transcripts involved in the control of CD23 expression (Matsui *et al.*, 1993).

1.5.3-1 ***EBV-regulation of CD23 expression***: EBV exists in a latent state once it transforms B cells and the viral genome expresses only a few proteins. The proteins most closely related to the transformation process are EB nuclear antigen (EBNA)2 and latent membrane protein (LMP)1 (Rousselet & Tursz, 1992). As mentioned previously, EBV immortalisation of B cells induces expression of CD23; CD23a and CD23b are induced by EBNA2 and LMP1, respectively, and these proteins appear to synergise to induce membrane expression of CD23 (Wang *et al.*, 1990).

The enhancing effect of IL-4 on CD23 expression can be inhibited at the protein and mRNA levels by IFN-γ, IFN-α , TGF-β and anti-CD19 antibodies (Gordon *et al.*, 1991). Signals known to synergise with IL-4 in enhancing CD23 expression are contact with cognate T cells, and engagement of sIg, CD40 or CD72 (Keegan *et al.*, 1989; Gordon *et al.*, 1991; Katira *et al.*, 1992). The engagement of CD40 appears to override the suppressive effects of IFNs, TGF-β and anti-CD19 on the IL-4-induced CD23 expression (Gordon *et al.*, 1991) and CD40 ligation induces CD23 on human B cell precursors (Saeland *et al.*, 1993). Ligation of sIgM counteracts the negative influence of IFN and TGF-β on CD23 production, and ligation of CD72 overrides inhibition of this change due to TGF-β. Thus, the expression of CD23 on B cells is a complex and tightly regulated process.

1.5.3-2 ***Other cell types:*** It has not yet been clearly established whether unstimulated T cells express CD23 but, on activated-T cells, CD23 expression is induced by IL-4; the activated phenotype of the T cells is in accordance with the observation that many HTLV-1$^+$ T cell lines express CD23 (Maekawa *et al.*, 1992). Under normal conditions, this expression is probably induced by coculture with B cells.

Human monocytes, or alveolar macrophages express little or no CD23 but in pathological conditions associated with increased IgE levels, such as allergic asthma, CD23-positive monocytes have been described (Spiegelberg, 1987). It appears that this CD23 is of the type b isoform and can be induced on normal macrophages by IL-4 (Yokota *et al.*, 1988). Unlike the situation in B cells, IFN-γ actually increases CD23 mRNA (Mayumi *et al.*, 1988) and also the release of sCD23, possibly by increasing cleavage from the cell surface (te Velde *et al.*, 1990). IL-6 may also increase CD23 expression on U937 cells in synergy with IL-4 (Boltz-Nitulescu *et al.*, 1990).

Thus, control of expression of the two CD23 isoforms is very complex and tightly regulated and may be a reflection of its multi-functional nature.

1.5.4 *Ligands for CD23 other than IgE*

The IgE-binding ability of CD23 is retained by all sCD23 fragments because they contain the lectin domain. However, the various cytokine effects of sCD23 (described later) appear to be mediated by a domain which is overlapping, but distinct from, the IgE-binding domain (Mossalayi *et al.*, 1992).

The existence of a different ligand for CD23 was suggested by the numerous reports of non-IgE related activities attributed to CD23, including promotion of B cell growth (Gordon *et al.*, 1988), germinal centre B cell survival (Liu *et al.*, 1991) and prothymocyte differentiation (Mossalayi *et al.*, 1990). A proposed role in antigen presentation (Flores-Romo *et al.*, 1990), together with the observation that CD23 is associated with the MHC class II molecule, HLA-DR on the B cell surface (Bonnefoy *et al.*, 1988), suggest that CD23 may be involved in T/B cell interactions. The discovery of a ligand for CD23 other than IgE was made by Pochon *et al.*, 1992 using recombinant CD23 incorporated into fluorescent liposomes. This group went on to show that CD21 is an alternative ligand for CD23.

CD21: Human CD21 is an approximately 140kD phosphoprotein found on B cells, some T cells, follicular dendritic cells and pharyngeal epithelial cells (Aubry *et al.*, 1992). It is a receptor for the complement degradation products, C3dg and iC3b (Weis *et al.*, 1984), IFN-α (Delcayre *et al.*, 1991) and also for the gp350/220 envelope glycoprotein of EBV (Tanner *et al.*, 1987). CD21 is part of a membrane complex on B cells which includes CD19, TAPA-1 and Leu13 and appears to form a pre-existing signal transduction complex of the B cell (Matsumoto *et al.*, 1993). As such, CD21 has been implicated in B cell activation on triggering by EBV, C3d fragments or anti-CD21 antibodies (reviewed by Aubry *et al.*, 1992).

Structurally, the extracellular domain of CD21 is composed of 15 or 16 short consensus repeats (SCR), which are repetitive units of 60 to 75 amino acids, and the amino acid sequence has 11 potential sites for N-linked glycosylation. There is a 24 amino acid transmembrane region and an intracytoplasmic region of 34 amino acids (Weis *et al.*, 1988). The interaction of CD23 with CD21 appears to be via a newly recognised, functional extracytoplasmic domain of CD21 which contains N-linked oligosaccharides (Aubry *et al.*, 1994). Using CD21 mutants with deletions of extracytoplasmic SCRs, Aubry and colleagues mapped the binding sites of a panel of anti-CD21 mAbs and then demonstrated that the strongest inhibitors of the CD21/CD23 interaction bound to SCRs 5 to 8. Point mutations of asparagine residues indicate that sugars in SCRs 5 to 8 are involved in binding to CD21; it was also demonstrated that SCRs 1 and 2 participate in the interaction (Aubry *et al.*, 1994). Interestingly, SCRs 1 and 2 are the binding sites previously described for EBV, C3d and C3g. (Lowell *et al.*, 1989; Molina *et al.*, 1991). Tunicamycin treatment of the CD21-transfected K562 cells does not completely inhibit binding of the CD23-containing liposomes suggesting that the binding site recognised by CD23 is not soley a carbohydrate structure (Aubry *et al.*, 1994). It does not appear that the contribution of SCRs 1 and 2 is via a carbohydrate structure but it may involve a conformational change. The CD23-CD21 interaction is thought to be of a low affinity that relies on oligomerisation of CD23 for binding avidity (Aubry *et al.*, 1992; Beavil *et al.*, 1992 and 1995; Dierks *et al.*, 1993).

It is not entirely clear whether all the reported functions of CD23 on B cells occur via CD21 but it has been suggested that,for example, in EBV-transformed cells, where there is increased expression of CD21 concomitant with that of CD23 (Cordier *et al.*, 1990), the CD23/CD21 interaction may well contribute to the autocrine growth stimulation of these cells (Aubry *et al.*, 1992).

CD11b / CD11c: Recent data have described the β_2-integrin molecules CD11b-CD18 and CD11c-CD18 as alternative ligands for CD23, with these interactions apparently able to regulate monocyte activation and cytokine release (Lecoanet-Henchoz *et al.*,1995). CD11b-CD18 (Mac-1) and CD11c-CD18 (p150,95) are members of the integrin superfamily; membrane glycoproteins involved in cell-cell adhesion which exist as heterodimers of distinct α subunits (M_r 17,000 and 15,000, respectively) and a common β subunit (M_r 95,000, in the case of the β_2-integrins) (Kurzinger & Springer, 1982; Corbi *et al.*, 1988). The third member of this group is leucocyte function-associated antigen-1 (LFA-1) which is found on almost all leukocytes and binds to ICAM-1. CD11b-CD18 and CD11c-CD18 are expressed on monocytes, granulocytes and some activated lymphocytes and, as well as their roles in cell-cell interactions, they are also complement receptors for C3bi (Anderson & Springer, 1987).

With respect to binding of CD23, the CD11b and CD11c α subunits appear to be involved, since LFA-1 (which shares the CD18 β subunit) does not bind CD23 (Lecoanet-Henchoz *et al.*,1995). As with binding of CD23 to CD21, the interaction with CD11b/c is dependent on sugars, but not sialic acid residues, and it appears to be a low affinity interaction. The DGR triplet of amino acids found on CD23, which in reverse orientation is a common recognition site for other integrin receptor families, does not appear to be involved in the interaction with CD11b/c since antibodies against the site do not inhibit CD23 binding. Thus, CD23 appears to be acting as a C-type lectin recognising carbohydrate and also protein structures. The result of sCD23 binding to activated monocyte is an increase in nitric oxide production along with release of IL-1β, IL-6 and TNFα - proinflammatory cytokines. (Lecoanet-Henchoz *et al.*,1995). Interestingly, sCD23 has previously been shown to potentiate the IL-1-induced secretion of IL-6 and IL-1-receptor antagonist by human monocytes, although the receptor through which it was acting was not known (Herbelin *et al.*, 1994). Thus sCD23 may help to modulate activation of monocytes in sites of inflammation.

1.5.5 *Functions of membrane-bound CD23 - with respect to B cells*

Like most receptor molecules, CD23 has multiple functions. Early studies showed that antibodies to CD23 were co-stimulatory with PMA in induction of proliferation of resting B cells (Gordon *et al.*, 1986). As the low-affinity IgE receptor, CD23 has been implicated in IgE regulation after the demonstration that, *in vitro*, specific anti-CD23 antibodies block the spontaneous release of IgE by PBL from atopic patients (Sarfati and Delespesse, 1988), as well as IgE production by IL-4 treated normal peripheral blood lymphocytes (PBL) (Pene *et al.*, 1988). Bonnefoy and colleagues, 1990, demonstrated that this inhibition is restricted to certain CD23 epitopes involved in IgE-binding; they suggest that the anti-CD23 antibodies bind to surface CD23 and interfere with the T cell/B cell interaction necessary for Ig production. T cell/B cell conjugate formation is now known to involve interaction between CD23 and CD21 (Aubry *et al.*, 1992 and 1993) and IgE production is increased on engagement of CD21 by recombinant soluble CD23 or by anti-CD21 antibody (Aubry *et al.*, 1992). Also, data from *in vivo* studies show inhibition of an antigen-specific IgE response using a polyclonal anti-CD23 antibody (Flores-Romo *et al.*, 1993). These data all suggest a central role for CD23 in IgE production with two possible mechanisms:

1) That CD23 expressed on B cells interacts with CD21 on T cells as an adhesion molecule to enhance the signals for IgE production (Fischer *et al.*, 1991; Pochon *et al.*, 1992)

2) Since triggering of CD21 on B cells enhances IgE production even in the absence of T cells (Aubry *et al.*, 1992) and allergen-activated T cells can be induced to express CD23 by IL-4 (Prinz *et al.*, 1990), it is possible that T cell-associated CD23 interacts with CD21, expressed on B cells, to enhance IgE production.

CD23 has been shown to be physically associated with the MHC class II molecule, HLA-DR, on B cells which indicates that it could act as an accessory molecule in T cell interactions with antigen-presenting cells (Bonnefoy *et al.*, 1988; Flores-Romo *et al.*, 1990). Flores-Romo *et al.*, 1990 demonstrated that occupancy of CD23 prevents B cells from stimulating allogeneic T cells, and a recent investigation showed that the CD23/CD21 interaction is required for presentation of conventional, soluble protein antigens by B cell lines to $CD4^+$ T cell clones (Grosjean, 1994). Thus, CD23 may not only strengthen B-T conjugation but simultaneously provide a co-stimulatory signal to the antigen-primed T cell.

Homotypic aggregation of B cells is thought to be important in the later stages of activation and differentiation, possibly to facilitate the exchange of autocrine factors (Bjork & Paulie, 1993). CD23 and CD21 function as adhesion molecules in homotypic aggregation of human B cells (Bjork *et al.*, 1993) and if antibodies to CD23 are used in conjunction with anti-LFA-1 then aggregation is often completely inhibited, suggesting that LFA-1/ICAM-1 and CD23/CD21 are the major molecules involved in homotypic aggregation of human B cells.

The question of homotypic interactions between CD23 molecules is subject to controversy. Several reports that CD23-transfected B lymphoma cells do not form aggregates and that neither purified recombinant CD23, nor CD23-liposomes, bind to CD23-transfected cell lines (Pochon *et al.*,1992) are in contrast to a recent report by Moulder *et al.*, 1993 where they demonstrate clustering of RPMI-8866 cells mediated solely via CD23. The consolidating factor may be that the CD23-mediated adhesion occurs via the portion of CD23 remaining on the cell surface after cleavage of sCD23. Apparently, proteolysis is required to induce CD23-mediated cell adhesion and the proteolysis does not occur in the absence of serum, unless sCD23 (which has inherent proteolytic activity) is added (Moulder *et al.*, 1993).

That CD23 can function as an adhesion molecule is consistent with its classification as a C-type lectin since other members of this family, such as CD72, are involved in endocytosis and adhesion reactions.

1.5.4 *Functions of soluble CD23 - with respect to B cells*

While the soluble fragments of CD23 retain IgE-binding activity, they have also been ascribed numerous cytokine-like functions. With respect to IgE, sCD23 has been described as an IgE regulant which, in synergy with IL-4, can upregulate IgE synthesis. This property, however, may be confined to transient intermediate cleavage fragments (Delespesse *et al.*, 1989) and thus depend on the proteolytic capabilities of the fragment.

As mentioned previously, sCD23 has been implicated as an autocrine growth factor for normal receptor-activated B cell blasts and EBV-transformed B lymphoblasts (Swendeman & Thorley-Lawson, 1987). However, the 25kDa form of sCD23 co-purifies with a 12kDa protein which may be either a further cleavage product of CD23 or else a separate protein which tightly co-purifies with CD23. The presence of this 12kDa molecule was used by Uchibayashi and colleagues to explain their contradictory observation that sCD23 has no B cell growth-promoting ability (Uchibayashi *et al.*, 1989). Using sCD23 derived from a construct containing cDNA which coded directly for the 25kDa secretory form of CD23, as opposed to a cleavage product of the membrane receptor, they demonstrated that sCD23 could bind IgE but had no B cell growth activity. Other reports disagree and support the observations of Swendeman and Thorley-Lawson (Gordon *et al.*, 1988; Armitage & Goff, 1988). To add to the confusion, in 1990, Cairns and Gordon reported that only the intact, 45kDa form of CD23, purified from lysates of RPMI 8226 cells, was consistently mitogenic for pre-activated normal and transformed B lymphoblasts. They found that isolates of sCD23 species were of highly variable efficiency in stimulation assays. In an attempt to consolidate the situation, several explanations are proposed :

1) That the BCGF activity is highly labile; thus, the BCGF activity of CD23 is present in the 45kDa form, and retained in a transient initial cleavage product (35kDa) but quickly degrades to the 25kDa fragment.

2) That the 45kDa form is actually converted to a lower molecular weight form, with growth-promoting effects. This idea is supported by the fact that stimulatory activity of 25kDa CD23 was only observed in already substantially activated cells and may explain why Uchibayashi *et al.*, 1989, (who used B cells triggered with sub-mitogenic amounts of anti-Ig) saw no growth promoting effects.

For whatever reasons these discrepancies exist, sCD23 has since been attributed with several other B-cell-related biological functions, albeit effects which are usually seen in synergy with IL-1.

Early work showed that sCD23 can promote differentiation of $CD19^{+}$ $CD20^{-}$ antibody-producing B cells (Kagan *et al.*, 1989) and then, in 1991, Liu *et al.* discovered that recombinant 25kD sCD23, in synergy with IL-1α, promotes the survival of germinal centre B cells (Liu *et al.*, 1991a). There are two distinct populations of B cells in germinal centres - rapidly dividing centroblasts, which populate the dark zone, and their progeny, centrocytes, which populate the light zone. The light zone is also home to specialised antigen presenting cells called follicular dendritic cells (FDC) and in the 'apical' light zone, but not in the 'basal' light zone, FDC have high levels of cytoplasmic CD23 (Gordon *et al.*,

1989). Freshly isolated germinal centre B cells die by apoptosis when placed into tissue culture and this process can be prevented if they are activated both through their antigen receptors and CD40 or by sCD23 and IL-1α (Liu *et al.*, 1989 & 1991a). Prevention of apoptosis provides a way to select for cells which have somatically-mutated antigen receptors with a higher affinity for antigen since they will compete for the limited antigen on the FDC (Liu *et al.*, 1989). The finding that sCD23 and IL-1α also drive B cells towards a plasmacytoid pathway of differentiation suggests a bifurcation in the development of centrocytes after rescue from apoptosis. The suggested model is that centrocytes are initially rescued from apoptosis through re-encounter with antigen, and then they develop along one of two pathways; signalling through CD40 induces memory cell development whereas sCD23 and IL-1α signal for plasmacytoid differentiation (Liu *et al.*, 1991a). Thus, sCD23 can prevent apoptosis and promote differentiation of germinal centre B cells.

It is assumed that the sCD23 in germinal centres signals B-cells through CD21 to prevent apoptosis. This assumption has the support of a recent report from Bonnefoy *et al.*, 1993 who showed that a subset of anti-CD21 antibodies prevent apoptosis of germinal centre B cells. These antibodies also promote a plasmacytoid appearance in the rescued cells and upregulate expression of the *bcl-2* proto-oncogene. However, CD21 also binds C3d, which is abundant on immune complexes held by FDC, and C3 activation is required for germinal centre formation (Klaus & Humphrey, 1977). In addition, CD21 is also a receptor for IFN-α (Delcayre *et al.*, 1991) and IFN-α can also rescue germinal centre B cells from apoptosis (Holder *et al.*, 1992). Thus, it will be difficult to determine the physiological role of these interactions and whether CD23 is, indeed, binding to CD21 or another receptor to prevent apoptosis of centrocytes. Another unanswered question is whether the CD23 on FDC acts as a membrane-anchored or a soluble molecule. It would be inappropriate for CD23 in germinal centres to influence 'nonselected' cells; thus, it may be membrane bound to localise the effects, or the highly labile nature of sCD23 may ensure it is active only in the immediate proximity of its production (Shields *et al.*, 1992).

At present there has been no role described for CD23 in the antigen-independent phase of B cell development in the bone marrow, but most of the other cytokine functions ascribed to sCD23 involve myeloid and T cell precursors suggesting a role for CD23 in haematopoiesis. It is, however, difficult to determine if the true functions would be performed by soluble or membrane-bound CD23.

1.5.6 *CD23 expression and function in the bone marrow and thymus*

Fourcade *et al.* (1992) examined the expression of CD23 by human bone marrow stromal cells. They showed that freshly isolated BM-cells have very low levels of CD23 expression but a subset of long-term BM-culture (LTBMC)-derived stromal cells express

CD23 mRNA at high levels and secrete soluble CD23 into their culture supernatants. The adherent stromal cells in LTBMCs are a heterogeneous population consisting of mesenchymal cells (mainly fibroblasts) and monocytes/macrophages (Fourcade *et al.*, 1992). It appears that only a subset of these cells, probably the monocytes/macrophages express high levels of CD23 on their surface but it is possible that these cells are activated by the *in vitro* culture conditions. However, Fourcade and colleagues demonstrated that addition of neutralising anti-CD23 antibodies to LTBMC leads to a significant decrease in total numbers of haematopoetic cells and CFU-GM recovery (Fourcade *et al.*, 1992). Also, proliferation and maturation of human BM-derived $CD34^+$ myeloid precursors is induced following sequential incubation with IL-1α and sCD23. The sCD23 induced colonies are composed of 20-30% basophilic cells, which could have implications during the allergy process (Mossalayi *et al.*, 1990a). Thus, CD23 expression by human stromal cells appears to regulate haematopoietic cell development and sCD23 has an effect on myeloid precursors, although whether physiological effects are mediated through membrane bound or soluble CD23 is not clear.

Previous work has indicated an effect of sCD23 on human T-cell precursors. Mossalayi *et al.* (1990b) showed that $CD7^+CD2^-CD3^-CD4^-CD8^-$ precursor T cells, which appear to represent recent thymic immigrants, can be induced to mature by sequential incubation with recombinant IL-1α and recombinant sCD23. Le *et al.*, 1987, have documented IL-1 production by human thymic epithelium after *in vitro* incubation with autologous thymocytes. The source of CD23 in the thymus is thought to be thymic epithelial cells in the outer cortex region (Mossalayi *et al.*, 1991) - the point of entry for early thymocytes (Janossy *et al.*, 1981). Of interest, *in situ*, cortical epithelial cells also express IL-4 receptors, thus CD23 expression by these cells may be increased by IL-4 (Mossalayi *et al.*, 1991).

Soluble CD23, in synergy with IL-1α, also increases the proliferation of mature $CD4^+$ T cells in response to mitogens such as PHA (Bertho *et al.*, 1991). Membrane-bound CD23 may also contribute to $CD4^+$ T cell proliferation by enhancing adhesion between antigen-presenting cells and $CD4^+$ T cells, possibly because CD23 is linked to HLA-DR (Bonnefoy *et al.*, 1988).

The role of CD23 in haematopoiesis was recently disputed by Fujiwara and colleagues, 1994, who demonstrated that in CD23-knockout mice, there is normal lymphocyte differentiation and the mice can mount normal IgE responses. Germinal centre formation and the *in vitro* response of B cells are not affected in the mutant mice. The only phenotype that they noted is a severe impairment of antigen-specific IgE-mediated enhancement of the antibody response. This controversy may, however, be explained by redundancy in the immune system or by differences between murine and human CD23. As

discussed previously, the expression of CD23 differs, with mice lacking the human CD23b isoform, and murine CD23 is a larger molecule which lacks the 'RGD' domain. Bartlett and Conrad (1992) in their article entitled 'Murine soluble FcεRII: a molecule in search of a function' discuss the fact that they were unable to repeat any of the assays for sCD23 function observed in the human system with murine sCD23. They suggest that differences in ability of human and murine sCD23 to bind IgE could reflect a broader difference in function. In the mouse, cleavage of sCD23 from the cell surface removes regions of the stalk which are required for IgE binding as well as for oligomerisation (Dierks *et al.*, 1993 & 1995). It is possible that formation of multimers is important for biological activity of sCD23.

Thus, there appear to be differences between human and murine CD23 which have caused controversy about the *in vivo* role of CD23. However, there is overwhelming evidence that human CD23 is a biologically active molecule which can function both as an adhesion molecule and as a soluble cytokine. This is reflected by the implication of CD23 in several disease states.

1.5.7 *The role of CD23 in disease*

CD23 has been implicated in various pathological conditions, including allergy, rheumatoid arthritis and B-chronic lymphocytic leukaemia, mainly due to the presence of elevated serum levels of sCD23.

Allergic diseases: Atopic diseases are associated with production of specific IgE which appears to interact with the high affinity IgE receptor (FcεR1) present on mast cells and basophils. Cross-linking of IgE by allergen activates this receptor, leading to degranulation of the cells and release of various inflammatory mediators, including cytokines such as IL-4 and IL-5, and to immediate allergic symptoms (Dugas *et al.*, 1992). CD21 expressed on basophils is involved in histamine release triggered by CD23, suggesting that CD23 may have a direct role in mediator release (Lecoanet-Henchoz *et al.*, 1995). However, these mediators could also play a role in the late allergic reaction since IL-4 can induce CD23b expression on various cells including monocytes and macrophages, allowing these cells to bind IgE and thus release more inflammatory cytokines. Indeed, the number of CD23-positive peripheral blood monocytes and B cells is increased in atopic individuals compared to normal subjects (Melewicz *et al.*,1981; Spiegelberg, 1987; Borish *et al.*, 1990). Serum sCD23 levels are reported to be significantly higher in atopic compared to normal individuals (although this has recently been disputed (Wilhelm *et al.*, 1994)) and are weakly but significantly correlated with levels of IgE (Yanagihara *et al.*, 1990). Both soluble and membrane forms of CD23 could contribute to the pathogenesis of atopic disease by: 1) regulation of IgE production (Sarfati *et al.*, 1984 & 1988; Pene *et al.*,1988; Flores-Romo *et al.*, 1993; 2) direct

interaction with CD21 to induce histamine release from basophils (Bonnefoy *et al.*, 1993); 3) IgE-dependent release of inflammatory mediators by monocytes, eosinophils etc.; 4) activation of monocytes and macrophages via the CD11-CD18 family of intergrins and the consequent release of inflammatory cytokines (Lecoanet-Henchoz *et al.*, 1995).

Rheumatoid arthritis: Patients with rheumatoid arthritis (RA) have an increased number of circulating CD23-positive B cells. Also, elevated levels of sCD23 have been discovered in the serum and synovial fluid of these patients (Ikizawa *et al.*, 1993; Bansal *et al.*, 1993). It has been suggested that $CD5^+$ B cells of RA patients may be specifically activated leading to the constitutive expression of CD23a mRNA and accelerated release of sCD23 (Ikizawa *et al.*, 1993). That sCD23 plays a role in arthritis is supported by data from Plater-Zyber and Bonnefoy (submitted) who demonstrated that administration of anti-CD23 IgG in a murine arthritis model results in amelioration of established disease.

Chronic Lymphocytic Leukaemia: Chronic lymphocytic leukaemia (CLL) is defined as the proliferation and accumulation of monoclonal $CD5^+$ B cells "arrested" at the mature $sIgM^+/sIgD^+$ stage of differentiation (Sarfati, 1993). Sera from CLL patients can contain up to 500-fold more sCD23 than that from normal individuals or patients with other B cell lymphoproliferative disorders (Sarfati *et al.*, 1988). As well as increased numbers of CD23-positive B cells, B-CLL cells also overexpress CD23 on their surface and TNFα enhances the expression but has no effect on normal B lymphocytes (Sarfati, 1993). It has been proposed that deregulation of CD23 expression may not be simply a marker of CLL but may also be involved in the proliferation of the leukaemic B cells (Sarfati, 1993). Cross-linking of CD23 by anti-CD23 antibodies suppresses normal and leukaemic B cell proliferation (Fournier *et al.*, 1992; Luo *et al.*, 1991) with consequent biochemical changes compatible with cell activation (Kolb *et al.*, 1990). However, it is also possible that sCD23 could act to allow the CLL cells to proliferate as is the case with EBV-transformed B cells. The balance of CD23a and CD23b forms, induced by cytokines such as IL-2, IL-4 and IFN-γ, may be important in CLL proliferation allowing accumulation of long-lived and slowly-dividing malignant B cells arrested at the G0/G1 stage of the cell cycle (Sarfati, 1993).

The clinical significance of CD23 in these cases is not absolutely clear, however, there is strong circumstantial evidence that CD23 plays role in the pathogenesis of these diseases and is clinically important.

1.6 *Apoptosis - Programmed cell death*

Views on cell death were, until relatively recently, dominated by the idea that death was a degenerative phenomenon caused by injury (Virchow, 1858). The fact that cell death can occur as a controlled event in normal, healthy animals and, moreover, that this 'normal cell death' might be a process of active self destruction was not highlighted until 1972, by Kerr and colleagues. It is now possible to categorise most dying cells into one of two discrete and distinctive patterns of morphological change; either *necrosis* or *apoptosis*. The first of these, *necrosis*, is characterised by cell swelling, rupturing of plasma and organelle membranes and finally disintegration of the cell (Wyllie *et al.*, 1980). This is usually caused by severe and sudden injury e.g. hyperthermia, or physical or chemical trauma, which results in an inability of the cell to maintain homeostasis and subsequently uncontrolled osmotic pressure causes cell swelling. After rupture of the cell, the contents are spilled into the surrounding tissue provoking an inflammatory response which may aid removal of debris and repair of the damaged tissue (Cohen & Duke, 1992).

Apoptosis involves a more ordered cell death mechanism. It is associated with normal processes of tissue regulation and maintenance of a steady-state condition, since if cell division is not exactly matched by cell death, the tissue or organ will enlarge - representing uncontrolled growth i.e. neoplasia. This is of particular importance in the human immune system where, for example, 5×10^9 polymorphonuclear leukocytes (neutrophils) are in circulation at any given time. Neutrophils have a life expectancy of only one day after which they undergo apoptosis, apparently under the control of an "internal clock", allowing numbers of neutrophils to remain remarkably constant (Cohen & Duke, 1992). Some workers refer to apoptosis as programmed cell death but that term may be more appropriately used to describe situations, for example in invertebrates, where the death of individual cells can be accurately predicted (Ellis & Horvitz, 1986; Malayapa & Sawada, 1991). In vertebrates, most demonstrations of apoptosis are more random with respect to the individual cells affected, even though the internal death programme they follow is ordered. Examples of apoptosis include morphogenetic death of cells during embryonic development, elimination of self-reactive T and B cells and death of short-lived cells such as neutrophils (Cohen, 1993). In each case, the trigger for apoptosis is probably different, for example, withdrawal of a growth factor or some form of internal clock, but the final mechanism appears the same. Not all apoptosis-inducing signals seem physiological, for example, thymocytes will apoptose if exposed to ionising radiation (Sellins & Cohen, 1987), or to certain cytotoxic drugs (McConkey *et al.*, 1988). It is possible that these situations reflect a broader role for apoptosis - removal of damaged cells which may have harmful mutations.

1.6.1 *The role of apoptosis in B cell development*

During B cell development there are several stages of differentiation at which cells are susceptible to apoptosis.

Pro-B: When a pro-B cell undergoes Ig gene rearrangement there is a significant chance that the rearrangement will be non-productive. It has been estimated that about 75% of developing B cells are eliminated at the transition from the pro-B cell to the small pre-B cell stage (Osmond *et al.*, 1992). It is assumed that the remainder of the cells die, probably by apoptosis - a view supported by the presence of macrophages in the bone marrow which phagocytose apoptotic cells (Cohen & Duke, 1992).

Immature B cell: The next stage of development where B cells are subject to apoptosis is at the immature B cell stage when they begin to express surface immunoglobulins. These cells are subject to clonal deletion by antigen to avoid expansion of anti-self reactive B cells - a process termed negative selection (Nossal, 1983). This has been clearly demonstrated using mice bearing transgenes for rearranged Ig because when the mice co-express the cognate antigen, they have very few B cells (Nemazee & Burki, 1989a and 1989b). That this deletion occurs by apoptosis has not been shown conclusively but cell lines "frozen" at the immature B cell stage can be induced to apoptose by anti-IgM antibodies (Benhamou *et al.*, 1990). It will be interesting to determine the differences in signal coupling between immature and mature B cells which determine that immature cells apoptose and mature cells proliferate in response to the same stimulus.

Germinal centre B cells: B cells are also subject to apoptosis in germinal centres, where antigen-driven positive selection occurs. To produce high affinity antibodies during secondary responses to T-cell-dependent antigens, the Ig genes undergo a process referred to as somatic hypermutation. Hypermutation occurs in rapidly dividing centroblasts within the dark zone of the germinal centre and these cells subsequently move to the light zone and become non-dividing centrocytes which are subject to selection on the basis of their ability to receive a positive signal from antigen (MacLennan & Gray, 1986). If the centrocytes do not receive the positive signal then they rapidly die by apoptosis. The signal which saves centrocytes from apoptosis is delivered via the antigen receptors and the surface antigen, CD40, and causes differentiation to resting, memory B cells (Liu *et al.*, 1989). Apoptosis of centrocytes is also prevented by a combination of soluble CD23 and IL-1α, which induce a plasmablast phenotype (Liu *et al.*, 1991). This whole process allows selection of B cells expressing mIg with high affinity for the antigen and elimination of cells with low affinity for the antigen and, potentially, mutations which could result in specificity for self.

Therefore, apoptosis is of extreme importance in the immune system to avoid development of leukaemia or autoimmune disease. This theory was described by John Cohen as "altruistic suicide" where the cell's suicide insures the gamete's survival (Cohen, 1993). Indeed, apoptosis was the ancient Greek term for leaves falling off trees in the autumn and the word was chosen to suggest that cell loss is desirable for the survival of the host (Touchette & Fogle, 1991).

It has become obvious that apoptosis induced by different stimuli may proceed through different pathways. In the case of glucocorticoid-induced and activation-induced apoptosis, each mode of induction mutually inhibits the apoptosis induced by the other (Zacharchuk *et al.*, 1990) but all apoptotic death can be characterised both morphologically and biochemically.

1.6.2 *Morphology of apoptotic cells*

It is still not clear exactly which of the numerous morphological changes in apoptotic cells are directly associated with death. One of the first features is that the cells appear to shrink, possibly due to structural changes in the cytoskeleton. The plasma membrane becomes 'untethered' and undergoes rapid blebbing, or "zeiosis" - a characteristic sign of apoptosis (Cohen & Duke, 1992). Amenta *et al.*, 1989, described a generalised increase in the level of internal proteolysis in apoptotic cells which may lead to non-specific degradation of the cytoskeleton. Another hallmark of apoptosis is the collapse of the nucleus; the other organelles remain intact but are compacted by cytoplasmic "shrinking". The overall compaction of the cytoplasm is associated with development of translucent cytoplasmic vacuoles (Wyllie *et al.*, 1980). The nucleus shrinks and its chromatin becomes very condensed often collapsing into crescents around the nuclear envelope before, in some cell types, finally forming very dense spheres. At this stage, cells may break up into membrane-bound apoptotic bodies which are still in a state of osmotic balance and able to exclude vital dyes. Some investigators believe that formation of apoptotic bodies helps ingestion of the apoptotic cell by phagocytes (Cohen, 1993).

Biochemical Changes: the timing of biochemical events in relation to morphological changes is not fully understood. Synthesis of RNA and protein appears to decrease rapidly with a concomitant increase in their degradation (Cidlowski, 1982). However, there are reports which demonstrate a requirement for new gene expression in the apoptotic process and indeed, in these systems, apoptosis can be blocked if mRNA or protein synthesis are inhibited. Examples include apoptosis induced by growth factor removal, and activation-induced apoptosis in T cell hybridomas (Shi *et al.*, 1992). These models have been referred to as "induction" mechanisms and led to the idea of "death genes" which are activated during apoptosis (Cohen, 1993). A candidate 'death gene' is transglutaminase (Owens *et al.*, 1991). Another model, referred to as "release", has been described for the

situation where, for example, HL-60 cells are triggered to apoptose by the inhibition of mRNA or protein synthesis (Martin *et al.*, 1990). The cells respond as if the suicide program or "death genes" are constitutively expressed but held in check by survival factors with short half-lives.

The condensation of chromatin indicates that apoptosis results in extensive damage to DNA. DNA exists as double strands wound around histones to form nucleosomes. One of the original demonstrations of apoptosis in thymocytes showed that chromatin is degraded by random cleavage at the linker regions between nucleosomes (Wyllie, 1980). Since nucleosomes are spaced regularly at 180-200 base pair intervals along eukaryotic chromatin, a characteristic pattern of DNA cleavage into multiples of 200 bp oligonucleosome fragments (a DNA ladder) is produced on gel electrophoresis of DNA from apoptotic cells. This internucleosomal cleavage of DNA is considered to arise enzymatically from the activation of an endonuclease(s). Cohen and Duke, 1984, showed that thymocytes contain detectable endogenous endonuclease activity which, when activated in isolated nuclei by addition of Ca^{2+} and Mg^{2+}, forms 180bp nucleosomal fragments indistinguishable from those seen *in vivo*. Whether this is the precise enzyme involved in apoptosis is not clear and there are numerous other enzymes purported to have similar effects. Recently, it has been suggested that the endonuclease initiating apoptosis may be NUC-18 (Caron-Leslie *et al.*, 1991), DNase I (Ucker *et al.*, 1992; Peitsch *et al.*, 1993) or DNase II (Barry & Eastman, 1993). The relationship between the appearance of condensed chromatin and the endonucleolytic cleavage of DNA is still uncertain. A number of studies have shown cell types where there is chromatin condensation but no evidence of a classical DNA ladder, and flow cytometric analysis of apoptotic thymocytes has suggested some changes in chromatin structure which precede the initiation of internucleosomal DNA cleavage (Oberhammer *et al.*, 1993).

Conventional gel electrophoresis of DNA, which shows the laddering pattern of cleavage in apoptosis, is restricted to analysis of DNA fragments of approximately 20 kbp and below. Walker *et al.* (1991) showed that using field inversion gel electrophoresis (which allows analysis of fragments up to 2 Mbp) they could detect distinctive fragment of 50 and 300 kbp in apoptotic thymocytes. This work involved the use of DNA-damaging drugs and it has been suggested that the 300 kbp fragments were formed by the interaction of the drugs with topoisomerase II molecules located at approximately 300 kbp along chromatin (Filipski, 1990). However, the 50 kbp fragments appear to be generated by endonucleolytic activity at the sites of the anchorage of loop domains of chromatin to the nuclear membrane and matrix (Oberhammer *et al.*, 1993). Subsequently, this group have shown that in epithelial cells, cleavage of DNA to 300 and/or 50 kbp fragments occurs before or even in the absence of internucleosomal fragmentation. This supports the idea that the degradation of DNA to oligonucleosome fragments is a late event, or even absent

in apoptosis. DNA degradation may serve to limit potentially dangerous viral genetic information from spreading from dying cells (Sellins & Cohen, 1989).

1.6.3 *Genes involved in apoptosis*

As mentioned previously, there are cases where the *de novo* expression of mRNA is required for apoptosis to occur i.e. the cells actively "commit suicide". Several genes have been the subject of interest regarding a possible role in apoptosis including *c-myc*, *Fas* and *p53*.

p53: has been termed an anti-oncogene and is associated with apoptosis. The *p53* gene product is a transcription factor and may downregulate expression of genes, such as *c-fos* and *c-jun*, but induce expression of other genes such as WAF1/p21-a potent inhibitor of cyclin-dependent kinases (Prokocimer & Rotter, 1994; El-Diery *et al.*, 1994). Thus, *p53* arrests proliferation of cells, possibly switching the cell to differentiation, but more likely, the role of *p53* is to hold a damaged cell in G1 while the damage is repaired. Cells which try to oppose the G1 block may activate the apoptosis pathway (Cohen, 1993). Mutations in *p53* are found in almost 50% of human primary tumours and these mutated products are inactive with respect to arresting cell growth (Prokocimer & Rotter, 1994). In some reports of Abelson murine leukaemia virus (Ab-MuLV)-transformed pre-B cells, the *p53* gene is inactivated by integration of viral sequences into the *p53* gene (Wolf *et al.*, 1983). However, the expression of wild type *p53* can occur in transformed cells but it is inactive due to complex formation with viral proteins, for example, large T antigen of SV-40 or EBNA of Epstein-Barr virus (Levine & Momand, 1990; Szekely *et al.*, 1993). Apoptosis involving induction of *p53* is mainly associated with stress or exposure to agents which damage DNA (El-Diery *et al.*, 1994).

c-myc: the *c-myc* proto-oncogene appears to have a role in regulating the choice between cell proliferation and apoptosis. *c-myc* is a potential candidate for a gene able to influence the induction of apoptosis since its induction is an early response to mitogenic stimuli from multiple growth factors, including IL-3, and its expression diminishes as cells enter a quiescent state (Dean *et al.*, 1987: Cleveland *et al.*, 1989). Askew *et al.* (1991) used an IL-3-dependent myeloid precursor cell line, which was incapable of autonomous growth, to demonstrate that constitutive expression of murine *c-myc* suppresses G1 arrest and accelerates apoptosis on withdrawal of IL-3. Overexpression of *c-myc* may override cell cycle regulatory controls via constitutive induction of the ornithine decarboxylase (ODC) gene required for entry into S-phase (Bowlin *et al.*, 1986). Apparently, *c-myc* can only provide a partial proliferative signal to the cells which is insufficient to replace the additional mitogenic stimuli provided by IL-3, or other growth factors.

In 1992, Evan and colleagues demonstrated that fibroblasts, which usually undergo growth arrest in low serum, are unable to arrest growth if they constitutively express *c-myc*. In addition, the cells die by apoptosis, the amount of death correlates with the level of *c-myc* expression and death occurs at various points in the cell cycle. Activation-induced apoptosis of T-cell hybridomas is prevented by antisense oligonucleotides corresponding to *c-myc* but this treatment does not inhibit another outcome of activation - the production of lymphokines (Shi *et al.*, 1992). With respect to the observation that dexamethasone can inhibit activation-induced apoptosis, this may be due to the ability of DMSO to inhibit *c-myc* expression (Yuh & Thompson, 1989). Finally, a recent paper, by Cherney and colleagues, suggests a role for deregulated *c-myc* expression in apoptosis of EBV-immortalised B cells (Cherney *et al.*, 1994). These cells, if deprived of autocrine growth factors, undergo apoptosis. It was shown that, unlike normal cells which down-regulate *c-myc* expression, the EBV-immortalised cells have levels of *c-myc* comparable to those found before factor starvation. The cells continue to proliferate until death, which occurrs randomly throughout the cell cycle. Anti-sense oligonuclotides to *c-myc* prevent apoptosis of the starved cells and suppress growth of non-starved EBV-immortalised cells. One suggestion made by these investigators was that the expression of *c-myc*, by promoting proliferation of the cells despite factor deprivation leads to faster consumption of essential growth factors present in limiting concentrations. They suggest that antisense to *c-myc*, which inhibits cell growth, leads to a decreased demand for the essential factor and thus limit or delays cell death.

The *c-myc* protein has two distinct properties:
1) It is a DNA binding protein, and after forming a heterodimer with other proteins, such as MAX, it can regulate gene transcription (Shi *et al.*, 1992; Fanidi *et al.*, 1992)
2) It can bind to retinoblastoma protein, which in turn regulates cell cycle events through cyclins (Rustgi *et al.*, 1991).

It is not entirely clear exactly which function is most important in induction of apoptosis and although expression of *c-myc* is necessary for activation-induced apoptosis it does not appear to be sufficient.

The induction of *c-myc* in cells grown in the presence of appropriate growth factors (or co-expressing other "survival" genes, such as *bcl-2*) causes cell proliferation. In the absence of these factors, *c-myc* expression causes cell death.

Fas: the *Fas* antigen, also known as APO-1 or CD95, is a 36kD transmembrane receptor that is a member of the growing tumour necrosis factor (TNF) receptor/ nerve growth factor (NGF) receptor superfamily, along with CD40. Cells which express *Fas* can be induced to apoptose by cross-linking with anti-*Fas* antibody (Itoh *et al.*, 1991). The *Fas*

Ag is found on numerous cell types, including activated T-, B- and NK cells, thymocytes, myeloid cells and fibroblasts (Eischen *et al.*, 1994). In the thymus, *Fas* antigen is purported to have an important role in differentiation and maturation by inducing apoptosis. Peripheral activated T cells from *lpr* (lymphoproliferation) mutant mice have a reduced number of APO-1 receptors and a defect in TCR-induced apoptosis (Watanabe-Fukunaga *et al.*, 1992). It is possible that their lymphoproliferation is more correctly "lymphoaccumulation" because the cells die less readily than normal T cells (Cohen, 1993). The involvement of *Fas* in T cell apoptosis has been supported recently by two reports demonstrating that TCR crosslinking induced *Fas*, and *Fas* ligand expression, with the ensuing engagement of *Fas*/*Fas* ligand activating the cell death programme (Dhein *et al.*, 1995; Shyr-Te *et al.*, 1995). This cell death could be prevented by a soluble *Fas*-Ig fusion protein or F(ab')2 anti-*Fas* which are unable to induce apoptosis because of insufficient crosslinking of *Fas*. *Fas* ligand, produced in a soluble form by Jurkat T cells, can bind to cell surface *Fas* in an autocrine fashion - with apoptosis occurring even in single cell cultures but it is also possible that T-cell apoptosis may occur as 'fratricide' by interaction of a membrane bound form of the *Fas* ligand with *Fas* expressed on neighbouring cells (Dhein *et al.*, 1995). These data strongly implicate *Fas* and *Fas* ligand as "death genes" whose interactions account for the apparently "autocrine suicide" mechanism of activation-induced T-cell death. This correlates with the requirement for RNA and protein synthesis in T cell apoptosis. Interestingly, adult T-cell leukaemia cells, which have an activated T-cell phenotype, express high levels of functional *Fas* antigen raising the possibility of anti-*Fas* antibody treatment in this form of leukaemia (Kotani *et al.*, 1994).

One possibility for pharmacological intervention in tumours is drugs which can induce apoptosis. It is possible that resistance of certain neoplastic cells to apoptosis could explain the inherent drug resistance of many tumours. Numerous gene products have been implicated in prevention of apoptosis, including A20, a zinc finger protein. A20 is inducible by TNF, IL-1 and cross-linking of CD40 on B cell lines (Tewari *et al.*, 1995) resulting in a TNF-sensitive fibroblast cell line becoming resistant to TNF-induced apoptosis (Opipari *et al.*, 1992); it also prevents apoptosis of serum-deprived B cell lines. Expression of A20 is induced by the latent membrane protein 1 gene product of EBV (Laherty *et al.*, 1992) and may play a role in the resistance of these cells to apoptosis.

Another molecule which is thought to play a major role in rescue of cells from apoptosis is *bcl-2*.

bcl-2: the putative oncogene *bcl-2* is juxtaposed to the Ig heavy chain locus by the t(14:18) chromosomal translocation typical of human follicular B-cell lymphomas (Fukuhara *et al.*, 1979). The *bcl-2* gene product is not altered by the translocation but its expression is deregulated leading to constitutively high levels of protein (Cuende *et al.*, 1993). *Bcl-2* has properties of an 'anti-apoptosis' gene; for example, when transfected into an IL-3-dependent B cell line, on removal of IL-3 the cells do not cycle but are suspended at G_0/G_1, when they would normally undergo rapid apoptosis (Marvel *et al.*, 1994). Experiments carried out in Chinese hamster ovary (CHO) cells transfected with *c-myc* show that elevated *c-myc* expression resultsin cell death but that cells co-expressing *bcl-2* are relatively resistant to *c-myc* induced death (Merino *et al.*, 1994). A 'two signal' model may exists where *c-myc* can provide the first signal, leading either to apoptosis or to proliferation. Certain growth factors may provide a second signal, to inhibit apoptosis and allow *c-myc* to drive the cells into cell cycle. It is suggested that *bcl-2* may substitute for the putative survival signal. This would provide a mechanism whereby cells can express *c-myc* without undergoing apoptosis, and may explain why some investigators have shown a synergistic relationship between *bcl-2* and *c-myc* in cell transformation (Strasser *et al.*, 1990).

As well as preventing apoptosis through regulation of cell cycle events, two other models for bcl-2 function are: enhancement of antioxidant function and resistance to apoptosis in instances of oxidative stress; and regulation of enzymes e.g. interleukin-1β-converting enzyme (ICE) alleged to be involved in a protease cell death pathway (Hockenbery, 1995).

1.6.4 *Bcl-2 expression during B cell development*

As mentioned previously, apoptosis occurs at several stages throughout B cell development; as pro-B cells rearrange their Ig genes, immature B cells express their surface Ig and in germinal centres after centroblasts have undergone somatic hypermutation. At all these stages the cells appear to be susceptible to apoptosis as a safety measure to ensure that B cells which do not have a normal phenotype or are specific for self, are removed. The molecular signals which control B cell survival are largely unknown but Merino *et al.*,1994, suggest that *bcl-2* expression may play a major role. They demonstrated that there is developmental regulation of *bcl-2* protein in B cells with high levels present in pro-B and mature B cells but down-regulation at the pre-B and immature B cell stages of development. In their hands, susceptibility to dexamethasone-induced apoptosis correlates with low levels of *bcl-2* and this susceptibility is reversed by transfection of the cells with *bcl-2*. It has been shown previously that *bcl-2* expression is absent or diminished in germinal centres (Pezzella *et al.*, 1990; Hockenbery *et al.*, 1991) and cross-linking of surface Ig, CD40 antibody and sCD23 plus IL-1α (signals known to prevent apoptosis of these cells) all cause germinal centre cells to express *bcl-2* protein

(Liu *et al.*, 1991). These data suggest that *bcl-2* is a physiological signal controlling B cell death. It could act to protect pro- and mature B cells from a variety of apoptosis-inducing signals or, conversely, down-regulated expression at specific stages may facilitate apoptotic death of non-functional or anti-self B cells.

However, in addition to *bcl-2*, three recently described genes, *bcl-x*, *bax*, and *bak* appear to control cell death and *bcl-2* function (Boise *et al*, 1993; Oltavi *et al.*, 1993; Farrow *et al.*, 1995); thus, regulation of expression of these genes may also have a role in determining susceptibility to apoptosis at different stages of development.

1.6.5 *The bcl-2 gene family*

Briefly, *bcl-x* is a *bcl-2*-related gene which exists in two forms, *bcl-*x_L (large) and *bcl-*x_S (small). The large form appears to function like *bcl-2* to inhibit apoptosis, whereas the small molecule inhibits the ability of *bcl-2* to enhance survival of growth factor-deprived cells. *Bcl-*x_S mRNA is expressed at high level in cells with a high rate of turnover, such as immature thymocytes undergoing negative selection. In contrast, *bcl-*x_L is found in mature, post-mitotic neural structures in the absence of *bcl-2*. Thus, *bcl-x* may have an important function in both positive and negative regulation of apoptosis (Boise *et al.*, 1993).

Bax, a *bcl-2* homolog, is able to dimerise with itself and with *bcl-2*. Korsmeyer and colleagues (1993) demonstrated that overexpression of *bax* promotes apoptosis and thus it is a putative "death gene". It has recently been demonstrated that *bcl-2* must heterodimerise with *bax* to function (Yin *et al.*, 1994). It appears that the ratio of *bcl-2* to *bax* may predetermine the susceptibility of a cell to a given apoptoic stimulus since the presence of *bax* homodimers would promote death but if *bcl-2* levels were high, and *bcl-2*/*bax* heterodimers existed, the cell would survive.

Finally, *bak* or '*bcl-2* homologous antagonist/killer' adds greater complexity to the issue. This protein is found in several cell types and, if overexpressed, it accelerates apoptosis induced by growth-factor withdrawal. It appears that *bak* may act in a similar fashion to *bax* but by binding to *bcl-*x_L and not to *bcl-2* (Farrow *et al.*, 1995).

In the WEHI-231 B cell lymphoma, anti-membrane IgM-induced apoptosis is thought to be a model for clonal deletion of immature B cells by antigen. Cuende *et al.* (1993) showed a specific down-regulation of both *bcl-2* RNA and protein on apoptosis induced by anti-IgM. However, they failed to show rescue of the cells from apoptosis by stable transfection of *bcl-2*, although the transfected cells were more resistant to death induced by heat-shock. These observations, along with others, indicate that there are *bcl-2*-dependent and -independent mechanisms of apoptosis. The reason that *bcl-2* cannot

protect in every setting may be partly explained by competing interactions among the expanding *bcl-2* family which could set switches in individual cells that dictate resistance or suscepibility to 'death' signals. It is also possible that some stimuli, such as the *Fas*-ligand-receptor interaction, are so strong they may break through the checkpoint (Oltavai & Korsmeyer, 1994). Thus, regulation of apoptosis appears to be very complex, involving numerous induction signals and multiple interacting and opposing gene products which promote either cell death or survival.

1.7 *Acute Lymphoblastic Leukaemia*

As discussed above, lymphocyte populations are subject to selection through apoptosis at various stages of their development, and it has become apparent that aberrations in the capacity to undergo apoptosis can lead to the formation of tumours. African Burkitt lymphoma (BL) and follicular centre cell (FCC) lymphoma are two such examples, thought to arise from germinal centre B cells. Constitutive activation of *bcl-2*, brought about by chromosomal translocation (14;18) of *bcl-2* to the Ig heavy chain gene, probably contributes to the inability of FCC cells to undergo apoptosis. Burkitt lymphoma cells also have deregulated *bcl-2* expression possibly brought about by the latent membrane protein-1 of resident EBV (Henderson *et al.*, 1991). Some confusion may arise, however, because FCC lymphoma can sometimes undergo a leukaemic blast crisis, with misdiagnosis of the tumour as acute lymphoblastic leukaemia. (Kramer *et al.*, 1991).

Acute leukaemia, although a rare disease, is a major form of malignancy in early B cell development, and is the most common form of childhood cancer. It is characterised by an arrest of lymphocyte maturation and the subsequent accumulation of these cells in the bone marrow, blood and other tissues (reviwed by Pui *et al.*, 1993). Traditionally, acute leukaemias are classified according to their morphological and/or cytochemical features and are named according to the assumed normal counterparts which they most closely resemble. Greaves *et al.*, 1981 originally postulated that leukaemic lymphoblasts are the neoplastic counterparts of normal lymphopoietic progenitors "frozen" at various stages of development.

Classification of childhood B-ALL depends on identification of several B cell differentiation antigens such as CD19, CD22, CD10 and the state of *VpreB* expression (Bauer *et al.*, 1991) and Ig gene rearrangement, although there are leukaemias termed null-ALL because they expess neither T or B cell markers. Null-ALL cells are thought to represent tumours of stem cells which are not yet committed to the lymphoid lineage, and they are often $CD34^{+}$. CD10, also known as the common ALL antigen (CALLA) was among the first differentiation antigen used in classification of acute leukaemias but CD10-negative acute leukaemias do exist (Pui *et al.*, 1993).

Early pre-B-ALL cells account for about 60% of ALL and tend to represent a more favourable outcome than pre-B ALL (Crist *et al.*, 1985). The pre-B immunophenotype accounts for about 25% of childhood ALL and 94% of cases are $CD10^+$. B-ALL refers to acute leukaemia of a more mature B cell stage, which is sIg^+, and represents approximately 2% of childhood ALL. In adult ALL there is a high frequency of T-cell (found in the anterior mediastinum) and CD10-negative B-lineage ALL phenotypes (Thiel *et al.*, 1980).

As with normal haematopoiesis, the majority of leukaemic cells are thought to descend from a relatively small number of progenitor cells with a high proliferative activity. In most cases the leukaemic blast cells do not actually divide once they leave the original mass and enter the blood (Lowenberg & Touw, 1993) and, although the cells may be able to proliferate on addition of exogenous mitogens, they do not usually differentiate any further (Salem *et al.*, 1989). Some myeloid cell lines, for example HL-60, can be induced to differentiate by addition of GM-CSF or G-CSF *in vitro* (Begely *et al.*, 1987), with the cells progressively losing self-renewal capabilities until they are terminally-differentiated, when they undergo apoptosis (Yamaguchi *et al.*, 1991).

Acute leukaemia is an extremely heterogeneous group of disorders, both at the clinical and biological level and numerous mechanisms have been implicated in the disease process. It has been suggested that infection by bacteria or viruses *in utero* or early infancy may greatly increase the chance of developing leukaemia (Greaves & Alexander, 1993). It is possible that viruses may activate proto-oncogenes which can then transform cells from the normal to the malignant phenotype. The majority of B-cell leukaemic lymphoblasts have non-productive Ig gene rearrangements of heavy or light chains and this is thought to hinder normal development (Greaves, 1986). However, there must be other genetic defects present in the cells since non-productive Ig gene rearrangements usually result in cells undergoing apoptosis and leukaemic cells are able to survive. The mechanisms of leukaemic cell survival are not entirely clear but may involve deregulated growth factor production or changes in the ability to receive growth factor receptor signals i.e. increased numbers of receptors, expression of receptors not usually present on the cells, increased affinity of cell surface receptors or deregulation of intracellular signalling pathways. Examples of some of these mechanisms will be discussed below.

More that 90% of childhood ALL cases have clonal chromosomal rearrangements, with the karyotypic abnormalities more commonly found in B-lineage leukaemias than in those of T cell origin. There are an increasing number of apparently non-random chromosomal abnormalities associated with specific immunophenotypes, suggesting a relationship to leukaemogenesis (Pui *et al.*, 1993).

1.7.1 *Translocations*

Up to 50% of childhood ALL have gene translocations and these have helped to identify genes with a possible function in malignant transformation and proliferation. Most B-cell ALL cases have translocations involving Ig genes suggesting that Ig recombinase (RAG-1) or class-switch enzymes are involved in the interchromosomal movement (Cleary *et al.*, 1991). Common translocations include the *myc* proto-oncogene on chromosome 8(q24) being juxtaposed to the Ig heavy chain enhancer on chromosome 14 or the κ light chain promotor on chromosome 2, leading to abberant activation of *myc* (Erikson *et al.*, 1983; Taub *et al.*, 1984).

Numerous translocations implicated in acute leukaemias involve transcription factors. The most common translocation of childhood pre-B ALL (approx. 25%) is the t(1;19)(q23;p13). This translocation involves the E2A gene which encodes a transcription factor thought to be essential for expression of Ig genes (Staudt & Lenardo, 1991) and initiation of Ig gene recombination (Schlissel *et al*, 1991); E2A is mandatory for B lymphopoiesis. The E2A gene is fused into a homeobox gene known as PBX1 to form a chimeric protein. As well as effects on Ig genes, pre-B cells with t(1;19) have insulin receptors with 2 fold-higher affinity for insulin and 4 -fold higher basal and insulin-stimulated receptor kinase activity compared to receptors on a B-cell line (with no translocations), from the same patient (Greaves, 1981).

