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B LYMPHOPOIESIS AND B CELL SELECTION BY APOPTOSIS IN BONE MARROW OF NORMAL AND GENE-MODIFIED MICE

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ABSTRACT

B lymphopoiesis in mouse bone marrow (BM) represents a delicate balance between B cell production and loss. The present study has aimed to examine the differentiation and selection of B lineage cells during their development in BM. A comparison has been made between two major B cell differentiation models, one based on BM cell fractions sorted by immunofluorescence labeling and flow cytometry of cell surface markers and the other using fluorescence microscopy of the B lineage differentiation markers, TdT and μ heavy chains of IgM. The results have revealed a heterogeneity of B cell differentiation stages within each sorted BM fraction, contributing toward a unified model of B cell development. To examine the nature of selection during B cell development, a variety of criteria have been used to demonstrate that B cell loss in BM takes the form of apoptotic cell death. The apoptotic incidences of B cells at various developmental stages *ex vivo* and the rates of entry into apoptosis analyzed by flow cytometry, have demonstrated that B cells are particularly prone to apoptosis at two differentiation stages, known to follow Ig V_H gene rearrangement and antigen receptor expression, respectively. It has been revealed that intracellular levels of Bcl-2 and Bax proteins are developmentally regulated in precursor B cells and are involved in the control of B cell death in both normal and RAG-2 gene-deficient mouse BM. The use of IL-7 transgenic mice, IL-7 gene-deficient mice and stromal cell cocultures demonstrates that the stromal cell-derived cytokine, IL-7, exerts an anti-apoptotic effect, associated with changes in Bax/Bcl-2 ratio. Accelerated apoptosis in op/op mutant mice suggests an augmented B cell loss in the absence of CSF-1. The results demonstrate that the loss of B lineage cells in mouse BM involves programmed cell death at critical quality control checkpoints in B cell development, influenced by both intrinsic cellular mechanisms and microenvironmental factors.

RÉSUMÉ

La génération de lymphocytes B dans la moelle osseuse de la souris repose sur une balance délicate entre la production de lymphocytes B et leur perte. Cette étude a eu pour objectif d'examiner la différentiation et la sélection de cellules de la lignée des lymphocytes B durant leur développement dans la moelle osseuse. Deux principaux modèles de différenciation de lymphocytes B furent comparés; L'un est basé sur la caractérisation des populations de cellules de la moelle osseuse à l'aide de marqueurs de surface en utilisant des techniques de marquage par immunofluorescence et de cytometrie de flux. Le second modèle repose sur l'analyse des marqueurs cytoplasmiques de différenciation propres aux lymphocytes B, il s'agit de TdT et de Mu, la chaîne lourde de l'immunoglobuline IgM. Les résultats ont démontré une hétérogénéité d'étapes de différenciation des lymphocytes B dans les deux approches, contribuant ainsi vers un modèle unifié de lymphocytopoïèse des cellules B. Afin d'examiner la nature de la sélection durant le développement des lymphocytes B, une variété de critères furent établis démontrant ainsi que la perte de lymphocytes B dans la moelle osseuse survient par mort par apoptose. L'incidence de cellules B en état d'apoptose à des stages variés de développement *ex vivo* et la fréquence d'entrée en apoptose analysées par cytometrie de flux ont démontré que les lymphocytes B sont particulièrement prône à l'apoptose à deux stages de différenciation, l'un suivant le réarrangement du gène IgVH et l'autre suivant l'expression du récepteur d'antigène respectivement. Il fût révélé que les niveaux intracellulaires des protéines Bcl-2 et Bax sont régulés durant le développement des précurseurs de lymphocytes B et sont impliqués dans le contrôle de la mort de ces cellules B à la fois dans la moelle osseuse de la souris normale et RAG-2 déficiente. L'usage de souris transgéniques pour IL-7, de souris déficientes pour IL-7 et les cocultures de cellules du stroma démontrent qu'IL-7, la cytokine dérivée des cellules du stroma, exerce un effet anti-apoptique associé à un changement du ratio Bax/Bcl-2. L'accélération de l'apoptose chez la souris mutante op/op suggère une augmentation de la perte de

lymphocyte B dans l'absence de CSF-1. Ces résultats démontrent que la perte de cellules de la lignée des lymphocytes B dans la moelle osseuse de la souris implique la mort par apoptose. Cette selection est influencée à la fois par des mécanismes intrinsèques à la cellule et à des facteurs environmentaux et survient à des stages critiques, contrôlant ainsi la qualité de la génération des lymphocytes B.

To my parents and my wife, Sharon

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ABBREVIATIONS

Ab	Antibody
Ag	Antigen
BCR	B cell receptor
BSA	Bovine serum albumin
CAM	Cell adhesion molecule
CED	Cell death gene
CSF-1	Colony stimulating factor
c μ	Cytoplasmic μ chains
Dex	Dexamethasone
FACS	Fluorescence activated cell sorter
FADD	Fas-associating protein with death domain
FasL	Fas ligand
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
FLICE	FADD-like ICE
Fr.	Fraction
gld	Generalized lymphoproliferative disease
HSA	Heat stable antigen
HSC	Hemopoietic stem cell
ICE	Interleukin-1β- converting enzyme
IgH	Immunoglobulin heavy chain
IgL	Immunoglobulin light chain
IL-7	Interleukin-7
lpr	Lymphoproliferation
MEM	Minimal essential medium
NCS	Newborn calf serum
pBCR	Precursor B cell receptor

PE	Phycoerythrin
PI	Propidium iodide
RAG	Recombination activating gene
ROS	Reactive oxidative species
RT	Room temperature
SCF	Stem cell factor
scid	Severe combined immunodeficiency
sIgM	Surface IgM molecule
TdT	Terminal deoxynucleotidyl transferase
TNF	Tumor necrosis factor
TNFR	Tumor necrosis factor receptor
TRADD	TNFR1-associated death domain protein
VCAM-1	Vascular cell adhesion molecule-1
VLA	Vary late antigen

CONTENTS

Chapter 1 GENERAL INTRODUCTION

The mammalian immune system consists of lymphocytes, macrophages and other accessory cells such as the dendritic cells. These cells are normally found in anatomically organized lymphoid organs and as scattered cells in virtually all tissues as well as circulating in the blood and lymph. B lymphocytes are the only specialized eukaryotic cells capable of producing antibodies (Ab), molecules which can recognize a wide diversity of antigens (Ag) to mediate humoral immune responses throughout life. During mouse embryonic development, lymphopoietic progenitors, along with other lineages of blood cells, are originally derived from primordial stem cells in the intraembryonic tissue and subsequently migrate to the blood islands of the yolk sac once the blood circulation develops at day 8 of gestation (Cumano et al., 1996). Their progeny are found next in the fetal liver, then briefly in the fetal spleen, and finally in the bone marrow (BM) (Palacios et al, 1984; Owen et al., 1975; Ogawa et al., 1988; Rolink, 1991). In postnatal life, BM becomes the primary site of B lymphopoiesis (Osmond and Everett, 1964). Intimate contact of marrow stromal cells with developing hemopoietic precursors provides a supportive nurturing microenvironment for the continuous production of large numbers of B lymphocytes to maintain the peripheral immune system in a dynamic steady state (Osmond, 1990; Kincade, 1991). B cell genesis in mammalian BM represents a delicate balance between cell proliferation and cell loss (Osmond et al., 1994). Factors modifying either one of these processes may profoundly influence the kinetics of B lymphopoiesis. The present work concentrates on B cell differentiation and selection in mouse BM.

1. 1. B Cell Development in BM

1. 1. 1. B Lineage Commitment

Hemopoietic stem cells (HSCs) are defined to have the capacity to produce multiple hemopoietic lineages. The intraembryonic aorta-gonad-mesonephros region generates the first definitive HSC (Sanchez et al., 1996). Though still controversial due to difference between *in vitro* and *in vivo* experimental systems, there is evidence that HSCs become committed to the B lineage first in the embryonal body rather than in yolk sac, then in fetal liver and adult BM and are then maintained throughout life by self-renewal (Ogawa et al., 1988; Uchida et., 1993). Recently, targeted mutagenesis techniques have been used to identify several genes critical for lineage commitment (Tsai et al., 1994; Scott et al., 1994; Opstelten, 1996). The Ikaros gene, a strong candidate for the role of master regulator of lymphocyte specification and differentiation, encodes a family of early hemopoietic and lymphocyte-restricted transcription factors (Georgopoulos et al., 1992). Analysis of the hemopoietic compartments in Ikaros homozygous mutant mice shows a complete absence of lymphoid cells but almost normal erythropoiesis and myelopoiesis, implying that Ikaros promotes differentiation of pluripotential HSCs into the lymphoid pathway (Georgopoulos et al., 1994). Helix-loop-helix (HLH) proteins are a class of transcription factors often involved in regulating cell type differentiation (Kadesch, 1992). It has been demonstrated that the HLH gene E2A is specifically required for B cell differentiation because only B cell genesis is completely blocked while other lineages are intact in $E2A^{-/-}$ mice (Bain et al., 1994). Similar results have been obtained from $EBF^{-/-}$ (early B-cell factor) mice, in which very few $B220^{-}$ cells

with immunoglobulin heavy chain (IgH) genes in germline configuration were found (Lin, et al., 1995). Thus, transcription factors E2A and EBF play critical roles in B lineage commitment and differentiation. Recent studies show that a single CD34 $^{low/-}$, c-kit^{+}, Sca-1^{+} and Lin^{-} (lineage marker) HSC purified by fluorescence-activated cell sorting can provide long term reconstitution of the lymphohemopoietic system *in vivo* (Osawa et al., 1996). However, the process by which pluri-potential HSCs become lineage committed, an irreversible progress, remains largely unknown. Surface phenotypic markers to characterize the earliest B lineage cells in the BM have been extensively investigated, but still remain elusive due to the difficulty of defining the stage of B lineage commitment indicated by the initiation of Ig H gene rearrangement (McKearn et al., 1985; Cumano et al., 1992; Li et al., 1996). It has become evident that the regulation of B lineage differentiation in BM is a complex process that involves a variety of molecules both within the B cell itself and in various accessory cells.

1. 1. 2. B Cell Differentiation

Precursor B cells arising from self-renewing stem cells undergo a programmed process of differentiation and proliferation which involves an ordered rearrangement of Ig gene loci for generating Ag-recognition receptors. The rearrangement mechanism involves an excision of the DNA between various gene segments followed by ligation of these segments mediated by recombinases (Rajewsky, 1996; Lewis and Wu, 1997). Two genes required for Ig gene recombination, termed recombination activating genes 1 and 2 (RAG-1 and RAG-2), have been identified in precursor B cells (Oettingeret al., 199?);

Schatz et al., 1989). Ig gene rearrangement is initiated by the specific binding of RAG-1 protein to the nonamer recombination signal sequence motif (van Gent et al., 1996). Rag-2 is then recruited through its interaction with RAG-1 to form a complex, which is required for the formation of double strand breaks and ligation of gene segments (Spanopoulou et al., 1996). During B cell ontogeny, VH, DH and JH segments of IgH locus are initially rearranged, firstly by DH-JH joining and then by VH-DHJH joining, which combined with the constant region gene locus, allows expression of a μ chain. In postnatal BM, nucleotides are added to the junctions of rearranged DJ or VDJ genes by an enzyme termed terminal deoxynucleotidyl transferase (TdT) to generate N region diversification (Desiderio et al., 1984). More diversity is generated by imprecision between gene segments. As soon as the IgH is productively rearranged, the Ig light chain (IgL) gene is assembled by rearranging the V and J segments of Igκ or Igλ loci. N region diversification is absent during IgL recombination due to a developmentally related downregulation of TdT activity. Once L chains are expressed and associated with μ chains, the assembled IgM molecules together with associated glycoproteins Ig-α, Ig-β are expressed on the cell surface as the Ag receptor and further Ig gene rearrangements are stopped (Rajewsky, 1996). Finally, surface IgM^+ ($sIgM^+$) immature B lymphocytes leave the BM and a fraction of them are selected by unknown mechanisms to enter the peripheral mature $sIgM^+/sIgD^+$ cell compartment.

1. 1. 3. Phenotypic Stages

B cell development in the BM can be considered to pass through a series of

developmental compartments defined by sequential changes of their phenotypic markers or molecular events. A differentiation sequence has been proposed by Osmond et al. based on double immunofluorescence labeling (Osmond, 1990). Six distinct developmental stages are defined by phenotypic markers including lymphoid-specific intranuclear TdT, the B lineage-associated B220 glycoprotein (CD45 RA isoform) and μ chains of IgM. Three cell populations comprise pro-B cells including the stage of IgH gene rearrangement before the expression of μ chains, 1) early pro-B cells (TdT^+B220^-), 2) intermediate pro-B cells (TdT^+B220^+), and 3) late pro-B cells (TdT^-B220^+). The latter give rise to pre-B cells defined by expression of cytoplasmic μ chains, subdivided into large dividing and small postmitotic forms that finally become $sIgM^+$ B lymphocytes. The population sizes and proliferative properties of each precursor B cell stage have been analyzed by a combination of immunofluorescence and mitotic arrest techniques (Osmond, 1986). After sublethal γ-irradiation a sequence of proliferation and expansion of B lineage cells passes in turn through the six phenotypic compartments in accord with this model, supporting the proposal that the scheme represents successive stages through which individual precursor B cells pass with time during their differentiation (Park and Osmond, 1989). Osmonds's model of B cell development forms the basis of current work (Fig. 1.1).

Several other models of B lymphopoiesis in mouse BM have been subsequently proposed using differing phenotypic markers and terminologies. In Rolink and Melchers' model, the different stages of B cell development were initially defined by the status of Ig gene rearrangement and then by further addition of phenotypic markers (Rolink and

Melchers, 1991; Rolink and Melchers, 1993). Pro-B cells have all their Ig gene loci in the germline configuration (Melchers et al., 1995). They express the membrane-bound receptor tyrosine kinase c-kit, the surrogate L chain encoded by V_{preB} and $\Lambda 5$ associated with a complex of glycoproteins (gp130/gp35-65) within the cytoplasm and on the cell surface, and also the specific elements of the VDJ recombination machinery (RAG-1 and RAG-2) (Karasuyama et al., 1993; Rolink and Melchers, 1993; Winkler et al., 1995). The second stage cells, termed pre-B I cells have rearranged D_HJ_H on both alleles but L chain loci in germline. The next stage, called pre-B II cells, is composed of large and small pre-B II cells. Large pre-BII cells are a cycling population and have one productively rearranged H chain allele ($V_HD_HJ_H$). At this stage, surrogate L chains are expressed in association with μ H chains on the cell surface forming the pre-B receptor. These cells start to express CD25 (IL-2 receptor α chain), but have lost the expression of c-kit, RAG-1 and RAG-2 (Rolink et al., 1994). Small pre-B II cells represent a resting population and are now rearranging the L chain loci (V_LJ_L). Accordingly, RAG-1 and RAG-2 are turned on again. These cells are able to undergo secondary L chain rearrangements (Tiegs et al., 1993; Rolink et al., 1995). Finally, $sIgM^+$ immature B cells are formed when both H and L chains have been successfully rearranged. In general, the sequential rearrangement of Ig H and L chain and progressive expression of IgM molecules form a common basis for the two proposed models. The only exception is that early pro-B cells are not included in the Melcher/Rolink model which uses B220 as an inclusive B lineage marker. Pro-B cells and pre-B I cells correspond with intermediate and late pro-B cells in Osmond's model, while large and small pre-B II cells are

equivalent to large and small pre-B cells, consisting of populations with successful IgH rearrangement but still in the process of Ig L chain rearrangement. Subsequent expression of cell surface IgM molecules is the common criterion of immature B lymphocytes. Thus, apart from the minor differences in terminology, these two models are essentially identical and may be combined to provide a unified phenotypic model of B cell development in BM (Fig. 1.1).

Hardy and colleagues have separated $B220^+$ precursor B cells into fractions based on flow cytometric analysis of cell surface markers: B220, CD43 (S7, leukosialin), BP-1, and heat stable antigen (HSA). Among $B220^+CD43^+$ populations, the earliest B cells (Fr. A) are $HSA^-BP\text{-}1^-$. Fr. B cells are characterized by expression of HSA without BP-1, while Fr.C cells express both HSA and BP-1. Among $B220^+CD43^-$ populations, IgM^- cells are defined as Fr. D, which subsequently give rise to Fr. E cells expressing IgM without IgD. Fractions A-D correspond with pro-B cells and pre-B cells, while Fr. E consists of immature IgM^+ B lymphocytes. However, since the surface markers distinguishing fractions A-D do not appear to be related to lineage specificity, the question whether these fractions represent developmental compartments in a strict linear sequence requires further study. This has been the first objective of the current thesis.

1. 1. 4. B Cell Proliferation

Using mitotic-arrest techniques *in vivo* to measure the numbers of cells passing through mitosis per unit time, it becomes possible to quantitate the population dynamics of B cells at each differentiation stage in BM (Park and Osmond, 1987). The production

rate can be determined to estimate the outflow of one compartment and the inflow of the next in a concatenated series of differentiation stages. The kinetic studies indicate that there is at least one mitosis within each of the pro-B cell and large pre-B cell compartments, but no further mitoses among small pre-B or immature B lymphocytes (Osmond, 1991). The population dynamics data suggest a sequence of at least six mitoses during B cell development, leading to a clonal expansion in the BM. It has been calculated that 3.5 days elapse between the onset of VH gene rearrangement and the formation of $sIgM^+$ lymphocytes with total production of 20×10^6 B cells per day per mouse (Osmond, 1990). Although many newly formed B cells are known to migrate to the peripheral lymphoid tissues, mainly to spleen, some of them may die locally. It has been suggested that only a small fraction of the immature B lymphocytes that do reach the peripheral lymphoid tissues is selected every day to remain in the peripheral B cell pool, but it is not known how these B cells are selected (Rolink et al., 1995).

1. 2. B Cell Selection in BM

1. 2. 1. B Cell Loss

The first *in vivo* evidence of substantial cell selection has been provided by population dynamics studies (Opstelten and Osmond, 1983). A considerable discrepancy is observed between the predicted outflow from the dividing large pre-B cell population and the much smaller observed turn over rate of postmitotic small pre-B cells, suggesting that 70% of cells are actually lost at this stage since cells with pre-B phenotype are not normally detected elsewhere in the immune system. However, the

possibility of additional cell loss at other stages of B cell differentiation can not be excluded from the kinetic data. It is worthy of note that similarly high degrees of B cell loss have been observed in rat BM, sheep Peyer's patch and chicken bursa of Fabricius (Deenen et al., 1990; Motyka et al., 1991; Paramithiotis et al., 1995).

Electron microscopy of mouse BM sections has revealed that certain $B220^+$ cells, immunolabeled *in situ*, exhibited apoptotic characteristics and could soon be observed within the cytoplasm of resident macrophages (Jacobsen and Osmond, 1990). Thus, B cell death in the BM appears to be accomplished by apoptosis. Because of the speed and efficiency of macrophage-mediated deletion, however, the true magnitude and rate of cell loss at various stages of B lymphopoiesis remain unclear.

1. 2. 2. Apoptotic Cell Death

1. 2. 2. 1. General Features of Apoptosis

Apoptosis is a morphologically defined cell death process that plays indispensable roles in the embryonic and postnatal development and homeostatic maintenance within multicellular organisms (William and Smith, 1993; Ameisen, 1996; Jacobson et al., 1997). The word apoptosis was originally coined to describe the stereotypic sequence of events in a cell death process which involves cell shrinkage, nuclear fragmentation, membrane blebbing, and finally uptake of the cell by phagocytes (Kerr et al., 1972; Wyllie et al., 1980). Apoptosis is often used as synonym for programed cell death since it is generally accepted that dying cells activate an intrinsic death program that actively contributes to their own demise. As a fundamentally important element of cell biology,

apoptosis has been widely described in all classes of higher eukaryotes: plants, slime molds, nematodes, insects and vertebrates (Vaux et al., 1994).

Although the induction phase is characterized by great variety of potential death-triggering signals, apoptosis is a highly regulated process in which the numerous induction stimuli converge into a few stereotypical pathways and death execution becomes ultimately irreversible (Wyllie et al., 1980; Cohen et al., 1992; Osmond et al., 1994). Once the apoptotic process is initiated, cellular structure changes rapidly. The nucleus condenses and chromatin forms dense caps under the intact nuclear membrane; the endoplasmic reticulum dilates and peripheral cisternae fuse with the plasma membrane, while other cytoplasmic organelles including mitochondria remain generally unaffected. The cell surface membrane starts blebbing, there is a striking decrease in cell volume and increase in cell density due to loss of water and ions, the nucleus breaks up into multiple fragments and the cell finally splits into several membrane-bound apoptotic bodies which are phagocytosed immediately by neighboring phagocytic cells. Thus, apoptotic cell death avoids both the release of intracellular contents into the tissues and the resulting inflammatory response which characterize cell death by necrosis. Cells undergoing apoptosis exhibit a virtually intact external membrane until a late phase of the suicide process. In contrast, even at an early stage, necrotic cells lose the integrity of internal and external membranes, resulting in cellular swelling due to the uptake of water and release of cytoplasmic content into intercellular space.

Biochemical features of apoptosis have also been extensively studied (Kroemer et al., 1995; Trump and Berezesky. 1995; Patel et al., 1996). One well-characterized event

is internucleosomal DNA cleavage, often used as a definitive apoptotic marker. This pattern of DNA degradation occurs by activation of an endogenous endonuclease that cleaves the DNA in the linker region between histones. Since the DNA wrapped around the histones comprises around 180-200 bp, multiples of this interval are characteristically observed as the "apoptotic ladder" by electrophoresis. Recently, other types of DNA degradation have been reported during apoptosis, including fragmentation into 50-300 kbp lengths and single-strand cleavage events (Brown et al., 1993; Oberhammer et al., 1993; Bortner et al., 1995). The size of large DNA fragments correlates well with the size of chromatin loop domain of the nuclear scaffold, implying that enzymes may produce nuclear cleavage at higher level of DNA organisation (Fipipski, 1990). However, it has been reported that DNA fragmentation is not involved in the cell death of germinal centre B cells (Nakamura et al., 1996). Therefore, both morphological and biochemical criteria need to be examined collectively to determine that cell death occurs by apoptosis.

1. 2. 2. 2. Genes Involved in Apoptosis

An evolutionally conserved cell death program appears to have developed early in metazoan (Vaux et al., 1994). The Caenorhabditis elegans has become a very useful model for studying gene regulation of cell death (Ellis et al., 1991). During embryonic development, 131 of a total 1090 somatic cells undergo programmed cell death in every organism. Since the worms are transparent, cell death can be observed by microscopy. It becomes possible to examine mutant nematodes with defects in developmental cell

death due to the predictability of dying cell numbers and availability of extensive genetic information. 14 genes, termed CED (cell death defective) genes, have been identified (Yuan, 1995). Two "killer" genes, CED-3 and CED-4, are required for the execution of the death machinery in the Caenorhabditis elegans (Yuan and Horvitz, 1990). Mutations in either of these genes block all programmed cell death in the organism. However, CED-9, the "protector" gene, is able to prevent cell death by mitigating the actions of the killer genes. Gain-of-function mutations in CED-9 result in a blockade of almost all cell death, while loss-of-function mutations are lethal to the worms due to severe cell loss (Hengartner and Horvitz, 1994; Hengartner et al., 1992). The genetic identification of these mutations has led to the molecular cloning and characterization of cell death-related genes. The CED-9 sequence reveals a significant homology with Bcl-2, a negative regulator of cell death in mammals. The CED-3 product is structurally and functionally homologous to mammalian interleukin-1β-converting enzyme (ICE) (Yuan et al., 1993). No mammalian homolog for CED-4 has been identified, yet it is clear now that ectopic expression of CED-4 in mammalian cells can simultaneously interact with CED-3 and ICE to induce apoptosis (Chinnaiyan et al., 1997). Moreover, CED-9 has been determined as an interacting partner of CED-4 to regulate its subcellular localization and inhibit apoptosis (Wu et al., 1997). Such experiments have provided a fundamental understanding of programmed cell death in nematodes and a great deal of insight into cell suicide machinery in mammals. There is increasing evidence that all nucleated mammalian cells constitutively express the cell death machinery (Weil et al., 1996).

1. 2. 2. 3. Membrane Alterations during Apoptosis

At early stage of apoptosis, the cell membrane displays changes including increased fluidity (Jourd'heuil et al., 1996). Some specific features serve as surface markers that ensure their rapid recognition and deletion by neighbouring phagocytic cells.

Phosphatidylserine is normally located in the inner plasma membrane, whereas the neutral sphingomyelin and phosphatidylcholin are found on the outer membrane. During apoptosis, phosphatidylserine is exposed on the outer membrane surface, which triggers specific recognition and removal by macrophages (Fadok et al., 1992).There is evidence suggesting that interactions between the macrophage thrombospondin receptor, $\alpha_v \beta 3$ vitronectin receptor integrin and an unidentified thrombospondin binding moiety on the apoptotic cell are involved in the phagocytic binding, which can be enhanced by macrophage-secreted thrombospodin forming a molecular bridge (Savill et al.,1993; Savill, 1995; Ren et al., 1995). Apoptotic cells may also expose some sugar residues such as N-acetylglucosamine and N-acetylgalactosamine by loss of terminal sialic residues, which could then interact with a lectin on the macrophage surface to elicit the phagocytic recognition (Duvall et al., 1985). However, it is possible that cell-specific mechanisms for macrophage-mediated phagocytosis exist in order to prevent the leakage of cell components which could cause inflammation and tissue damage (Hall et al., 1994; Dini et al., 1995).

Since these clearance mechanisms are so efficient, apoptotic cells are rarely seen in tissue sections, suggesting that the extent of cell death *in situ* is likely to be underestimated. Accordingly, this raises a fundamental question how to quantitate or

estimate the magnitude of apoptosis of a given cell type *in vivo*.

1. 2. 2. 4. Surface Receptor-Mediated Signalling Pathway

Apoptosis can be mediated through interaction between cell surface receptors and cytokines such as tumour necrosis factor (TNF). Fas ligand (FasL) belongs to the TNF family and is synthesized as a type II membrane protein, from which the soluble form is generated via proteolytic cleavage (Suda et al., 1993; Tanaka et al., 1996). Fas (Apo-1 or CD95), the receptor for FasL, is a type I-membrane protein and a member of the TNF receptor (TNFR) family including TNFR1 and TNFR2 (Itoh et al., 1991; Nagata and Golstein, 1995). Recent studies on the primary structure of Fas and related proteins have revealed that Fas and TNFR1 both contain a cytoplasmic region of 60-70 amino acids which is critical in inducing apoptosis and has been termed the "death domain"(Itoh and Nagata, 1993; Tartaglia et al., 1993). The death domain region has been demonstrated to be homologous to the protein Reaper that is essential for most programmed cell death in Drosophila (White et al., 1994; Golstein et al.,1995). This region has a tendency to self-aggregate and is involved in protein-protein interactions based on homo- and heterodimerization, which has enabled several novel death-domain-containing proteins to be identified (Huang et al., 1996). They are FADD(Fas-associating protein with death domain) or MORT1 (Boldin et al., 1995; Chinnaiyan et al., 1995), TRADD(TNFR1-associated death domain protein) (Hsu et al., 1995), and RIP (receptor interaction protein)(Stanger et al., 1995), which can use their own death domains to interact with Fas and/or TNFR and may form part of the intracellular pathway induced by Fas/FasL

interaction. Over-expression of any of these proteins induces apoptosis. MORT1/FADD is found to link Fas directly to the ICE-like enzymes, MACH (MOAT1 associated-CED-3/ICE homolog) and FLICE (FADD-like ICE), which may activate other members of the ICE family leading to cell death (Muzio et al., 1996). Since there is no evidence yet that TNFR1 is directly linked to MORT1/FADD, TRADD's death domain may serve as an adaptor to link TNFR1 to other proteins in the death pathway.

Mutation of the Fas or FasL genes causes abnormal lymphoproliferation and autoimmune response in *lpr* (lymphoproliferation) and *gld* (generalized lymphoproliferative disease) homozygous mice, respectively, suggesting that Fas-mediated apoptosis is a critical event in the regulation of the immune response (Roths et al., 1984; Watanabe-Fukunaga et al., 1992; Takahashi et al., 1994). Furthermore, the accelerated autoimmune disease and lymphoproliferative phenotype can be corrected when Fas is expressed as a transgene in the lymphocytes of *lpr* mice (Wu et al., 1994). Thymic clonal deletion is apparently normal, whereas activated T cells accumulate in the lymph nodes and spleen of these mice (Singer and Abbas, 1994). Accumulation of B lymphocytes and elevated levels of immunoglobulins are also observed in *lpr* and *gld* mice, suggesting that Fas/FasL system involves in the deletion of activated or autoreactive B lymphocytes (Cohen and Eisenberg, 1991). B cells that encounter self-Ags may express Fas and could be induced to apoptosis by FasL expressed on helper T cells through cognate T cell- B cell interactions (Rathmell et al., 1995). Thus, the Fas/FasL system plays little role in selecting developing lymphocytes, but is important in down-regulating the immune response and/or eliminating self-reactive mature

lymphocytes to maintain peripheral tolerance.

Lymphocytes undergoing apoptosis are found to have increased expression of inositol 1, 4, 5-trisphosphate (IP3R) receptors on plasma membrane, which mediate Ca^{++} entry into lymphocytes to trigger signal transduction of death pathway. Conversely, expression of IP3R antisence constructs can block dexamethasone-induced apoptosis, implying that IP3R is critical in mediating cell death (Khan et al., 1996).

1. 2. 2. 5. Endonucleases in Apoptosis

The generation of nuclear fragmentation in cells undergoing apoptosis has led to the investigation of involved endonuclese(s). Early evidence has suggested that endogenous endonuclease is activated in apoptotic thymocytes induced by glycocorticoid (Wyllie, 1980). Further observations reveal that Ca^{++}/Mg^{++}-dependent endonuclease activation occurs in dying cells and contributes to the cleavage of nuclear DNA (Gaido and Cidlowski, 1991; Montague et al., 1994; Sokolova et al., 1992; Wyllie et al., 1992). Moreover, the enzyme activity is induced and enhanced by addition of Ca^{++} and Mg^{++} ions, while Zn^{++} ions can inhibit both Ca^{++} and glycocorticoid triggered DNA fragmentation *in vitro* (Cohen and Duke, 1984; Arends et al., 1990). Other signalling events leading to endonucleolytic activity during apoptosis include endoplasmic reticulum Ca^{++} release, activation of protein kinase C or tyrosine kinase, production of ceremide and accumulation of cAMP (McConkey et al., 1991; Lotem et al., 1991). Due to the heterogeneity of nuclear endonucleases and the existence of different types of Ca^{++}/Mg^{++} -dependent endonuclease, it is difficult to determine which enzyme(s) is (are)

selectively activated, especially among various cell types. Two cytoplasmic DNases, DNases I and DNases II, have been well characterized as candidates for the apoptotic endonuclease (Barry and Eastman, 1992; Peitsch et al., 1993; Alnemri and Litwack, 1990). Recent studies have demonstrated an inducible endonuclease associated with lymphocyte apoptosis (Khodarev and Ashwell, 1996). It seems likely that the endonuclease(s) activated in apoptosis may vary depending on cell types, death signals and other factors.

Although endonucleolytic DNA cleavage is still widely used as a chemical hallmark of apoptotic cell death, the typical morphological features of apoptosis can still occur even in the absence of nuclear fragmentation, as caused by inhibitors of DNA degrading enzymes (Cohen et al., 1992; Schulze-Osthoff et al., 1994).

1. 2. 2. 6. Proteases in Apoptosis

Evidence that the activation of nuclease and the presence of DNA fragmentation or even the nucleus are not absolutely required for cells to undergo morphologically typical apoptotic cell death has prompted research on the role of proteases in the mediation of apoptosis (Jacobson et al., 1994; Steller, 1995). The most important clue in understanding the molecular death machinery has come from genetic studies showing that CED-3 is homologous to ICE, suggesting a central role for ICE in apoptosis. However, the discovery of an expanding CED-3 protease family in mammals indicates the complexity of the cellular regulation of these enzymes in the death process.

ICE has been shown to cleave the 33-kDa IL-1β precursor (pro-IL-1β) at an

aspartic acid residue into the biologically active form (17.5kD) of IL-1β, a cytokine that plays an important role in inflammation and in a variety of other physiological and pathological processes including B lymphopoiesis in BM (Ikejima et al., 1990; Dorshkind, 1988; Fauteux and Osmond, 1996). ICE is synthesized as a precursor molecule of 45kDa and subsequently processed to form an active heterodimeric enzyme of 20 KDa and 10 KDa chains, both of which are essential for enzymatic activity. Subsequent enzymatic, mutagenetic, and crystallographic studies have shown that ICE contains a cysteine in its active site, a conserved QACRG motif, which participates in substrate binding and catalysis. In a physiological context, only precursor ICE p45 is detectable in resting monocytes, and activation triggers proteolytic processing to the active species by an unknown mechanism. While both precursor ICE and processed ICE are localized in the cytoplasm, only processed ICE is detectable on the plasma membrane (Singer et al., 1995). It is now clear that ICE is the prototype of a new emerging family of cysteine proteases, termed the caspase family (Alnemri et al., 1996). At least 10 members have now been identified in humans and a number of them have been implicated in apoptosis (Chinnaiyan and Dixit, 1996). Indeed, overexpression of ICE causes fibroblasts to undergo apoptosis, which can be inhibited by Bcl-2 and CrmA, a cowpox virus protein that inhibits ICE-like cysteine proteases (Miura et al., 1993; Gagliardini et al., 1994). Mutation in the catalytic cysteine residue of ICE completely abolishes its activity of inducing apoptosis (Miura et al., 1993). During apoptosis, mature IL-1β is found to be generated among several cell lines, induced by various factors, implying ICE is activated in the process (Hogquist et al., 1991; Miura

at al., 1995). Pro-IL-1β is the only known substrate of ICE but many cells that undergo apoptosis do not appear to produce IL-1β, suggesting that there may be other substrates and that IL-1β is not required for apoptosis among certain cell types. Mice deficient in ICE cannot synthesize mature IL-1β, but their thymocytes are able to undergo apoptosis induced by dexamethasone and *r*-irradiation, suggesting either that ICE is not essential for the cell death process or that other ICE homologues are involved (Kuida et al., 1995). CPP32, an ICE homologue, has been demonstrated to be activated in mammalian cells undergoing apoptosis and specific inhibition of CPP32 activity can attenuate apoptosis *in vitro* (Wang et al., 1996; Tewari et al., 1995; Nicholson et al., 1995). It has been shown that CPP32 can cleave poly (ADP-ribose) polymerase and DNA-dependent protein kinase that function in the splicing of mRNA and the repair of double-strand DNA breaks, which may represent a fundamental feature ensuring the rapid irreversibility of the apoptotic process (Tewari et al., 1995; Casciola-Rosen et al., 1996). Though the specificity of the proteolytic cleavage of ICE suggests that the number of targets for the ICE family proteins may be limited to certain crucial proteins, different ICE proteases may fulfill different physiologic roles, which may also reflect different cell lineage specificity or different substrate specificity. Thus, the complexity and hierarchical relationships between ICE family members require extensive studies to understand the critical role of proteolysis in apoptosis (Henkart, 1996; Patel et al., 1996; Abastado, 1996).