As well as transcription factors, some translocations involve haematopoieitc growth factors. In ALL, the translocation (5;14)(q31;q32) has been associated with autocrine growth stimulation due to juxtaposition of the IL-3 gene with the J region of the IgM gene and subsequent overexpression of IL-3 (Grimaldi & Meeker, 1989).

However, in at least two types of leukaemia, Philadelphia-positive ALL (mostly of pre-B or early pre-B cell) and chronic myeloid leukaemia (CML), deregulation of a tyrosine kinase activity is actively involved in leukaemogenesis (Katz *et al.*, 1994). This translocation - (9;22) - juxtaposes the c-*abl* gene on chromosome 9 with the breakpoint cluster region (*bcr*) on chromosome 22. The c-*abl* encodes a tyrosine kinase activity and the result of the translocation is a chimeric protein increased in size from 145kDa to 210kDa, and with enhanced activity (Konopka *et al.*, 1984).

The Abelson murine leukaemia virus (Ab-MLV) encodes *v-abl*, an oncogene derived from the cellular gene, *c-abl*, but lacking the Src homology (SH) 3 domain. The protein tyrosine kinase encoded by *v-abl* allows the virus to transform B lymphocyte precursors, mostly $CD45R^+$, $CD43^+$, HSA^+ cells which are actively cycling (Hardy *et al.*, 1991; Rosenberg & Kinade, 1994), and the cells become arrested at the stage of light chain gene rearrangement. Aberrant *abl* expression in man, due to the t(9:22) translocation, also

involves differentiation arrest of pre-B cells at the same stage. Temperature-sensitive Abelson virus mutants have been used to demonstrate that virus-transformed cells undergo light chain rearrangement once they are switched to the non-permissive temperature (Takemori *et al.*,1987). *V-abl* protein has lost localisation to the nucleus and is found at the plasma membrane of the B cell. The high level of phosphorylation events, especially with the altered subcellular localisation, must perturb the tyrosine kinase-regulated signalling pathways that normally control the growth and differentiation of B-cell precursors and expression of genes central to antigen receptor gene rearrangement, but the nature of these pathways is not understood (Rosenberg, 1994).

Thus, translocations allow identification of genes involved in leukaemogenesis and help to illustrate mechanisms whereby leukaemic cells can overcome normal growth regulation. The studies presented here describe one of those mechanisms - autocrine growth factor production by a pre-B acute lymphocytic leukaemia cell line called SMS-SB.

1.8 SMS-SB cell line

The SMS-SB cell line was characterised by Smith *et al.*, in 1981. The cells were derived from the leukocytes of a 16 year old girl in the leukaemic phase of a lymphoblastic lymphoma. Most lymphoblastic lymphomas are of T-cell origin and are found in the anterior mediastinum and cervical lymph nodes with subsequent spread into the bone marrow. The original tumour from patient SB was unusual because it was present as lytic bone marrow lesions and a paraspinal mass with no involvement in the anterior mediastinum (Smith *et al.*, 1981). This distribution of cells in the bone marrow and vertebrae is typical of lymphomas induced in mice by the Abelson murine leukaemia virus. Mice infected with this virus develop lymphomas that have no thymic involvement and frequently have solid paravertebral tumour masses (Abelson & Rabstein, 1970b). Cultured SMS-SB cells do not display T-cell specific surface markers and, unlike most lymphoblastic lymphomas have only very low levels of the enzyme TdT. The cells are designated as 'pre-B' by virtue of the presence of 70kDa cytoplasmic μ-chains and the absence of surface Ig, along with expression of several B cell specific markers. In several ways, SMS-SB cells bear a resemblance to normal bone marrow late pre-B cells; for example, they express only one specificity of μ heavy chain with no light chains and they do not express CD10 (CALLA), but they express HLA-DR surface antigens. These phenotypic features distinguish SMS-SB from most other cultured pre-B cells (Smith, 1984). Karyotypic analysis has shown that although SMS-SB cells contain two submetacentric marker chromosomes, there are no other chromosomal abnormalities, and the Philadelphia translocation is not present. It is also of interest that SMS-SB cells do not contain the EBV nuclear antigen, indicating that their transformation was not a result of infection by this virus (Smith, 1981).

The similarities between the SMS-SB tumour and Abelson virus induced disease led Ozanne *et al.* (1982) to investigate the genomic rearrangement and expression of the cellular homologue of the Abelson viral oncogene, *c-abl*, in SMS-SB cells. They discovered that there is no gross rearrangement of the human *c-abl* sequence but the gene is transcribed in an abberant fashion. The cells contain two extra *abl*-related transcripts of 6.2 and 6.8kb in addition to the 5.2 and 5.4kb mRNAs found in normal cells. These data suggest that altered expression of *c-abl* may have contributed to the transformation of SMS-SB cells.

On examination of SMS-SB cells it was discovered that they have normal expression of *c-myc*, *c-myb* and *c-jun* but overexpress the *c-fos* proto-oncogene when compared with other cell lines and to peripheral blood lymphocytes which normally express undetectable levels of *c-fos* mRNA (Tsai *et al.*, 1991). The *c-fos* gene does not appear to be grossly altered in SMS-SB cells and the transcription rate and mRNA levels can be up-regulated by serum.

The leukaemic cells, taken from SB, grew spontaneously in culture and did not go through a crisis phase. The cells can be cultured in serum- and protein-free media without the addition of exogenous mitogens. That the cells proliferated with high viability when first placed in culture was unusual for human leukaemias and the unusual growth characteristics prompted Ozanne and colleagues to characterize the growth promoting factors produced by the cells. They identified two activities, one which enhances the growth of SMS-SB cells plated at low cell density in serum-free media, and another, now termed transforming growth factor - leukaemia-derived (TGF-LD) which promotes the growth of mouse and human fibroblasts but does not act on hematopoietic cells. TGF-LD is secreted by various other B and T cell precursor ALL cell lines but at a lower level than secreted by SMS-SB cells (Zack *et al.*, 1987). From proliferation assay data it seems unlikely that TGF-LD has an autocrine function for SMS-SB.

1.9 *Research aims*

The original aim of this thesis was to investigate the growth characteristics of SMS-SB cells, identify the autocrine growth factor, and determine its exact role in SMS-SB and normal B cell growth and development. It was hoped to purify and characterise the autocrine growth factor (SB-AF), since preliminary studies performed by the *Genetics Institute*, Cambridge, Massachussets, had determined that SMS-SB cells do not produce any of the currently characterised interleukins; SB-AF appears to be a novel pre-B cell cytokine.

Numerous cytokines were tested for the ability to substitute for the SB-AF activity, and these studies have identified PDGF as a candidate autocrine growth factor. PDGF, and

PDGF receptors, are known to be expressed by SMS-SB cells. Unfortunately, further investigations will be required to fully understand the role of PDGF in SMS-SB cell growth, and whether PDGF has a role in normal B cell development.

Another interesting discovery made during preliminary attempts to identify the SB-AF components, was that sCD23 dramatically promotes thymidine incorporation by SMS-SB cells, although an autocrine role for this cytokine has subsequently been ruled out. The main focus of the research presented in this thesis was to identify the biological effects of sCD23 interaction with SMS-SB cells, and to determine exactly how this interaction takes place, after a demonstration that SMS-SB cells do not express any known CD23 receptors. SMS-SB cells appear to express a novel receptor for CD23.

SMS-SB cells provide an extremely interesting model to study autocrine factor activity and characterise the novel CD23 receptor. Future studies should help to clarify the role of autocrine growth factors and CD23 in both early B cell development and malignant transformation.

Chapter 2

Materials and Methods

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 *Chemicals*

The chemicals were of 'AnalaR' grade, and, with the exception of those listed below, were obtained from BDH Chemicals Ltd. or Sigma Chemical Co. Ltd., both of Poole, Dorset, England.

SUPPLIER	CHEMICAL
Amersham International Plc., Aylesbury, Bucks, UK.	α–[^{32}P]-dCTP (3000Ci/mmol) (Methyl-[^{3}H])-Thymidine (20-30Ci/mmol)
Avanti Polar Lipids, Inc., Alabaster, Alabama, USA.	1-palmitoyl-2-oleoyl-phosphatidyl-choline (POPC)
BRL (UK), Gibco Ltd., Paisley, Scotland.	Restriction enzymes / buffer concentrates. Select agar
Biogenesis Ltd., Bournemouth, England.	RNAzol B
Biorad Laboratories	Affigel-15 Affigel-10
Boehringer Mannheim UK Ltd., Lewes, East Sussex, England.	Caesium chloride DNA molecular weight markers IV and VI Proteinase K Neuraminidase
J. Burrough (FAD) Ltd., Witham, Essex, England.	Ethanol
Central Services, Beatson Institute.	Sterile distilled water and PBS L-broth
Difco Labs., Detroit, Michigan, USA.	Bacto-agar
Gibco (Life Technologies), Paisley, Scotland.	Uncut Lambda DNA
Molecular Probes Ltd.	3,3'-Dioctadecyloxacarbocyanine percholate ($DiOC_{18}(3)$)
Oxoid Ltd., Basingstoke, England.	PBS tablets
Pfansteihl Europe Ltd., Davenham, England.	n-Octyl-β-D-glucopyranoside (analytical grade)
Pharmacia Biotech Ltd., Milton Keynes, England.	NICK™ columns Sephadex G50

2.1.2 *Kits.*

SUPPLIER	KIT
Amersham International PLC., Amersham, Bucks, England.	ECL Western Blotting analysis system.
Bio 101 Inc., Stratech Scientific, Luton, England.	Geneclean kit
Cruachem Ltd., Glasgow, Scotland.	Cruachem oligonucleotide purification cartridges.
Perkin Elmer Cetus Ltd., Beaconsfield, England.	GeneAmp RNA PCR kit.
Pharmacia Biotech Ltd., Milton Keynes, Bucks, England.	Oligo-labelling kit.
Promega Ltd., Southampton, England.	PolyATract mRNA Isolation System IV Magic Minipreps DNA Purification system

2.1.3 *Equipment and Plasticware*

Main pieces of equipment are referred to in the appropriate sections. The following companies provided the most commonly used items:

SUPPLIER	EQUIPMENT
Becton Dickinson Labware, Plymouth, Devon, England.	Falcon tubes (15 and 50 ml) Polystyrene round bottom tubes (12x75mm and 17x100mm)
Bibby-Sterilin Ltd., Stoney, Staffs., England.	Bacteriological dishes (90mm)
Chance Propper Ltd., Warley, England.	Gold star microslides (76x26mm) Glass coverslips (22x50mm)
Costar, Cambridge, Massachusetts, USA.	96 well plates Disposable cell scrapers
Eastman Kodak Co., Rochester, N.Y., USA.	X-ray film (XAR-5)
Fuji Photo Co. Ltd., Japan	X-ray film (RX)
NUNC, Gibco, Life Technologies Ltd., Paisley, Scotland.	Tissue culture flasks (25, 80 and 175 cm^3) Cryotubes (1 and 1.5 ml)
Griener Labortechnik Ltd., Dursley, England.	Eppendorf tubes
Labsystems, Basingstoke, England.	Pipette tips (200 and 500µl)
Whatman International Ltd., Maidstone, England.	3mm chromatography paper.

2.1.4 *Plasmids*

pBSCD21	Contains an Xho1-Cla1 subfragment of CD21 recloned in pBluescriptIISK-. A gift from Dr. J-Y. Bonnefoy.
pCDL SRα296CD23	The full length CD23 cDNA in expression vector pCDL SRα. A gift from Dr. J-Y. Bonnefoy.
Sp65	The full length *c-fos* cDNA in a pBR322 expression vector . A gift from Mr.Joseph Winnie.

2.1.5 *cDNA Probes*

CD21	Approximately 1000bp Xho-1/Not-1 fragment of pBSCD21
CD23	A 1.6kbp BamHI fragment of human CD23 from pCDL SRα296CD23.
c-fos	A 2.1kbp HINDIII - EcoRI fragment of pSp65.
GAPDH	0.7kb PCR fragment of rat GAPDH

2.1.6 *Water*

Distilled water for solutions was obtained from a Millipore MilliRO 15 system and for nucleic acid procedures it was further purified on a Millipore MilliQ system.

2.1.7 *Cell Culture Materials*

SUPPLIER	MATERIAL
Biological Industries (Kibbutz Beth Heamek Israel).	RPMI-1640 medium
Life Technologies Ltd (Gibco Europe), Paisley, Scotland.	2.5% (w/v) Trypsin Protein free hybridoma medium II Penicillin / streptomycin Glutamine
Northumbria Biologicals.	Foetal calf serum
Seromed, Biochrom KG,	HAM's F-12 Medium Iscove medium
Sigma Chemical Co., St Louis, USA.	Trypan blue

2.1.8 *Cytokines*

SUPPLIER	HUMAN RECOMBINANT CYTOKINES
Glaxo Institute for Molecular Biology, Geneva, Switzerland.	IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-8, IL-13, GM-CSF
British Biotechnology Ltd., Abingdon, UK	IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, TNF-α, TNF-β, PDGF-$\alpha\alpha$
Genetics Institute,	IL-11
Pepro Tech Inc., Rocky Hill, NJ, USA.	IL-15
R&D Systems, Abingdon, UK.	IL-1α

2.1.9 *Recombinant CD23*

Full length 45kDa CD23 and affinity-purified 25, 29 and 37 kDa forms of sCD23 were produced in baculovirus-infected Sf9 cells and in *E.Coli,* as described previously (Jansen *et al.*, 1991; Pochon *et al.*, 1992; Flores-Romo *et al.*, 1993). All forms were a gift from Glaxo IMB in Geneva, courtesy of Dr. Pierre Graber.

Gel filtration-purifed 25kDa sCD23 was produced from baculovirus-infected Sf9 cell supernatant using an AcA44 column, and then SDS-PAGE/western blotting with anti-CD23 antibodies to detect the CD23-containing fractions. These fractions were then pooled and dialysed against PBS for 48 hrs, before the protein concentration was calculated.

2.1.10 *Antibodies*

SUPPLIER	ANTIBODY
Alexis Corporation, (Ancell Ltd.), Nottingham, UK.	Mouse monoclonal anti-85kD (Human B Cell)/ R-PE
Glaxo IMB, Geneva, Switzerland (gifts from Dr. J-Y. Bonnefoy)	Anti-CD23 antibodies: Mab25 (mouse IgG monoclonal), EBVCS4 (mouse IgM monoclonal), Rb55 (rabbit polyclonal)
Monosan Biologicals, Bradsure Biologicals Ltd., Loughborough, UK.	Mouse anti-CD6, -CD16, -CD18, -CD43, -CDw49d, -CD54, -CD71, -CD76, -CD105
Pharmingen, San Diego, CA, USA.	Mouse anti-human *bcl-2* monoclonal Ab.
R&D Systems Europe Ltd., Abingdon, UK.	Mouse anti-human platelet derived growth factor (PDGF), neutralising antibody

Serotec Ltd., Kidlington, Oxford, UK.	Fluorescein-isothiocyanate (FITC) - conjugated mouse anti-CD11a, -CD11b, -CD11c, -CD19, -CD20, -CD21, - CD22, -CD23, -CD25, -CD45 and anti-CD72 Anti-*c-myc* (mouse monoclonal) $F(ab)_2'$ Rabbit anti-mouse IgG : FITC Sheep anti-mouse IgG : FITC Normal rabbit IgG
The Binding Site, Birmingham, UK.	Biotinylated anti-CD23
Scottish Antibody Production Unit (SAPU)	Normal rabbit serum

2.2 Methods

2.2.1 *Cell Culture of Human B Lymphocyte Cell Lines*

SMS-SB cells were maintained in Protein Free Hybridoma Medium II (PFHMII) and also in RPMI-1640 medium supplemented with 10% (v/v) foetal calf serum and 2mM L-glutamine. All other cell lines (described below) were maintained in RPMI-1640 supplemented with 10% (v/v) foetal calf serum and 2mM L-glutamine. Cells were routinely sub-cultured every 3 days unless otherwise described in the text or figure legends. The cells were cultured in 75cm^2 Costar flasks at 37^{o}C in a 7% CO_2 atmosphere. Maintenance of the cells was carried out in a Laminar-flow-hood.

All of the cell lines were routinely examined for mycoplasma infection (Mr. Joseph Winnie, Beatson Institute) and were consistently negative.

2.2.1a *Cell lines*

Daudi - Human thyroid Burkitts lymphoma (gift from Dr. M.M. Harnett, Dept. Biochemistry and Molecular Biology, Univ. of Glasgow).

DOK- Pre-malignant human keratinocyte (gift from Dr. K. Parkinson, Beatson Institute).

Jijoye - Human B lymphoblastoid cell line (gift from Prof. CM Steel, Dept. of Medical Biology, University of St.Andrews).

Raji - Human, EBV-negative, Burkitts lymphoma (gift from Dr. J-Y. Bonnefoy, Glaxo IMB, Geneva).

Ramos - Human Burkitts lymphoma (gift from Dr. M.M. Harnett)

RPMI-8226 - Human EBV-transformed multiple myeloma (gift from Dr. J-Y. Bonnefoy)

SKW - IgM-secreting human B-lymphoblastoid line (gift from Prof. C.M. Steel)

SMS-SB- Human Pre-B acute lymphoblastic leukaemia (gift from Dr. B. Ozanne, Beatson Institute).

2.2.1b *Frozen Cell Stocks*

A frozen stock of each cell line was maintained in liquid nitrogen. From a logarithmically growing culture, cells were centrifuged and resuspended in 90% FCS with 10% DMSO at approximately $1x10^6$ cells/ml. The cells were rapidly aliquoted into 1ml Nunc cryotubes and frozen at -70°C overnight. The tubes were then placed into liquid nitrogen for long term storage.

2.2.1c *Large Scale Culture of Cell Lines*

To obtain sufficient cells for preparation of large scale cell lysates, cells were grown in spinner-fermenters until at least 10^9 cells were available for harvest. This process was carried out for Raji, RPMI 8226 and SMS-SB cells to provide a negative, positive and test sample, respectively. Numerous test cultures were carried out until it was decided that, in spinners, SMS-SB cells grow optimally in a mixture of 3 parts Iscoves plus 1 part Ham's F-12 medium (Raji and RPMI-8226 cells grow equally well in most media tested). The cultures were grown in successively larger spinners until at least 4 litres of cells at approximately 10^6 cells per ml were available for harvest. The cells were incubated at 37°C and approximately 2 weeks elapsed before the cells were ready to harvest. Prior to harvest, an aliquot of each cell type was removed and tested in a liposome binding assay to ensure that no alteration in liposome binding ability had occurred during culture under the new conditions. After centrifugation and two washes with ice-cold PBS, aliquots of cells were biotinylated (see section 2.2.8a) and then kept at -70°C until required.

2.2.1d *Determination of cell viability*

Cell viability was determined by mixing 25µl of cell suspension with 75 µl of 0.05% (w/v) trypan blue in PBS. The membranes of dead cells lose their integrity and so allow trypan blue to enter the cell. The numbers of live and dead cells were counted using a Neubauer haemocytometer. The total number of cells in 4 x 16 square grids was multiplied by 10^4 to estimate the number of cells per ml of culture.

$$\% \text{ viability} = \frac{\text{number of live cells}}{\text{total number of cells}} \times 100$$

2.2.1e *Preparation of Conditioned Medium*

To prepare conditioned medium, SMS-SB cells were grown for 2 days at an initial concentration of $5x10^5$ per ml in PFHMII medium. The cells were then removed by centrifugation at 3000 rpm for 10 minutes, and the media was collected and sterile filtered through a 0.22μm filter before being aliquoted and stored at -20°C.

2.2.2 *Proliferation Assay*

Tritiated-thymidine uptake assays were performed to assay for SB-AF activity and to assess the response of SMS-SB cells to various recombinant human cytokines.

The appropriate number of SMS-SB cells (2500 cells, unless otherwise stated) harvested from log phase cultures (without centrifugation), were dispensed as 80 μl aliquots (in PFHMII) to individual wells of 96 well flat-bottomed tissue culture plates. A further 20μl of PFHMII supplemented with the appropriate concentration of cytokine or conditioned medium (as described in the figure legends) was then added. The plates were incubated at 37°C for 24 or 48 hrs (see individual experiments) in a humidified 5% CO_2 atmosphere. After this time, each culture was pulsed with 0.33 μCi of tritiated methyl thymidine ([^{3}H]-TdR) for 6 hrs at 37°C. The cells were harvested onto glass fibre mats using a LKB Wallac 1295-001 Cell Harvester. The mats were air-dried and counted using an LKB 1205 Betaplate Liquid Scintillation Counter. All determinations were made in triplicate and at least 3 repeats were performed for each experiment.

2.2.3 *Flow Cytometric Analysis*

2.2.3a *Cell phenotyping*

Flow cytometric techniques were used to analyse the surface expression of various cluster of differentiation (CD) antigens by SMS-SB cells. Aliquots of between $5x10^5$ and $1x10^6$ cells were removed from cultures, centrifuged and then washed twice with ice-cold PBS. The cells were resuspended at a concentration of 10^7 cells per ml in PBS, 1μg of the appropriate mouse anti-human MAb was added to a 100μl sample of cells (see text and figure legends for details). Samples where the antibodies were directly conjugated to a fluorescent marker were incubated for 30 min on ice and protected from light. After incubation, the cells were centrifuged, washed twice with ice-cold PBS and finally resuspended in 0.5 ml of PBS for analysis by flow cytometry. In cases where the primary antibody was unconjugated, a secondary conjugated antibody was employed to allow detection. Staining with the primary Ab was as described for the directly conjugated Ab, but after the final wash step, 1μg of the secondary Ab (e.g. FITC anti-mouse IgG) was added to the cells for 30 min. After incubation, the cells were washed twice in ice-cold PBS and resuspended in 0.5 ml of PBS for analysis.

In each experiment, a control was included where no antibody was added, to correct for autofluorescence of the cells. Where a secondary antibody was used, a control of cells stained with only the second Ab was included to account for any non-specific binding of the antibody.

Propidium iodide (PI) was added to each sample at a final concentration of 5μg/ml, immediately prior to data collection. Ab staining was analysed using a FAC*Scan* flow cytometer (Becton-Dickinson, Mountain View, Ca., USA) with an argon laser tuned at 488 nm. Cells were gated on forward and side light scatter, as well as for the PI emitted fluorescence to facilitate exclusion of non-viable cells from the acquisition. All histograms represent data from 10^4 viable cells for each sample.

2.2.3b *Flow cytometric determination of apoptosis*

Flow cytometric discrimination and quantification of viable, early and late apoptotic and necrotic cells is based on their differential permeability and fluorescence following staining with the DNA-binding fluorochromes Hoechst 33342 and propidium iodide (Dive *et al.*, 1992).

Preparation of the cells was the same as in section 2.2.3a but instead of adding an Ab, the cells were stained with 20 μl of propidium iodide (50 μg/ml in PBS, stock) and 100 μl of Hoechst 33342 (10μg/ml in PBS, stock). Both fluorochrome solutions were freshly prepared for each experiment. An unstained population of cells was analysed initially to allow settings to be adjusted for forward and side scatter then both dyes were added individually to give an estimate of which populations were being stained. For the dual staining, cell suspensions were incubated with the dyes for 1 min. before 10^4 cells were analysed by a dual laser Coulter *Elite* flow cytometer. The laser excitation frequency for propidium iodide was 488nm and the ultraviolet emitting laser (351.1-363.8nm) was used to excite Hoechst 33342. Both fluorescence signals were subjected to logarithmic amplification - propidium iodide fluorescence (red) was detected with a 575 nm band pass and Hoechst fluorescence (blue) between 420 and 470 nm. In all experiments cell debris was excluded by electronic gating.

2.2.3c *Flow cytometric analysis of cell cycle and intracellular proteins*

Flow cytometry was used to examine expression of the intracellular proteins bcl-2 and c-myc during the cell cycle and apoptosis of SMS-SB cells. Saponin was used to permeabilise the cells and allow entry of the antibodies.

For each stain, 10^6 cells were washed in 0.5% (w/v) BSA / 0.1% (w/v) saponin/PBS (SBP buffer) and then incubated in SBP supplemented with 10% (v/v) normal rabbit serum for 15 minutes at 4°C. The cells were then washed twice with SBP buffer before addition of 1μg of the primary antibody (mouse anti-human *bcl-2* or anti-human *c-myc*) in

SBP buffer to a final volume of 100 µl, and incubation at 4°C for 20 min. After another 2 washes in SBP buffer, 10µl of a 1/100 dilution of the secondary antibody (sheep anti-mouse IgG - FITC) were added in SBP buffer for 20 min at 4°C. The cells were washed, as before, and fixed in 2% paraformaldehyde / 0.5% BSA in PBS for 15 minutes, washed another twice with PBS, and kept at 4°C until analysis.

To analyse the cell cycle position of the cells after various culture treatments (see individual experiments), propidium iodide was used to stain the DNA. After the cells were stained with antibody, fixed and washed, they were suspended in a solution of 0.1% Triton X-100 / 0.1% sodium citrate (citrate buffer) plus 100µg/ml propidium iodide, prior to analysis on a Coulter *Elite* flow cytometer. Cells were incubated with SBP buffer alone to control for any change in forward or side scatter or in autofluorescence due to the saponin. Cells incubated with saponin plus secondary antibody were used to control for fluorescence due to non-specific binding of the secondary antibody.

2.2.3d *Fluorescent liposome FACS*

The cells were prepared as before (section 2.2.3a) but instead of adding fluorescent Ab to stain the cells, 50µl of CD23-containing fluorescent liposomes (see section 2.2.4) were added and the cells incubated at 4°C for 2 hrs. After incubation, the cells were washed three times with liposome buffer (20mM HEPES pH7 / 140mM NaCl / 200mM $CaCl_2$ / 0.5% BSA / 0.1% Na Azide) before being resuspended in 100 µl of liposome buffer and analysed. The tubes were kept at 4°C at all times prior to analysis.

i) For antibody inhibition assays, either the liposomes or the cells (see individual experiments) were pre-incubated with the given antibody (at 10µg/ml, unless otherwise stated) for 1 hr at 4°C before the liposomes were added.
In all cases, glycophorin A-containing liposomes were used as a negative control and normal rabbit IgG or mouse IgG (as detailed in the figure legends) were used as controls in the antibody blocking experiments.

ii) To investigate the nature of the cell-liposome interaction, various sugars were tested for their ability to inhibit the binding of CD23-liposomes to SMS-SB cells. Glucose-1-phosphate and fucose-1-phosphate (from Sigma Chemical Co.) were pre-incubated with the liposomes for 1 hour, at final concentrations between 0.02 and 50 mM in liposome buffer, before the liposomes were added to the cells.

iii) To assess the sensitivity of the novel receptor to neuraminidase treatment, cells at 10^6 cells per ml were washed in PBS and incubated in PBS containing 0.1 U/ml of one of the following bacterial neuraminidase :
a- *Vibrio cholerae*

b- *Clostridium perfringens*

c- *Arthrobacter ureafaciens.*

The cells were incubated for 1 hr at 37°C then washed with ice-cold PBS before being incubated with the liposomes as before.

iv) To investigate whether the CD23 / novel receptor interaction was calcium-dependent, a liposome FACS assay was performed as before but 5mM EDTA / PBS was added to the incubation of cells plus liposomes.

2.2.4 Liposome Preparation

Preparation of fluorescent liposomes containing 45kDa CD23 or Glycophorin-A was carried out as follows : 200 µl (50mM in chloroform stock) of POPC (10 µmoles) and 100 µl (0.5 mM in Dimethylformamide stock) of DiO18 (50 nmoles) were vortexed in a glass tube at room temperature to give a lipid ratio of 1/200, DiO18/POPC. The solvent was evaporated by flushing with N_2 followed by 30 min drying with a Speedivac. After drying, 50µl of 1x PBS/ 800 mM n-Octyl-β-D-glucopyranoside (OGP) (analytical grade) was added and the solution vortexed until clear (15 - 30 min). 150 µl of 1x PBS were then added to make the volume 200µl and the final concentration of OGP 200 mM. The ideal ratio of detergent to lipid is 100 mM OGP to 10mM lipids or 1/10.

At this point, the protein was added to the lipids - either CD23 or glycophorin A. The protein was dissolved in 1x PBS/50mM OGP at 20-25µg/ml. All the work was carried out at 4°C. Approximately 15µg of protein was added to the tube and 50mM OGP / PBS was added to give a final volume of 800µl. The tube was protected from the light, vortexed for 10 seconds and then incubated on ice for 1 hr with occasional vortexing. The ratio of protein to lipid should be 1 / 50000 moles (1/25000 minimum). The final volume was 1 ml and OGP concentration was 50 mM.

12-14 kD cut off, 0.0011", 1 cm dialysis tubing was use to dialyse the liposomes against 4 x 1L of 20mM HEPES pH 7.0 / 140 mM NaCl (liposome buffer base) Firstly, the tubing was boiled in 1mM EDTA, rinsed well with dH_2O, and dried with a paper towel. The fresh liposomes were filtered through a 0.22 µm filter and then loaded into 25 cm long dialysis tubing using a 0.8 x 80mm needle (Socochim).

After dialysis, 0.1% Na Azide was added to the solution and 100 µl of the concentrated liposomes were kept at 4°C without protease inhibitors to test for binding. The remaining concentrated liposomes were diluted 1/10 with liposome buffer containing 1mM Nα-*p*-tosyl-L-lysine chloromethyl ketone (TLCK), 10mM iodoacetamide and stored at 4°C in 5 ml aliquots.

2.2.5 *Nucleic acid preparation*

2.2.5a *Transformation of competent cells*

To grow up sufficient plasmid and extract fragments for use as nucleic acid probes, competent *E.coli* DH5α cells were thawed on ice and to each 20 µl aliquot, 100ng of appropriate plasmid DNA were added. Following a 20 minute incubation on ice, the cells were heat-shocked for 2 min at 42°C, placed on ice for 2 minutes and finally incubated in SOC medium (2% bacto-tryptone, 0.5% bacto-yeast extract, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 10mM $MgSO_4$ and 20 mM glucose) for 1 hr at 37°C with constant shaking at 225rpm to allow expression of antibiotic resistance markers. The transformed cells were micro centrifuged for 5 minutes and the pellet resuspended in 100 µl of L-broth. Dilutions of 1:10 and 1:100 (in L-broth) of the resuspended cells were spread onto separate agar plates (1.5% agar in L-broth containing 100µg/ml ampicillin). The plates were incubated overnight at 37°C. Representative colonies were inoculated into 12ml of L-broth containing 100mg/ml ampicillin to be incubated overnight at 37°C with constant shaking. For expansion of the cultures and DNA extraction, see below.

2.2.5b *Small scale preparation of plasmid DNA*

Modification of the alkali lysis method, proposed by Sambrook *et al.,* 1989, was used to extract plasmid DNA from small bacterial cultures. Following on from section 2.2.5a, 1.5 ml aliquots of a confluent overnight culture were spun in eppendorf tubes at 14000rpm for 5 minutes, the pellet was resuspended in 200µl of 2 mg/ml lysozyme in 50 mM glucose, 25 mM Tris and 10 mM EDTA pH 8.0 and left at room temperature for 5 minutes. Cell lysis was continued by the addition of 400µl of 0.2M NaOH, 1% (v/v) SDS and incubation on ice for 10 minutes. Lysis was terminated by the addition of 300ml of 3M NaOAc pH 4.8, vortexing and incubation on ice for 10-15 minutes. The tubes were centrifuged at 14000 rpm for 5 min, 800µl of the supernatant was mixed with 480µl of isopropanol to induce DNA precipitation and the samples kept at -20°C overnight. The precipitated DNA was pelleted by centrifugation for 20 minutes at 4°C, washed with ice-cold 70% ethanol and finally lyophilised under vacuum and resuspended in 50µl of TE (10mM Tris, pH7.4, 0.1mM EDTA). Before gel electrophoresis, RNase treatment was performed by incubating the sample with 50µg/ml RNase for 30 minutes at 37°C. Aliquots of 1µl were subjected to the appropriate restriction enzyme digestion (section 2.2.5d) and electrophoretic analysis (section 2.2.7a) while the DNA concentration was determined by spectrophotometry. The DNA was stored at -20°C.

2.2.5c *Large scale preparation of plasmid DNA.*

To obtain large amounts of plasmid DNA, the alkali lysis method proposed by Sambrook *et al.,* 1989 was used, after analysis of mini-prep samples to ensure the correct plasmid and fragment were being cultured. Overnight cultures (1 litre) were collected by centrifugation (6000 rpm) for 5 minutes at 4°C. The pellets were washed in 100ml of

bugwash solution (150mM NaCl, 10mM TrisHCl and 1mM EDTA pH 8.0), respun and then resuspended in 20 ml of solution 1 (50mM glucose, 25mM TrisHCl pH8.0 and 10mM EDTA pH8.0). After a 5 minute incubation on ice, 80 ml of solution II (0.2M NaOH and 1% (w/v) SDS) were added and mixed by gentle inversion. The samples were left at room temperature for 5-10 minutes and then the lysis terminated by addition of 30 ml of cold solution III (3M Na acetate, 11.5% (v/v) glacial acetic acid). The samples were shaken vigorously, left on ice for 10 minutes followed by centrifugation at 4000 rpm, 4°C for 15 minutes; at the end of the run, the centrifuge was allowed to stop without braking. The supernatant was filtered through a gauze and then 0.6 volumes of ice-cold isopropanol were added, the sample mixed well and the DNA precipitated at room temperature for 15 minutes. The DNA was pelleted at 5000 rpm, 15 minutes at room temperature, the supernatant removed and the pellet washed with ice-cold 70% ethanol. The pellet was dried a room temperature, resuspended in 12 mls of TE buffer and then the plasmid DNA separated out by a $CsCl_2$ gradient as follows:

12ml of resuspended DNA were mixed with 12.9g $CsCl_2$ and 0.8ml of ethidium bromide (stock solution 10mg/ml). The refractive index was adjusted to 1.39 with $CsCl_2$ or TE buffer and the solutions transferred to centrifuge tubes. After centrifugation at 40,000rpm for 48 hours at 20°C in an ultracentrifuge, the closed, circular plasmid DNA was removed using a UV lamp and a blunt ended needle. The ethidium bromide was removed by vigorously mixing equal volumes of sample and $CsCl_2$ saturated isopropanol, removing the top red layer and repeating the process until the top layer was clear. Plasmid DNA was precipitated in glass Corex tubes by adding 3M NaOAc - 1/10 the volume, 100% ethanol - 2.5 volumes and leaving the samples overnight at -20°C. The precipitates were pelleted by centrifugation at 8000 rpm for 30 min, washed with 70% ethanol, vacuum dried and resuspended in 0.5ml TE buffer to be stored at -20°C.

2.2.5d *Restriction endonuclease digestion of DNA.*

For every 1μg of plasmid DNA to be digested, 1 unit of the appropriate restriction enzyme was added in the presence of reaction buffers supplied by the manufacturers. The total reaction volume did not exceed 10μl and the ratio of enzyme to volume was 1:10. The samples were incubated for 1 hr at 37°C before the reaction was terminated by addition of DNA loading buffer and the samples run on an agarose gel (see section 2.2.7a).

Genomic DNA (25-50μg) was digested in the same way but in a proportionally larger volume to a maximum of 100μl, and incubation was at 37°C overnight.

2.2.5e *Extraction of Genomic DNA from cell lines.*

Genomic DNA was extracted using a method described by Laird *et al.,* 1991. Briefly, 10^7 cells were washed with PBS and then digested with 0.5 ml of lysis buffer at 37°C for 2 hrs with agitation.

Lysis buffer: 100mM Tris HCl (pH8.5)
5mM EDTA (pH8)
0.2% SDS
200mM NaCl
100µg Proteinase K per ml

One volume of isopropanol was added to the lysate and the sample swirled until precipitation was complete. The aggregated precipitate was lifted from the solution and washed with ethanol before being dissolved in TE by agitation at 55°C for 2 hrs. The concentration was determined by spectrophotometer and the samples stored at -20°C.

2.2.5f *Total and PolyA RNA extraction from cell lines.*

Total RNA was isolated using the *RNAzol B* method, according to the manufacturer's instructions. Briefly, 10^6 - 10^7 cells were pelleted, washed in ice-cold PBS, lysed with 0.2-2 ml RNAzol B and the RNA solubilised by passing the lysate through a pipette a few times. Chloroform was added to 1/10 the volume, the tube was shaken vigorously for 15 seconds, left on ice for 5 minutes and then centrifuged at 12,000g / 4°C for 15 minutes. The colourless upper aqueous phase (where the RNA is found) was removed from the blue phenol-chloroform phase. To precipitate the RNA, the aqueous phase was added to a tube containing an equal volume of ice-cold isopropanol and the sample was stored at -20°C overnight. The RNA was then pelleted, washed with 70% ethanol, air-dried and resuspended in 20-50µl of DEPC-treated dH_2O. Sample concentrations were determined by spectrophotometer readings on 1/200 dilutions with samples having a 260/280 ratio greater than 1.9.

PolyA mRNA obtained using the *PolyATract mRNA Isolation System IV* according to manufacturer's instructions. Briefly, 0.1 -1.0mg of total RNA were brought to a final volume of 500µl in RNase-Free water. The RNA was denatured at 65°C before the biotinylated-oligo(dT) probe and 20xSSC were added. The annealing mixture was cooled at room temperature while the streptavidin-paramagnetic beads were washed with 0.5xSSC. The contents of the annealing reaction were added to the beads and incubated for 10 minutes. After several washes with 0.1xSSC, the mRNA was eluted with a total of 250µl of RNase-free water and the concentration and purity determined by spectrophotometer.

2.2.6 *Polymerase chain reaction (PCR)*

2.2.6a *Oligonucleotide preparation*

Designed oligonucleotides were synthesised on an Applied Biosystems mode 381A DNA synthesiser. After incorporation of the final nucleotide, the 5' methoxy-trytil group was left in place to allow efficient purification of the oligonucleotides on trytil affinity columns. Elution from the synthesis resin and removal of side chain protective groups was achieved by overnight incubation in 2ml of concentrated ammonia solution (29%) at 55°C. The Cruachem trytil affinity columns were washed with 2ml of concentrated acetonitrile and then 2ml of 2M triethylamine acetate pH7.0 before the oligonucleotide samples were added. The sample was passed through the column twice to ensure efficient capture. 3ml of dilute ammonium hydroxide in water (1:10), 2ml of MilliQ water, 2ml of 2% trifluoroacetic acid and then 2ml of MilliQ water were flushed through the column to remove any unbound material. The purified oligonucleotides were eluted with 1ml of 20% acetonitrile and the DNA lyophilised in a Speedivac before being resuspended in 50µl of MilliQ water and stored at -20°C.

The following oligonucleotides were designed, produced and used as primers in PCR reactions to examine the appropriate gene expression in various cell lines:

CD21a	5'-CCT ATG ATC CAC AAT GGA CAT CAC-3'
CD21b	5'-AGA TTT ACA GCG TGC CTC TTC ACA-3'
CD23a	5'-GCA GCT GAA CAG CAG AGA TTG AAA-3'
CD23b	5'-TGC CCT TGC CGA AGT AGT AGC ACT-3'
c-fos a	5'-TCC AAC CGC ATC TGC AGC GAG CA-3'
c-fos b	5'-GAC AGG CGA GCC CAT GCT GGA GAA-3'

2.2.6c *Reverse-transcriptase PCR Amplification*

The Perkin-Elmer Cetus *GeneAmp* RNA PCR kit was used to detect and analyse gene expression at the RNA level. This involved reverse transcription of total RNA to cDNA with subsequent amplification of the cDNA by PCR with *AmpliTaq* DNA Polymerase.

For the reverse transcription step, a master mix was prepared as follows : 5mM $MgCl_2$, 1xPCR buffer II, 1mM of dCTP, dGTP, dATP and dTTP, 1µM RNase inhibitor, 1 unit/ml of reverse transcriptase, and 2.5µM random hexamers. To 18µl of this mix was added 1µg of the appropriate total RNA in a volume of 2µl. To reduce evaporation, the mix was overlaid with 100µl of mineral oil and to allow extension of the hexameric primers by reverse transcriptase, they were incubated at room temperature for 10 minutes. The tubes were incubated at 42°C for 15 minutes, 99°C for 5 minutes and 5°C for 5 minutes to achieve reverse transcription. To each reaction tube, 78µl of a prepared PCR master mix

(2mM $MgCl_2$, 1xPCR bufferII, 2.5 units/100µl of *Amplitaq* DNA polymerase and water to a final volume of 78µl per sample) was added along with the appropriate "upstream" and "downstream" primers (as detailed on figures) each at a final concentration of 0.15µM and sterile dH_2O was added to make the total reaction volume 100µl. The tubes were spun briefly in a microcentrifuge and then PCR amplification was carried out in a Perkin Elmer Cetus DNA Thermal Cycler as follows:

2 minutes at 94°C for 1 cycle
1 minute at 94°C,1 minute at 55°C and 1 minute at 72°C for 35 cycles
10 minutes at 72°C for 1 cycle
storage at 5°C until retrieval

The samples were examined by gel electrophoresis (section 2.2.7a), purified (section 2.2.7b) and then probed in a Southern blot (section 2.2.7c) to demonstrate the presence or absence of specific products.

2.2.7 *Nucleic acids - separation, purification and hybridisation.*

2.2.7a *Agarose gel electrophoresis.*

Non-denaturing 0.8% (w/v) agarose gels were used to visualise DNA fragments of >200bp. Agarose was dissolved in 50ml (mini-gel) or 200ml (maxi-gel) of 1xTBE buffer (10x stock contains 108g Tris HCl, 55g boric acid and 9.5g of EDTA in 1 litre of dH_2O) for fragment resolution or 1xTAE (10x stock contains 48.8g Tris, 11.4ml glacial acetic acid and 40ml of 0.25M EDTA pH8.0 in 1 litre of dH_2O) for DNA purification, in the case of restriction fragments. Ethidium bromide was added to a final concentration of 0.4µg/ml. TAE gels were set at 4°C and TBE gels at room temperature. Electrophoresis buffer was the same as that used in gel formation. Prior to loading, the DNA samples were mixed with 3µl of DNA loading buffer (0.25% bromophenol blue and 30% glycerol in dH_2O) and then electrophoresed at 1-5V/cm with the appropriate sized DNA markers. The DNA was visualised on a 312nm transilluminator.

2.2.7b *Purification of DNA restriction fragments*

To purify DNA fragments from low melting point TAE agarose gels, a Promega *Magic Miniprep DNA purification system* was used. The fragment of DNA was cut out with the gel and the agarose melted in an Eppendorf tube before the addition of 1ml of DNA purification resin. After vortexing for 1 minute, the resin with the bound DNA was drawn slowly through a mini-column with a syringe. The column was washed with 2ml of 70% isopropanol, the tube microfuged for a few seconds to remove residual wash solution, and then left at room temperature for 10 minutes to allow evaporation of isopropanol. Bound DNA was eluted with 50µl of dH_2O at room temperature for 1 minute and the eluate

collected by microcentrifuge for 20 seconds. The purified fragments were analysed by gel electrophoresis and stored at -20°C.

2.2.7c *Southern Blot Analysis*

Genomic DNA was digested with either BamHI, HINDIII or ECoRI restriction enzymes (see section 2.2.5d). After digestion, samples of 20µg per well were size fractionated by electrophoresis on 1% agarose / TBE gels at a constant 30V (see section 2.2.7a). Transfer of the DNA onto Hybond N+ membrane was achieved under vacuum (50-60 mbar pressure). Once the gel was on the vacuum blotter and the edges sealed with agarose to ensure a vacuum, it was treated to depurinate and denature the DNA and allow transfer to the nylon. After each step, the solution was removed from the blotter before addition of the next one. The steps used were as follows :

1. 0.25M HCl for 30-45 min (until bromophenol blue of the dye front turned yellow).
2. 0.5M NaOH / 1.5M NaCl for 30-45 min.
3. 3M NaCl / 0.5M Tris pH8 for 30-45 mins.

At this point the gel was completely covered with 20xSSC (3M NaCl, 0.3M sodium citrate, pH 7.0) and transfer was continued for 1.5 - 2 hrs. After the transfer, the membrane was UV cross-linked in a Stratalinker 1800.

2.2.7d *Northern blot analysis*

Total RNA was loaded at 20-30µg/well and polyA RNA loaded at 3-6µg/well. Before loading, volumes of approximately 5µl were mixed with 3 volumes (15µl) of RNA loading buffer (350µl formaldehyde, 1µl formamide, 150µl DNA loading buffer, 10µl ethidium bromide [stock 10mg/ml] and 200µl 5x MOPS buffer containing 0.1M MOPS, 40mM CH_3COONa and 5mM EDTA, pH7.0) and heated for 15 minutes at 65°C. The samples were then loaded onto a 150ml flat-bed 1% (w/v) agarose gel containing 30ml of 5x MOPS buffer and 26.3ml of formaldehyde. The electrophoresis buffer was 1x MOPS buffer and this was recirculated during an overnight run at 30V. The gel was washed in dH_20 for 15 minutes prior to blotting to remove the formaldehyde. RNA was transferred to nylon membrane (Hybond N+) either by traditional overnight capillary blotting (described by Sambrook *et al.*, 1989) or by $1^1/_2$ hours of vacuum blotting, both methods using 20xSSC buffer. The RNA was UV-crosslinked using a UV Stratalinker 1800 and the positions of 18S and 28S ribosomal RNA were marked. Hybridisation to specific probes was carried out as described in section 2.2.7.

2.2.7e *Pulse Field Gel Electrophoresis*

The method for running pulse field gels was adapted from the LKB 2015 Instruction Manual from Pharmacia.

i) **Preparation of agarose blocks**- DNA was prepared from cells suspended in agarose blocks to prevent shearing. 10^5-10^6 cells (approximately 0.6-6μg of DNA) were added to each block (ensuring same number of cells in all samples) after they had been washed with ice-cold PBS to remove any traces of serum. Cells were resuspended in approximately 50μl of L-buffer (0.1M EDTA pH8.0, 0.01M Tris Cl pH7.6, 0.02M NaCl) and then 50μl of 1.2% low melting point agarose in PBS (equilibrated to 45°C) were added. The 100μl sample was pipetted into the plastic block mould provided and allowed to solidify for 20 minutes. Once solidified, the blocks were pushed out into a universal containing 1ml of Proteinase K digestion buffer (100mM EDTA pH8.0, 0.2% (w/v) sodium deoxycholate, 1% (w/v) sodium lauryl sarcosime, 2mg/ml Proteinase K) and incubated at 55°C for 24-48 hrs. After incubation, the blocks were stored at 4°C for 1 hour to firm the agarose and then they were washed 4x30 minutes in Wash buffer (20mM Tris pH8.0, 50mM EDTA) on a shaker.

ii) **Pulse field gels**- A 200ml 0.8% agarose / TBE solution was prepared and equilibrated to 42°C. To load the gel, the DNA blocks were placed onto the teeth of the comb and then the comb placed in position in the 20x20 cm gel mould. The cooled agarose was slowly poured into the gel mould to cover the blocks. After the gel had set, the comb was removed and the blocks sealed with the remainder of the agarose. 2 litres of 0.25x TBE were prepared, poured into the pulse field tank and the thermostatic circulator was run for 30 minutes to cool the buffer to 14°C before initiating the run. The gel was removed from the frame, clipped into the tank and then the apparatus programmed with the desired settings - 16 hr run with 30 second pulses of 6V/cm. After the run, the gel was removed and stained in 0.4μg/ml ethidium bromide in 0.25x TBE for 20-30 minutes and then washed for 20 minutes (or as long as necessary) in dH_20 before being photographed under UV light.

2.2.7f *Radiolabelling of cDNA probes.*

For Southern and Northern blot analyses, $\alpha[^{32}P]$dCTP labelled DNA fragments were produced using the random priming technique. An *Oligo-labelling* kit (Pharmacia) was used to synthesise new cDNA strands in the presence of radioactively labelled dCTP. For each probe, 50-100ng of appropriate DNA, in a volume of 34μl with dH_20, was linearised by boiling, cooled on ice and then 10μl of reaction buffer (containing random primer sequences, dATP, dGTP and dTTP, at the required salt concentrations) were added along with 5μl of $\alpha[^{32}P]$dCTP and 1μl of Klenow fragment. The reaction was incubated at 37°C for 1 hour. After incubation was complete, the unincorporated nucleotides were

37°C for 1 hour. After incubation was complete, the unincorporated nucleotides were removed by centrifugation at 2500 rpm for 4 min through a column made of Sephadex G50/TE (pH8.0), supported by glass wool, in an Eppendorf tube. The unincorporated nucleotides remained in the beads while the labelled probe was collected in a clean Eppendorf. The degree of $\alpha[^{32}P]$dCTP incorporation was determined by analysis of 1µl aliquots in a Beckman LS5000CE counter and was 1-2 x 10^9 dpm/µg for each probe. The probe was boiled for 10 mins, placed on ice for 5 min and then added to the hybridisation solution.

2.2.7g Hybridisation with ^{32}P-Labelled cDNA

2.2.7g-1 Southern blots were hybridised using 10 - 20ml of *Rapid-Hyb* buffer (*Amersham*; used as per manufacturer's instructions), in Hybaid bottles. Pre-hybridisation was performed at 42°C for 30 minutes, in a Hybaid oven, prior to addition of the ^{32}P-labelled cDNA probe (see section 2.2.7f) for 1.5 hours.

2.2.7g-2 Northern blots:- the nylon membranes were pre-hybridised at 42°C in Hybaid bottles or in plastic bags for 6-16 hrs in hybridisation buffer (5x SSPE, 10x Denhardt's, 2% SDS, 50% deionised formamide and 1mg/ml yeast RNA). Fresh hybridisation buffer was then added and the blot hybridised for 16 hrs with the relevant ^{32}P-labelled cDNA probe (see section 2.2.7f) at 42°C.

Denhardt's solution (50X)	5g Ficoll, 5g polyvinyl pyrrolidone, 5g BSA plus H_20 to final volume 500ml
SSPE (20X)	174g NaCl, 27.6g NaH_2P0_4-$2H_20$, 7.4g EDTA plus H_20 to 1 litre, pH7.4

After hybridisation, both Southern and Northern blot membranes were washed as follows:-

2 x SSC / 0.1% SDS	4 washes of 15 min. each at room temperature
1 x SSC / 0.1% SDS	1 wash of 20 min at 65°C
0.1 x SSC / 0.1% SDS	1 wash of 15 min at 65°C

The membranes were then exposed to Kodak XAR-5 film for 1 to 7 days at -70°C, with an intensifying screen.

After hybridisation, the membranes were stripped by placing them into a solution of 0.1% SDS at 100°C and leaving the solution shaking until it had cooled to room temperature. The membranes were reprobed with GAPDH cDNA, as a loading control.

2.2.8. *Isolation and Purification of novel receptor*

The approach chosen to purify the novel CD23 receptor was to biotinylate the surface proteins of SMS-SB cells, prepare a cell lysate, and then run the lysate over a pre-adsorption column (to reduce non-specific binding) followed by a CD23 affinity column. Any bound material was eluted, dialysed, run on an SDS-PAGE gel, Western blotted to nylon membrane and probed with streptavidin-peroxidase to be detected by ECL.

2.2.8a *Biotinylation of cell surface proteins*

10^8 cells were harvested and washed with 50ml of PBS. The cells were resuspended at 10^7 cells/ml in 5mM sulfosuccinimidyl-6-(biotinamido) hexanoate (NHS-LC-Biotin) / PBS and incubated on a roller for 30 - 60 minutes at room temperature, in the dark. The cells were then washed three times with 50ml of ice-cold PBS before a cell extract was made.

2.2.8b *Preparation of cell lysate*

2.2.8b-1 *Small scale cell lysate*

After biotinylation, the cells were resuspended in 15ml of Extraction buffer (50mM HEPES, 5mM $CaCl_2$, 140mM NaCl, 1% n-Octyl-β-D-glucopyranoside (OGP), 1mM PMSF, 1mM TLCK). The mixture was vortexed for approximately 4 minutes before being ground in a glass homogeniser twenty times, to break up the cells. The extract was left on ice for 30 - 60 minutes then centrifuged at 100,000g for 30 minutes at 4°C, before the supernatant was added to the pre-adsorption column.

2.2.8b-2 *Large scale cell lysate*

After biotinylation, the 10^8 biotinylated cells were mixed with 1-5x10^9 non-biotinylated cells, produced in large scale spinner cultures (see section 2.2.1c). The cells were resuspended in 50-100ml (depending on the viscosity of the mixture) of Extraction buffer before being homogenised using a Polytron homogeniser, passaged twice through a French Pressure Cell, sonicated and then left on a roller, at 4°C, for 2 - 18 hours. Before the extract was loaded onto the pre-adsorption column it was centrifuged at 45K, 4°C, for 45 minutes and the supernatant was collected.

2.2.8c *Preparation of affinity columns*

Since 25 kD CD23 is an acidic protein (pI = 5.3), Affigel-15 was used to produce an affinity column. The protein was dialysed overnight with 6-8 kD cut-off tubing, against 2 litres of coupling buffer (0.1M MOPS pH7.5). The reaction volume for the coupling was 1.5 - 4.5 ml of protein per ml of resin and the ratio was 3mg protein per ml resin. The resin was mixed and then added to a scintered glass funnel to remove the liquid phase. Taking care to avoid drying out the resin, it was washed with 3 volumes of ice-cold dH_2O and transferred to a polypropylene tube where the prepared protein was added. After

overnight incubation on a roller, the unbound protein was removed with coupling buffer on a scintered glass funnel - the eluate was retained. The remaining active sites of the resin were blocked by incubation for 1 hour at room temperature with 0.1M Ethanolamine pH8.0 (five x column volumes) and then the resin was washed in 1xPBS. Pre-elution was performed with two x column volumes of 3M NH_4SCN followed by a wash with PBS. The unbound protein, eluted material and all the washes were pooled and dialysed overnight against 5 litres of PBS before spectrophotometer readings were taken to determine the coupling efficiency. BSA / IgG or BSA alone columns were used as pre-affinity columns to remove proteins which would bind non-specifically to the CD23 column (as detailed in figure legends). These were made with Affigel-10 resin and BSA / IgG (3:1) to give 3mg protein per ml of resin.

2.2.8d *CD23 affinity chromatography*

2.2.8d-1 *Small scale affinity columns*

Pre-adsorption affinity column - to remove a 0.5ml sample of BSA/IgG or BSA column resin from the tube, 2ml of PBS were added and the tube was tapped until a 'slurry' had formed. An approximately 1ml sample (depending on the initial amount of resin) was transferred to a clean 15ml Falcon tube, centrifuged at 1000rpm for 2 minutes and the PBS removed. 2ml of Extraction buffer were added and mixed with the resin before it was centrifuged, as before, and the process with extraction buffer was repeated; the 2ml of extraction buffer was removed after centrifugation. The pre-adsorption column was now equilibrated and ready for use.

The supernatant from the cell extract preparation (section 2.2.8b-1) was loaded onto the resin and the tube was sealed, put onto a roller at 4°C, and left overnight.
After incubation, the resin was centrifuged, as before, and the unbound material removed with a pipette. The final fraction of extract was removed with a needle, taking care not to aspirate any resin. The unbound material was retained to load onto the CD23-affinity column resin.

CD23 affinity column - the column resin was equilibrated, as described for the pre-adsorption column, and the unbound material from the pre-adsorption was added to the CD23 column. The column was incubated overnight at 4°C, as described for the pre-adsorption column. Again, after centrifugation, the unbound material was collected and retained to load onto the gel.

Elution of bound material - the resin was washed at 4°C, on a roller, as follows: 1 x 15 minutes with 14ml of Extraction buffer; 1x 20 minutes and 2 x 10 minutes with Wash Buffer 1; 1 x 10 minutes with Wash Buffer 2.

Wash buffer 1: 50mM HEPES
5mM $CaCl_2$
0.5M NaCl
plus 0.1% Triton X 100 (for pre-adsorption columns)
or 30mM OGP (for CD23-affinity columns).

Wash Buffer 2: 50mM HEPES
5mM $CaCl_2$
0.5M Urea
plus 0.1% Triton X 100 (for pre-adsorption columns)
or 30mM OGP (for CD23-affinity columns).

After the washes, the bound material was eluted by resuspending the 0.5ml of resin in 0.5ml of SDS-PAGE sample buffer (as described in 2.2.8e-1). The sample was boiled for 5 minutes, centrifuged at 1000 rpm for 3 minutes, and then the 0.5ml sample was removed to be run on the gel.

Since the same pre-adsorption and CD23 columns were used for each cell line sample, a small sample of the resin was removed after each elution, and boiled in SDS sample buffer. This sample was run on a *Phastgel* and a silver stain was performed, using the automated *PhastGel* system, by Pharmacia. From the silver stain it could be determined that the resin was clean before addition of the next cell sample.

2.2.8d-2 *Large scale*

Pre-adsorption affinity column - 5ml of IgG/BSA resin were equilibrated with Extraction buffer, as described in section 2.2.8d-1, but in a Pharmacia XK26 column. The prepared cell extract (50ml) (section 2.2.8b-2) was added to the column, and the column connected to a pump to allow recirculation of the extract. The recirculation was carried out at 4°C, overnight, before the unbound material was collected a measuring cylinder to add to the CD23-affinity column.

CD23 affinity column - 5ml of CD23-affinity resin were equilibrated, as described above. The unbound material from the pre-adsorption column was added to the CD23-column and recirculated overnight, at 4°C, as described above. After recirculation, a measuring cylinder was used to collect the unbound material as it was pumped through the column.

Elution - for both columns, the resin was washed whilst in the column:- 100ml of Extraction buffer (20 x column volume) were pumped through the column for 30 minutes; 100ml of Wash buffer 1, flowed through for 1hour; 100ml of Wash buffer 2, flowed

through for 1 hour. The progress of the washes was followed with a Pharmacia, UV1 Single Path Monitor.

To elute the bound material, five x column volumes (15 ml) of elution buffer were added to the column, and the elution followed using the UV single path monitor.
Elution buffer:- 100mM Tris pH8.5
20% Ethyleneglycol
0.5M NaCl
3M NH_4SCN
plus 0.1% Triton X 100 (pre-adsorption column)
or 30mM OGP (CD23-affinity column)

The fractions which contained protein were collected and dialysed (8-12kDa cut-off) overnight against 500ml of the following buffer:-50mM HEPES, 2mM $CaCl_2$, plus either 0.1% Triton X 100 (pre-adsorption columns) or 30mM OGP (CD23 columns). After dialysis, the sample was concentrated from 50ml to approximately 1ml with a *Filtricon* centrifuge concentrator. The concentrators were filled with 0.1M NaOH for 10 minutes then washed with H_2O, before the samples were loaded and centrifuged at 6K until the volume was sufficiently reduced. 40µl of each sample was removed and mixed with 10µl of SDS-PAGE non-reducing sample buffer (see section 2.2.8e-1).

2.2.8e *SDS-PAGE gel electrophoresis, Western blotting and ECL detection system*

2.2.8e-1 **SDS-PAGE** sample buffer (non-reducing) - 25mM Tris pH 6.8, 20% glycerol, 10% SDS, 5mM EDTA, bromophenol blue - 1 part sample buffer was added to four parts sample, before it was loaded onto the gel. A 7.5% acrylamide gel was prepared using a Biorad mini-gel gel rig; the gel was made as follows:- 25% acrylamide (30% acrylamide / 0.8% bis-acrylamide, therefore final percentage is 7.5%), 0.375M Tris pH8.8, 0.1% SDS, 0.5% ammonium persulphate and 0.05% Temed, made up to 10ml with dH_2O. The gel was set with a methanol overlay before a stacking gel (0.125M Tris, pH6.8, 0.1% SDS, 17% acrylamide (as above), 1% ammonium persulphate, 0.13% TEMED) was poured. After 40µl of each sample was loaded, the gel was run at 200V, maximum mA for 45 minutes in running buffer (1% SDS, 30g/l Tris, pH 6.8, 144g/l Glycine).

2.2.8e-2 **Western blot:** the protein was transferred from the gel to nitrocellulose (Biorad, Transblot, 0.45µm), in transfer buffer (10mM 3-(cyclohexylamino)-1-propane sulphonic acid (CAPS), 10% methanol) at 90V, 300mA for 25 minutes. After transfer, the nitrocellulose was incubated for 1 hour in blocking buffer (1xPBS, 0.1% Triton x100, 1% caesin, 1% gelatine), then washed for 15 minutes in PBS/0.05% Tween.

For the CD21 Western, 10ml of a cocktail of primary anti-CD21 MAbs (IoB1a (8μg/ml), CR2 (8μg/ml), MCA664 (8μg/ml), THB5 (20μg/ml) and BU33 (10μg/ml) in PBS / 0.05% Tween 20) were added to the blot for 4 hours at room temperature, on a shaker. The blot was then washed 3 times with blocking buffer (over 1 hour) before 20ml of 4μg/ml goat anti-mouse peroxidase IgG (in PBS/0.05% Tween 20) was added for 1 hour, at room temperature, on a shaker. The blot was washed three times with blocking buffer over 1 hour and then washed twice with PBS / 0.05% Tween 20 for 10 then 5 minutes.
For Western blots of biotinylated proteins, the blot was blocked (as described for the CD21 Western) and then put into a bag with 10ml of 2μg/ml streptavidin-peroxidase in PBS / 0.05% Tween 20 for 1 hour, at 37°C, on a shaker. Excess streptavidin-peroxidase was kept to be re-used, and the blot was washed three times with blocking buffer (over 1 hour) and then twice with PBS / 0.05% Tween 20 for 10 then 5 minutes.

2.2.8e-3 ECL detection system- To develop the blots, an Amersham, *Enhanced chemilluminescence (ECL)* kit was used, according to manufacturers instructions. 10ml of Solutions 1 and 2 were mixed in a dish, and the blot submerged for 1 minute before excess liquid was removed with a tissue and the blot wrapped in Saran wrap. The blot was exposed to Fuji RX film for between 1 second and 20 minutes, depending on the strength of the signal, to detect antibody binding.

Chapters 3, 4 and 5

Results

Results

Chapter 3

Autocrine growth factor production by SMS-SB cells

3.1 *Introduction*

The proliferation and differentiation of B lymphocytes is under the control of numerous growth factors; most act in a paracrine fashion but some are known to be produced by the B cells themselves and to have autocrine effects. Most examples of B cell autocrine growth factors come from EBV-transformed or neoplastic B-cell lines, although it is now apparent that normal cells, if appropriately activated and cycling, produce cytokines which act in an autocrine fashion to promote their growth (Blazar *et al.*, 1983; Gordon, 1985; Jurgensen *et al.*, 1986). Autocrine factor production has been implicated in the development of tumours, allowing cells to grow autonomously and avoid normal growth regulation, but it is apparent that production of an autocrine factor is not sufficient for transformation (Young *et al.*, 1991). Most of the information regarding autocrine growth factors comes from studies of mature B cells and there is very little evidence of a role for autocrine growth regulation in the early stages of B cell development.

The data presented in this chapter demonstrate a role for autocrine factor production by a pre-B cell line, SMS-SB, and attempt to identify the cytokine responsible.

The cells are derived from a patient, SB, whose acute leukaemia had the very unusual characteristic that the leukocytes were actively dividing in her blood and, when leukaemic cells were taken from the patient, they grew spontaneously in culture without going through a crisis phase. These cells are routinely cultured in serum-containing and protein-free media without the addition of exogenous mitogens. The results demonstrate that SMS-SB cells are density-dependent for growth, and that conditioned media from SMS-SB cultures can promote the growth of those cells cultured at low cell density - data which demonstrate the production of an autocrine growth factor by SMS-SB cells.

3.2 *Results*

3.2.1 *Phenotype of SMS-SB cells*

SMS-SB cells express the following B cell antigens: CD5, CD19, CD22, CD45; and are MHC (HLA-DR) class II positive. The cells are CD10- and CD20-negative with only low expression of the enzyme TdT. They have cytoplasmic μ chains but no surface immunoglobulin and, as such, have a phenotype similar to that of most normal pre-B cells (see table 5.1).

3.2.2 *SMS-SB cells proliferate in protein-free media*

SMS-SB cells proliferate under conditions of low serum and so it was decided to investigate the growth characteristics of SMS-SB cells in a medium completely devoid of protein - PFHMII. The graphs of figure 3.1 compare cell number (in cells/ml x 10^5) against days of incubation, from initial seeding densities of 10^5, $5x10^5$ and 10^6 cells per ml, in 10% FCS/DMEM (panel A) and protein-free (panel B) grown cultures. The doubling time of the cells is approximately 48 hours, at all seeding densities and, initially, the cells appear to grow at approximately the same rate in serum-containing and protein-free conditions. At an initial seeding density of 10^5 cells, the number of cells doubles after 48 hrs but the growth for the subsequent 3 days is slower than that of cells seeded at higher density. The main difference between SMS-SB cell growth in protein-free and 10% FCS medium is the final concentration to which the cells grow. In protein-free medium, the cells grow to a maximum of 2.9 x 10^6 cells/ml whereas in 10% FCS medium they reach 4.3 x 10^6 cells/ml, suggesting that essential nutrients in protein-free medium are depleted more quickly than in 10% FCS medium. However, the growth of SMS-SB cells is not adversely affected by culture in protein-free medium and this medium was chosen for further experiments because it eliminates the possibility of synergistic reactions between serum proteins and cytokines being tested.

3.2.3 *SMS-SB cells are density-dependent for growth*

SMS-SB cells were seeded at various initial densities to examine their ability to proliferate in protein-free media. The data in figure 3.2 represent incorporation of [^{3}H]-thymidine ([^{3}H]-TdR) by cells seeded at 10^2, 10^3, 10^4 and 10^5 cells per well and incubated for 1, 2 or 3 days. SMS-SB cells seeded at 10^5 and 10^4 cells per well continue to grow at the characteristic rate, with a two-fold increase in incorporated [^{3}H]-TdR after 2 days. Cells seeded at 1000 cells/well show a lag period of 2 days and then begin to proliferate; cells seeded at 100 cells/well do not proliferate and actually show decreased [^{3}H]-TdR incorporation, suggesting that they are dying. Thus, SMS-SB cells are density-dependent for growth. The most likely explanation for these observations is that SMS-SB cells produce an autocrine growth factor which is required at a minimum concentration to maintain growth of the cells. The presence of 10^5, 10^4 cells per well appears sufficient to maintain the concentration of the autocrine factor, whereas the lag phase seen with 1000 cells/well may be explained by the time taken for the cells to produce a sufficient quantity of the autocrine factor to allow proliferation. Apparently, at 100 cells/well, there is not enough autocrine factor produced to 'condition' the media and the cells lose the ability to grow autonomously.

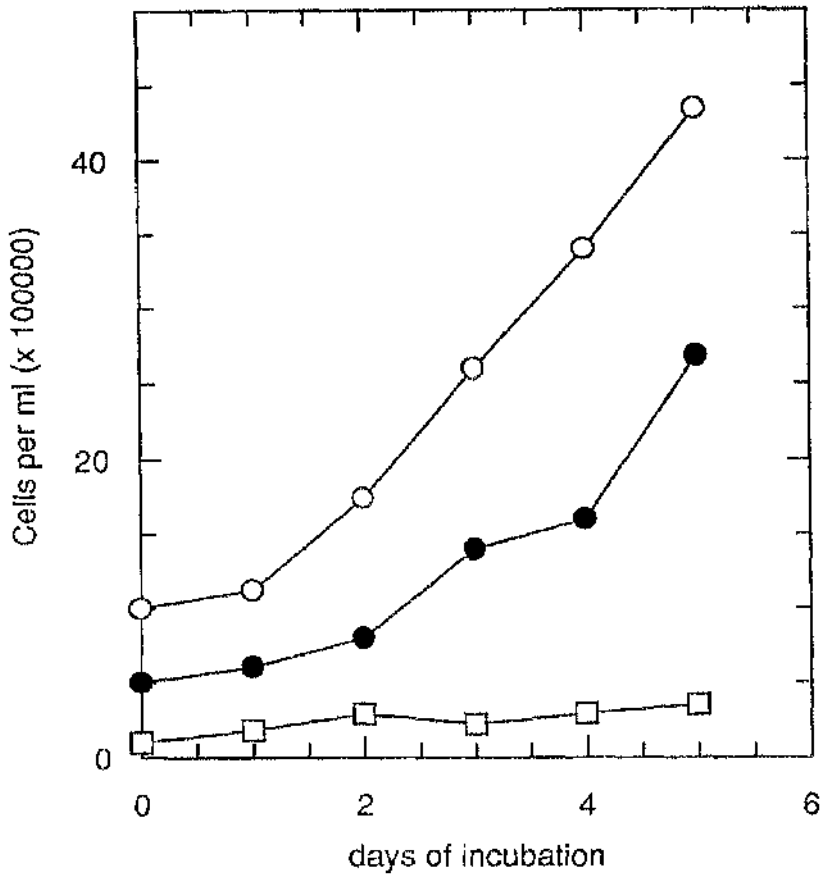
3.2.4 *SMS-SB cells produce an autocrine growth factor*

To test the hypothesis that SMS-SB cells produce an autocrine growth factor, medium from cultures of SMS-SB cells grown at the 'normal' cell density of $5x10^5$ cells/ml (known

Fig.3.1. SMS-SB cells grow in protein-free medium.

For each of the experiments illustrated in figure 3.1., SMS-SB cells were harvested from log-phase cultures in either 10% FCS/DMEM (panel A) or PFHMII (panel B) and seeded at 10^5 (□), $5x10^5$ (●) and 10^6 (○) cells per ml in the same medium. The number of cells per ml in each culture was counted every day for 5 days of incubation, using trypan blue to determine the viability of the cells. The graphs compare cell number against days of incubation, for the different initial seeding densities.

a)


Cells per ml (x 100000)
40
20
0
0
2
4
6
days of incubation

b)

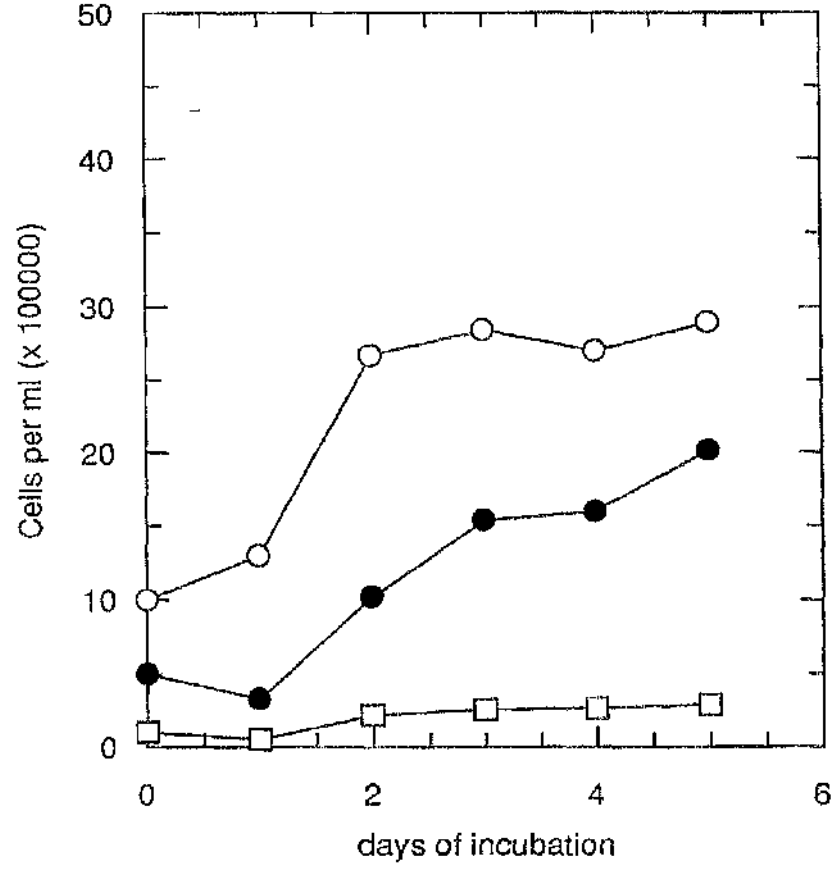

Cells per ml (x 100000)
50
40
30
20
10
0
0
2
4
6
days of incubation

Fig.3.2 SMS-SB cells are density dependent for growth

SMS-SB cells were harvested from log-phase cultures in PFHMII and seeded at 10^2, 10^3, 10^4 and 10^5 cells per well, as shown in the legend, in the same medium. Cultures were pulsed with 0.3μCi/well [^{3}H]-TdR for six hours prior to harvest, on days 1,2 and 3. All cultures were established in triplicate and the error bars represent the standard deviation of the triplicate data. This is a single experiment, representative of four independent repeats.

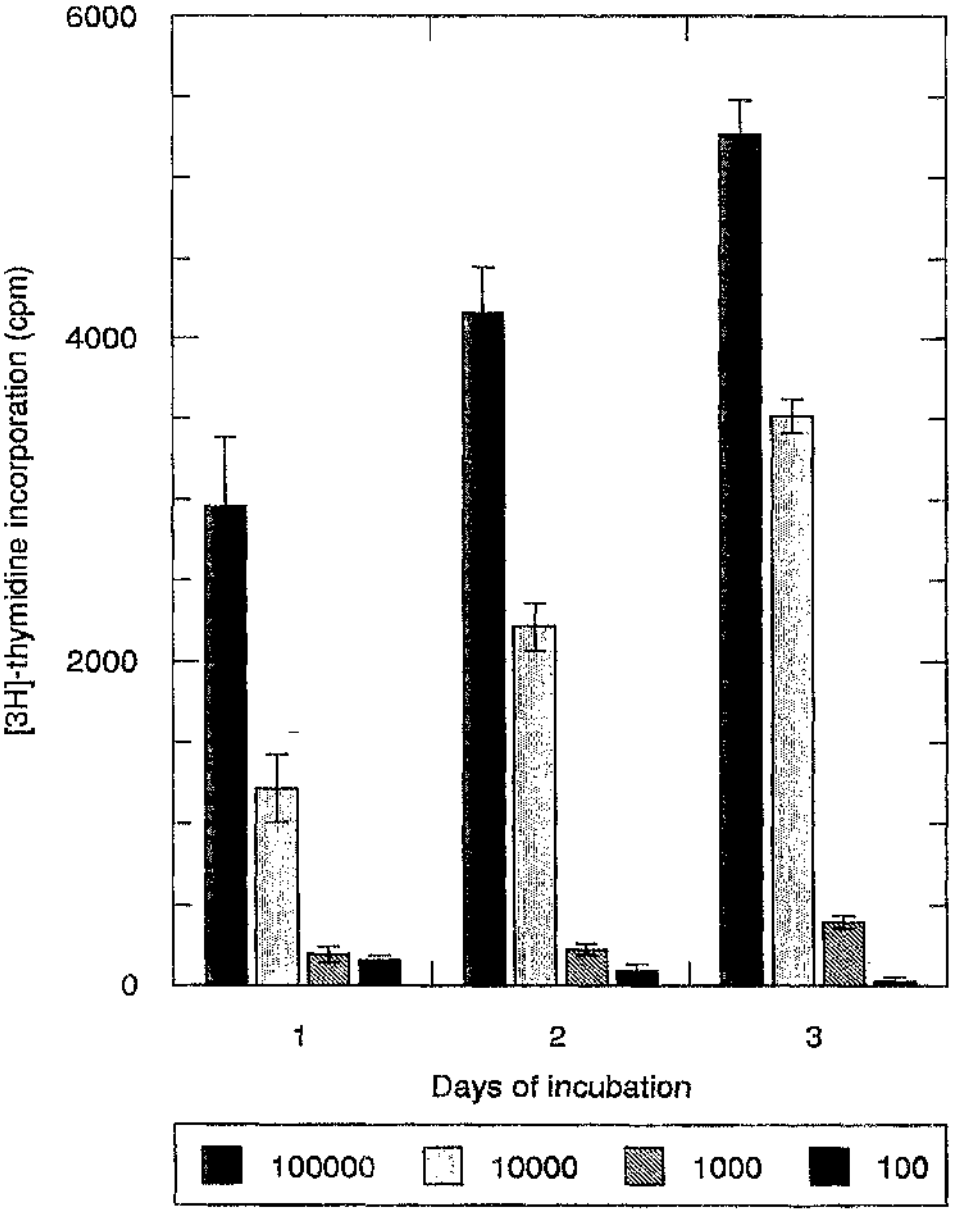

6000
4000
2000
0
[3H]-thymidine incorporation (cpm)
1
2
3
Days of incubation
100000
10000
1000
100

Fig.3.3 SMS-SB cells produce an autocrine growth factor

SMS-SB cells were seeded at 2500 cells per well in PFHMII; PFHMII conditioned medium (as described in materials and methods) was included at 1, 5, 10, 15 and 20% of the final culture volume of 100µl. The cultures were incubated at 37°C for 48hrs and pulsed with 0.3µCi/well [^{3}H]-TdR for six hours prior to harvest. All cultures were established in triplicate, and the experiment is representative of more than five independent repeats. The error bars represent the standard deviation of triplicate data.

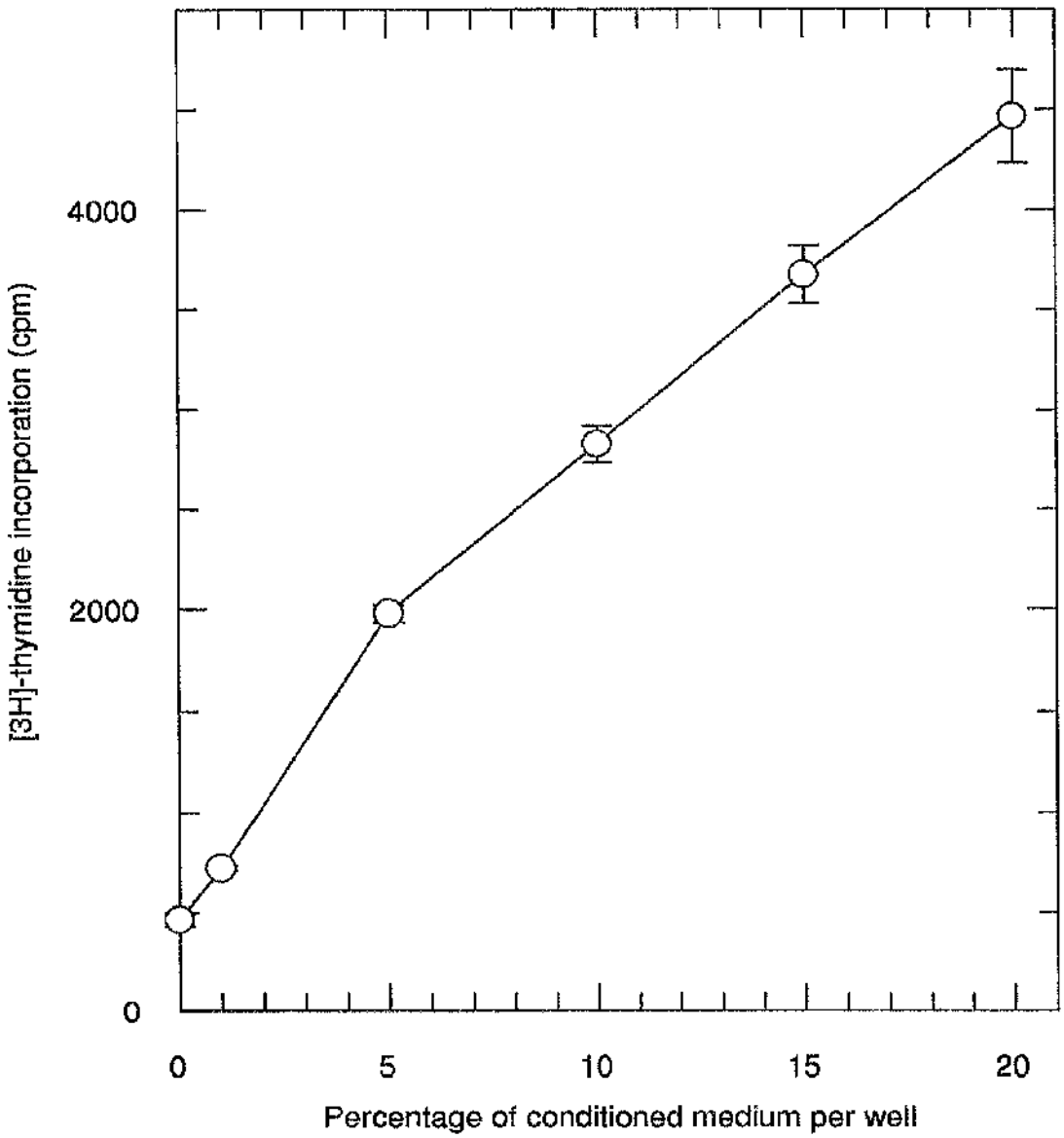

[3H]-thymidine incorporation (cpm)
4000
2000
0
0
5
10
15
20
Percentage of conditioned medium per well

Fig.3.4 Growth of SMS-SB cells in the presence and absence of conditioned medium.

The cells were prepared as described for figure 3.3; however, they were seeded in the culture wells at 1000, 2500 and 5000 cells/well, in a final volume of 100μl. Cells were cultured in the presence and absence of conditioned medium (CM) at 20% of the final culture volume. The data legend illustrates which bar on the chart represents each seeding density (cells per well), and whether or not conditioned medium was present in the culture (+ CM). The cultures were incubated for 24, 48 and 72 hours, and pulsed with 0.3μCi/well [^{3}H]-TdR for six hours prior to harvest. All cultures were established in triplicate, and the experiment is representative of more than five independent repeats. The error bars represent the standard deviation of triplicate data.

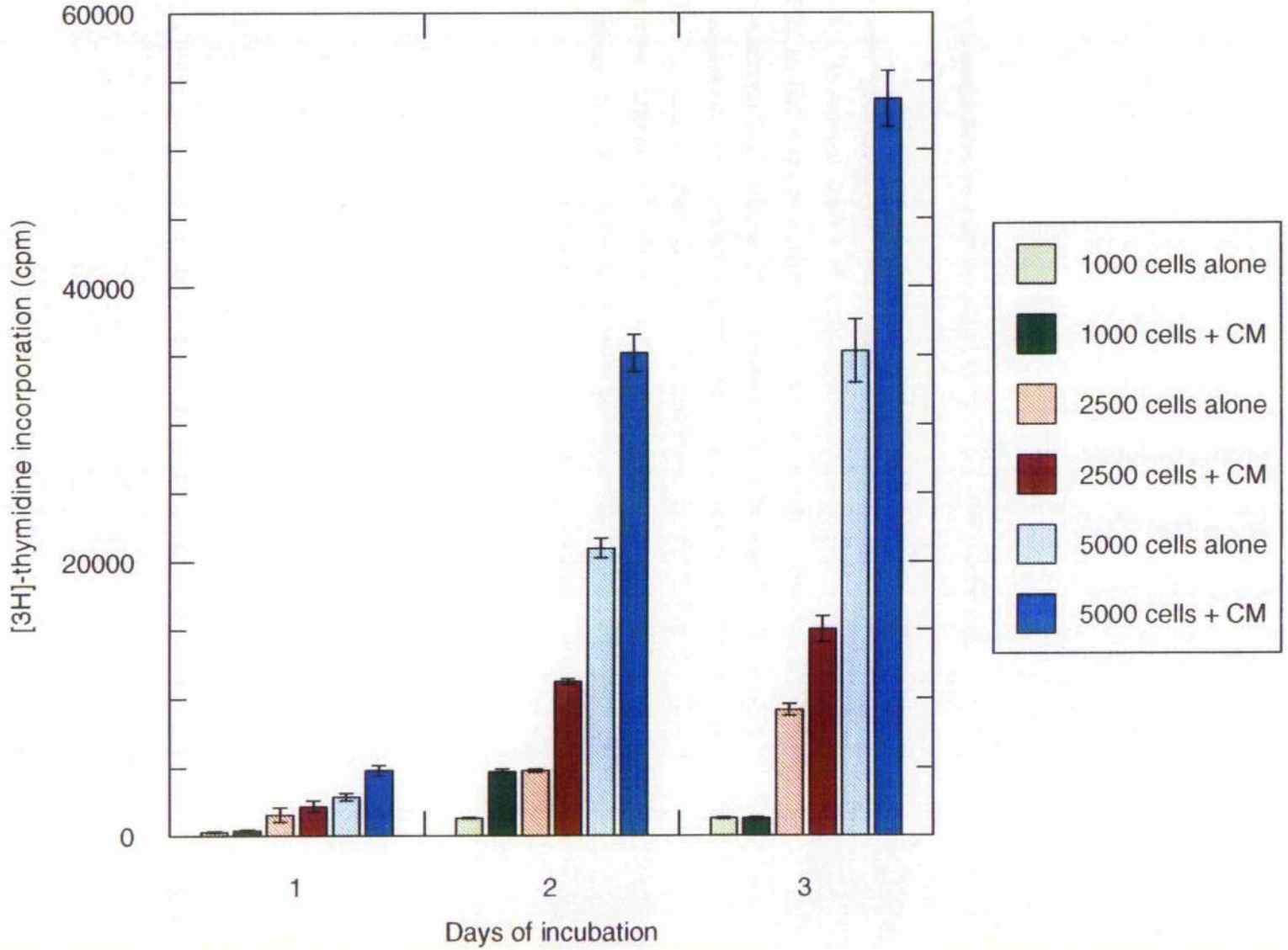
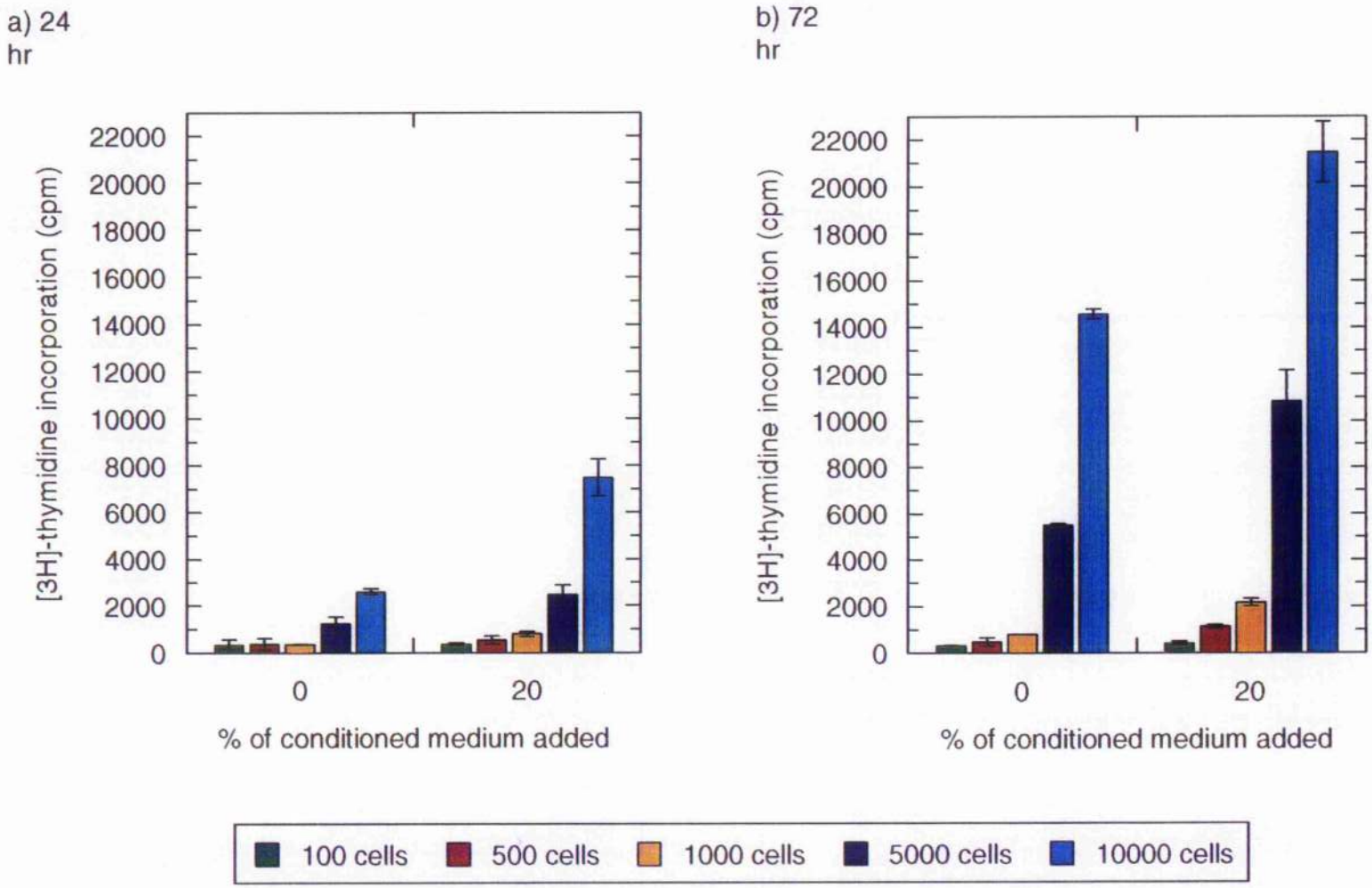

60000
40000
20000
0
[3H]-thymidine incorporation (cpm)
1
2
3
Days of incubation
1000 cells alone
1000 cells + CM
2500 cells alone
2500 cells + CM
5000 cells alone
5000 cells + CM

Fig.3.5 [^{3}H]-TdR incorporation by SMS-SB cells cultured at low cell density in the presence and absence of conditioned medium.

The experiment was performed as described in figure 3.4. but the cells were seeded at 10^2, $5x10^2$, 10^3 $5x10^3$ and 10^4 cells per well. The cultures were harvested after a) 24 hrs and b) 72hrs, only. The graphs represent incorporation of [^{3}H]-TdR for various seeding densities of cells (in cells per well), with and without conditioned medium. The data legend illustrates which bar represents each initial seeding density (cell number per well) and whether the cells were cultured in the presence of conditioned medium (+ CM). The cultures were pulsed with 0.3μCi/well [^{3}H]-TdR for six hours prior to harvest. All cultures were established in triplicate, and the experiment is representative of four independent repeats. The error bars represent the standard deviation of triplicate data.


a) 24 hr
b) 72 hr
[3H]-thymidine incorporation (cpm)
22000
20000
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
0
20
% of conditioned medium added
100 cells
500 cells
1000 cells
5000 cells
10000 cells

as conditioned medium (CM), was added back to cells propagated at lower cell density and growth was compared in the presence and absence of conditioned medium. Figure 3.3 shows a graph of [^{3}H]-TdR incorporation by 2500 cells, versus the final percentage of CM in the culture well, after an incubation period of 48 hours. CM augments the growth of the cells in a dose-dependent manner - in wells where the cells were cultured in fresh medium alone, there was a mean [^{3}H]-TdR incorporation of approximately 500 cpm compared with a maximum mean of 4500 cpm in wells where 20% CM was present. The data of figure 3.4 are from experiments where different numbers of SMS-SB cells were incubated in the presence or absence of conditioned medium for 1,2 or 3 days. The results demonstrate that CM promotes the growth of SMS-SB cells at 2500 and 5000 cells/well, although, at these densities, the cells can grow in media alone. At 1000 cells per well, conditioned media appears to promote growth of the cells for 48 hours and then the [^{3}H]-TdR incorporation decreases at 72 hours suggesting that the CM effect is short-lived. It is possible that the exogenous conditioned media has been depleted and the cells are unable to produce enough of the factor to condition their own media. At 2500 and 5000 cells per well, the exogenous conditioned media may have sustained cell growth long enough for the cells to produce the necessary concentration of CM and continue growth. If there is a lag when cells are unable to proliferate, between the initial seeding and the time when the minimum required concentration of CM is reached, it could explain why the addition of CM produces higher incorporation; it would remove the lag phase of growth and allow continued proliferation.

Conditioned media appears to promote the growth of SMS-SB cells cultured at densities as low as 500 cells per well but if only 100 cells are seeded per well then even addition of CM cannot promote their growth (figure 3.5). It is likely that the exogenous CM is acting to 'save' the cells until they can produce sufficient autocrine factor to condition their own media. Presumably, cultures of 100 cells/well are unable to attain the required concentration of CM in 72 hours, before the exogenous CM is depleted and the cells can no longer proliferate.

Thus, SMS-SB are density-dependent for growth due to the production of an autocrine growth factor which, if present at a high enough concentration, allows them to grow autonomously under protein-free conditions.

3.2.5 *Preliminary identification and characterisation of the autocrine growth factor*

Initial studies to characterise the SMS-SB autocrine growth factor (SB-AF) involved the use of *Centricon* filters which allow concentration of molecules above a specific molecular weight. The autocrine activity can be concentrated over 10kDa, 30kDa and 100kDa filters (figures 3.6, 3.7 and 3.8 respectively), suggesting that SB-AF is either a large protein of

over 100kDa in size or that it is a 'sticky' molecule which can bind to other proteins produced by SMS-SB cells and thus be concentrated above the 100kDa filter. The data of figures 3.6-8 demonstrate the growth promoting effect of normal CM and how this effect is removed once the media is passed through various sized filters. If the material retained above the filters is added to the cells then it greatly enhances proliferation of the cells - above that seen with normal CM - presumably because the SB-AF has been concentrated. That SB-AF is actually removed from CM by the filters is shown by the graph representing the material contained in the filtrate. The material which has passed through the filters does not promote SMS-SB cell growth, indicating that the autocrine factor has been removed. To demonstrate that the filters themselves have no effect on the SB-AF, samples of the retentate and filtrate were mixed to reconstitute the CM. From the graphs it can be seen that reconstituted CM gives results almost identical to those of normal CM, showing that the activity is not altered by concentration/separation over the filters. Therefore, the SB-AF can be concentrated above an 100kDa filter but gel filtration chromatography will be required to determine the native molecular weight of SB-AF.

One problem encountered during proliferation assays performed with SMS-SB cells in PFHMII is that addition of protein, for example BSA or HSA, promotes growth of the cells. As can be seen from figure 3.9, the protein effect does not occur until addition of approximately 10μg/ml of BSA and seems to plateau at 40-100μg/ml. It is possible that this effect contributes to the enhanced proliferation seen on addition of the *Centricon* retentates to SMS-SB cells, although the filters do appear to remove SB-AF, since filtrates alone have no growth promoting effect compared to normal CM.

In an effort to identify candidates for the SB-AF, a range of recombinant human cytokines were analysed for the capacity to promote the proliferation of SMS-SB cells cultured at low cell density in PFHMII (figure 3.10). The majority of cytokines tested had no ability to promote SMS-SB cell growth and gave a stimulation index of approximately 1, suggesting that they do not represent the SB-AF. IL-5 gave a stimulation index of 2.4, sCD23 gave a stimulation index of 5.6, and PDGF-$\alpha\alpha$, an index of 2.2. However, data from experiments performed by the *Genetics Institute*, Cambridge, Massachusetts, using cytokine bioassays and analysis of SMS-SB RNA, have shown that none of IL-1α to IL-7, or TNFα or β, are actually produced by SMS-SB cells (data not shown); thus, IL-5 does not represent the SB-AF. The effect of sCD23 on SMS-SB cells will be discussed in chapter 4A. No analysis has been made for production of IL-10, IL-11, IL-13 or IL-15, although the recombinant forms of IL-11, IL-13 and IL-15 do not promote SMS-SB cell proliferation, in the range of concentrations tested (figure 3.10). The growth promoting effect of IL-10 on SMS-SB cells remains to be tested.

Fig.3.6 SMS-SB cell autocrine activity can be concentrated over a 10kDa filter.

Centricon, 10kDa molecular weight cut-off filters were used to concentrate the SMS-SE cell conditioned medium (as described in materials and methods). 48 hour proliferation assays were set up with 2500 cells per well in a volume of 100µl PFHMII (as described in materials and methods) in the presence of 0, 1, 5, 10, 15 and 20% conditioned medium either: normal CM (O); retentate CM- material concentrated above the 10kDa filter-(●); filtrate CM-material which had passed through the filter-(open triangles); CM which had been reconstituted by mixing the retentate and the filtrate together (closed triangles). The cultures were pulsed with 0.3µCi/well [^{3}H]-TdR for six hours prior to harvest. All cultures were established in triplicate, and the experiment is representative of three independent repeats. The error bars represent the standard deviation of triplicate data.

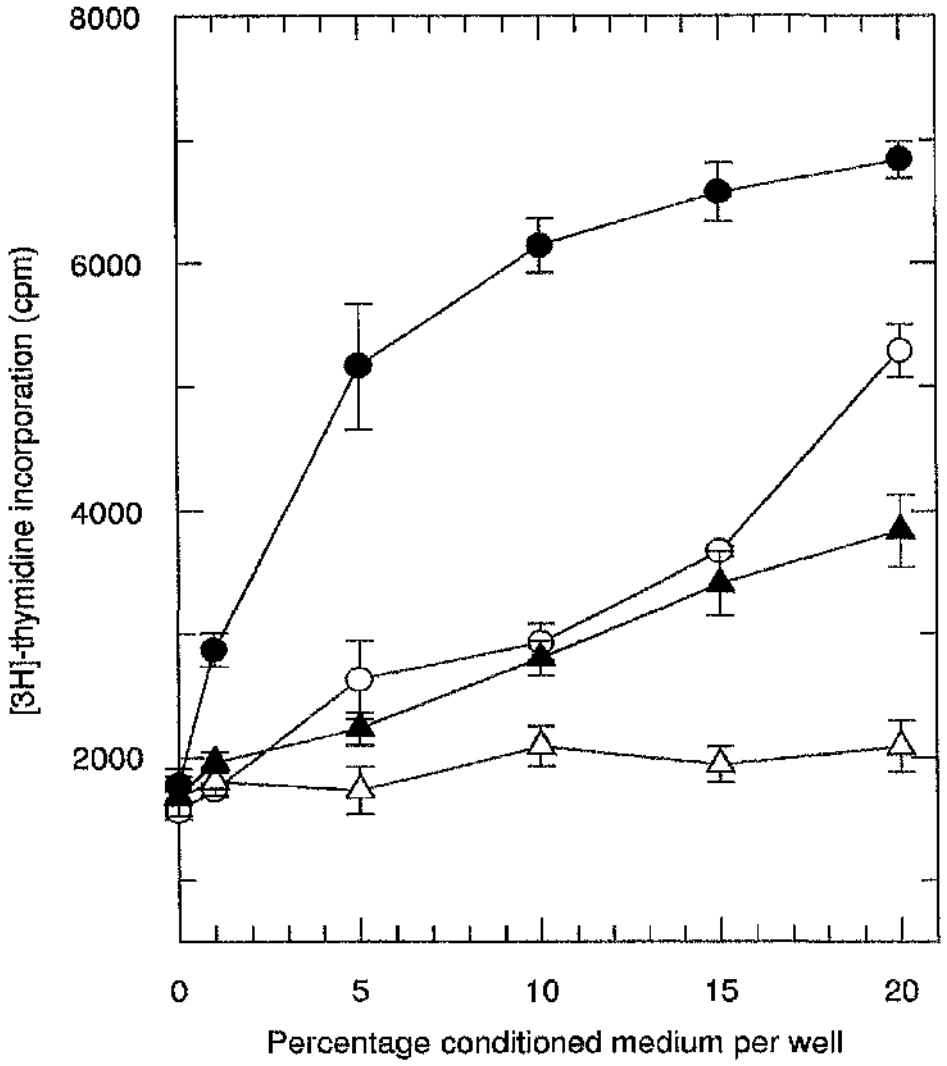

8000
6000
4000
2000
[3H]-thymidine incorporation (cpm)
0
5
10
15
20
Percentage conditioned medium per well

Fig.3.7. SMS-SB cell autocrine activity can be concentrated over a 30kDa filter.

The experiment was performed exactly as described in the figure legend of figure 3.6 but a 30kDa cut-off *Centricon* filter was used to concentrate the conditioned medium.

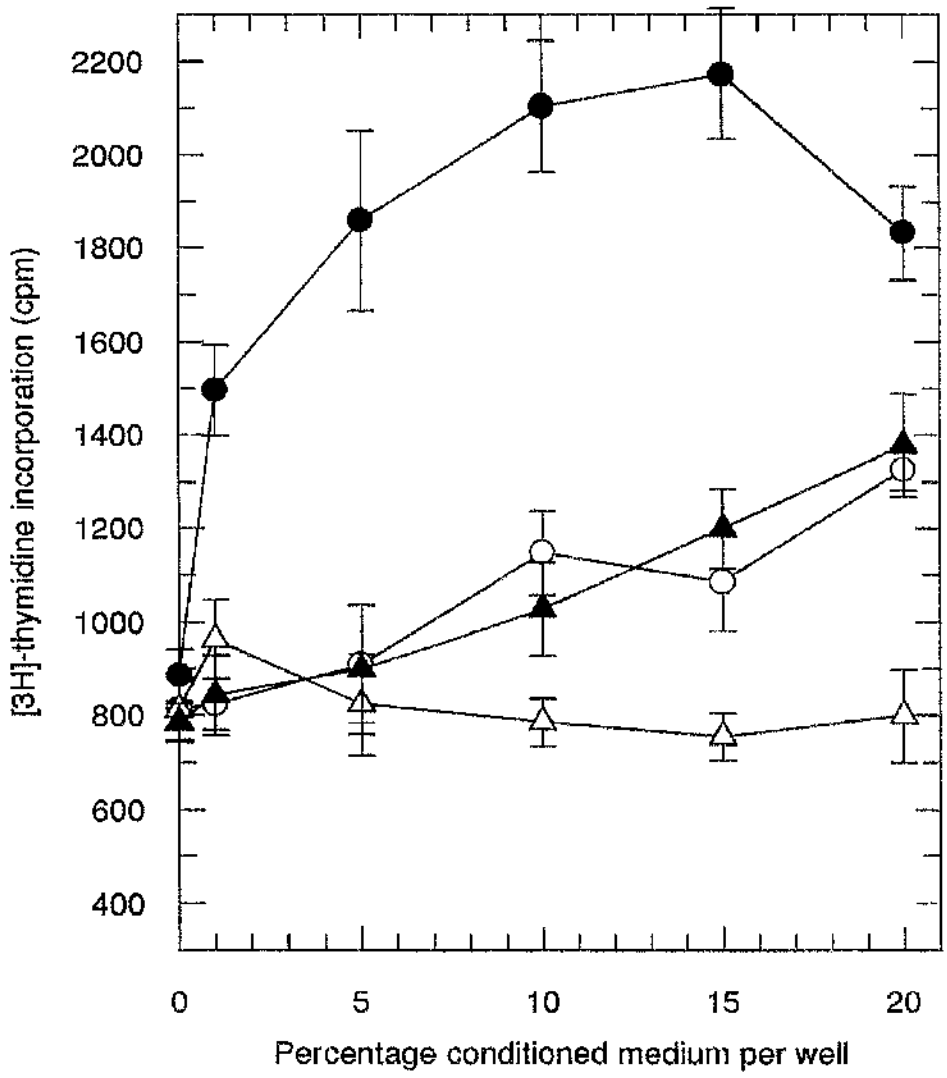

2200
2000
1800
1600
1400
1200
1000
800
600
400
[3H]-thymidine incorporation (cpm)
0
5
10
15
20
Percentage conditioned medium per well

Fig.3.8 SMS-SB cell autocrine activity can be concentrated over a 100kDa filter.

The experiment was performed exactly as described in the figure legend of figure 3.6 but an 100kDa cut-off *Centricon* filter was used to concentrate the conditioned medium.

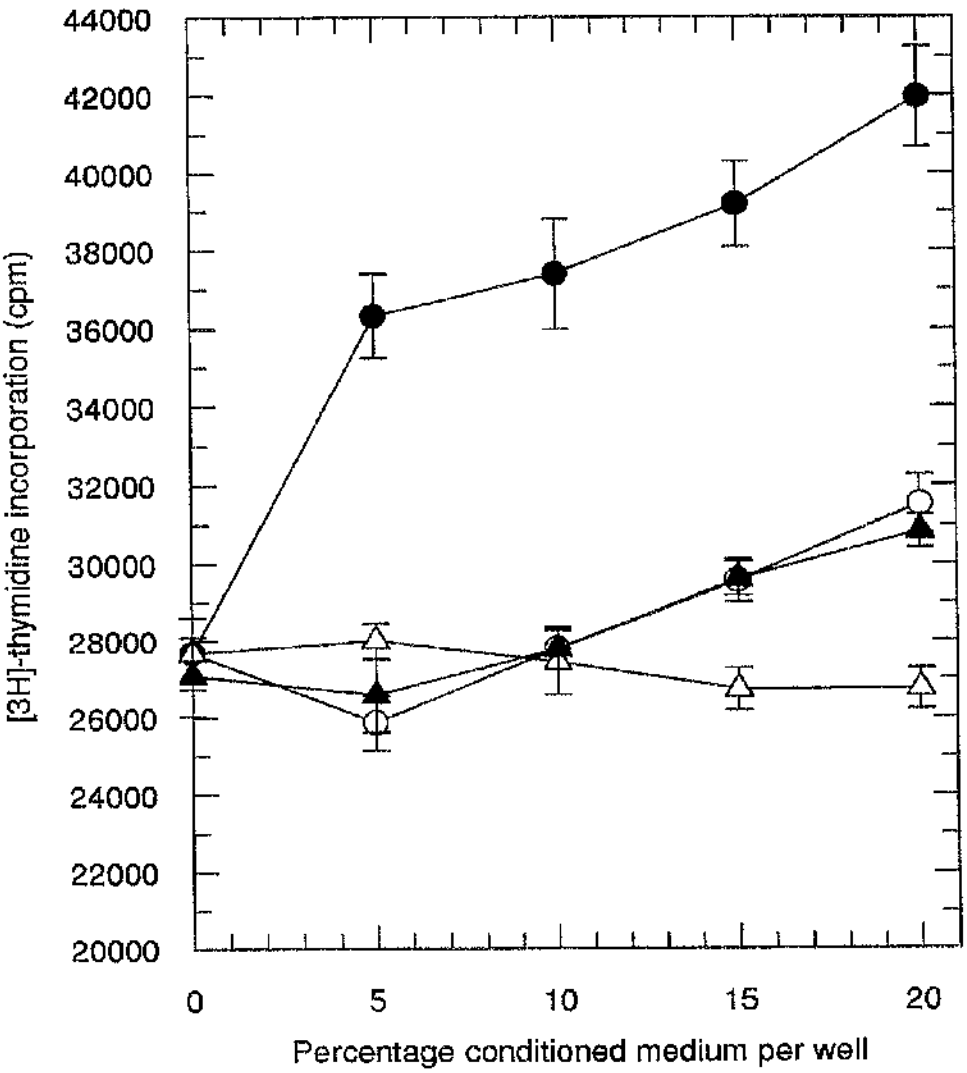

44000
42000
40000
38000
36000
34000
32000
30000
28000
26000
24000
22000
20000
[3H]-thymidine incorporation (cpm)
0
5
10
15
20
Percentage conditioned medium per well

Fig.3.9. Effect of protein upon [^{3}H]-TdR uptake by SMS-SB cells

A proliferation assay was performed using 2500 cells per well in PFHMII supplemented with increasing concentrations of bovine serum albumin (BSA). The graph represents the [^{3}H]-TdR incorporation recorded after the cells were incubated for 48hrs in either medium alone or in PFHMII supplemented with increasing concentrations of BSA, from 100pg/ml up to 100µg/ml BSA (shown on a log scale). All cultures were established in triplicate, and the experiment is representative of three independent repeats. The error bars represent the standard deviation of triplicate data.

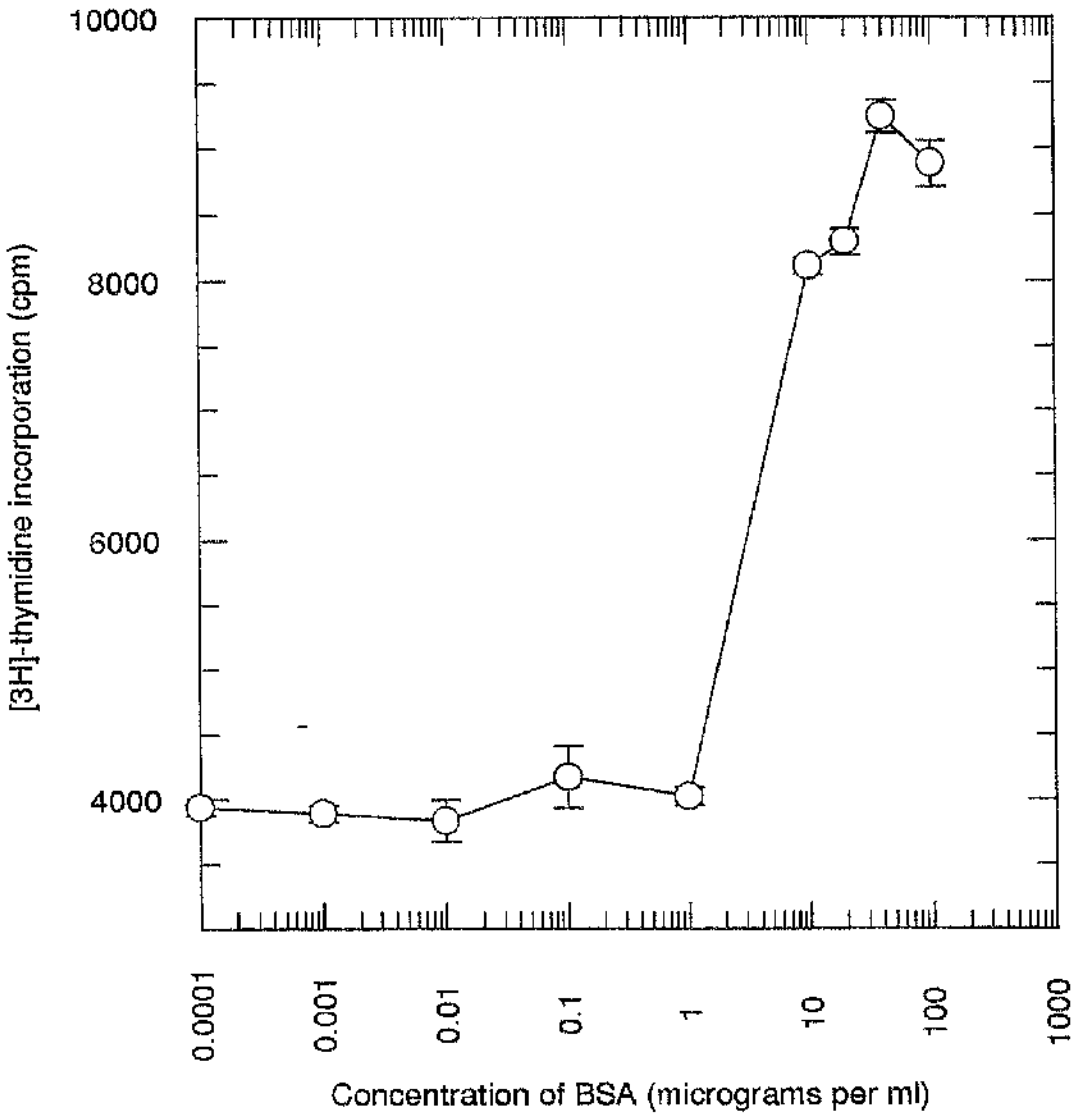
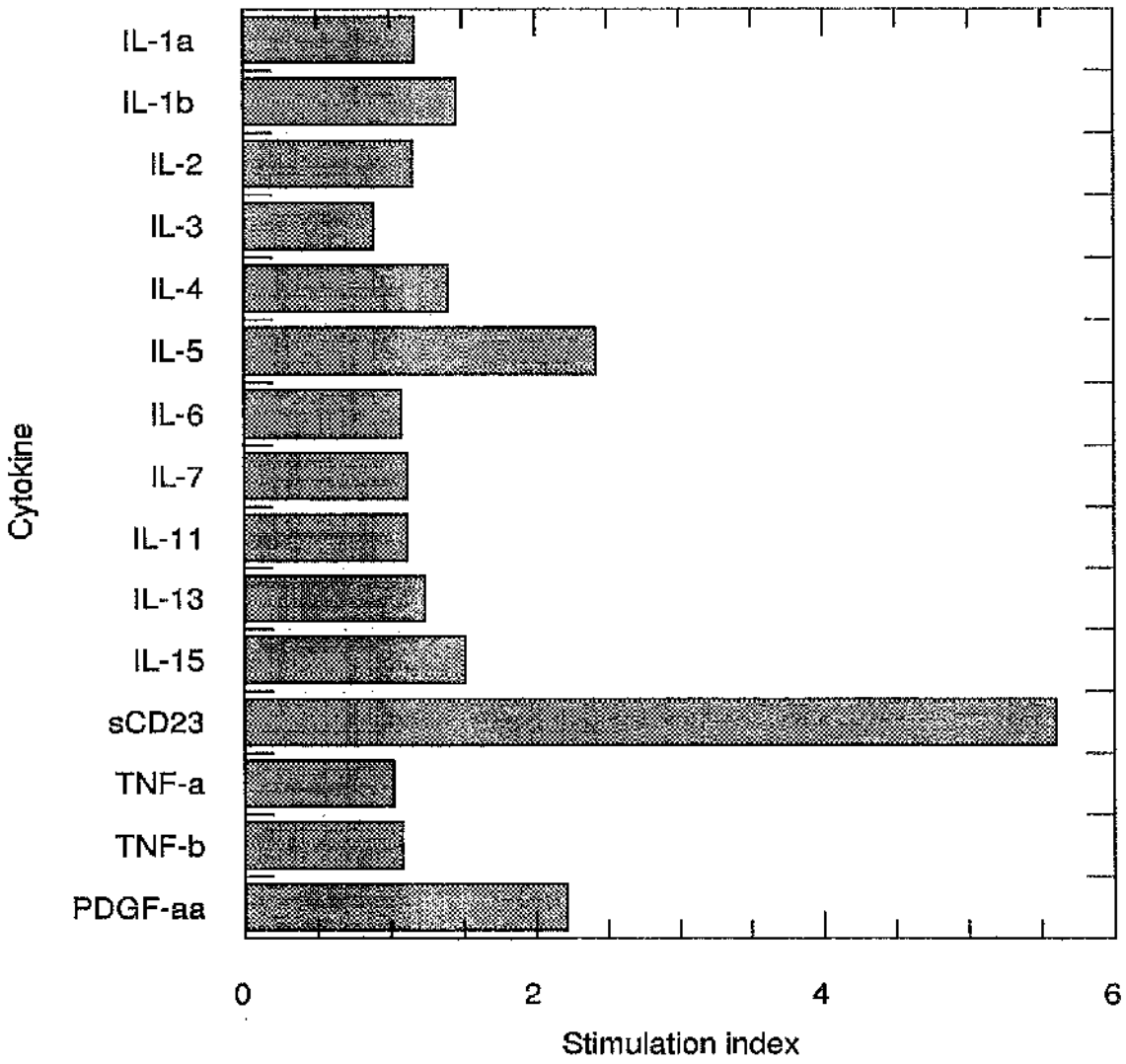

10000
8000
6000
4000
[3H]-thymidine incorporation (cpm)
0.0001
0.001
0.01
0.1
1
10
100
1000
Concentration of BSA (micrograms per ml)

Fig.3.10. Effect of recombinant cytokines upon [^{3}H]-TdR uptake by SMS-SB cells

Proliferation assays were performed, using 2500 cells per well, in PFHMII supplemented with recombinant cytokines, as detailed on the bar chart. The individual cytokines were included at several concentrations, however, the data presented represents the effect of 50U/ml of each cytokine, in a final culture volume of 100μl. The cultures were pulsed with [^{3}H]-TdR for six hours prior to harvest at 24hrs and each culture was established in triplicate. A stimulation index was calculated for each cytokine according to the equation:-

$$\text{Stimulation Index} = \frac{\text{Mean cpm incorporated in presence of cytokine}}{\text{Mean cpm incorporated in absence of cytokine}}$$

Each experiment illustrated for an individual cytokine is representative of at least three independent repeats.