1. 2. 3. Regulation of Apoptosis

The proper functioning of the immune system depends upon mechanisms ensuring the selection of appropriate lymphocytes for survival. Many developing lymphocytes die probably either because of the failure to receive a survival factor or due to the activation of a killing signal. A carefully tuned homeostatic system is critical and its disruption may cause lymphoid malignancies, lymphoproliferative diseases, and autoimmunity. Although signals that trigger the developmental death program during B lymphopoiesis remain unclear, it becomes increasingly evident that the *bcl-2* gene family plays a critical role in regulating apoptotic cell death with an evolutionarily conserved machinery (Cohen, 1992; Reed, 1994; Cory, 1995). The *bcl-2* proto-oncogene is discovered at the break-point region of the t(14; 18) chromosomal translocation involved in B cell malignancies, placing it into juxtaposition with enhancer elements in IgH locus. (Tsujimoto et al., 1984). Bcl-2 is an intracellular 26 KDa protein associated with mitochondrial, nuclear, and endoplasmic reticulum membranes. In a variety of physiological and pathological contexts, Bcl-2 blocks a final common pathway for apoptotic cell death (Reed, 1995; Motyka and Renolds, 1995; Boise and Thompson, 1996). Bcl-2 family proteins contain up to 4 conserved domains, termed Bcl-2 homology 1, 2, 3 and 4 (BH1, BH2, BH3 and BH4), among which BH1 and BH2 are required for Bcl-2 to inhibit apoptosis (Yin et al., 1994). Proteins of the Bcl-2 family contain a stretch of hydrophobic amino acids at their C-termini, which is important for attachment to intracellular membranes, while the N-terminus of the integrated protein is exposed to the cytosol (Tanaka et al., 1993; Nguyen et al., 1993). Mutant Bcl-2, lacking the C-terminal transmembrane domain, associates

inefficiently with mitochondria and displays an impaired ability to prevent cell death (Tanaka et al., 1993). Within an expanding family of Bcl-2 homologs exist positive and negative regulatory proteins. Bax, a 21 KDa protein with 21% homology to Bcl-2, appears to inhibit the function of Bcl-2 by forming Bcl-2-Bax complexes or by competing with other Bcl-2 family proteins. It has been revealed that heterodimerization between members of the Bcl-2 family of proteins is a key event in the regulation of apoptosis (Yang and Korsmeyer, 1996). The response to death signals in the cell is probably modulated by the differential expression and interaction of anti- and pro-apoptotic proteins. Interestingly, all antiapoptotic members have the BH4 domain (Zha et al., 1996), which is typically located near the first amphipathic α helix in the crystal structure of the Bcl-x_L protein, an antiapoptotic homologe of Bcl-2 (Muchmore et al., 1996). Conversely, the proapoptotic members lack BH4, with the exception of Bcl-x_s, a proapoptotic protein. Deletion mutants of Bcl-2 lacking the BH4 domain exhibit either loss of function or attenuated apoptotic-inhibitory activity, paradoxically promoting apoptosis (Hanada et al., 1995), thus indicating the functional significance of the BH4 domain. Recently, extensive studies have provided accumulating evidence of possible mechanisms of Bcl-2 family proteins in regulating apoptosis. It is found that Bcl-2 can inhibit cell death by modulating organellar Ca^{++} homeostasis and mitochondrial permeability transition. Since anucleated cells (cytoblasts) can be induced to undergo apoptosis, it has been suggested that a cytoplasmic death pathway may function independently from the nucleus (Jacobson et al., 1994; Schulze-Osthoff et al., 1994). The observations that mitochondria isolated from cells undergoing apoptosis can induce

apoptotic degradation of naive nuclei, whereas mitochondria purified from Bcl-2 overexpressing cells do not show the same effect suggest an important role for mitochondria in controlling apoptosis (Zamzami et al., 1996; Kroemer et al., 1997). In a cell-free system, induction of permeability transition is critical for isolated mitochondria to trigger nuclear apoptosis, which can be directly abolished by Bcl-2 to inhibit apoptotic process (Zamzami et al., 1996). Overexpression of Bcl-2 has been reported to prevent the dumping of mitochondrial Ca^{++} into the cytosol of cells caused by oxidative phosphorylation uncouplers and thus to block the induced apoptosis (Baffy et al,1993). Further studies demonstrate that Bcl-2 protects cells from apoptosis by targeting to the mitochondrial protein kinase Raf-1 to phosphorylate BAD, a proapoptotic protein (Wang et al., 1996). Cells undergoing apoptosis are found to have elevated cytochrome C in the cytosol and a corresponding decrease in the mitochondria (Martin et al., 1995).Moreover, cytosolic cytochrome C is necessary for the initiation of the apoptotic process. It has been further confirmed that overexpression of Bcl-2 prevents the efflux of cytochrome C from the mitochondria and inhibits apoptosis (Yang, et al., 1997). However, Bcl-2 can also block apoptosis in cells lacking mitochondrial DNA, implying that neither apoptosis nor the protective effect of Bcl-2 depends on mitochondrial respiration (Jacobson et al., 1993). It has been suggested that apoptosis involves the generation of reactive oxygen species (ROS) and that Bcl-2 suppresses cell death by inhibiting the production of ROS, but the intriguing observation remains that under anaerobic conditions apoptosis still occurs and can be protected by Bcl-2 (Buttke and Sandstrom, 1994; Jacobson and Raff, 1995). New evidence suggests that Bcl-2 and Bcl-

x_L function upstream of caspase family (Chinnaiyan et al., 1996). Interestingly, the three-dimensional structure of Bcl-x_L bears a certain similarity to the pore-forming domains of several bacterial toxins including colicin A and diphtheria toxin (Parker and Pattus. 1993; Jeanteur et al., 1994). Further studies show that Bcl-x_L can insert into a synthetic lipid membrane and form an ion channel, suggesting that Bcl-x_L may inhibit apoptosis by regulating the permeability of the intracellular membrane (Minn et al., 1997).

A tremendous increase in understanding of the cell death pathway has been gained in recent years (Golstein, 1997). However, the precise biochemical mechanisms by which Bcl-2 family proteins exert their effect on cell life and death remain far from clear. The biochemical nature of the cell death signals and the biological significance of protein interactions of Bcl-2 family proteins are unknown. The answer to these outstanding questions should provide great insights into the cell death process.

1. 2. 4. Mechanisms of B Cell Selection

Studies of mutant and transgenic mouse models have demonstrated that cell deletion can occur at various stages of B cell development. Mutant mice with severe combined immunodeficiency (*scid*), unable to repair DNA double-strand breaks (Bosma et al., 1983), show the normal number of early and intermediate pro-B cells in the BM, but a much reduced size of the late-pro B cell compartment and an absence of pre-B cells and $sIgM^+$ B lymphocytes (Osmond et al., 1992), implying that successful IgH gene recombination is critical for survival of precursor B cells *in vivo*. Similar results have been obtained from RAG-1 deficient and RAG-2 deficient mice, which can not initiate

IgH gene rearrangements (Shinkai et al., 1992; Mombaerts et al., 1992). When a μ transgene is introduced into *scid* mice, cytoplasmic-μ-chain-expressing pre-B cells appear which are then aborted at this stage (Reichman-Fried et al., 1990). Mice lacking the membrane exon of the μ chain do not produce B cells beyond the pre-B stage. Only when both μ and *k* transgenes are introduced into *scid* mice, do both pre-B cells and $sIgM^+$ B cells emerge in the BM (Reichman-Fried et al., 1990). Thus, the introduction of a functionally rearranged μ gene into the genome of *scid* mice enables B progenitors in the BM to synthesize cytoplasmic μ chains and differentiate into pre-B cells, whereas $sIgM^+$ B cell formation requires the introduction of both a functional μ and a functional *k* gene into the *scid* mouse germ line. Taken together, the evidence demonstrates that all B precursors are deleted either at the late pro-B cell stage if unable to rearrange IgH genes or at the pre-B cell stage if successful to express IgH but not IgL genes. During B cell ontogeny in the BM, Ig gene rearrangement is a stochastic process. Recent studies suggest that almost half of all large pre-B cells should not express a μ chain, if VH to DHJH rearrangements occur in- and out-of-frame in a random fashion (Melchers et al., 1995). Therefore, many B cell precursors that fail to assemble functional IgH and IgL chains may undergo cell death. However, the molecular signals and mechanisms that implement cell death remain unsolved. It has recently been proposed that successful expression of a pre-B cell receptor (pBCR) and the further engagement of the receptor by an unknown ligand presented possibly by the local microenvironment may deliver a survival signal to allow precursor cells to progress beyond the pro-B/pre-B transition stage for further differentiation (Rajewsky, 1996; Rolink et al., 1994). The pBCR,

expressed exclusively by precursor B cells, contains IgH chains, but the IgL chains are replaced by the surrogate L chains Λ5 and VpreB (Melchers et al., 1993; Sakaguchi and Melchers, 1986; Kudo and Melchers, 1987). Targeted disruption of the Λ5 gene results in remarkable reduction of precursor B cells (Kitamura et al., 1992), suggesting that surrogate L chain is required for the continuing differentiation and survival of precursor B cells (Karasuyama et al., 1994). Later in development, immature B lymphocytes start expressing surface Ag receptor (B-cell Ag receptor, BCR), a complex of Ag-binding sIg with the signal-transducing heterodimer Igα/Igβ (Reth, 1992). Newly formed B lymphocytes with autoreactive specificities are further selected, by either physical elimination or functional inactivation (Nemazee and Burki, 1989; Goodnow et al., 1988; Nossal, 1996), which underscores the clonal selection theory that the cells express their Ab as a surface receptor and can then be selected by Ag (Burnet, 1959). In mice treated with Abs to IgM from birth, the differentiation and proliferation of precursor B cells are essentially normal, but no $sIgM^+$ B lymphocytes are detected in the BM or peripheral lymphoid tissues and humoral immune responsiveness is lacking (Lawton and Cooper, 1974; Fulop et al., 1983; Opstelten and Osmond, 1985). Subsequent *in situ* studies show a remarkable accumulation of macrophages containing many ingested bodies displaying apoptotic morphology and concentrated in central areas of BM where $sIgM^+$ normally congregate, suggesting that newly formed B cells are physically eliminated following BCR ligation by anti-IgM Ab (Jacobsen and Osmond, 1990). There is evidence that certain immature $sIgM^+$ lymphocytes having encountered autoantigens in BM are capable of escaping deletion by "editing" their receptors through

secondary Ig L rearrangements (receptor editing) (Radic et al, 1993; Rolink et al., 1993; Tiegs et al.,1993). Therefore, the demise of developing B cells in the BM is a highly regulated process that serves to select cell populations expressing functional antigen receptors, and to remove aberrant or autoreactive cells.

1. 3. B Lymphopoietic Microenvironment in BM

1. 3. 1. Cytoarchitectural Organization

The hemopoietic cells in BM are in intimate association with stromal reticular cells, located in the extravascular space between venous sinusoids. *In vivo* immuno-labeling techniques have made it possible to reveal the relationship between developing B lineage cells and the BM stroma by systemically administering ^{125}I-labeled mAbs to phenotypic markers (Batten and Osmond, 1984). B lineage cells are normally retained in the BM parenchyma until they reach the terminal stage of maturation before being released into the blood circulation. Many precursor B cells make extensive contact with the processes of reticular cells and macrophages. Moreover, early B precursors have a particular subosteal localization and tend to move along a network of stromal cells to the more central region of the marrow during the developmental process, so that sIgM$^+$ immature B lymphocytes are present mainly in perisinusoidal locations around the central venous sinus (Jacobsen and Osmond, 1991; Jacobsen et al., 1992). Studies have shown that gap junctions exist between stromal cells to mediate cell-cell communication, which can be regulated by cytokine treatment (Dorshkind et al., 1993). It has become clear that resident macrophages, extending reticular processes between adjacent hemopoietic cells

throughout marrow, play an important role in recognizing and phagocytosing apoptotic cells (Hume et al., 1983; Jacobsen et al., 1994; Savill et al., 1990; Osmond et al., 1994). Although it has been generally accepted that macrophages function as passive scavengers, recent studies have suggested that macrophages may also employ proapoptotic mediators including ROS, nitrogen species and TNF-α to kill target-cell by inducing apoptosis (Alipratis et al., 1996). The intricate BM architecture provides a morphological basis for molecular interactions between developing B cells and associated stromal cells.

1. 3. 2. Microenvironmental Regulation of B Lymphopoiesis

B cell genesis in the BM is modified by the local microenvironment as well as by certain systemic factors including steroid hormones (Kincade et al., 1987; Dorshkind, et al., 1993; Kincade, 1994). Innovative use of *in vitro* cell cultures, mAbs and recombinant cytokines has brought rapid progress in understanding lymphostromal interaction (Rosenberg and Kincade, 1994). New molecular cloning techniques contribute greatly to the identification of extracellular matrix molecules and cytokines in regulating both B cell proliferation and selection (Oritani and Kincade, 1996). Radioautographic studies by electron microscopy after systemically administering radiolabeled mAb M/K-2 have revealed that stromal cell processes in normal mouse BM express vascular cell adhesion molecule 1 (VCAM-1), a cell adhesion molecule (CAM) of the Ig superfamily of proteins and a ligand for very late antigen-4 (VLA-4) expressed on B lineage lymphocytes (Osmond et al., 1992). Precursor B cells are rapidly

detached from the adherent stromal layer of BM cocultures by addition of anti-VCAM-1 M/K mAbs (Miyake et al., 1991). Moreover, these Abs can selectively and completely block lymphopoiesis in long-term BM cultures (Miyake, et al., 1990). It has been well documented that disruption of the interaction between extracellular matrix and specific cognate integrins triggers apoptosis, a process termed anoikis, in epithelial and endothelial cells (Ruoslahti and Reed, 1994; Meredith et al., 1993; Frisch and Francis, 1994). Recent studies show that the ligation of VLA can induce apoptosis among certain hemopoietic cell lines (Sugahara et al., 1994). Therefore, the CAMs on stromal cells are important for signal transduction in regulating B lymphopoiesis as well as for mediating lymphocyte binding.

Access to essential growth factors is required for the differentiation and proliferation of hemopoietic progenitors, whereas competition for the limited microenvironmental factors may underlie the balance between B lyphopoiesis and other hemopoietic lineages *in vivo* (Kincade, 1991; Witte et al., 1993; Rico-Vargas et al., 1994). Precursor B cells develop in close association with stromal cells which produce a variety of regulatory cytokines (Jacobsen and Osmond, 1990; Kincade, 1991; Witte et al., 1993; Funk et al., 1995). Interleukin-7 (IL-7), a stromal cell-derived cytokine, has been identified to be crucial for the pre-B cell development in long-term mouse BM cultures (Namen et al., 1988), while stromal cells clones incapable of producing IL-7 fail to support B lymphopoiesis (Sudo et al., 1989). IL-7 receptors are detected on essentially all $B220^{+}sIgM^{-}$ precursor B cells, but downregulated among $sIgM^{+}$ immature B lymphocytes (Sudo et al., 1993; Loffert et al., 1994). Stromal cells also express kit

ligand (stem cell factor, SCF) that can augment the stimulatory effect of IL-7 on precursor B cells (Ogawa et al., 1991; Billips et al., 1992; Funk et al., 1993). It has further been revealed that distinctive growth requirements exist at various stages of B cell development. Pro-B cell development is dependent upon stromal cells. No defined cytokines or colony stimulating factors, including kit ligand and IL-7, can replace stromal cell conditioned medium in mediating this developmental process *in vitro*. Pre-B cells can be stimulated by IL-7 to proliferate in the absence of stromal cells, while newly formed $sIgM^+$ B lymphocytes lack responsiveness to IL-7 (Dorshkind, 1990; Dorshikind et al., 1992; Billips et al., 1992; Faust et al., 1993). A prelymphomatous B cell hyperplasia has been observed in the BM of IL-7 transgenic mice, indicating that excessive proliferation of precursor B cells caused by overexpression of IL-7 may predispose to preneoplastic condition (Mertsching et al., 1996; Valenzona et al., 1996). Conversely, B cell production is almost completely blocked in IL-7 gene-deleted mice (von Freeden-Jeffry et al., 1995). Although the critical function of stromal cells and IL-7 in B cell production has been extensively characterized, little information is available on their possible role in B cell selection. Thus, whether IL-7 can directly regulate B cell survival in the BM remains yet to be elucidated.

It has been clearly demonstrated that BM stromal cells constitutively express colony-stimulating factor-1 (CSF-1) as well as IL-7 and SCF (Witte, 1993). CSF-1 is a primary growth factor for the differentiation , proliferation and survival of the macrophage lineage (Wiktor-Jedrzejczak and Gordon, 1996). However, hemopoiesis of other lineages besides the monocytic lineage is severely impaired in BM of the CSF-1

deficient osteopetrotic (op/op) mouse (Wiktor-Jedrzejczak et al., 1982). It is unclear whether CSF-1 influences B cell differentiation and selection *in vivo*.

1. 4. Purpose of the Study and Experimental Design

This study has aimed to examine B cell differentiation and regulation, with special reference to the role of B cell selection by apoptosis in mouse BM.

During B lymphopoiesis, precursor B cells pass through a series of developmental compartments. Several models have been proposed to define these stages of B cell differentiation. However, the use of different phenotypic markers and terminologies has hampered a comparison of experimental results. Hardy's cell fractions based on flow cytometric analysis of cell surface expression of B220, CD43, HSA and BP-1 have been recently described as a method to separate subsets of precursor B cells, and the fractions have been used in a variety of single cell assays. Without lineage-specific markers, however, it has not been possible to assign a particular developmental stage to individual cells from any given fraction.

In Chapter 2, cell fractions sorted from mouse BM using a fluorescence activated cell sorter (FACS) according to Hardy's scheme have been examined for expression of intranuclear TdT and cytoplasmic μ chains by epifluorescence microscopy to correlate the cell populations within each fraction with B cell differentiation stages according to the scheme proposed by Osmond. The results reveal and quantitate a heterogeneity of B cell differentiation stages within each fraction. This study enables a direct comparison to be made between Hardy's and Osmond's models, contributing toward a unified model

of B cell differentiation in mouse BM.

B cell genesis in BM requires a delicate control of cell differentiation, proliferative expansion and cell selection. However, the nature of the high degree of B cell loss that occurs during development in BM is not well understood.

In Chapter 3, a variety of criteria including *in situ* DNA strand break labeling, nuclear fragmentation, hypodiploid DNA content, annexin-V labeling and electron microscopy have been used to demonstrate that B cell death takes the form of apoptosis in BM. The apoptotic incidences of B cells at various developmental stages *ex vivo* and the rates of entry into apoptosis during short term culture have been analyzed by flow cytometry to test the hypothesis that B cell selection by apoptosis is developmentally regulated. The findings demonstrate that B cells are particularly prone to apoptosis at two differentiation stages, soon after Ig V_H gene rearrangement and antigen receptor expression, respectively. Coculture of BM cells with stromal cells has evaluated the apoptotic rate and estimated magnitude of B cell loss under optimal conditions. The results lead to the formulation of the first dynamic model of B cell production and selection *in vivo*.

During development, aberrant, functionless and certain autoreactive B cells are eliminated. This constitutes an efficient culling process in controlling B cell production. Although the precise mechanisms remain unclear, Bcl-2 family proteins play crucial roles in regulating cell death in a variety of systems.

In Chapter 4, the hypothesis that Bcl-2 and Bax proteins are differentially expressed by B cells at various stages during development has been tested. Flow cytometric

quantitation of protein expression reveals that changes in the Bcl-2/Bax ratio are closely correlated with the apoptotic susceptibility of B cells at defined developmental stages. The results suggest that Bcl-2 and Bax proteins are involved in regulating B cell selection during development in BM.

B cell differentiation is closely regulated by intrinsic DNA recombinatorial events. Developing precursor B cells appear to be able to avoid being triggered into a programmed death pathway while Ig gene recombination is proceeding, but to abort whenever this process ceases to be successful. However, the molecular regulations that coordinate Ig gene expression and B cell survival remain essentially unknown.

In Chapter 5, B cell differentiation and apoptotic kinetics have been examined by flow cytometry in RAG-2$^{-/-}$ and control mouse BM. The results demonstrate that changes in Bcl-2 and Bax expression are implicated in controlling the demise of aberrant pro-B cells, unable to achieve productive rearrangement of IgH genes.

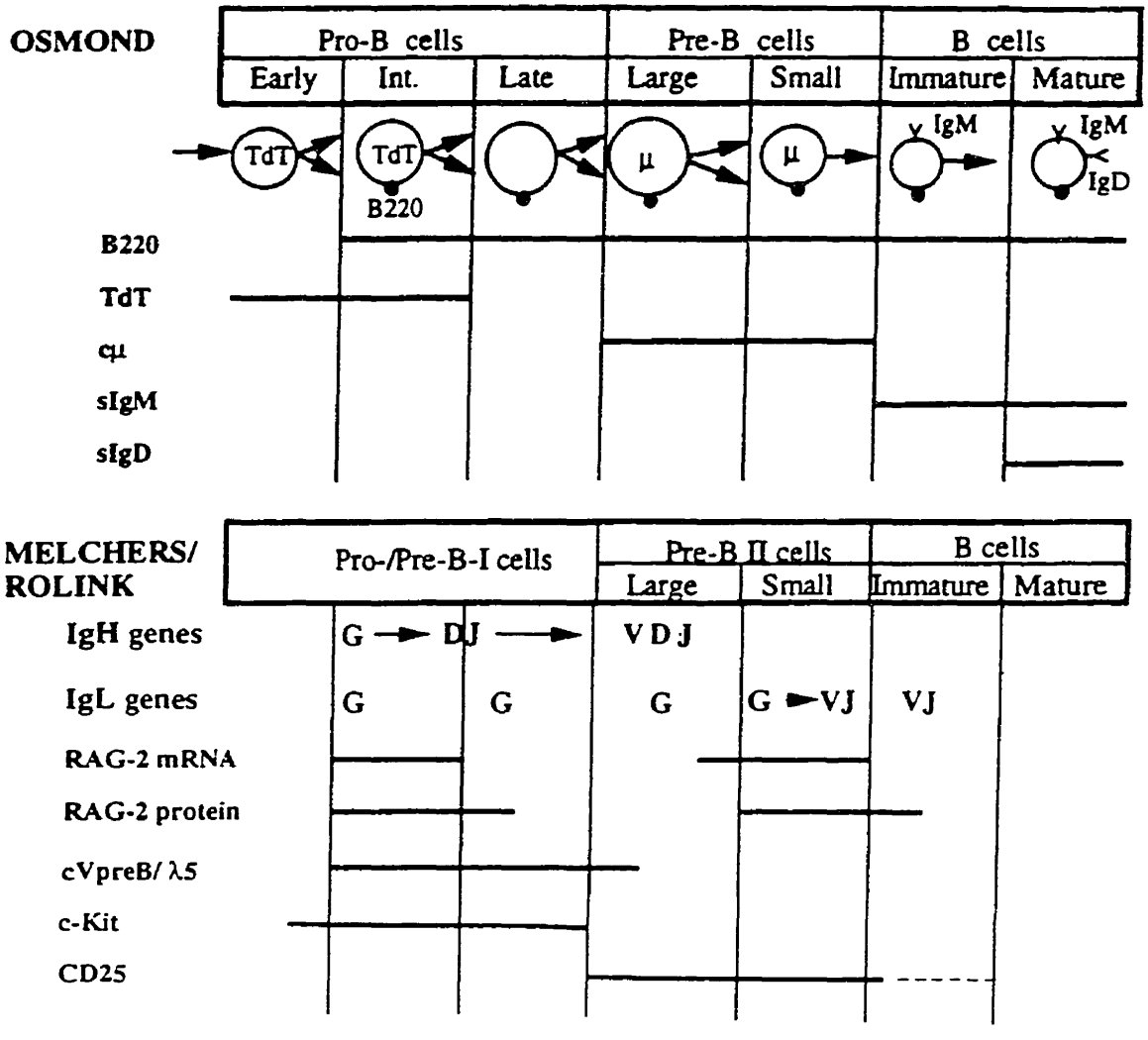
B lymphopoiesis is highly influenced by local microenvironmental factors. IL-7, a stromal cell derived cytokine, plays an essential role in B cell differentiation and proliferation in mouse BM.

In Chapter 6, flow cytometric studies on B cell apoptosis using IL-7 gene-deleted mice lacking IL-7, and IL-7 transgenic mice, overexpressing IL-7, indicate that IL-7 serves as a survival factor *in vivo* as well as a proliferative stimulus during precursor B cell development in BM. The results reveal that modulations of IL-7 gene expression *in vivo* are associated with altered protein levels of Bcl-2 and Bax and correspondingly marked changes in the degree of apoptotic B cell selection in BM.

The function of CSF-1 in regulating the development of mononuclear phagocyte system has been clarified by extensive studies on the CSF-1 deficient op/op mutant mouse. However, it is unknown whether CSF-1 has an *in vivo* role in B lymphopoiesis in BM.

In Chapter 7, B cell differentiation and apoptotic selection have been compared in op/op mutant and normal mouse BM. The results reveal that B cell development is severely impaired and B cell apoptosis is greatly accelerated in mutant mice, suggesting that CSF-1 normally plays an important role in B cell differentiation and selection in BM.

Fig. 1.1. Correlation of two phenotypic schemes of B cell development in mouse bone marrow.


OSMOND
Pro-B cells
Pre-B cells
B cells
Early
Int.
Late
Large
Small
Immature
Mature
TdT
TdT
B220
μ
μ
IgM
IgM
IgD
B220
TdT
cμ
sIgM
sIgD
MELCHERS/
ROLINK
Pro-/Pre-B-I cells
Pre-B II cells
B cells
Large
Small
Immature
Mature
IgH genes
G → DJ →
V D J
IgL genes
G
G
G
G → VJ
VJ
RAG-2 mRNA
RAG-2 protein
cVpreB/ λ5
c-Kit
CD25

Chapter 2 Flow Cytometric Analysis of B Cell Differentiation in BM and A Direct Comparison of Two Murine B Lymphopoiesis Models

2.1. Summary

During B cell genesis in mouse BM, precursor B cells pass through a series of developmental stages, which are defined by changes in phenotypic markers. The use of various experimental methods and dissimilar phenotypic criteria, however, has led to various models of B lymphopoiesis. An initial flow cytometric analysis has now been performed to examine the phenotypic populations of Osmond's model, previously based on epifluorescence microscopy. The resulting data are consistent with previous studies. In order to correlate this model with Hardy's model based on flow cytometric analysis of surface phenotypic markers, intranuclear TdT and intracytoplasmic μ chains (cμ) have been examined by immunofluorescence microscopy of cytocentrifuged cells from FACS-sorted B cell fractions based on expression of B220, CD43, HSA and BP-1. The results document the heterogeneity of B cell differentiation stages within each fraction and contribute an important step towards a unified model of B cell development in BM.

2.2. Introduction

In mouse BM throughout post natal life precursor B cells undergo processes of differentiation and proliferation influenced by microenvironmental control signals

(Osmond et al., 1994; Kincade, 1991). During this process, the precursor B cells can be considered to pass through a series of developmental compartments defined by sequential changes in expression of phenotypic marker molecules (Osmond, 1990; Hardy et al., 1991; Rolink and Melchers, 1993; Billips et al., 1995; Rajewsky, 1996).The precise definition of such differentiation compartments is an important prerequisite for the experimental analysis of genetic events, control signals and aberrations of B lymphopoiesis in BM. The use of a variety of phenotypic markers, however, has led to the formulation of schemes of B cell development that are based on dissimilar criteria and are thus difficult to correlate. Using double immunofluorescence labelling and stathmokinetic techniques, Osmond et al have proposed a B cell differentiation sequence of six phenotypically distinct populations based upon three markers associated with the B lineage; 1) surface membrane B220 glycoprotein (CD45RA isoform), 2) intranuclear TdT, and 3) μ chains of IgM (Osmond, 1990). In contrast, the model subsequently developed by Hardy and coworkers subdivides $B220^{+}$ cells into fractions based on flow cytometric analysis, exclusively using cell surface markers, CD43, HSA, and BP-1 (Hardy et al.,1991). The purpose of the present work has been to make a direct comparison between these two models in order to permit an integration of the extensive experimental literature that has developed around each one.

In the present study, the distribution of cells expressing intranuclear TdT protein and intracytoplasmic μ chains within cytocentrifuged cells of Fr.A to D sorted from normal mouse BM has been determined by immunofluorescence labelling and epifluorescene microscopy.

2.3. Materials and Methods

2.3.1. Animals and Cell Preparation

BALB/c mice bred in the Oklahoma Medical Research Foundation Animal Resource Centre were used at 8-9 wks of age. Femoral BM cell suspensions were pooled from 5-10 mice, as described (Park and Osmond, 1987).

2.3.2. Immunophenotypic Staining

BM cell suspensions were incubated with phycoerythrin (PE)-conjugated 6B2 (CD45RA, PharMingen, San Diego, CA), and -IgM Abs (Southern Biotechnology Associates, Birmingham, AL) for 20 min on ice. Samples were fixed with cold 70% ethanol (45 min, 4°C), followed by incubation with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse μ heavy chain mAb (Kirkegaard and Perry Laboratories, Gaithersburg, MD) to examine total μ expression. Large and small $c\mu^+$ cells were discriminated by forward scatter during flow cytometry. Similar results were obtained using mAb 14.8 (PharMingen) instead of mAb 6B2. To detect TdT^- pro-B cell subset, ethanol-fixed cells were incubated with normal goat serum (Gibco Laboratories) for 30 min at room temperature to block non-specific binding sites for Ab, then stained with rabbit anti-TdT (Supertechs Inc., Bethesda, MD) for 1 h and further incubated with FITC-conjugated goat anti-rabbit IgG (Southern Biotechnology Associates) for 30 min at room temperature.

2.3.3. Immunofluorescent Cell Surface Labeling and Cell Sorting

BM cells were suspended in staining buffer (PBS without Ca^{++} and Mg^{++} with 3% fetal calf serum (FCS) and 0.1% sodium azide). Three-colour analysis was performed to resolve subfractions of B lineage precursors using the modified procedures described by Hardy and colleagues (Hardy et al., 1991; Medina and Kincade, 1994). BM cells were incubated with FITC-conjugated M1/69 (HSA),PE-conjugated14.8 and biotinylated-S7 (CD43, PharMingen)and then stained with streptavidin-Cychrome to reveal the biotinlated Ab. Fraction (Fr.)A cells were discriminated by their lack of expression of HSA among the $B220^+CD43^+$ population. After an initial depletion of $sIgM^+$ cells by magnetic beads Fr. B cells were collected with the defined phenotype of $B220^+HSA^+BP\text{-}1^-$. When samples were stained with FITC-BP-1 instead of M1/69 and gated among the $B220^+CD43^+$ population, Fr.C and Fr.(A+B) were defined by positive and negative expression of BP-1, respectively. Fr.D ($B220^+IgM^-$) was resolved by staining with FITC-anti-IgM (PharMingen) as well as S7 and 14.8. In separate cell fractionation experiments, mAbs 6B2 and CD24 were substituted for 14.8 and M1/69, respectively, with identical results. Stained cells (5-10 x 10^7) were sorted using a dual laser flow cytometer (FACStar^{plus}, Becton Dickinson) by gating on forward *vs.* side scatter to exclude red blood cells and cell clumps. Fractions of B lineage cells (5-10 x 10^5) were collected into 3% heat inactivated FCS in PBS.

2.3.4. Stripping Surface-bound Abs and Cytocentrifugation

Cell surface bound Abs were proteolytically removed by using a modified method

(Ashman, 1980). Sorted B lineage cells were treated with 2 mg/ml pronase (Sigma Chemical Co.) at 37°C for 15 min, then intensively washed with cold PBS, and centrifuged at 200 g for 15 min. Cells were resuspended in medium (pH 7.4) containing 0.15M NaCl, 2.7 mM EDTA (Fisher Scientific Co. NJ) and 5% (w/v) bovine serum albumin (BSA, Boehringer Mannheim, Germany) and cytocentrifuged at 200 g for 5 min (Shandon Cytospin, Shadon Instruments, Sewickly, PA) onto glass slides previously coated by centrifugation (5min, 200 g) with 100μ l of PBS/BSA (3% w/v) and quickly air dried. In order to evaluate the effectiveness of stripping surface bound Abs, enzyme-treated samples from each sorted fractions were examined by either flow cytometry or fluorescence microscopy of cytocentrifuged preparations. No fluorescent residues were detected.

2.3.5. Intracellular TdT and μ Chain Staining and Fluorescence Microscopy

For TdT staining, slides of each fraction were fixed in cold absolute methanol for 30 min on ice and hydrated in gradually reducing concentrations of methanol in PBS. Cytospots were blocked with 50 μl normal goat serum, washed with PBS and incubated overnight with rabbit anti-TdT Ab (1:10 dilution with 0.25% saponin in PBS) in a humidified chamber at room temperature followed by incubation with FITC-conjugated goat anti-rabbit IgG for 30 min at room temperature. For μ chain staining, slides were fixed with cold 5% (v/v) glacial acetic acid in absolute ethanol for 30 min on ice , then washed and incubated with FITC-conjugated goat-anti-mouse μ chain mAbs for 30 min and washed thoroughly with cold PBS. The immunolabeled cytospot preparations were

mounted in 90% (v/v) glycerol in PBS (pH 8.0) containing 0.1% (w/v) para-phenylenediamine (Sigma Chemical Co.) to prevent quenching of fluorescein. Slides were examined using a phase-contrast, epifluorescence microscope equipped with a 100 x Neofluar PH3 objective (Carl Zeiss, Don Mills, Ontario, Canada). A minimum of 10,000 cells of each B cell fraction were scored for TdT and μ chain labeling.

2.4. Results and Discussion

2.4.1. *Immunophenotypic Analysis of B Cell Development in BM by Flow Cytometry*

An initial flow cytometric analysis of precursor B cell populations provided a comparison with previous studies by Osmond et al. based on epifluorescence microscopy (Osmond, 1990). BM cell suspensions were immunofluorescence-labeled for expression of intranuclear TdT, surface B220, cμ chains and surface IgM (Fig. 2.1). The incidences of precursor B cells of each defined phenotype detected by flow cytometry in whole BM (Fig.2.1, Table 2.1) were consistent with values derived from the previous cell population dynamics studies (Osmond, 1991).

2.4.2. Fluorescence Microscopy of TdT and μ Chain Expression Among Sorted B Cell Fractions

In order to examine the composition of Hardy's B cell fractions, immunolabeling of intranuclear TdT in cytocentrifuge preparations of sorted cell fractions provided clear-cut evidence of TdT expression by individual cells when analysed by epifluorescence

microscopy (Fig.2.2). TdT^+ cells were present throughout Fr. A-C but were absent from Fr.D (Table 2.2; Fig.2.3). The maximum incidence of TdT^+ cells occurred in Fr.B (Table 2.2), which contained almost two thirds of all the TdT^+ cells(Fig.2.3).

Cells expressing cμ, when similarly analysed by microscopy of cytocentrifuged cell fractions, were absent from Fr.A and infrequent in Fr.B, but they formed a majority of cells in Fr.C and accounted for almost all ells in Fr.D (Table 2.2). In relation to the populations as a whole, however, Fr.C represented only a small subset of the total $c\mu^+$ cells, 95% of which were recovered in Fr.D (Fig.3). Most Fr.C cells were large and in some cases exhibited mitotic figures by phase microscopy. Approximately 10% of Fr C cells, however, were small cells, less than 10 μm diameter in cytocentrifuged preparations. Fr.D cells were predominantly small cells by this criterion, though 7.8% were of larger diameter.

A substantial proportion of $B220^+$ cells in Fr.A-C lacked both TdT and c μ expression by epifluorescence microscopy, consistent with previous reports (Osmond, 1990). They formed the large majority of cells in Fr.A (Table 2.2),declining in both incidence and relative frequency from Fr.A to Fr.C (Fig.2.3). Table 2.3 shows the frequencies of TdT^+ and $c\mu^+$ cells in Fr.A-D as percentages of total BM cellularity, combining the recovery of BM cells in each fraction derived from flow cytometry and the percentage composition of each fraction analysed by fluorescence microscopy. The combined frequencies of $B220^+$ TdT^+ cells in Fr.A-C (0.7%) and of $B220^+$ cells lacking both TdT and c μ in Fr.A-D (3.3%) are in general accord with analyses of whole BM samples by epifluorescence microscopy (Osmond, 1990).