IL-1a
IL-1b
IL-2
IL-3
IL-4
IL-5
IL-6
IL-7
IL-11
IL-13
IL-15
sCD23
TNF-a
TNF-b
PDGF-aa
Cytokine
0
2
4
6
Stimulation index

3.2.6 *PDGF- a candidate for SB-AF*

PDGF-$\alpha\alpha$ is able to promote the growth of SMS-SB cells cultured at low cell density, and gives a stimulation index of approximately 2 (figure 3.10). This stimulation index is quite low, compared to the effect of soluble CD23 (discussed in chapter 4A), but the result is of great interest because SMS-SB cells are known to release PDGF-α chains and they also express PDGF-β receptors (Tsai *et al.*, 1994). Thus, PDGF-$\alpha\alpha$ is implicated as a candidate for the SB-AF. Figure 3.11 shows a dose-response titration of PDGF-$\alpha\alpha$ action on the proliferation of 2500 SMS-SB cells/well after a 48 hour incubation. The cells grew at the characteristic rate after addition of PDGF-$\alpha\alpha$, with incorporation of [^{3}H]-TdR at 48 hrs being twice the level recorded for cells in medium alone. The maximum response was seen on addition of 1ng/ml PDGF-$\alpha\alpha$, which is within the normal concentration range for PDGF activity. Expression, at the RNA level, of the PDGF-A chain gene has also been demonstrated in another pre-B cell line, NALM-6, which expresses the PDGF-A receptor and can bind PDGF-$\beta\beta$, but the cells do not secrete PDGF-$\alpha\alpha$ into the media (Tsai *et al.*, 1994). Unfortunately, the action of recombinant PDGF, or SMS-SB cell conditioned medium, upon Nalm-6 cell proliferation (or vice versa) could not be examined because Nalm-6 cells do not grow in protein-free media are not density-dependent for growth.

To investigate whether PDGF is responsible for the autocrine activity present in SMS-SB cell conditioned media, samples of CM were pre-incubated with a neutralising anti-human PDGF at 4°C for 3 hours (with gentle agitation) before addition to 1000 cells/well in a 48 hr proliferation assay (figure 3.12). Unfortunately, attempts to perform subtraction experiments by removing the antibodies, and any bound PDGF, from the CM prior to addition to the cells - using *Dyna-beads* with bound protein G - were unsuccessful because treatment of normal PFHMII with the beads made the media inhibitory for SMS-SB cell growth (data not shown). It is possible that residual azide was present in the beads, although thorough washing did not rectify the situation.

The results of titrating anti-PDGF (which is able to neutralise human PDGF-$\alpha\beta$, -$\alpha\alpha$, and -$\beta\beta$ forms) with a constant, sub-optimal amount (10% final concentration) of CM, in a proliferation assay, are shown in figure 3.12. If the mean [^{3}H]-TdR incorporation due to addition of antibody alone, is subtracted from the mean [^{3}H]-TdR incorporation recorded for a constant 10% CM plus anti-PDGF, the results suggest that anti-PDGF does remove some of the SB-AF activity (see figure legend for calculation used). However, the results are not significantly different to those observed on addition of 10% CM alone. The protein-effect caused by addition of the antibody at concentrations of over 10µg/ml may have affected the results, however, it is possible that the small inhibition of autocrine activity is real but PDGF represents one of several molecules which comprise SB-AF. It is also possible that PDGF has no real role to play, although the fact that the cells express

Fig.3.11. Effect of PDGF-$\alpha\alpha$ on [^{3}H]-TdR incorporation by SMS-SB cells

Proliferation assays were performed, as described previously, to examine the effect o PDGF-$\alpha\alpha$ on the growth of SMS-SB cells. 2500 cells were seeded per wells in PFHMI supplemented with increasing concentrations of PDGF-$\alpha\alpha$ (ng/ml), and incubated for 4 hrs; for the last 6hrs prior to harvest, the cells were pulsed with [^{3}H]-TdR. Each cultur was established in triplicate and the experiment is representative of four independen repeats.

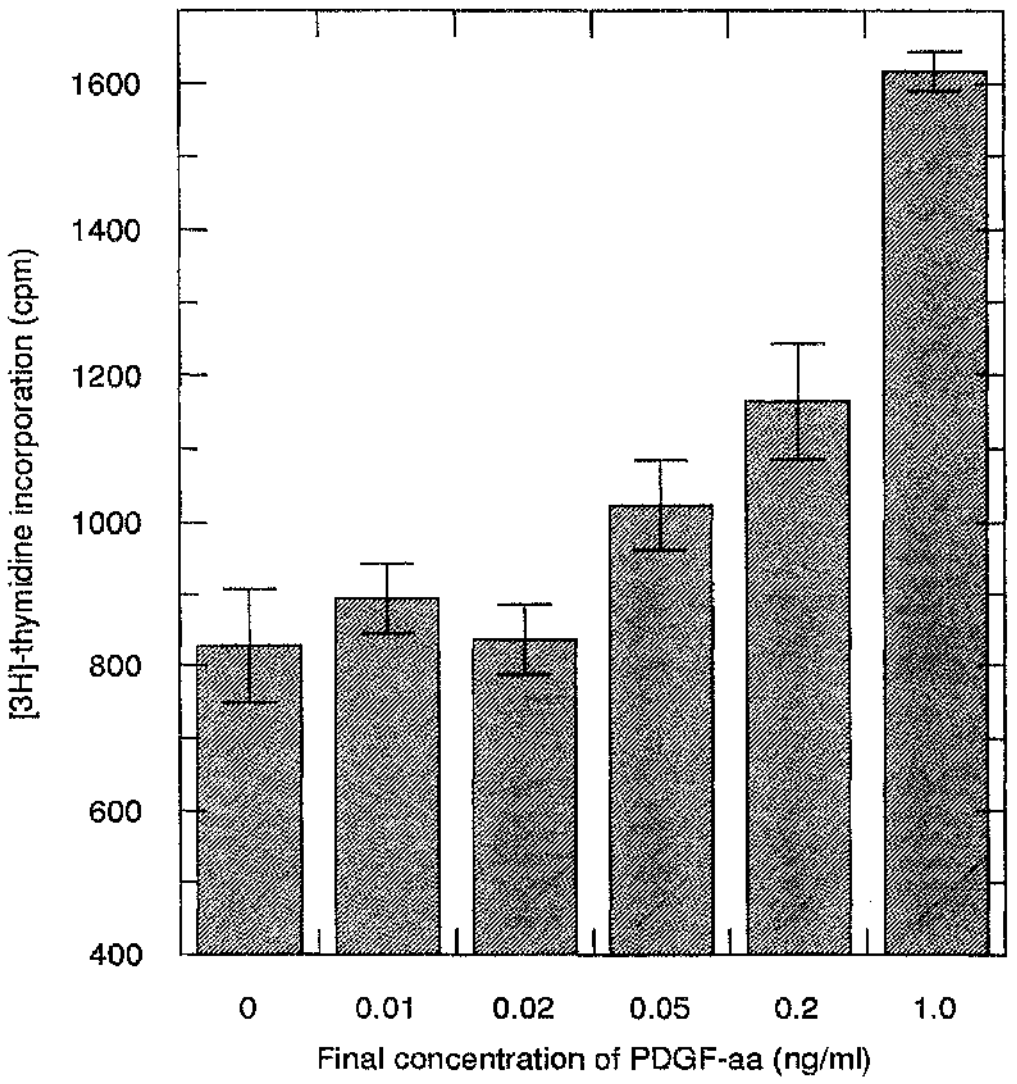

1600
1400
1200
1000
800
600
400
[3H]-thymidine incorporation (cpm)
0
0.01
0.02
0.05
0.2
1.0
Final concentration of PDGF-aa (ng/ml)

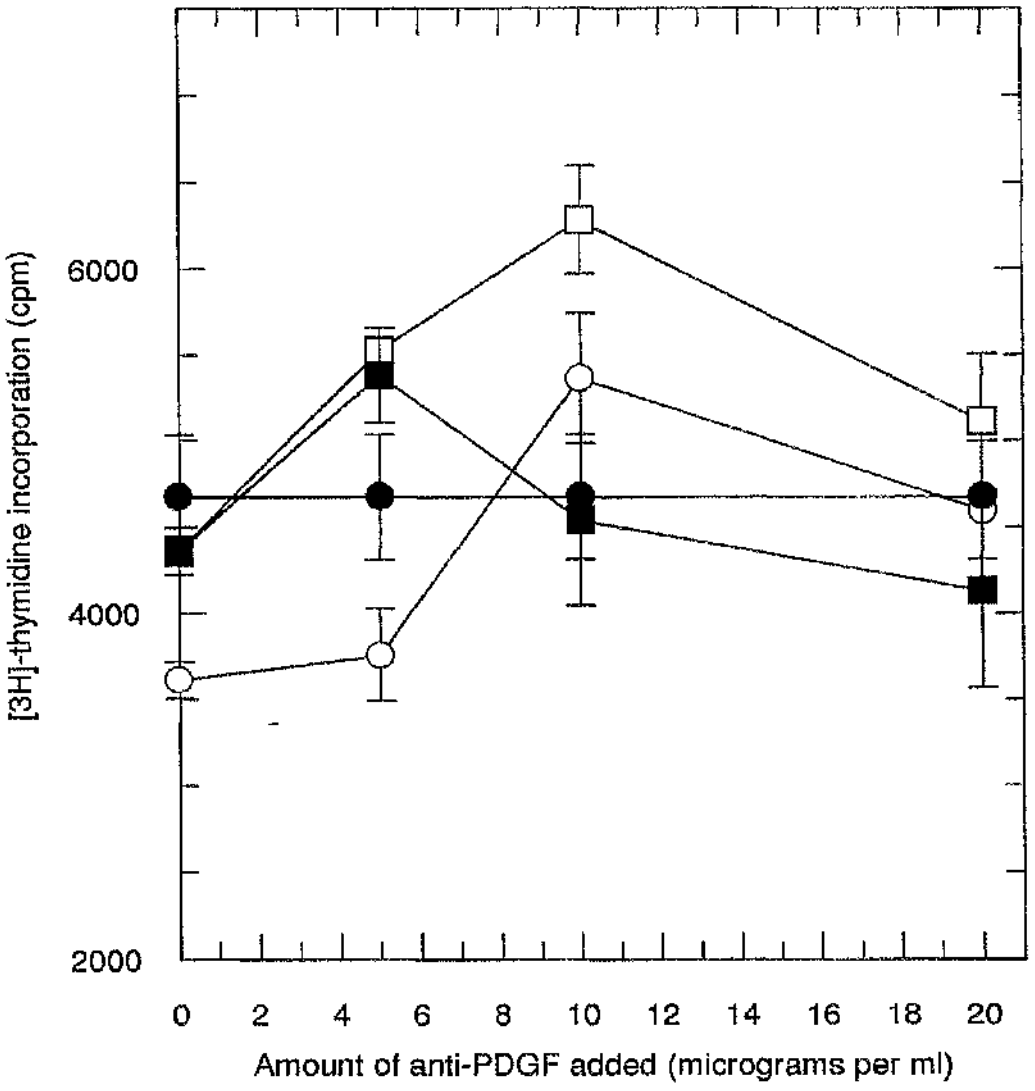
Fig.3.12. Effect of anti-PDGF on SMS-SB cell autocrine growth factor

Proliferation assays were performed (as described previously) using 2500 cells per wel and incubated for 48hrs. A comparison was made of [^{3}H]-TdR incorporation when cell: were cultured in: 10% conditioned medium (CM) (●); increasing concentrations o neutralising anti-PDGF-$\alpha\alpha$/PFHMII from 0 to 20μg/ml (○); a constant 10% CM plu increasing concentrations of neutralising anti-PDGF-$\alpha\alpha$/PFHMII from 0 to 20μg/ml (□) The fourth line (■) represents the incorporated cpm for 10% CM plus anti-PDGF-$\alpha\alpha$ after the data has been corrected for the protein effect due to addition of antibody alon (○). This correction was made by calculating the stimulation (cpm) due to antibody alon (cpm in the presence of antibody - cpm in PFHMII alone) and subtracting this value fro the incorporated cpm in the presence of both 10% CM and anti-PDGF-$\alpha\alpha$. The standar error of the corrected data was calculated using the equation:-

$$Sc = \sqrt{((S_1)^2 + (S_2)^2)/2)}$$

Where Sc represents the standard deviation (S.D.) of the corrected mean; S_1 represent the S.D.of mean 1; and S_2 represents the S.D. of mean 2.

Each culture was established in triplicate and the experiment is representative of thre independent repeats.


[3H]-thymidine incorporation (cpm)
6000
4000
2000
0
2
4
6
8
10
12
14
16
18
20
Amount of anti-PDGF added (micrograms per ml)

PDGF receptors and secrete PDGF-α chains suggests that it may contribute to the ability of SMS-SB cells to grow autonomously.

3.3 *Discussion*

The results detailed in this chapter demonstrate that the pre-B cell line, SMS-SB, can grow autonomously in protein-free medium and maintain the same growth rate as when cultured in serum-containing medium. The use of PFHMII allows the growth promoting effects of various cytokines to be examined without synergistic or inhibitory effects from growth factors present in serum. SMS-SB cells are density-dependent for growth and die when cultured at densities of less than 1000 cells per well in PFHMII (figure 3.2). The density of 1000 cells per well may be 'on the cusp' for cell survival since in some experiments the cells die at this density and, in others, the cells begin to proliferate after a lag phase of approximately 2 days (figure 3.5). This density-dependence for growth suggests that SMS-SB cells produce an autocrine factor to sustain and promote their growth. If the cells are seeded at low density, i.e. less than 1000 cells per well, then they may be unable to produce enough of this factor to condition the medium for growth. This hypothesis is supported by data which show that conditioned media from high density cultures of cells can augment the growth of SMS-SB cells cultured at densities where they would usually die. This augmentation of cell growth occurs even in cultures where cells can survive in the absence of CM (figure 3.4), but the exogenous SB-AF prevents the lag in growth seen when cells are initially seeded and are attempting to produce a sufficient concentration of autocrine factor. It is probable that some cells die in the low cell density cultures, which could account for the observed lag in growth; cells given CM would continue to grow at the normal rate and so cell numbers would be higher when the cultures were finally pulsed with [^{3}H]-TdR, compared to cultures where there had been a lag in growth.

Several cytokines are known to be involved in pre-B cell growth and development, including IL-3, IL-4, IL-5 and IL-7 (Dorshkind & Landreth, 1992); these are normally produced by stromal cells, not by the B cells themselves. However, since SMS-SB is a leukaemic cell line, it is possible that deregulated transcription of one of these cytokine genes has lead to autocrine production of the growth factor. Experiments performed by the *Genetics Institute* have shown that none of IL-1α to IL-7 or TNFα or-β, are produced by SMS-SB cells, and IL-11, IL-13 and IL-15 do not significantly promote SMS-SB cell growth (figure 3.10). The only cytokines tested which are candidates for SB-AF are PDGF and sCD23. The effects of sCD23 will be discussed in chapter 4A.

Initial experiments to determine the molecular weight of the SB-AF, using *Centricon* filters, suggest that it is over 100kDa in size. This sizing does not correspond with identification of PDGF as a candidate for SB-AF, since the molecular weight of PDGF

heterodimers is approximately 30kDa. However, PDGF is a very 'sticky' protein and it could attach to larger proteins produced by SMS-SB cells; PDGF-$\alpha\alpha$ would, therefore, be concentrated with them over the 100kDa filter.

PDGF-$\alpha\alpha$ is a candidate for SB-AF because SMS-SB cells release PDGF-α chains into the medium and also express the PDGF-B receptor. The PDGF-B receptor does not bind PDGF-$\alpha\alpha$ with very high affinity but the cells were shown to express low levels of PDGF-A receptor RNA, so it is possible that an autocrine system could exist (Tsai *et al.*, 1994). Experiments where neutralising anti-PDGF antibodies were added to conditioned media did not show a significant inhibition of SMS-SB cell growth; thus, the antibodies did not appear to affect the autocrine activity present in CM. Unfortunately, interpretation of these experiments is severely hampered by the protein-effect caused by addition of microgram amounts of protein to SMS-SB cells in PFHMII. Several investigators have demonstrated an autocrine growth mechanism for *v-sis*-transformed cells (the viral homologue of human PDGF-β chain) (Waterfield *et al.*, 1983; Doolittle *et al.*, 1983) but in some cases the mitogenic effect of the *v-sis* product ($p28^{v\text{-}sis}$) is only partially inhibited by anti-PDGF antibodies. There are several reports that PDGF may act as an internal autocrine factor by binding to, and activating, the PDGF-R inside the cell, but requiring surface expression of the receptor to couple with intracellular signalling pathways and have a mitogenic effect (Huang *et al.*, 1984; Fleming *et al.*, 1989 & 1992).

Thus, it is possible that PDGF-$\alpha\alpha$ does play a role in SMS-SB cell growth but that it binds to the PDGF receptors internally. That the receptors require to be expressed on the cell surface to be involved in the signalling pathways, could explain why some stimulation of growth is seen with recombinant PDGF. It is probable that some of the receptors expressed on the cell surface would not have bound PDGF internally and, therefore, would be free to bind exogenous PDGF and augment the growth of the cells. Although the other PDGF autocrine models are from non-haematopoietic tumours, there is a report of an internal autocrine factor effect in murine haematopoietic stem cells which produce erythropoietin (EPO) and express EPO receptors (Goldwasser *et al.*, 1991). Experiments which must be performed to establish the autocrine effect of PDGF include: addition of anti-sense for PDGF; examinination of PDGF-receptor tyrosine phosphorylation; and the effect of suramin on cell growth. The theory that PDGF acts as an internal autocrine factor for SMS-SB cells remains purely hypothetical.

It is possible that PDGF has no real role to play in SMS-SB cell growth, although it is unusual to find its expression in B cells. It is equally possible, if not probable, that PDGF plays only a minor role in SMS-SB cell growth with the majority of the activity being due to an, as yet unidentified, molecule. This would explain why the cells are density-dependent for growth; an internal autocrine system might suggest independent growth.

Normal pre-B cells are under the control of multiple growth factors, some of which may be produced in an autocrine fashion. Synergistic effects between several autocrine factors have been described for non-EBV-transformed B cell lines (Abken *et al.*, 1992), so it is possible that SMS-SB cells produce numerous factors to promote their own growth.

Another candidate for SB-AF activity, possibly in synergy with PDGF, is soluble CD23 (sCD23). Data from proliferation assays (figure 3.10) shows a stimulation index of 5.6 for sCD23 - nearly twice that of PDGF or IL-5. Soluble CD23 has been shown to act as an autocrine factor for several EBV-transformed mature B cell lines but no effects have been described during early B cell development. It is of interest to note that SMS-SB cells do not express EBVNA2 and, as pre-B cells, would not yet be expected to express CD23 - a mature B cell marker.

The focus of the next chapter is the effect of sCD23 on SMS-SB cell growth and death.

Chapter 4A
Proliferative effects of sCD23 on SMS-SB cells

4a.1 *Introduction*

The 25kDa soluble form of CD23, sCD23, has been ascribed numerous cytokine functions. Most of these functions involve synergy with IL-1α for the effects to be seen: hence, pro-thymocyte differentiation (Mossalayi *et al.*, 1990a), proliferation and maturation of myeloid progenitors (Mossalayi *et al.*, 1990b), and centrocyte survival and differentiation (Liu *et al.*, 1991) are all promoted by a combination of IL-1α and sCD23.

CD23 has also been implicated in B cell growth and activation. The expression of CD23 is a requirement for transformation of B cells by EBV, and it appears to be involved in growth stimulation of the cells (Kitner & Sugden, 1981). Normal, IgM^+/IgD^+ B cells also express CD23 when appropriately activated (Walker *et al.*, 1986), but transformation by EBV appears to cause super-induction and constitutive expression of CD23 (Thorley-Lawson *et al.*, 1985). The sCD23 which is released into the supernatant of EBV-transformed cells, acts in an autocrine fashion to promote proliferation of the cells (Swendeman & Thorley-Lawson, 1987). It is also a growth factor for normal, receptor-stimulated, B cell blasts (Swendeman & Thorley-Lawson, 1987; Gordon *et al.*, 1988), although this role is disputed by some investigators (Uchibayashi *et al.*, 1989). It is possible that the discrepancy in results is due to the state of activation of the B cells used in the proliferation assays; the growth of B cells triggered with sub-mitogenic amounts of anti-Ig is not stimulated by sCD23 (Uchibayashi *et al.*, 1989); sCD23 may act to promote the growth of appropriately activated or transformed B cells.

However, these growth-promoting effects of sCD23 on B cells have only been seen with mature cells and there are no reports of sCD23 affecting early B cell growth or differentiation. The data presented in this chapter demonstrate a potential role for sCD23 in promoting the growth of the pre-B cell line, SMS-SB, and indicate that this effect does not require synergy with IL-1α.

4a.2 *Results*

4a.2.1 *sCD23 promotes proliferation of SMS-SB cells, in the absence of IL-1α.*

The data in figure 3.10 demonstrate that sCD23 can promote the growth of SMS-SB cells, with a stimulation index of 5.6 - almost twice that seen with PDGF-αα or IL-5. The graph of figure 4.1 represents [^{3}H]-TdR incorporation by 2500 SMS-SB cells, after a 48 hr incubation with the range of concentrations of sCD23 indicated. The graph shows a bell-shaped, dose-response curve for gel filtration-purified recombinant sCD23, with the maximum growth stimulation seen on addition of approximately 200-500 ng/ml. The

concentration of sCD23 required is quite high, compared to the effects of sCD23 observed in other systems, where a concentration of approximately 100-200 ng/ml sCD23 is usually sufficient. However, the specific activity of the gel filtration-purified sCD23 was not determined due to its inherent instability; the material may contain other proteins. The protein concentration calculated for the gel filtration sample will also account for the other proteins, so the actual concentration of sCD23 added in the assays was probably somewhat lower than the values shown in figure 4.1.

To ensure that the effect of the gel-filtration purified material was, in fact, due to sCD23 and not to some other protein which had been co-eluted from the column, antibody-inhibition experiments were performed with a polyclonal anti-CD23 antibody, Rb55. Figure 4.2 shows [^{3}H]-TdR incorporation by 2500 SMS-SB cells/well incubated for 48 hrs. SMS-SB cells (panel A) were incubated with either; a sub-optimal, concentration of gel-filtration purified sCD23 (100ng/ml); Rb55, anti-CD23, alone; or constant, 100ng/ml concentration of sCD23, which had been pre-incubated for 1 hr at 4^{o}C with an increasing concentration of anti-CD23, as described in the figure legend. Due to the obvious protein effect caused by the addition of antibody alone, the difference in [^{3}H]-TdR incorporation due to antibody alone was subtracted from the values obtained for sCD23 plus anti-CD23. These values are recorded as the 'corrected' result for sCD23 plus anti-CD23 and demonstrate that anti-CD23 inhibits, by approximately 30%, the growth promoting effects of gel filtration purified sCD23. The error bars drawn for these corrected values were calculated from the standard deviations of the two mean [^{3}H]-TdR incorporation values which represent each antibody concentration. The formula used in the calculation is:

$$S_c = \sqrt{((S_1)^2 + (S_2)^2)/2}.$$

Where S_c = Standard deviation (S.D.) of the corrected mean; S_1 = S.D. mean 1; S_2 = S.D. mean 2.

To ensure that the observed inhibition was CD23-specific, a control experiment was performed using purified rabbit IgG in place of anti-CD23. The experiment was performed exactly as before and the results are shown in panel B of figure 4.2. From the graph, it is evident that even after correction for the protein effect due to antibody addition, IgG did not inhibit the growth stimulation due to sCD23. Therefore, it can be concluded that the effect of Rb55, anti-CD23 is truly CD23-specific, and that at least 30% of the growth stimulation by gel-filtration purified sCD23 is actually due to sCD23. It is likely that most, if not all, of this response is actually due to sCD23 because in later FACS experiments (figure 5.10), the maximum inhibition of CD23-binding observed with Rb55 was only approximately 50%. Experiments to further inhibit the stimulatory effect, by using higher amounts of anti-CD23, were hindered by an enhanced protein effect .

Fig.4.1 Effect of sCD23 on [^{3}H]-TdR incorporation by SMS-SB cells

A proliferation assay was performed with 2500 cells per well in PFHMII supplemented with increasing concentrations of gel-filtration purified 25kDa sCD23 from 20ng/ml to 10μg/ml, and incubated for 48 hrs. The dose response curve represents the mean incorporation of [^{3}H]-TdR stimulated by each concentration of sCD23; the error bars indicate the standard deviation of the data for each triplicate culture. The experiment is representative of more than five independent repeats.

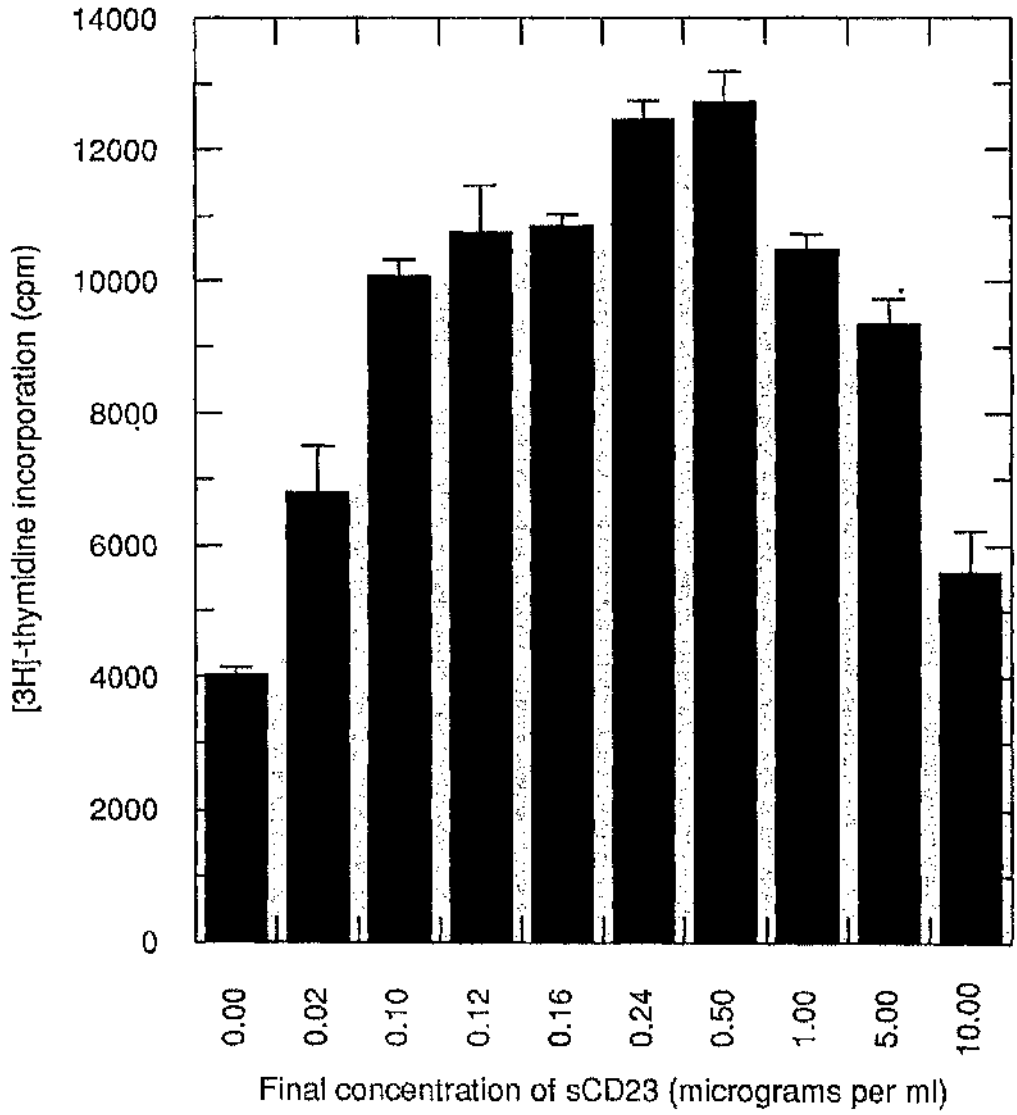

14000
12000
10000
8000
6000
4000
2000
0
[3H]-thymidine incorporation (cpm)
0.00
0.02
0.10
0.12
0.16
0.24
0.50
1.00
5.00
10.00
Final concentration of sCD23 (micrograms per ml)

Fig.4.2 Effect of anti-CD23 on the ability of sCD23 to promote SMS-SB cell growth.

The experiment in panel A was performed as described in Fig.4.1 but the cells were cultured in either: 100ng/ml of gel-filtration purified sCD23 (sCD23) (●); an increasing concentration of Rb55 anti-CD23 (μg/ml) (○); or a constant 100ng/ml sCD23 plus an increasing concentration of Rb55 anti-CD23 (μg/ml) (□). The fourth line (■) represents data for anti-CD23 plus sCD23 (□) corrected for the protein effect due to antibody alone. (○). The corrected data (■) was calculated by subtracting the stimulation (cpm) due to antibody alone (mean cpm of cells in the presence of antibody - background mean cpm of cells with PFHMII alone), from the values (mean cpm) obtained for sCD23 plus anti-CD23 (○). The errors for the corrected data were calculated by the equation:-

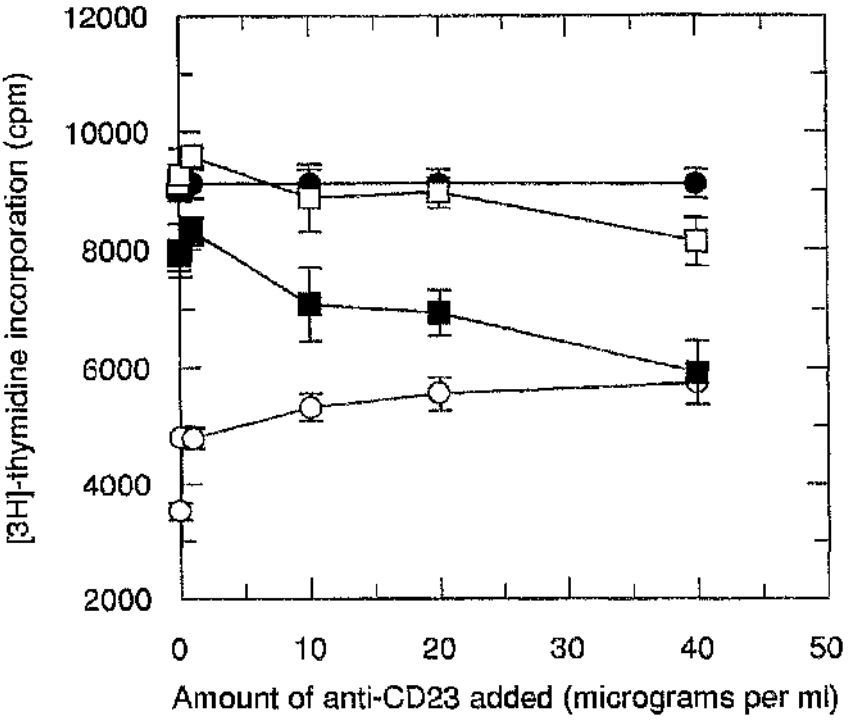
$$Sc = \sqrt{((S_1)^2 + (S_2)^2)/2)}$$

Where Sc represents the standard deviation (S.D.) of the corrected mean; S_1 represents the S.D.of mean 1; and S_2 represents the S.D. of mean 2.

Panel B demonstrates the results obtained when this experiment was repeated but with the substitution of purified normal rabbit IgG for the anti-CD23 antibody.

Each culture was established in triplicate and the graph shows the mean incorporated cpm; the error bars represent the standard deviation of the data or, in the case of the corrected data, the standard error of the difference. The experiment is representative of three independent repeats.

A)


12000
10000
8000
6000
4000
2000
[3H]-thymidine incorporation (cpm)
0
10
20
30
40
50
Amount of anti-CD23 added (micrograms per ml)

B)

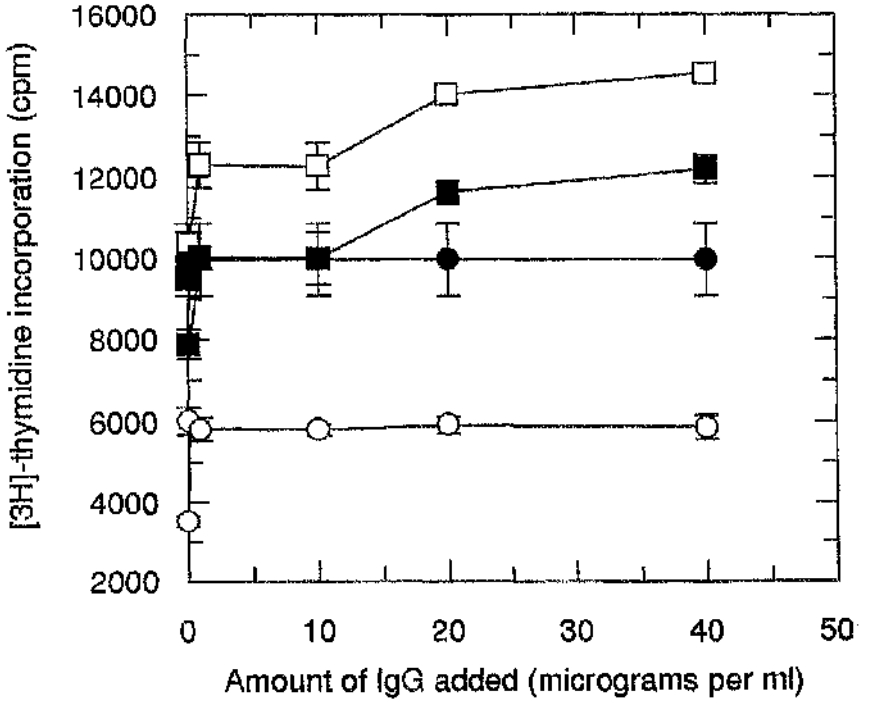
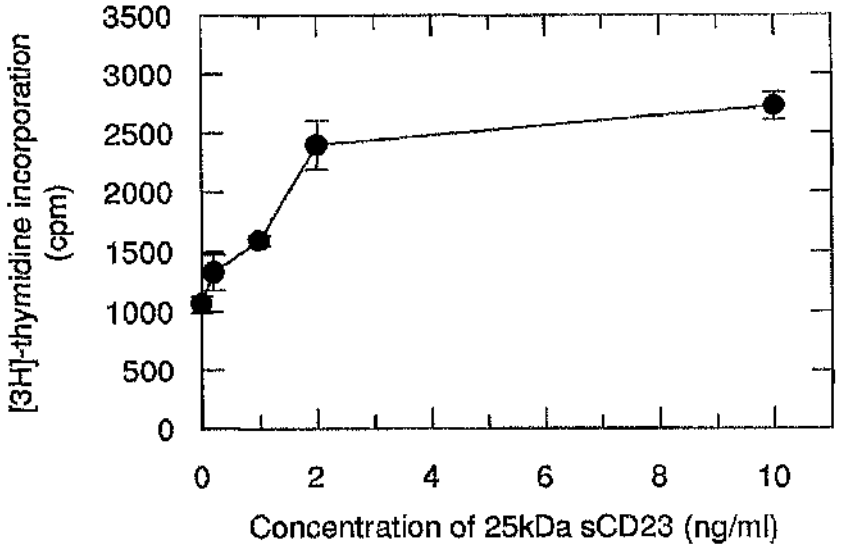

16000
14000
12000
10000
8000
6000
4000
2000
[3H]-thymidine incorporation (cpm)
0
10
20
30
40
50
Amount of IgG added (micrograms per ml)

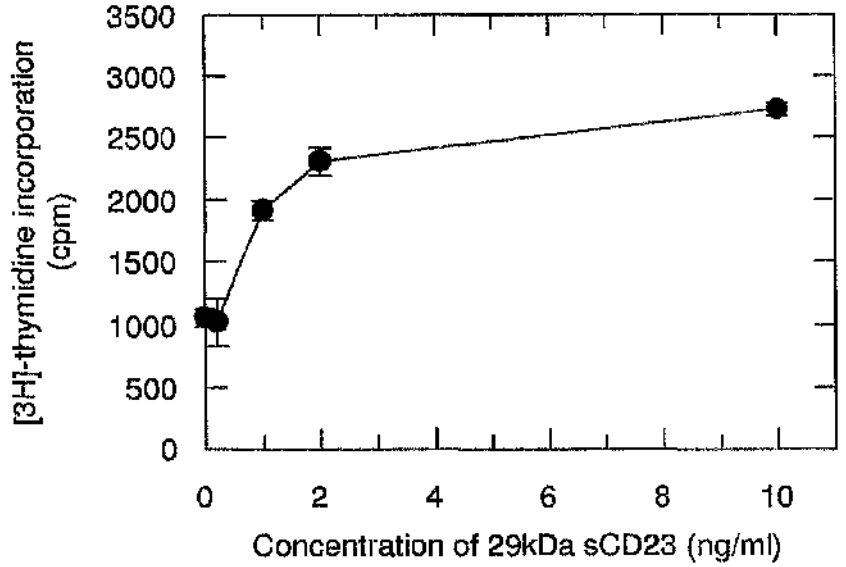
Fig.4.3 Effect of affinity-purified sCD23 fragments upon growth of SMS-SB cells.

Proliferation assays were performed as previously described (Fig.4.1) but the cultures were supplemented with either 25kDa (panel A), 29kDa (panel B) or 37kDa (panel C) baculovirus-produced affinity-purified sCD23 (ng/ml). The results represent the mean $[^3H]$-TdR incorporation of triplicate cultures with the standard deviation of the data shown as error bars.

A


[3H]-thymidine incorporation (cpm)
3500
3000
2500
2000
1500
1000
500
0
0
2
4
6
8
10
Concentration of 25kDa sCD23 (ng/ml)

B


[3H]-thymidine incorporation (cpm)
3500
3000
2500
2000
1500
1000
500
0
0
2
4
6
8
10
Concentration of 29kDa sCD23 (ng/ml)

C

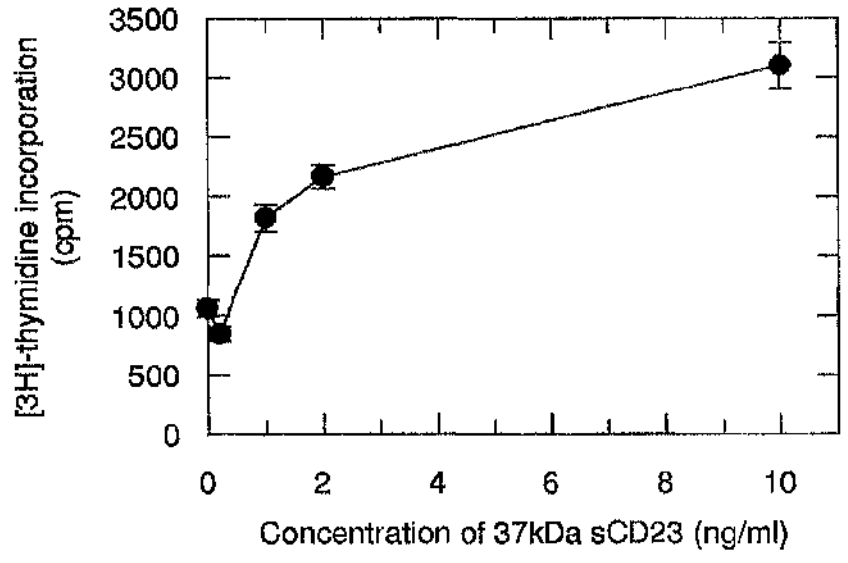

[3H]-thymidine incorporation (cpm)
3500
3000
2500
2000
1500
1000
500
0
0
2
4
6
8
10
Concentration of 37kDa sCD23 (ng/ml)

Fig.4.4 IL-1α does not enhance the growth-promoting effect of sCD23 with SMS-SB cells.

Proliferation assays were performed using 2500 cells per well in PFHMII supplemented with either increasing concentrations of affinity-purified 25kDa sCD23, from 20ng/ml to 200ng/ml (●), or increasing sCD23 concentrations plus a constant 1ng/ml IL-1α (○) or 10ng/ml IL-1α (□). The cultures were established in triplicate and incubated for 48hrs with a pulse of [^{3}H]-TdR for 6hrs prior to harvest. The data represent the mean [^{3}H]-TdR incorporation for each condition, with error bars showing the standard deviation of the data.

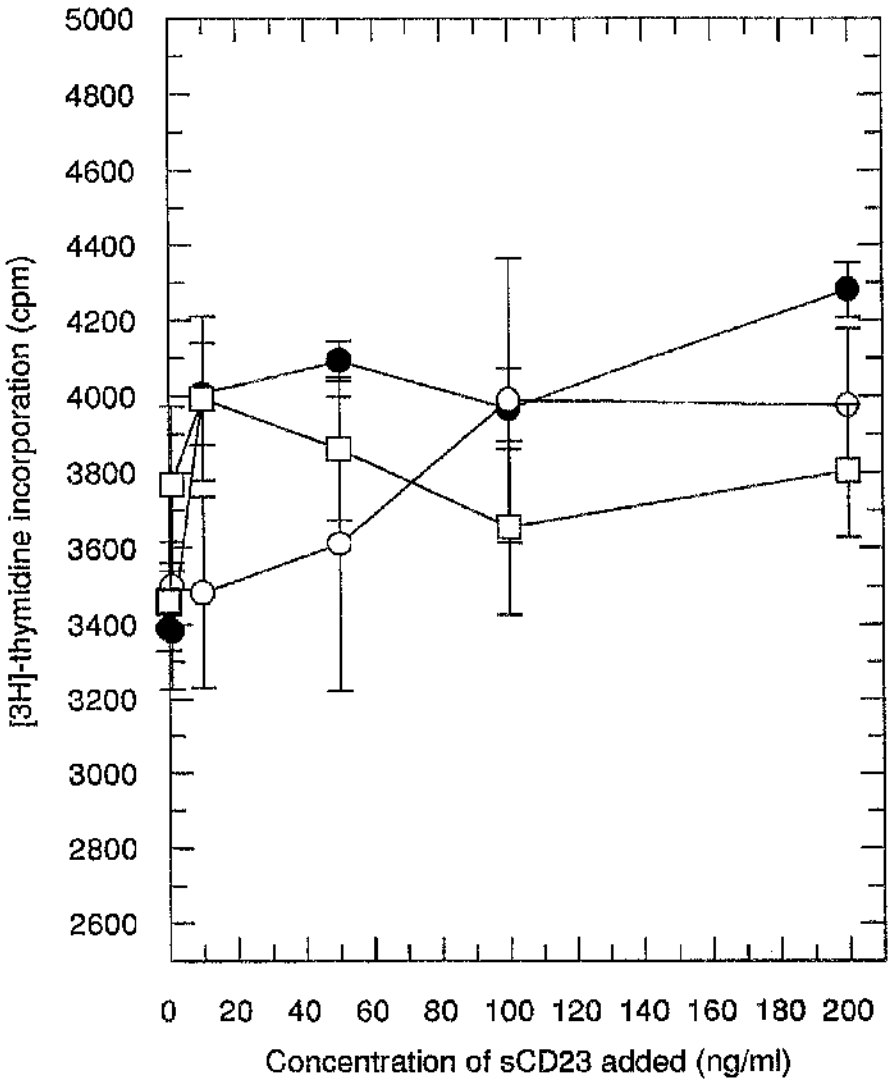
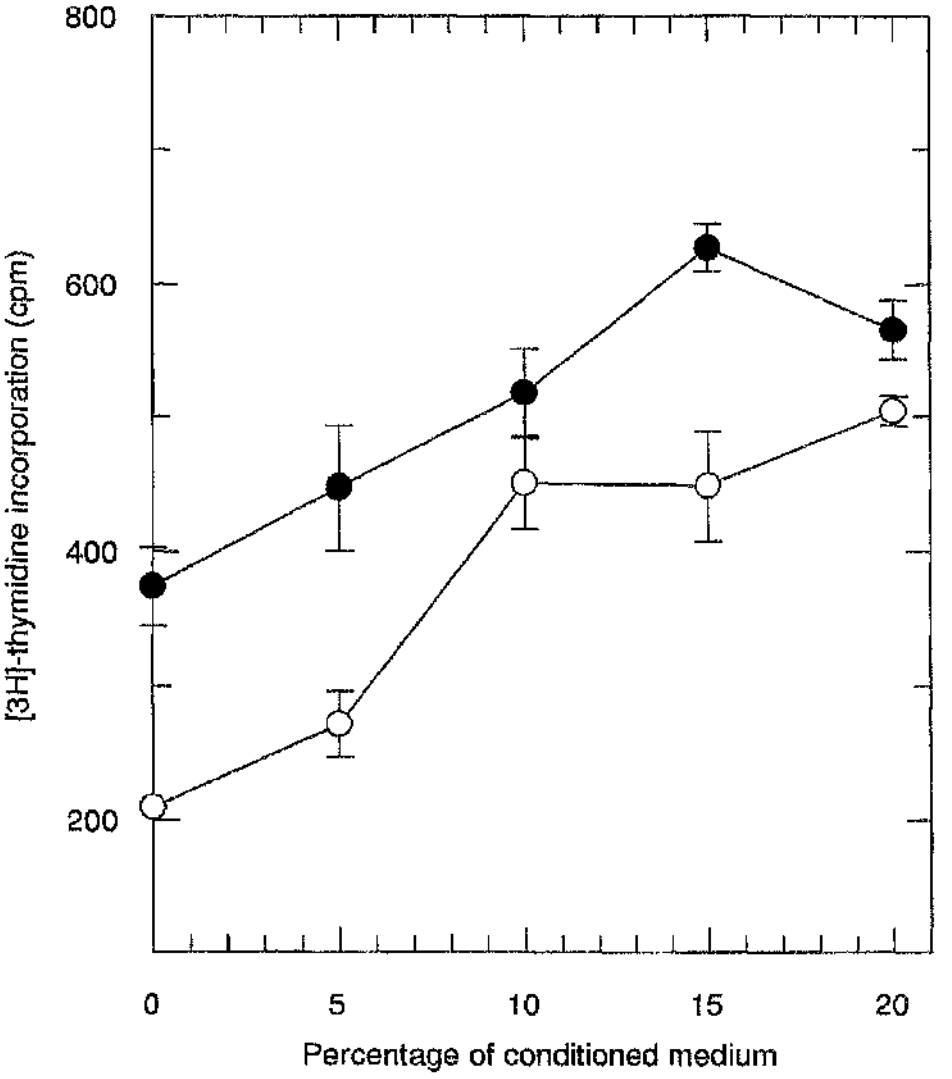

5000
4800
4600
4400
4200
4000
3800
3600
3400
3200
3000
2800
2600
[3H]-thymidine incorporation (cpm)
0
20
40
60
80
100
120
140
160
180
200
Concentration of sCD23 added (ng/ml)

Fig.4.5. Additive effect of sCD23 and conditioned medium on SMS-SB cell growth.

A proliferation assay was performed using 1000 cells per well in PFHMII supplemented with either: increasing amounts of conditioned medium (CM) (○); or a constant 100ng/ml gel-filtration purified 25kDa sCD23 plus increasing amounts of CM (●). The cultures were set up in triplicate, incubated for 48hrs, and pulsed with [^{3}H]-TdR for the last 6hrs prior to harvest. The results represent the mean [^{3}H]-TdR incorporation for each condition, with the standard deviation of the data shown as error bars. This is a single experiment, representative of three independent repeats.


800
600
400
200
[3H]-thymidine incorporation (cpm)
0
5
10
15
20
Percentage of conditioned medium

Due to the possibility that some of the other proteins in the gel filtration material might contribute, in a synergistic fashion, to the growth promoting effect of sCD23 on SMS-SB cells, affinity-purified sCD23 was also tested. The results of experiments using the 25, 29 and 37kDa fragments of CD23 are illustrated in figure 4.3, panels A, B and C, which represent [^{3}H]-TdR incorporation by 2500 cells/well, after a 48 hour incubation in the presence or absence of sCD23. All three, affinity-purified, fragments of CD23 stimulate the proliferation of SMS-SB cells, and the magnitude of the effect is similar to that seen with the gel filtration purified material, at equivalent concentrations. Affinity-purified sCD23 is able to promote the growth of SMS-SB cells, and this occurs in the absence of IL-1α. Neither pre-treatment of cells with recombinant IL-1α, nor addition of recombinant IL-1α to cells in the proliferation assays enhanced the effect of either gel-filtration or affinity-purified sCD23 - Figure 4.4 is representative of data obtained for all these various conditions. The graph represents [^{3}H]-TdR incorporation by 2500 SMS-SB cells per well, after 48 hour incubation with either affinity-purified sCD23, sCD23 plus 1ng/ml IL-1α or sCD23 plus 10ng/ml IL-1α, as indicated in the figure legend. It is clear that IL-1α does not act in synergy with sCD23 to promote the growth of SMS-SB cells since the mean [^{3}H]-TdR incorporation for cells incubated with a combination of sCD23 and IL-1α was not higher than the mean [^{3}H]-TdR incorporation recorded for cells with sCD23 alone. IL-1α alone added to SMS-SB cells has no effect, either positive or negative, on SMS-SB cell growth (refer to figure 3.10).

Figure 4.5 demonstrates that sCD23 and CM have an additive effect on SMS-SB cell growth. A constant, sub-optimal concentration of gel filtration-purified sCD23 (100ng/ml) was added to the cells, along with an increasing amount of CM (as described in the figure legend); the effect of both stimulants on SMS-SB cell growth was greater than either added alone.

4a.2.2 SMS-SB Cells Do Not Express CD23

Since sCD23 can promote the growth of SMS-SB cells, it was identified as a candidate for the SMS-SB autocrine activity and experiments were performed to determine whether SMS-SB cells actually express CD23. Southern blot analysis, using a CD23-specific probe, was performed on DNA from SMS-SB and SKW cells, digested with the restriction enzymes; BamHI, HINDIII and EcoR1 (figure 4.6). A sample of 25 pg of uncut CD23 plasmid was run on the gel to test the hybridisation capacity of the probe. The results of restriction digest and the hybridisation demonstrate that the CD23 gene is present in SMS-SB cells, as expected, and the restriction digest pattern is the same as that seen with SKW cells: the restriction enzymes utilised suggest that the CD23 gene in SMS-SB cells has no deletions or insertions.

Fig.4.6. Southern blot analysis of the CD23 gene in SMS-SB cells

Southern blots were carried out as described in materials and methods. The first lane contained 25pg of uncut CD23 plasmid, pCDL SRα296CD23, as a positive control for probe hybridisation. Lanes 2 and 3 were loaded with 20μg samples of BamHI-digested SMS-SB and SKW genomic DNA, respectively. The SMS-SB and SKW samples of each digest were loaded beside each other to allow comparison of the digest patterns. Lanes 4 & 5, and lanes 6 & 7, represent HINDIII and EcoRI digests of the two cell lines, respectively. After the digests were separated by agarose gel electrophoresis and blotted onto nylon-membrane, the blot was hybridised with a [^{32}P]-labelled, 1.6kbp, BamHI insert of human CD23 from the pCDL SRα296CD23 plasmid. Hybridisation was visualised by autoradiography.

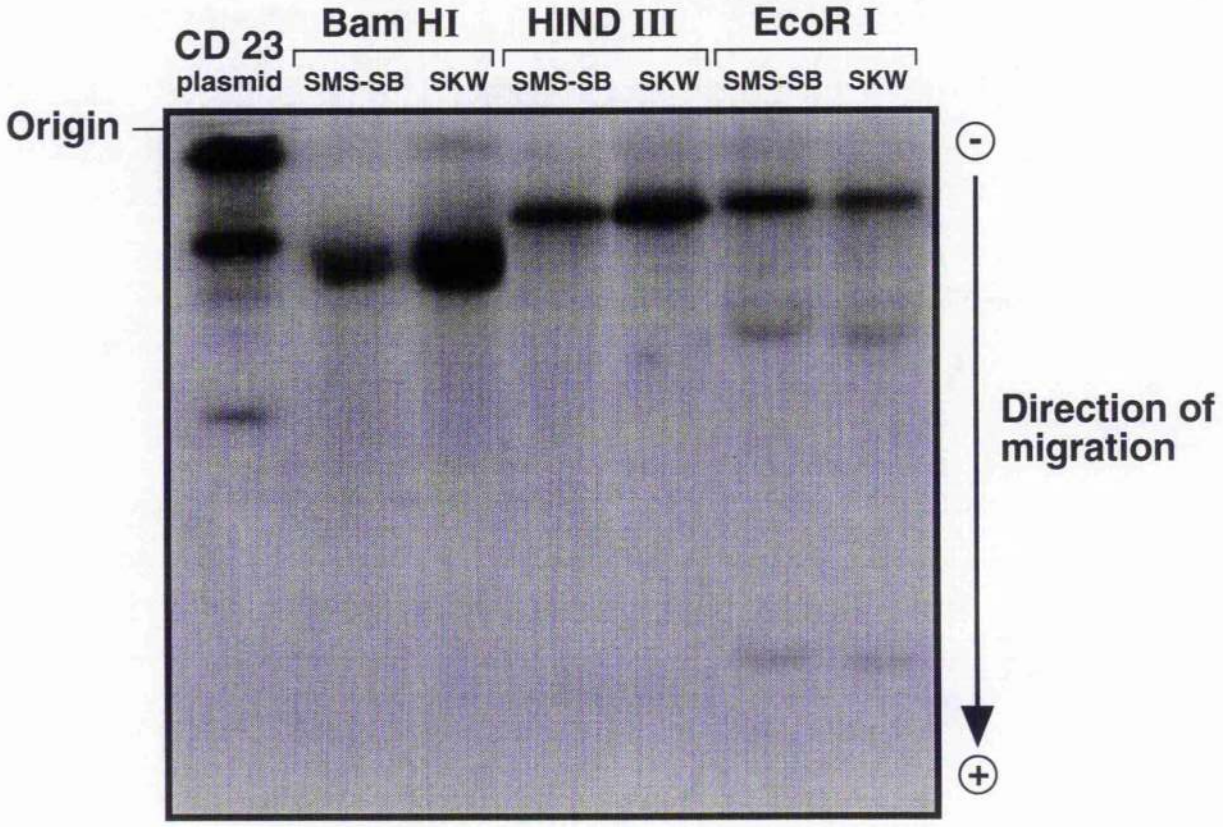

CD 23
plasmid
Bam HI
HIND III
EcoR I
SMS-SB
SKW
SMS-SB
SKW
SMS-SB
SKW
Origin
-
+
Direction of migration

Fig.4.7 Northern blot analysis of CD23 expression in SMS-SB cells and other B lymphoid cell lines.