2.4.3. Correlation Between Sorted BM Fractions and B Cell Differentiation Stages

Figure 4 compares the phenotypic composition of $B220^+$ cells in Fr.A-D with the scheme of B cell development based on the expression of B220, TdT and μ chains (Osmond, 1990). TdT and μ expression mark significant sequential steps in B cell differentiation, representing stages of μ H chain gene rearrangement and the successful synthesis of complete μ chains, respectively (Desiderio, et al). The tyrosine phosphatase, B220(CD45RA isoform), is also intimately associated with the B cell lineage implicated in B cell signalling (Justement, 1991), and is common to both the TdT/cμ phenotypic model and cell sorting of Fr. A-D. One limitation of B220 as a phenotypic marker recently revealed is that a subset of $B220^+$ cells in mouse BM lack CD19 and at least some of these cells appear to be of non-B lineage (Rolink, 1996). The $B220^+CD19^-$ cells are $TdT^-c\mu^-$ and account for approximately two thirds of the cells of this phenotype (D.G. Osmond, unpublished data). The other cell surface glycoproteins used to define Fr.A-D, the sialoglycoprotein CD43, phosphatidylinositol linked glycoprotein HSA and the ectopeptidase BP-1, provide a convenient combination of markers to separate samples of viable BM cells by fluorescence activated cell sorter (FACS) for experimental analysis. They do not appear, however, to be related to lineage specific events or stages of IgM synthesis and their possible functions and controlling factors during B lymphopoiesis remain unknown.

The present results show that cells in Fr.A-D are heterogeneous with respect to stages of B cell development. In general, the fractions advance in differentiation states from Fr.A to Fr.D, in accord with the difference in time taken for IgM^+ cells to develop from

each fraction in culture (Hardy, 1991). The TdT^+ and μ^+stage markers, however, are smeared across several fractions, overlapping in incidence so that each fraction contains cells at various differentiation stages (Hardy, 1991).

Fr.D, lacking CD43 and IgM, is phenotypically the most homogeneous fraction, consisting almost entirely of $c\mu^+$ pre-B cells. In addition, however, the subset of larger cells observed by microscopy of Fr.D and previous flow cytometric observations of cell size and DNA content (Hardy, 1991), indicate that Fr.D contains substantial numbers of actively proliferating large pre-B cells. All Fr.D cells reportedly express successful VHDHJH gene rearrangements, as well as some VLJL gene rearrangement (Ehlich et al., 1994; Loffert et al., 1994).

Fr.C consists predominantly of actively cycling large pre-B cells. Because the recovered Fr.C is of relatively low cellularity, however, it represents only 5% of the total number of $c\mu^+$ pre-B cells (Table 2.3, Fig.2.3). Furthermore, Fr.C contains a substantial minority of cells of pro-B cell phenotype, including TdT^+ cells, as well as some small pre-B cells. This accords with the finding that individual Fr.C cells may exhibit a range of Ig gene rearrangements, including DHJH, VHDHJH, unsuccessful VHDHJH and some VLJL (Ehlich et al., 1994; Loffert et al., 1994).

Fr.B contains the largest number of TdT^+ pro-B cells, presumably being cells in the process of IgH chain gene rearrangement (Dediderio et al., 1984), together with a minor component of $c\mu^-$ pre-B cells and substantial subset of $TdT^-c\mu^-$ cells (Table 2.2; Figs.2.3, 2.4), a phenotype that includes $CD19^+$ cells (D.G.Osmond, unpublished data). Such late pro-B cells may be postulated to represent cells in which TdT expression has been

switched off but before the synthesis of detectable levels of μ chains (Osmond, 1990). Thus, cells in Fr.B are predominantly DHJH rearranged, some are VHDHJH rearranged, and almost all have germline L chain genes (Ehlich et al., 1994; Loffert et al., 1994).

Fr.A contains one quarter of all the TdT^+ pro-B cells, (Table 2.3, Fig.2.3) but the large majority of cells in this fraction express neither TdT nor cμ (Fig.2.4). Since $B220^+CD19^-$ cells have Fr.A phenotypic characteristics (Rolink et al., 1996; Li et al., 1996), it may be predicted that the $TdT^-c\mu^-$ cells now detected in Fr.A would include $CD19^-$ cells, and that some such cells, though $B220^+$, may have non-B potentials including the NK cell lineage (Rolink et al., 1996; Li et al., 1996). The cells in Fr.A predominantly have germline Ig genes but some have DHJH rearrangements (Ehlich et al., 1994; Loffert et al., 1994). Some $B220^+CD19^-$ cells, nevertheless, include early B cell progenitors, as shown by a low expression of genes encoding pre-B and B cell receptor components and a capacity to generate B lineage cells under appropriate culture conditions (Rolink et al., 1996; Li et al., 1996). Hardy and his colleagues have recently characterized these cells in detail and reported that they reside in a subfraction of Fr. A expressing surface AA4.1 (Li., et al, 1996), a molecule found on early hemopoietic and B lineage precursor cells. Further studies are needed to establish the actual proportions of cells in Fr. A that either are B lineage-committed or have the potential to generate other lineages.

In conclusion, Fr. A-D represent overlapping mixtures of B cells at various differentiation stages, rather than individual developmental compartments in strict linear sequence. The reported variety of Ig gene rearrangement in Fr. A-D (Ehlich et al., 1994; Loffert et al., 1994) and the critical decision points at which B lineage cells abort in

various gene modified mice as characterized by either Fr. A-D or TdT/cμ phenotype are generally consistent with the present study. Thus, in *scid*, RAG-/-, Igβ-/-, λ5-/- and IL-7-/- mice B cells develop apparently normally through TdT^+ stages and Fr. A-B and continue partially into the late pro-B phenotype and Fr. C, but they then abort, either completely or nearly so, before expressing c μ or Fr. D phenotype (Osmond et al., 1994; Melchers et al., 1995; Rajewsky, 1996; Loffert et al., 1994; Osmond et al., 1992). While cell sorting of Fr. A-D represents a convenient method of fractionating BM cells for experimental analysis, caution is necessary in assigning a particular developmental stage to individual cells within any given fraction in the absence of additional lineage-specific criteria. We hope that the present results will be of value in evaluating experimental data derived from the use of Fr. A-D cell sorting and will represent a step towards a unified model of B cell development in mouse BM.

Table 1. B lineage cell populations in mouse BM detected by flow cytometry

	Cell Phenotype					
	TdT^+	$B220^+\mu^-$	$c\mu^+ s\mu^-$		$sIgM^+$	$B220^+$
			Large	Small		
Incidences (%)	2.2±0.4	6.5±1.2	3.4±0.6	8.1±0.9	7.5±0.6	25.5±1.4
Cells/femur($x10^{-5}$)	3.8±0.6	10.1±1.8	5.3±0.9	12.6±1.4	11.7±1.2	39.5±2.2

The BM nucleated cellularity totalled 15.5±0.1 x 10^6/femur. The values are based on results of 5 separate determinations (mean±SD).

Table 2. Incidence of TdT^+ and $c\mu^+$ cells in sorted $B220^+$ B lineage fractions (%)

Cell fraction (Phenotype)	TdT^+ cells	$c\mu^+$ cells	TdT^- $c\mu^-$ cells
A ($CD43^+$ HSA^- $BP\text{-}1^-$)	11.0±1.5	0	89.0±1.2
B ($CD43^+$ HSA^+ $BP\text{-}1^-$)	25.5±1.9	8.2±0.9	66.3±1.5
C ($CD43^+$ HSA^+ $BP\text{-}1^+$)	5.5±1.3	68.6±3.5	25.9±1.8
D ($CD43^-$ IgM^-)	0	96.6±2.5	3.4±1.4

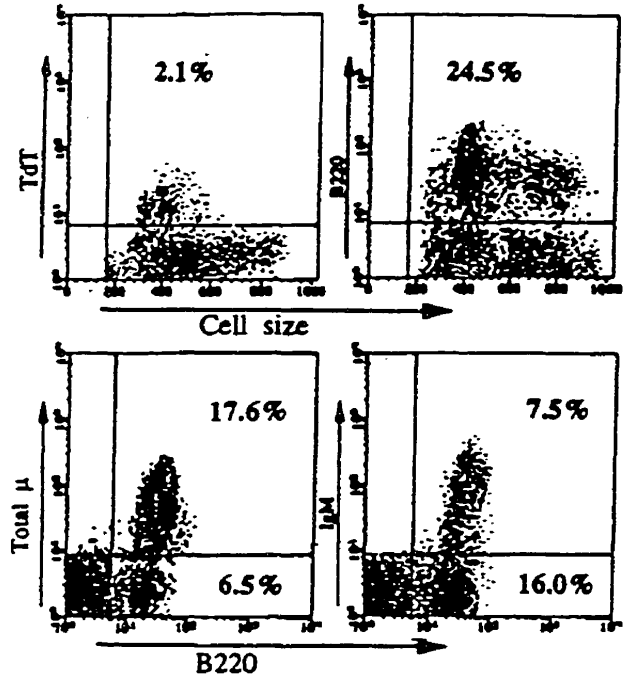
Incidence of TdT^+, $c\mu^+$ and $TdT^-c\mu^-$ cells in cytocentrifuged preparations of sorted B cell fractions examined by epifluorescence microscopy. Data represent five separate experiments (mean±SD).

Table 3. Incidence of TdT^+ and $c\mu^+$ cells in sorted B lineage fractions (% total nucleated BM cells)

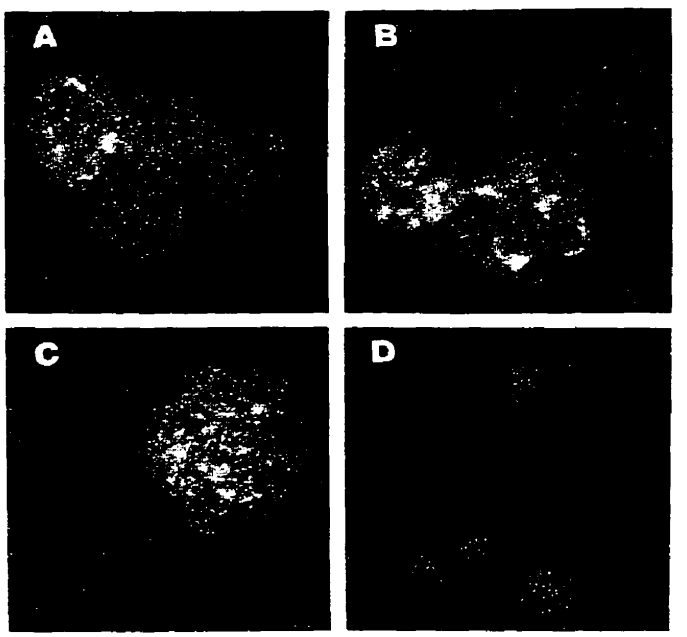
Cell fraction (Phenotype)	Total cells	TdT^+ cells	$c\mu^+$ cells	$TdT^-c\mu^-$ cells
A ($CD43^+$ HSA^- $BP\text{-}1^-$)	1.4	0.2	0	1.3
B ($CD43^+$ HSA^+ $BP\text{-}1^-$)	1.5	0.4	0.1	1.0
C ($CD43^+$ HSA^+ $BP\text{-}1^+$)	1.4	0.1	1.0	0.3
D ($CD43^-$ IgM^-)	21.0	0	20.3	0.7

Incidence of TdT^+, $c\mu^+$ and $TdT^-c\mu^-$ cells in sorted B cell fractions as a percentage of the total cellularity of the BM. Data represent the mean values derived from five experiments.

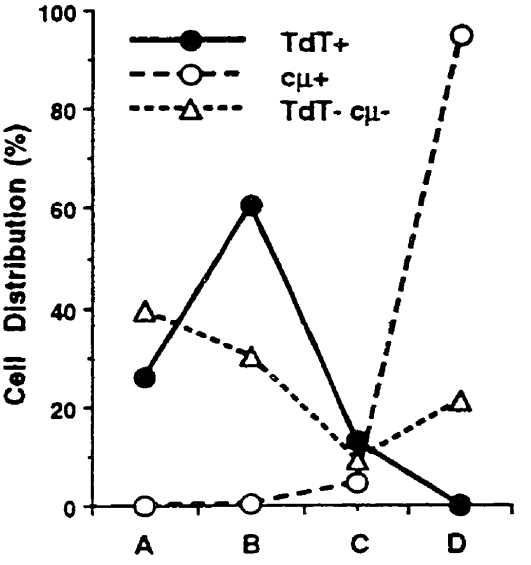
2.1. Immunophenotypic labeling of B lineage cells in BM cell suspensions analyzed by flow cytometry. The incidences of B lineage cells at various defined stages of differentiation from representative samples are given in the dot-plots.


2.1%
24.5%
TdT
B220
Cell size
17.6%
7.5%
6.5%
16.0%
Total μ
IgM
B220

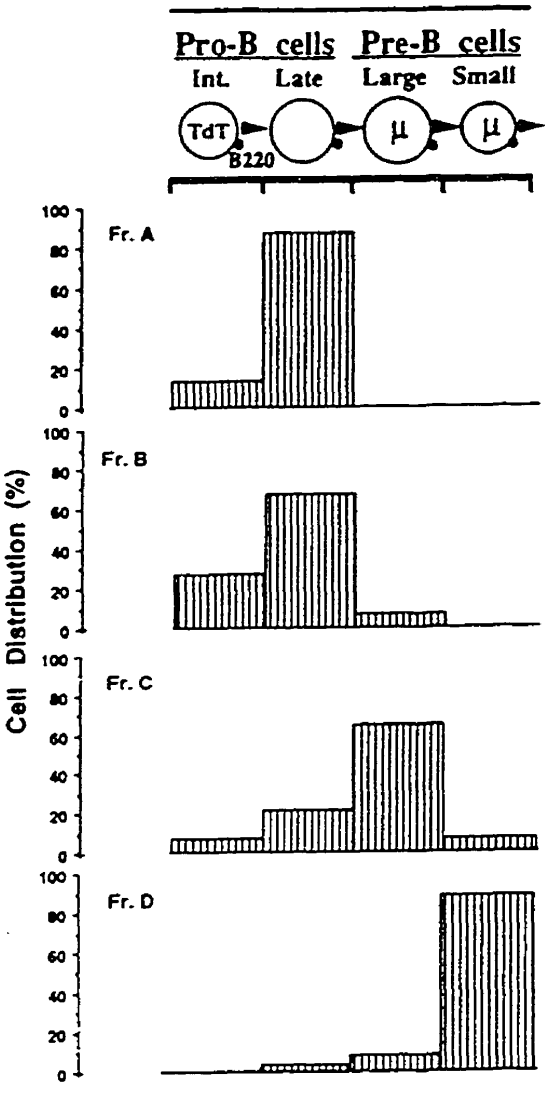
2.2. (A-D) TdT^+ cells detected by immunofluorescence microscopy in sorted B cell fractions A. B. C and D, respectively. (A, B, C: x 1,000; D: x 400).


A
B
C
D

2.3. Distribution of TdT^+, $c\mu^-$ and $TdT^-c\mu^-$ cell populations throughout sorted B cell fractions A-D. The data represent the mean proportions of cells with a given phenotype that are located within each of four fractions and are derived from 5 separate experiments.


100
80
60
40
20
0
Cell Distribution (%)
TdT+
cμ+
TdT- cμ-
A
B
C
D

2.4. Correlation between sorted BM fractions A-D and B cell differentiation stages.


Pro-B cells
Pre-B cells
Int.
Late
Large
Small
TdT
B220
μ
μ
Cell Distribution (%)
Fr. A
Fr. B
Fr. C
Fr. D
100
80
60
40
20
0

Chapter 3 Apoptosis During B Lymphopoiesis in Mouse BM

3.1. Summary

During B cell proliferation in mouse BM, many seemingly aberrant or undesirable cells appear to die and undergo rapid ingestion by local macrophages. To evaluate the nature of this process and its role in B lymphopoiesis, we have examined the incidence and rate of apoptosis within successive phenotypic compartments of B cell development in BM. FACS-sorted $B220^+$ cells exhibited a low incidence of morphologically apoptotic cells by electron microscopy. In freshly prepared BM suspensions, the fraction of apoptotic cells, showing hypodiploid DNA by multiparameter flow cytometry, was greater among large dividing $B220^+sIgM^-$ precursor B cells and immature $sIgM^{Low}$ B lymphocytes, than TdT^+ pro-B cells, small nondividing $B220^+sIgM^-$ cells and mature IgD^+ B lymphocytes. During short term culture, apoptotic cells, identified by both DNA content and *in situ* DNA strand break labeling, increased in number in linear fashion without being ingested by macrophages, providing an assay for the rate of entry into apoptosis. $B220^+$ B lineage cells accumulated in apoptosis more rapidly than cells of other lineages. The apoptotic rate of $B220^+sIgM^-$pre-B cells, was considerably greater than that of $sIgM^+$ B cells and was accelerated more rapidly by Dexamethasone. Co-culture with stromal cell reduced the apoptotic rate of $B220^+sIgM^-$ pre-B cells to a greater extent than of immature $sIgM^+$ B lymphocytes. The results lead to estimates of the actual number of cells undergoing apoptosis and demonstrate that, while influenced

by local microenvironmental factors, apoptotic cell death occurs most markedly at two phenotypic stages, soon after Ig VH gene rearrangement and antigen receptor expression, respectively.

3.2. Introduction

B cell genesis in mammalian BM represents a delicate balance between cell production and loss. Factors modifying either one of these processes may determine the number and functional properties of B cells delivered into the immune system. Proliferative events and factors have been extensively studied. However, the magnitude and nature of cell loss at successive stages of B lymphopoiesis *in vivo* have been unclear (Osmond et al., 1994).

The first *in vivo* evidence of substantial cell loss among precursor B cells in normal mouse BM was provided by population dynamics data (Opstelten and Osmond, 1983). A combination of immunofluorescence and mitotic arrest techniques was used to measure cell production and turnover rates at successive stages of B cell development *in vivo* (Osmond, 1990). Six phenotypic compartments were defined by the expression of TdT, B220 glycoprotein (CD45RA isoform) and the μ H chain of IgM. Early progenitor B cells before the expression of μ chains termed pro-B cells. The subsequent population of pre-B cells, expressing cμ , includes both large dividing and small post-mitotic forms that develop finally into immature B lymphocytes expressing surface IgM (sIgM). Measurements of cell production and turnover in successive phenotypic cell

compartments revealed a considerable discrepancy between the kinetics of large and small pre-B cells, indicating that a large fraction of cells are actually lost and rapidly eliminated in the BM at this stage (Opstelten and Osmond, 1983). The possibility of cell loss at other stages was also raised but could not be proven by cell production data. An elevated cell loss at various stages of B lineage differentiation has been observed in perturbed situations which increase the proliferation of precursor B cells. These conditions include the influence of the E μ *-myc* transgene (Jacobsen et al., 1994; Sidman et al., 1993) and macrophage activation associated with pristane-induced granuloma and murine malaria (Rico Vargas et al., 1995). Complete or almost complete cell loss occurs under *in vivo* conditions in which developing B cells cannot successfully rearrange IgH chain genes (Shikai et al., 1992; Elich et al., 1993), or are unable to express surrogate L chains (Kitamura et al., 1991) or are deprived of IL-7 (von Freeden-Ieffry et al., 1995). The physical deletion of certain autoreactive newly-formed B cells in BM is also now generally accepted (Melchers et al., 1995). Little is known, however, of the extent and significance of apoptosis at the various stages of primary B cell differentiation and selection in BM of normal mice (Osmond et al., 1994; Schwartz and Osbone, 1993)

Apoptosis is a highly regulated process of ell death, triggered by a variety of intrinsic and extrinsic signals (Allen et al.,1993; Cohen, 1992). Salient features of the process include an early surface expression of phosphatidylserine that is normally confined to the inner aspect of the plasma membrane (Vermes et al., 1995), chromatin condensation, nucleosomal DNA fragmentation (Bortner et al., 1995), and hypodiploid DNA content (Nicoletti et al., 1991), accompanied or followed by recognition and ingestion by local

tissue phagocytes (Savill et al., 1993). Morphological evidence provided the first indication that B ell loss in BM involves apoptotic cell death. It was found by electron microscopy that certain $B220^+$ cells in BM, immunolabeled *in situ*, exhibited apoptotic characteristics (Jacobsen and Osmond, 1990; Allen et al., 1993; Golstein et al., 1991; Kerr et al., 1972; Wyllie et al., 1984; Cohen, 1993) and could soon be observed within the cytoplasm of resident macrophages (Jacobsen and Osmond, 1991). Because of the rapidity with which apoptotic cells in BM are recognized and engulfed by macrophages the number of apoptotic cells found at any given time is small. Thus, measurement of the incidence of apoptosis alone cannot accurately assay the true magnitude of apoptosis *in vivo*.

The present study has aimed to evaluate the nature and kinetics of cell death occurring during the course of normal B lymphopoiesis in mouse BM. Apoptotic criteria have been established by electron microscope morphology, and evidence of DNA fragmentation using BM cell suspensions and FACS-sorted cell fractions. Annexin-V has been used to label the surface phosphatidylserine on early apoptotic B cells. Short-term cultures of BM cell suspensions and fractions have been analysed by immunofluorescence labeling and multiparameter flow cytometry in conjunction with DNA content staining and *in situ* DNA strand break labeling to examine the *ex vivo* incidence of apoptosis and the relative rate at which cells enter apoptosis at phenotypic stages of B cell differentiation. This system has been used to compare the susceptibility of precursor B cells to induction of apoptosis by dexamethasone (Dex) and the protective effect of coculturing BM cells with a stromal cell clone capable of supporting both B

lymphopoiesis and myelopoiesis (Ogawa et al., 1988). Combined with previous population dynamics data, the findings contribute to a provisional kinetic model of B cell production and selection in mouse BM.

3.3. Materials and Methods

3.3.1. Mice

Male C3H/HeJ mice (Jackson Laboratories, Bar Harbor, ME) were used at 8-10 wk of age.

3.3.2. Cell Suspension and Short Term Culture

Femoral marrow cells were flushed and pooled from groups of 3-5 mice as described (Park and Osmond, 1987). After washing with newborn calf serum (NCS; Gibco Laboratories, Grand Island, NY), nucleated cells were counted using an electronic particle counter (Coulter Electronics, Burlington, ON). Cell suspensions were adjusted to 40 x 10^6 cells/ml in minimal essential medium (MEM) with L-glutamine (Gibco Laboratories), supplemented with 10%(v/v) NCS. Aliquots of cell suspensions(100 μ l) were either assayed immediately or incubated in MEM-NCS with or without the addition of 1 μ M Dex (Sigma Chemical Co, St. Louis, MO) for time intervals up to 8 h at 37°C in a humidified atmosphere with 5% CO_2.

3.3.3. Panning Separation of BM B220⁺ Cells

Polystyrene petri dishes were coated with goat anti-rat IgG mAb (Kirkegaard and Perry Laboratories, Gaithersburg, MD) in 0.05M Tris-HCl , 0.15 M NaCl buffer (pH 9.5), then coupled with affinity purified rat mAb 14.8 from cultures of hybridoma cells (American Tissue Type Collection, Rockville, MD) as described (Jacobsen and Osmond, 1991). BM cells were evenly placed in coated dishes and incubated at 4°C for 30 min. After extensive rinsing of plate with cold PBS, adherent B220⁺ cells were gently recovered using a plastic scraper (Costar, Cambridge, MA).

3.3.4. Detection of DNA Fragmentation by Gel Electrophoresis

5 x 10^6 panning-purified B220⁺ cells and untreated BM samples were cultured in MEM-NCS for 24 h, and washed by centrifugation in ice-cold PBS. Cells were lysed by incubating at 37°C for 1 h with extraction buffer (10 mM Tris·HCl, pH 8.0, 0.1 M EDTA, 20 μg/ml pancreatic RNAase, 0.5% SDS). Proteinase K (100 μg/ml) was added to the suspensions in a water bath for 3 h at 50°C. The lysates were extracted 3 times with phenol/chloroform (1:1). Samples were adjusted to 0.3 M of sodium acetate and DNA was precipitated with 2 volumes of cold ethanol. After overnight incubation at -20°C, DNA was recovered by centrifugation (13,000 g, 15 min, 4°C). The pellets were washed with 70% ethanol, vacuum dried and resolved in 50 μl TE buffer. 2 μg DNA were loaded into wells of 1% agarose gel containing ethidium bromide. After electrophoresis, the gel was examined under UV light and photographed with a Polaroid camera.

3.3.5. Stromal Cell Line and Co-culture

The stromal cell clone ST2 used in this study was established in Dr.Kincade's Laboratory (Oklahoma Medical Research Foundation, Oklahoma, USA). ST2 stromal cells in 6-well plates were grown to confluence in RPMI 1640 (Gibco Laboratories) supplemented with 5% FCS. The culture medium was then removed and 2 ml MEM with 10% FCS was added into each well. Freshly prepared BM cells (1×10^6) were placed either on the stromal monolayer or in medium alone and incubated at 37°C for 0-8 h. Finally, BM cells were collected first by gently pipetting, then 2 ml 1 mM EDTA in PBS was used to harvest the remaining BM cells without detaching the adherent stromal layer by microscopic examination.

3.3.6. Immunophenotypic Staining of Precursor B Cells

BM cell samples were incubated with FITC-conjugated anti-mouse-B220 (clone: RA3-6B2), -IgM and -IgD Abs (Southern Biotechnology Associates, Birmingham, AL), respectively (30 min, 4°C). To identify TdT^+ pro-B subsets, cells were fixed with 70% ethanol (50 min, 4 °C), resuspended with 200 µl normal goat serum (Gibco Laboratories) for 30 min at room temperature (RT) to block non-specific binding sites for Ab, then stained with rabbit anti-TdT (Supertechs Inc., Bethesda, MD) for 1 h at RT and further incubated with FITC-conjugated goat anti-rabbit IgG (Southern Biotechnology Associates) for 30 min at RT. Samples stained with irrelevant isotype Abs were used as negative controls. To examine $B220^+sIgM^-$ precursor B cells, samples were stained with FITC-anti B220 and PE-anti IgM (PharMingen, San Diego, CA). Large and small

$B220^+sIgM^-$ cell populations were separated according to forward scatter scale using a cell sorter (FACS Vantage, Becton Dickinson, Mountain View, CA). To examine cell size, some samples were deposited (1500 rpm, 5 min) on glass slides using a cytocentrifuge (Cytospin, Shandon Southern Instruments, Sewickly, PA). An epifluorescence microscope (Carl Zeiss of Canada, Don Mills, ON) equipped with phase-contrast optics was used for cell diameter analysis.

3.3.7. Flow Cytometric Analysis of Apoptosis

After immunofluorescent phenotypic staining, cell samples were washed with NCS and PBS (Ca^{++} and Mg^{++} free, Gibco Laboratories), fixed dropwise with ice-cold 70% ethanol (50 min, 4°C) and washed extensively with PBS. Samples were suspended in 50 μg/ml RNase (Boehringer Mannheim Biochemia, Mannheim, Germany) and 50 μg/ml propidium iodide (PI; Sigma Chemical Co.) in PBS and kept on ice in the dark until analysis. Using a FACScan flow cytometer equipped with a doublet discrimination module, FITC and PI were excited with the 480 nm line of an argon laser and emissions were detected at 550 nm and 650 nm, respectively. A minimum of 10,000 events was collected for each sample, gated on forward scatter *vs.* side scatter to exclude debris and cell clumps. Cell cycle was analysed using FACScan LYSIS II software (Becton Dickinson). The coefficient of variation for the G_0/G_1 peak of whole BM cells was normally less than 3%. Apoptotic cells were identified in the hypodiploid region of DNA content profiles, as described (Telford et al., 1994).

3.3.8. Evaluation of Apoptosis by Electron Microscopy

Freshly prepared BM samples were immunostained with FITC-anti mouse B220, then fixed with 70% ethanol (50 min, 4 °C). After washing with PBS, $B220^+$ cell fractions were separated by cell sorting and further fixed with 2.5% glutaraldehyde in 0.1M cacodylate buffer (pH 7.4) for 2 h. The centrifuged cell pellets were post-fixed in 1% osmium tetroxide in buffer, dehydrated in ethanol and embedded in Epon-812. Thin sections, counterstained with uranyl acetate and lead citrate were examined with a Philips 301 electron microscope.

3.3.9. In situ Assay of Apoptosis by DNA Strand Break Labeling

Apoptotic cells show new 3'-OH DNA ends generated by nuclear DNA fragmentation, which can be identified in nuclei and apoptotic bodies by direct immunoperoxidase detection of digoxigenin-labeled genomic DNA (Schmitz, 1991; Gavrieli et al., 1992). Freshly prepared and cultured BM cell suspensions were fixed with 4% neutral buffered formalin (20 min, RT). After extensive washing in PBS, cells were deposited on glass slides by cytocentrifugation. Hydrogen peroxide (2%) was then used to quench endogenous peroxidase (15 min, RT). *In situ* DNA strand break labeling was carried out using an apoptosis detection kit (ApopTag, Oncor, Gaithersburg, MD). Briefly, samples were incubated with reaction buffer, anti-digoxigenin-peroxidase was applied and the staining color was developed by adding 0.05% (w/v) diaminobenzidine (Sigma Chemical Co.) in PBS and 0.02% (v/v) hydrogen peroxide. Sham staining was performed simultaneously by substituting distilled water for TdT enzyme and continuing

with the staining procedure. 0.5% (w/v) methyl green (BDH Laboratory supplies, Poole, England) in 0.1 M sodium acetate (pH 4.0) was used for nuclear counterstain.

3.4. Results

3.4.1. B Cell Death in BM Takes the Form of Apoptosis

$B220^+$ B lineage populations from mouse BM purified by cell sorting were examined by electron microscopy. Certain $B220^+$ cells presented morphological characteristics of apoptosis. The cells showed chromatin condensation, forming electron-dense crescentic masses lying against an intact nuclear envelope (Fig.3.1A). Other features included nuclear shrinkage and plasma membrane blebbing (Fig.3.1B). These ultrastructural alterations clearly indicated that some B lineage cells die by apoptosis in normal mouse BM.

3.4.2. Incidence of Apoptosis ex vivo at Defined Stages of B Cell Development

Samples of freshly prepared BM cell suspensions were immunofluorescence-labeled for expression of intranuclear TdT, cμ and the cell surface determinants, B220, IgM and IgD, in conjunction with PI staining of DNA content. Flow cytometry using this small panel of markers thus provides a rapid quantitative analysis of phenotypic compartments representing meaningful events in B cell development. As shown by DNA profiles (Fig.3.2), TdT^+ pro-B cells were a cycling population. Large $B220^+sIgM^-$ cells were particularly rapidly dividing, 45% being in S, G2 and M phases of cell cycle. Small

$B220^+sIgM^-$ cells, in contrast, were postmitotic, 90% being in G0/G1 phase. $sIgM^+$ cells and $sIgD^+$ cells were also non-dividing postmitotic forms.

A small number of cells with hypodiploid DNA content were detected within each defined stage of B cell development (Fig.3.2). After double immunolabeling, $B220^+sIgM^-$ precursor cells were sorted into large and small $B220^+sIgM^-$ cell fractions according to their forward scatter (Fig.3.3). The cell size distribution was verified by microscopy of cytocentrifuged preparations, 95% of cells in the large $B220^+sIgM^-$ fraction being larger than 10 μm in diameter and 96% of small $B220^+sIgM^-$ cells being less than 10 μm, agreeing closely with previous data (Osmond, 1990). As shown in Fig. 3.4A, large $B220^+sIgM^-$ showed a high incidence of apoptosis (3.2%), approximately 3-fold greater than apoptotic incidences among the other precursor B cell populations. A further study within this differentiation stage before the expression of cμ ($B220^+\mu^-$), gave an even higher apoptotic incidence (3.5%). The next highest apoptotic incidence was that of immature $sIgM^{low}$ B lymphocytes (1.5%). In contrast, TdT^+ pro-B cells and $sIgM^{high}$ mature B lymphocytes both displayed relatively low apoptotic incidences (0.9% and 0.7%, respectively). Converting the percentage of apoptotic cells within each defined precursor cell compartment into number relative to whole BM (Fig.3.4B) revealed that apoptotic B lineage cells were present in mouse BM cell suspensions in a total frequency of 45 apoptotic cells per 10,000 BM cells. Almost half of the apoptotic cells were large $B220^+sIgM^-$ cells. This frequency of apoptotic cells is a minimal estimate of *in vivo* apoptosis, representing only the number of B lineage ells detectable during the brief time period between the onset of apoptosis and their removal by macrophages (Osmond et

al., 1994).

The incidence of annexin V-binding apoptotic cells among fresh panning-purified $B220^+$ cells (2.1%±0.5) was somewhat higher than that of hypodiploid $B220^+$ cells (1.3%±0.3%), consistent with the detection of an earlier stage of apoptosis (Fig.3.5).

3.4.3. Entry of BM Cells into Apoptosis in Short Term Culture without Macrophage-Mediated Deletion

To provide a more sensitive quantitation of the rate at which precursor B cells may become apoptotic we have examined a short term culture system designed to allow apoptotic cells to accumulate without being phagocytosed. BM single cell suspensions were cultured for various time intervals from 0 h to 8 h at 37°C, after which both DNA strand break *in situ* labeling and PI DNA staining were performed on respective aliquots.

Apoptotic cells were detected by microscopy of DNA stand break labeling on cytocentrifuged cell preparations (Fig.3.6). Positive labeling was identified by enzyme reaction product in apoptotic nuclei. In addition, apoptotic bodies of various sizes, typical features of nuclear fragmentation, showed intense staining after 6-8 h incubation. Some apoptotic cells also showed cytoplasmic staining, suggesting leakage of DNA fragments from the nucleus. Microscopic examination demonstrated that the labeled apoptotic cells accumulated as single cells in suspension without any evidence of being ingested by macrophages throughout the 8 h culture period.

By flow cytometry, the incidence of hypodiploid BM cells increased progressively during culture (Fig.3.7). The hypodiploid population showed decreased forward scatter

from 4 h onward, reflecting the reduced volume of cells undergoing apoptosis (Fig.3.8A,B). To check whether cultured BM cells displayed the major biochemical hallmark of apoptotic cell death, the cleavage of chromosomal DNA at inter-nucleosomal sites into fragments, genomic DNA from panning-purified $B220^+$ cells and untreated BM cells after 24 h culture was examined by gel electrophoresis. DNA fragmentation was evident as nucleosomal " laddering " without evidence of random-sized fragments, most marked among $B220^+$ cells (Fig.3.9). Freshly prepared BM cells showed no such DNA degradation (Fig.3.9). The incidence of apoptosis at each incubation time interval derived from the specific method of DNA strand break labeling agreed closely with the results of hypodiploid DNA content analysis (Fig.3.10), demonstrating the reliability of flow cytometric analysis of DNA content as a measure of apoptotic cells in the present work.

3.4.4. B Lineage Cells, Notably $B220^+sIgM^-$ Precursor Cells, are Particularly Prone to Apoptosis

To compare the susceptibility to apoptosis at various stages of B cell differentiation, BM cultures were performed, measuring the apoptotic incidence by flow cytometry of DNA content at 2-hourly intervals from 0 h to 8 h. In freshly prepared suspensions, the apoptotic incidence of $B220^+$ B lineage cells (1.2%) was somewhat higher than that of whole BM cells (0.9%) and of total μ^+ cells (0.9%). After 8 h incubation, the incidence of apoptosis among $B220^-$ B lineage cells (28.1%) considerably exceeded that of $B220^-$ "non-B" cells (6.8%) (Fig. 3.11A). Among $B220^+$ cells the percentage of apoptotic cells

in the B220$^+$sIgM$^-$ precursor population at 0 h (1.6%)was greater than that of sIgM$^+$ B cells (1.0%) and increased to much greater values by 8 h (34.5% and 15.1%, respectively) (Fig. 3.11B). Thus, B lineage cells are considerably more prone to undergo apoptosis than non-B lineage cells in BM and B220$^+$sIgM$^-$ precursor cells become apoptotic more extensively than sIgM$^+$ B cells. To dissect further the B precursor populations, total μ chain immunolabeling was performed to distinguish pro-B cells (B220$^+$μ$^-$) from mixed pre-B and B cell populations (B220$^+$μ$^+$). The B220$^+$μ$^-$ population became apoptotic much more rapidly than B220$^+$μ$^+$ cells in short term culture (Fig. 3.11C).