Total cellular RNA was prepared (as described in materials and methods) from a range of B lymphoid cell lines and from SMS-SB cells cultured in either PFHMII or in 10% FCS/ RPMI 1640 medium. From total RNA, the polyA mRNA fraction was isolated using the *PolyATract mRNA Isolation System IV*. 5μg of each cell line mRNA were loaded per lane, on an agarose / formaldehyde gel. Lanes 1 & 2 contained mRNA from SMS-SB cells grown in PFHMII and serum-containing medium, respectively. Lane 3, 4 and 5 contained mRNA from the B cell lines SKW, Jijoye and Ramos, respectively. After separation by electrophoresis, the RNA was blotted onto nylon membrane and hybridised with a [^{32}P]-labelled, 1.6kbp, BamHI insert from the pCDL SRα296CD23 plasmid. Hybridisation, demonstrating the existence of the 1503bp CD23 RNA, was visualised by autoradiography. The experiment is representative of five independent repeats.

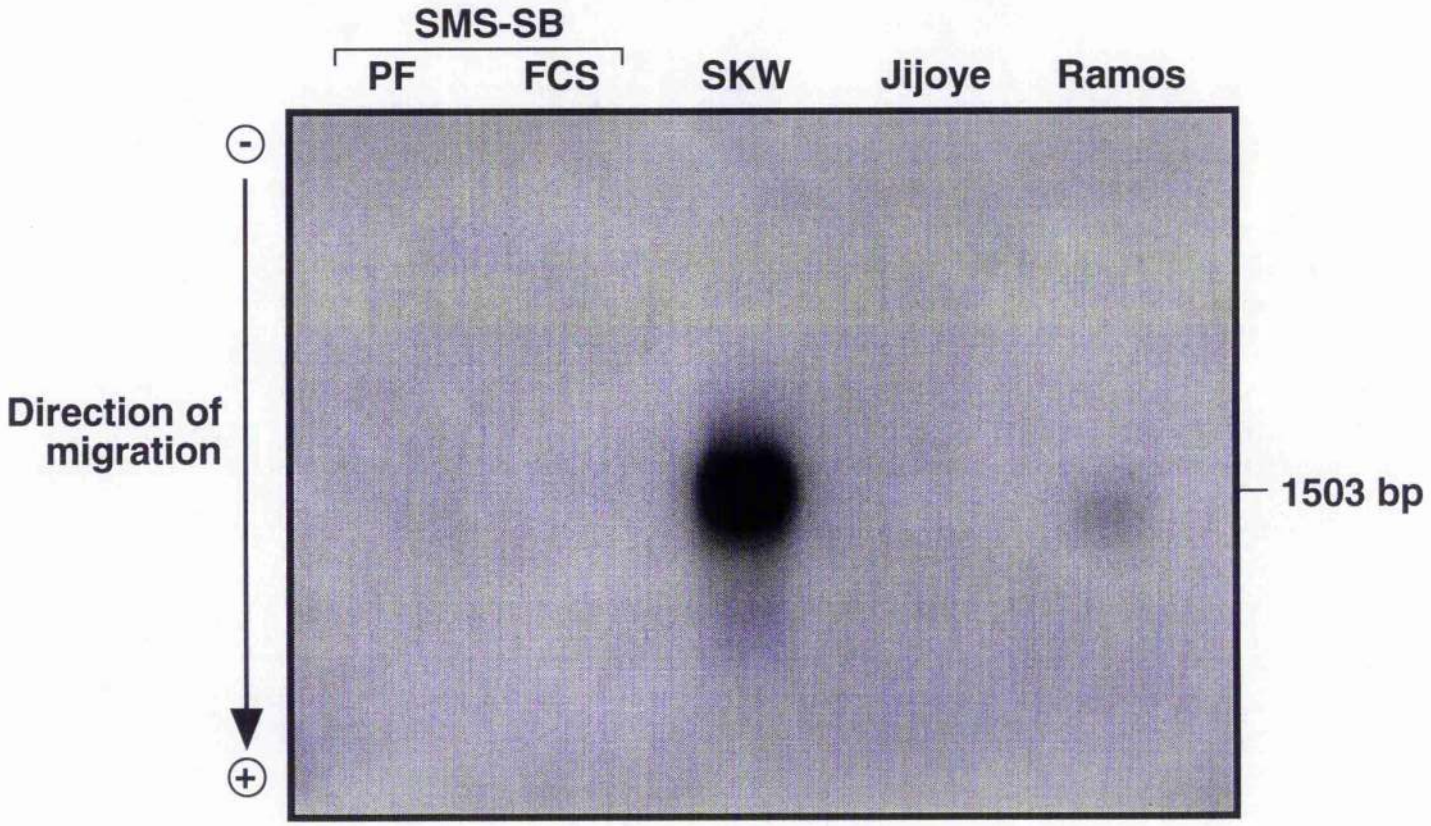

SMS-SB
PF
FCS
SKW
Jijoye
Ramos
Direction of migration
1503 bp

Fig.4.8 GAPDH loading control for CD23 Northern analysis

The Northern blots from the experiments described in Fig.4.7 were stripped (as described in methods) and re-hybridised with a [^{32}P]-labelled, 0.7kb GAPDH probe. Hybridisation was visualised by autoradiography.

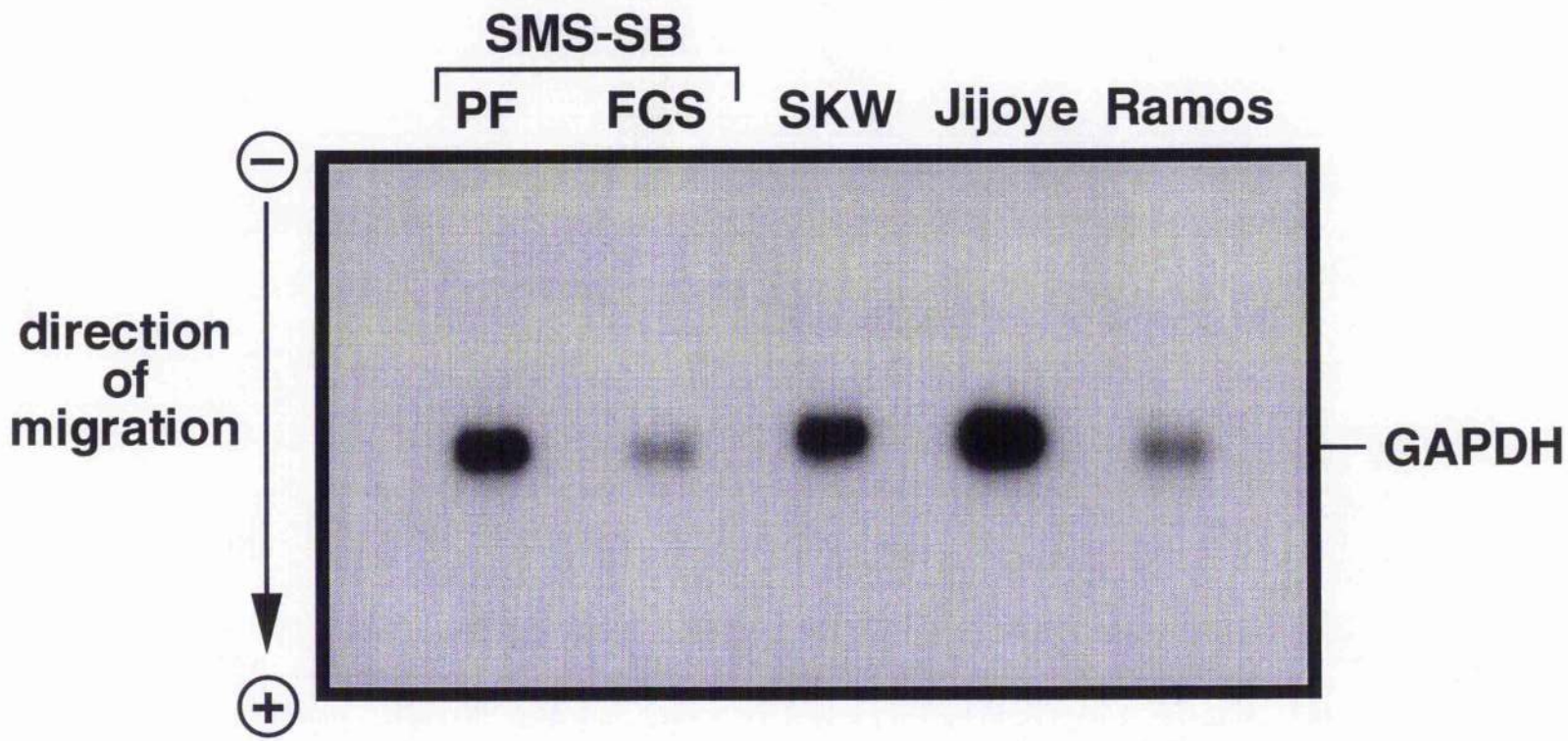

SMS-SB
PF
FCS
SKW
Jijoye
Ramos
direction of migration
GAPDH

Fig.4.9 PCR analysis of CD23 expression in SMS-SB and other B lymphoid cell lines.

Total cellular RNA was prepared (as described in materials and methods) from a range of B lymphoid cell lines, a keratinocyte cell line, and from SMS-SB cells cutured in either PFHMII or in 10% FCS/ RPMI 1640 medium. The total cellular RNA was used as a template for reverse-transcriptase (RT)-PCR using the primers, CD23a and CD23b, indicated on the figure. After the PCR reactions were complete, the products (256bp) were separated by agarose gel electrophoresis, Southern blotted onto nylon membranes, and probed with a [^{32}P]-labelled, 1.6kbp insert from the plasmid pCDL SRα296CD23. The gel was loaded as shown on the figure: lanes 1 & 2 were PCR products from SMS-SB cells cultured in PFHMII and in serum-containing medium; lanes 3, 4 and 5 contained PCR products from SKW, Jijoye and Ramos B cell lines, respectively; lanes 6 & 7 were negative controls of keratinocyte cells (DOK) and a PCR reaction performed with no template RNA, respectively. Hybridisation was visualised by autoradiography. The experiment is representative of four independent repeats.

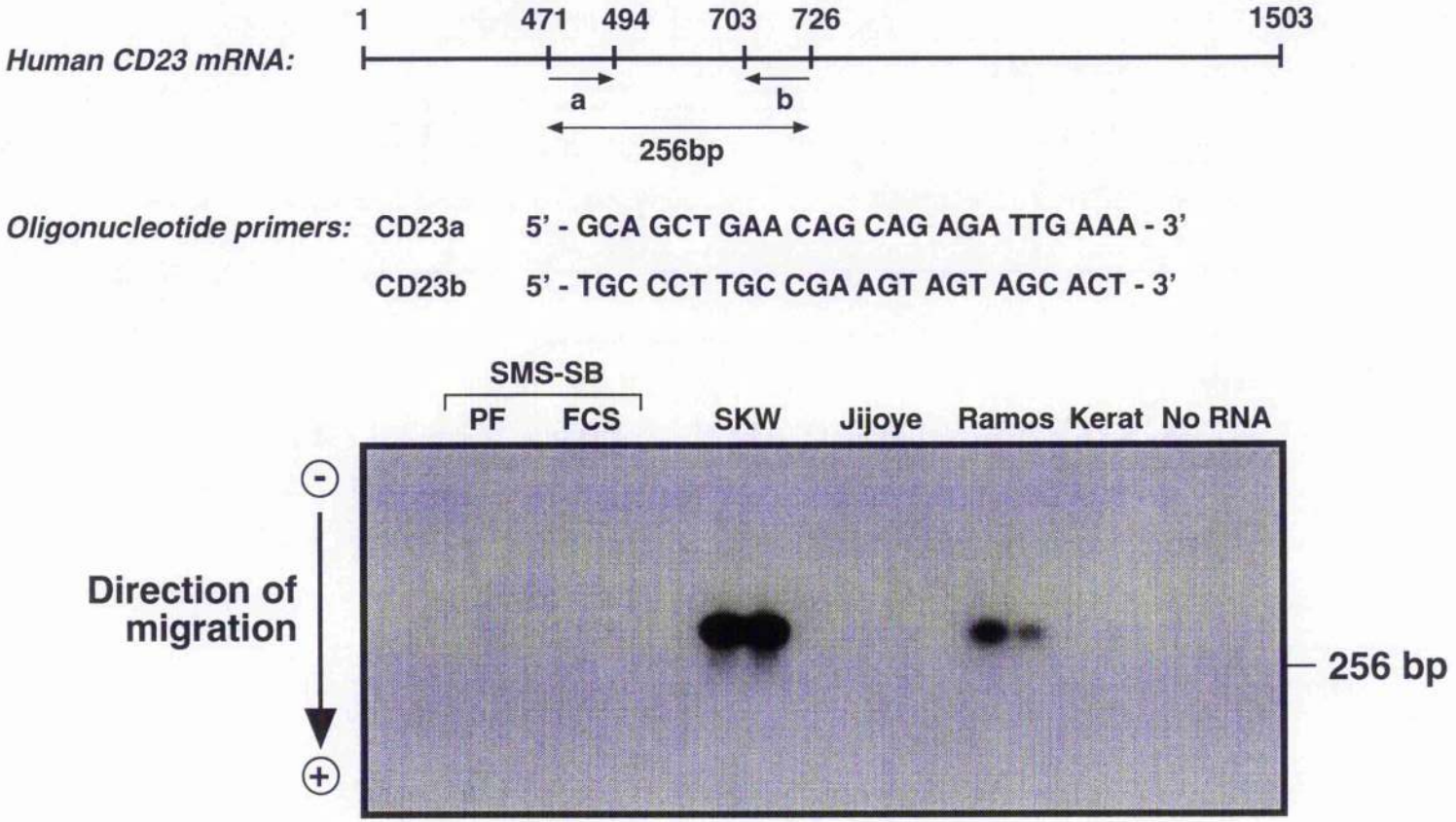
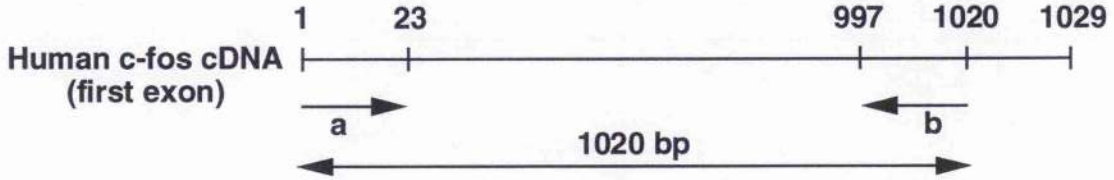

Human CD23 mRNA:
1
471
494
703
726
1503
a
b
256bp
Oligonucleotide primers:
CD23a
5' - GCA GCT GAA CAG CAG AGA TTG AAA - 3'
CD23b
5' - TGC CCT TGC CGA AGT AGT AGC ACT - 3'
SMS-SB
PF
FCS
SKW
Jijoye
Ramos
Kerat
No RNA
Direction of migration
256 bp

Fig.4.10. PCR analysis of *c-fos* expression - an RNA quality control.

The RNA samples used in the experiments described in Fig.4.9. were also used as templates for an RT-PCR reaction with primers specific for *c-fos* (as detailed on the figure). The products of PCR reactions (1020 bp) using RNA from SMS-SB cells cultured in PFHMII and in serum-containing medium, SKW, Jijoye, Ramos and keratinocyte cells were loaded onto an agarose gel as described above. After electrophoresis, the DNA was Southern blotted onto nylon membranes, and probed with a [^{32}P]-labelled *c-fos* insert from the plasmid Sp65. Hybridisation was visualised by autoradiography.



Oligonucleotide primers: c-fos a 5' - TCC AAC CGC ATC TGC AGC GAG CA - 3'

c-fos b 5' - GAC AGG CGA GCC CAT GCT GGA GAA - 3'

After the discovery that SMS-SB cells are negative, by flow cytometry, for surface expression of CD23 (data not shown), the next stage was to examine the expression of CD23 RNA. After numerous attempts, it was realised that the amount of CD23 RNA present in B cells, even in cell lines known to be CD23-positive by flow cytometry, is too low to be detected by a Northern blot of total RNA (data not shown). Therefore, messenger RNA (mRNA) was prepared from total RNA using the *PolyATract mRNA Isolation System IV*, from *Promega*. The results of the Northern blot of mRNA (figure 4.7) demonstrate that the cell line SKW, known to be CD23-positive by FACS analysis, express large quantities of CD23 mRNA and the Burkitt's lymphoma cell line, Ramos, which appears to be negative for CD23 expression by FACS, contains a small amount of CD23 mRNA. The cell lines Jijoye and SMS-SB cells, both negative for surface CD23 expression by FACS, do not express any CD23 mRNA. The Northern blot was stripped of radioactivity and reprobed with a GAPDH probe (figure 4.8) demonstrating that loading of the mRNA was approximately equal and that the mRNA of the negative cell lines could, in fact, be hybridised with a probe. That the loading of mRNA from SMS-SB cultured in serum-containing medium was quite low, compared with SKW cells, is not a concern because approximately the same level of Ramos mRNA was loaded and CD23 message could be detected.

Although it appears that neither SMS-SB cells cultured in 10% FCS/DMEM, nor PFHMII, express any CD23 mRNA, it was decided to perform reverse transcriptase - polymerase chain reaction (RT-PCR) with CD23-specific oligonucleotide primers to allow the most sensitive detection of CD23 RNA (figure 4.9). RNA was isolated from SMS-SB cells and from SKW, Ramos and Jijoye B cell lines and a keratinocyte line, as a negative control. A line drawing (not to scale) of the human CD23 cDNA is given, showing the positions of the two CD23 oligonucleotides, CD23a and CD23b; the sequences of which are shown on the figure. After reverse-transcription and 35 cycles of PCR amplification, products of the individual reactions were electrophoresed and detected by Southern blotting and hybridisation with a CD23-specific probe (figure 4.9). SKW and Ramos both generated a strong signal of the expected 256bp; results analogous to those obtained from the Northern blot analysis. No signals were detected using SMS-SB, Jijoye or keratinocyte RNA and there was no product from a reaction performed without template RNA, demonstrating that no contamination of reagents had occurred. As a positive control for the condition of the RNA and the reverse transcriptase reaction, an RT-PCR reaction was performed using *c-fos*-specific oligonucleotide primers, as illustrated in figure 4.10. It is clear that the RNA from all cell lines tested had been successfully reverse-transcribed giving clear bands of *c-fos* product. These data demonstrate that SMS-SB cells do not contain detectable levels of CD23-RNA and, thus, that they do not express CD23, thereby negating the hypothesis that SMS-SB cells utilise sCD23 as an autocrine growth factor.

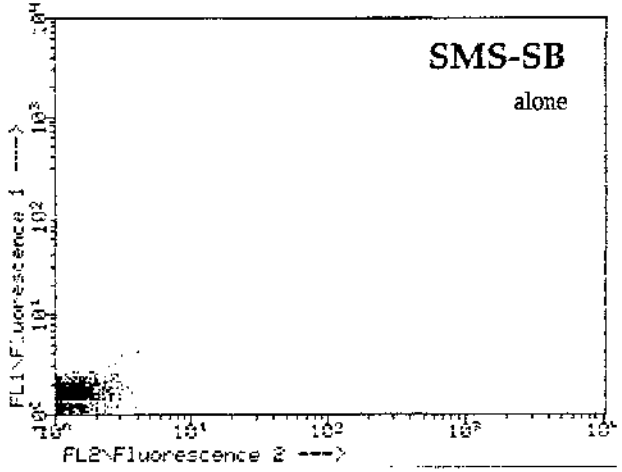
Fig.4.11 The effect of recombinant IL-4 on CD23 expression by SMS-SB cells.

SMS-SB cells were cultured in $25cm^3$ flasks at $5x10^5$ cells per ml in PFHMII, either in the presence or absence of recombinant human IL-4 at 10, 50 and 100U/ml. A culture of SKW cells was also prepared as a positive control for CD23 expression. After 18hrs, the cells were harvested, washed in ice-cold PBS and stained with FITC-conjugated anti-CD23 plus PE-conjugated anti-CD19, for 45 minutes. The cells were then washed and subjected to two-colour flow cytometric analysis - each graph represents data from 10^4 cells.

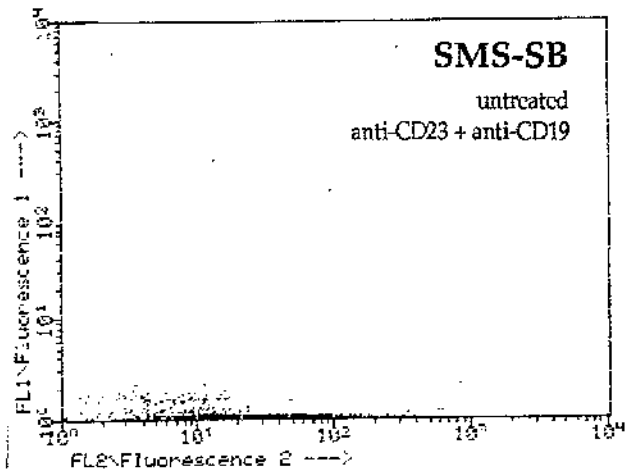
Panel A represents untreated SMS-SB cells without antibody; panel B represents untreated SMS-SB cells with both anti-CD23 and anti-CD19; panel C represents SMS-SB cells treated with 50U/ml IL-4 and then stained with both anti-CD23 and anti-CD19 (this panel also represents results obtained from cells treated with 10 and 100U/ml IL-4); panel D represents SKW cells stained with both anti-CD23 and anti-CD19 antibodies.

In the dot-plots illustrated, the x-axis shows staining with anti-CD19 (fluorescence 2), and the y-axis shows staining with anti-CD23 (fluorescence 1). The experiment is representative of three independent repeats.

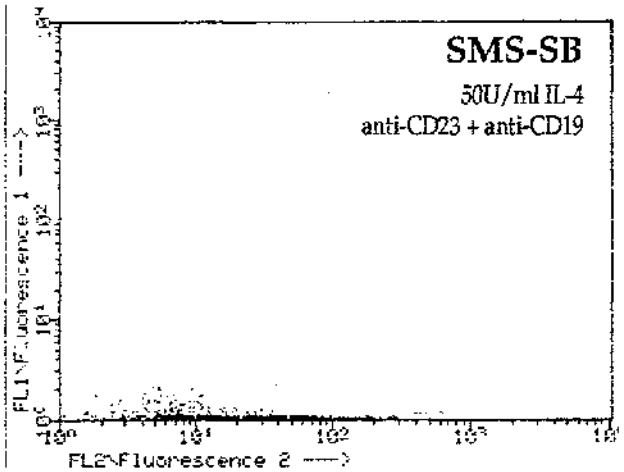
A)


SMS-SB
alone
FL1\Fluorescence 1 ---->
FL2\Fluorescence 2 ---->

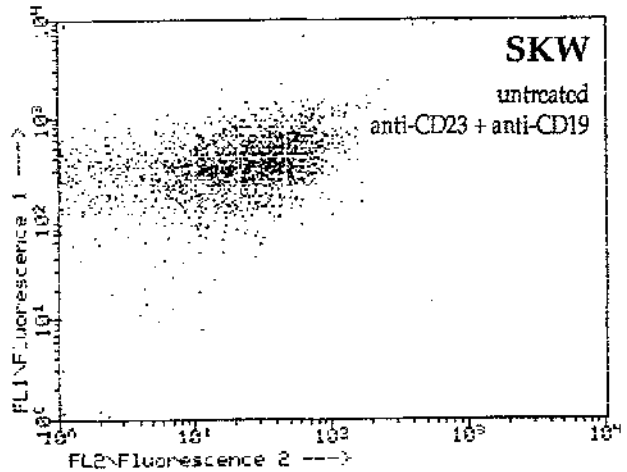
B)


SMS-SB
untreated
anti-CD23 + anti-CD19
FL1\Fluorescence 1 ---->
FL2\Fluorescence 2 ---->

C)


SMS-SB
50U/ml IL-4
anti-CD23 + anti-CD19
FL1\Fluorescence 1 ---->
FL2\Fluorescence 2 ---->

D)


SKW
untreated
anti-CD23 + anti-CD19
FL1\Fluorescence 1 ---->
FL2\Fluorescence 2 ---->

4a.2.3 *CD23 expression, by SMS-SB cells, is not induced by IL-4*

Normal, peripheral B cells express a small amount of the CD23a isoform but addition of IL-4 to these cells induces expression of substantial amounts of CD23a and, especially, CD23b. (Yokota *et al.*, 1988). IL-4 can induce expression of CD23b on several cell types, including normal human T cells (Delespesse *et al.*, 1992), and macrophages (Yokota *et al.*, 1988). In human B cells, the expression of CD23 appears to be associated with that of IgD, and most reports suggest that IL-4 cannot induce CD23 expression on IgD-negative cells (Kikutani et al., 1986). However, malignant pre-B cells, from ALL patients, have been shown to express CD23 after stimulation with IL-4 (Law *et al.*, 1991), so it was decided to investigate whether CD23 expression could be induced on SMS-SB cells.

To examine the effect of IL-4 on CD23 expression, SMS-SB cells were cultured in the presence of varying concentrations of IL-4, for 18 hours before being washed, incubated with FITC-labelled anti-CD23 and PE-labelled anti-CD19 MAbs and analysed by flow cytometry. The results of figure 4.11 demonstrate that IL-4 does not induce surface CD23 expression on SMS-SB cells at 10, 50 or 100U/ml, although the cells stain with anti-CD19 antibody; levels of CD23 RNA were not examined. SKW cells were used as a positive control for staining of cells by the FITC-labelled anti-CD23 antibody.

Ligation of CD40 also induces expression of CD23 on normal human pre-B cells (Saeland *et al.*, 1993). SMS-SB cells were analysed, by flow cytometry, for expression of CD40 antigen but it was discovered that SMS-SB cells do not express CD40 (data not shown) and so experiments attempting to induce CD23 expression by ligation of CD40, were not performed.

4a.3 *Discussion*

The data presented in this chapter illustrate the ability of sCD23 to promote the growth of SMS-SB cells but demonstrate that it is a paracrine, not autocrine, response since the cells do not express CD23.

Gel filtration (GF) purified sCD23 augments the growth of SMS-SB cells and to test the specificity of this effect it would have been useful to use a control insect cell supernatant and collect the same fractions for testing, but, unfortunately, this was not available. However, the growth promoting effect of the gel filtration-purified sCD23 was partially inhibited by a polyclonal anti-CD23 antibody, but not by control rabbit IgG. Due to the protein effect seen on addition of antibodies to the cultures, the results had to be corrected but, after the correction, it was shown that there was a significant difference between values obtained on addition of sCD23 and those obtained when the anti-CD23 was present. The dose-response curve obtained during proliferation assays with GF-purified sCD23 was bell-shaped, suggesting that the growth promoting effect was lost at higher

concentrations of sCD23. There was evidence of some precipitated material in those culture wells during proliferation assays and it is possible that precipitation of sCD23, due to formation of oligomers in solution (Beavil *et al.*, 1995), had occurred. Precipitation of oligomeric complexes would reduce the available sCD23 and could account for the decreased growth-promoting activity observed in the cultures.

Affinity-purified sCD23 was capable of promoting SMS-SB cell growth, although difficulty was encountered due to its apparent instability; often a repeat of the same experiment showed no enhancement of SMS-SB cell growth. Several batches of sCD23, provided by GIMB, were tested, with some batches never showing any growth-promoting activity and others showing activity for a few weeks and then becoming inactive.

However, the 25, 29 and 37kDa forms of affinity-purified sCD23 have the ability to promote SMS-SB cell growth, suggesting that the stalk region, which is absent from the 25kDa form, is not involved in binding to SMS-SB cells. These fragments have all been shown to exist as trimers, after chemical cross-linking, and the 25kDa form may also form dimers of trimers which aid in binding to CD23 receptors (Beavil *et al.*, 1995). There is no obvious difference in the ability of the three forms of sCD23 to promote SMS-SB cell growth. The affinity-purified material promotes SMS-SB cell growth to a level equivalent with GF-purified sCD23 which also suggests that there were no synergistic effects due to contaminating proteins found in the GF material. One concern had been that IL-1α (the cytokine most frequently described to synergise with sCD23), or an IL-1-like molecule, were contaminants in the gel filtration sCD23. Although inhibition experiments with a neutralising anti-IL-1α antibody were not performed, this explanation appears unlikely since affinity-purified sCD23 alone augments SMS-SB cell growth, and no synergy was observed on addition of recombinant IL-1α. Thus, sCD23 alone promotes the growth of SMS-SB cells.

Analysis of CD23 expression by Northern blot and RT-PCR demonstrates that SMS-SB cells do not express CD23, although CD23 message could be detected in several other B cell lines which were examined. Also, CD23 expression could not be induced by culture of the cells with IL-4, as has been noted for some pre-B cells from ALL patients (Law *et al.*, 1991).

There is a negative regulatory element near the murine CD23 transcription start site (Dierks *et al.*, 1994), and CD23 gene expression may be controlled by the presence or absence of a factor able to bind to this site, thus, some pre-B ALL cells may have lost this negative signal, or, alternatively express transcription factors which override the negative signals - possibly in the same way that a signal through CD40 can induce CD23 expression on normal pre-B cells (Saeland *et al.*, 1993). That IL-4 alone does not induce

CD23 expression by SMS-SB cells suggests that regulation of CD23 expression may be similar to that seen in normal pre-B cells; however, the lack of CD40 expression by SMS-SB cells makes it difficult to compare the two regulatory mechanisms directly.

CD23 is not produced by SMS-SB cells so it cannot account for the autocrine growth activity present in conditioned media from SMS-SB cells; sCD23 is not the SB-AF. Unfortunately, no further work has been carried out to characterise the SB-AF. The remaining section of chapter 4 describes experiments which attempt to determine the mechanism of action whereby sCD23 promotes SMS-SB cell growth; the data demonstrate that sCD23 prevents apoptosis of SMS-SB cells.

Chapter 4B
Anti-apoptotic effect of sCD23 on SMS-SB cells

4b.1 *Introduction*

Haematopoietic precursor cells are strictly dependent upon the presence of haematopoietic growth factors for growth, survival and differentiation (Askew *et al.*, 1990). IL-3 is one such factor, and its ability to enhance the survival of haematopoietic precursors is thought to be accomplished by the suppression of apoptosis (Askew *et al.*, 1990). Thus, myeloid precursor cells, deprived of IL-3, will undergo apoptosis. There are several genes which may be involved in promotion or suppression of apoptosis. The normal response of most IL-3-dependent cells, to cytokine withdrawal, is down-regulation of *c-myc* expression, followed by cell cycle arrest in G_1, and eventually apoptosis. However, over-expression of *c-myc* accelerates apoptosis of IL-3-dependent cells upon growth factor withdrawal (Askew *et al.*, 1990), possibly by 'forcing' the cells into cycle but providing only a partial proliferative signal which fails to replace those provided by IL-3. An imbalanced mitogenic signal, induced by inappropriate expression of cell cycle genes, such as *c-myc*, appears to induce apoptosis (Evan *et al.*, 1992; Cherney *et al.*, 1994); this mechanism may aid in the elimination of cells which sustain mutations that perturb their proliferative potential.

Bcl-2 has properties of an anti-apoptotic gene and, for example, when transfected into an IL-3-dependent B cell line, on factor deprivation, the cells do not undergo apoptosis but arrest in G_0/G_1 (Marvel *et al.*, 1994). Even in cells which over-express *c-myc*, the co-expression of *bcl-2* allows resistance to apoptosis (Merino *et al.*, 1994), suggesting that *bcl-2* may substitute for a 'survival' signal, normally provided by the growth factor, to allow the cell cycle to proceed.

Thus, in the absence of necessary growth factors, many cells will undergo growth arrest and/or apoptosis. With respect to SMS-SB cells, the data from proliferation assays show that cells cultured in the absence of CM have very low [^{3}H]-TdR incorporation which suggests that the cells die at low cell density. The density-dependence of SMS-SB cells could be explained if the inability to condition their own medium leads to factor deprivation and consequently to apoptosis of the cells. In this respect, the increased [^{3}H]-TdR incorporation observed on addition of sCD23 to the cultures may reflect the ability of sCD23 to save the cells from apoptosis and allow their continued growth, rather than a role for sCD23 as an overt mitogen.

A role for sCD23 in the prevention of apoptosis has already been demonstrated, since addition of sCD23 and IL-1α to centrocytes prevents apoptosis and drives the cells towards a plasmacytoid phenotype (Liu *et al.*, 1991). Therefore, experiments were

performed to examine whether SMS-SB cells undergo apoptosis when plated at low cell density, and whether sCD23 acts to prevent apoptosis and promote survival of the cells.

4b.2 *Results*

4b.2.1 *SMS-SB cells undergo apoptosis at low cell density*

One of the biochemical hallmarks of apoptosis is DNA cleavage in the linker regions between nucleosomes, which results in a characteristic pattern of fragmentation into 180bp (or multiples thereof) oligonucleosomes (a DNA 'ladder') when the DNA is subject to conventional gel electrophoresis (Wyllie, 1980).

To investigate whether SMS-SB cells undergo apoptosis at low cell density, the cells were plated at $2x10^4$ cells per ml and incubated for 24 hrs before preparation of DNA and gel electrophoresis with ethidium bromide to visualise the DNA. Unfortunately, clear DNA laddering was not visible with SMS-SB cells at low cell density, and only a smear of DNA was present on the gel (data not shown). There are reports which suggest that internucleosomal cleavage of DNA is not an essential step in apoptosis, but in cell lines which do not show DNA laddering, there are distinctive fragments of 50kbp generated. These fragments appear to be caused by endonucleolytic activity during apoptosis which releases loops of chromatin (Oberhammer *et al.*, 1993).

To examine SMS-SB cells for the presence of the 50kbp band, indicative of apoptosis, the cells were cultured at $2x10^4$ cells/ml in PFHMII and at various time points, 10^5 cells were harvested and washed before being embedded in agarose blocks and the DNA released by a 48 hr digestion with proteinase K solution. The blocks were then subjected to pulse-field gel electrophoresis and the gel stained with ethidium bromide. A 46kbp fragment of lambda DNA, generated by heating to 65°C, was used as a size marker. The visible 50kbp band (figure 4.12) demonstrates that SMS-SB cells do undergo apoptosis at low cell density, with the maximum DNA cleavage apparent between 18 and 24 hours. The disappearance of the 50kbp band after 24 hours may be due to further DNA degradation, although DNA laddering was not observed.

The time course experiment suggests that, in future experiments, incubation for between 18 and 24 hours should allow examination of the cells in the process of undergoing apoptosis.

Fig 4.12 Pulse-field gel electrophoresis - to examine apoptosis of SMS-SB cells after culture at low cell density.

SMS-SB cells were cultured at $2x10^4$ cells/ml in PFHMII and, at times of 0, 4, 6, 8, 12, 18, 24, 36 and 48 hours, 10^5 cells were harvested (using trypsin, if necessary, to remove the cells), washed and embedded in agarose blocks, before being subjected to digestion with a proteinase K solution. A block containing lambda DNA (previously heated to 65°C) was run in lane one of the pulse-field gel as a size marker of 46kbp. The SMS-SB cell sample blocks were loaded in chronological order. After 16 hours of pulse-field gel electrophoresis, the gel was stained with a 0.4μg/ml solution of ethidium bromide and then washed in dH_20 before being photographed under UV light. The experiment is representative of two independent repeats.

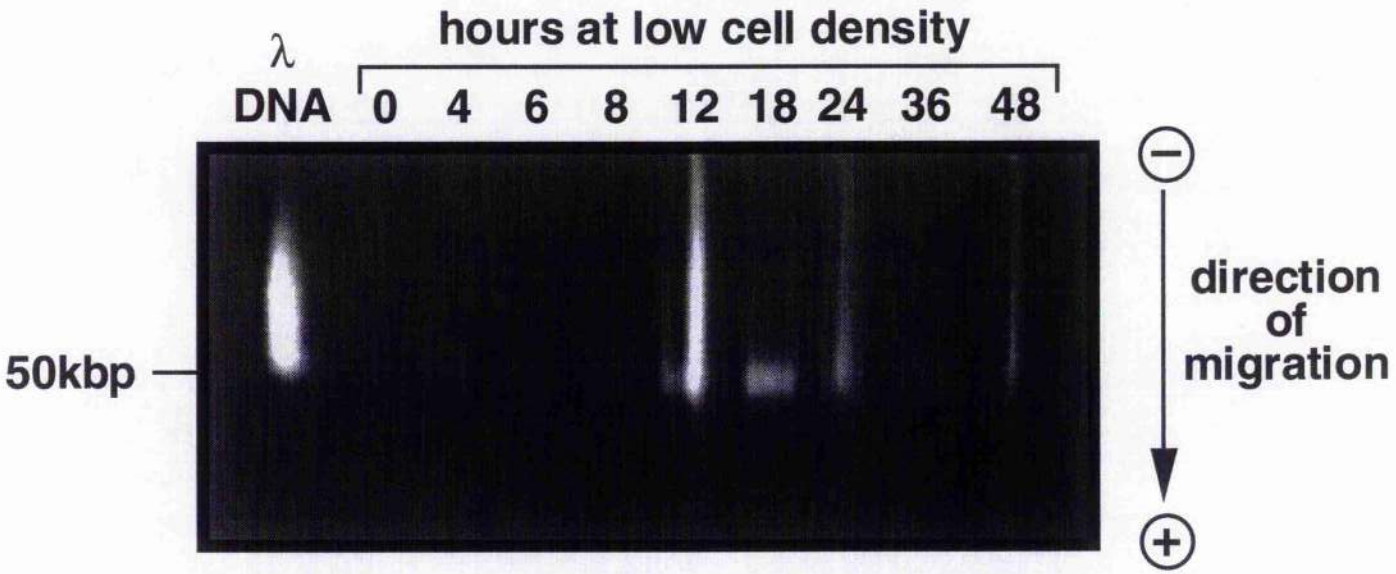

hours at low cell density
λ
DNA
0
4
6
8
12
18
24
36
48
50kbp
direction
of
migration

Fig.4.13 Photomicrographs of SMS-SB cells.

Cultures of SMS-SB cells were established in PFHMII at normal cell density ($5x10^5$ cells/ml; Panel A), or at low cell density ($2.5x10^4$ cells/ml) in the absence (Panel B) or presence of 10ng/ml (Panel C) or 100ng/ml (Panel D) gel-filtration purified 25kDa-sCD23. After 24 hours of culture, the cells were examined by phase-contrast light microscopy using a 40X magnification lens.

A
B
C
D

4b.2.2 *sCD23 prevents apoptosis in low cell density cultures of SMS-SB cells*

To test the hypothesis that sCD23 inhibits apoptosis of SMS-SB cells, cells were cultured at normal and low cell density (2 x 10^4 cells per ml) in PFHMII for 24 hr in the presence and absence of sCD23 and then examined by light microscopy (figure 4.13).

Cells cultured at normal cell density (5 x 10^5 cells per ml) showed few apoptotic characteristics and grew in suspension (figure 4.13, panel A), whereas cells cultured at low cell density (panel B) exhibited a more spread morphology and became so adherent to the tissue culture flask that trypsinisation was required to remove them. The cell membranes were blebbed and the cytoplasm was vacuolated, all features characteristic of apoptotic cells (Cohen, 1993), in contrast with the few, remaining viable cells which appear smaller and are still spherical (compare cell morphology in panel B). In several instances, apoptotic bodies were visible (data not shown), representing the final stage of apoptosis; *in vivo* these would probably have been phagocytosed by macrophages. Addition of sCD23 produced cells with a morphology indistinguishable from that of normal cell density cultures; fewer apoptotic cells were evident as the concentration of sCD23 was increased from 10ng/ml to 100ng/ml (panels C & D), and the cells grew in suspension. Incubation of SMS-SB cells in the presence of 100ng/ml sCD23 was sufficient to maintain the morphology of the cells exactly as those in culture at normal cell density.

4b.2.3 *Flow cytometric analysis of apoptotic cells*

A two-colour flow cytometric technique has been described which discriminates and quantifies viable, apoptotic and necrotic cells via measurement of forward and side light scatter (proportional to cell diameter and granularity, respectively), and the DNA-binding flurochromes Hoechst 33342 and propidium iodide (Dive *et al.*, 1992; Darzynkiewicz *et al.*, 1992). Cells in the early stages of apoptosis have changes in membrane permeability such that propidium iodide is excluded from the cells (as normal), but the Hoechst fluorochrome begins to stain the cells. The increase in Hoechst 33342 fluorescence seen in apoptotic, compared with viable, cells may reflect a decreased ability of apoptotic cells to actively pump out the dye. Viable cells exclude propidium iodide and efflux Hoechst 33342, therefore are stained with neither fluorochrome but, in contrast, late stage apoptotic or necrotic cells stain with both dyes (Dive *et al.*, 1992).

This method was employed to confirm the morphological criteria which suggest that SMS-SB cells undergo apoptosis when cultured at low cell density but addition of sCD23 can prevent the apoptosis. Light scatter measurements were used initially to gate out debris from the analysis. Two sub-populations were apparent - one with high forward scatter/ low side scatter (viable cells) and the second with low forward scatter and high side scatter (apoptotic cells). That these populations represent viable and apoptotic cells was confirmed by analysis of the fluorochrome binding patterns of the cells (figure 4.14).

Cells were analysed after a 24hr incubation at normal or low cell density either alone or in the presence of sCD23 or IL-4. The cells were stained with propidium iodide and Hoechst 33342 and, after gating on the basis of scatter parameters, analysed by two-laser flow cytometry. In cultures propagated at normal cell density (figure 4.14, panel A and Table 4.1), >90% of the cells were routinely located in the lower left quadrant of the dot-plots - the quadrant which represents viable cells, not stained by either dye. In the experiment presented, 91.5% of cells cultured at normal cell density were viable with very few dead/necrotic cells evident (lower right quadrant). There were a total of 8.9% apoptotic cells in the normal cell density cultures; 2.8% were stained only with Hoechst 33342 (upper left quadrant) and represent early apoptotic cells whereas 6.1% of the cells stained with both fluorochromes and were scored as late apoptotic cells (upper right quadrant).

These values contrast sharply with values obtained for cells cultured at low cell density (panel B) where 68.3% of the cells were viable, 3.3% early apoptotic, and 25.1% late apoptotic. The inclusion of IL-4 in these cultures (panel D) results in a small decrease in late apoptotic cells, with a slight increase in the number of dead cells present, but the percentage of viable cells remains low and comparable with that seen in cultures of cells at low cell density alone. In striking contrast, the presence of sCD23 in the low cell density cultures (Figure 4.14, panel C) restores the percentage of viable cells in the culture to 89.1%, and reduces the percentage of late apoptotic cells to 6.3%; values which are essentially identical to those obtained for normal cell density cultures.

Table 4.1 Flow cytometric analysis of apoptosis in SMS-SB cells

CULTURE CONDITIONS	% of cells: VIABLE	% of cells: EARLY APOPTOTIC	% of cells: LATE APOPTOTIC	% of cells: DEAD
NCD	91.5	2.8	6.1	0.3
LCD	68.3	3.3	25.1	4.3
LCD + sCD23	89.1	0.9	6.3	3.9
LCD + IL-4	64.5	0.8	17.5	20.0

Where, NCD = normal cell density, LCD = low cell density. For experimental detail see legend to figure 4.14

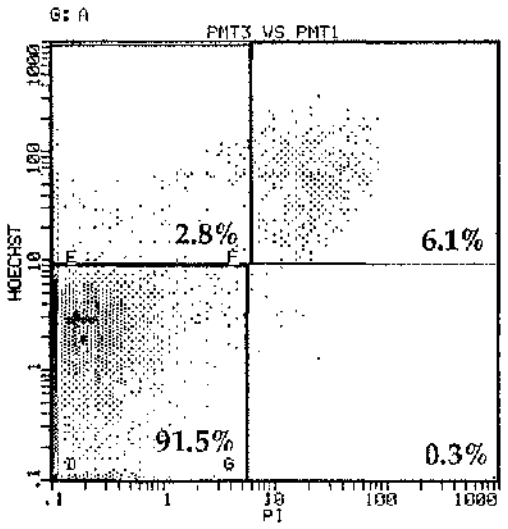
In individual experiments, the actual percentages of viable and apoptotic cells varied in both normal and low cell density cultures but the restoration of values to those observed with normal cell density cultures, on addition of sCD23, was consistent. Figure 4.15 describes the results of an individual experiment, representative of 3 others, demonstrating that the capacity of sCD23 to block apoptosis in low cell density cultures is concentration dependent. Addition of 20ng/ml gel filtration purified sCD23 reduces the late apoptotic population to 5.9%, from the 97% present in low cell density cultures, and 100ng/ml sCD23 reduces the percentage of late apoptotic cells to only 1.6% - comparable with the

Fig.4.14 Flow cytometric analysis of apoptosis in SMS-SB cells.

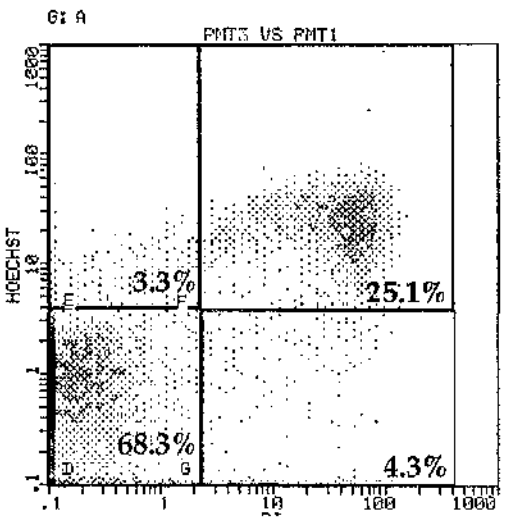
Cultures of SMS-SB cells were established in PFHMII at normal cell density ($5x10^5$ cells/ml; Panel A), or at low cell density ($2.5x10^4$ cells/ml) in the absence (Panel B) or presence of 100ng/ml gel filtration-purified 25kDa sCD23 (Panel C), or 20U/ml IL-4 (Panel D). After 24 hours of culture, the cells were harvested (using trypsin, if necessary, to remove the cells), washed with ice-cold PBS and then stained simultaneously with propidium iodide and Hoechst 33342; to detect apoptotic and dead cells. Cell debris was gated out on forward and 90° scatter parameters, and the cells analysed by two-laser flow cytometry.

On the individual dot-plots, the percentage figures shown on the lower left quadrant represent viable cells and those on the lower right represent dead cells. The values in the upper left quadrant refer to early apoptotic cells, while those in the upper right quadrant represent late apoptotic cells. The experiment shown is representative of more than five independent repeats.

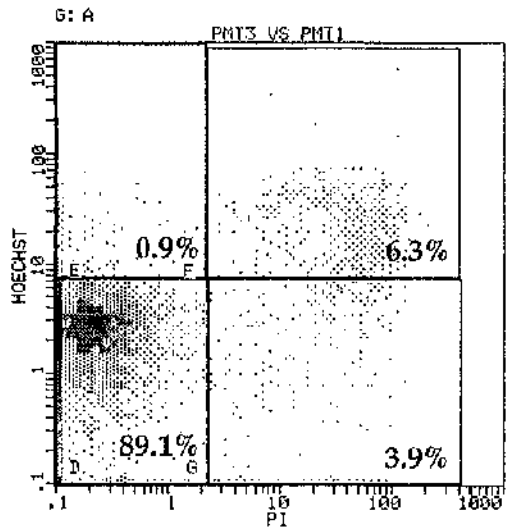
a) Normal Cell Density



b) Low Cell Density (LCD)



c) LCD +sCD23



d) LCD +IL-4

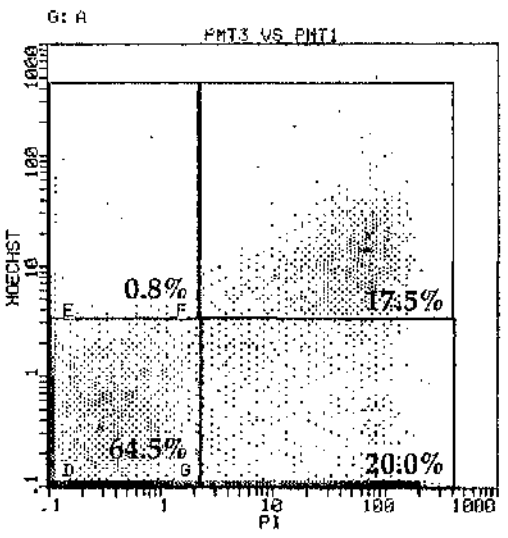


Fig.4.15 Apoptosis of SMS-SB cells - effect of sCD23 and anti-CD23.

The experiments were performed exactly as described in the legend to figure 4.14, but different concentrations and forms of sCD23 were used. The bar chart represents the percentage of apoptotic cells (early plus late apoptotic cells), as measured by the two-laser flow cytometric technique. From the first bar downwards, the chart demonstrates the percentage of apoptotic cells in SMS-SB cells cultured at:- low cell density (LCD) ($2.5x10^4$ cells/ml); LCD plus 200ng/ml affinity purified, *E.coli* produced, 25kDa sCD23; LCD plus 200ng/ml affinity purified, *E.coli* produced, 25kDa sCD23, plus 10ng/ml IL-1α; LCD plus 20ng/ml gel-filtration purified 25kDa sCD23, plus 40μg/ml Rb55, polyclonal, anti-CD23; LCD plus 20ng/ml gel-filtration purified 25kDa sCD23; LCD plus 100ng/ml gel-filtration purified 25kDa sCD23; and finally, normal cell density ($5x10^5$ cells/ml).

The data presented is from a single experiment, representative of at least four independent repeats.

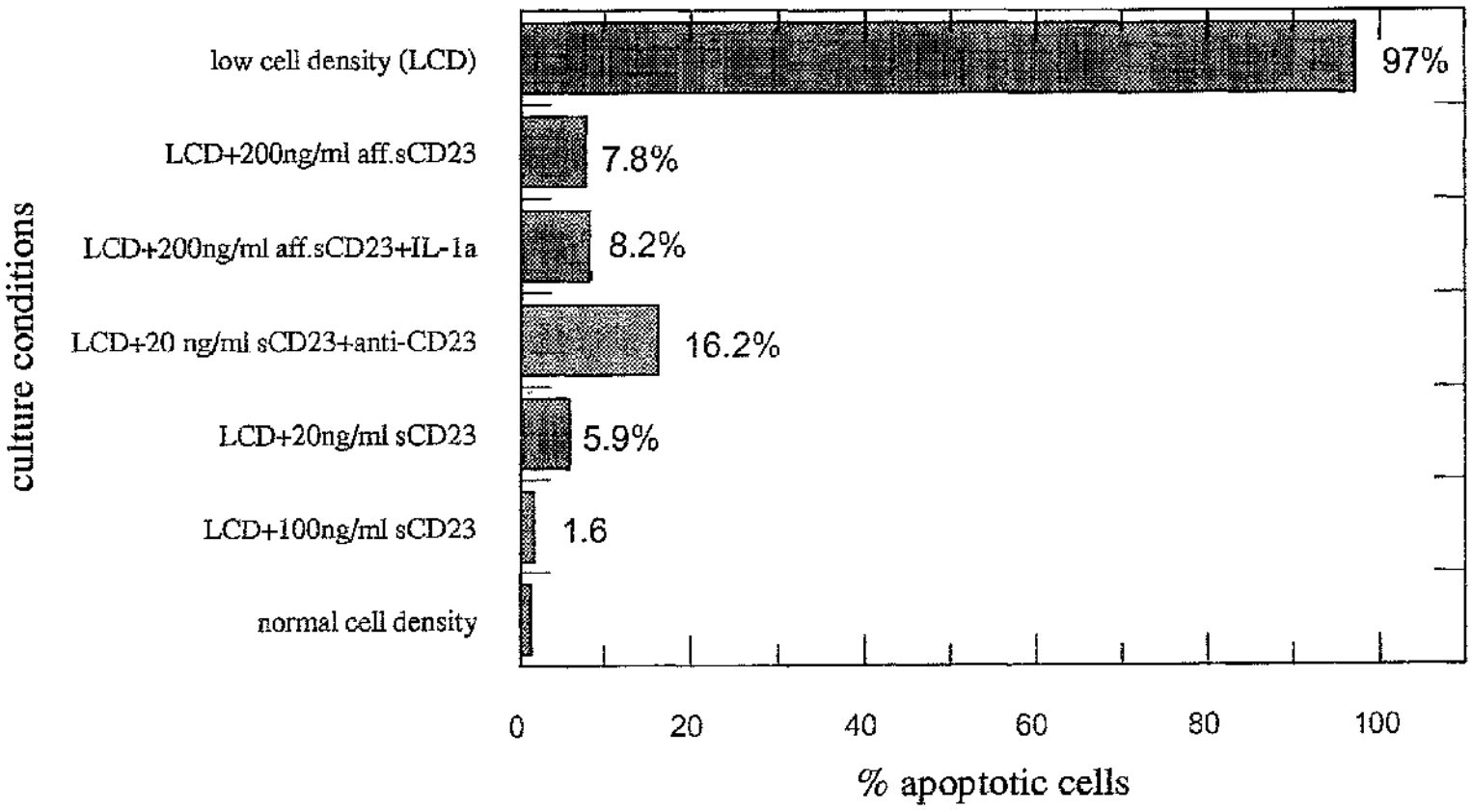
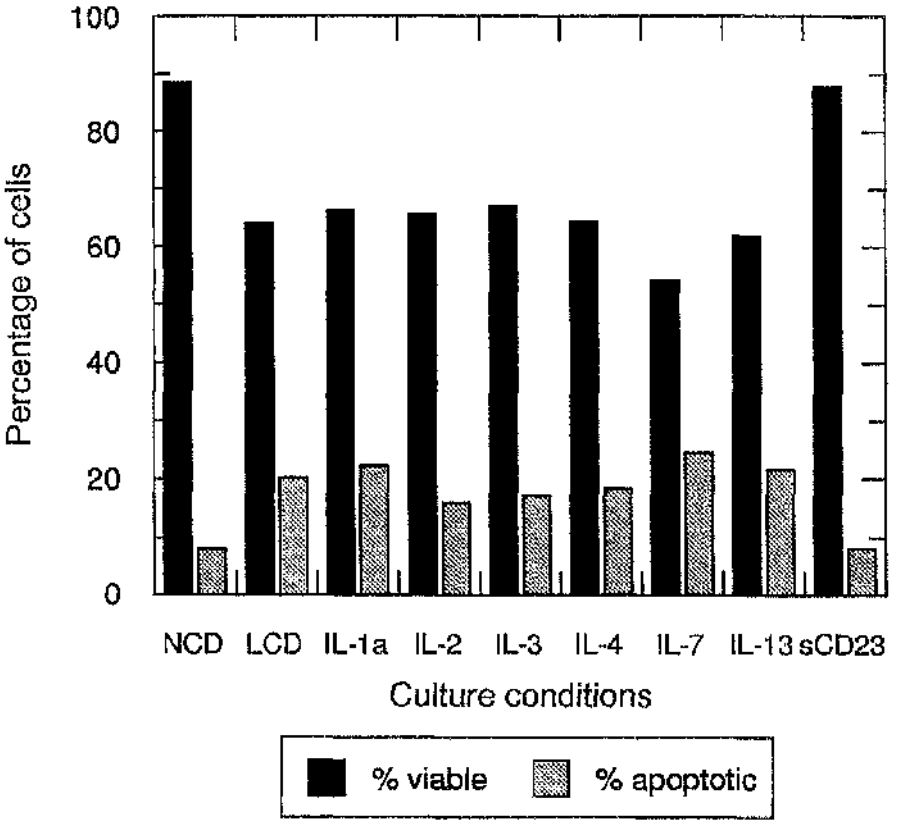

culture conditions
low cell density (LCD)
LCD+200ng/ml aff.sCD23
LCD+200ng/ml aff.sCD23+IL-1a
LCD+20 ng/ml sCD23+anti-CD23
LCD+20ng/ml sCD23
LCD+100ng/ml sCD23
normal cell density
97%
7.8%
8.2%
16.2%
5.9%
1.6
0
20
40
60
80
100
% apoptotic cells

Fig.4.16 Apoptosis of SMS-SB cells - the effect of various cytokines.

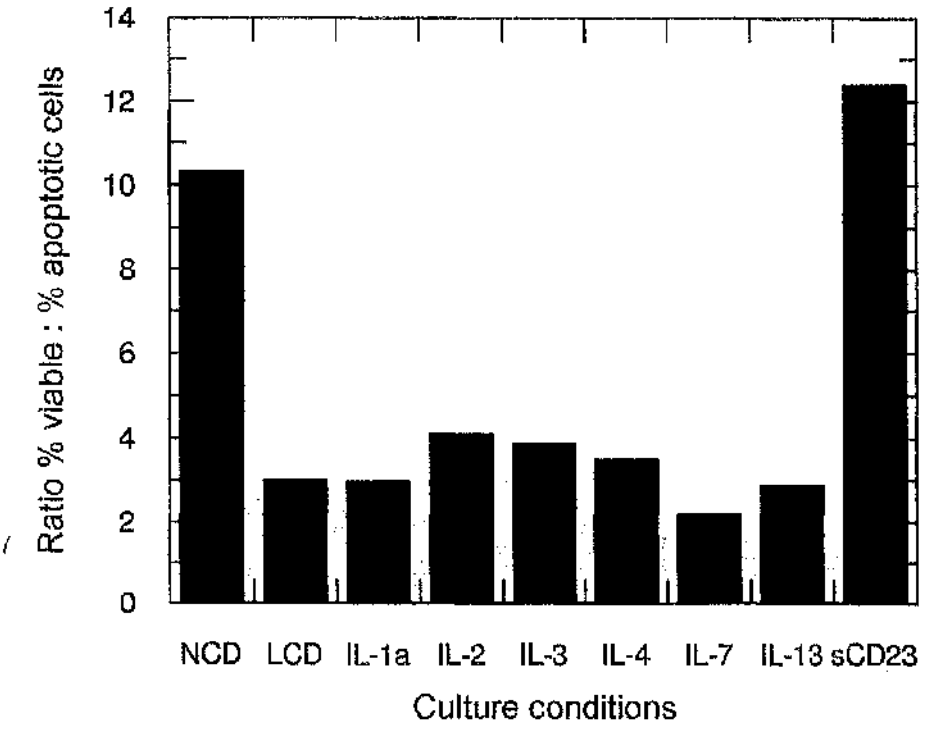
The experiments were performed exactly as described in the legend to figure 4.14, but several cytokines were tested for the ability to prevent apoptosis of SMS-SB cells at low cell density. The column chart (A) demonstrates the percentage of viable cells and apoptotic cells (as calculated by two-laser flow cytometry) present in a culture at normal cell density, compared with low cell density cultures in the presence of various cytokines. The data legend demonstrates which column represents viable versus apoptotic cells. The effect of addition of 10 ng/ml IL-1α, IL-2, IL-3, IL-4 (50U/ml), IL-7 or IL-13 (both 10 ng/ml) only, to the LCD cultures is shown, and compared to the effect of addition of 100ng/ml gel-filtration purified 25kDa sCD23.

The bottom panel represents the same data but shown as the ratio of % viable : % apoptotic cells. The results demonstrated are from a single experiment but are representative of three independent repeats.

A


100
80
60
40
20
0
Percentage of cells
NCD
LCD
IL-1a
IL-2
IL-3
IL-4
IL-7
IL-13
sCD23
Culture conditions
% viable
% apoptotic

B


14
12
10
8
6
4
2
0
Ratio % viable : % apoptotic cells
NCD
LCD
IL-1a
IL-2
IL-3
IL-4
IL-7
IL-13
sCD23
Culture conditions

1.3% seen in cultures propagated at normal cell density. The ability of 20 ng/ml sCD23 to prevent apoptosis of SMS-SB cells was partially inhibited by the addition of 40µg/ml Rb55 anti-CD23 to the cultures. The anti-CD23 increased the percentage of apoptotic cells to 16.2% - approximately a three fold increase. The data of figure 4.15 also demonstrate the ability of affinity purified sCD23 to prevent apoptosis of SMS-SB cells and show that there is no synergistic effect on addition of IL-1α in combination with affinity-purified sCD23. Addition of IL-1α, in combination with a sub-optimal concentration of gel filtration purified sCD23 (data not shown), did not decrease the percentage of apoptotic cells obtained, thus sCD23 does not appear to synergise with IL-1α for prevention of apoptosis of SMS-SB cells.

Several other cytokines were tested for the ability to prevent apoptosis of SMS-SB cells at low cell density (figure 4.16). The data are presented both as absolute percentages obtained (panel A) and as a ratio of the percentage viable cells divided by the percentage apoptotic cells (panel B). None of IL-1α, IL-2, IL-3, IL-4, IL-7 or IL-13 were able to prevent apoptosis of SMS-SB cells, at the concentrations tested. The results obtained on addition of these cytokines were almost exactly the same as those for cells cultured at low cell density alone, as is apparent from the ratio bar chart (figure 4.16, panel B).

Other than IL-1α, these cytokines have not been tested for synergistic effects with sCD23, but the fact that sCD23 alone can reduce the percentage of apoptotic cells to the values obtained from cultures at normal cell density, suggests that sCD23 alone is sufficient to prevent entry of SMS-SB cells into apoptosis.

4b.2.4 *sCD23 promotes bcl-2 expression in SMS-SB cells*

In several models of apoptosis, the proto-oncogenes *c-myc* and *bcl-2* are implicated in regulating the induction of apoptosis - in a positive and negative fashion, resepctively.

Normal B cells appear to down-regulate *c-myc*, after growth factor deprivation, and undergo growth arrest but, in EBV-immortalised B cells, over-expression of *c-myc* seems to force cells into the cell cycle and in the absence of a second signal they undergo apoptosis (Cherney *et al.*, 1994). SMS-SB cells were examined, by flow cytometry, for the expression of *c-myc* protein, after culture at normal and low cell density, in the presence and absence of sCD23. No fluorescence was observed, suggesting that the cells contain extremely low levels of *c-myc* protein, and there was no increase of *c-myc* observed in cells cultured at low cell density. Due to the short half life of *c-myc* protein, there was concern that the protein was being degraded before antibody staining was achieved. However, fixing the cells after initial permeabilisation with saponin did not allow detection of any *c-myc*. Another concern was that the anti-*c-myc* antibody may not have been binding effectively to the *c-myc* protein. This explanation was also eliminated after

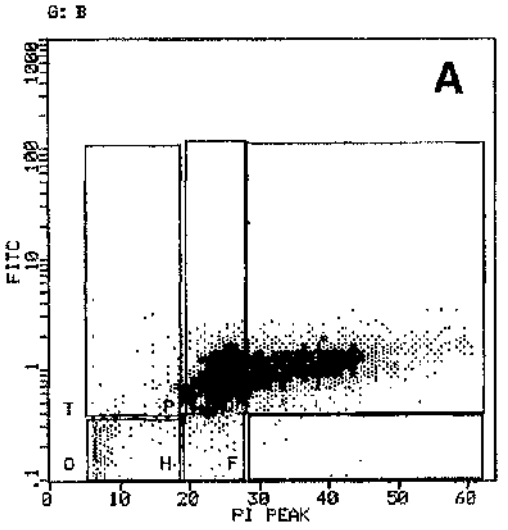
the B cell lines Raji and RPMI 8226 were tested with anti-*c-myc*. Although Raji cells did not have detectable *c-myc* protein, RPMI 8226 cells showed strong binding of the anti-*c-myc* antibody, demonstrating that the flow cytometric technique did allow detection of the intracellular protein (data not shown). Thus, SMS-SB cells do not appear to overexpress *c-myc*, and there was no visible increase in the level of *c-myc* in the cells after culture at low cell density. However, to further investigate the role of *c-myc* in apoptosis of SMS-SB cells, Northern blots would have to be performed.

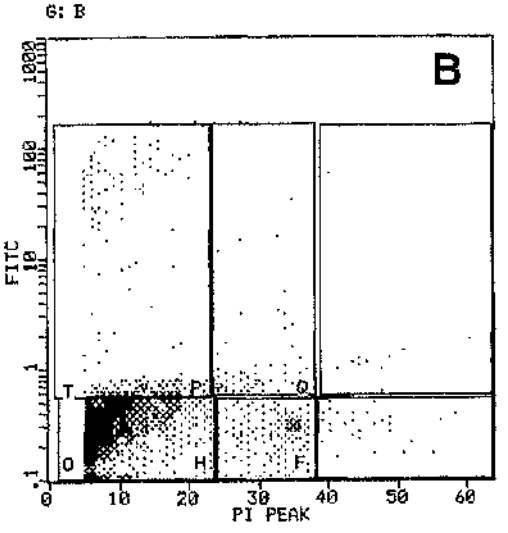
As mentioned previously, in the mature B lymphoid system the induction (or maintenance) of *bcl-2* appears to be critical for the survival of germinal centre cells, and sCD23 is one of a number of stimuli which have been shown to induce *bcl-2* expression in centrocytes in *in vitro* studies (Liu *et al.*, 1991). Thus, SMS-SB cells were examined for expression of *bcl-2* under a variety of culture conditions. A flow cytometric technique was employed, which allows analysis of *bcl-2* expression and cell cycle parameters of the cell populations. After culture, the cells were gently permeabilised with saponin, stained with anti-*bcl-2* antibody (and FITC-labelled sheep anti-mouse IgG), fixed with 1% paraformaldehyde, and then stained with propidium iodide, to visualise cell cycle parameters. In normal cell density cultures (figure 4.17, panel A), the cells show a strong G1 peak, with a significant proportion of cells in or G2/M phases; i.e., the cells are still cycling. There is strong staining for *bcl-2* expression in all phases of the cell cycle, and the level may increase slightly in the G2/M population; over 97% of cells scored positive for *bcl-2* expression. Approximately 2% of the cells were present in the far left area, which represents apoptotic cells. The cells were all in the lower half of this area, demonstrating that they have low levels of *bcl-2*.

After culture at low cell density (figure 4.17, panel B), the majority of the cells are in a pre-G1 peak (far left area) - known to represent the late apoptotic population - and show weak staining for *bcl-2*, with only 22% of the cells scoring positive for *bcl-2* expression. However, in the presence of sCD23 (figure 3.2.20, panel C), there is a restoration of the cell cycle profile - characteristic of asynchronously growing cells - and strong *bcl-2* expression occurs in all phases of the cell cycle, with 92% of the cells scoring as *bcl-2*-positive. Figure 4.18 is an example from a separate experiment showing *bcl-2* expression during cell cycle phases, after culture of the cells for 12 hours at low cell density. The dot-plot clearly shows the pre-G1, apoptotic population (far left area) but, in this example, some cells appear to be in earlier stages of apoptosis and are still expressing *bcl-2* (upper left section). There is an obvious downwards trend in *bcl-2* expression as the cells proceed through apoptosis until, at the late apoptotic stage (lower left section) they are *bcl-2*-negative.

Fig.4.17 Flow cytometric analysis of *bcl-2* expression in SMS-SB cells.

SMS-SB cells were cultured as described in the legend to figure 4.14, harvested (using trypsin, if necessary, to remove the cells) and washed. The cells were gently permeabilised in 0.1% saponin-containing buffer, blocked with normal rabbit serum and then treated with monoclonal anti-*bcl-2* antibody. After a 20 minute incubation on ice, the cells were washed and an aliquot of FITC-conjugated, sheep anti-mouse IgG added to visualise the primary MAb. After further incubation and washing, the cells were fixed in 2% paraformaldehyde, treated with 100μg/ml propidium iodide in citrate buffer and then analysed by flow cytometry. In the dot-plots illustrated, the x-axis shows propidium iodide staining in linear units of fluorescence, while the y-axis represents *bcl-2* staining on a logarithmic scale. The three panels represent the following culture conditions: Panel A - normal cell density; Panel B - low cell density; Panel C - low cell density + 100ng/ml gel-filtration purified sCD23. The experiment shown is representative of four independent repeats.


G: B
A
FITC
PI PEAK
T
P
O
H
F


G: B
B
FITC
PI PEAK
T
P
O
H
F

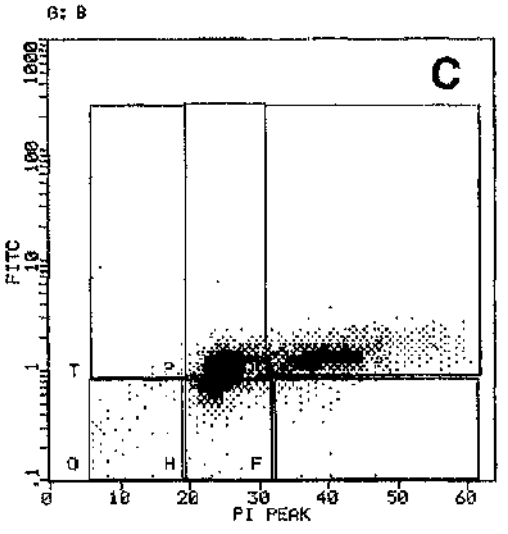
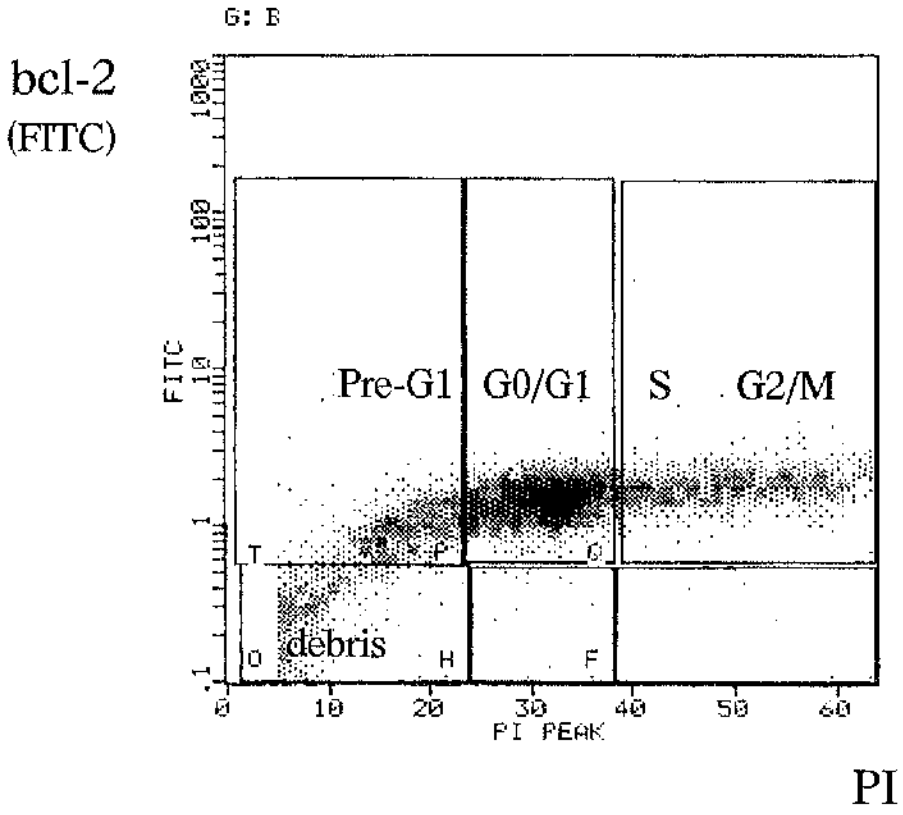

G: B
C
FITC
PI PEAK
T
P
O
H
F

Fig.4.18 Flow cytometric analysis of *bcl-2* expression throughout the cell cycle and apoptosis.

SMS-SB cells were cultured at low cell density for 12 hours, stained and treated exactly as described in figure 4.17. In the dot-plot illustrated, the x-axis shows propidium iodide staining in linear units of fluorescence, while the y-axis represents *bcl-2* staining on a logarithmic scale. The dot-plot demonstrates that levels of *bcl-2* expression fall as cells enter apoptosis (the pre-G1 peak) but are relatively constant throughout the rest of the cell cycle (Go/G1, S and G2/M phases). The cell cycle phases were determined as compared with a population of cells growing at normal cell density. The experiment shown is representative of four independent repeats.


G: B
bcl-2
(FITC)
FITC
1000
100
10
1
.1
Pre-G1
G0/G1
S
G2/M
T
P
Q
O
debris
H
F
0
10
20
30
40
50
60
PI PEAK
PI

4b.3 *Discussion*

The results presented in this section demonstrate that SMS-SB cells do, indeed, undergo apoptosis when they are cultured at low cell density; this effect is possibly due to deprivation of the SB-autocrine factor, and provides an explanation for the initial lag phase seen during growth of the cells (refer figure 3.2), and also for the low [^{3}H]-TdR incorporation achieved when SMS-SB cells are cultured at low cell density in the absence of CM (refer figure 3.3). Entry into apoptosis appears to begin at approximately 12 hours after initial seeding at low cell denisty, and continues until approximately 24 hours. Both morphological and flow cytometric results indicate that SMS-SB cells do undergo apoptosis at low cell density and that sCD23 is able to prevent entry of the cells into apoptosis. IL-1α, the cytokine which has numerous synergistic effects with sCD23, including the prevention of apoptosis of germinal centre B cells, does not synergise with sCD23 in the prevention of SMS-SB cell apoptosis. None of the other cytokines tested were able to promote the survival of SMS-SB cells. In fact, sCD23 alone appears sufficient to maintain the low cell density population at the viability of normal cell density cultures.

The precise role of *c-myc* in the apoptotic process is not known, although, by flow cytometry, SMS-SB cells do not appear to over-express *c-myc* and the levels do not appear to increase as the cells enter apoptosis. However, a role for *c-myc* in the apoptosis of SMS-SB cells cannot be ruled out until more sensitive analysis of expression is performed by Western or Northern blots.

One proto-oncgene which appears to be involved in prevention of SMS-SB cell apoptosis is *bcl-2*. The conclusion reached, from the FACS experiments, is that sCD23 either sustains, or actively promotes, the expression of *bcl-2* in SMS-SB cells cultured at low cell density, and this event may provide a molecular explanation for the ability of sCD23 to rescue the cells from apoptosis. It is possible that *bcl-2* substitutes for the signal provided by the SB-AF, and provides a 'survival' signal to allow the cells to continue proliferation under the control of other cell cycle genes.

SMS-SB cells, as pre-B cells, may be expected to undergo apoptosis since at the pre-B cell stage rearrangement of Ig genes occurs and there is a considerable chance that they will rearrange in a non-productive fashion. The productive rearrangement of Ig genes is essential for further differentiation of B cells and, to ensure that only cells with a normal phenotype are released into the periphery, cells with non-productive rerrangements die in the bone marrow - probably by apoptosis (Cohen & Duke, 1992). It has become apparent that at the pre-B stage of B cell development the cells are 'susceptible' to apoptosis, and levels of *bcl-2* present in the cells appear to correlate with this susceptibility. Merino *et al.*, 1994, demonstrated developmental regulation of *bcl-2* levels with high levels present

in pro-B and mature B cells, but low levels in pre-B, immature B and germinal centre cells - the stages where cells are prone to apoptosis.

Some human, and murine, malignant pre-B cell lines have been reported to express higher levels of *bcl-2*, compared with mature B cell lines, although normally the opposite is seen (Graninger *et al.*, 1987; Gurfinkel *et al.*, 1987). When SMS-SB cells are compared with Raji and RPMI 8226 cells (both mature B cell stages), the levels of *bcl-2* detected by flow cytometry are almost exactly the same (data not shown). Unfortunately, without analysis of *bcl-2* levels in other pre-B and mature B cell lines it is difficult to conclude whether SMS-SB cells do over-express *bcl-2*. It will also be important to investigate the possible involvement of other members of the *bcl-2* family, such as *bcl-x* and *bax*, since they may act to regulate *bcl-2* function.

However, assuming that *bcl-2* does have a direct effect on prevention of apoptosis, it will be interesting to discover what causes the down-regulation of *bcl-2* when SMS-SB cells are cultured at low cell density. A recent report has identified two pre-B cell-specific enhancer sites (π1 sites) in the 5' negative regulatory region of the *bcl-2* gene which have no significant function in mature B cells. The protein(s) which binds to these regions are thought to be a members of the Ets family of proteins, several of which are expressed at a higher levels in pre-B cells than in mature B cells (Chen & Boxer, 1995). It is possible that, as pre-B cells, SMS-SB cells still express the negative regulatory protein(s) but that a positive regulatory signal normally over-rides the negative signal. If SB-AF provides a positive regulatory signal for *bcl-2* transcription, then removal of SB-AF, by culture of the cells at low cell density, may remove this signal and allow the negative regulatory protein to act, or there may be a signal which induces transcription of the negative factor. The level of *bcl-2* would, therefore, decrease and the cell would undergo apoptosis.

Another interesting feature of the *bcl-2* gene is the identification of a negative regulatory element (NRE) in the 5' untranslated region which contains a *p53*-dependent element. Thus, *p53* may act to down-regulate *bcl-2* expression and promote apoptosis of SMS-SB cells. Investigation into the induction of *p53* in low cell density cultures of SMS-SB cells would also be interesting.

From the data presented, it is apparent that sCD23 can prevent the down-regulation of *bcl-2* expression in SMS-SB cells and so it will be interesting to examine the signals transduced by the CD23-receptor. In germinal centre B cells, sCD23 is thought to prevent apoptosis by signalling through CD21, the B cell-expressed, non-IgE ligand for CD23 (Aubry *et al.*, 1992). Since CD21 is not normally expressed on pre-B cells, it was decided to investigate which CD23-receptor mediates the biological effects of sCD23 on SMS-SB cells. The finding of these experiments are presented in chapter 5.

Chapter 5
SMS-SB cells express a novel CD23-receptor

5.1 *Introduction*

The reported receptors for CD23 are CD21 (Aubry *et al.*, 1992) and CD11b & CD11c (Lecoanet-Henchoz *et al.*, 1995). CD21, also a receptor for C3d, C3g and iC3b complement proteins, interferon-α and EBV, is an approximately 140kDa glycoprotein expressed on B lymphocytes, some T lymphocytes, follicular dendritic cells and pharyngeal epithelial cells (Weis *et al.*, 1984; Delcayre *et al.*, 1991; Tanner *et al.*, 1987). The CD23/CD21 interaction appears to involve the C-type lectin activity of CD23 since binding can be inhibited by fucose-1-phosphate, and by tunicamycin treatment of the RPMI 8226 cell line (Pochon *et al.*, 1992). CD21 and CD23 appear to function as adhesion molecules (Aubry *et al.*, 1992; Bjorck *et al.*, 1993; Aubry *et al.*, 1994), and the interaction is thought to have a role in enhancing antigen presentation, possibly because CD23 also associates with MHC class II antigens (Bonnefoy *et al.*, 1988; Flores-Romo *et al.*, 1990). Since CD21 is expressed by follicular dendritic cells, the ability of sCD23 to prevent apoptosis in germinal centre cells (Liu *et al.*, 1991) has been postulated to occur via CD21, a hypothesis somewhat supported by data from Bonnefoy *et al.* (1993) that a subset of anti-CD21 antibodies are able to prevent apoptosis of germinal centre B cells, *in vitro*. However, CD21 is not expressed until the late pre-B cell stage of differentiation, raising the possibility that other, as yet unidentified, receptors for CD23 might exist within the B cell compartment.

CD11b-CD18 and CD11c-CD18 are members of the β2-integrin family of adhesion proteins, and act as CD23-receptors on monocytic cells (Lecoanet-Henchoz *et al.* 1995). Binding of CD23 to CD11b-CD18/CD11c-CD18 is also inhibited by tunicamycin treatment of the cells, suggesting that sugars are involved in the interaction. Addition of sCD23 to activated monocytes results in production of inflammatory cytokines including IL-1β, IL-6 and TNF-α, indicating a possible role for CD23 in inflammatory responses. The only member of the β2-integrin family normally found on B cells is LFA-1 (CD11a-CD18), although activated B cells may express CD11b and/or CD11c; expression of LFA–1 is not usually noted on pre-B cells and it does not bind CD23 (Lecoanet-Henchoz *et al.* 1995).

This chapter of results describes experiments performed to investigate the CD23-receptor on SMS-SB cells which is responsible for the biological effects of sCD23. The data demonstrate that none of CD21, CD11b or CD11c are expressed by SMS-SB cells yet the cells can bind CD23-containing liposomes in a CD23-specific fashion. Initial results from CD23 affinity-columns, used in an attempt to isolate the novel CD23-receptor, are also presented.

5.2 *Results*

5.2.1 *SMS-SB cells do not express known CD23-receptors*

The expression of known CD23 receptors was assessed by flow cytometry as shown in figure 5.1. SMS-SB cells and SKW cells were stained with FITC-labelled MAb to each of the β2-integrins, CD11a, CD11b and CD11c, represented by the data of panels A-C respectively. The data clearly illustrate that while SKW cells express high levels of all three CD11 species (solid line), the level of staining of SMS-SB cells (closely spaced dotted line) was the same as the autofluorescence profile of SMS-SB cells (widely spaced dotted line). Thus, SMS-SB cells do not express any of CD11a, CD11b or CD11c antigens on their surface, and staining with FITC-anti-CD18 (figure 5.1, panel D) illustrates that SMS-SB cells also lack this component of the β2-integrin complex. Panel E, shows flow cytometric analysis of CD21 expression by SMS-SB cells compared with the mature B cell lines, Daudi and Ramos. Daudi cells are strongly positive for CD21 expression while Ramos are either negative, or very weakly positive. SMS-SB cells are negative for surface expression of CD21.

Southern blot analysis of the CD21 gene in SMS-SB cells, compared to SKW cells, was performed on DNA digested with the restriction enzymes BamHI, HINDIII and EcoRI (figure 5.2). A sample of 25pg of uncut plasmid was run on the gel to test the hybridisation capacity of the probe. The results of the restriction digest hybridisation demonstrate that the CD21 gene in SMS-SB cells has no deletions or insertions, compared with SKW cells.

More sensitive techniques, to detect expression of the CD23 receptors, were only performed for CD21 since the data on CD11b and CD11c was published quite recently (Lecoanet-Henchoz *et al.* 1995). As for detection of CD23 RNA (Figure 4.7), no transcripts for CD21 were detected by Northern blots of total RNA and preparation of mRNA was necessary. The results of Northern blots of mRNA (Figure 5.3) demonstrate that the cell lines Daudi and Ramos both express transcripts for CD21 but none of SMS-SB cells (cultured either in protein-free or serum-containing medium), SKW or Jijoye cells have detectable levels of CD21 mRNA. That Ramos cells express CD21 mRNA is interesting due to the very low, almost undetectable, staining seen by FACS (figure 5.1, panel E). The Northern blot was stripped of radioactivity and reprobed with a GAPDH probe (Figure 5.4) to demonstrate that loading of the mRNA was approximately equal and that the mRNA of the cell lines which were negative could be hybridised with a probe.

To ensure that there was no transcription of CD21 in SMS-SB cells, RT-PCR was performed with CD21-specific oligonucleotide primers (Figure 5.5). RNA was isolated from SMS-SB cells and from SKW, Ramos and Jijoye B cell lines and a keratinocyte line,

Fig.5.1 Expression of CD21, CD18 and CD11 antigens by SMS-SB cells.

Aliquots ($5x10^5$ cells) of SMS-SB cells and Ramos, SKW and Daudi B lymphoid cell lines were harvested, washed in ice-cold PBS and stained with FITC-conjugated MAbs specific for the CD11a, CD11b, CD11c, CD18, CD19 and CD21 antigens. After a 45 minute incubation, the cells were washed and analysed by flow cytometry; non-viable cells were excluded from acquisition by propidium iodide counter-staining. On each graph, FITC-staining is shown along the x-axis on a logarithmic scale (fluorescence 1), with the number of events recorded per channel shown on a linear scale along the y-axis. For each plot, data was collected from 10^4 live-gated cells. The line styles, which represent each MAb staining, are indicated on the individual panels; 'auto' refers to the autofluorescence of unstained cells.

Panel A - CD11a expression on SKW and SMS-SB cells

Panel B - CD11b expression on SKW and SMS-SB cells

Panel C - CD11c expression on SKW and SMS-SB cells

Panel D - CD18 and CD19 expression on SMS-SB cells

Panel E - CD21 expression on Daudi, Ramos and SMS-SB cells

The experiment is representative of three independent repeats.

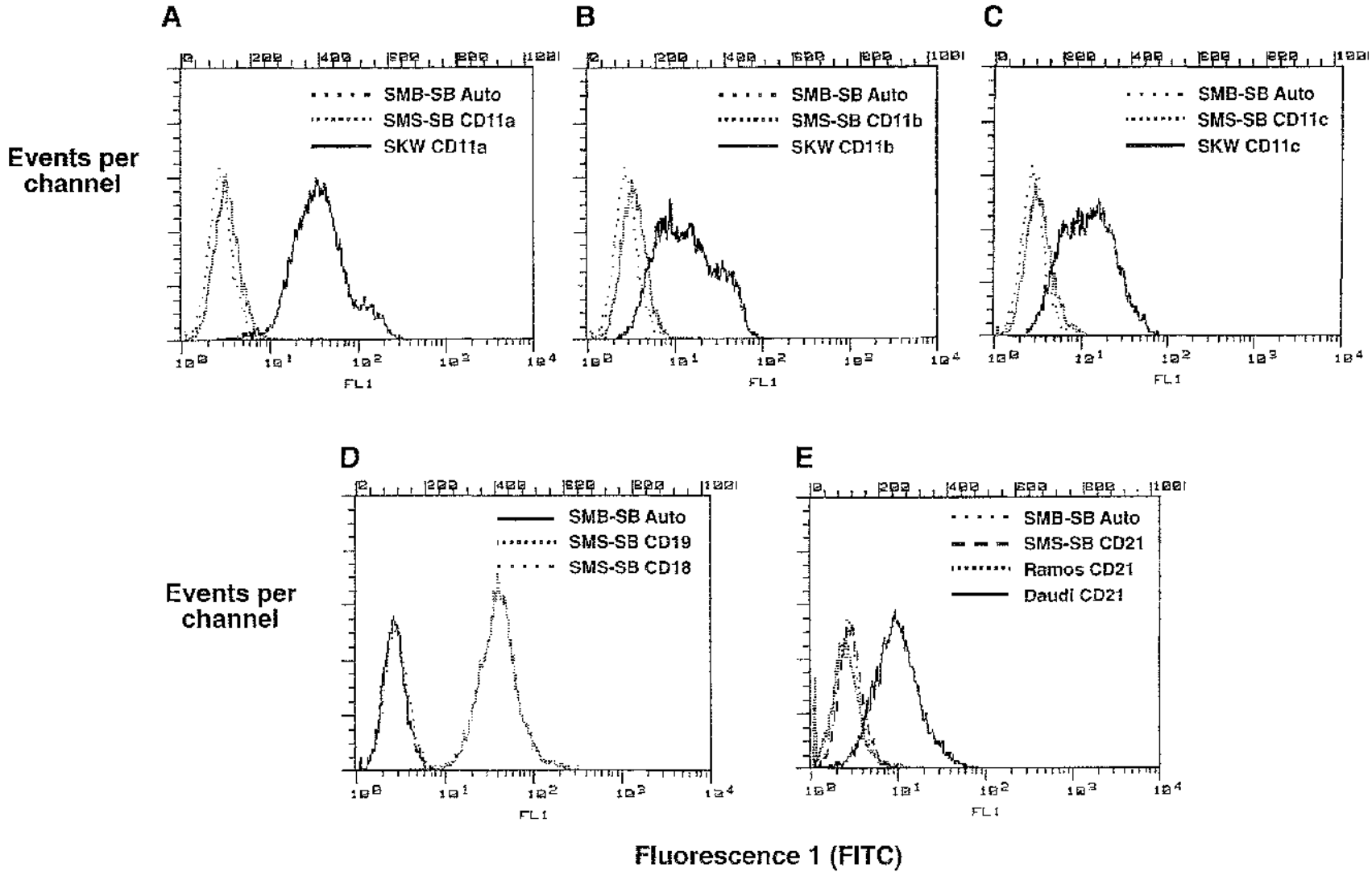

A
B
C
D
E
Events per channel
Events per channel
SMB-SB Auto
SMS-SB CD11a
SKW CD11a
SMB-SB Auto
SMS-SB CD11b
SKW CD11b
SMB-SB Auto
SMS-SB CD11c
SKW CD11c
SMB-SB Auto
SMS-SB CD19
SMS-SB CD18
SMB-SB Auto
SMS-SB CD21
Ramos CD21
Daudi CD21
FL1
Fluorescence 1 (FITC)

Fig.5.2 Southern blot analysis of the CD21 gene in SMS-SB cells

Southern blots were carried out as described in materials and methods. The first lane contained 25pg of uncut CD21 plasmid, pBSCD21, as a positive control for probe hybridisation. Lanes 2 and 3 were loaded with 20μg samples of BamHI-digested SMS-SB and SKW genomic DNA, respectively. The SMS-SB and SKW samples of each digest were loaded beside each other to allow comparison of the digest patterns. Lanes 4 & 5, and lanes 6 & 7, represent HINDIII and EcoRI digests of the two cell lines, respectively. After the digests were separated by agarose gel electrophoresis and blotted onto nylon membrane, the blot was hybridised with a [^{32}P]-labelled, 1.0kbp, Xho-1/Not-1 insert of human CD21 from the pBSCD21 plasmid. Hybridisation was visualised by autoradiography. The experiment is representative of three independent repeats.

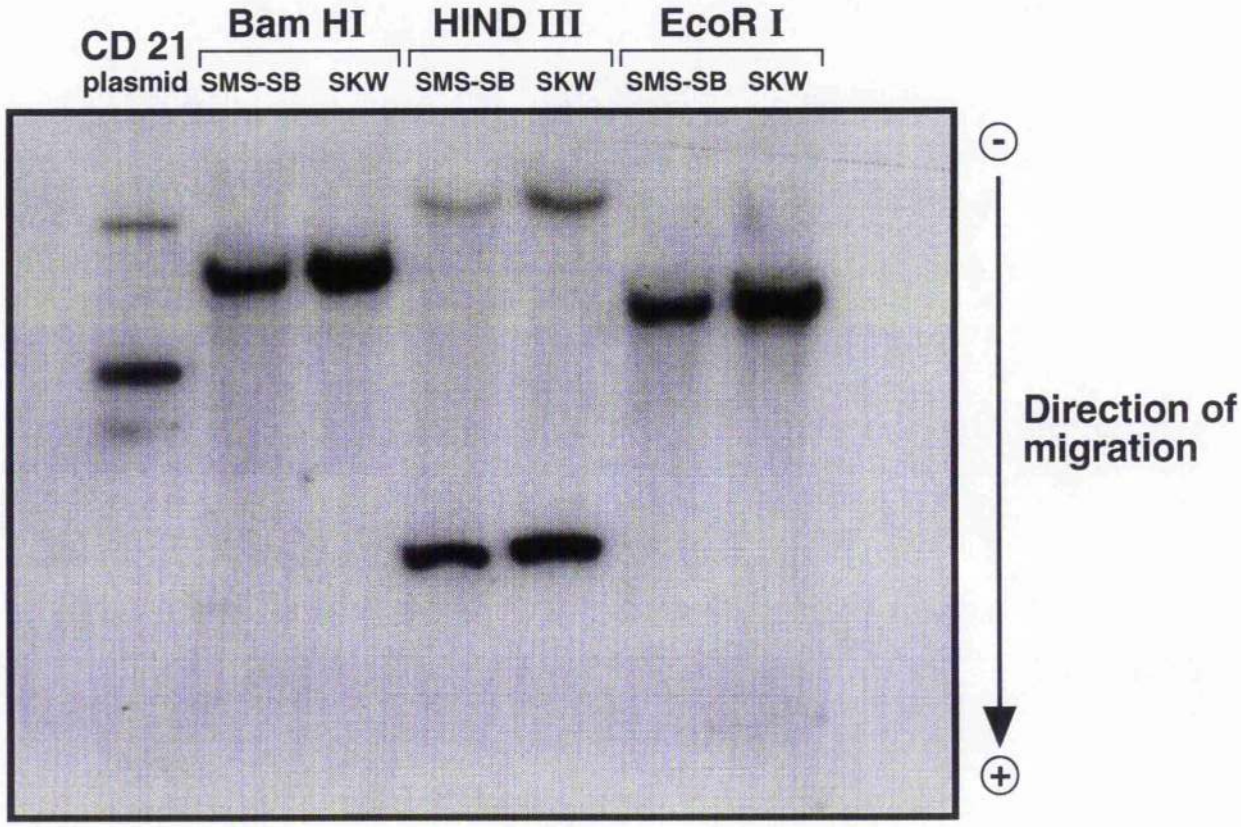

CD 21
plasmid
Bam HI
HIND III
EcoR I
SMS-SB
SKW
SMS-SB
SKW
SMS-SB
SKW
Direction of migration

Fig.5.3 Northern blot analysis of CD21 expression in SMS-SB cells and other B lymphoid cell lines.

Total cellular RNA was prepared (as described in materials and methods) from a range of B lymphoid cell lines and from SMS-SB cells cutured in PFHMII and in 10% FCS/ RPMI-1640 medium. From total RNA, the polyA mRNA fraction was isolated using the *PolyATract mRNA Isolation System IV*. 5μg of each cell line mRNA were loaded per lane, on a formaldehyde gel. Lanes 1 & 2 contained mRNA from SMS-SB cells grown in PFHMII and serum-containing medium, respectively. Lane 3, 4, 5 & 6 contained mRNA from the B cell lines SKW, Jijoye, Ramos, and Daudi respectively. After separation by electrophoresis, the RNA was blotted onto nylon membrane and hybridised with a [^{32}P]-labelled, 1.0kbp, Xho-1/Not-1 insert of human CD21 from the pBSCD21 plasmid. Hybridisation, demonstrating the existence of the 3934bp CD21 RNA, was visualised by autoradiography. The experiment is representative of five independent repeats.

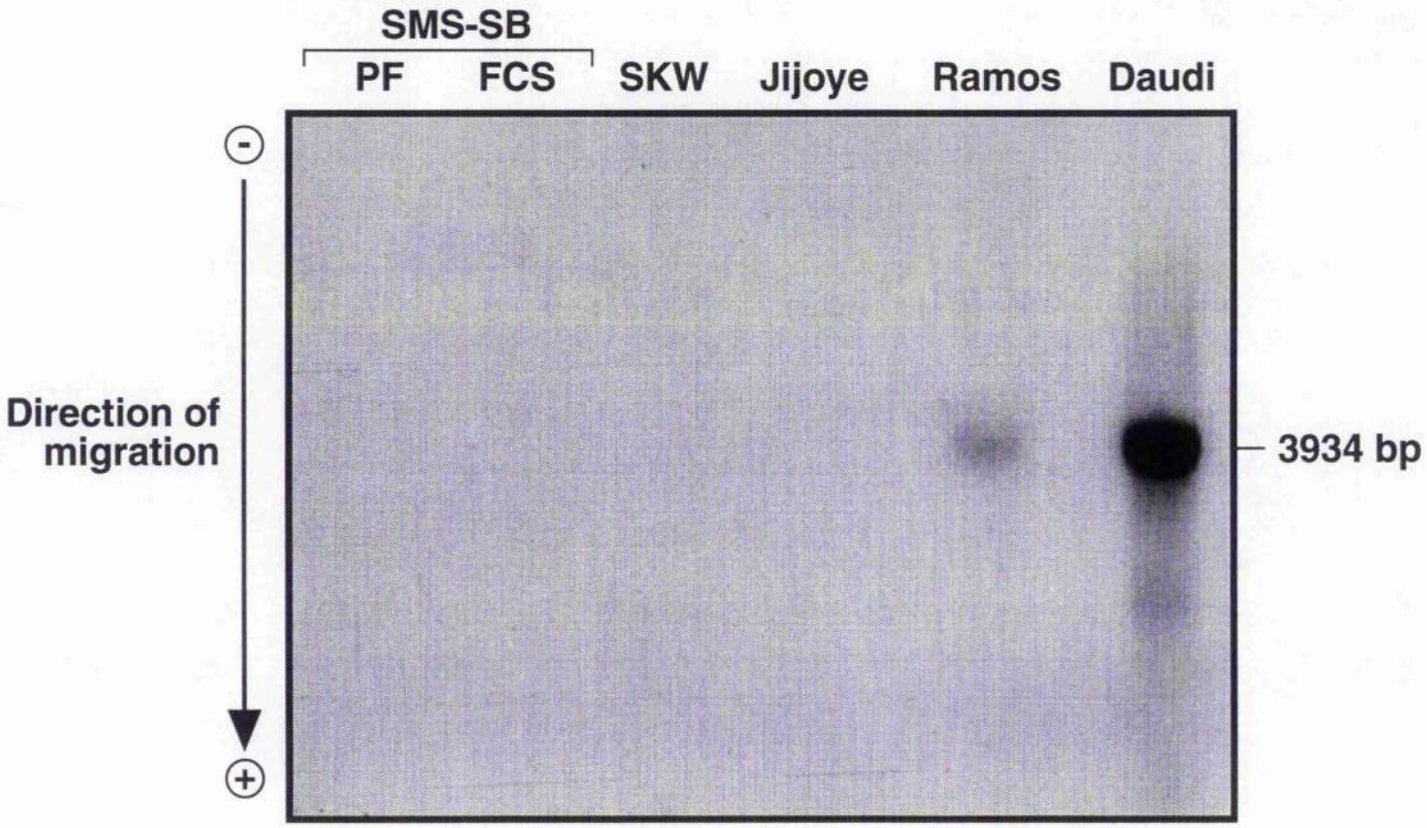

SMS-SB
PF
FCS
SKW
Jijoye
Ramos
Daudi
Direction of migration
3934 bp

Fig.5.4 GAPDH loading control for CD21 Northern analysis

The Northern blots from the experiments described in Fig.5.3 were stripped (as described in methods) and re-hybridised with a [^{32}P]-labelled, 0.7kb GAPDH probe. The lane which represented Daudi cells was removed from the blot before it was exposed to the film, since the strength of the signal obtained interfered the other lanes. Hybridisation was visualised by autoradiography.

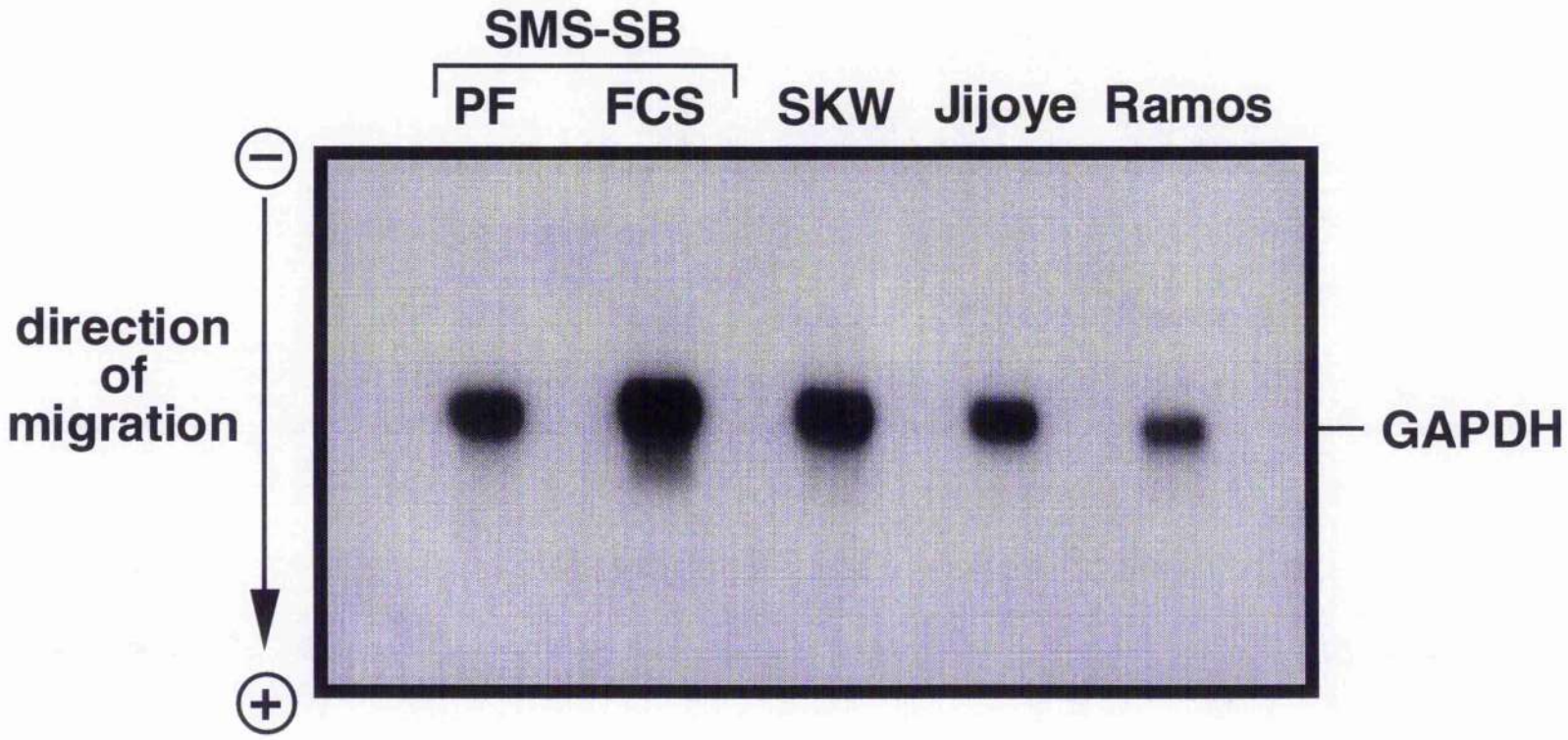
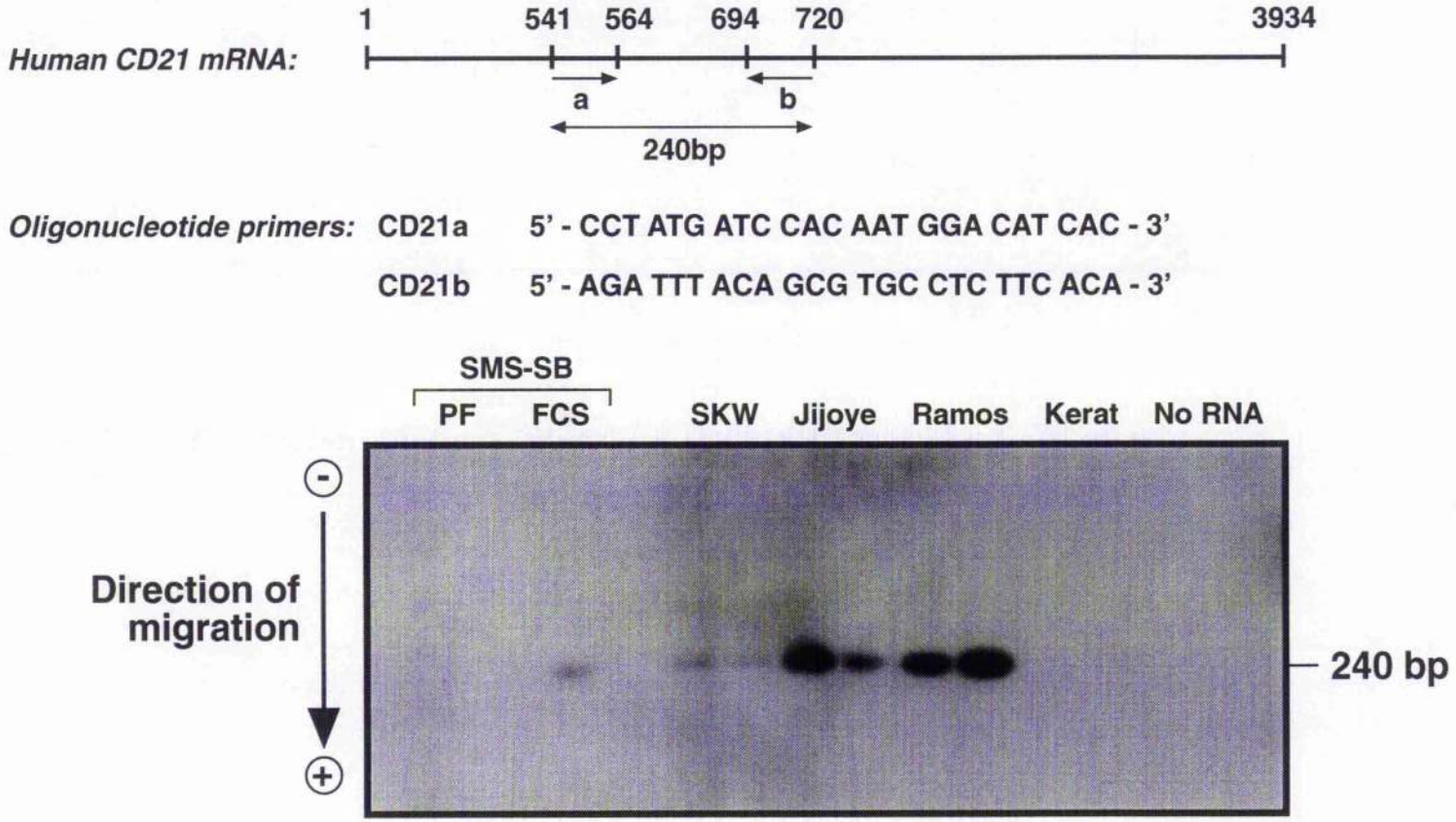

SMS-SB
PF
FCS
SKW
Jijoye
Ramos
direction of migration
GAPDH

Fig.5.5 PCR analysis of CD21 expression in SMS-SB and other B lymphoid cell lines.

Total cellular RNA was prepared (as described in materials and methods) from a range of B lymphoid cell lines, a keratinocyte cell line, and from SMS-SB cells cultured in PFHMII and in 10% FCS/ RPMI 1640 medium. The total cellular RNA was used as a template for reverse-transcriptase (RT)-PCR using the primers, CD21a and CD21b, indicated on the figure. After the PCR reactions were complete, the products (240bp) were separated by agarose gel electrophoresis, Southern blotted onto nylon membranes, and probed with a 1.0kbp, Xho-1/Not-1 insert from the pBSCD21 plasmid. The gel was loaded as shown on the figure: lanes 1 & 2 were PCR products from SMS-SB cells cultured in PFHMII and in serum-containing medium; lanes 3, 4 and 5 contained PCR products from SKW, Jijoye and Ramos B cell lines, respectively; lanes 6 & 7 were negative controls of keratinocyte cells (DOK) and a PCR reaction performed with no template RNA, respectively. Hybridisation was visualised by autoradiography. The experiment is representative of four independent repeats.


1
541
564
694
720
3934
Human CD21 mRNA:
a
b
240bp
Oligonucleotide primers: CD21a 5' - CCT ATG ATC CAC AAT GGA CAT CAC - 3'
CD21b 5' - AGA TTT ACA GCG TGC CTC TTC ACA - 3'
SMS-SB
PF
FCS
SKW
Jijoye
Ramos
Kerat
No RNA
Direction of migration
240 bp

DOK, as a negative control. A line drawing of the human CD21 cDNA is given, showing the position of the two CD21 oligonucleotide primers, CD21a and CD21b; the sequences of these primers are given on the figure. After reverse-transcription and 35 cycles of PCR amplification, products of the individual reactions were electrophoresed and detected by Southern blotting with a CD21-specific probe. Ramos, Jijoye and SKW cells all gave a product of 240bp, demonstrating that they do contain transcripts for CD21. This shows that the RT-PCR technique is much more sensitive than mRNA Northern analysis, since both Jijoye and SKW had no detectable signal by Northern blot (figure 5.3). Figure 5.5 illustrates that SMS-SB cells do not express CD21, although in 2 out of five experiments, one of the duplicate reactions gave a product of similar (but not identical) size to the products from the other cell lines. That this product could hybridise with the CD21-specific probe suggests that it is CD21, although the amount of product, after 35 cycles of PCR, was very small. Developmental regulation of gene expression is unlikely to be an 'all or nothing' process so very low levels of CD21 transcript may be expected since expression of CD21 begins at the late pre-B cell stage.

The keratinocyte RNA and reaction performed with no RNA, both gave negative results demonstrating that the reagents were not contaminated. As a positive control for the reverse-transcriptase reaction, *c-fos*-specific oligonucleotides were used (refer to figure 4.10).

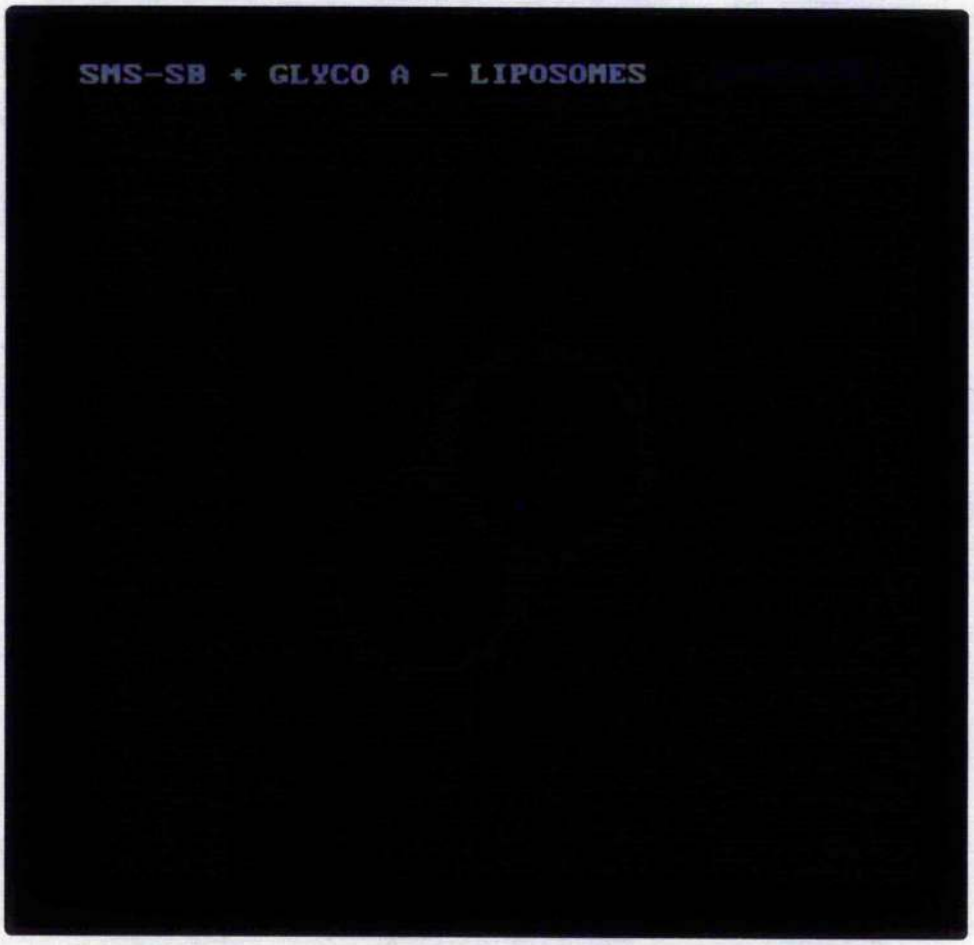
5.2.2 *Specific binding of CD23-containing liposomes to SMS-SB cells*

SMS-SB cell do not express any of the currently characterised receptors for CD23 and so a formal demonstration of CD23 binding to SMS-SB cells was sought. Liposomes containing full-length (45kDa) CD23, and control liposomes containing glycophorin A, were prepared and incubated with SMS-SB cells before the cells were examined by confocal microscopy. Propidium iodide was added prior to the examination, to allow detection of dead cells; the nuclei of dead cells appear red under the confocal microscope. CD23-containing liposomes bind to SMS-SB cells, with an apparently random distribution on the surface of the cells (figure 5.6, panel A); control glycophorin-A containing liposomes do not bind to the cells (panel B). Binding of CD23-liposomes to the cell line RPMI 8226, which binds CD23 via CD21 (Aubry *et al.*, 1992), is shown in figure 5.7. Liposome binding to RPMI 8226 cells does not appear to be evenly distributed on the cell surface; a possible reason for this is that the cells had been left for approximately two hours before analysis and some 'capping' or clustering of the receptor/liposomes may have occurred. This effect was observed with SMS-SB cells if the cells were left with the liposomes for over an hour before analysis (data not shown).