When cell suspensions were incubated with 1μM Dex, the apoptotic incidence at each time point was markedly increased among both B220$^+$sIgM$^-$ precursor cells and sIgM$^+$ B lymphocytes (Fig. 3.11D, E).

3.4.5. Kinetics of Precursor B Cell Apoptosis during Short Term Culture

For all phenotypic cells examined, the incidence of apoptosis showed an initial lag during the first 2 h incubation, followed by an approximately linear increase from 2 h to 6 h (Fig. 3.11). Apoptotic cells continued to increase in incidence at a similar or somewhat reduced rate from 6 h to 8 h. Measurements of the slope of the apoptotic curve during the linear phase at 2-6 h showed that the rate at which cells were accumulating in apoptosis was much greater among B220$^+$ B lineage cells (5.5%/h) than B220$^-$ non-B BM cells (1.0%/h). Among B lineage cells, the apoptotic rate among B220$^+$sIgM$^-$ precursor cells (7.3%/h) was 3 times higher than that of sIgM$^+$ B cells

(2.2%/h), while the highest apoptotic rate was that of $B220^+ \mu^-$ pro-B cells (9.0%/h)

Incubation with Dex had virtually no effect on apoptosis of $B220^-$ non-B lineage cells (1.0±0.2%/h *vs.* 1.3±0.4%/h), but increased the apoptotic rate of $B220^+$ cells from 5.5±0.6%/h to 8.4±0.8%/h (Fig. 3.12). The apoptotic rates of both $B220^+sIgM^-$ precursor cells and $sIgM^+$ B cells were increased to a similar extent, approximately two-fold (from 7.3±0.5%/h to 12.5±0.7%/h and 2.2±0.3%/h to 4.4±0.2%/h, respectively) (Fig. 3.12).

3.4.6. Apoptosis of Precursor B Cells is Modified by Co-culture with Stromal Cells

Freshly prepared BM cell suspensions were co-cultured on a ST2 stromal cell monolayer for periods of 0 h to 8 h (Fig. 3.13). From 0.5 h onwards, most BM cells formed clusters adhering to stromal cells. Since the adhesion could be disrupted only partially by agitation, EDTA treatment was used to detach essentially all the adherent cells for representative sampling.

The incidences of hypodiploid B lineage cells at each time interval were decreased in the presence of ST2 cells compared to cells cultured in medium alone (Fig. 3.13). However, precursor B cells and B lymphocytes were affected to markedly different extents. The apoptotic index of $B220^+sIgM^-$ cells after 8 h co-culture with ST2 cells was reduced considerably (from 4.3%/h to 2.3%/h). In contrast, the apoptotic index of $sIgM^+$ cells at 8 h showed less reduction (from 14.7% to 10.9%) and apoptotic rate remained almost unchanged (1.8%/h). Thus, ST2 stromal cells partially protected $B220^+sIgM^-$ precursor cells from apoptosis but had little effect on apoptosis of $sIgM^+$ B lymphocytes.

3.5. Discussion

The present study identifies apoptotic cells within the B cell lineage in mouse BM and reveals distinctive incidences of apoptosis at successive stages of normal B cell development and selection. We have determined the absolute number of apoptotic cells within phenotypic precursor B cell compartments and obtained a measure of the rates at which the cells appear to enter the apoptotic pathway. These results can be added to our previous model of B cell genesis, incorporating estimates of the fraction of cells undergoing apoptosis at critical decision points in B lymphopoiesis.

The loss of precursor B cells predicted from population dynamics data has been proposed to involve cell death by apoptosis (Osmond et al., 1994; Jacobsen et al., 1994; Rico Vargas et al., 1995; Rajewsky, 1996). This has now been verified by the multiple criteria of electron microscopy, nucleosomal DNA fragmentation, DNA strand break labeling, hypodiploid DNA content and annexin V labeling. Of these techniques, hypodiploid DNA content analysed by flow cytometry provides a convenient indicator of apoptosis in B lineage cells. The correlation between results obtained by this method and by specific *in situ* labeling of DNA strand breaks in cytocentrifuged preparations demonstrates the reliability of using hypodiploid DNA content to monitor apoptosis among cultured BM cells.

$B220^+$ B lineage cells in both freshly prepared and cultured BM suspensions exhibit a higher incidence of apoptosis than non-B lineage cells. Features of B lineage cells in BM that make them especially susceptible to apoptosis include the rearrangement of Ig

H and L chain genes, and cross linking of surface receptors by autoantigens. The present results show that apoptosis does not occur randomly at various stages of differentiation. The incidence of apoptosis at an early stage expressing TdT, representing cells undergoing Ig H chain gene rearrangement (Desiderio et al., 1984), is closely similar to that of BM cells as a whole. However, at the next stage of large $B220^+sIgM^-$ cells, a phenotype that includes both late μ^- pro-B cells and large cytoplasmic μ^+ pre-B cells, the apoptotic incidences are considerably higher than at any other stage. Thus, assuming constant kinetics of clearance of apoptotic cells by resident macrophages, large $B220^+sIgM^-$ cells appear to be particularly susceptible to apoptosis. This result, in agreement with observations of *in vivo* cell loss (Opstelten and Osmond, 1983), suggests an extensive elimination of precursor B cells soon after Ig H chain gene rearrangement. This accords with the findings in abnormal conditions including the *scid* mutation and RAG-deficiency, in which only a few or no successful Ig H chain gene rearrangements are possible (Bosma et al., 1983; Mombaerts et al., 1992). It has been shown that *scid* mutant BM shows extensive morphologic B cell apoptosis and macrophage-mediated deletion (Jacobsen and Osmond, 1991). The transition from μ^- pro-B to large μ^- pre-B stage is characterized by downregulation of c-kit, TdT and RAG (Melchers et al., 1995). Large pre-B cells have at least one of their IgH loci VHDHJH-rearranged and they express μ H chains, whereas their IgL chain gene loci remain in germline configuration (Chen and Alt, 1993; Hardy et al., 1991). It is estimated that almost half of all large pre-B cells should not express a μ H chain, if VH to DHJH rearrangements occur in- and out-of-frame in a random fashion (Melchers et al., 1995). Therefore, a selection of precursor

B cells expressing productively rearranged μ H chains within the pre-B compartment appears to occur before the expression of L chains. The present results support the view that apoptotic cell death is the fate of precursor B cells that generate non-productive or aberrant IgH chain gene rearrangements during normal B lymphopoiesis *in vivo* (Osmond et al., 1994).

The signals determining precursor B cell apoptosis or survival are not known. It may be noted, however, that surrogate L chain complexes are expressed transiently by pro-B and large pre-B cells (Karasuyama et al., 1994; Winkler et al., 1995; Lassoued et al., 1993; Karasuyama et al., 1993; Melchers et al., 1993), while targeted disruption of the Λ5 gene results in massive depletion of pre-B cells (Rajewsky, 1996; Loffert et al., 1994), and preliminary studies suggest that cells expressing surrogate L chain complex on their surface exhibit exceptionally low incidences of apoptosis (L. Fauteux, L. Lu, J. Canton, F. Melchers, and D. G. Osmond, unpublished data). The possible functional role of surrogate L chains in blocking apoptosis in B cells with successful Ig H chain gene rearrangements remains to be further elucidated.

The second highest incidence of apoptosis among B lineage cells in BM is observed among immature $sIgM^{low}$ B lymphocytes, almost 3 times higher than that of mature $sIgD^{+}$ B cells whose apoptotic incidence resembles that of BM cells as a whole. This is consistent with studies suggesting that newly-formed B lymphocytes are subjected to selection in establishing self-tolerance. Findings in transgenic mice show that immature B cells with autoreactive specificities can be either eliminated (clonal deletion) or inactivated (clonal anergy) within BM (Lawton and Cooper, 1974; Nemazee and

Burki, 1989; Nemazee and Burki; 1989; Goodnow et al., 1988). In mice treated with anti-IgM Ab from birth, cross-linkage of surface Ag receptor by Ab leads to the elimination by apoptosis and macrophage-mediated deletion of all immature $sIgM^+$ B cells as soon as they develop in BM (Jacobsen et al., 1994; Jacobsen and Osmond, 1991; Fulop et al., 1983). Such deletion of newly-formed B lymphocytes appears to require cross-linking of Ag receptor above a critical threshold (Nossal, 1996). Less intense cross-linking results in delayed elimination of $sIgM^+$ B lymphocytes either during $sIgD^-$ to $sIgD^+$ transition (Carsetti et al., 1995) or later, after a period of functional anergy (Fulcher and Basten, 1994). Only a fraction of immature B lymphocytes appear to be able to escape apoptosis when they encounter autoantigens in BM by further rearrangements of their Ig L chain genes to alter their Ag receptor specificity (receptor editing) (Radic et al., 1993; Rolink et al., 1993; Tiegs et al., 1993). Thus, the present finding of extensive apoptosis among immature $sIgM^{low}$ B lymphocytes seems likely to represent the negative selection of cells encountering autoantigens presented in high concentration in BM, and cells that have undergone faulty L chain gene rearrangements.

B cell genesis *in vivo* can be modified by both systemic factors and the local microenvironment (Kincade, 1994; Garvy et al., 1993; Wyllie, 1980; Kincade, 1991; Jacobsen, 1992). We now show the suitability of a short term culture system to assay the influence of potential modifiers of apoptosis at various stages of B cell development. Both $B220^+sIgM^-$ precursor cells and $sIgM^+$ B lymphocytes show increased rates of entry into apoptosis when exposed to Dex, a corticosteroid known to trigger cell death (Wyllie, 1980). In contrast, coculture with IL-7 producing ST2 stromal cells reduces the

rate of apoptosis among $B220^+sIgM^-$ precursor cells but has virtually no effect on apoptosis of $sIgM^+$ B cells. This accords with findings in IL-7 deficient mice that survival of cells at the large pro-B cell stage *in vivo* depends critically upon the presence of IL-7 (von Freeden-Jeffry et al., 1995; Grabstein et al., 1993; Sudo et al., 1989; Hayashi et al., 1990), while in IL-7 transgenic mice apoptosis of large $B220^+sIgM^-$ cells occurs at a much reduced rate but apoptosis of $sIgM^+$ B cells is unaffected (L. Lu, P. Chaudhury and D. G. Osmond, unpublished data). The results suggest that stromal cell-dependent processes are involved in promoting survival as well as proliferation and differentiation of precursor B cells *in vivo*, but not in selecting immature B cells (Oritani and Kincade, 1996).

Only a small number of apoptotic cells are detected in freshly prepared BM cell suspensions, reflecting the rapidity with which dying cells are removed by macrophages *in vivo* (Jacobsen and Osmond, 1991). During short term culture of BM cell suspensions, however, apoptotic cells accumulate and small differences in the initial incidence of apoptosis become substantial. The initial lag in increase of apoptotic incidence during the first 2 h of culture may represent the time taken to recover from metabolic inhibition caused by preparing the sample on ice. The subsequent linear phase at 2-6 h reflects accumulation of apoptotic cells as they enter the apoptotic pathway without loss by disintegration or macrophage ingestion. The reduced rate at which apoptotic cells then continue to accumulate may reflect some disintegration of apoptotic cells or reduced initiation of apoptosis. Presuming that coculture with stromal cells can mimic supportive *in vivo* conditions, at least for short periods of time, and that the culture conditions

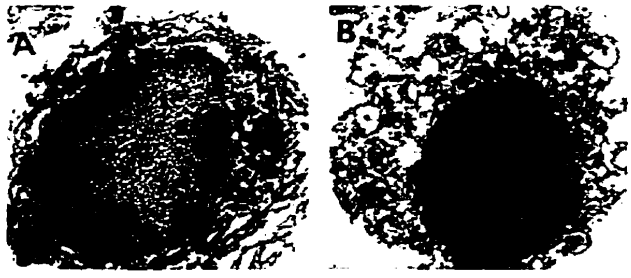
themselves do not induce apoptosis, the linear phase of the apoptotic curve would appear to reflect the rate at which cells have already been programmed for cell death in the BM. The slope of the linear apoptotic curve thus provides an assay to compare quantitatively the kinetics of entry into apoptosis at defined stages of cell development and under various experimental conditions *in vivo*.

Our previous kinetic studies have measured the rates of B cell production in proliferative compartments and turnover in nondividing compartments in mouse BM (Osmond, 1990). The data on apoptosis derived from the coculture system can now be added to make a provisional dynamic model of B cell generation and death (Fig.3.14). The rate at which cells of a given phenotype enter apoptosis combined with the total number of cells in the same phenotypic compartment *in vivo* give the actual number of cells apparently entering apoptosis per unit time. In the case of $B220^+sIgM^-$ cells, the rate of entry into apoptosis derived from cocultures with stromal cells in the present work (2.3%h) and the previously determined population size (76×10^6 cells) in mice of the same inbred strain and age (Jacobsen et al., 1994), indicate that 51×10^6 $B220^+sIgM^-$ cells would be deleted daily by apoptosis throughout the entire BM of the mouse. This loss by apoptosis would thus represent 67% of the cells being generated within the large dividing $B220^+sIgM^-$ population, leaving only the remaining 33% to continue along the differentiation pathway (Fig.3.14). From the ratios of the respective apoptotic incidences in freshly prepared BM suspensions, reflecting the frequency of apoptosis *in vivo*, it is likely that most of the loss is occurring at or near the late pro-B/large pre-B transition. Despite the variety of data sources represented in Figure 3.14, the value for the onward

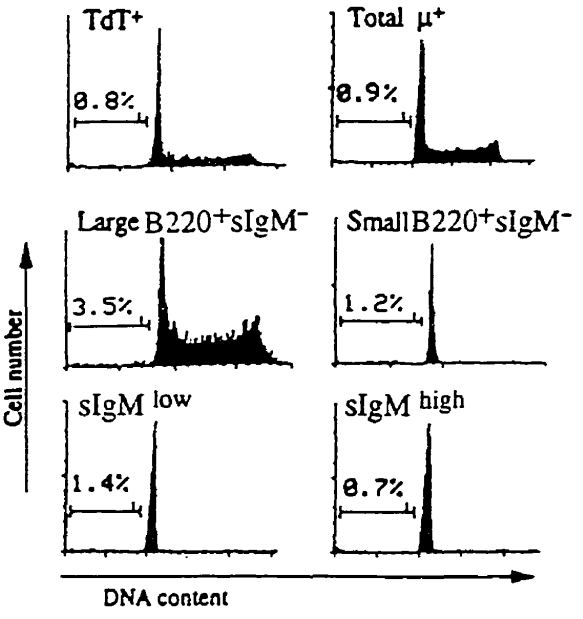
flow of cells derived by subtracting the numbers of cells undergoing apoptosis from those being generated is in reasonable concordance with the turnover of small pre-B cells and B lymphocytes, previously measured directly (Jacobsen et al.,1994). Similar calculations for $sIgM^+$ B lymphocytes (apoptotic rate, 1.8%/h; population size, $16x10^6$ cells (Jacobsen et al., 1994) indicate a daily apoptotic deletion of $7x10^6$ cells. If so, only 9 $x10^6$ surviving $sIgM^-$ B lymphocytes may actually leave the BM each day (Fig. 3.14). This scheme is a first model of B lymphopoiesis incorporating direct measurement of rates of cell production, turnover and apoptosis at phenotypically defined stages of B cell development *in vivo*.

In conclusion, the use of a range of techniques to detect apoptosis *ex vivo* and to measure kinetics of apoptosis in short term culture has demonstrated distinctive rates of apoptosis at defined phenotypic stages of B cell differentiation in mouse BM, two major stages prone to apoptosis, large $B220^+sIgM^-$ precursor B cells and immature $sIgM^{low}$ B lymphocytes. The results provide baseline data for studies of apoptosis-regulatory signals and role of apoptosis in perturbations of B lymphopoiesis.

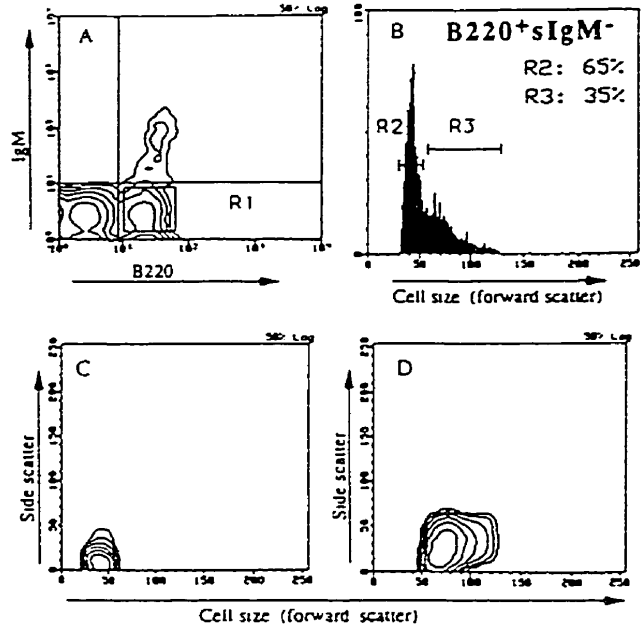
3.1. Apoptotic cells in $B220^{+}$ cell fraction sorted from BM cell suspension and examined by electron microscopy. Note (A) nuclear chromatin compaction into crescentic masses lying against the nuclear envelope and cytoplasmic vaculation and (B) membrane blebbing and nuclear shrinkage.


A
B

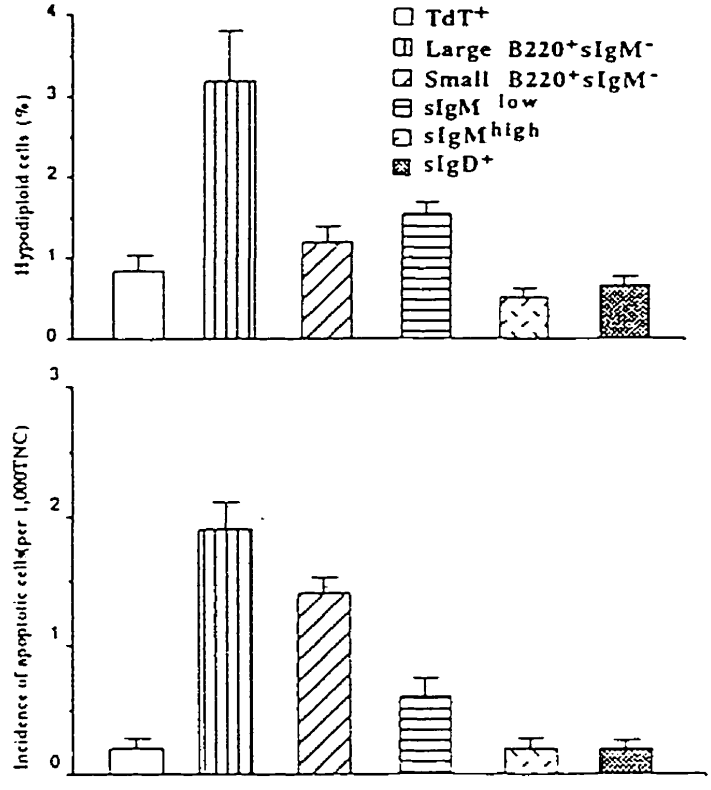
3.2. Cell cycle histograms of freshly prepared BM cell suspensions immunofluorescence-lebeled with B lineage phenotypic markers in conjunction with PI DNA content staining , indicating the incidence of hypodiploid cells at each defined differentiation stage.


TdT+
0.8%
Total μ+
0.9%
Large B220+sIgM-
3.5%
Small B220+sIgM-
1.2%
sIgM low
1.4%
sIgM high
0.7%
Cell number
DNA content

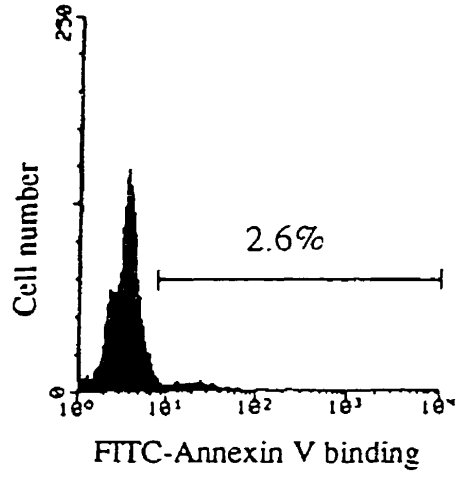
3.3. (A) BM cell suspensions immunolabeled with FITC- anti B220 and PE-anti-IgM mAbs. The rectangle within R1 encloses the gated region of $B220^{+}sIgM^{-}$ cells. (B) Cell size distribution of $B220^{+}sIgM^{-}$ precursor cells. R2 and R3 represent the proportion of small and large cells among the $B220^{+}sIgM^{-}$ population. (C) and (D) show separation of $B220^{+}sIgM^{-}$ cells into fractions of small and large $B220^{+}sIgM^{-}$ precursor cells, respectively.


A
IgM
R1
B220
B
B220+sIgM-
R2: 65%
R3: 35%
R2
R3
Cell size (forward scatter)
C
Side scatter
D
Side scatter
Cell size (forward scatter)

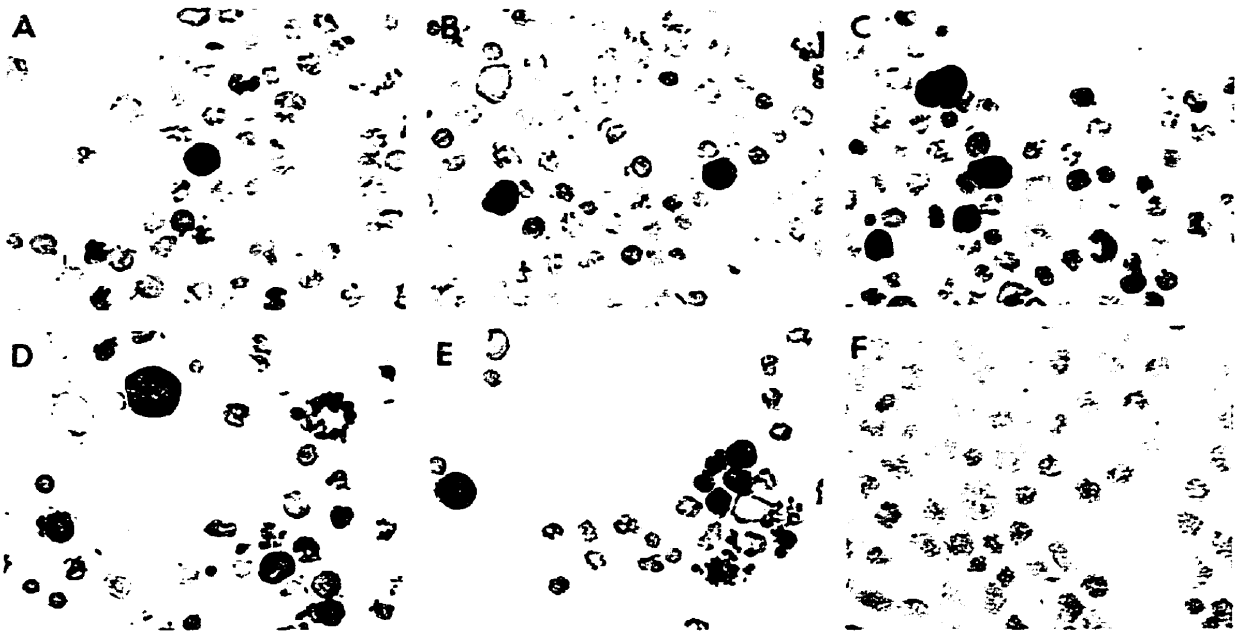
3.4. Incidence of hypodiploid cells at various phenotypic stages of B cell development in freshly prepared BM cell suspensions detected by flow cytometry. (A) incidence of apoptotic B cells within each defined compartment. (B) frequency of apoptotic B cells per 1, 000 total nucleated BM cells. Data were derived from 4 separate experiments (mean±SD).


TdT+
Large B220+sIgM-
Small B220+sIgM-
sIgM low
sIgM high
sIgD+
Hypodiploid cells (%)
Incidence of apoptotic cell(per 1,000TNC)
0
1
2
3
4

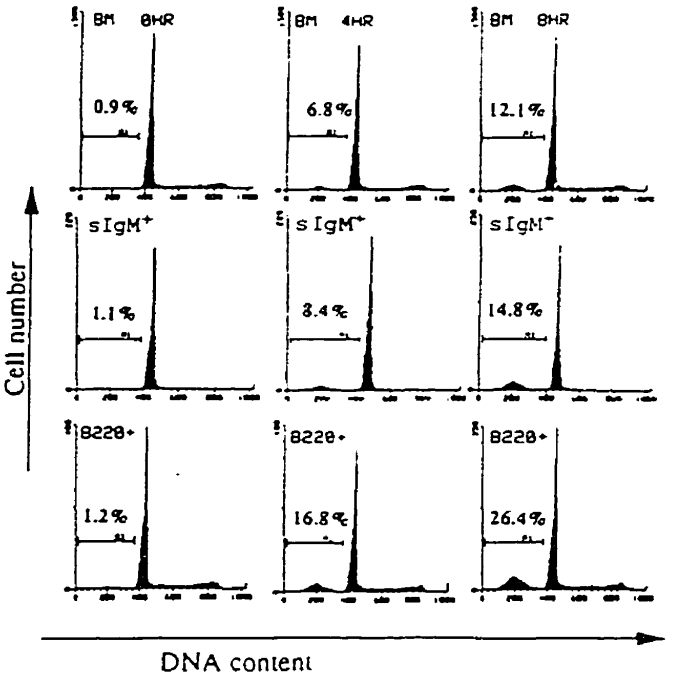
3.5. Representative histograms of freshly prepared BM $B220^+$ cells examined by flow cytometry for FITC-annexin V labeling.


Cell number
2.6%
FITC-Annexin V binding

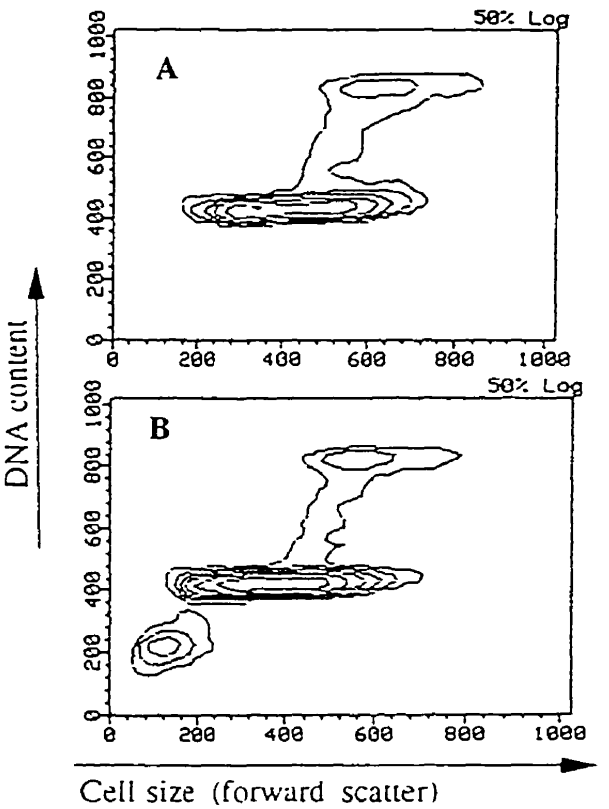
3.6. (A-E) Apoptotic cells stained by *in situ* labeling of DNA strand breaks in BM cell suspensions after 2, 4, 6, 8 h incubation, respectively, showing positive reaction product in cell nuclei and, after 6-8 h incubation, in apoptotic bodies of various sizes. (F) Negative control, showing only counterstain (x 400).


A
B
C
D
E
F

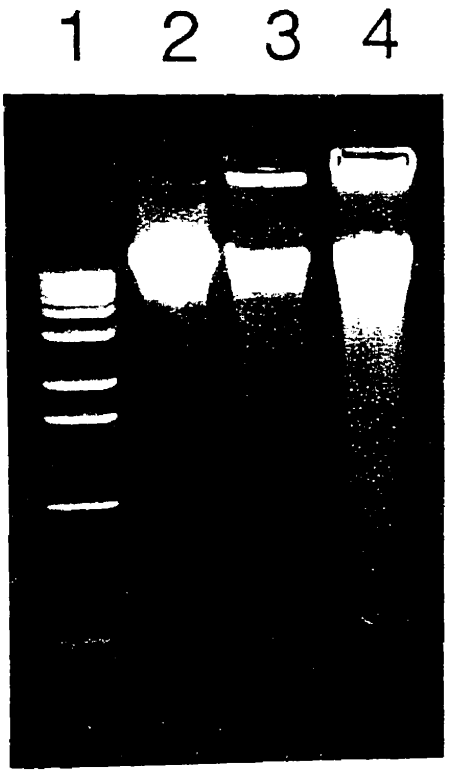
3.7. Hypodiploid apoptotic cells, among total BM cells (top), IgM^- cells (middle) and $B220^-$ cells (bottom) in BM cell suspensions after various periods of incubation (0-8 h), detected by flow cytometry. Incidences of apoptotic cells are indicated.


BM 8HR
0.9%
BM 4HR
6.8%
BM 8HR
12.1%
sIgM+
1.1%
sIgM+
3.4%
sIgM-
14.8%
B220+
1.2%
B220+
16.8%
B220+
26.4%
Cell number
DNA content

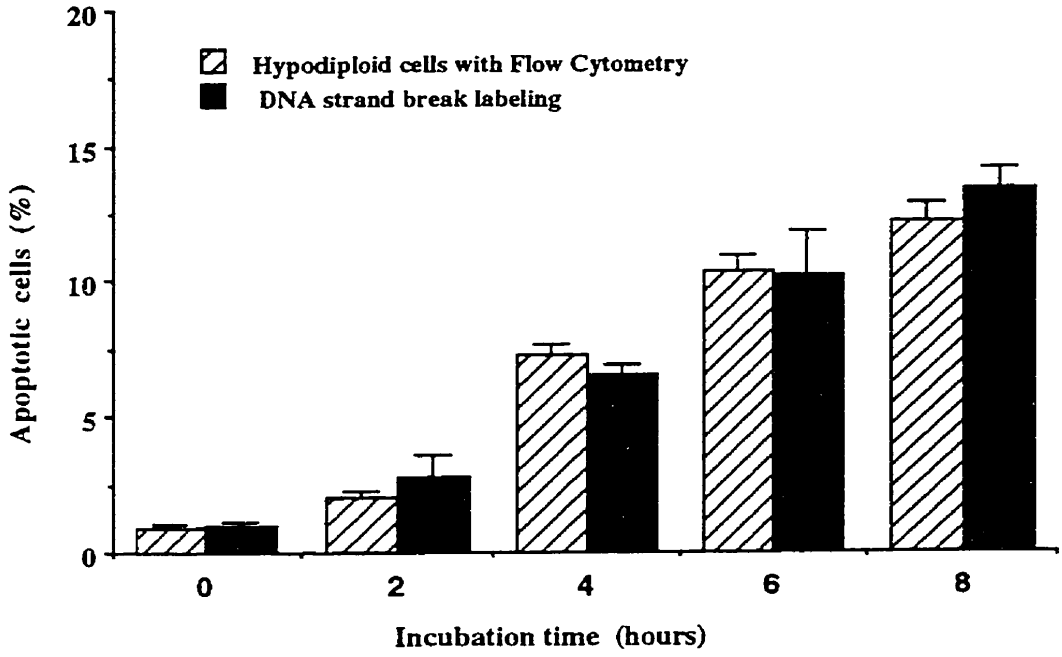
3.8. Contour plots of the DNA content and size of BM cells analysed by flow cytometry, showing (A) freshly prepared BM cell suspension. (B) after 6 h incubation, the presence of a substantial population of cells with both hypodiploid DNA and decreased cell volume.


50% Log
A
0
200
400
600
800
1000
50% Log
B
DNA content
Cell size (forward scatter)

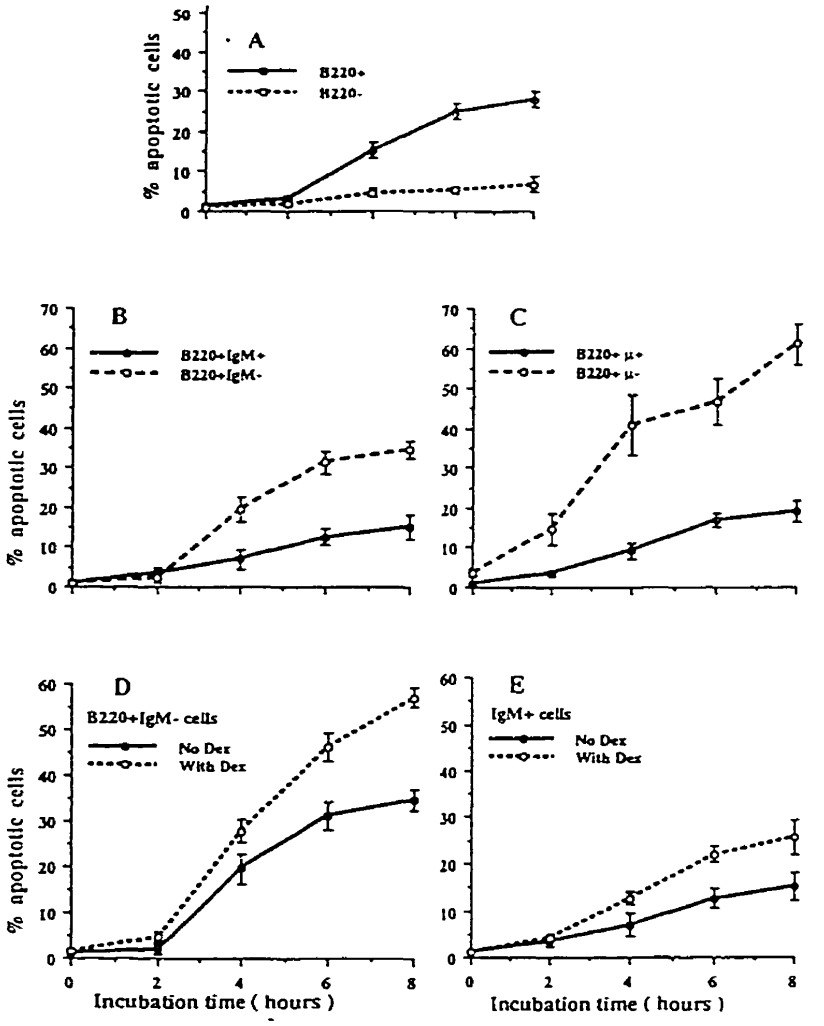
3.9. Gel electrophoresis of genomic DNA extracted from BM samples. Lane 1: 1 kb MW marker (Gibco BRL); lane 2: freshly prepared BM cells; lane 3 and 4: total BM cells and panning-purified BM $B220^-$ cells, respectively, after 24 h culture.