Fig.5.6 CD23-containing liposomes bind to SMS-SB cells.

Fluorescent liposomes containing either full length (45kDa) CD23 or glycophorin-A were prepared as described (materials and methods), and 50µl were added to each washed aliquot of 10^5 SMS-SB cells, at 4°C. After a 2hr incubation, the cells were washed three times in ice-cold liposome buffer and the suspensions analysed by confocal microscopy. Propidium iodide was included to identify dead cells which stain red in the analysis (for an example, see panel A). Panel A illustrates binding of CD23-containing liposomes, and panel B the binding of control, glycophorin-containing liposomes, to SMS-SB cells. A photomicrograph was taken at a magnification of 160X.

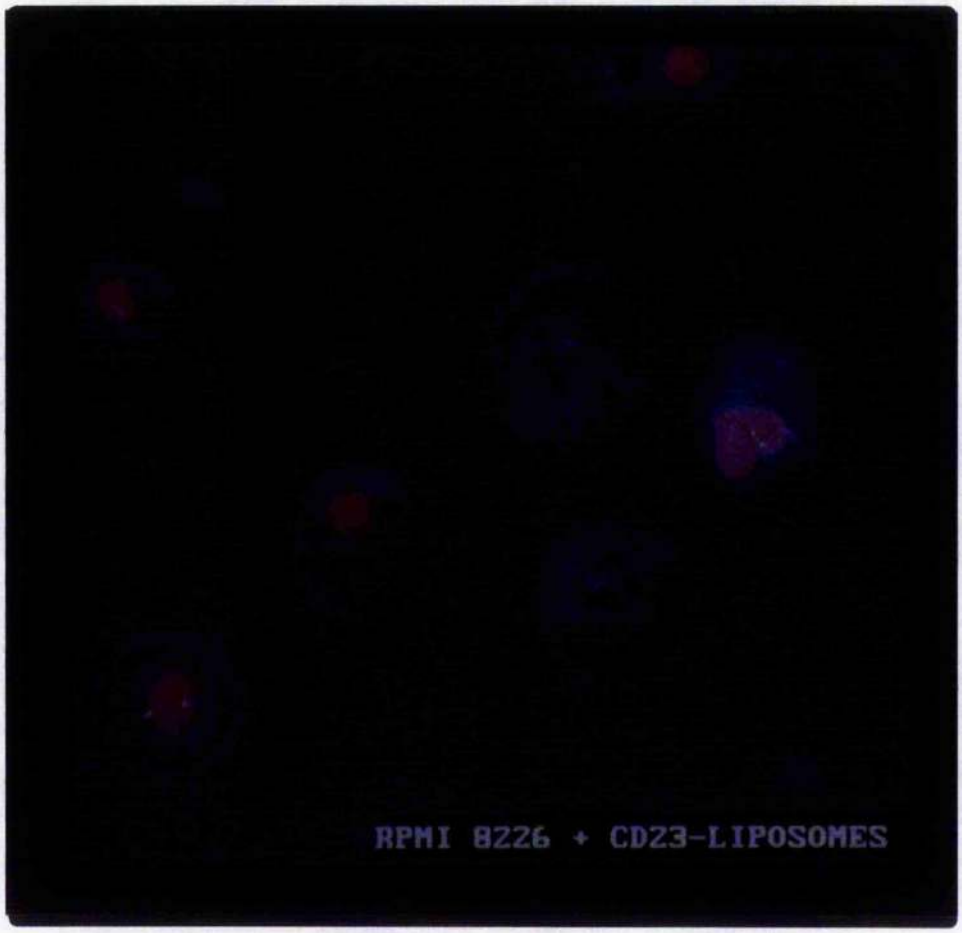
A



B

Fig.5.7 CD23-containing liposomes bind to RPMI 8226 cells.

This experiment was performed exactly as described in the legend of figure 5.6. but with the B cell line RPMI 8226, and the stained cells were left on ice for approximately 1.5 hrs prior to examination under the confocal microscope. The confocal photomicrograph was kindly provided by Dr. J-P. Aubry, Glaxo IMB, Geneva.


RPMI 8226 + CD23-LIPOSOMES

To quantitate liposome binding, the cells were stained with the liposomes, as before, and analysed by flow cytometry. Figure 5.8 demonstrates that CD23-containing liposomes bind strongly to both SMS-SB (panel B) and RPMI 8226 (panel C) cells but not to Raji cells (panel A); glycophorin-A-containing liposomes failed to bind to any of the cell lines. Liposome binding to SMS-SB cells, via the novel CD23 receptor, is of a level similar to that observed for RPMI 8226 cells which bind CD23 via CD21.

Several cytokines were tested for the ability to modulate the level of CD23-liposome binding to SMS-SB cells. None of IL-1α to IL-8, or GM-CSF have any effect on CD23-liposome binding to SMS-SB cells (figure 5.9), suggesting that they do not affect the expression of the novel CD23 receptor. IL-1α has previously been shown to increase the binding of CD23-liposomes to monocytes, which bind CD23 via CD11b and/or CD11c (Lecoanet-Henchoz *et al.*, 1995), suggesting that it increases either the number, or affinity, of receptors on the surface of the cells.

One interesting result obtained during these experiments is that culture of SMS-SB cells in PFHMII supplemented with 50U/ml IL-4 increases the size of SMS-SB cells from approximately 17μm diameter to approximately 24μm, as determined by confocal microscopy (figure 5.10); IL-4 has no effect on the level of CD23-liposome binding (figure 5.9 & 5.10). This result demonstrates that SMS-SB cells do express functional IL-4 receptors but IL-4 does not act to increase the number of CD23 receptors or prevent apoptosis of SMS-SB cells (refer figure 4.14, panel D).

The specificity of liposome binding was demonstrated by pre-incubating the liposomes for 1 hour at 4°C, with various concentrations of the IgG fraction of Rb55 polyclonal anti-CD23 antibody or normal rabbit IgG, before addition to the cells. The data of figure 5.11 illustrate that anti-CD23 inhibits binding of CD23-containing liposomes to both SMS-SB and RPMI 8226 cells, but normal rabbit IgG is unable to prevent liposome binding. There is no binding of control glycophorin-A containing liposomes to either cell line and this is not affected by either antibody (data not shown). The monoclonal antibodies EBVCS4 and MAb25, murine IgM and IgG1 respectively, are also able to inhibit binding of CD23-liposomes to SMS-SB cells and to RPMI 8226 cells (figure 5.12); control mouse IgG1 and IgM antibodies have no effect on liposome binding (data represented by line for CD23 liposomes + control, top panel). Since both Mab25 and EBVCS4 can inhibit binding of CD23-containing liposomes to SMS-SB and to RPMI 8226 cells, CD23 must bind the receptor present on SMS-SB cells via epitopes also used in binding to CD21.

One difference in the CD23 interaction with SMS-SB compared to RPMI 8226 cells is that 25kDa sCD23 may partially inhibit the CD23-liposome binding to SMS-SB but not to RPMI 8226 cells (figure 5.13). The addition of 10μg/ml BSA has no effect on liposome

Fig.5.8 Quantitation of CD23-containing liposome binding to SMS-SB and other B lymphoid cell lines.

Aliquots of 10^5 Raji (panel A), SMS-SB (panel B) and RPMI 8226 cells (panel C), were incubated with either CD23-containing (solid line) or glycophorin-A-containing (broken line) fluorescent liposomes, for 2 hours at 4°C. After incubation, the cells were washed three times in liposome buffer and then analysed by flow cytometry for liposome binding. The x-axis of each panel represents the FITC-fluorescence (due to liposome binding), on a logarithmic scale. The y-axis represents the number of events per channel, on a linear scale. The experiment is representative of more than five independent repeats.

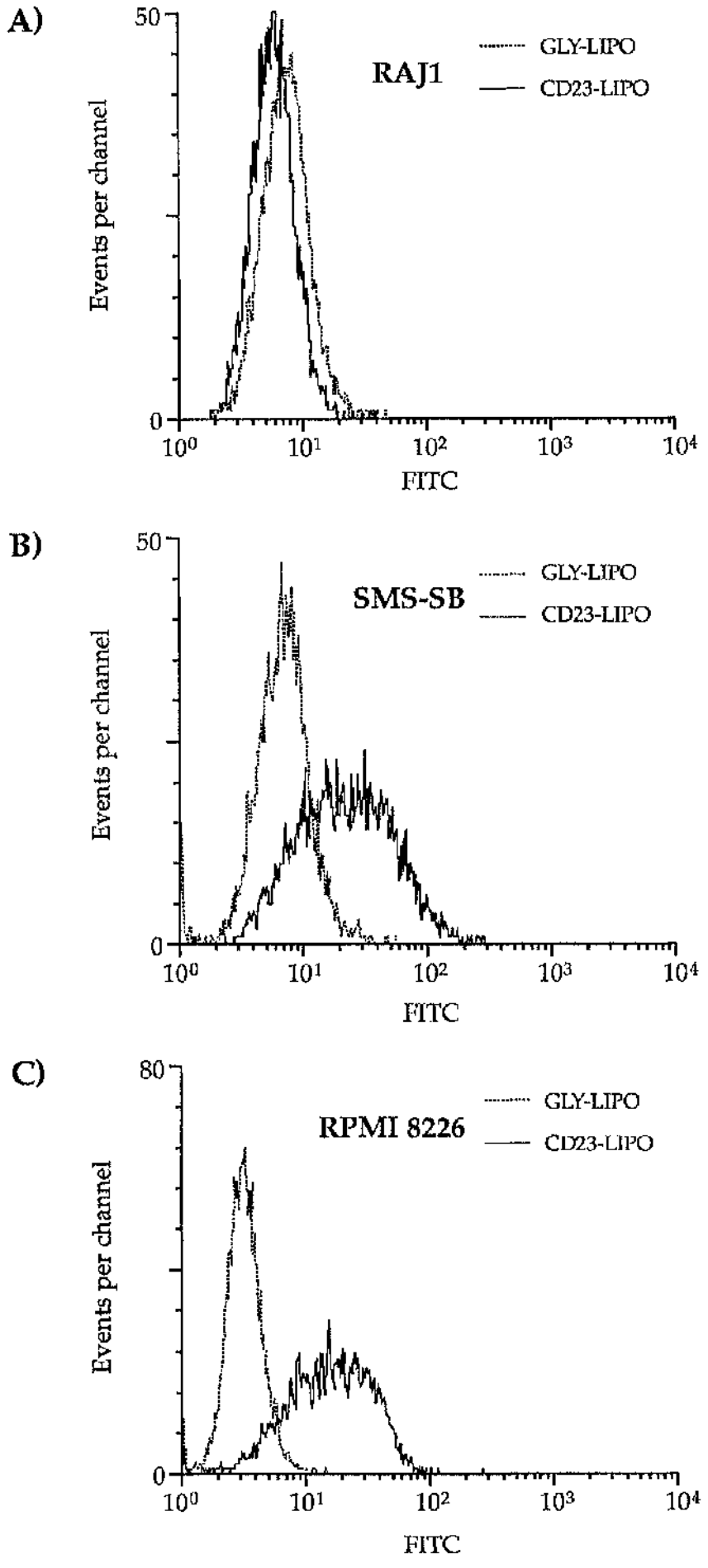

A)
50
0
Events per channel
RAJ1
GLY-LIPO
CD23-LIPO
10^0
10^1
10^2
10^3
10^4
FITC
B)
50
0
Events per channel
SMS-SB
GLY-LIPO
CD23-LIPO
10^0
10^1
10^2
10^3
10^4
FITC
C)
80
0
Events per channel
RPMI 8226
GLY-LIPO
CD23-LIPO
10^0
10^1
10^2
10^3
10^4
FITC

Fig.5.9 Effect of various cytokines on the level of CD23-containing liposome binding to SMS-SB cells.

SMS-SB cells were incubated in PFHMII supplemented with 5ng/ml of one of the cytokines IL-1α to IL-8 or GM-CSF. After 18 hours, the cells were washed and then incubated for 2 hours with 50µl of either glycophorin-A-containing liposomes or CD23-containing liposomes. The cells were then washed three times and analysed by flow cytometry. The chart shows the average fluorescence obtained after treatment of the cells with various cytokines, as a percentage of the CD23-liposome binding to untreated SMS-SB cells (the positive control for binding is given as 100%). The error bars represent the standard error of the mean, giving the maximum and minimum difference in fluoresence from the control CD23-liposome binding. The data from three experiments was averaged and then the standard error calculated.

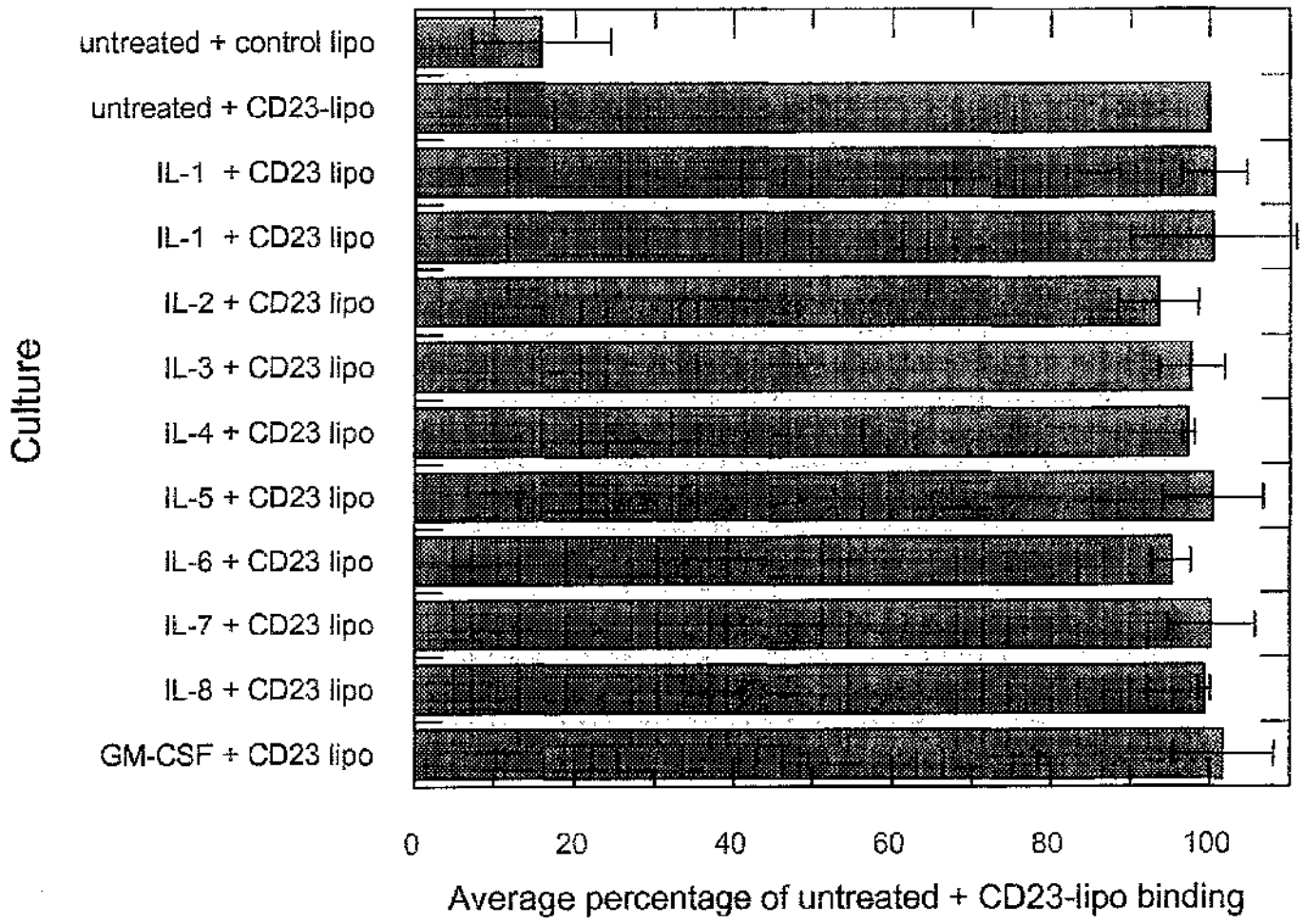
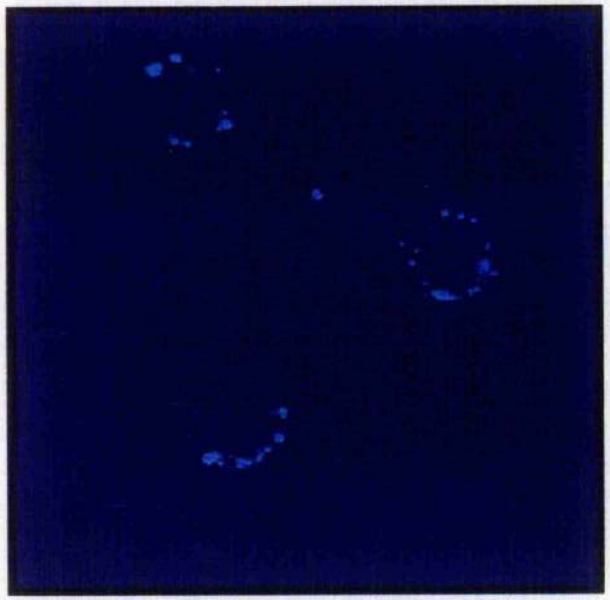
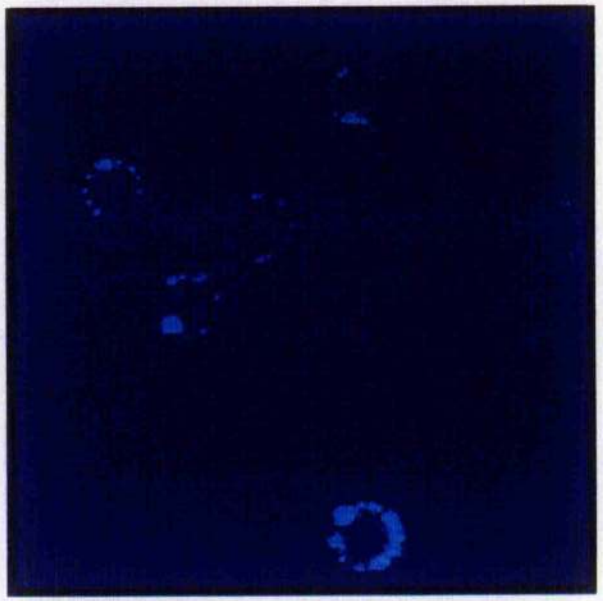

untreated + control lipo
untreated + CD23-lipo
IL-1 + CD23 lipo
IL-1 + CD23 lipo
IL-2 + CD23 lipo
IL-3 + CD23 lipo
IL-4 + CD23 lipo
IL-5 + CD23 lipo
IL-6 + CD23 lipo
IL-7 + CD23 lipo
IL-8 + CD23 lipo
GM-CSF + CD23 lipo
Culture
0
20
40
60
80
100
Average percentage of untreated + CD23-lipo binding

Fig.5.10 IL-4 increases the size of SMS-SB cells but does not increase the level of CD23-containing liposome binding.

SMS-SB cells were incubated in PFHMII in the presence or absence of 50U/ml IL-4 for 18 hrs. The cells were then washed and mixed with CD23-containing liposomes for 2 hours. After three washes with ice-cold liposome buffer, the cells were analysed by confocal microscopy. Binding of CD23-containing liposomes to IL-4-treated cells (left hand panel, IL-4), demonstrates the increased size of the cells compared to untreated SMS-SB cells (right hand panel, control). Both photomicrographs were taken at a magnification of 160X. The cells were also analysed by flow cytometry and the mean fluorescence observed were the same whether or not the cells had been treated with IL-4 (see figure 5.9). The experiment is representative of three independent repeats.



IL-4



Control

Fig.5.11 Specificity of CD23-containing liposome binding to SMS-SB and RPMI 8226 cell lines.

CD23-containing liposomes were pre-incubated for 1 hour with 40μg/ml of either normal rabbit IgG or Rb55 anti-CD23 polyclonal IgG fraction. Aliquots (10^5 cells) of SMS-SB and RPMI 8226 cells were incubated with the pre-treated liposomes for 2 hours before being washed three times with liposome buffer and analysed by flow cytometry. Panel A illustrates binding to SMS-SB cells; panel B illustrates binding to RPMI 8226 cells. In both panels:- the broken line represents the autofluorescence of the cell line; the dotted line represents binding of CD23-containing liposomes pre-incubated with normal rabbit IgG; the solid line represents binding of CD23-containing liposomes pre-incubated with Rb55 anti-CD23 IgG. The data is illustrated as described in the legend for figure 5.8., 'EPC' represents 'events per cell'. The experiment is representative of three independent repeats.

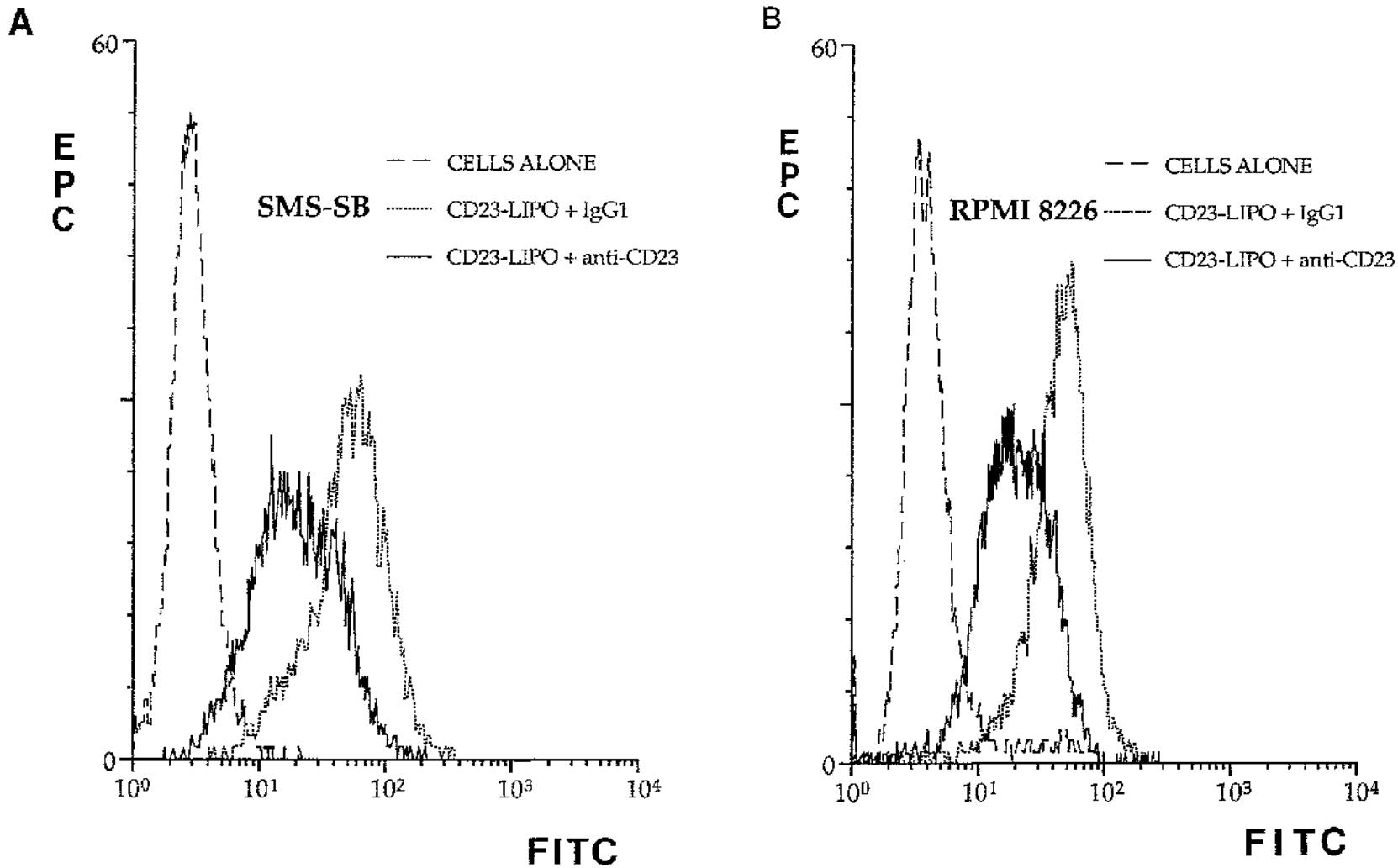
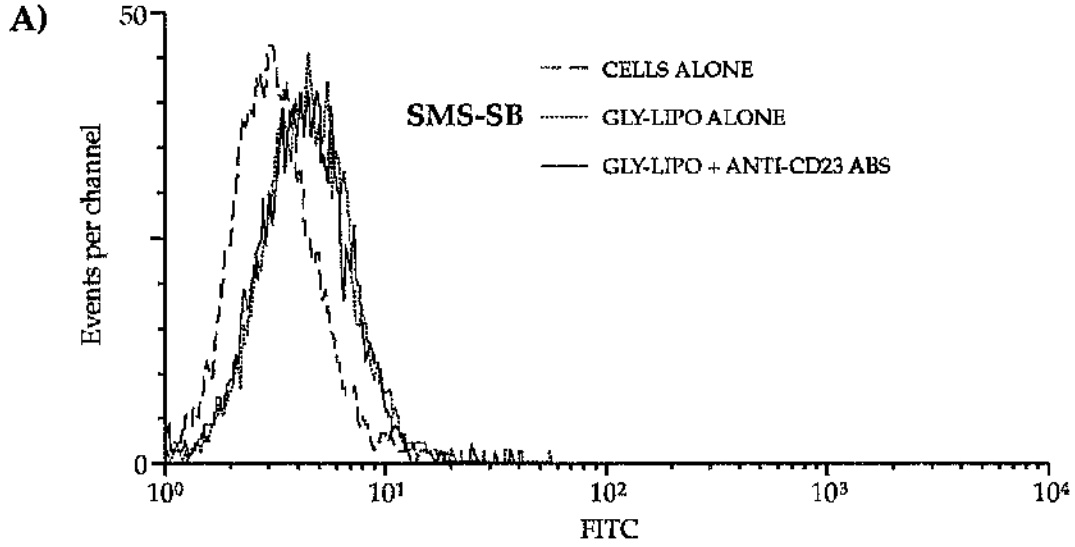
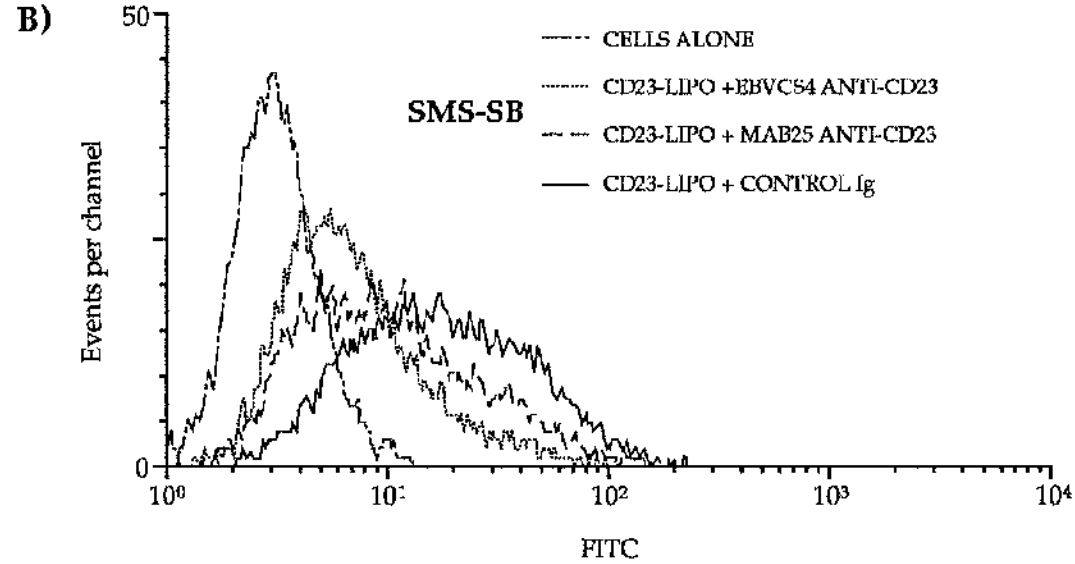

A
60
EPC
0
SMS-SB
CELLS ALONE
CD23-LIPO + IgG1
CD23-LIPO + anti-CD23
10^0
10^1
10^2
10^3
10^4
FITC
B
60
EPC
0
RPMI 8226
CELLS ALONE
CD23-LIPO + IgG1
CD23-LIPO + anti-CD23
10^0
10^1
10^2
10^3
10^4
FITC

Fig.5.12 Differential ability of monoclonal anti-CD23 antibodies to inhibit CD23-containing liposome binding to SMS-SB cells.

The experiment was performed as described in the legend of figure 5.11, but using only SMS-SB cells, and with addition of CD23- and glycophorin-A-containing liposomes which had been pre-incubated for 1 hour with 10μg/ml of EBVCS4 or Mab25 anti-CD23 MAbs, or control mouse IgG, or mouse IgM. Panel A represents the results obtained on addition of control, glycophorin-A-containing liposomes to SMS-SB cells (dotted line); the solid line represents control liposomes plus all control and anti-CD23 antibodies tested. The evenly broken line represents the autofluorescence of SMS-SB cells

Panel B represents SMS-SB cells incubated with CD23-containing liposomes pretreated with either:- EBVCS4 anti-CD23 (dotted line); Mab25 anti-CD23 (broken line); or control mouse IgG (solid line). The unevenly broken line represents the autofluorescence of SMS-SB cells. The experiment is representative of four independent repeats.


A)
50
0
Events per channel
SMS-SB
CELLS ALONE
GLY-LIPO ALONE
GLY-LIPO + ANTI-CD23 ABS
10^0
10^1
10^2
10^3
10^4
FITC


B)
50
0
Events per channel
SMS-SB
CELLS ALONE
CD23-LIPO +EBVCS4 ANTI-CD23
CD23-LIPO + MAB25 ANTI-CD23
CD23-LIPO + CONTROL Ig
10^0
10^2
10^3
10^4
FITC

binding to either cell type, as represented by the line for CD23-liposomes alone (figure 5.13). Binding of sCD23 to CD21 is thought to be a low affinity interaction (Aubry *et al.*, 1992) and this result confirms that sCD23 is unable to compete with the binding of the full length CD23 contained in the liposomes. However, the CD23 receptor present on SMS-SB cells appears to bind sCD23 with a higher affinity since sCD23 can partially inhibit the binding of full length CD23 to SMS-SB cells.

Pre-incubation of SMS-SB cells with a panel of monoclonal anti-CD21 MAbs, including BU-33, a strong inhibitor of CD23 binding to RPMI 8226 cells, does not inhibit binding of CD23-liposomes to SMS-SB cells; and no inhibition is seen on pre-incubation of SMS-SB cells with several anti-CD11a, -CD11b and -CD11c, or anti-CD18 antibodies (table 5.1). The data all support the hypothesis that SMS-SB cells do not bind CD23 via CD21, CD11b or CD11c, the currently characterised CD23 receptors.

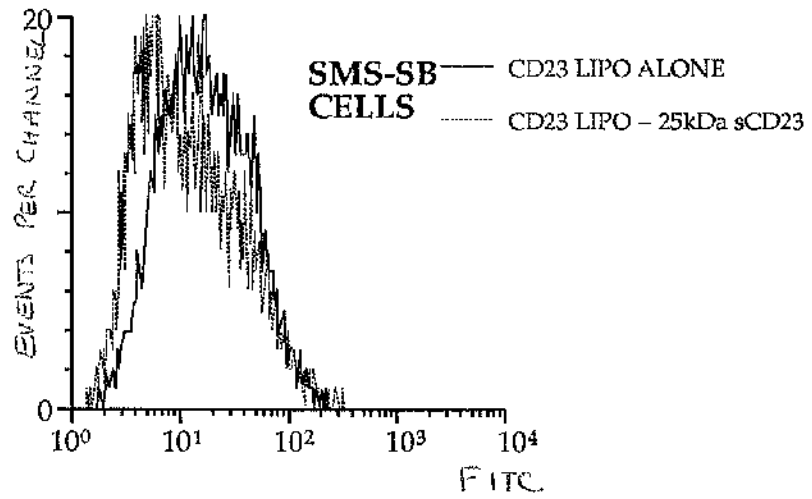
Table 5.1 shows the range of MAbs tested for their ability to bind to SMS-SB cells and the ability to inhibit CD23-liposome binding. The majority of the monoclonals were chosen due to the molecular weight of the CD molecule they were raised against; preliminary data from affinity-purification columns suggests that the novel receptor may have a molecular weight of approximately 85kDa (see below). CD5 is a candidate receptor because its known ligand, CD72, is a member of the same family as CD23 and the two molecules have a very similar structure (Gould *et al.*, 1991). However, none of the monoclonals tested, except anti-CD23 Mabs, are able to inhibit binding of CD23-containing liposomes to SMS-SB cells (table 5.1). Several of the MAbs against CD molecules expressed by SMS-SB cells (such as CD19, CD54, CD71 and CD105) gave ambiguous results, since addition of the antibodies increased the level of liposome binding. This effect was shown to be non-specific, since it also occurred with glycophorin-A-containing liposomes. Thus, several CD molecules cannot be excluded as candidate CD23 receptors, although no decrease in liposome binding was observed even on co-incubation with anti-CD23 MAbs, so it appears that they are not involved in CD23-binding to SMS-SB cells. Fresh liposomes will be necessary to examine any possible role of these molecules in CD23-binding by SMS-SB cells.

The liposome binding and inhibition studies are consistent with the hypothesis that SMS-SB cells possess a novel and, as yet, unidentified receptor for CD23.

Fig.5.13 Effect of 25kDa sCD23 on CD23-containing liposome binding.

In this experiment, aliquots (10^5 cells) of SMS-SB and RPMI 8226 cells were pre-treated with 10μg/ml of *E.coli*-produced 25kDa sCD23 for 30 minutes, on ice. 50μl of CD23-containing liposomes were then added and the cells incubated for 2 hours at 4°C before they were washed three times with liposome buffer and analysed by flow cytometry. Panel A illustrates CD23-liposome binding to SMS-SB cells untreated (solid line) or pre-treated with sCD23 (dotted line). A control of addition of 10μg/ml BSA was also performed; the results are also represented by the solid line. Panel B illustrates the results obtained with RPMI 8226 cells, represented as for panel A. The x-axis of each panel represents the FITC-fluorescence (due to liposome binding), on a logarithmic scale. The y-axis represents the number of events per channel, on a linear scale. The experiment is representative of three independent repeats.

A)


SMS-SB CELLS
CD23 LIPO ALONE
CD23 LIPO – 25kDa sCD23
EVENTS PER CHANNEL
20
0
10^0
10^1
10^2
10^3
10^4
FITC

B)

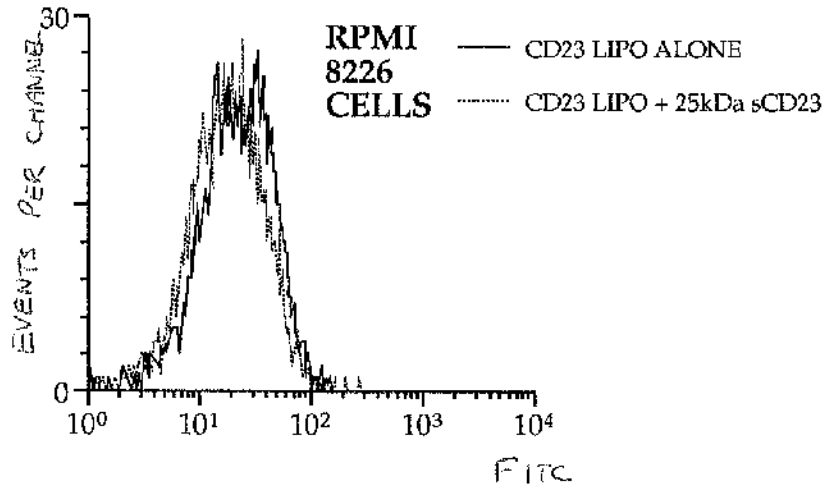

RPMI 8226 CELLS
CD23 LIPO ALONE
CD23 LIPO + 25kDa sCD23
EVENTS PER CHANNEL
30
0
10^0
10^1
10^2
10^3
10^4
FITC

Table 5.1 Antibody inhibition of CD23-containing liposome binding.

SMS-SB cells were harvested, washed and pre-incubated with 10 μg/ml of the antibodies listed in the table, for one hour at 4°C. The table shows whether the antigens were detectable by FITC-MAbstaining of SMS-SB cells (both FITC conjugated and unconjugated MAbs were used; a FITC-conjugated secondary MAb was used where the primary MAb was unconjugated).The cells were then stained with CD23-containing liposomes as described previously (Fig.5.8), and liposome binding analysed by flow-cytometry. Binding was scored in relation to cells incubated with CD23-liposomes alone. '-' represents no binding to SMS-SB cells, or no inhibition of liposome binding (maximal / normal CD23-liposome binding), depending on the column heading. '+' represents binding to SMS-SB cells or inhibition of CD23-liposome binding equivalent to the inhibition seen in figure 5.11. with Rb55 anti-CD23.

* the liposomes were pre-incubated with anti-CD23 antibody rather than SMS-SB cells; as described previously, EBVCS4, Mab25 and Rb55 all inhibited liposome binding, although to slightly different extents (see figures 5.11 and 5.12)

\# the anti-CD21 MAbs tested were: BU33 (The Binding Site), THB5 (GIMB), MCA644 (Serotec), CR2 (Becton Dickinson) and IOBIa (Immunotech).

nd this represents a situation where the results were not clearly defined due to non-specific enhancement of liposome binding.

Each individual experiment was repeated at least three times.

ANTIBODY ADDED	BINDING TO SMS-SB CELLS	INHIBITION OF CD23-LIPOSOME BINDING
control IgG/ IgM	-	-
MHC class II	+	nd
CD5	+	-
CD6	-	-
CD9	-	-
CD10	-	-
CD11a	-	-
CD11b	-	-
CD11c	-	-
CD16	-	-
CD18	-	-
CD19	+	-
CD20	-	-
CD21 #	+	-
CD22	+	-
CD23 *	-	++
CD25	-	-
CD32	-	-
CD37	-	-
CD38	-	-
CD40	-	-
CD43	+	nd
CD45	+	nd
CD49d	+	nd
CD54	+	nd
CD64	-	-
CD71	+	nd
CD72	+	nd
CD76	-	-
CD105	-	nd

5.2.3 *Characterisation of the CD23-novel receptor interaction*

CD23 may be acting as a C-type lectin in its interaction with the novel receptor, as has been observed for its interaction with CD21 and CD11b/CD11c. Figure 5.14, panelA, demonstrates that EDTA decreases CD23 binding to SMS-SB cells by chelation of Ca^{2+} in the liposome buffer. Panel B shows the same result for binding of CD23-liposomes to RPMI 8226 cells. Ca^{2+} is, apparently, essential for CD23 binding to SMS-SB cells. The involvement of sugars in the CD23 / SMS-SB cell interaction is not clear; technical difficulties have prevented a clear demonstration of whether fucose-1-phosphate or glucose-1-phosphate can inhibit liposome binding to either SMS-SB cells or to RPMI 8226 cells. Fucose-1-phosphate is known to inhibit CD23 / CD21 binding, as does tunicamycin treatment of RPMI 8226 cells. Tunicamycin treatment of monocytes also inhibits CD23 binding, suggesting that sugar structures are involved in the binding of CD23 to CD11b/CD11c; unfortunately, tunicamycin treatment of SMS-SB cells was not performed. However, it seems likely that a sugar structure will be involved since sCD23 is comprised almost exclusively of the lectin head.

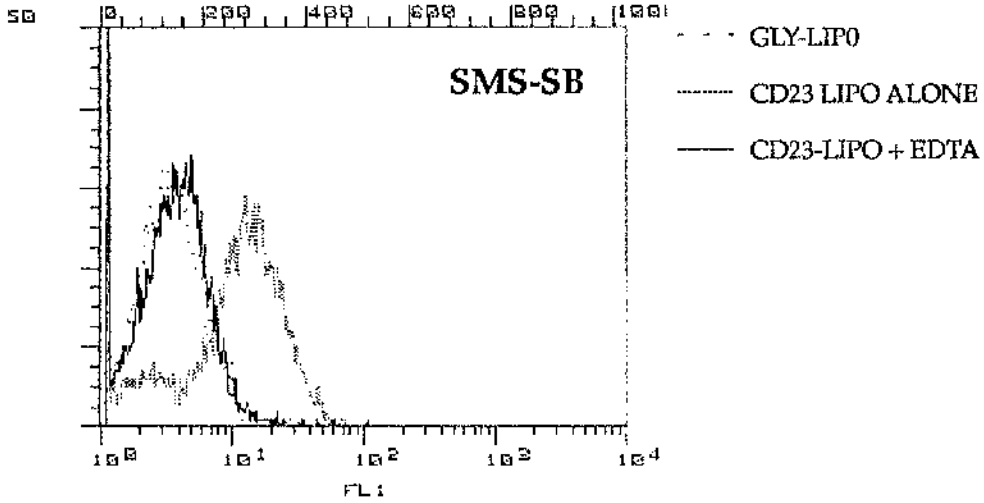
The involvement of sialic acid structures was investigated to examine whether CDw75 or CD76 are candidates for the novel receptor. These differentiation antigens have a molecular weight of approximately 85kDa, and CDw75 is expressed by germinal centre B cells (Bast *et al.*, 1992). Although neither antigen is reported to be expressed by pre-B cells, the antigens are carbohydrate-rich and neuraminidase-sensitive. SMS-SB cells were treated with bacterial neuraminidase for 1 hour at 37°C, before incubation with the liposomes (figure 5.15). Neuraminidase treatment of the cells does not inhibit CD23-liposome binding, demonstrating that sialic acid is not involved in the interaction between CD23 and SMS-SB cells. This is consistent with CD23 binding to CD11b and CD11c, where neuraminidase has no effect on liposome binding (Leconaet-Henchoz *et al.*, 1995).

At present, the binding characteristics of CD23 to the novel receptor are not fully characterised; numerous experiments require to be performed to understand the interaction. Tunicamycin treatment of the cells would be useful to identify whether sugars are involved in the binding, although tunicamycin can sometimes affect the intracellular transport or cell surface expression of ligands (Aubry *et al.*, 1992), so results may not be entirely conclusive. Involvement of N-linked oligosaccharides could be further investigated by treatment of the cells with plant alkaloids such as castanospermine (CSP) and/or swainsonine (SW). These compounds can be used to inhibit N-linked oligosaccharide processing (Hashim & Cushley, 1988) and would allow determination of the role of these sugars in binding of CD23 to the novel receptor. The role of O-linked sugars could also be investigated using the enzyme O-glycanase.

Fig.5.14 Effect of EDTA on CD23-liposome binding to SMS-SB and RPMI 8226 cells.

SMS-SB and RPMI 8226 cells were stained with CD23-containing or glycophorin-A-containing liposomes, as described in the legend of figure 5.8. In addition, an aliquot of each cell type was incubated with 5mM EDTA / PBS along with the CD23-containing liposomes. After 2 hours, the cells were washed three times and liposome binding analysed by flow cytometry. Panel A illustrates liposome binding to SMS-SB cells and panel B shows the results obtained with RPMI 8226 cells. The lines illustrate:- glycophorin-A-liposome binding (wide-spaced dotted line); CD23-liposome binding (dotted line); and the binding observed with CD23-liposomes plus EDTA (solid lines). The x-axis of each panel, FL1, represents the FITC-fluorescence (due to liposome binding), on a logarithmic scale. The y-axis represents the number of events per channel, on a linear scale. The experiment is representative of three independent repeats.

A)


SMS-SB
GLY-LIP0
CD23 LIPO ALONE
CD23-LIPO + EDTA
FL1

B)

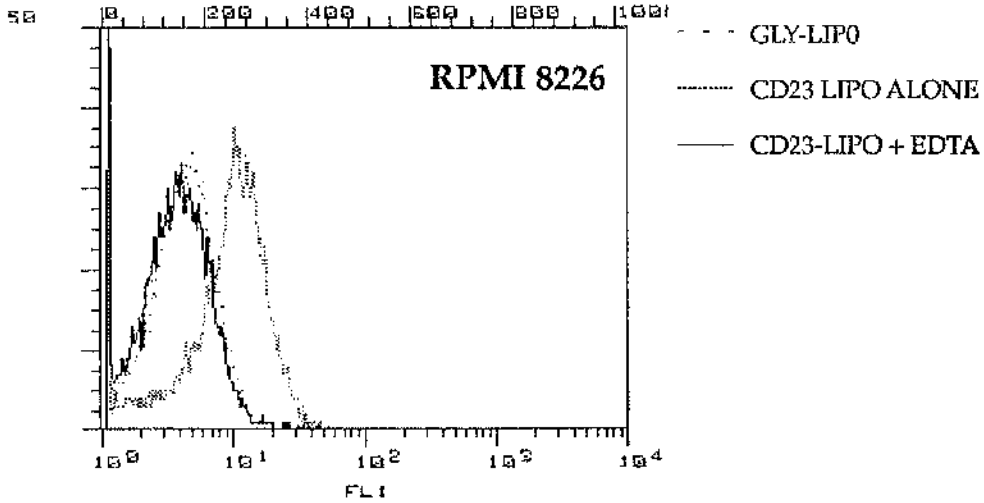
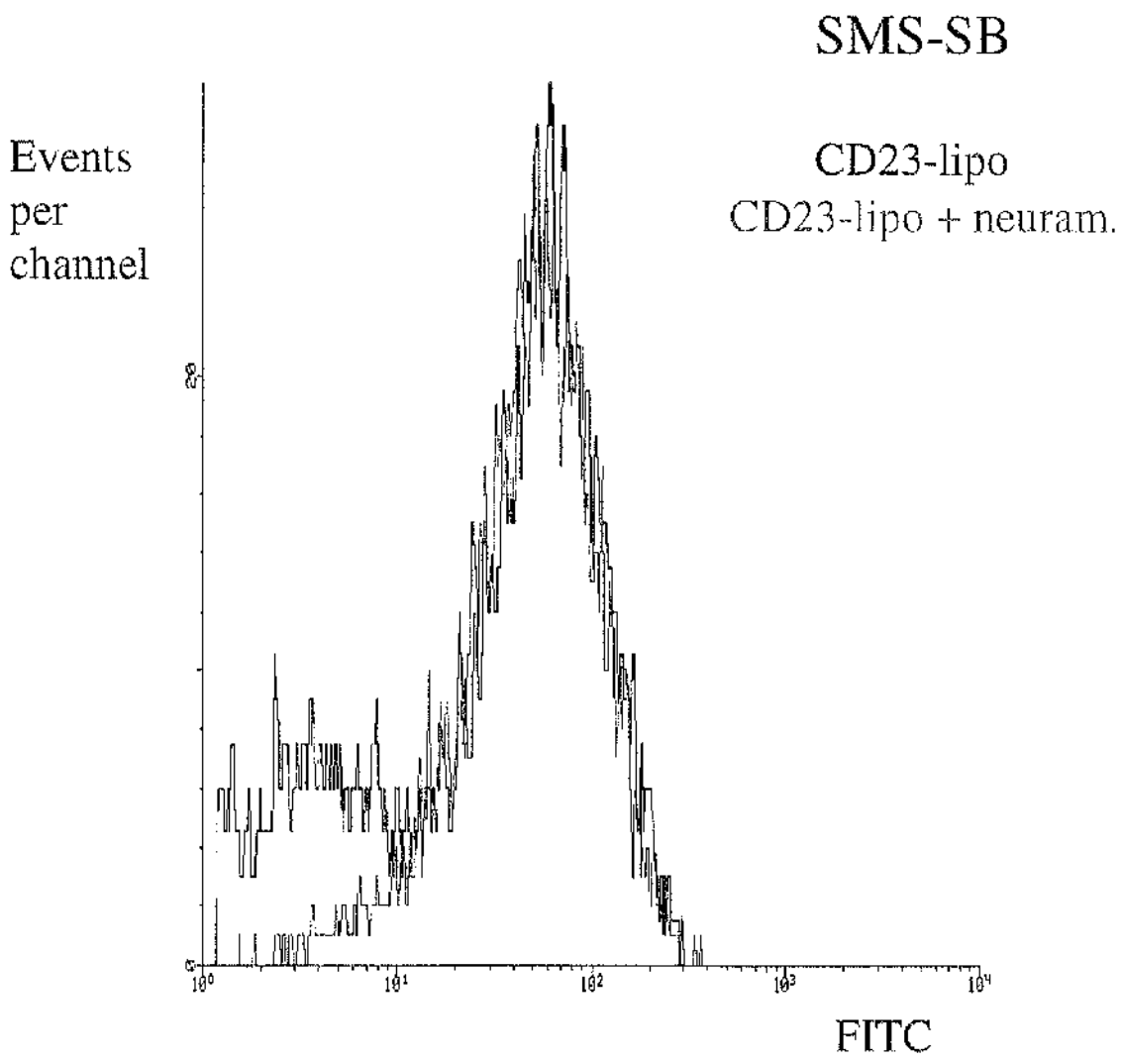

RPMI 8226
GLY-LIP0
CD23 LIPO ALONE
CD23-LIPO + EDTA
FL1

Fig.5.15. Neuraminidase treatment of SMS-SB cells does not affect binding of CD23-containing liposomes.

SMS-SB cells were treated with 50µl of 0.1 U/ml neuraminidase from *Arthrobacter ureafaciens, Clostridium perfringens* or *Vibrio cholerae*, for 1 hour at 37°C. 50µl of CD23-containing liposomes were then added and the cells incubated for 2 hours before being washed three times (with liposome buffer) and analysed by flow cytometry. On the FACS plot, the black line shows binding of CD23-containing liposomes and the red line shows binding of CD23-containing liposomes in the presence neuraminidase from *Arthrobacter ureafaciens* (this line also represents the results obtained with *Clostridium perfringens* and *Vibrio cholerae*). The experiment is representative of three independent repeats.


SMS-SB
Events per channel
CD23-lipo
CD23-lipo + neuram.
FITC

Identification of the epitopes recognised by anti-CD23 MAbs able to inhibit the interaction would give information about the domains of CD23 involved in binding, and once the novel receptor is isolated, monoclonal antibodies raised against it can be screened for the ability to inhibit liposome binding. After epitope mapping, this would provide information about regions on the novel receptor which are involved in binding of CD23.

5.2.4 *CD23- affinity purification of the novel receptor- preliminary results*

The method chosen to identify and isolate the novel CD23 receptor on SMS-SB cells was a CD23-affinity column. The sCD23 is very strongly bound to the *Affigel* column but, for the first few elutions of a new column, some 25kDa sCD23 elutes from the resin, raising the possibility that some of the bound receptor may also be lost; the resin becomes more efficient after a few uses.

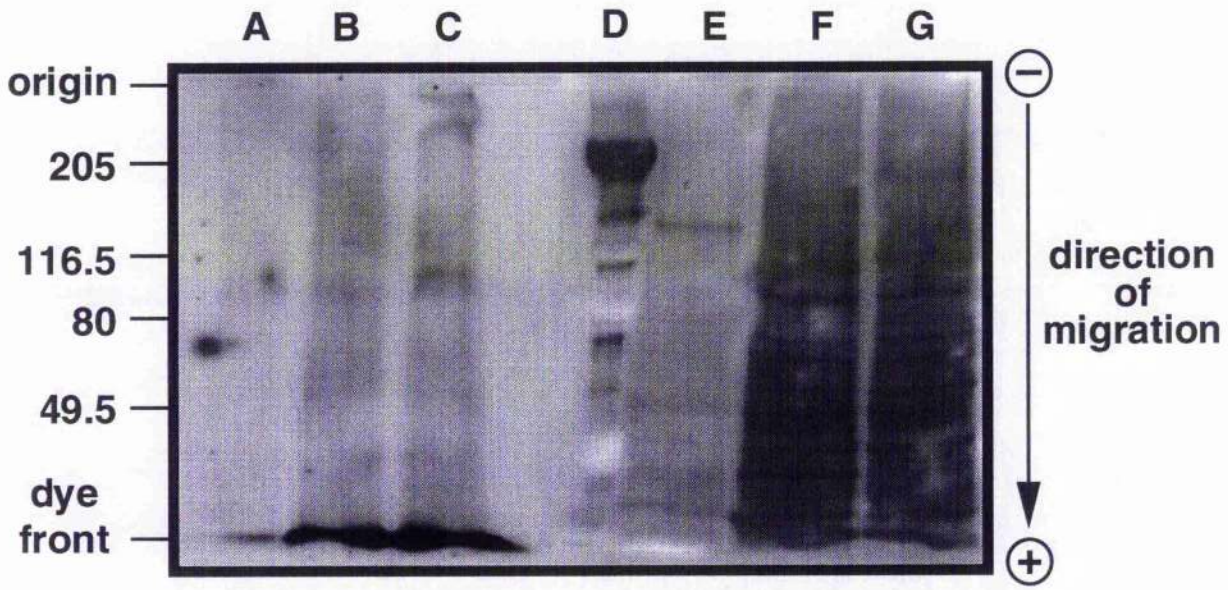
To examine the efficiency of the CD23 affinity column in the isolation of CD21, from RPMI 8226 cells, and to further demonstrate that CD21 is not involved in binding of CD23 to SMS-SB cells, cell extracts were made from SMS-SB cells, Raji cells (as a negative control) and RPMI 8226 cells. After passing all the extracts over a BSA pre-adsorption, and then a CD23-affinity column, the eluted material from the CD23-column, and the flow-through of each cell line was electrophoresed, Western blotted and then incubated with a cocktail of anti-CD21 MAbs. A peroxidase-labelled goat-anti-mouse antibody was used as a secondary antibody and detected by ECL (figure 5.16). As expected, there is a band visible for RPMI 8226 cells (lane C), although, compared to the molecular weight markers, it appears to be smaller than expected for CD21. However, comparison of this blot with those prepared using an anti-CD21 affinity column, shows that the band does represent CD21 (Dr. Pierre Graber, personal communication); no band is visible for SMS-SB cells (lane B). Raji cells were used as a negative control and, indeed, no bands are visible in the eluted material from the CD23-affinity column (lane A). However, the flow-through from the Raji column has a very strong band of approximately 116kDa, thought to represent Raji CD21 (lane D). It is known that only a subtype of CD21 molecules binds CD23 (Aubry *et al.*, 1992), and although Raji cells do express high levels of CD21 they do not bind CD23.

The results of the CD21 Western suggest that the binding of CD21 to sCD23 may be a low affinity interaction. The ECL reaction, used to detect anti-CD21 staining, had to be left for 20 minutes before the RPMI 8226 band (lane C) was visible. Lanes D, E & F represent the same blot but with the film exposed for only 1 second. Thus, either RPMI 8226 cells only express a tiny amount of the CD21 isoform or the efficiency of CD21 capture by sCD23 is very low. The latter explanation appears most likely since results using anti-CD21 affinity columns give very strong bands for RPMI 8226, suggesting that

Fig.5.16 CD23 affinity column - CD21 Western of eluates from SMS-SB, Raji and RPMI 8226 cells.