1 2 3 4

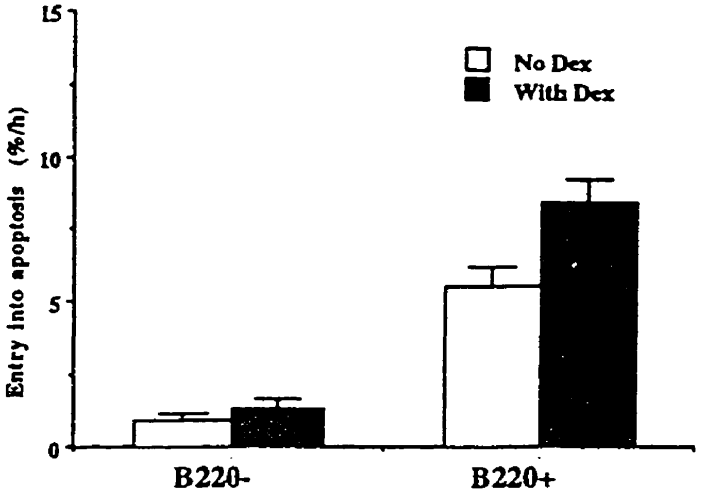
3.10. Comparison of incidence of apoptotic cells in mouse BM suspensions detected by flow cytometric analysis of DNA content and *in situ* DNA strand break staining. Data were derived from 4 separate experiments (mean±SD).

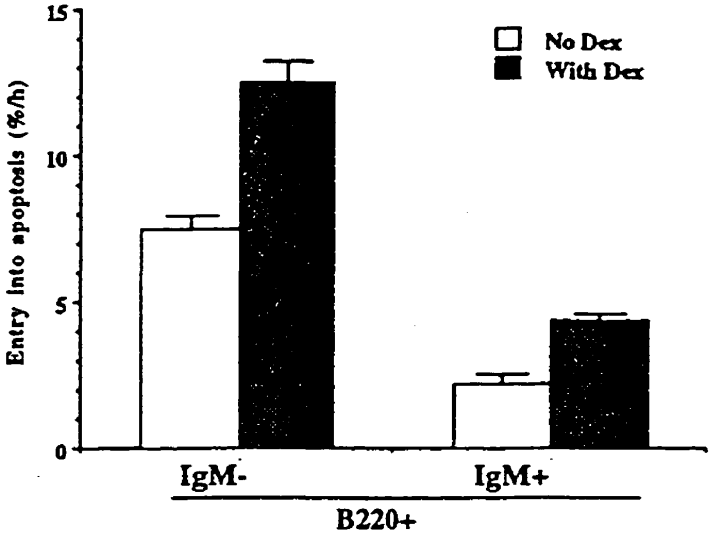

20
15
10
5
0
Apoptotic cells (%)
Hypodiploid cells with Flow Cytometry
DNA strand break labeling
0
2
4
6
8
Incubation time (hours)

3.11. Incidence of hypodiploid cells, detected by flow cytometry inmouse BM suspensions during 0-8 h incubation. (A, B, C) Comparison of apoptosis among $B220^+$ B lineage cells *vs.* $B220^-$ "non B" lineage cells, $B220^+sIgM^-$ precursor B cells *vs.* $sIgM^+$ B lymphocytes and $B220^+\mu^-$ pro-B cells *vs.* $B220^+\mu^+$ cells. (D, E) Comparison of apoptosis within $B220^+sIgM^-$ and IgM^+ population when incubated either with or without 1 μ M Dex. Data represent 4 separate experiments (mean±SD).

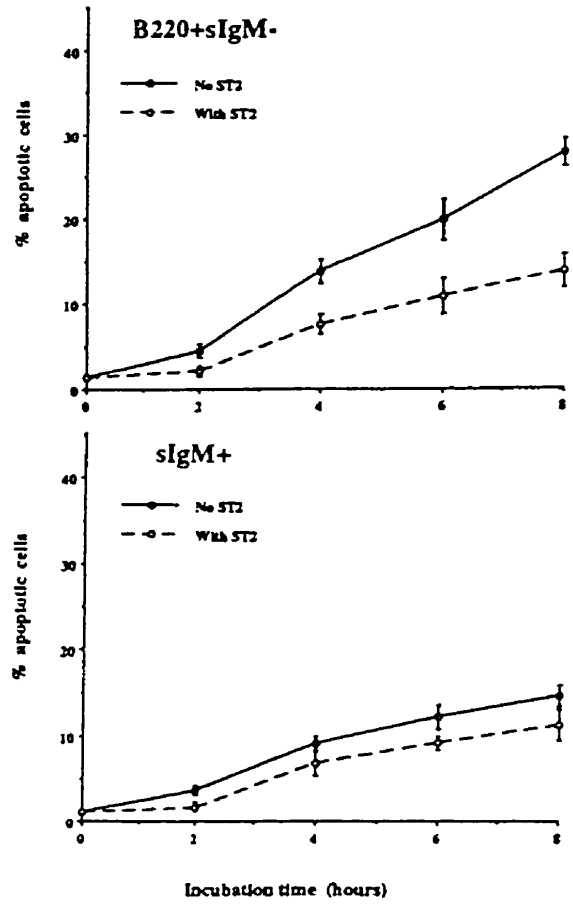

A
B220+
B220-
% apoptotic cells
B
B220+IgM+
B220+IgM-
C
B220+ μ+
B220+ μ-
D
B220+IgM- cells
No Dex
With Dex
E
IgM+ cells
No Dex
With Dex
Incubation time (hours)
Incubation time (hours)

3.12. Rates of BM cells entering into apoptosis during short-term culture. The slopes are calculated from the incidences of hypodiploid cells from 2 to 6 h incubation with and without Dex as shown in Fig. 3.11.

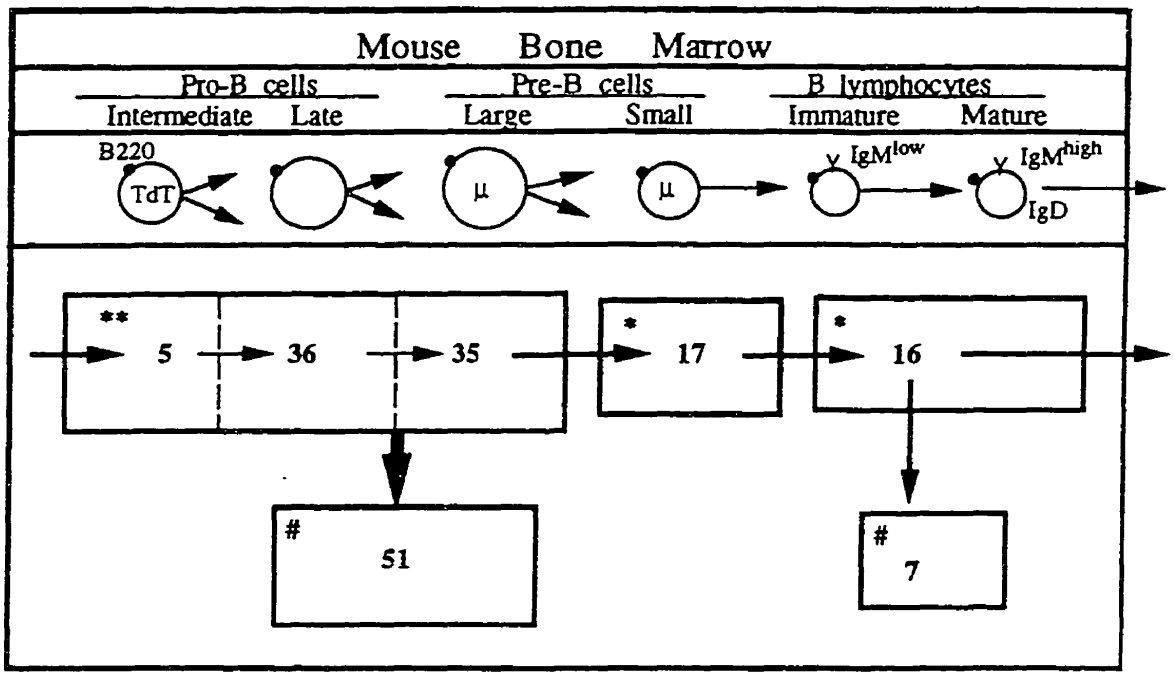

15
10
5
0
Entry into apoptosis (%/h)
No Dex
With Dex
B220-
B220+


15
10
5
0
Entry into apoptosis (%/h)
No Dex
With Dex
IgM-
IgM+
B220+

3.13. Incidence of hypodiploid cells detected by flow cytometry in mouse BM suspensions during 0-8 h incubation with or without amonolayer of ST2 stromal cells. Data represent 3 separate experiments (mean±SD).


B220+sIgM-
No ST2
With ST2
% apoptotic cells
40
30
20
10
0
0
2
4
6
8
sIgM+
No ST2
With ST2
% apoptotic cells
40
30
20
10
0
0
2
4
6
8
Incubation time (hours)

3.14. Provisional scheme of kinetics of cell production and apoptosis during B lymphopoiesis in mouse BM. The indicated rates of cell production (**) and turnover (*) are as previously described (Osmond, 1986; Osmond, 1990), while apoptotic rates (#) are from present results (see text for details). The large $B220^{+}sIgM^{-}$ cell phenotype defines a dividing transit compartment in which precursor B cells undergo a series of mitoses as they pass through the stages of $B220^{+}\mu^{-}$ pro-B cells and c μ^{+}s μ^{-} large pre-B cells. In contrast, both small $B220^{+}sIgM^{-}$ cells, and $sIgM^{+}$ B cells constitute simple transit compartments of nondividing cells.



** Cell production or * Cell turnover ($x10^6$ /day)

Cells entering apoptosis ($x10^6$ /day)

Chapter 4 Developmental Regulation of Bcl-2 and Bax Expression During B Lymphopoiesis in Normal Mouse BM

4.1. Summary

B cell genesis in BM represents a finely controlled balance between cell production and cell selection, modulated by signals that may be either external or intrinsic to the cell. During their development, B cells undergoing nonfunctional Ig gene rearrangements and unable to express functional surface Ag receptors are eliminated by apoptosis. In many biological systems, Bcl-2 and related proteins are involved in regulating the cell death process. However, the molecular signals that modulate B cell selection during normal development in BM are unclear. In the present study, Bcl-2 and Bax protein expression has been examined among B cells at various phenotypic differentiation stages in mouse BM cell suspensions using Western blotting and flow cytometry. The results reveal that Bcl-2 and Bax expressions among B lineage cells is developmental stage related and that the ratio of Bax/Bcl-2 among B cells generally correlates with the respective apoptotic incidence detected *ex vivo* at defined differentiation stages. These findings suggest that competitive interactions of these two proteins play a critical role in controlling cell survival, and that Bcl-2 family proteins are normally implicated in regulating the apoptotic selection of B cells during their development in mouse BM.

4.2. Introduction

During B cell development in mouse BM, an extensive cell loss takes place, reflecting an efficient culling process by which only those B cells undergoing successful assembly of IgH and IgL chains and functional expression of surface Ag receptors are allowed to proceed into the peripheral B cell pool (Osmond et al., 1994). Aberrant B lineage cells die by apoptosis *in situ*, a process that plays a pivotal role in qualitative and quantitative control of B cell production. Little is currently known about the molecular signals that regulate B cell death or survival during *in vivo* development. A candidate molecule, however, is the product of the *bcl-2* protooncogene.

In a variety of biological systems, overexpression of *bcl-2* inhibits apoptotic cell death (Cory, 1995). The ability of Bcl-2 protein to enhance cell survival was initially suggested by the observation that its overexpression increased the *in vitro* viability of IL-3-dependent pro-B cell lines and myeloid cells upon cytokine deprivation (Vaux et al., 1988; Nunez et al., 1990). The *in vivo* effects of Bcl-2 have been examined using transgenic mice expressing *bcl-2* under the control of an IgH enhancer (McDonnell et al., 1989; Strasser et al., 1991), in which expanded B cell populations, follicular hyperplasia, prolonged Ab responses and autoimmune phenomena are observed. Moreover, introduction of an Eμ-*bcl-2* transgene into Eμ-*myc* transgenic mice markedly accelerates the onset of lymphoma, demonstrating a synergistic relationship between these two oncogenes for transforming potential (Strasser et al., 1990).

The Bcl-2 protein family consists of both suppressors and promoters of apoptosis.

These proteins can interact with each other by forming homo- and heterodimers (Sedlak et al., 1995; Sato et al., 1994). Bax heterodimerizes *in vivo* with Bcl-2 to accelerate apoptotic cell death. High levels of Bax can antagonize the protective effect of Bcl-2 (Oltvai et al., 1993). Although the precise biochemical mechanisms by which Bcl-2 family proteins regulate the apoptotic process are still far from clear, it has been suggested that the ratio of pro- and anti-apoptotic members determines the cell's susceptibility to a wide range of death signals (Yang and Korsmeyer. 1996). Bcl-2, being able to inhibit apoptosis resulting from various signals, may act after the convergence of several different intracellular pathways. However, the developmental regulation of intracellular levels of various members of the Bcl-2 protein family and their correlation with cell survival during B cell genesis are largely unclear.

In the present study, the expression of Bcl-2 and Bax proteins among B cells at various differentiation stages has been analysed by Western blotting and multi-color flow cytometric analysis, suggesting that alterations in the Bax/Bcl-2 ratio are implicated in B cell deletion during B cell development in mouse BM.

4.3. Materials and Methods

4.3.1. Mice

Male C3H/HeJ mice (Jackson Laboratories, Bar Harbor, ME) were used at 8-10 wk of age.

4.3.2. Cell Suspensions

Femoral BM cell suspensions were prepared as described in Chapter 2. Splenic and thymic cell suspensions were prepared by passing through a stainless steel sieve in ice-cold MEM/NCS.

4.3.3. Immunophenotypic Labeling

BM cell samples were labeled with PE-conjugated anti-B220 and -IgM as described in Chapter 2. To examine the total μ chain expression, cells were fixed with cold 70% ethanol and then stained with biotin-conjugated anti-μ chain Ab, revealed by streptavidin-Red 670™ (Gibco-BRL Research Laboratories).

4.3.4. Immunofluorescent Staining of Intracytoplasmic Bcl-2 and Bax Protein Expression

Phenotypically labeled cells were incubated either with hamster anti-Bcl-2 Ab (PharMingen, San Diego, CA) or rabbit anti-Bax Ab (Oncogene Research Products, Cambridge, MA) in PBS with 0.1% saponin for 30 min on ice, followed by FITC-conjugated goat-anti-hamster IgG(Cedarlane Laboratories Limited) or goat-anti-rabbit IgG (Southern Biotechnology Associates) Abs, respectively. Samples stained with isotype control Abs were used to determine fluorescence background.

4.3.5. Flow Cytometric Analysis

Immunostained cells were analysed with a FACScan flow cytometer (Becton

Dickinson) using Lysys II software. A minimum of 10,000 events per sample were collected from various B cell subpopulations with defined phenotypes. Cell debris and clumps were excluded by setting a gate on forward scatter *vs.* side scatter. Measurement of the ratio of Bcl-2 and Bax protein expression among defined subpopulations of B lineage cells was based on the mean fluorescence intensity per cell, evaluated as the mean fluorescence index (mean fluorescence intensity of Bax relative to Bcl-2 mean fluorescence intensity).

4.3.6. Panning Separation of BM B Lineage Cells

Polystyrene petri dishes were coated with goat anti-rat IgG mAb (Kirkegaard and Perry Laboratories, Gaithersburg, MD) in 0.05M Tris-HCl , 0.15 M NaCl buffer (pH 9.5), then coupled with affinity-purified rat mAb 14.8 from cultures of hybridoma cells (American Tissue Type Collection, Rockville, MD), as described (Jacobsen and Osmond, 1991). BM cells were evenly placed in mAb14.8-coated dishes and incubated at 4°C for 40 min. After extensive rinsing of the plate with cold PBS, adherent $B220^-$ B lineage cells were gently recovered using a plastic scraper (Costar, Cambridge, MA). The cells were then transferred to anti-IgM-coated dishes and incubated at 4°C for 40 min. Finally, the adherent $sIgM^-$ B cells and non-adherent $B220^+sIgM^-$ precursors were separately collected. The sample purity was further examined by flow cytometry.

4.3.7. SDS-polyacrylamide Gel Electrophoresis and Western Blot Analysis

Lysates from freshly panning-purified $B220^+sIgM^-$ precursor B cells and $sIgM^+$ B

lymphocytes were prepared by incubation in sample buffer containing 62.5 mM Tris-HCl (pH6.8), 2% SDS, 10%glycerol, 5% 2-mercaptoethanol and 0.01% bromophenol blue. After heating at 100^{0}C for 5 min, sample proteins were loaded and separated on 12% polyacrylamide gel under constant current of 30 mA using a mini-protein apparatus (Bio-Rad Laboratories, Hercules, CA). Proteins were then transferred to nitrocellulose membrane (0.45 um, Pierce, Rockford, Illinois) by electroblotting. After blocking with 5% skim milk in TBS (10mM Tris-HCl, 150 mM NaCl, pH 7.4) for 1 h at room temperature and washing 3 times with TTBS (TBS containing 0.05% Tween-20), the membranes were incubated with hamster-anti-Bcl-2 Ab (PharMingen) or rabbit anti-Bax Ab (Oncogene Research Products) at 1 μg/ml in TTBS containing 2% skim milk for 2 h at room temperature and washed 3 times for 5 min each in TTBS buffer.

Detection of Western blot reactions was performed by incubation with horseradish peroxidase-conjugated goat-anti-hamster-IgG or anti-rabbit-IgG (Jackson ImmunoResearch Laboratories Inc.) Abs at 1:2500 dilution on a rotating table for 90 min at room temperature, followed by thoroughly washing with TBS, and development with an enhanced chemiluminescence ECL™ system (Amersham Life Science, Buckinghamshire, England).

4.4. Results

4.4.1. Expression of Bcl-2 and Bax Protein Among B220^{+}sIgM^{-} Precursor B Cells and sIgM^{+} B Lymphocytes

To investigate the normal expression of Bcl-2 and Bax during B cell development, whole-cell lysates from panning-purified $B220^{+}sIgM^{-}$ precursors and sIgM $^{+}$B lymphocytes were studied by Western blot analysis. As shown in Figure 4.1A , the 26 KDa Bcl-2 protein was constitutively expressed by B lineage and non-B cells in BM. Within the B lineage, samples of precursor B cells expressed a much greater amount of Bcl-2 than B lymphocytes. Similarly, a higher level of Bax expression was also observed among precursor B cells than B lymphocytes (Fig. 4.1B). Interestingly, splenic B lymphocytes, as control samples, displayed a large amount of Bcl-2, but only a barely detectable level of Bax. Thymocytes expressed extremely high levels of both Bcl-2 and Bax proteins.

4.4.2. Differential Expression of Bcl-2 at Various Stages of B Cell Differentiation

To examine the developmentally regulated expression of Bcl-2, protein levels were examined among B lineage cells by three-color flow cytometric analysis (Fig. 4.2). Populations of large $c\mu^{-}$ $s\mu^{-}$ pre-B cells expressed the highest amount of Bcl-2, followed by $B220^{+}\mu^{-}$ pro-B cells. Small $c\mu^{+}s\mu^{-}$ pre-B cells and IgM^{high} mature B cells showed similar intermediate levels of Bcl-2 expression, whereas IgM^{low} immature B lymphocytes displayed the lowest level of Bcl-2 protein.

4.4.3. Bax Expression at Various Stages of B Cell Development

Flow cytometric analyses were simultaneously performed to examine Bax expression among B lineage cells (Fig. 4.3). Populations of large $c\mu^{+}s\mu^{-}$ pre-B cells exhibited much

the highest amount of Bax protein, followed by $B220^{+}\mu^{-}$ pro-B cells expressing only half the amount. Small $c\mu^{+}s\mu^{-}$ pre-B, IgM^{low} and IgM^{high} cell populations all displayed similar and much lower levels of Bax expression.

4.4.4. Developmentally Regulated Bax/Bcl-2 Ratios During B Lymphopoiesis

The ratio of Bax to Bcl-2 expression at each phenotypically defined stage of B cell development was quantitated by comparing the mean fluorescence intensities of staining of the 2 proteins (Fig. 4.4). Large $c\mu^{+}s\mu^{-}$ pre-B cells showed the highest ratio(2.7), followed by IgM^{low} immature B lymphocytes (2.3) and $B220^{+}\mu^{-}$ pro-B cells (2.0); while IgM^{high} mature B lymphocytes displayed the lowest value (1.6) and small $c\mu^{+}s\mu^{-}$ pre-B cells the second lowest ratio (1.8). Thus, the data suggested that Bax/Bcl-2 ratios were differentially modulated among the cell populations at various stages during B cell development.

4.5. Discussion

Cell death by apoptosis is a significant feature of B lymphopoiesis in BM (Osmond et al., 1994). Although the mechanisms that trigger cell death remain poorly understood, Bcl-2 family proteins have been recognized as important modulators (Reed, 1994; Cory, 1995). In the present study, the expression of Bcl-2 and Bax protein among B lineage cells at various stages of development has been extensively examined. Western blot analysis has demonstrated that BM B lineage cells and splenic B lymphocytes

constitutively express Bcl-2 and Bax proteins. However, samples of $B220^+sIgM^-$ precursor B cells in BM express larger amounts of Bcl-2 protein than $sIgM^+$ B lymphocytes. Three-color flow cytometric analysis confirms these findings and allows further dissection of B cell differentiation compartments in order to determine the relative levels of expression of Bcl-2 and Bax during B cell development. The results show that the highest ratios of Bax to Bcl-2 are displayed by large $B220^+c\mu^+$ pre-B cells and $sIgM^{low}$ immature B cells, whereas $sIgM^{high}$ mature B lymphocytes show the lowest Bax/Bcl-2 ratio. Our studies (Chapter 3) have shown that the highest apoptotic incidences *ex vivo* are found among large $B220^+sIgM^-$ precursors and $sIgM^{low}$ immature B lymphocytes, while the lowest apoptotic incidence is shown by mature IgM^{high} cells. Thus, the Bax/Bcl-2 ratio correlates closely with the apoptotic incidence at each phenotypically defined B cell stage. Previous studies have shown that Bcl-2 is highly expressed by both $B220^+CD43^+$ pro-B cells and IgM^+IgD^+ mature B cells, but is downregulated in $B220^+CD43^-$ cells, thus exhibiting a biphasic pattern during development (Merino et al.,1994). In the present study, an important B lineage marker, $c\mu$, has been employed to examine pre B cells. The results reveal a changing pattern of Bcl-2 expression and differential levels of Bcl-2 and Bax during this stage as the cells progress from large cycling cells to small postmitotic forms.

It has been proposed that susceptibility to glucocorticoid-induced cell death in developing B cells correlates with the amount of endogenous Bcl-2 protein expressed by these cells (Merino et al., 1994). There is evidence, however, that treatment of leukemic cells with glucocorticoid hormones downregulates Bcl-2 expression but

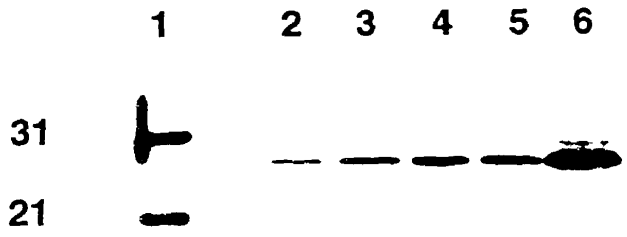
nevertheless does not sensitize the cells to induction of apoptosis because of simultaneous modulations of Bax and Bcl-x_L (Lotem and Sachs, 1995). Among an expanding family of Bcl-2 homologues, the ratio between Bcl-2 and Bax has been suggested to determine whether a given cell will undergo suicide or resist an apoptotic stimulus (Oltvai et al., 1993). The present results show that large pre-B cells express the highest levels of both Bcl-2 and Bax, yet they also display the highest apoptotic incidence *ex vivo* among all B lineage cells in BM. With the premise that the mean protein levels and ratios observed for each phenotypically defined population reflect the values for individual cells in the population, the findings argue strongly that the Bcl-2/Bax ratio can provide a more valuable insight into the regulation of B cell death in BM than the absolute amounts of either Bcl-2 or Bax alone.

Recent evidence indicates that Bcl-x_L, an anti-apoptotic member of the Bcl-2 protein family, is also highly regulated during B cell development, being especially upregulated at the pre-B cell stage (Grillot et al., 1996). The findings in Bcl-2 deficient mice have shown that although Bcl-2 is critical for the maintenance of lymphoid homeostasis, it is not an absolute requirement for B cell genesis. In contrast, Bcl-x_L is highly expressed during embryonic development and a massive cell death of hematopoietic precursors is observed in Bcl-x deficient mice. It appears that Bcl-2 and Bcl-x_L play distinctive roles in suppressing cell death at different developmental stages. The yeast two-hybrid system has recently been used to examine the specificity of heterodimer formation among Bcl-2 family proteins, demonstrating that Bax can form a common partner for heterodimerizing with either Bcl-x_L or Bcl-2 (Sato et al., 1994; Sedlak et al., 1995). It is possible,

therefore, that cell selection during B cell development may be modulated by interactions of several Bcl-2 related proteins, though the signals that regulate the expression of these proteins remain to be established. Under physiological conditions, the differential expression of Bcl-2 family proteins may autonomously regulate the death signals that ensure the selection of B cells with successfully rearranged Ig genes and functional Ag receptors, and the demise of aberrant or autoreactive B cells.

Fig. 4.1. (A). Western blotting analysis of endogenous Bcl-2 protein expression. Lane 1 showed the low range MW markers (Bio-Rad Laboratories). Whole-cell lysates were prepared from panning-purified BM $sIgM^+$ B lymphocytes (lane 2), $B220^+sIgM^-$ precursors (lane 3) and $B220^-$ non-B lineage cells (lane 4). Splenic $sIgM^+$ B lymphocytes and thymocytes were in lane 5 and 6, respectively. Lysates of 1x 10^6 cells were loaded in each lane (photographic exposure time:45 seconds).

(B). Western blotting analysis of endogenous Bax protein expression. Lane 1 showed the low range MW markers (Bio-Rad Laboratories). Whole-cell lysates were prepared from panning-purified BM $sIgM^+$ B lymphocytes (lane 2) and $B220^+sIgM^-$ precursors (lane 3). Splenic $sIgM^+$ B lymphocytes and thymocytes were in lane 4 and 5, respectively. Lysates of 1x 10^6 cells were loaded in each lane (photographic exposure time:25 seconds).


1 2 3 4 5 6
31
21

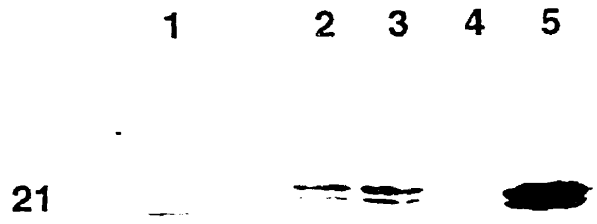

1 2 3 4 5
21

Fig. 4.2. Flow cytometric analysis of endogenous Bcl-2 expression among B lineage cells at various phenotypically defined stages in freshly prepared BM cell suspensions. Control samples of whole BM cell suspensions were incubated with hamster IgG instead of hamster anti-Bcl-2 Ab. The profiles are representative of 3 separate experiments.

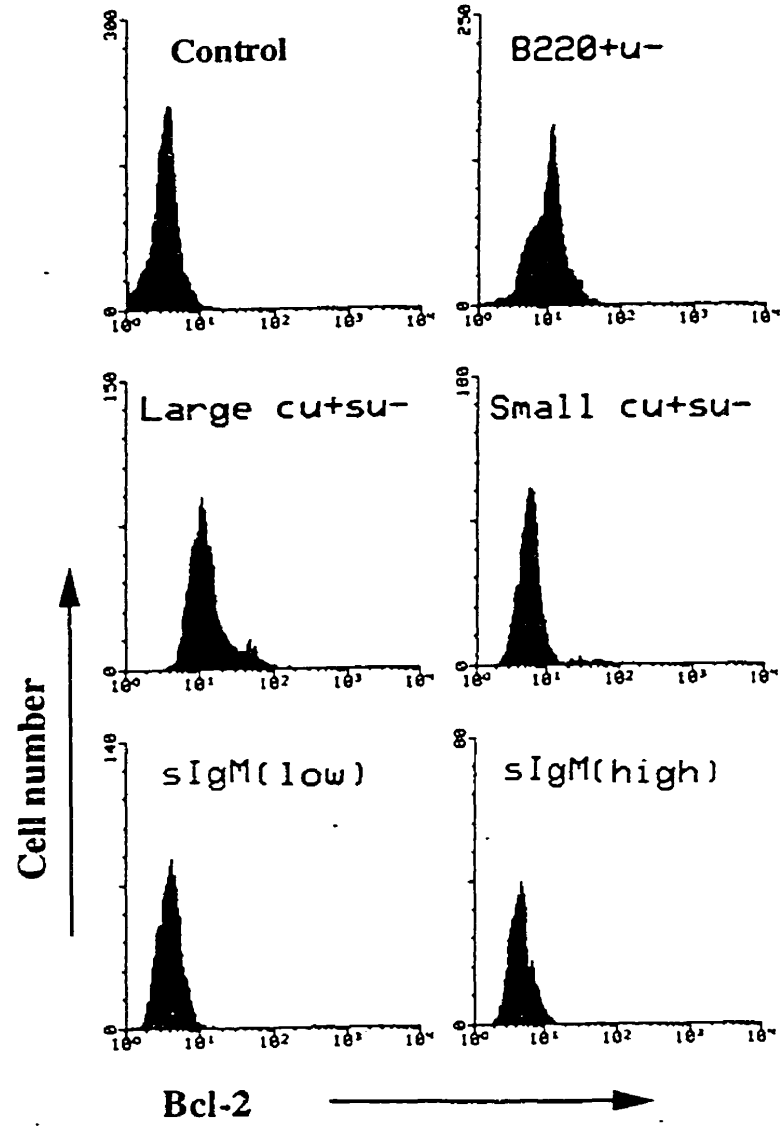

Control
B220+u-
Large cu+su-
Small cu+su-
sIgM(low)
sIgM(high)
Cell number
Bcl-2

Fig. 4.3. Flow cytometric analysis of endogenous Bax expression among B lineage cells at various defined stages in freshly prepared BM cell suspensions. Control samples of whole BM cell suspensions were incubated with rabbit IgG instead of rabbit anti-Bax Ab. The profiles are representative of 3 separate experiments.

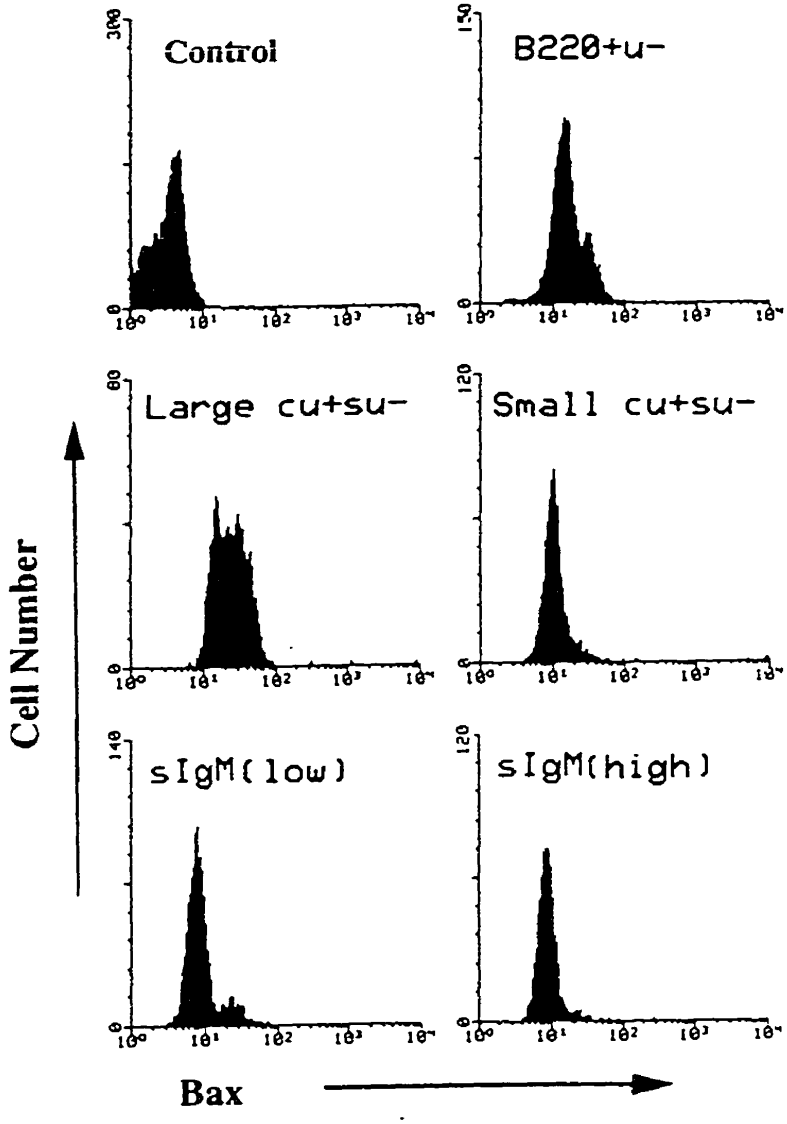
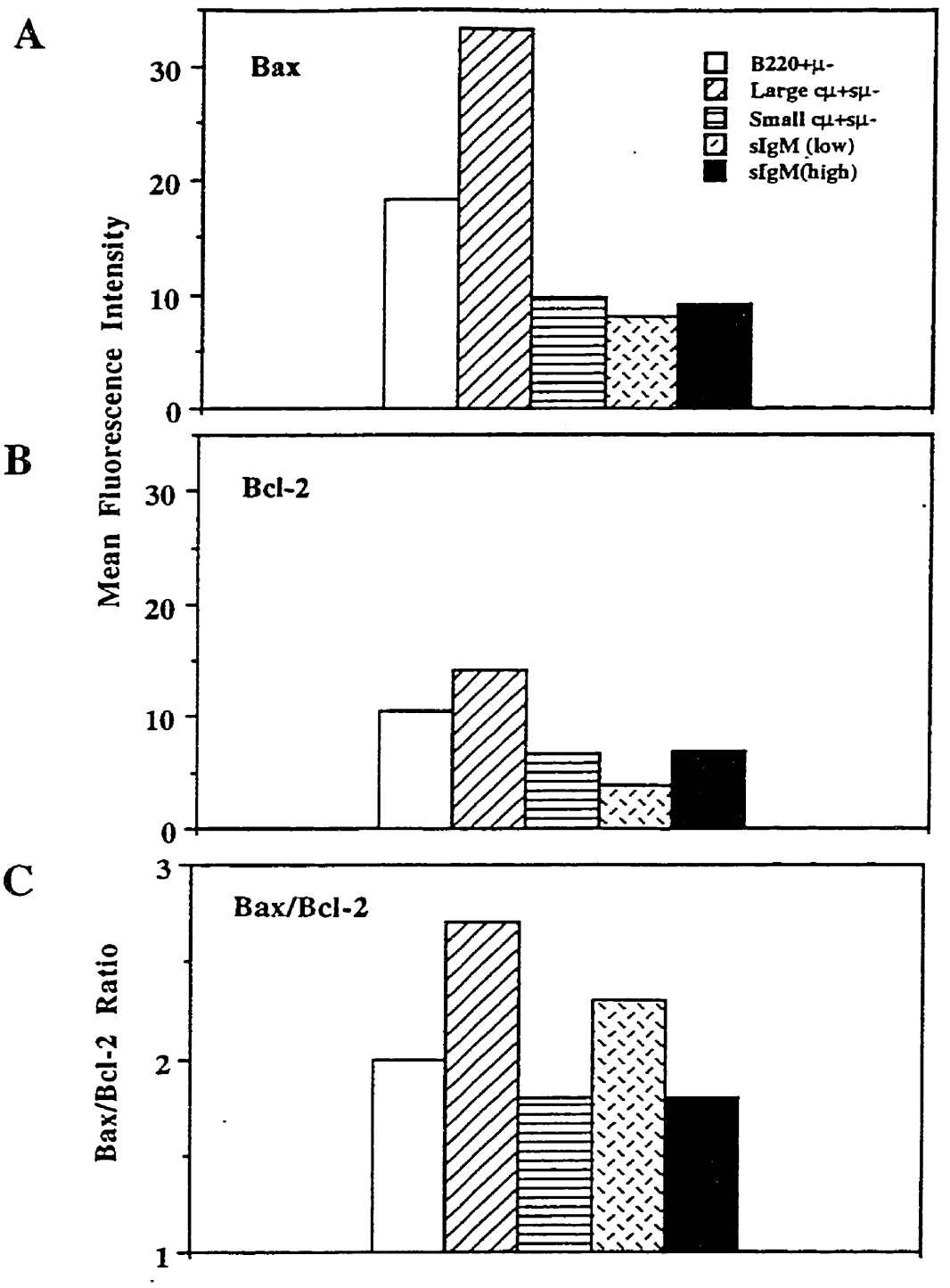

Control
B220+u-
Large cu+su-
Small cu+su-
sIgM(low)
sIgM(high)
Cell Number
Bax

Fig. 4.4. Expression of Bax (A) and Bcl-2 (B) proteins among B lineage cells at various defined differentiation stages, each measured as the mean fluorescence intensity. (C) Ratio of Bax to Bcl-2 protein expression among B cells at various defined differentiation stages, each measured as the mean fluorescence level relative to respective background level. Data are mean values from 3 separate experiments.


A
Bax
B220+μ-
Large cμ+sμ-
Small cμ+sμ-
sIgM (low)
sIgM(high)
30
20
10
0
Mean Fluorescence Intensity
B
Bcl-2
30
20
10
0
C
Bax/Bcl-2
3
2
1
Bax/Bcl-2 Ratio

Chapter 5 B Cell Differentiation and Selection in RAG-2-/- Mouse BM

5.1. Summary

During B cell development in mouse BM, RAG genes are crucial for initiating V(D)J recombination, while homozygous mutations of RAG can completely prevent B cell production *in vivo*. In order to examine the influence of RAG gene mutations on B cell differentiation and selection, phenotypic and apoptotic analyses have been performed by flow cytometry in RAG-2 gene-deleted mice. The results demonstrate that all B lineage cells in BM of RAG-2-/- mice abort at a $B220^{+}\mu^{-}$ phenotypic stage, but the incidence and absolute number of the $B220^{+}\mu^{-}$ cells, themselves, are increased. The incidence of hypodiploid apoptotic cells *ex vivo* and the rate at which cells enter the apoptotic pathway in short term culture are much higher among $B220^{+}\mu^{-}$ cells in RAG-2-/- mouse BM than in controls. Bax protein expression by $B220^{+}\mu^{-}$ cells in RAG-2-/- mice is markedly increased while the Bcl-2 protein level remains comparable with controls. The findings provide evidence that the Bcl-2/Bax ratio is instrumental in mediating selective cell death of early precursor B cells during B lymphopoiesis in BM. The RAG-2-/- mouse also provides a model to estimate the normal transit time and apoptotic fraction of early precursor B cells *in vivo*.

5.2. Introduction

During B cell differentiation, Ab diversity is generated mainly by the somatic DNA rearrangement known as Ig gene recombination, which occurs at H and L chain loci and is developmentally regulated (Tonegawa, 1983). Two genes, RAG-1 and RAG-2, have been identified by their ability to activate V(D)J recombination on an artificial substrate in fibroblasts (Oettinger et al., 1990; Schatz and Baltimore, 1988; Schatz et al., 1989). Both genes are expressed in cell lines displaying Ig recombination activity. They are restricted in expression to primary lymphoid organs and are evolutionarily conserved in vertebrates (Oettinger et al., 1990; Carlson et al., 1991; Chun et al., 1991). Although the precise functions of the RAG genes have not been fully elucidated, it is generally accepted that they encode tissue-specific components of the Ig recombination machinery (Lewis, 1994). *In vitro,* RAG-1 and RAG-2 proteins are sufficient to mediate signal-dependent, site-specific DNA cleavage (McBlane et al., 1995; van Gent et al., 1995). The importance of RAG genes in lymphocyte development *in vivo* has been demonstrated in homozygous RAG mutant mice. Mature B and T lymphocytes cannot be generated in these RAG-deficient mice and the arrest of lymphocyte differentiation occurs at an early stage correlating with the inability to perform V(D)J recombination (Shikai et al., 1992; Mombaerts et al., 1992). No recombination intermediates are found in RAG-/- lymphoid cells, implying that RAG-1 and RAG-2 act at the initial stages of Ig recombination (Schlissel et al., 1993).

Ig recombination thus constitutes a critical event permitting precursor B cells to

differentiate and to generate mature B lymphocytes. Moreover, the complete blockade of B cell development in RAG-deficient mutant mice provides an ideal model to study the kinetics and mechanisms of B cell selection among early precursor B cells. In the present study, apoptotic incidences and expression of Bcl-2 and Bax proteins among precur or B cells from RAG-2-/- and control mouse BM have been examined *ex vivo* and in short term culture by flow cytometry. The results suggest that changes in Bax/Bcl-2 ratio are involved in determining the apoptotic selection of RAG-2 deficient precursor B cells and lead to estimates of the transit time and normal apoptotic fraction of early precursor B cell populations *in vivo*.

5.3. Materials and Methods

5.3.1. Mice

Male RAG-2 mutant (RAG-2-/-) mice and age-and-strain-matched wild type mice (129/sv) were obtained from Jackson Laboratories and used at 8-10 wk of age.

5.3.2. BM Cell Suspensions

Femoral BM cell suspensions were prepared as described in Chapter 3.

5.3.3. Immunophenotypic Staining

BM cell suspensions were immunofluorescent labeled with B lineage markers including B220, sIgM and total μ chains, as described in Chapter 3.

5.3.4. Immunofluorescent Staining of Protein Expression

Bcl-2 and Bax protein expression by B cells at defined stages of development were immunolabeled, as described in Chapter 4.

5.3.5. Flow Cytometric Analysis

Immunostained samples were analysed for phenotype and assayed for apoptotic rates, as described in Chapter 3.

Measurements of Bcl-2 and Bax protein levels at each defined B cell stage were based on their mean fluorescence intensity, expressed as a mean fluorescence index relative to values from control mice.

5.3.6. Statistics

Statistical analysis was performed using Student's t test. $P < 0.05$ was considered significant.

5.4. Results

5.4.1. B Cell Development in BM of RAG-2-/- Mice

In RAG-2-/- mice, the total BM cellularity was 80% of the normal control value. No $c\mu^+$ pre-B cells or $s\mu^+$ B cells were detected (Table 5.1; Fig. 5.1). However, $B220^+\mu^-$ pro-B cells were increased more than 2 fold in incidence while the incidence of TdT^+ pro-B cells increased more than 5 fold compared to controls. Double immunofluorescent

labeling experiments further revealed that the $B220^{-}TdT^{+}$ population comprised 3.7% of BM cellularity and $B220^{+}TdT^{+}$ cells, 6.3%. Thus, the pro-B cell compartment was expanded in RAG-2-/- mouse BM, but B cell development proceeded no further than cells of late pro-B cell phenotype.

5.4.2. B Cell Apoptosis in RAG-2-/- Mice

In freshly prepared BM suspensions from RAG-2-/- mice, $B220^{+}$ B lineage cells showed a higher hypodiploid apoptotic incidence than $B220^{-}$ non-B cells. The apoptotic incidence among $B220^{+}\mu^{-}$ pro-B cells in RAG-2-/- mice was significantly greater than in controls ($p < 0.01$, Fig. 5.2). During short term culture for 2-4 h, the apoptotic incidence among $B220^{+}\mu^{-}$ B lineage cells increased linearly. In RAG-2-/- mice, the $B220^{+}\mu^{-}$ pro-B cells became apoptotic more extensively than usual, with an overall apoptotic rate of 5.8%/h compared to 4.0%/h in controls. After 4 h incubation, the hypodiploid incidence among $B220^{+}\mu^{-}$ pro-B cells reached 26% in RAG-2-/- mice, but only 18% in controls (Fig. 5.3).

5.4.3. Upregulated Bax but Unchanged Bcl-2 Expression Among Pro-B Cells in RAG-2-/- Mice

Using three-color flow cytometric analysis, intracellular Bcl-2 and Bax protein levels were evaluated based on mean fluorescence index, as described (Chapter 4). Interestingly, Bax expression was upregulated among $B220^{+}\mu^{-}$ pro-B cells from RAG-2-/- BM samples, to almost 2.5 fold control values, while Bcl-2 protein remained at a level comparable with

controls (Fig. 5.4).

5.5. Discussion

In RAG-2-/- mouse BM, no cμ-expressing pre-B cells or $sIgM^+$ B cells are found, confirming that B cell development is completely blocked at the $B220^+\mu^-$ late pro-B cell stage. It has been reported that introduction of a μ H chain transgenic into RAG-/- mice leads to the appearance of proliferating pre-B cells (Spanopoulou et al., 1994; Young et al., 1994). Thus, it has become clear that Ig H gene recombination and μ chain expression are crucial for the survival and further differentiation of pro-B cells in BM.

Our data show that the pro-B cell population in RAG-2-/- BM is significantly augmented, including each of the early, intermediate and late pro-B cell compartments. There is no proliferative or kinetic mechanism yet known to interpret this expansion. Other models in which developing B cells abort at the late pro-B cell stage, including the *scid* mutation, have failed to show hyperplasia of the pro-B cells that might indicate a feedback stimulation of early precursor B cells (Osmond et al., 1992; Oamond, et al., 1994).

The pro-B cell population in RAG-2-/- mice shows a higher apoptotic incidence *ex vivo* than controls. We have employed a previously established short term culture system to determine the rate of accumulation of apoptotic cells when not removed by macrophage-mediated phagocytosis (Lu and Osmond, 1997). As expected, pro-B cells from RAG-2-/- mutant mice display accelerated apotosis, confirming that RAG-2-/- pro-B

cells are more prone to cell death than normal controls. We also observe that Bax protein expression among pro-B cells is significantly upregulated in RAG-2-/- BM, though the Bcl-2 protein level remains comparable with control values. Thus, the results suggest that an elevated Bax/Bcl-2 ratio can determine the susceptibility of pro-B cells to selection in RAG-2-/- mouse BM, as proposed in normal B cell development (Chapter 4). It is plausible to propose that successful rearrangement of μ H genes delivers a survival signal to permit pro-B cells to undergo further differentiation, arguing for a mechanism of positive selection (Melchers et al., 1995). Otherwise, pro-B cells that fail to achieve IgH recombination due to either RAG-2 mutation or other naturally occurring events will die by apoptosis *in vivo*.

Since every RAG-2-/- pro-B cell is destined to undergo cell death in BM, this provides an attractive model to estimate the *in vivo* transit time of cells passing through the $B220^{+}\mu^{-}$ phenotypic stage in RAG-deficient mice and also the fraction of cells that normally undergo apoptosis at this critical checkpoint in B cell development. Cells may be visualised as entering the $B220^{+}\mu^{-}$ differentiation compartment from proliferating progenitor cells and leaving the compartment after a given transit time by undergoing apoptosis. First, given the premise that the rate of accumulation of apoptoses during the brief period of the *in vitro* assay approximates to the rate at which cells had been signalled to initiate the cell death program *in vivo*, the observed data indicate that 100% of $B220^{+}\mu^{-}$ cells in RAG-2-/- mice would pass through the compartment and become apoptotic in a period of 18 hours. Second, given the evident fact that the observed rate of apoptosis among $B220^{+}\mu^{-}$ pro-B cells in RAG-2-/- mice represents the result of a complete

apoptotic deletion, comparison with the observed apoptotic rate in normal control mice suggests that the latter represents the deletion of 70% of $B220^{+}\mu^{-}$ cells. Although this figure can constitute only an approximation, it is generally consistent with the estimates proposed for the high frequency of nonfunctional Ig H chain gene rearrangements normally generated (Melchers et al., 1995; Rajewsky, 1996) and the fraction of precursor B cell production normally resulting in apoptosis, as previously proposed in our kinetic model (Chapter 3).

In conclusion, all B lineage cells abort at a $B220^{+}\mu^{-}$ phenotypic stage of development in RAG-2 deficient mouse BM. $B220^{+}\mu^{-}$ pro-B cells show a higher apoptotic incidence *ex vivo* and an accelerated rate of entry into apoptosis in short term culture. Bax expression by RAG-2-/- pro-B cells is markedly increased whereas Bcl-2 protein remains at control levels providing further evidence that the Bax/Bcl-2 protein ratio is implicated in regulating early precursor B cell death in mouse BM.

Table 5.1 Incidence of B lineage cells in BM of RAG-2$^{-/-}$ and control mice*

	TdT^{+}	B220$^{+}\mu^{-}$	Total μ^{+}
RAG-2$^{-/-}$	10.1 $\pm$ 1.4	20.2 $\pm$ 2.1	0
Control	1.8 $\pm$ 0.5	10.1 $\pm$ 2.2	24.1 $\pm$ 2.6

* Percentage of B cells of each defined phenotype relative to total nucleated BM cells. Values (Mean $\pm$ SD) were derived from 4 separate experiments.

Fig. 5.1. Flow cytometric analysis of immunolabeled BM cell suspensions from RAG-2-/- and control mice. Incidences of various defined populations of precursor B cells are indicated.

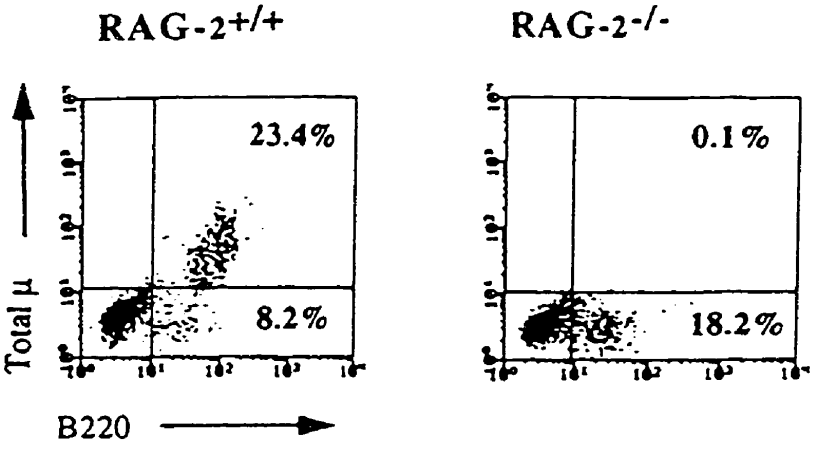

RAG-2+/+
RAG-2-/-
23.4%
8.2%
0.1%
18.2%
Total μ
B220

Fig.5.2. Incidences of hypodiploid apoptotic $B220^{+}\mu^{-}$ precursor B cells in freshly prepared BM cell suspensions from RAG-2-/- and control mice. Values derived from 3 separate experiments (Mean±SD).

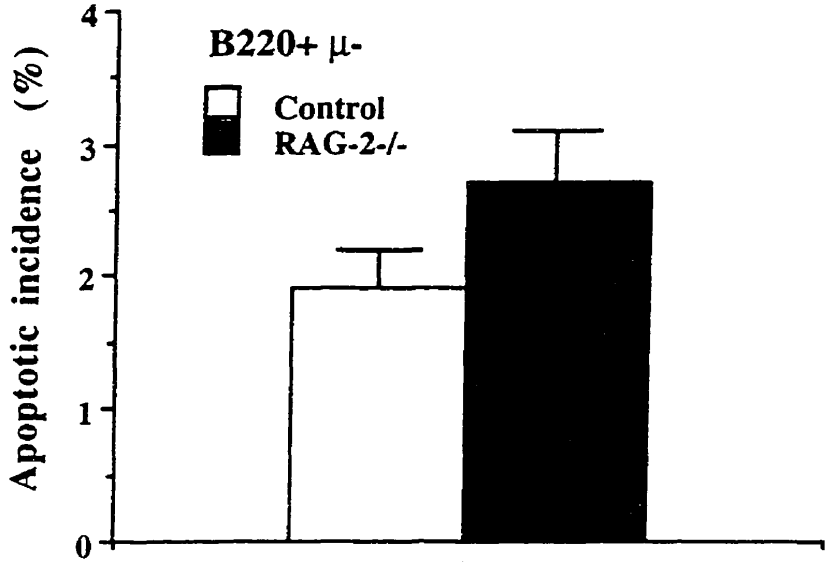

B220+ μ-
Control
RAG-2-/-
Apoptotic incidence (%)
0
1
2
3
4

Fig. 5.3. Incidence of hypodiploid cells among $B220^{+}\mu^{-}$ cells in BM suspensions from RAG-2-/- and control mice after 0-4 h incubation. Data represent 3 separate experiments (Mean±SD).

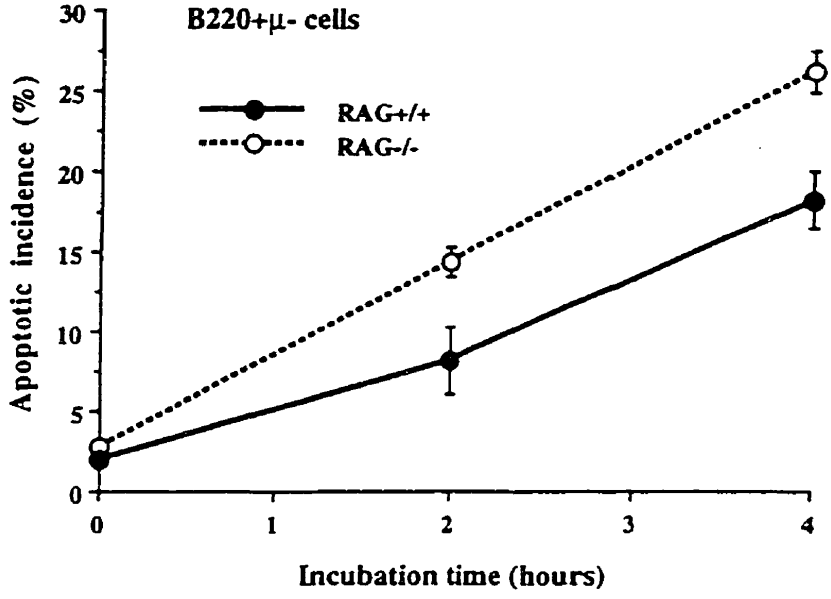
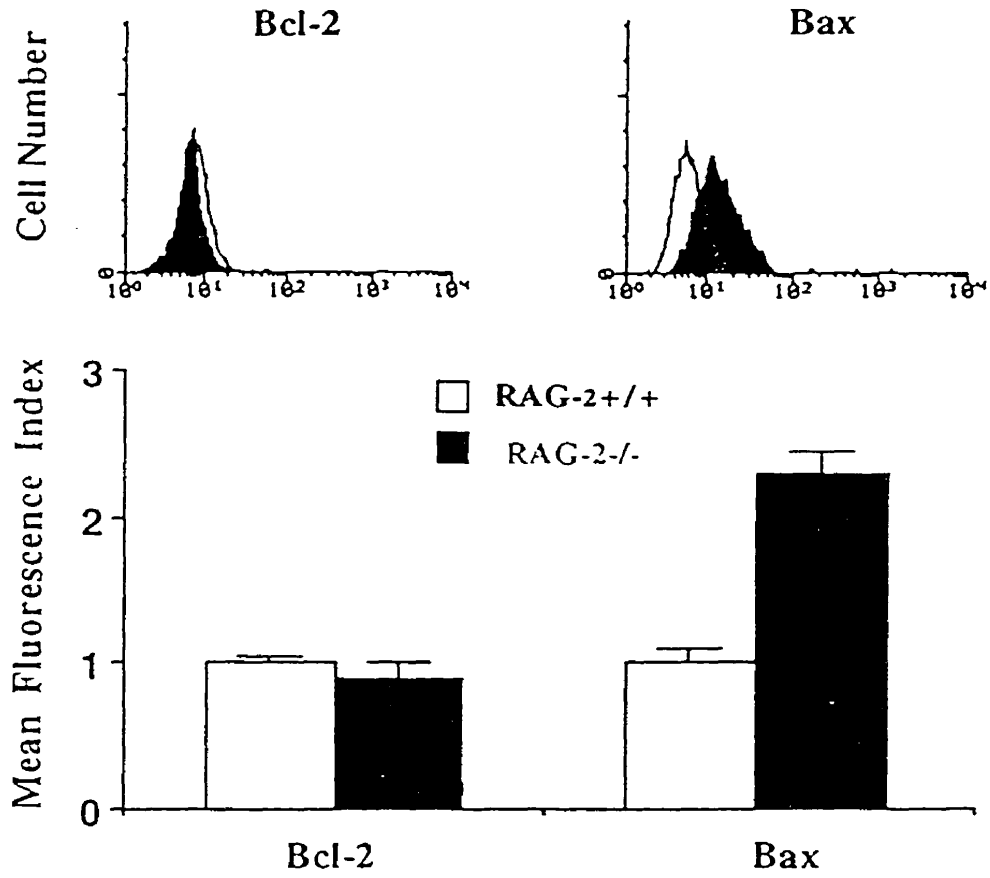

B220+μ- cells
RAG+/+
RAG-/-
Apoptotic incidence (%)
30
25
20
15
10
5
0
0
1
2
3
4
Incubation time (hours)

Fig. 5.4. Representative histograms (above) showing expression of endogenous Bcl-2 and Bax proteins among $B220^{+}\mu^{-}$ B cells in freshly prepared BM cell suspensions from RAG-2-/- and control mice.

Bcl-2 and Bax expressions (below) were measured as mean fluorescence index by flow cytometry, expressed relative to control values. Data derived from 3 separate experiments (Mean$\pm$SD).


Bcl-2
Bax
Cell Number
Mean Fluorescence Index
RAG-2+/+
RAG-2-/-
0
1
2
3
Bcl-2
Bax

Chapter 6 B Cell Apoptosis and its Regulation in IL-7 Transgenic and IL-7 Gene Deleted Mouse BM

6.1. Summary

B cell genesis in mouse BM is critically influenced by local microenvironmental factors. IL-7, a stromal cell derived cytokine, plays an essential role in B cell differentiation and proliferation. Little is known, however, about its possible effect on the selection of B cells during their development. It has been reported that stromal cells can influence Bcl-2 and Bax expression in a pro-B cell line and that clonogenic IL-7-dependent precursor B cells, ultrasensitive to apoptotic induction, express only low levels of Bcl-2 protein. It remains unclear, nevertheless, whether IL-7 itself can regulate the expression of Bcl-2 family proteins in B cells. In the present study, IL-7 gene-deleted and IL-7 transgenic mice have been examined to determine the effect of IL-7 on B cell selection *in vivo*. Flow cytometric analysis has revealed that modifications of IL-7 gene expression greatly affect B cell differentiation and proliferation in BM. Further, apoptosis of precursor B cells was enhanced by IL-7 gene deletion and suppressed by IL-7 transgene overexpression both *ex vivo* and in short term culture, associated with corresponding alterations in Bcl-2/Bax ratios. The findings suggest that IL-7 plays an important role in regulating precursor B cell selection in mouse BM.

6.2. Introduction

During development in mouse BM, precursor B cells pass through stages of differentiation, proliferation and selection in close association with stromal cells (Osmond, 1990; Jacobsen and Osmond, 1990). Molecular interactions between B lineage cells and BM stroma play key roles in B lymphopoiesis (Kincade, 1994). IL-7, a stromal cell-derived cytokine, regulates B lymphopoiesis and induces proliferation of precursor B cells both *in vitro* and when administered systemically *in vivo* (Namen et al., 1988; Takeda et al., 1989; Morrissey et al., 1991). IL-7 receptors are expressed by $B220^{+}sIgM^{-}$ precursor B cells, but become downregulated on $sIgM^{+}$ B lymphocytes (Sudo et al., 1993). Overexpression of IL-7 in IL-7 transgenic (IL-7 Tg) mice produces excessive proliferation and expansion of precursor B cell populations in BM and predisposes to B cell neoplasia (Valenzona et al., 1996). Conversely, in IL-7 gene-deleted (IL-7-/-)mice, only small numbers of B lineage cells develop beyond an early precursor stage of development (von Freeden-Jeffry et al., 1995). A similar result is produced in mice treated with anti-IL-7 mAb *in vivo* (Grabstein et al., 1993). While this result might be expected as a consequence of the lack of proliferative expansion of precursor B cell populations, the effect could also be due to enhanced precursor B cell death under conditions of IL-7 deprivation. The requirement for IL-7 in precursor B cell proliferation has been extensively characterized but little information is available on the possible role of IL-7 in B cell selection as an *in vivo* survival factor (Boise and Thompson, 1996).

B cell genesis in mouse BM represents a delicate balance between cell proliferation and cell death throughout postnatal life (Osmond et al., 1994), but the mechanisms determining survival or death of B lineage cells in BM are incompletely understood. Members of the Bcl-2 gene family commonly serve as positive and negative regulators of lymphoid cell death (Cory, 1995). The pairing of Bcl-2 and Bax proteins provides a prominent example. The balance between Bcl-2/Bax heterodimers, acting to suppress apoptosis, and Bax homodimers, favoring apoptosis, determines susceptibility to a given death signal in a variety of cell systems (Oltvai and Korsmyer, 1994). Stromal cells cultured from BM influence Bcl-2 and Bax expression in a pro-B cell line (Gibson et al., 1996). IL-7-dependent precursor B cells, particularly prone to apoptosis, normally express only low levels of Bcl-2 protein (Griffiths et al., 1994), while IL-7 withdrawal induces rapid cell death of IL-7 dependent B cell lines *in vitro* (Borzillo et al., 1992). Whether IL-7 itself can influence *in vivo* expression of Bcl-2 family proteins by B lineage cells in BM remains unknown.

In the present study, rates of apoptosis and levels of Bcl-2 and Bax proteins among B lineage cells in BM have been examined in IL-7-/- mice and IL-7 Tg mice. The results reveal that apoptosis of precursor B cells is enhanced by IL-7 gene deletion, but suppressed by IL-7 transgene and that these effects are correlated with alterations in Bcl-2/Bax ratio.

6.3. Materials and Methods

6.3.1. Mice

8-12-wk-old IL-7 Tg mice were provided by Dr. R. Ceredig (INSERM, Strasbourg, France). They had been generated by backcrossing (57BL/6 x DBA2) F1 mice carrying a high copy number of murine IL-7 cDNA under the control of MHC class II (Ea) promoter to C57BL/6 mice, as described (Mertsching et al., 1995). Age-and strain-matched normal mice served as controls.

8-12-wk-old IL-7-/- and wild type controls (IL-7+/+) were provided by Dr. R. Murry (DNAX, Palo Alto, California). To generate IL-7-/- mice, cells of the embryonic stem cell line, E14.1, were transfected with an IL-7 gene-targeting vector. The selected mutated clones were injected into blastocysts from C57BL/6 mice and implanted into pseudopregnant (B6 x CBA) F1 females. Heterozygous offspring were crossed to obtain homozygous animals (von Freeden-Jeffry et al., 1995).

6.3.2. BM Cell Suspension and Cell Culture

Femoral BM cell suspensions were prepared as described in Chapter 2. Samples were either processed immediately or cultured in MEM/NCS at 37^{0}C in a humidified incubator with 5% CO_2 for 4 h.

6.3.3. Phenotypic Labeling

BM cell samples were labeled with PE-conjugated anti-B220 and -IgM, as described

in Chapter 1. To examine total μ chain expression, cells were fixed with cold 70% ethanol and then stained with biotin-conjugated anti-μ chain Ab, revealed by streptavidin-Red 670™ (Gibco-BRL Research Laboratories).

6.3.4. Cell Sorting

Samples of pooled BM cell suspensions from IL-7 Tg mice were immunolabeled for B220 and IgM expression. Large and small $B220^{+}sIgM^{-}$ precursor B cells were purified by a FACS sorter, as described in Chapter 3. The cells were either processed immediately or cultured for apoptotic assays.

6.3.5. Apoptotic Assay

After phenotypic labeling, ethanol-fixed cells were stained with PI for DNA content analysis, as described in Chapter 3.

6.3.6. Immunofluorescent Staining of Protein Expression

Phenotypically labeled cells were incubated with hamster anti-Bcl-2 Ab (PharMingen, San Diego, CA) or rabbit anti-Bax Ab (Oncogene Research Products, Cambridge, MA) at 1:50 dilution in PBS with 0.1% saponin for 30 min on ice, followed by FITC-conjugated goat-anti-hamster IgG (Cedarlane Laboratories Limited) or goat-anti-rabbit IgG (Southern Biotechnology Associates) Abs at 1:50 dilution in PBS, respectively. Samples stained with isotype control Abs were used to determine fluorescence background.

6.3.7. Flow Cytometric Analysis

Immunostained cells were analysed with a FACscan flow cytometer (Becton Dickinson) using Lysys II software. A minimum of 10,000 events per sample were collected from various B cell subpopulations with defined phenotypes. Cell debris and clumps were excluded by setting a gate on forward scatter *vs.* side scatter. Measurement of Bcl-2 and Bax protein expression among defined subpopulations of B lineage cells was based on the mean fluorescence intensity, expressed as a mean fluorescence index relative to a control mean fluorescence intensity equal to 1.

6.3.8. *Statistics*

Statistical analysis was performed using Student's t test. $P < 0.05$ was consided significant.

6.4. Results

6.4.1. Altered B Cell Development in IL-7-/- and IL-7 Tg Mouse BM

In IL-7-/- mice the total BM cellularity was reduced to about 65% that of controls, attributable mainly to severely impaired B cell production. Only 7.6±1.2% $B220^+$ B cells were detected among total nucleated BM cells, compared with 35±2.1% in controls. In addition to surface B220, the more specific B lineage phenotypic markers of intracytoplasmic μ chains and sIgM molecules were examined to determine the stage at which B lymphopoiesis was defective. The incidence of $B220^+\mu^-$ pro-B cells was within

the normal range, but both cμ$^+$sIgM$^-$ pre-B cells and sIgM$^+$ B cells were profoundly reduced to about one tenth normal incidence (Fig.6.1A). Thus, B cell development was abruptly inhibited at the pro-B/pre-B transition, in agreement with previous findings (von Freeden-Jeffry et al., 1995). Conversely, in IL-7 Tg mouse BM (Fig. 6. 1B) B220$^+$μ$^-$ pro-B cells and cμ$^+$sIgM$^-$ pre-B cells were markedly increased in both incidence and absolute numbers, though numbers of sIgM$^+$ B lymphocytes were augmented to a lesser extent (Fig.6.1), consistent with recent epifluorescence microscopic studies (Valenzona et al., 1996).

6.4.2. Higher Incidence of Precursor B Cell Apoptosis in BM of IL-7-/- Mice than IL-7 Tg Mice

Apoptosis of B lineage cells was evaluated by the criterion of hypodiploid DNA content, analysed by flow cytometry. Freshly prepared BM cell suspensions were examined at phenotypically defined stages of B cell development. The hypodiploid B cells thus detected *ex vivo* represented only the number of apoptotic B lineage cells present in BM during the brief period between the onset of apoptosis and clearance by macrophages. This value, nevertheless, appeared to reflect the relative frequency of apoptosis within the defined cell populations *in vivo*.

In IL-7-/- mice, the incidence of apoptosis among B220$^+$sIgM$^-$ precursor B cells was significantly higher (2.6±0.2%) than that in controls (2.1±0.4%; $p < 0.05$), whereas the few surviving sIgM$^+$ B lymphocytes in IL-7-/- mice showed essentially the same apoptotic incidence (1.3±0.4%) as in wild type mice (1.2±0.2%) (Fig. 6.2A).

In IL-7 Tg BM suspensions, dissection of the $B220^+sIgM^-$ precursor compartment demonstrated that large cells were rapidly dividing, 54±4.2% being in S, G2 and M phases of cell cycle, whereas small cells were postmitotic, 92±3.5% being in G0/G1 phase, determined from PI-DNA-labeling. The apoptotic incidence among the large cycling $B220^+sIgM^-$ precursors was significantly lower in IL-7 Tg mice than control mice (1.3±0.2% *vs.* 1.9±0.3%; $p<0.05$). In contrast, no significant differences in apoptotic incidence between IL-7 Tg and control BM were detected among either $sIgM^-$ B lymphocytes or small $B220^+sIgM^-$ precursors (Fig. 6.2B).

6.4.3. Apoptosis of $B220^+sIgM^-$ Precursors is Accelerated in IL-7-/- Mice but Suppressed in IL-7 Tg Mice

In order to examine the kinetics of B cell apoptosis, a short term culture system was used to reveal the rate of accumulation of apoptotic cells without macrophage-mediated deletion (Osmond et al., 1994). Among B lineage cells from both IL-7-/- and control IL-7+/+ mouse BM, $B220^+sIgM^-$ precursors became apoptotic more extensively than $sIgM^+$ B lymphocytes during a 4h culture period (Fig.6.3), consistent with our previous findings (Chapter 3).

The apoptotic incidence of $B220^+sIgM^-$ precursor B cells from IL-7-/- mice increased steeply during incubation, in a linear fashion to reach 22.5±1.8% by 4 h. In IL-7+/+ control mice, $B220^+sIgM^-$ precursors also showed an approximately linear increase in apoptotic incidence, but at a much lower rate (Fig. 6. 4). In contrast to precursor B cells, $sIgM^+$B cells showed similar apoptotic kinetics in IL-7-/- and IL-7+/+ control mice (Fig.

6.4).

Unlike IL-7-/- mice, FACS-sorted $B220^+sIgM^-$ precursor B cells from IL-7 Tg mice showed a much lower apoptotic incidence than controls during *in vitro* culture (Fig. 6.5). In particular, large $B220^+sIgM^-$ precursor B cells displayed only half the apoptotic incidence of controls after 6 h incubation. Small $B220^+sIgM^-$ cells and $sIgM^+$ cells, on the other hand, displayed similar apoptotic incidences in IL-7 Tg mice and controls (Fig. 6. 5).

6.4.4. Downregulated Bcl-2 Expression, but Upregulated Bax Protein Among Precursor B Cells in IL-7-/- Mice

Three-color flow cytometric analysis was performed to determine the intracellular expression of Bcl-2 and Bax by B lineage cells from freshly prepared BM samples. As shown in Fig. 6. 6 among $B220^+sIgM^-$ precursor B cells from IL-7-/- mice, the mean Bcl-2 protein content per cell was reduced to half normal levels, while Bax protein was increased 1.5-fold. On the other hand, the levels of Bcl-2 and Bax among $sIgM^-$ B lymphocytes from IL-7-/- mice remained comparable with control values (Fig. 6.7).

6.4.5. Reciprocal Changes of Bcl-2 and Bax Protein Levels among Large $B220^+sIgM^-$ Precursor B cells in IL-7 Tg Mice

The mean Bcl-2 protein content determined by flow cytometric analysis was increased among large $B220^+sIgM^-$ precursors from IL-7 Tg mice as compared to controls (Fig. 6.8). Conversely, Bax expression was reduced to half normal levels among these cells

(Fig.6.8). Lesser degrees of Bax protein reduction were shown by small $B220^+sIgM^-$ cells and $sIgM^+$ cells in IL-7 Tg BM (Fig. 6.9).