10^9 SMS-SB, Raji and RPMI 8226 cells, produced in large scale cultures, were washed and then 1/10th of the cells were biotinylated. Cell extracts were made from the cells (biotinylated and non-biotinylated cells mixed together) and run through an IgG/BSA pre-adsorption column and then the flow-through run through a CD23 affinity column (as described in materials and methods). After extensive washes, any material bound to the CD23 column was eluted using a NH_4SCN elution buffer, and the eluted material dialysed extensively. The eluted material and the flow-through samples were diluted 4 parts to one part with non-reducing SDS-PAGE sample buffer, boiled for 5 minutes, and 40µl were loaded per well of a 7.5% acrylamide gel. After the gel was run, the protein was Western blotted onto nitrocellulose and the blot incubated with 10ml of a cocktail of anti-CD21 MAbs (as detailed in the legend of table 5.1) at 10.8mg/ml in PBS/0.05% Tween. Antibody binding was detected with 4mg/ml goat anti-mouse-peroxidase in PBS/0.05% Tween and an ECL detection system with Fuji RX film.

Lanes A, B and C contained the material eluted from the CD23 affinity column for Raji, SMS-SB and RPMI 8226 cells respectively. High molecular weight standard markers were run in lane D and lanes E, F, and G were the flow-through from the CD23 affinity columns of Raji, SMS-SB and RPMI 8226 cells respectively. The band at approximately 120kDa (lane C) is RPMI 8226 cell CD21, and the band seen in the Raji flow-through (lane E), although a different molecular weight, also represents CD21, as judged by comparison with Western blots previously performed using anti-CD21 affinity columns (courtesy of Dr. Pierre Graber, GIMB). The experiment is representative of three independent repeats.


A
B
C
D
E
F
G
origin
205
116.5
80
49.5
dye
front
direction
of
migration

CD21 is expressed at quite a high level by RPMI 8226 cells (Dr. Pierre Graber, personal communication).

The data in figure 5.17 show a strepdavidin-peroxidase, ECL Western blot of biotinylated SMS-SB cell proteins. The surface of the cells was biotinylated and then a cell extract prepared using the detergent octyl-β-D-glucopyranoside (OGP), which solubilises membrane-bound proteins in their native state. After complete homogenisation, the cell extract was run through a BSA, pre-adsorption column to remove proteins which bind non-specifically. The flow-through of the BSA, pre-adsorption column was then passed over the CD23-affinity column and the flow-through of this was also collected. Due to the small volume of these preliminary affinity columns, it was possible to elute the bound material by boiling the resin with non-reducing SDS-sample buffer; thus the material could be loaded directly onto an SDS-gel, electrophoresed and then a Western blot performed with streptavidin-peroxidase to detect the biotinylated protein. Figure 5.17 shows the biotinylated proteins present: in the total cell extract (lane A); eluted from the BSA column (lane B); eluted from the CD23-affinity column (lane C); in the flow-through of the CD23 affinity column (lane D). A sample of biotinylated sCD23 was run on the gel as a positive control for the biotinylation reaction and strepdavidin-peroxidase binding (lane E). The CD23-affinity column reduces the number of bands seen on the gel but there are still numerous bands present. Two particularly strong bands are visible, one of 65-70kDa and the other of approximately 85kDa molecular weight. These bands are present in the material which has bound to the CD23-affinity column but, unlike most of the other bands, are not present in the flow-through, suggesting that they have bound specifically to the CD23 column. To examine if other, non-biotinylated, proteins also bound to the affinity column, a silver stain was performed using the *Pharmacia, PhastGel* system. Several other protein bands are visible by silver stain (data not shown) so care must be taken if attempts are made to extract bands from the gel for sequencing. It may be necessary to perform 2-D gel elecrophoresis to check for contaminating proteins.

In an attempt to reduce the number of proteins bound non-specifically to the column, the volume of resin used was increased to 5ml and and sodium thiocyanate was used to elute the bound material, as opposed to boiling the resin. To extract sufficient material from the blot to attempt sequencing, it was decided to use $2x10^9$ cells instead of the 10^8 cells used previously. After large scale culture of the cells, only a proportion (1/10th) of the cells were biotinylated since preliminary blots had shown that this was sufficient for detection of biotinylated proteins, and the labelling procedure would be very costly for such a large number of cells. The results of figure 5.18 show a strepdavidin-peroxidase ECL Western of biotinylated material eluted from the large scale IgG/BSA pre-adsorption column (after the flow-through had been collected to be passed over the CD23 column), and CD23 affinity column; the flow-through of the CD23-column was collected before the material

Fig.5.17 CD23 affinity column - attempt to identify the novel CD23 receptor on SMS-SB cells.

A cell extract was prepared from 10^8, surface-biotinylated SMS-SB cells, run through an IgG/ BSA pre-adsorption column and then the flow-through from this column was run-through a CD23 affinity column. After extensive washes, both the BSA and the CD23 affinity resin were boiled in non-reducing SDS-PAGE sample buffer to elute any bound material. Samples of the initial cell extract and the flow-through from both columns were prepared as described in the legend of figure 5.16. The samples were loaded on a 7.5% acrylamide gel:- lane A = initial cell extract; lane B = material bound to the BSA column; lane C = material eluted from the CD23 affinity column; lane D = the flow-through from the CD23 affinity column; lane E = a sample of biotinylated 25kDa CD23. High and low molecular weight standard markers were also run, as demonstrated on the figure. After electrophoresis and Western blotting, the blot was incubated with streptavidin-peroxidase and then an ECL detection system was used to visualise any biotinylated proteins. The experiment is representative of two independent repeats.

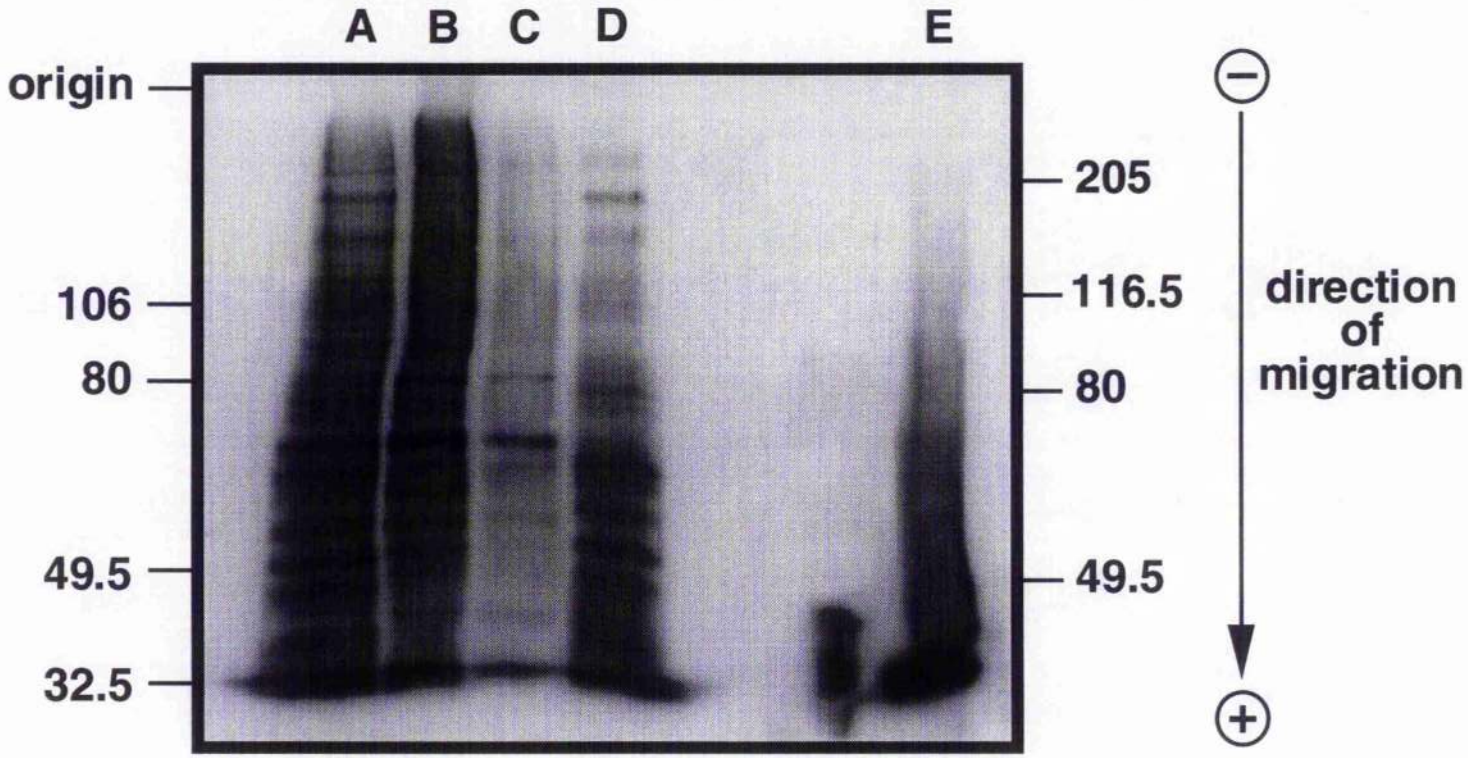
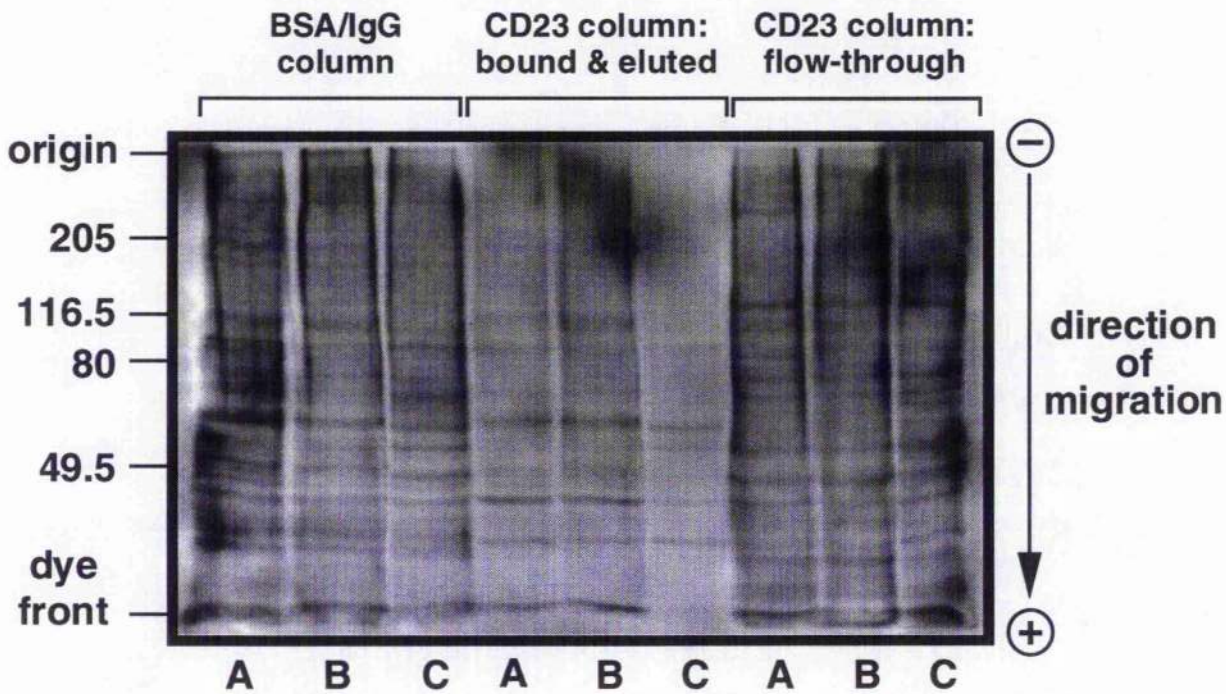

A
B
C
D
E
origin
106
80
49.5
32.5
205
116.5
80
49.5
direction
of
migration

Fig.5.18 CD23 affinity column - attempt to isolate the novel CD23 receptor.

Aliquots of 10^8 SMS-SB (cultured in both PFHMII and 10% FCS/RPMI 1640) and Raji cells were biotinylated, mixed with $1.4x10^9$ unbiotinylated SMS-SB cell, before cell-extracts prepared, and run through an IgG/BSA pre-adsorption column. The flow-through of this was run through a CD23 affinity column (as described in materials and methods). After extensive washes, any material bound to the IgG/BSA or CD23 columns was eluted using a NH_4SCN elution buffer, and the eluted material dialysed extensively. The eluted material, and the material which passed through the CD23 column, were mixed 4 parts to 1 part with non-reducing SDS-PAGE sample buffer, boiled for 5 minutes, and 40µl were loaded per well of a 7.5% acrylamide gel. After electrophoresis and Western blotting, the blot was incubated with streptavidin-peroxidase and an ECL detection system was used to detect the biotinylated proteins.

Lanes 1, 2 and 3 contained the material eluted from the IgG/BSA column:- A, represents SMS-SB cells cultured in PFHMII; B, represents SMS-SB cells cultured in 10% FCS/RPMI 1640; C, represents Raji cells. Lanes 4, 5 and 6 contained the material eluted from the CD23-affinity column, and lanes 7, 8 and 9 contained the material which passed through the CD23 affinity column; with A, B and C, as before. High molecular weight standard markers were also run, as represented on the figure. The experiment is representative of two independent repeats.



A) SMS-SB; protein free
B) SMS-SB; FCS
C) Raji

bound to the CD23-column was eluted. This procedure was performed for SMS-SB cells grown in protein-free medium (A), SMS-SB cells grown in serum-containing medium (B) and Raji cells (C). Several bands are visible in the material eluted from the SMS-SB cell CD23-affinity columns, and although weaker, most of the bands are also visible in the material eluted from the Raji cell CD23-affinity column. The results suggest that the 65-70kDa band visible in figure 5.17 is also present in the Raji cell extract, and therefore, is unlikely to represent the novel CD23 receptor - although it may represent part of a receptor complex, the other components of which are missing from Raji cells. One band which does appear to be specific to SMS-SB cells is approximately 85kDa, which corresponds with the initial result (figure 5.17). There is no difference in the banding patterns seen with SMS-SB cells cultured in serum-containing or protein-free medium, removing the concern that some serum proteins may be biotinylated and detected by this method.

Several further attempts were made to isolate the SMS-SB cell receptor by comparing the CD23-affinity bound material from large numbers ($2x10^9$ cells) of SMS-SB (test), Raji (negative control) and RPMI 8226 (positive control) cells, to allow a complete comparison of cell extracts. Unfortunately, the results obtained were disappointing; either the gels were overloaded or no bands were visible at all.

However the CD23-affinity-column method does indicate that an 85kDa protein represents a candidate for the novel CD23 receptor and, with minor adjustments, this large scale procedure should allow enough protein to be isolated for the band to be removed and, hopefully, sequenced.

5.3 *Discussion*

The main findings presented in this chapter are that SMS-SB cells do not express any of the known CD23-receptors, namely CD21, CD11b-CD18 or CD11c-CD18, but bind CD23-containing liposomes specifically, demonstrating the existence of a novel CD23-receptor on SMS-SB cells. Preliminary results from CD23-affinity columns suggest that the novel receptor may have a molecular weight of approximately 85kDa, although attempts to inhibit CD23-containing liposome binding - using MAbs raised against known CD antigens of approximately 85kDa - have, so far, failed to identify the novel receptor.

CD21 is not expressed by SMS-SB cells, as shown by several techniques including the extremely sensitive technique of RT-PCR, and also by the fact that none of a cocktail of anti-CD21 antibodies are able to inhibit the binding of CD23-containing liposomes to SMS-SB cells. That SMS-SB cells do not express CD21 helps to characterise them as pre-B cells since CD21 expression usually occurs at the late-pre-B / immature B cell stage of development. The expression of CD11b-CD18 and CD11c-CD18 was only examined

by flow cytometry, which does not exclude the possibility of very low level expression at the cell surface. However, none of a panel of anti-CD11b, anti-CD11c or anti-CD18 MAbs are able to inhibit-liposome binding, demonstrating that these molecules are not involved in binding of CD23 to SMS-SB cells. These β2-integrin molecules are normally expressed by cells of the myeloid lineage (Lecoanet-Henchoz *et al.*, 1995) and not by pre-B cells, so this result was as expected.

The only available antibodies which are able to inhibit the binding of CD23-liposomes to SMS-SB cells are Rb55, a polyclonal anti-CD23, and the MAbs, EBVCS4 and MAb25. These monoclonal antibodies are also able to prevent binding of CD23-liposomes to RPMI 8226 cells, suggesting that binding of CD23 to the novel receptor involves at least two epitopes which are involved in binding of CD23 to CD21. Although the results with the monoclonal antibodies suggest that the interaction of CD23 with CD21 and with the novel receptor involve some common epitopes, binding does appear to differ, at least with respect to soluble CD23. The binding of full length CD23 to SMS-SB cells is partially inhibited by pre-incubation of the cells with sCD23, however, very little inhibition of CD23-liposome binding is observed for RPMI 8226 cells treated in the same way. These results demonstrate that the two receptors bind in a similar but substantially different fashion, and the novel receptor on SMS-SB cells may bind soluble CD23 with higher affinity than does CD21.

None of a range of cytokines tested are able to influence the binding of CD23-liposomes to SMS-SB cells which suggests that the numbers and / or affinity of the novel receptor are not affected. This is of particular interest after the report demonstrating that IL-1α increases the number of IL-2, IL-3 and GM-CSF receptors present on cells (Mossalayi *et al.*, 1991). IL-1α also appears to affect either the number or affinity of CD11b / CD11c molecules present on monocytes, since it increases the level of CD23-liposome binding to the cells (Lecoanet-Henchoz *et al.*, 1995). However, IL-1α does not affect the binding of CD23-liposomes to SMS-SB cells, consistent with the apoptosis studies which also failed to demonstrate a role for IL-1α.

CD23-binding to SMS-SB cells is Ca^{2+}-dependent and not sensitive to neuraminidase treatment, so sialic acid residues are not involved in binding. These results are consistent with the interactions involved in binding of CD23 to CD21 and CD11b/CD11c (Aubry *et al.*, 1992; Lecoanet-Henchoz *et al.*, 1995), although unlike these interactions, inhibition of CD23-binding to SMS-SB cells by fucose-1-phosphate has not been clearly demonstrated. If fucose-1-phosphate is shown to inhibit the SMS-SB / CD23 interaction then the lectin-activity of CD23 is involved in binding; this appears likely since sCD23 consists almost entirely of the lectin domain (Delepesse *et al.*, 1991).

The results of the CD23-affinity columns support the suggestion that the sCD23/CD21 interaction is of low affinity, since very little CD21 is captured by the 25kDa sCD23 column. It is also possible that RPMI 8226 cells have only low level expression of CD21 or that the specific isoform of CD21 which binds CD23 is not continually expressed. However, from the results of anti-CD21 affinity columns it appears that RPMI 8226 cells do express high levels of CD21, therefore it may be reasonable to suggest that CD21 does not bind to sCD23 with very high affinity (Dr. Pierre Graber, personal communication).

If the SMS-SB cell-specific 85kDa band eluted from the CD23-affinity column does represent the novel CD23 receptor, the results suggest that its interaction with CD23 is of a higher affinity than that between CD23 and CD21 since much more of the 85 kDa protein is captured by the CD23 column, compared with CD21. The main concerns with this method of receptor capture and identification are that the biotinylation technique is biased towards detection of proteins which are lysine-rich and so some proteins may not be labelled and others will be very strongly labelled, giving the impression that they are expressed at high levels. There will also be problems in isolation if the receptor consists of more than one component.

However, preliminary results suggest that there is a protein of approximately 85kDa which can be detected in material eluted from a CD23-affinity column of SMS-SB cell extract but not Raji cell extract, a cell line which does not bind CD23. Secondly, this protein appears to be captured specifically by the CD23 column because no 85kDa band is visible in the flow-through of the column, unlike the majority of protein bands seen.

Numerous MAbs specific for CD molecules of between 65 and 100kDa molecular weight, have been tested for the ability to inhibit CD23-liposome binding to SMS-SB cells. Several of these antibodies, known to bind to SMS-SB cells, require to be re-tested due to non-specific enhancement of liposome binding, although they do not appear to have any ability to inhibit liposome binding. At present, the novel CD23 receptor present on SMS-SB cells remains to be fully characterised but its function, in prevention of pre-B cell apoptosis, makes it an extremely interesting molecule for future investigation.

Chapter 6

Discussion

Chapter 6

General Discussion

6.1 *Main conclusions*

The main findings of this thesis are that the pre-B cell line, SMS-SB, is density-dependent for growth, possibly due to the production of an autocrine growth factor. Attempts to identify the SB-AF have excluded the interleukins normally associated with normal pre-B cell growth and differentiation, namely, IL-3, IL-4, IL-5 and IL-7, since these cytokines are not produced by the cells. One candidate component of the SB-AF activity is PDGF-$\alpha\alpha$; SMS-SB cells express both PDGF receptors, secrete PDGF-α chains, and recombinant PDGF-$\alpha\alpha$ promotes the growth of the cells (Tsai *et al.*, 1994). The presence of autocrine PDGF activity in conditioned medium from SMS-SB cells could not be proven using anti-PDGF neutralising MAbs, leading to the hypothesis that PDGF might be acting as an internal autocrine factor. The ability of conditioned medium to promote the growth of SMS-SB cells cultured at low cell density suggests the existence of another component of the SB-AF activity, but at present this factor has not been identified.

During experiments to identify the SB-AF, it was noted that sCD23 can promote the growth of SMS-SB cells cultured at low cell density. It has been demonstrated that SMS-SB cells do not express CD23 and, therefore, it does not represent the SB-AF. Subsequent studies have shown that, when cultured at low cell density, SMS-SB cells undergo apoptosis; sCD23 can prevent apoptosis of the cells. This effect of sCD23 is independent of IL-1α, the cytokine which normally synergises with sCD23 during its cytokine activities. The mechanism of action of sCD23 appears to be via induction, or maintenance, of *bcl-2* expression. No role for *c-myc* expression has, so far, been demonstrated in SMS-SB cell apoptosis.

SMS-SB cell do not express any of the known receptors for CD23, namely CD21, CD11b-CD18 or CD11c-CD18, but the cells do bind CD23-containing liposomes in a specific manner and the binding is inhibited by a polyclonal anti-CD23 antibody and at least two anti-CD23 MAbs. Thus, SMS-SB cells express a novel receptor for CD23 receptor. Unlike RPMI 8226 cells, which bind CD23-liposomes via CD21 (Aubry *et al.*, 1992), the binding of CD23-liposomes to SMS-SB cells is partially inhibited by 25kDa sCD23, suggesting that the novel receptor has a higher affinity for sCD23 than does CD21. CD23-affinity columns were used to identify the novel receptor, and preliminary results suggest that the novel receptor has a molecular weight of 85kDa. Of several MAbs tested (which have specificity for CD molecules with molecular weights of approximately 85kDa, and/or expressed by B cells) none have the ability to inhibit binding of CD23-

liposomes to SMS-SB cells. Therefore, at present, the novel CD23-receptor present on SMS-SB cells has not been fully characterised.

There is very little information available about autocrine factor production during early B cell development and so the discovery of the cell line, SMS-SB, a pre-B cell ALL, was of great interest. The leukaemia from which the cell line was derived had the unusual characteristic that the leukaemic cells were actively dividing in the blood. Most leukaemias originate from a proliferating progenitor cell; the progeny may differentiate somewhat but they do not continue to divide once they are removed from their source (Salem *et al.*, 1989; Lowenberg & Touw, 1993). Usually, a differentiation block is involved in leukaemia formation which prevents the cell from continued development, leading to an accumulation of cells which 'spill' into the blood as undifferentiated B cells. However, in the case of SMS-SB cells, the progeny of the stem cell have retained the capacity to divide, due to the production of an autocrine growth factor. Consequently, it was a very acute and aggressive leukaemia and the patient had a very high white blood cell count.

Normal, mature B cells, if appropriately activated, produce autocrine growth factors. There are also several reports which demonstrate production of negative autocrine growth factors; thus, a normal B cell could independently regulate its growth (Gordon & Cairns, 1991). Autocrine growth stimulation is a potentially dangerous situation, since if a cell can continually promote its own growth, and the control mechanisms become deregulated, there is a risk that a tumour may occur. It is known that production of an autocrine growth factor is not usually sufficient for transformation of a cell; multiple mutations appear to be required (Young *et al.*, 1991). However, autocrine factor production can cause a more aggressive tumour, and is thought to be a contributing factor in tumour progression (Overell *et al.*, 1991). Some investigators believe that autocrine factor production may act as a tumour progression factor by increasing the size of the pool of potential tumour cells, where another genetic perturbation is required for full transformation (Sporn & Todaro, 1985). Thus, autocrine factor production could increase the chance of a tumour occurring.

There are several mechanisms with the potential to cause constitutive autocrine activity and uncontrolled growth of SMS-SB cells. Normal pre-B cells may express receptors for SB-AF but the factor is usually produced by the stromal cell population, to control pre-B cell growth. In this case, aberrant expression of the gene for the autocrine factor would leave SMS-SB cells independent of stromal cell regulation, leading to development of the lymphoma/leukaemia.

However, SB-AF may well represent a normal pre-B cell autocrine factor, the effects of which, in the case of SMS-SB cells, could have become deregulated by:

1) increased production of the SB-AF
2) enhanced cellular responsiveness due to an aberrant increase in receptor expression
3) enhanced affinity of the receptor, possibly due to amplification of intracellular signalling pathways (in the case of SMS-SB cells, the over-expression of *c-fos* may be implicated in this role)
4) loss of action of a negative autocrine growth factor and/or loss of ability to respond to a negative factor produced by stromal cells.

6.2 *SMS-SB cell autocrine activity*

With respect to SMS-SB cells, the only SB-AF candidate so far identified is PDGF-$\alpha\alpha$. However, it does not appear that PDGF-$\alpha\alpha$ can account for all of the SB-AF activity, consistent with previous accounts of B cell autocrine factors where several cytokines are known to synergise to produce the autocrine growth factor activity (Abken *et al.*, 1992). With respect to PDGF, other investigators have demonstrated that, for example, transformation of non-neoplastic rat fibroblasts by growth factors, requires the concerted action of TGF-α, TGF-β and PDGF (Assoian *et al.*, 1984). PDGF has been described as a progression factor (Heldin & Westermark, 1990) and studies using the *v-sis* product of Simian sarcoma virus (SSV), the viral homologue of the PDGF-β chain, have demonstrated that cells are transformed but not immortalised by the gene product (Johnsson et al., 1986). Other factors or genetic alterations appear to be necessary for the fully malignant phenotype.

It is known that PDGF signalling is important for commitment to DNA synthesis, probably via tyrosine phosphorylation of intracellular proteins (Ek & Heldin, 1984), and it appears to induce specific genes, including *c-myc* (Kelly *et al.*, 1983) and *c-fos* (Greenberg & Ziff, 1984). This is of particular interest in the case of SMS-SB cells since they are known to over-express *c-fos;* this may be due to constitutive activation of PDGF receptors via an autocrine loop. It would be interesting, during investigations into the possible internal autocrine role of PDGF, by suramin treatment or anti-sense to PDGF, to examine the effect that inhibition of PDGF function has on *c-fos* expression by the cells.

No investigations appear to have been undertaken to examine expression of PDGF by normal B cells. At present, the only documented role for PDGF, with respect to B cells, is the expression of the α-chain by SMS-SB cells and another pre-B ALL cell line Nalm-6 (Tsai *et al.*, 1994). Although Nalm-6 does not actually secrete the PDGF α-chain, it does possess the PDGF-α receptor; SMS-SB cells express the PDGF-β receptor. Since PDGF is a fibroblast mitogen, it is possible that pre-B cells could also use PDGF to stimulate

stromal cells to produce other B cell growth or differentiation factors. Thus, the role of PDGF in normal B cell development must be investigated more thoroughly since it seems intuitively unlikely that two pre-B cell lines would express both PDGF and PDGF receptors after random genetic mutations.

If SB-AF has a role in normal B cell development, then it must have become deregulated for the leukaemic SMS-SB cells to continue cycling in the blood; autocrine mechanisms are normally tightly regulated to avoid such a devastating scenario. Of the mechanisms previously mentioned, there is circumstantial evidence to support several explanations.

The alteration in *c-abl* expression by SMS-SB cells may have contributed to development of malignancy. The cells contain two extra *abl*-related transcripts, and since *abl* encodes a tyrosine kinase activity, it is possible that signals transduced via the autocrine factor receptors are amplified by this constitutive activity. The expression of *c-abl* has also been associated with a block in light chain rearrangement, proposed as a reason that mice infected with Abelson virus develop pre-B cell leukaemias (reviewed by Rosenberg, 1994). This event appears to act as a differentiation block, with the cells continuing to proliferate instead of proceeding along the differentiation pathway. SMS-SB cells are known to have non-productively rearranged κ and λ light chain genes, which may act as a differentiation block, allowing deregulated expression of the autocrine growth factor to lead to tumour formation. It seems unlikely that the *abl*-product would have caused this differentiation block, since the effect usually prevents any light chain rearrangement from occurring, although SMS-SB cells are still CD43-positive (a pre-B cell marker commonly found on *v-abl* transformed cells) and several groups have shown low levels of light chain rearrangement in Abelson-transformed murine B cell lines (Muller & Reth, 1988).

A recent paper demonstrates that, after growth factor withdrawal, *v-abl* protein tyrosine kinase activity prevents apoptosis in a murine mast cell line (Evan *et al.*, 1993). SMS-SB cells are known to undergo apoptosis when cultured at low cell density suggesting that *c-abl* cannot 'save' the cells; however, it is possible that during the early stages of tumour development, the aberrant *abl* expression gave the cells a selective growth advantage over normal pre-B cells.

It is possible that light chain gene rearrangement occurred at a later stage during development of the tumour and the proposed differentiation block was due to some other alteration. Due to their continued proliferation, and movement away from bone marrow stromal cells, SMS-SB cells may have been deprived of the normal differentiation signals, and this, with the concomitant growth promoting signals from the SB-AF, may have prevented any further differentiation. This model could be tested by co-culture of early

SMS-SB cells in contact with various stromal cell lines, to examine whether differentiation could occur.

6.3 *Hypothetical model for SMS-SB leukaemogenesis and general lymphopoiesis*

SMS-SB cells are known to produce another fibroblast mitogen - TGF-LD- which apparently has no autocrine growth-promoting effects. In long-term bone marrow cultures, TGF-LD inhibits lymphopoiesis, although this effect appears to occur via macrophage-like cells since when these are removed, lymphopoiesis is stimulated (Dr. B. Ozanne, personal communication). A hypothetical model for the role of TGF-LD, SB-AF and sCD23 in lymphopoiesis and leukaemogenesis is demonstrated in figure 6.1. The model suggests that in normal bone marrow, TGF-LD is produced by pre-B cells and activates stromal fibroblasts to produce pre-B cell growth factors. As the cells divide, they are pushed away from the fibroblast-containing niche in the bone marrow to interact with surrounding macrophage-like cells; the TGF-LD acts to induce production of pre-B cell growth inhibitors by these cells and so allows differentiation to occur.

In the case of leukaemia, tumour cell growth would be enhanced by TGF-LD production if the leukaemic cells were somehow resistant to the inhibitory factors; the cells would receive the growth promoting signals derived from the fibroblasts but not the subsequent growth inhibitory signals from the macrophage-like cells. SB-AF may have been a progression factor for the leukaemic pre-B cells if it allowed them to continue dividing even after they were removed from the stromal cells, and hence their supply of pre-B cell growth factors. It is reasonable to suggest that, at this stage in development, normal pre-B cells also produce autocrine factors to allow their continued growth as they differentiate; however, they are probably also under the control of inhibitory factors which regulate the growth. For SMS-SB transformation, it seems likely that a differentiation block must also have occurred, to prevent further development of the cells and promote their continued proliferation. Some differentiation of SMS-SB cells did occur, as evidenced by the light chain gene rearrangements but for some reason the cells were not eliminated when the rearrangement was non-functional and the cells failed to express surface IgM. As mentioned previously, *v-abl* has been implicated in preventing apoptosis of mast cell precursors, so it is possible that some alteration in *c-abl* transcription helped early SMS-SB progenitor cells to survive. Eventually, the continued proliferation of resistant leukaemic SMS-SB cells, with concomitant inhibition of normal pre-B cell development due to production of TGF-LD, would lead to exclusion of normal B cells from the bone marrow, and to the release of proliferating leukaemic cells into the blood.

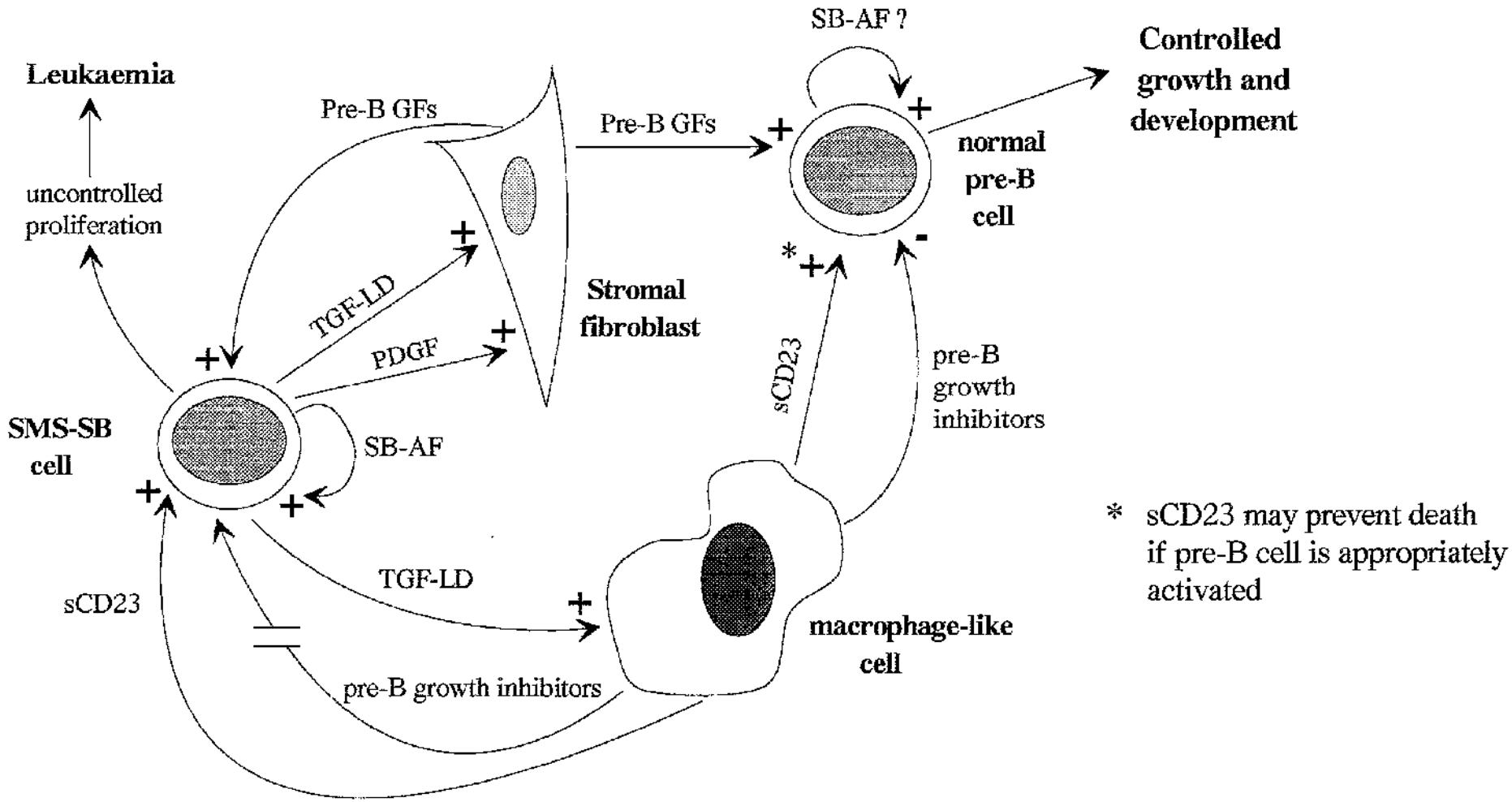


Fig 6.1 Model for SMS-SB leukaemogenesis - a role for sCD23? The abbreviation used in the diagram are as described in the text. 'GF' represents 'growth factor'; the '+' and '-' symbols refer to growth-promoting / survival signals, and growth-inhibitory signals, respectively. The // symbol refers to a break in the signal pathway.

Once the SB-AF activity has been fully characterised, it will be important to explore its role in normal B cell development, and to examine whether normal pre-B cells produce the autocrine growth factor or usually respond to SB-AF in an paracrine fashion. Hopefully, SMS-SB cells will provide a useful model to examine control of the early stages of B cell development in the bone marrow, which is, at present, poorly understood. SB-AF may be able to sustain the growth of immature B cells in long-term bone marrow cultures. If other normal or leukaemic cells do possess the capacity to respond to SB-AF, it will be interesting to examine the potential negative regulatory cytokines which normally control cell growth; these may offer scope for therapeutic intervention in some leukaemias.

6.5 *A role for sCD23 in leukaemogenesis ?*

There is another explanation for the continued survival of SMS-SB cells after non-functional rearrangement of the light chain genes. It is now known that sCD23 prevents apoptosis of SMS-SB cells and it is possible that the expression of the novel CD23 receptor allowed survival of the cells even after a differentiation block. If normal pre-B cells express the novel CD23 receptor, sCD23 may have a role in normal B cell development to keep pre-B cells alive as they differentiate.

CD23 expression by bone marrow stromal cells has already been documented (Fourcade *et al.*, 1992), although it occurs at a very low level and is thought to occur mainly on activated macrophage-like cells. It is conceivable that TGF-LD produced by SMS-SB cells (or another bone marrow-derived cytokine) can induce CD23 expression in addition to the production of growth inhibitory factors, by macrophage-like cells (see figure 6.1); however, this has not yet been examined. Assuming that normal pre-B cells express the novel CD23 receptor, this would allow tight regulation of pre-B cell development, preventing further proliferation of the cells but ensuring their survival as they proceed along the differentiation pathway.

If SMS-SB cell growth was not prevented by the inhibitory factors, the cells would continue to divide, possibly with some differentiation. However, due to expression of the novel CD23 receptor, after the non-functional rearrangement of the light chain genes, when normal pre-B cells would undergo apoptosis, SMS-SB cells would survive and continue to divide. In this respect, the involvement of sCD23 in maintenance or promotion of *bcl-2* expression by SMS-SB cells is of great interest. Normal pre-B cells express only low levels of *bcl-2*, leaving them prone to apoptosis as a protection mechanism against cells of abnormal phenotype entering the periphery (Merino *et al.*, 1994). It will be important to investigate the expression of other members of the *bcl-2* family, such as *bcl-x* and *bax*, since they may regulate *bcl-2* function. It is possible that negative-regulatory transcription factors, possibly members of the Ets family, which are normally functional in

pre-B but not mature B cells, act to down regulate *bcl-2* expression in pre-B cells by binding to π1 sites in the 5' negative regulatory region of the *bcl-2* gene (Chen & Boxer, 1995). sCD23 may act to induce transcription of a protein which can inhibit binding of the Ets-like protein to the π1 site, or to induce a protein which will bind to enhancer sites in the *bcl-2* promoter and over-ride any negative regulatory signals.

A comparison of SMS-SB cells with the mature B cell lines Raji and RPMI 8226 showed approximately equal levels of *bcl-2* expression in all three cell lines. This suggests that SMS-SB cells express abnormally high levels of *bcl-2*; possibly expression of the negative regulatory factors has been lost in SMS-SB cells, or some positive transcription signal, induced, for example, by the autocrine factor, over-rides the negative signals, leading to high levels of *bcl-2* expression. Thus, abnormal regulation of *bcl-2* expression may have contributed to the ability of SMS-SB cells to survive after a differentiation block, for example, non-functional light chain gene rearrangement.

If this hypothesis is correct, the continued action of the autocrine growth factor would prevent SMS-SB cells from dying; *in vitro*, removal of this factor by culture at low cell density causes apoptosis of the cells, possibly due to down regulation of *bcl-2*. Soluble CD23 can maintain the *bcl-2* levels in SMS-SB cells under these conditions. The *in vivo* significance of the sCD23 action is unclear but it may have aided survival of the cells as the tumour developed in the bone marrow. It would have been very interesting to measure the levels of sCD23 in the blood of the patient, since it seems likely that after escaping into the blood, the cells would have been exposed to much lower concentrations of SB-AF than were present in the tumour mass. In several leukamias, e.g. chronic lymphocytic leukaemia, the patients have high levels of sCD23 in their blood (Sarfati, 1993). Since SMS-SB cells do not express CD23, it seems unlikely that high levels of sCD23 would have been present; however, the production of the various stromal cell mitogens may have lead to sCD23 production by bone marrow cells. Equally plausible is that the role of CD23 was entirely localised to prevention of apoptosis of cells in the tumour mass; the levels of SB-AF in the blood may have been sufficiently high for the continued proliferation of the leukaemic cells, especially since other plasma proteins would have been present.

6.5 *The novel CD23 receptor*

The discovery of a novel CD23-receptor on SMS-SB cells is extremely exciting because it suggests that CD23, whether in the membrane bound or soluble form, has a role in early B cell development. It is likely that the novel receptor binds CD23 by mechanisms very similar to those involved in the interaction of CD23 with CD21, CD11b and CD11c. Since fucose-1-phosphate can inhibit CD23 binding to CD21, CD11b and CD11c, the lectin domain of CD23 must interact with carbohydrate motifs on these molecules. However,

certain anti-CD23 MAbs, known to have specificity for protein epitopes, can also inhibit the binding demonstrating that protein-protein interactions are also involved. Several interactions are likely to occur which all help to stabilise the binding. In this respect, the ability of CD23 to form oligomers has profound implications for its ability to bind to receptors since with CD21, CD11b and CD11c, the low affinity binding appears to be enhanced by oligomerisation (Aubry *et al.*, 1992; Beavil *et al.*, 1995). Pochon and colleagues (1992) failed to demonstrate any binding of natural or recombinant soluble CD23 to CD21-positive B cells, suggesting that the cells require the multivalency created by surface expression of the full-length molecule for CD23 binding. This explanation is somewhat complicated by data which shows that 25kDa form of sCD23 (which is composed almost entirely of the lectin domain) does form oligomers, even though it lacks the α-helical coiled stalk region; trimers of 37kDa sCD23 and hexamers of the 25kDa form were found after chemical cross-linking (Beavil *et al.*, 1995). The hexameric form of 25kDa sCD23 is most likely composed of two trimers, possibly due to end-on interactions of the lectin heads, a situation which could not occur on the cell surface. The low level of binding of sCD23 to CD21 may be due to steric hindrance of the necessary binding sites. Several effects of sCD23 are thought to occur via CD21, for example, prevention of germinal centre B cell apoptosis (Liu *et al.*, 1991). Also, sCD23 specifically increases IL-4-induced IgE production by blood mononuclear cells, the suggestion being that the interaction occurs via CD21, since it can be mimicked by anti-CD21 antibodies (Aubry *et al.*, 1992). Thus, it has been assumed that the roles attributed to the soluble form of CD23 occur via CD21 and CD11b, CD11c. Although no formal demonstration has been made of binding of sCD23 to these molecules it does appear likely that a low affinity interaction can occur. A molecule with six sites for contact would overcome an inherent low affinity interaction of any one site by the high avidity of multiple sites.

During isolation of the novel CD23 receptor on SMS-SB cells, a 25kDa sCD23 affinity column was used. This method is not very successful for isolation of CD21, as shown by the very low levels of protein isolated. The CD11b and CD11c molecules were, however, isolated by this method, suggesting that the binding of sCD23 to these molecules is a higher affinity interaction, than binding to CD21 (Lecoanet-Henchoz *et al.*, 1995). Indeed, recombinant soluble CD23 induces pro-inflammatory cytokine production by activated monocytes. It is interesting to note the existence of an 80kDa band in the material eluted from the monocyte CD23-affinity column (Lecoanet-Henchoz *et al.*, 1995). CD11b and CD11c are 170 and 150kDa, respectively, and the other component of the receptor, CD18, is 95kDa. The nature of this protein was not discussed by the authors but it appears to be of a similar size to the protein isolated in preliminary work with SMS-SB cells.

One hypothesis is that the novel CD23 receptor represents the true soluble CD23 receptor, responsible for the cytokine effects attributed to sCD23 such as survival of germinal centre B cells, and thymocyte and myeloid precursor differentiation (Liu *et al.*, 1991; Mossalayi *et al.*, 1990 & 1991). Once characterised, it will be interesting to examine the expression of the novel receptor on these cell types. It is possible that CD21, CD11b and CD11c bind CD23 due to the carbohydrate-lectin interaction but that the signals are actually transduced via the novel receptor, which associates with these molecules on the cell surface; such a situation occurs with receptors for both basic fibroblast growth factor and IL-6 (Taga *et al.*, 1986). The existence of the 80kDa band eluted from the CD23-affinity column of the monocyte cell extract would support such a theory, if the approximately 85kDa band isolated from SMS-SB cells turns out to be the novel CD23 receptor.

6.6 *A soluble CD23 receptor?*

The receptor present on SMS-SB cells is known to bind full length CD23 and the three soluble forms - 37, 29 and 25kDa sCD23. There is no real difference in effect of the different sized sCD23 fragments in proliferation assays. Unfortunately, problems were encountered during comparison of the ability of the sCD23 fragments to prevent apoptosis of SMS-SB cells, due to the inherent instability of sCD23; conclusive results were only obtained for the 25kDa form. If the 25kDa form does exist as a hexamer then the *effective* concentration of sCD23 used in the assays would have been much lower than that of the other sCD23 fragments yet it gave a similar response. The novel receptor appears to have an increased affinity for soluble, as opposed to full length CD23, when compared with CD21. Evidence which supports this hypothesis is that 25kDa sCD23 partially inhibits binding of CD23-containing liposomes to SMS-SB cells, but not to RPMI 8226 cells. It will be interesting to examine whether sCD23 could inhibit CD23-liposome binding to monocytes.

It is likely that the main role of CD21, and possibly CD11b & CD11c, are as adhesion molecules binding to membrane-bound CD23, for example, on follicular dendritic cells in germinal centres (Liu *et al.*, 1991), and in B/T cell interactions, to regulate IgE production, since CD23 and CD21 are expressed by both T and B cells (Flores-Romo *et al.*, 1993). If CD21 and CD11b/CD11c are really receptors for the full-length, stable trimer form of CD23 (binding may be enhanced by a high concentration of these trimers on the cell surface), this may explain why the responder cells require pre-activation before the effects of soluble CD23 are seen in these systems. Usually, sCD23 functions are only seen after pre-treatment of the responder cells with IL-1α (Mossalayi *et al.*, 1990; Liu *et al.*, 1991) or, in the case of monocytes, after activation of the cells (Lecoanet-Henchoz *et al.*, 1995). Several explanations may be advanced for this. Firstly, it is possible that cells such as centrocytes require two distinct signals, from IL-1α and sCD23 before survival

and differentiation can occur; i.e. the signal via CD21 is not sufficient to prevent apoptosis. Although anti-CD21 antibodies can 'save' germinal centre cells from apoptosis, this affect may have been enhanced by cross-linking of CD21 molecules by the antibodies. It appears that binding of soluble CD23 to CD21 is not of sufficient affinity to transduce the anti-apoptotic signal.

The second hypothesis is supported by reports that IL-1α can increase the number and /or affinity of cytokine receptors on cells (Mossalayi *et al.*, 1991); it is possible that IL-1α acts to pre-activate the cells, ready for the signal from sCD23. Monocytes must be pre-activated before an effect of sCD23 is seen in the absence of IL-1α (Lecoanet-Henchoz *et al.*, 1995) and treatment of resting monocytes with IL-1α increases the binding of CD23-containing liposomes to the cells, suggesting that IL-1α may increase the number and /or affinity of cytokine receptors on cells. Therefore, for the low affinity interaction of CD21 and CD11b/CD11c with soluble CD23 to exert its effect, the cells probably require pre activation. The hypothesis is that, *in vivo*, these molecules act as receptors for full length, membrane-bound CD23, which can either bind with sufficient affinity for signalling to occur, or the cells will be activated by co-stimulation from other receptor/ligand interactions which occur on the surface of the cells.

With respect to SMS-SB cells, sCD23 alone is sufficient for the biological effects to be seen; however, as a leukaemic cell line, SMS-SB may already be in an activated state and possibly constitutively expresses the novel CD23 receptor. A good working hypothesis is that the novel receptor is the *soluble* CD23 receptor and interaction of the novel receptor with sCD23 is of high enough affinity to negate the requirement for IL-1α; until the novel receptor has been identified and its function on normal pre-B cells investigated, this remains to be tested.

6.7 *Identity of the novel receptor?*

Attempts to identify the novel CD23 receptor by antibody inhibition of CD23-liposome binding were not successful; however, several candidate molecules exist. It is striking that the three known CD23 receptors, CD21, CD11b and CD11c are all receptors for complement proteins. SMS-SB cells should be examined for expression of the fourth complement receptor, CD35, and if it is present, the ability of anti-CD35 to inhibit liposome binding must be tested. However, CD35, or complement receptor 1, although found on most haematopoietic cells, is a large protein of 190-280kDa (depending on the isoform); no band of this size was visible on the Western blots from the affinity columns. Complement is known to be essential for germinal centre formation and is involved in activation of monocytes in sites of inflammation. Thus, a low affinity interaction of complement receptors with the 'sticky' sCD23 molecule may just mimic the normal cellular responses to complement. Bonnefoy and colleagues (1993) showed that anti-CD21

antibodies can prevent apoptosis of centrocytes, and suggest that this mimics the *in vivo* effects of CD23 in germinal centres. Unfortunately, the fact that germinal centres are full of complement proteins and that IFN-α, another ligand for CD21, is also essential for germinal centre formation, mean that the role of CD23/CD21 interaction, at least in germinal centres, is far from clear.

The novel CD23 receptor could also represent another IFN-α receptor, since CD21 binds IFN-α. Although IFN-α has no apparent role in B cell development, it must remain a candidate for the novel CD23 receptor.

The final candidate to be discussed is CD5 because it is the ligand for CD72, a member of the C-type lectin family which has significant homology with the structure of CD23. Antibodies against CD5 fail to inhibit CD23-liposome binding to SMS-SB cells, suggesting that CD5 is not the novel receptor for CD23, but it is possible that the epitopes blocked by the MAbs are not critical for CD23 binding or that, as with CD21, only a specific glycosylated form of the molecule actually binds CD23. It is of interest that CD23-liposomes do not bind to Raji cells even though these cells are strongly CD21 positive. Raji cells are also strongly positive for CD5 expression, suggesting that it is not involved in the interaction with CD23, but Raji cells may lack a specific enzyme required for the appropriate glycosylation of these molecules.

It seems likely that, due to the similarities in CD23 and CD72 structure, that the novel receptor, if not CD5, may be a CD5-like molecule. It would be useful to design oligonucleotides against CD5 homology domains to see whether SMS-SB cells express any related molecules. Western blots, using anti-CD5 antibodies, of material eluted from the CD23-affinity columns would also be interesting since CD5 is a 67kDa protein - approximately the same size as the lower band detected in the SMS-SB cell extract.

It might also be useful, for future experiments to re-design the CD23-affinity column. The use of chemically-cross-linked sCD23 hexamers, bound to an affinity column, may allow higher affinity binding of the novel receptor and, thus, aid isolation and identification of the protein.

6.8 *Role of the novel CD23 receptor in haematopoiesis*

The results demonstrate that sCD23 prevents apoptosis of a pre-B cell line; in parallel to studies where a combination of IL-1α and sCD23 promote proliferation and maturation of both thymocytes and myeloid precursors (Mossalayi *et al.*, 1990a & 1990b). This suggests that CD23, whether in the membrane-bound or soluble form, has a role in haematopoiesis; supported by the demonstration of CD23 expression by stromal cells in the bone marrow (Fourcade *et al.*, 1992), and by human thymic epithelium in the outer cortex (Mossalayi *et*

al., 1991). Once the novel receptor has been fully characterised, it will be interesting to examine its expression on other cell lines and primary cells from all stages of B cell, and other haematopoietic lineage, development. At present, all the demonstrated *in vitro* effects attributed to sCD23 are assumed to occur through CD21 or CD11b/CD11c. However, pro-thymocytes do not express CD21 or CD11b/CD11c, suggesting that the novel receptor may also be involved in early T cell development.

As mentioned previously, IL-1α is necessary for these sCD23 effects to occur, suggesting that cells require to be pre-activated. It was suggested earlier that the novel receptor on SMS-SB cells may bind sCD23 with sufficient affinity to negate the requirement for IL-1α. However, in light of the requirement for IL-1α by pro-thymocytes, and assuming that the novel CD23-receptor is actually present on pro-thymocytes, it appears likely that, *in vivo,* an element of pre-activation is required for the effects of sCD23 to be seen. As a leukaemic cell line, SMS-SB cells exist in a constitutively activated state by virtue of SB-AF, which could explain their ability to respond to sCD23 alone. The requirement that cells are pre-activated would protect against CD23 having non-specific effects, especially in areas such as the bone marrow, thymus and germinal centres where massive cell proliferation and negative selection occur.

The role of CD23 during haematopoiesis was recently disputed after the development of a CD23 knockout mouse which has apparently normal haematopoietic development; the only phenotype noted is a severe impairment of antigen-specific IgE-enhancement of the antibody response - a process entirely attributable to membrane-bound CD23 (Fujiwara *et al.*, 1994). It is possible that the apparently normal phenotype occurred due to redundancy in *in vivo* immunoregulatory systems. However, murine and human CD23 differ in several ways, including the apparent lack of the CD23b isoform in mice, and this could also explain the differences. It can be hypothesised that, since the CD23a isoform is only expressed by B cells, human bone marrow and thymic stromal cells will only express the CD23b isoform. The soluble forms of both isoforms are identical, due to the inverted membrane orientation, but early B cells do not express CD23 and so the only source of CD23 in the bone marrow is stromal cells; thus, the CD23b isoform is the molecule to which the roles in haematopoiesis are attributed. As such, it is no surprise that CD23 has no role in murine haematopoiesis, since they lack this isoform.

Another factor which may help to explain the contradictory results is the state of oligomerisation of murine soluble CD23 - 'A molecule in search of a function' according to Bartlett and Conrad, 1992. Murine sCD23 can only interact with IgE in a univalent manner, suggesting that it exists as a monomer (Dierks *et al.*, 1993). A recent paper supports this idea with studies where recombinant 28kDa CD23 (equivalent to the human 25kDa form) interacts with IgE in a monomeric fashion but a soluble form of full-length

CD23 has increased capacity for IgE binding, suggesting a multivalent interaction (Bartlett *et al.*, 1995). These data contrast with the discovery of hexameric sCD23 in the human (Beavil *et al.*, 1995); if murine sCD23 is unable to form oligomers then the absence of biological activity may be explained by lack of avidity required for binding to receptors.

6.9 *Coda*

The SMS-SB cell line provides a very useful model for the study of several aspects of early B cell development. The identity of the pre-B cell autocrine growth factor, SB-AF, and its role in normal and leukaemic B cells should be investigated further; the role of negative regulatory cytokines should also examined. This may shed some light onto how pre-B cell growth is regulated, and the processes involved in early B cell leukaemogenesis - possibly leading to targets for therapeutic intervention.

The novel CD23 receptor present on the pre-B cell line, and the ability of sCD23 to prevent apoptosis of these cells, suggests a role for soluble and/or full-length CD23 in haematopoiesis; roles in T cell and myeloid development have already been demonstrated. Examination of expression of the novel receptor on cells from all stages of haematopoietic development will help to clarify:- the role of CD23; the CD23-receptors which are involved at different developmental stages; and whether the novel CD23-receptor is, indeed, a functional receptor for *soluble* CD23.

SMS-SB cells will be extremely useful in these investigations because they do not express any of the known CD23 receptors and so the mechanisms of CD23 action can be examined in the absence of interference from other receptor types. The role of CD23 in regulation of *bcl-2* expression should also be examined - aided by fact that SMS-SB cells have no autocrine production of CD23, but possibly hindered if the cells aberrantly express *bcl-2* as a consequence of their leukaemic phenotype.

Ultimately, the precise expression and importance of SB-AF and the novel CD23 receptor in B cell development, will have to be examined in long term bone marrow cultures with 'normal' pre-B cells. However, SMS-SB cells will hopefully provide answers to several of the intriguing questions which have been raised.

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