6.5. Discussion

The present study has confirmed that IL-7 gene deletion results in a profound impairment in production of $c\mu^+$ pre-B cells and $sIgM^+$ B lymphocytes in mouse BM (von Freeden-Jeffry et al., 1995) . This finding is consistent with an effect upon the populations of precursor B cells that constitutively express IL-7 receptors, as reported (Sudo et al., 1993). However, the observation that the number of $B220^+\mu^-$ pro-B cells remains almost unaffected in IL-7-/- mice implies that these early progenitor B cells themselves do not require IL-7 for proliferation and survival. Flow cytometric analysis of the *in vivo* effect of IL-7 on B cell selection now reveals that $B220^+sIgM^-$ precursor B cells from IL-7 gene-deleted mice display an elevated initial incidence of apoptosis and increased apoptotic rate in short term culture, whereas large $B220^+sIgM^-$ precursors from IL-7 overexpressing mice show depressed apoptotic incidences both initially and after incubation. To some extent, such effects might simply be attributable to fluctuations in the degree of proliferative expansion of precursor B cells, depending upon the overexpression or lack of IL-7 relative to normal levels of IL-7 *in vivo*. It could be postulated that the substantial fraction of pro-B cells normally destined to undergo apoptotic selection is diluted to a lesser or greater extent by other precursor B cells

proliferating under the influence of IL-7, thus resulting in apparent changes in the apoptotic rates. This may constitute a contributory factor in the case of IL-7-/- mice, in which $B220^+\mu^-$ pro-B cells form a greater proportion of the $B220^+sIgM^-$ phenotype (80%) than normal (35-45%). It is inadequate, however, to account fully for the greatly enhanced apoptosis observed among $B220^+sIgM^-$ cells in these IL-7-/- mice and can in no way explain the suppressed apoptosis in IL-7 Tg mice, in which the proportion of $B220^+\mu^-$ pro-B cells among $B220^+sIgM^-$ precursors is near normal. Hence, the data demonstrate that IL-7 gene modification *in vivo* can profoundly affect the survival of $B220^+sIgM^-$ precursors in BM, their apoptotic cell death being partially blocked by overexpression of IL-7 and enhanced by lack of IL-7. The cytokine appears not to be an absolute requirement for survival of differentiating B cells, however. Even after IL-7 gene deletion, approximately one tenth of the normal number of pre-B and B cells are present in BM and display normal apoptotic rates, while the apoptotic kinetics of these cells are also relatively little affected by overexpression of IL-7. Thus, the level of IL-7 in BM microenvironment appears to be capable of modulating B cell selection at the early precursor B cell stage, when many cells with nonfunctional Ig V_H gene rearrangements are normally deleted, but not at the immature B cell stage, when cells with certain autoreactive specificities or defective Ig light chain rearrangements abort (Lu and Osmond, 1997).

The ability of IL-7 to block apoptosis among pro-B cells in BM may have implications for other B cell dysregulations, notably neoplasia. Many IL-7 Tg mice ultimately develop malignant lymphomas of early B lineage phenotype (Fisher et al., 1993). It may be

speculated that the oncogenic process could in some instances be initiated among early precursor B cells in BM. Cells that sustain genetic errors during Ig gene recombination, including chromosomal translocations, and that normally would undergo apoptotic selection at the pro-B/pre-B cell transition may be permitted to survive past this checkpoint. Subsequent genetic modifications in one or more such cells could result in a monoclonal or oligoclonal B lineage tumour, as described (Osmond, et al., 1990; Osmond et al., 1994). Thus, the effect of IL-7 on precursor B cell apoptosis could help to explain the association between prolonged hyperplasia of pro-B cells and increased incidence of B cell neoplasms. Such conditions may be produced by more usual conditions than an IL-7 transgene. Prolonged systemic activation of macrophages, as occurs in chronic granulomata and malarial infection, are associated with sustained elevations of pro-B cell proliferation and the development of B cell tumours (Rico-Vargas et al., 1995).The effect on pro-B cell proliferation appears to be mediated by circulating macrophage-derived cytikines acting via receptors on the BM stromal cells that in turn are presumed to be induced to increase IL-7 secretion (Fauteux and Osmond, 1996). Thus, an increase in IL-7-driven production of B lymphocytes in BM may be achieved at the cost of some reduction in the efficiency of quality control.

To determine whether IL-7 expression can influence the Bcl-2/Bax ratio among B lineage cells, three-color flow cytometric analysis has been performed. In IL-7-/- mice, Bcl-2 protein expression among $B220^{+}sIgM^{-}$ precursor B cells is downregulated and Bax expression is increased, while converse effects occur in IL-7 Tg mice. This is the first evidence that altered degrees of apoptosis among BM precursor B cells induced by IL-7

gene modifications *in vivo* are directly correlated with alteration of endogenous Bcl-2/Bax ratio. The results accord with recent studies that when removed from stromal cell cultures, precursor B cells rapidly downregulate Bcl-2 mRNA but increase Bax expression, correlating with the initiation of apoptosis (Gibson et al., 1996). In addition to IL-7, stromal cell lines also produce c-kit ligand and insulin-like growth factor-1, cytokines that can potentiate the proliferative effect of IL-7 on precursor B cells (Landreth et al., 1992). Nevertheless, it appears from the present work that IL-7, itself, plays a critical role in apoptosis by regulating Bcl-2 family proteins among precursor B cells.

Previous observations (Loffert et al., 1994) that IL-7 receptor is mainly expressed on $B220^+sIgM^-$ precursors accord with our finding that IL-7 gene deletion or IL-7 transgene overexpression *in vivo* affect the apoptosis of precursor B cells to a much greater extent than that of $sIgM^+$ B lymphocytes. We have noted, however, that Bax expression among $sIgM^+$ B lymphocytes is somewhat downregulated, though to a lesser extent than among $B220^+sIgM^-$ precursors in mice which carry an IL-7 transgene under the control of MHC class II promoter (Fisher et al., 1993). In these IL-7 Tg mice, some $sIgM^+$ B cells as they begin to express MHC II may be subject to both autocrine and paracrine stimulation by the IL-7 overexpression *in vivo*. IL-7 stimulates cells through a receptor complex, composed of the IL-7R α chain and the IL-2R *r* chain (Goodwin et al., 1990). Recent studies have shown that various cytokines, including IL-2, IL-4, IL-7 and IL-15, can signal through the *r* chain of IL-2R to prevent apoptosis of activated T cells in response to growth factor withdrawal by selectively inducing expression of Bcl-2 and Bcl-x_L, but

not Bax and Bcl-x_S (Danescu et al., 1992; Akbar et al., 1996). Pre-B cells and immature B cells in BM display the IL-2R chain (CD25) at the cell surface (Melchers et al., 1995). Possibly, therefore, signalling through IL-2R *r* chains on developing B cells may partially account the *in vivo* effect of IL-7 on B cell survival in mouse BM.

Cell type-specific sensitivity to apoptosis indicates the existence of an autonomous regulation of the apoptotic program during cell development, which is not solely determined by extracellular death signals (Oltvai and Korsmeyer, 1994; Yang and Korsmeyer, 1996). It has been suggested that the Bcl-2/Bax ratio can predetermine susceptibility to apoptosis rather than the absolute level of either protein. The present findings demonstrate that alterations in the balance between Bcl-2 and Bax expressions, due to either IL-7 gene deletion or IL-7 transgene overexpression, are associated with corresponding alterations in survival of precursor B cells. It is plausible that upregulation of Bax protein would favour the formation of Bax homodimers countering the inhibitory effect on apoptosis by Bcl-2/Bax heterodimers, and *vice versa*. We speculate that IL-7, derived from BM stromal cells and signalling via receptors on precursor B cells, may exert its effect upstream from Bcl-2 family proteins to regulate B cell selection, as well as its much studied role in stimulating proliferation and differentiation. The molecular mechanisms involved in this signalling pathway remain to be elucidated (Maraskorsky et al., 1996).

Further studies are required to assess whether other proteins of the Bcl-2 family, such as Bcl-x_L and Bcl-x_S, are regulated among B lineage cells by IL-7 gene modifications *in vivo*. Nevertheless, our observations on Bcl-2 and Bax provide insights into the

regulation of apoptosis during B cell development in BM that may ultimately be more fully characterised in terms of the balance between pro- and anti-apoptotic signals.

Fig. 6.1. Flow cytometric analysis of phenotypically immunolabeled BM cell suspensions from (A)IL-7-/- and control IL-7+/+ mice, and (B)IL-7 Tg and nontransgenic control mice. Incidences of various defined B cell populations are indicated. The profiles are representative of 3 separate experiments.

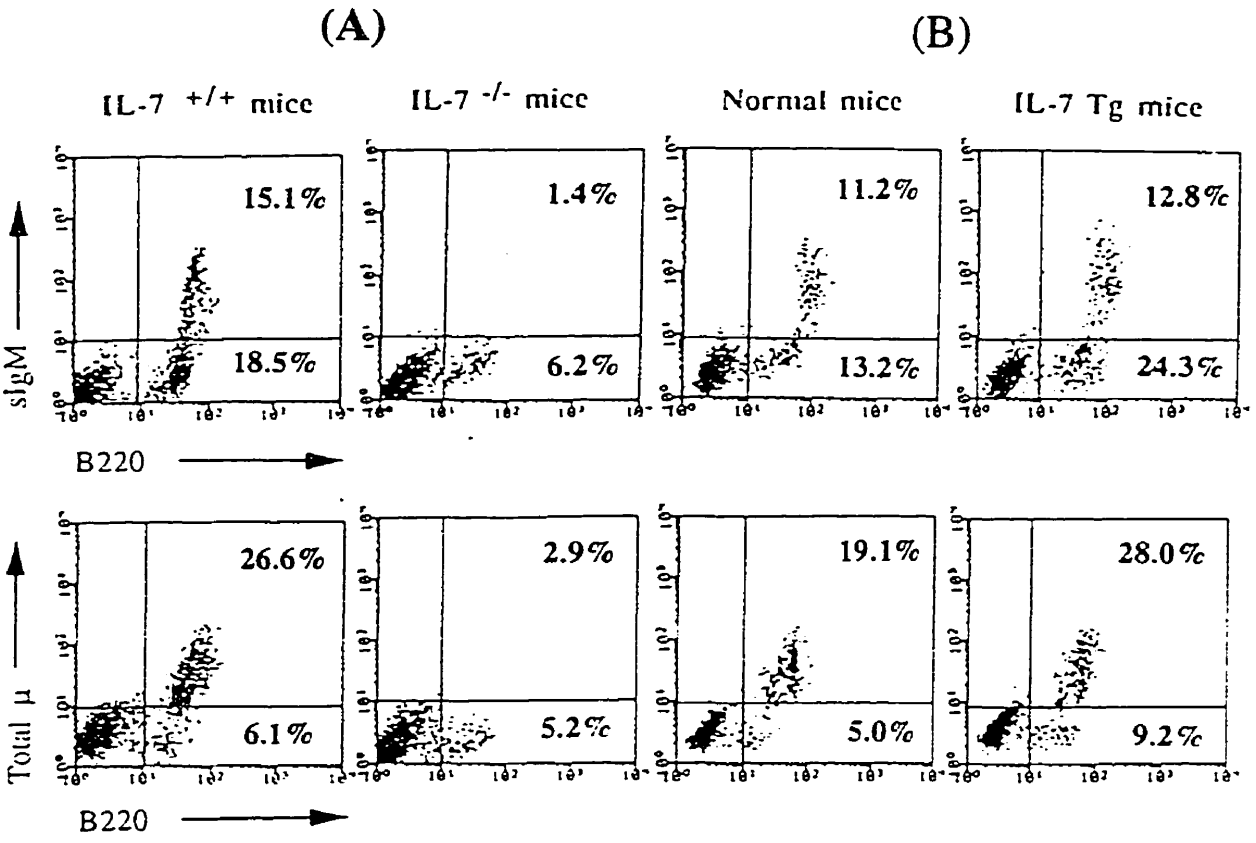
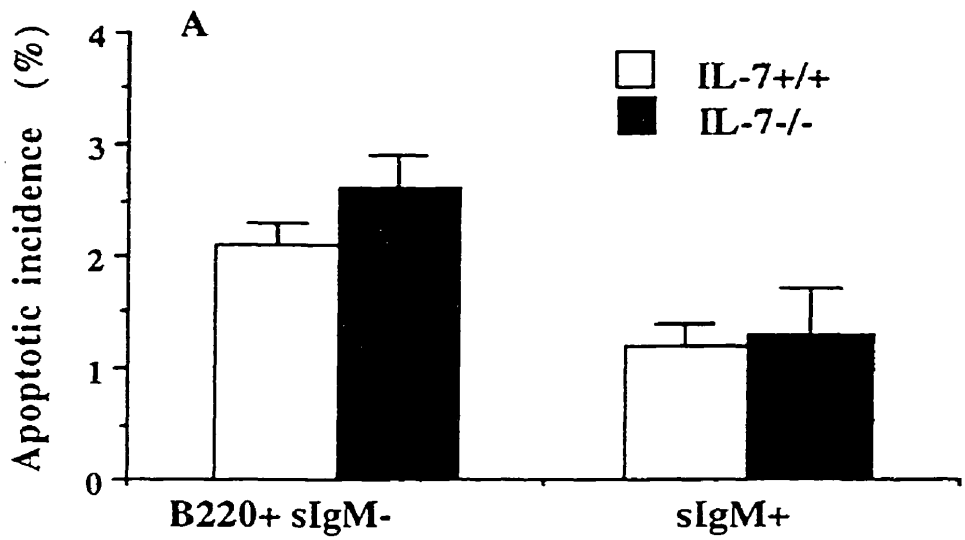

(A)
(B)
IL-7 +/+ mice
IL-7 -/- mice
Normal mice
IL-7 Tg mice
15.1%
18.5%
1.4%
6.2%
11.2%
13.2%
12.8%
24.3%
sIgM
B220
26.6%
6.1%
2.9%
5.2%
19.1%
5.0%
28.0%
9.2%
Total μ
B220

Fig. 6.2. Incidence of hypodiploid apoptotic B cells at defined phenotypic stages in freshly prepared BM cell suspensions from (A)IL-7-/- and IL-7+/+ control mice, and (B) IL-7 Tg and nontransgenic control mice. Values derived from 3 separate experiments (Mean $\pm$ SD).


A
Apoptotic incidence (%)
4
3
2
1
0
IL-7+/+
IL-7-/-
B220+ sIgM-
sIgM+

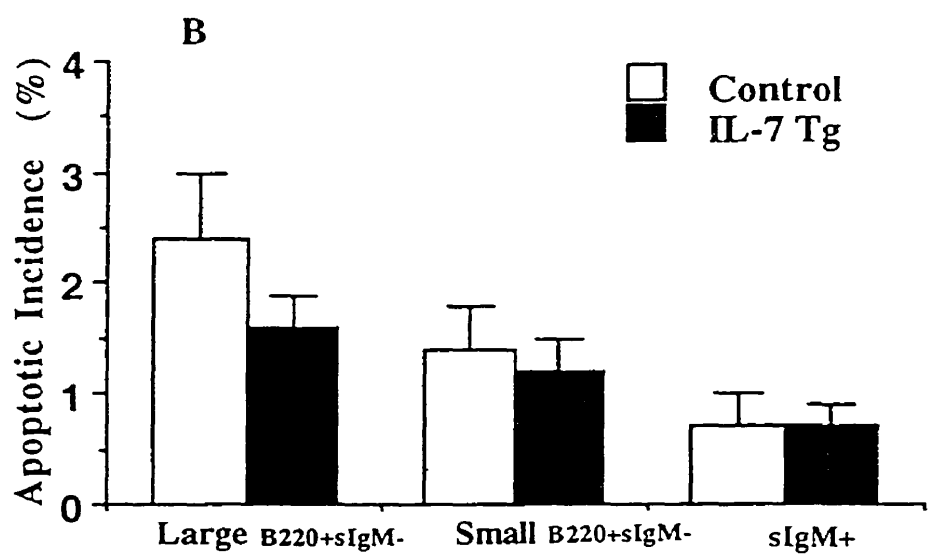

B
Apoptotic Incidence (%)
4
3
2
1
0
Control
IL-7 Tg
Large B220+sIgM-
Small B220+sIgM-
sIgM+

Fig. 6.3. Representative histograms showing apoptotic cells among $B220^+$ populations in BM suspensions from IL-7-/- and control IL-7+/+ mice after various periods of *in vitro* incubation. Hypodiploid incidences are indicated.

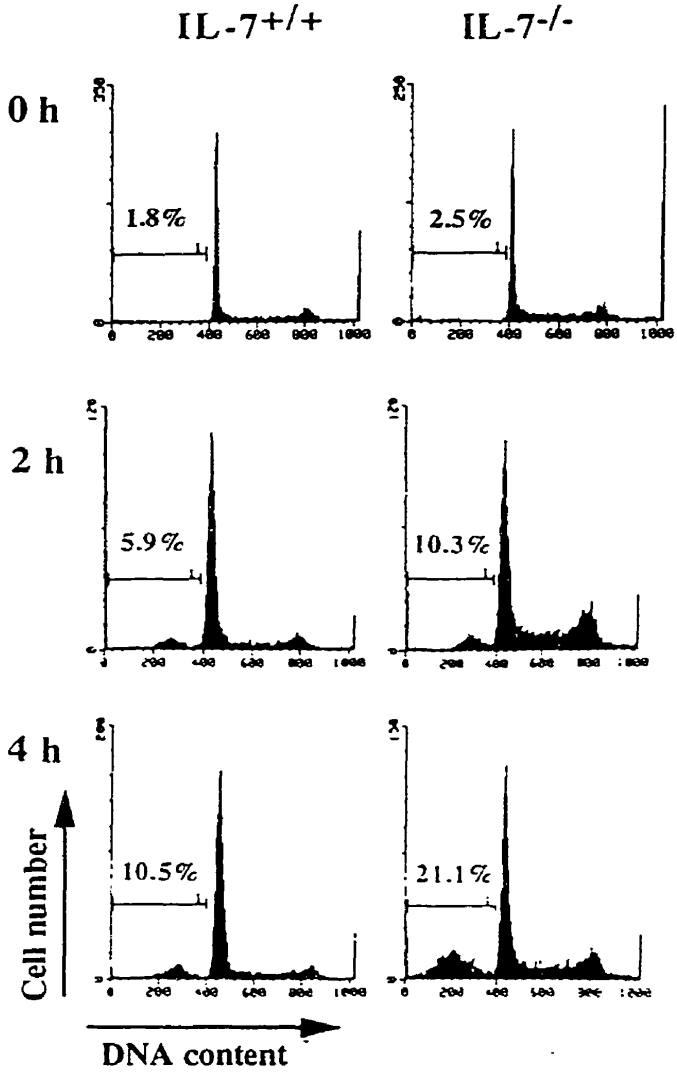

IL-7+/+
IL-7-/-
0 h
1.8%
2.5%
2 h
5.9%
10.3%
4 h
10.5%
21.1%
Cell number
DNA content

Fig. 6.4. Incidence of hypodiploid cells among $B220^{+}sIgM^{-}$ precursors (above) and $sIgM^{+}$ B lymphocytes (below) in BM suspensions from IL-7-/- and control IL-7+/+ mice during 0-4 h *in vitro* incubation. Values derived from 3 separate experiments (Mean $\pm$ SD).

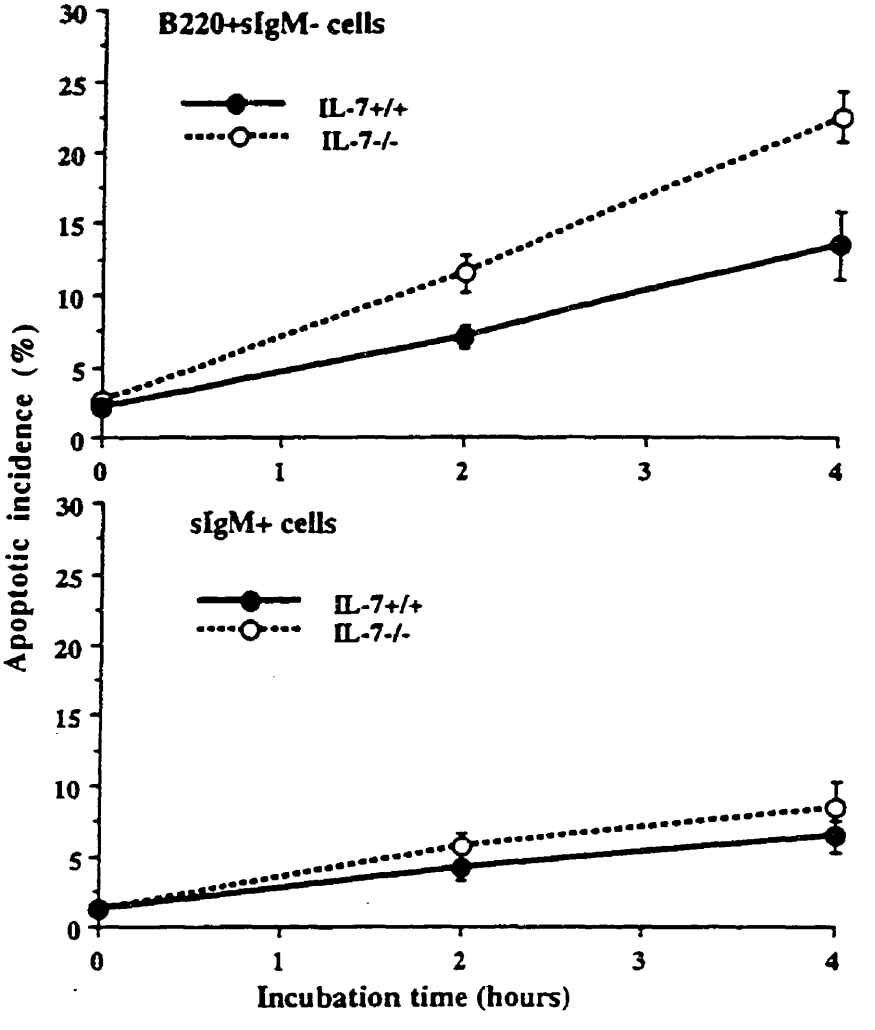

B220+sIgM- cells
IL-7+/+
IL-7-/-
sIgM+ cells
IL-7+/+
IL-7-/-
Apoptotic incidence (%)
Incubation time (hours)
0
5
10
15
20
25
30
0
1
2
3
4

Fig. 6.5. Incidences of hypodiploid B cells at various defined stages of development in BM cell suspensions from IL-7 Tg and control mice after *in vitro* 6 h incubation. Values derived from 3 separate experiments (Mean ± SD).

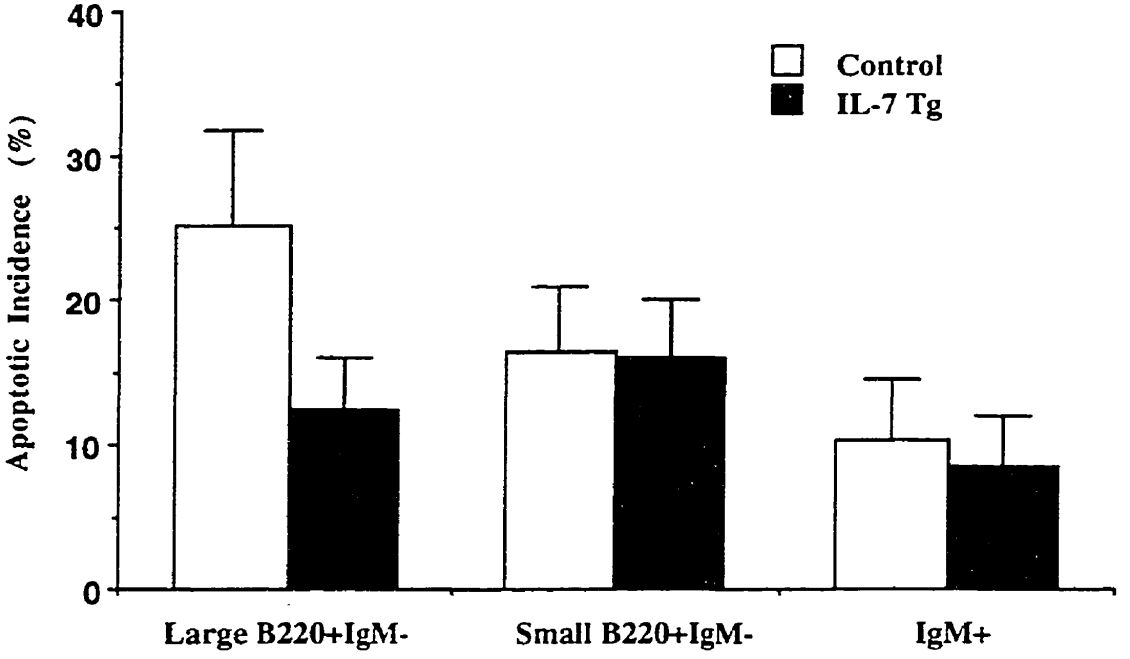

40
30
20
10
0
Apoptotic Incidence (%)
Control
IL-7 Tg
Large B220+IgM-
Small B220+IgM-
IgM+

Fig. 6.6. Expression of Bcl-2 and Bax among $B220^{+}sIgM^{-}$ precursor cells in freshly prepared BM cell suspensions from IL-7-/- and control IL-7+/+ mice. Histograms (above) are representative of 3 separate experiments. Protein expression (below) was measured as mean fluorescence intensity per cell,. Representing the mean values from IL-7+/+ control mice as equal to 1 (Mean $\pm$ SD).

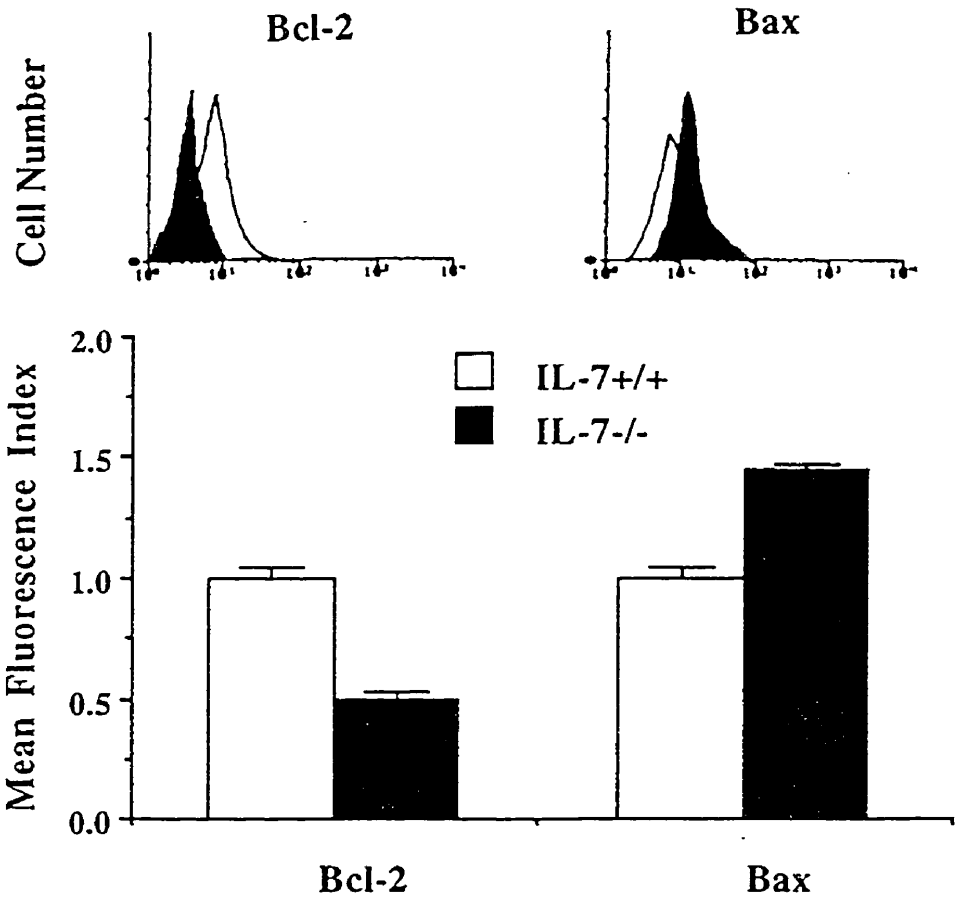

Bcl-2
Bax
Cell Number
Mean Fluorescence Index
2.0
1.5
1.0
0.5
0.0
IL-7+/+
IL-7-/-
Bcl-2
Bax

Fig. 6.7. Expression of Bcl-2 and Bax among $sIgM^+$ B lymphocytes in freshly prepared BM cell suspensions from IL-7-/- and control IL-7+/+ mice. Histograms (above) are representative of 3 separate experiments. Protein expression (below) was measured as mean fluorescence intensity per cell, representing the mean values from IL-7+/+ control mice as equal to 1 (Mean $\pm$ SD).

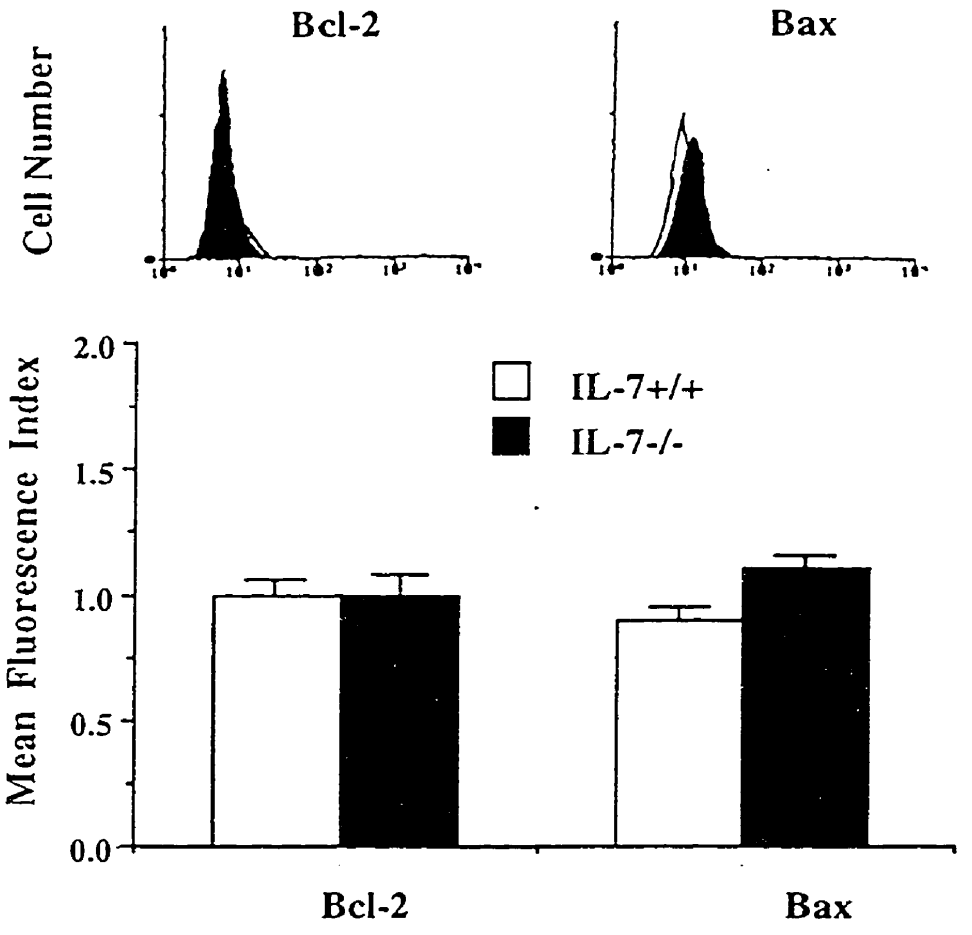

Bcl-2
Bax
Cell Number
Mean Fluorescence Index
2.0
1.5
1.0
0.5
0.0
IL-7+/+
IL-7-/-
Bcl-2
Bax

Fig. 6.8. Expression of Bcl-2 and Bax among large $B220^{+}sIgM^{-}$ precursor B cells in freshly prepared BM cell suspensions from IL-7 Tg and control mice. Histograms (above) are representative of 3 separate experiments. Protein expression (below) was measured as mean fluorescence intensity per cell, representing the mean values from normal control mice as equal to 1 (Mean $\pm$ SD).

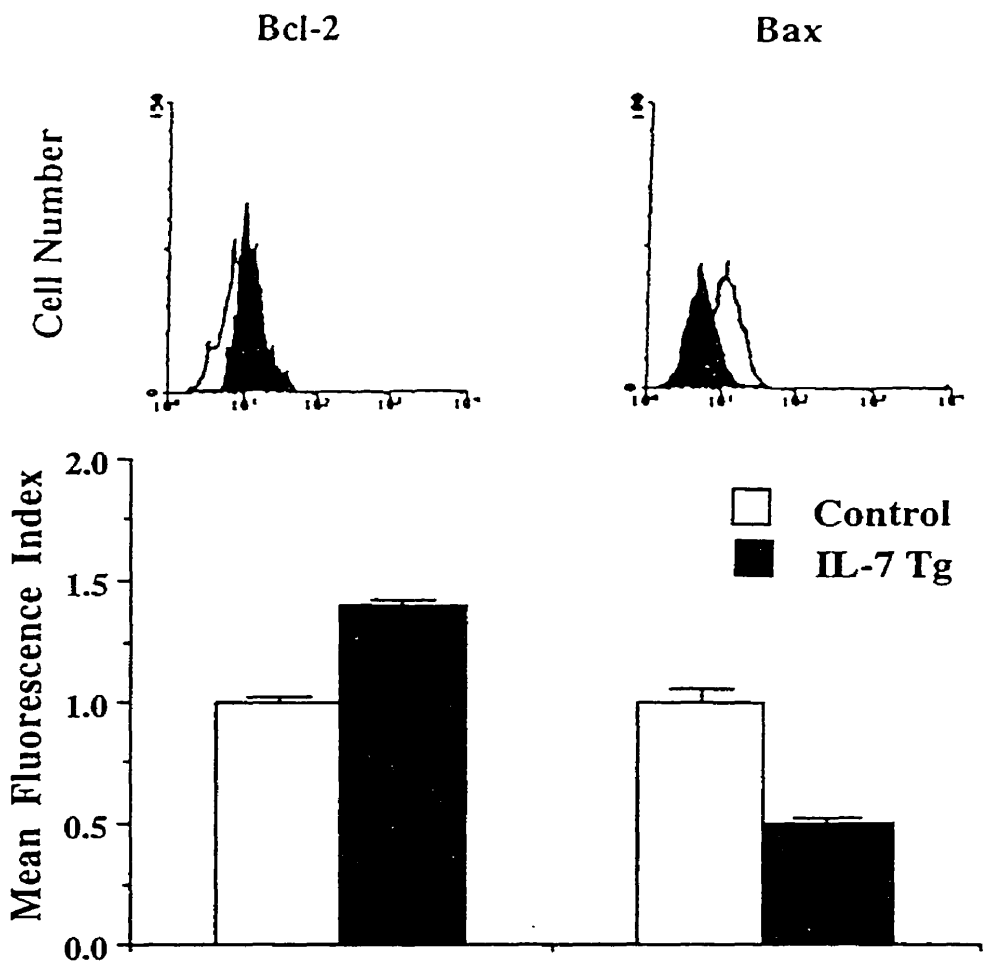
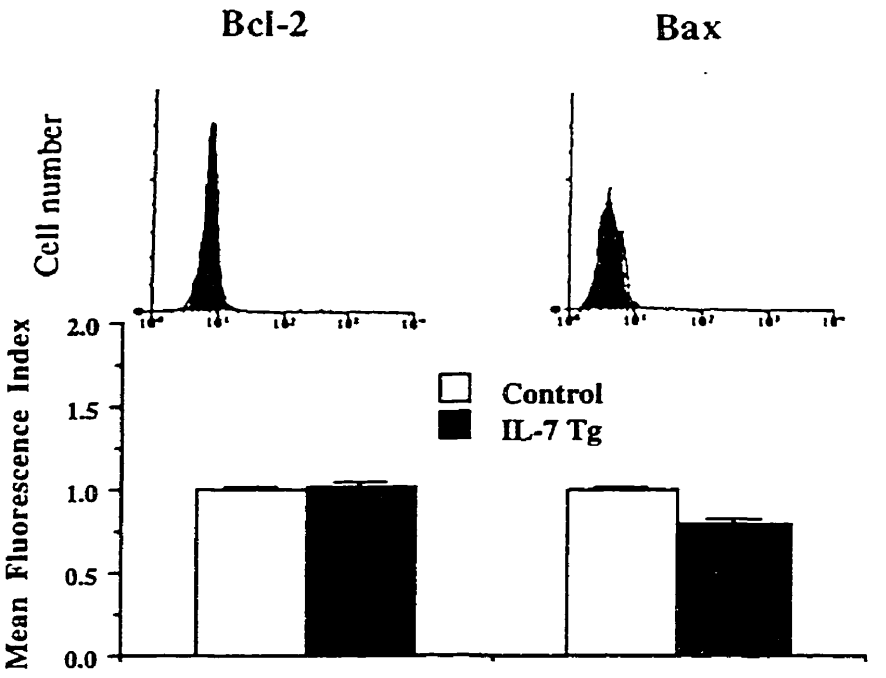
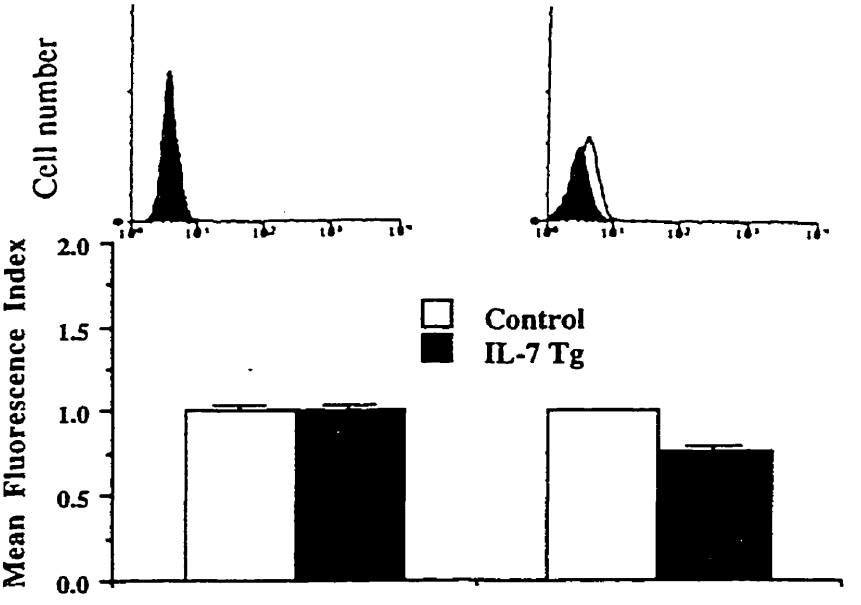

Bcl-2
Bax
Cell Number
Mean Fluorescence Index
2.0
1.5
1.0
0.5
0.0
Control
IL-7 Tg

Fig. 6.9. Expression of Bcl-2 and Bax among (A) small $B220^{+}sIgM^{-}$ precursor B cells and (B) $sIgM^{+}$ B cells in freshly prepared BM cell suspensions from IL-7 Tg and control mice. Histograms (above) are representative of 3 separate experiments. Protein expression (below) was measured as mean fluorescence intensity per cell, representing the mean values from normal control mice as equal to 1 (Mean $\pm$ SD).

A


Bcl-2
Bax
Cell number
Mean Fluorescence Index
2.0
1.5
1.0
0.5
0.0
Control
IL-7 Tg

B


Cell number
Mean Fluorescence Index
2.0
1.5
1.0
0.5
0.0
Control
IL-7 Tg

Chapter 7 B Cell Differentiation and Selection in Osteopetrotic Mouse BM

7.1. Summary

In osteopetrotic (op/op) mice, a CSF-1 gene mutation causes a severe deficiency of macrophages and osteoclasts. To examine whether CSF-1 may also play an *in vivo* role in B lymphopoiesis, B cell phenotypic populations and apoptoses have been analysed in BM of op/op mice. BM cellularity was reduced to 10% of the value in normal littermates. B cells at various defined differentiation stages were significantly decreased both in incidence and absolute number in op/op mice, most markedly at the pre-B cell stage. Flow cytometric analysis further showed that op/op precursor B cells displayed elevated apoptotic incidences, particularly among pre-B cells, both *ex vivo* and in short term culture. The findings provide evidence that CSF-1 is involved in the regulation of B cell differentiation and selection during B lymphopoiesis in mouse BM.

7.2. Introduction

Osteopetrotic (op/op) mice suffer from congenital osteopetrosis due to the recessive mutation of colony-stimulating factor-1 (CSF-1) gene locus on chromosome 3. The mice exhibit a variety of abnormalities in BM cavity development and tooth eruption as well

as in hemopoiesis, notably a deficiency of monocytes/macrophages and osteoclasts (Yoshida et al., 1990; Wiktor-Jedrzejczak et al., 1982; Wiktor-Jedrzejczak, et al., 1990; Wiktor-Jedrzejczak and Gordon, 1996). Young op/op mutant mice have reduced bone resorption, decreased BM volume and cellularity and defective immunological functions (Wiktor-Jedrzejczak et al., 1982; Yamamoto et al., 1994). CSF-1 is known mainly as a primary growth factor for proliferation, differentiation and survival of the macrophage lineage (Wiktor-Jedrzejczak and Gordon, 1996). Most observations in op/op mice have focussed on impaired development of the mononuclear phagocyte system and related dysfunctions. Since macrophages are clearly not the only cells to be reduced in number in op/op mice, CSF-1 might have a wider range of action *in vivo* upon hemopoietic stem cells in BM beyond that on monocytic precursors. Little information is available, however, about the possible effect of CSF-1 deficiency on B lymphopoiesis in BM.

In the present study, B cell differentiation and selection have been examined in op/op mice. The results reveal that B lymphopoiesis is severely impaired and B cell apoptosis is greatly accelerated, suggesting that CSF-1 is normally involved in the regulation of B cell development in BM.

7.3. Materials and Methods

7.3.1. Mice

Breeding pairs of heterozygous (op/±) mice were obtained from The Jackson Laboratory, Bar Harbor, ME. The mutant op/op offsprings were clearly identified by 10

d of age by failure of eruption of incisor teeth and by a domed skull. Homozygous mutant (op/op) and heterozygous littermates were used at 4 wk of age.

7.3.2. BM Cell Suspensions

Femoral marrow cells were prepared and aliquots of samples were either assayed immediately or incubated for 4 h, as described in Chapter 2.

7.3.3. Phenotypic Staining

BM cell suspensions were immunolabeled to determine B220, total μ chains and sIgM expressions, as described in Chapter 2.

7.3.4. Apoptotic Assay

Phenotypically labeled and ethanol-fixed BM cells were treated with RNase and stained with PI for DNA content analysis, as described in Chapter 2.

7.3.5. Flow Cytometric Analysis

Phenotypic and apoptotic analyses were examined by flow cytometry, as described in Chapter 3.

7.3.6. *Statistics*

Statistical analysis was performed using Student's t test. $P < 0.05$ was consided significant.

7.4. Results

7.4.1. B Cell Differentiation in op/op Mouse BM

In op/op mouse BM, hemopoiesis was severely impaired and the total cellularity was only 10% of the value in normal littermates. Flow cytometric analysis revealed that the incidence of $B220^+$ B lineage cells among the few residual BM cells was even further reduced to 12% as compared to 36% in controls (Fig.1). $C\mu^+s\mu^-$ pre-B cells were decreased 5-fold in incidence and 51-fold in absolute number in op/op BM whereas $sIgM^+$ B lymphocytes were reduced 2-fold and 25-fold, respectively (Table 7.1; Fig. 2). A relatively smaller decline was found among $B220^+\mu^-$ pro-B cells. In separate experiments, intranuclear TdT was immunostained and examined by epifluorescence microscopy. The incidence of TdT^+ pro-B cells was actually higher in op/op BM mouse than in controls (2.2% *vs.* 1.4%). No cells with precursor B cell phenotype were detected in the spleen (data not shown).

7.4.2. Precursor B Cells in op/op Mouse BM Display Increased Apoptotic Incidences ex vivo

In freshly prepared cell suspensions, similar incidences of hypodiploid apoptotic cells were detected in BM of op/op and control mice (1.4% *vs.* 1.2%). The B lineage cells, however, displayed significantly higher hypodiploid incidences in op/op BM than controls ($p<0.05$), particularly among pre-B cells ($p<0.01$; Fig. 3).

7.4.3. Apoptosis of Precursor B Cells from BM of op/op Mice is Accelerated in Short Term Culture

Short-term culture of BM cells was performed to examine apoptotic kinetics. After 4 h incubation, $B220^+$ B cells from op/op mouse BM became apoptotic more extensively than those from controls. However, while the apoptosis of $c\mu^+$ pre-B cells was greatly accelerated, the apoptotic incidence of $sIgM^-$ B lymphocytes was only slightly greater than of controls (Fig. 4).

7.5. Discussion

It has been established that a CSF-1 gene mutation is the primary cause of a severe deficiency of macrophages and osteoclasts in op/op mouse BM (Yoshida et al., 1990; Wiktor-Jedrzejczak, et al., 1990; Wiktor-Jedrzejczak and Gordon, 1996). In the present study, B lymphopoiesis has been examined in op/op mice to evaluate the possible roles of CSF-1 during B cell development *in vivo*. The results show that total BM cellularity in op/op mice is greatly reduced, due in large part to the reduced size of medullary cavity in long bones and thus a reduced BM volume. B lineage cells, however, are disproportionately decreased in op/op BM. B cell precursors are greatly diminished in both incidence and absolute number at various defined differentiation stages. Flow cytometric analysis also demonstrates that precursor B cells in op/op mouse BM have elevated apoptotic incidences both *ex vivo* and in short term culture, suggesting that precursor B cells are eliminated by apoptosis more extensively than usual under

conditions of CSF-1 deficiency. The most severe reduction in number and the highest rate of apoptosis are observed among pre-B cells. The data thus demonstrate that CSF-1 deficiency in op/op mice is associated with severe impairment of the normal *in vivo* process of B cell production and selection in BM. This supports the notion that CSF-1 has wide effects on hemopoietic cell development, other than the monocytic lineage (Wiktor-Jedrzejczak and Gordon, 1996). Although the present findings cannot exclude the possibility that the suppression of B lymphopoiesis in op/op mice might represent an indirect effect of the CSF-1 gene mutation due to other secondary factors, the results provide evidence that CSF-1 acts as an important local regulator of differentiation and selection of precursor B cells, particularly at the pre-B cell stage.

Ablation of functional BM, as produced by irradiation from the bone-seeking radioisotope, ^{89}Sr, can normally result in the development of extramedullary B lymphopoiesis, notably from hemopoietic stem cells in the spleen (Phillips and Miller, 1974). It has been reported that splenic hemopoiesis is increased in young op/op mice (Begg et al., 1993). However, no evidence of splenic lymphopoiesis has been detected in the present study. The failure of op/op mice to initiate splenic B lymphopoiesis despite the greatly limited BM volume, raises the possibility that, like BM B lymphopoiesis, B cell development in postnatal spleen may require CSF-1.

Recent studies suggest that synergistic interactions occur between CSF-1 and stem cell factor (SCF) during hemopoiesis, in addition to their individual activities (Van'T Hof et al., 1997). The CSF-1 receptor, a member of the Ig superfamily with tyrosine kinase activity, has homology to the SCF receptor, c-kit. Previous studies have shown that SCF

and IL-7 act synergistically in the clonal proliferation of B cells (McNiece et al., 1991; Funk et al., 1993). New evidence suggests that SCF stimulates osteoclast activity *in vitro*, possibly by synergizing with CSF-1 (Van'T Hof et al., 1997). It appears possible, similarly, that CSF-1 may play an auxiliary role in B cell development as a synergist with other hemopoietic cytokines. These usually manifest their biological activity through binding to specific high-affinity receptors (Wiktor-Jedrzejczak and Gordon, 1996; Mufson, 1997). It remains to be determined whether precursor B cells express receptors for CSF-1.

In freshly prepared BM samples, the apoptotic incidences of whole cell suspensions from both op/op and control mice are generally comparable with previous results obtained from normal C3H/HeJ mice, suggesting that macrophages, though reduced in numbers, can still efficiently remove apoptotic cells in op/op mouse BM. This accords with the recent observation that certain phagocyte subpopulations, including BM macrophages, are less affected than others by CSF-1 deficiency during postnatal development in op/op mice (Cecchini et al., 1994).

There is evidence that op/op mice can undergo an age-related hemopoietic recovery (Begg et al., 1993), suggesting that the adult hemopoietic system can develop the capacity to use alternative mechanisms to compensate for the CSF-1 deficiency. Nevertheless, the present study demonstrates that the presence of CSF-1 is important for B cell development and selection in BM during early postnatal life.

Fig. 7.1. Flow cytometric analysis of phenotypically immunolabeled BM cell suspensions from op/op and control mice. Incidences of various defined B cell populations are indicated.

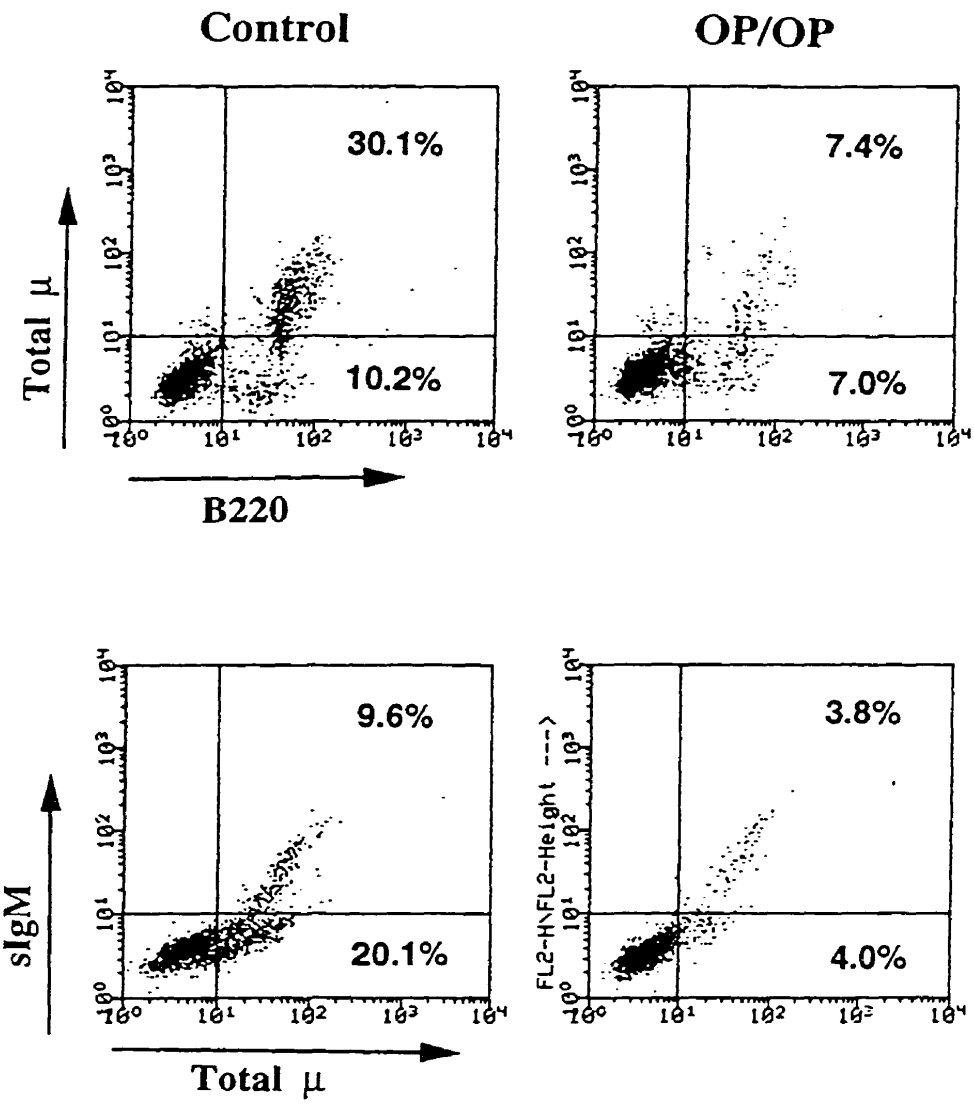

Control
OP/OP
30.1%
10.2%
7.4%
7.0%
Total μ
B220
9.6%
20.1%
3.8%
4.0%
sIgM
Total μ
FL2-H\FL2-Height --->
10⁰
10¹
10²
10³
10⁴

Table 7.1. B lineage cells in BM of op/op and control mice

Cell Phenotype	Cells/ femur (10^5)*	
	Control	op/op
TdT^+	2.8 ± 0.1	0.5 ± 0.05
$B220^+\mu^-$	24.1 ± 2.0	0.4 ± 0.02
$c\mu^+s\mu^-$	41.0 ± 3.2	0.8 ± 0.01
$sIgM^+$	20.1± 1.1	0.7 ± 0.03

* Values derived from 3 separate experiments (Mean ±SD)

Fig. 7.2. B cells at various phenotypic stages in the BM of op/op and control mice, indicating the number of cells per femoral shaft. Data represent 4 separate experiments (Mean $\pm$ SD).

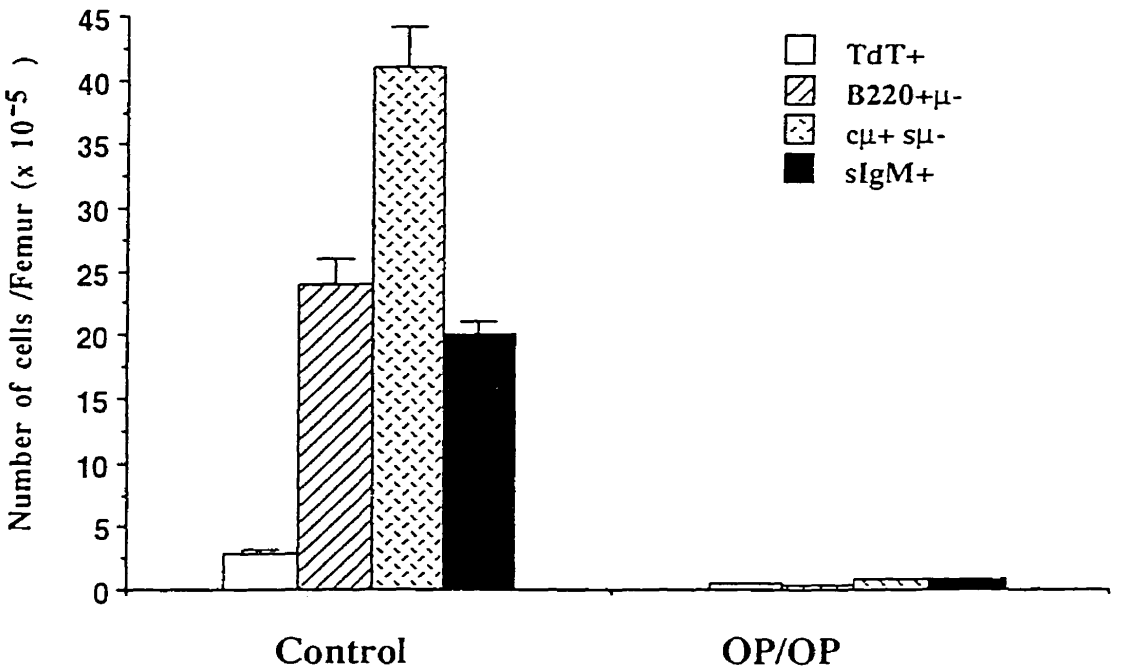

Number of cells /Femur (x 10^-5)
45
40
35
30
25
20
15
10
5
0
TdT+
B220+μ-
cμ+ sμ-
sIgM+
Control
OP/OP

Fig. 7.3. Flow cytometric analysis of apoptotic B lineage cells at defined phenotypic stages in freshly prepared BM cell suspensions from op/op and control mice. Hypodiploid incidences are indicated, representative of 3 separate experiments.

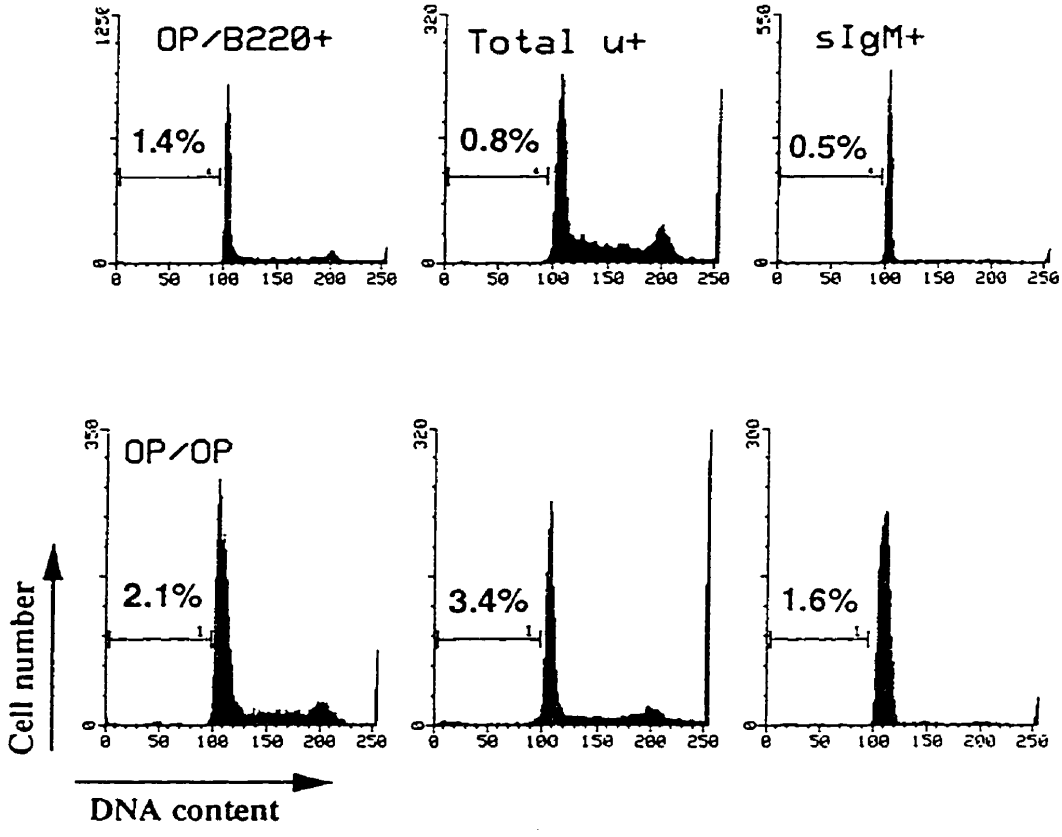
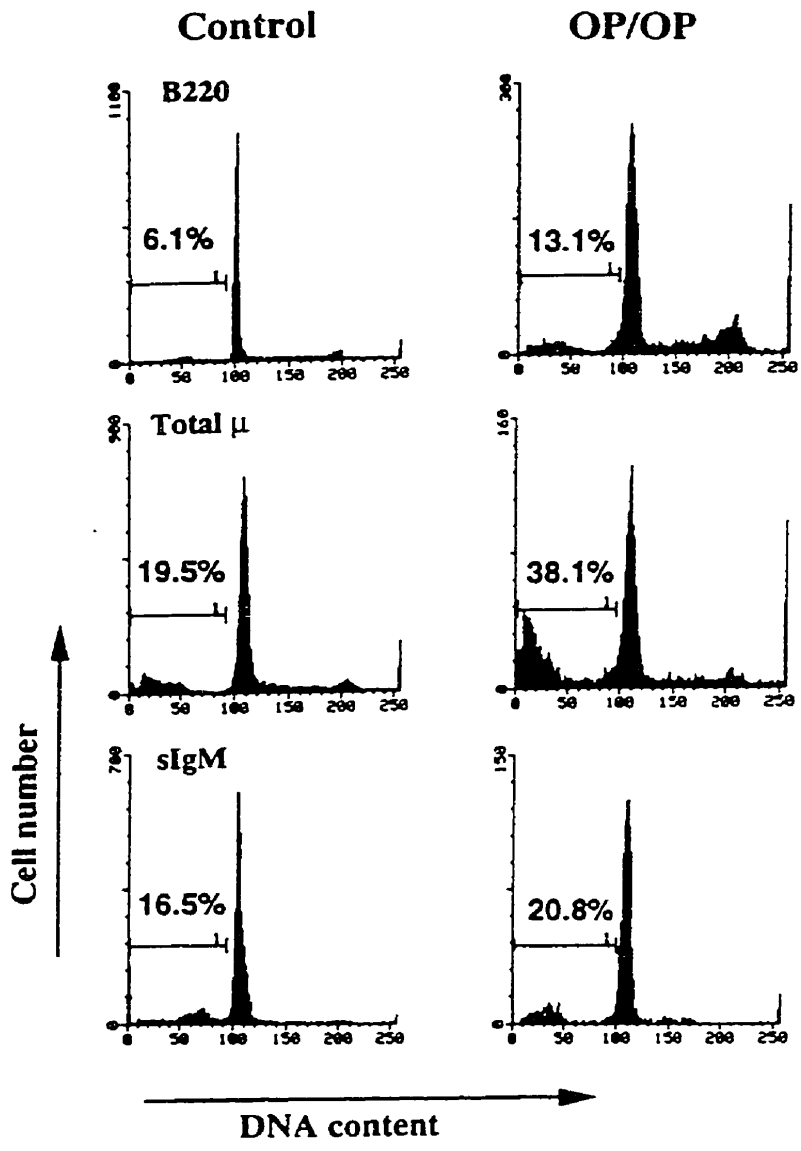

OP/B220+
1.4%
Total u+
0.8%
sIgM+
0.5%
OP/OP
2.1%
3.4%
1.6%
Cell number
DNA content

Fig. 7.4. Representative histograms showing apoptotic B lineage cells at defined phenotypic stages in BM suspensions from op/op and control mice after 4 h incubation. Hypodiploid incidences are indicated, representative of 3 separate experiments.


Control
OP/OP
B220
6.1%
13.1%
Total μ
19.5%
38.1%
sIgM
16.5%
20.8%
Cell number
DNA content

Chapter 8 General Summary and Original Contributions

During development in mouse BM, precursor B cells undergo a programmed process of differentiation, proliferation and selection to generate functional B cell clones. Developing B cells can be considered to pass through a series of phenotypically distinctive compartments. Several models have been proposed to define B cell differentiation stages. However, the use of different phenotypic markers and terminologies has hampered the understanding of the B cell developmental process in BM. In order to provide a direct comparison between two major models, Hardy's cell fractions based on flow cytometric analysis of cell surface expression of B220, CD43, HSA and BP-1 have been sorted by fluorescence activated cell sorter and examined for expression of intranuclear TdT and cytoplasmic μ chains by epifluorescence microscopy to examine the correlation between the cell surface marker phenotypes and the B cell differentiation stages defined by Osmond. The results have revealed and quantitated a heterogeneity of precursor B cells at various developmental stages within each fraction, showing that caution is necessary in assigning a particular differentiation stage to individual cells from any given fraction without additional lineage-specific criteria. The findings contribute toward a unified model of B cell development in mouse BM.

B lymphopoiesis in BM represents a delicate balance between B cell production and B cell selection. To determine the nature of B cell death during development, a variety of criteria including *in situ* DNA strand break labeling, nuclear fragmentation, hypodiploid DNA content, annexin-V labeling and electron microscopy have been used to demonstrate

that B cell death takes the form of apoptosis in BM. The apoptotic incidences of B cells at various developmental stages *ex vivo* and the rates of entry into apoptosis during short term culture have been analyzed by flow cytometry to test the hypothesis that B cell selection by apoptosis is developmentally regulated. The findings demonstrate that B cell apoptosis does not occur randomly during development and that B cells are particularly prone to apoptosis at two differentiation stages, soon after Ig V_H gene rearrangement and antigen receptor expression, respectively. Coculture of BM cells with stromal cells has evaluated the apoptotic rate and estimated magnitude of B cell loss under optimal conditions, leading to the formulation of the first dynamic model of B cell production and selection *in vivo*.

Western blotting and flow cytometric analyses have further demonstrated that Bcl-2 and Bax proteins are differentially expressed by B cells at various developmental stages. Flow cytometric quantitation of protein expression reveals that changes in the Bcl-2/Bax ratio are closely correlated with the apoptotic susceptibility of B cells at defined developmental stages, suggesting that Bcl-2 and Bax proteins are involved in regulating B cell selection during development in BM.

To determine the molecular regulations that coordinate Ig gene expression and B cell survival, B cell differentiation and apoptotic kinetics have been examined by flow cytometry in RAG-$2^{-/-}$ and control mouse BM. The results demonstrate that changes in Bcl-2 and Bax expressions are implicated in controlling the demise of aberrant pro-B cells, unable to achieve productive rearrangement of Ig H genes.

B cell development in BM is highly influenced by local microenvironmental factors.

To determine the effect of IL-7 on B lymphopoiesis, B cell differentiation and apoptosis have been analyzed by flow cytometry using IL-7 gene-deleted mice and IL-7 transgenic mice, demonstrating that modulations of IL-7 gene expression *in vivo* can greatly affect B cell production and selection and that the resulted changes of precursor B cell apoptosis are correspondingly associated with altered protein levels of Bcl-2 and Bax .

Finally, the possible *in vivo* role of CSF-1 in B lymphopoiesis in BM has been examined in op/op mutant and normal mouse BM. The results reveal that B cell development is severely impaired and that B cell apoptosis is greatly accelerated under conditions of the CSF-1 deficiency, suggesting that CSF-1 normally plays an important role in B cell differentiation and selection in BM.

The results presented in this thesis represent the following original contributions to the knowledge of B cell differentiation and selection in mouse BM:

1. A direct comparison provides a definitive correlation between Hardy's and Osmond's models, contributing towards a unified scheme of B cell development in mouse BM.

2. B cell death takes the form of apoptosis in mouse BM. B cells are particularly prone to apoptosis at two differentiation stages, known to follow Ig V_H gene rearrangement and antigen receptor expression, respectively.

3. The formulation of the first dynamic model of B cell production and selection *in vivo* provides baseline data for studies of B cell selection in perturbations of B lymphopoiesis.

4. During B lymphopoiesis, intracellular Bcl-2 and Bax protein levels are developmentally

regulated. Bax/ Bcl-2 ratios correlate closely with apoptotic incidence *ex vivo* of B cells at defined differentiation stages during development in mouse BM.

5.The demise of pro-B cells with an inability to initiate Ig H chain gene rearrangement in RAG-2-/- mice is controlled by intrinsic signals and associated with differential expression of Bcl-2 and Bax proteins.

6. Stromal cells can suppress apoptosis among precursor B cells, suggesting that BM microenvironment plays a critical role in B cell selection.

7. Modulations of IL-7 gene expression *in vivo* in IL-7 Tg and IL-7-/- mice can greatly influence B cell differentiation and proliferation and result in marked changes of B cell apoptosis that are associated with altered protein levels of Bcl-2 and Bax.

8. Op/op mutant mice, lacking CSF-1 gene, show aberrations of B lymphopoiesis and apoptosis, as well as the well known monocyte/macrophage deficiency, suggesting that CSF-1 may play a role in regulating B cell production and selection in mouse BM.

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IMAGE EVALUATION
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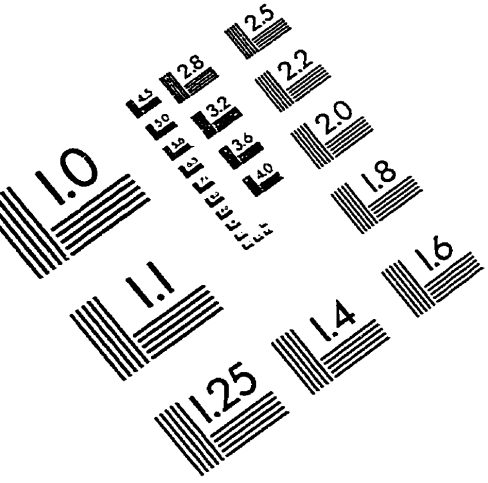

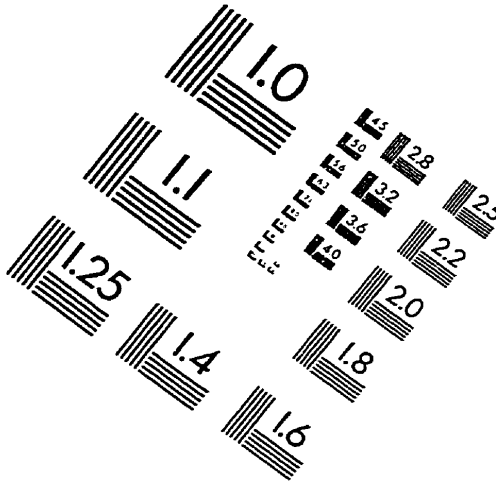

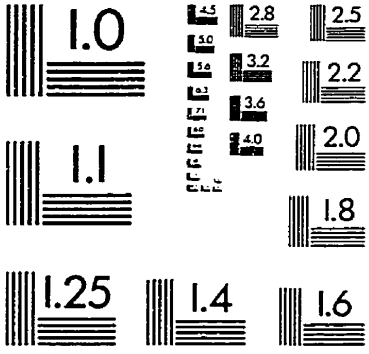

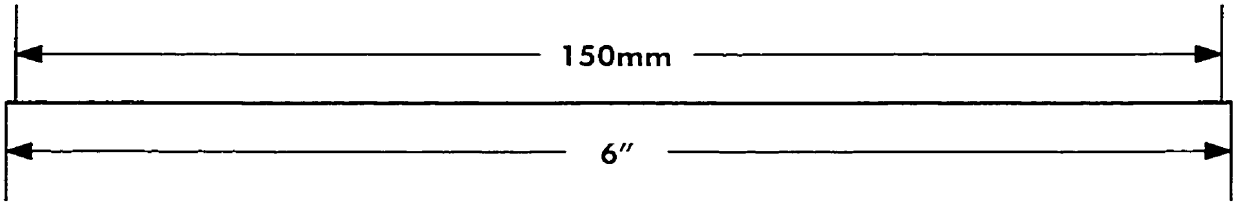

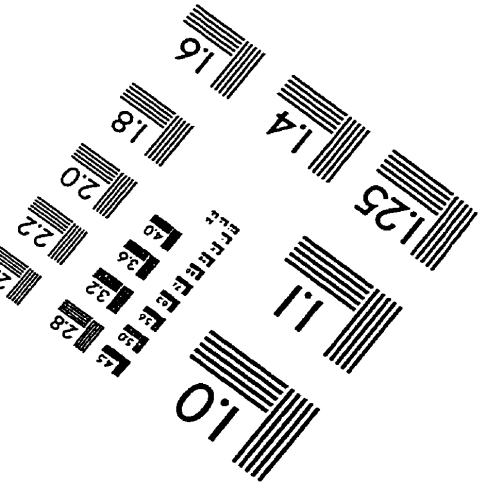

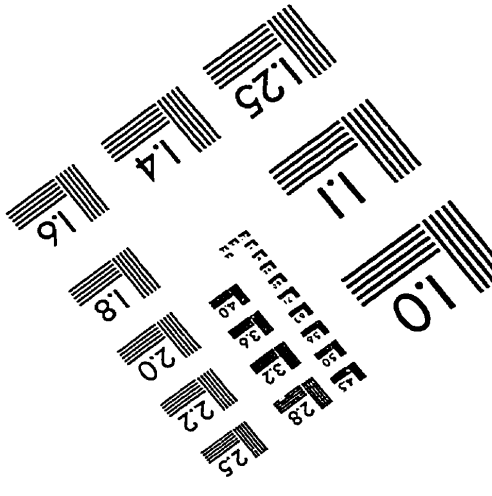

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