Regulation and dysregulation of B lymphopoiesis in mouse bone marrow: *in vivo* role of macrophage activation (pristane-treatment and malaria-infection), *c-myc*, *c-kit*, and immunoglobulin genes

by

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To Gely, Lucy and Jean Yves

CONTENTS

ABSTRACT

To examine factors influencing normal and disordered genesis of the B lymphocyte lineage in mouse bone marrow, precursor B cell dynamics have been analysed in conditions predisposing to B cell neoplasias and deficiencies. Double immunofluorescence labeling and stathmokinetic techniques have been used to quantitate the population size and mitotic activity of pro-B cells before μ chain expression, pre-B cells expressing cytoplasmic μ chains, and B lymphocytes expressing surface IgM. Two conditions associated with prolonged macrophage activation and B cell neoplasia, pristane oil-treatment and malaria infection, have been found to stimulate the proliferation of pro-B cells but to produce increased cell loss at later cell differentiation stages, suggesting that the stimulation of cells undergoing Ig gene rearrangement may predispose to genetic errors leading to cell death or oncogenesis. During a pretumorous period in Eμ-*myc* transgenic mice a marked stimulation of pro-B and pre-B cells is associated with much subsequent cell loss, suggesting that additional mutations are needed to promote B cell survival and the emergence of a tumorigenic clone. Many early precursor B cells express *c-kit* but their development is not blocked by a neutralizing anti-*c-kit* antibody *in vivo*, suggesting that the role of *c-kit* can be replaced by alternative signalling systems. The introduction of Ig transgenes in *scid* mutant mice, unable to rearrange endogenous Ig genes, shows that the survival of successive stages in precursor B cell development depends upon the successful progressive expression of the IgM molecule. The work demonstrates that processes influencing both cell proliferation and loss can be critical in regulating the genesis of both normal and potentially neoplastic B cells in the bone marrow.

RÉSUMÉ

Dans le but d'examiner les facteurs influençant la genèse normale et anormale des lignées lymphocytaires B dans la moelle osseuse de la souris, la différentiation des cellules précurseurs des lymphocytes B a été analysée dans des conditions prédisposant à des déficiences et aux néoplasies des lymphocyte B. Des techniques de double immunofluorescence et d'arrêt mitotique (stathmokinétique) ont été utilisées afin de quantifier l'ampleur et l'activité mitotique des populations des cellules pro-B avant le développement des chaînes μ, des cellules pré-B présentant des chaînes μ intracytoplasmiques et des lymphocytes B présentant des IgM de surface. Deux conditions associées à une activation prolongée des macrophages et aux néoplasies des lymphocytes B, soit le traitement avec l'huile minérale pristane et l'infection par la malaria, se sont avérées en mesure de stimuler la prolifération des cellules pro-B, conduisant toutefois à une perte cellulaire augmentée au niveau des stades ultérieurs de différentiation. Ceci suggère que la stimulation des cellules, au moment où elles sont en processus de réarrangement des gènes des immunoglobulines, prédisposerait à des erreurs génétiques conduisant à la mort cellulaire ou à l'oncogenèse. Au cours de la phase prétumorale chez des souris transgéniques Eμ-*myc*, une stimulation marquée des cellules pro-B et pré-B est associée à une perte cellulaire augmentée au niveau des stades subséquents. Ces résultats suggèrent que des mutations additionelles sont nécessaires pour permettre la survie des cellules de la lignée B et l'émergence d'un clone tumorigénique. Plusieurs cellules précurseurs précoces des lymphocytes B présentent *c-kit* à leur surface, mais ceci n'est pas empêché par un traitement *in vivo* avec un anticorps neutralisant anti-*c-kit*, suggérant que le rôle du *c-kit* peut être remplacé par des systèmes de signaux alternatifs. L'introduction d'immunoglobulines transgéniques chez des souris mutantes *scid*, incapables de réarrangement endogène des gènes des immunoglobulines, montre que la survie des stades successifs dans la différentiation des cellules précurseurs du lymphocyte B dépend de la capacité progressive de développer les molécules IgM. Les résultats de ce travail de recherche démontrent que les processus influençant à la fois la prolifération et la perte cellulaire sont d'une importance critique dans la régulation de la genèse des lymphocytes B normaux et potentiellement néoplasiques, au niveau de la moelle osseuse.

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Abbreviations used in this thesis

C	constant region of immunoglobulin genes
c μ	cytoplasmic μ chains
CFU-S	spleen colony forming unit
D	diversity region of immunoglobulin genes
FITC	fluorescein isothiocyanate
H chain	immunoglobulin heavy chain
Ig	immunoglobulin
IL	interleukin
J	joining region of immunoglobulin genes
L chain	immunoglobulin light chain
mAb	monoclonal antibody
MHC	major histocompatibility complex
PYNL	*Plasmodium yoelii* non-lethal strain
PYL	*Plasmodium yoelii* lethal strain
Sl	*Steel locus*
sIgM	surface class M immunoglobulin
s μ	surface μ chains
SRBC	sheep red blood cells
TdT	Terminal deoxynucleotidyl transferase
TNF	Tumor necrosis factor
TRITC	tetramethyl deoxynucleotidyl transferase
V	variable region of immunoglobulin genes
W	*White spotting locus*
+	positive
-	negative

CLAIM FOR ORIGINAL WORK

The results presented in this thesis represent the following original contributions to the knowledge of the regulation and dysregulation of B cell genesis in the bone marrow.

Firstly, this thesis demonstrates that the widely disparate agents pristane oil and malaria parasites, both exert marked effects on the genesis of B cells in mouse bone marrow. The earliest defined stages of B cell genesis, ie, TdT^+ cells, show sustained increases in population size and proliferation rate. On the other hand, the later populations show only normal or decreased levels of production. Thus, the elevated level of cell production at the TdT^+ cell stage is followed by an unusually high level of cell loss at the subsequent stages of development in the bone marrow.

Secondly, studies in Eμ-*myc* transgenic mice during the pretumorous phase of lymphoma development, demonstrate a marked increase in numbers and rates of generation of earlier precursor B cells in the bone marrow, without an increase in mature B lymphocytes.

Thirdly, double labeling procedures to detect the expression of the *c-kit* proto-oncogene on B cell precursors, as well as their proliferation dynamics demonstrate that many actively proliferating early precursor B cells express *c-kit in vivo*. However, *c-kit*-mediated signals are not essential for precursor B cell development *in vivo*.

Finally, this thesis provides a phenotypic analysis of precursor B cell

progenitors and their proliferation dynamics in Ig-transgenic scid mice. This study demonstrates that the cells are not in a state of developmental arrest but continue to enter the B cell lineage to differentiate and proliferate actively until they abort, thus allowing the stage of abortion to be identified and correlated with the state of Ig gene rearrangement.

CHAPTER 1

Introduction

INTRODUCTION

CONTENTS

The present thesis analyzes the *in vivo* genesis of B cell precursors in mouse bone marrow with special emphasis on conditions which can alter the delicate balance between cell proliferation and cell loss, and which may predispose to B cell neoplasias.

1. Hemopoietic Stem Cells.

The various hematopoietic lineages are derived and constantly renewed from hematopoietic stem cells. In order to achieve this task, stem cells must possess the capacity of self renewal, differentiation and proliferation, and be able to respond to regulatory signals.

In mammals, the hemopoietic stem cells migrate from blood islands in the yolk sac to the fetal liver, from here to fetal spleen, and finally to the bone marrow where they remain (Metcalf and Moore 1971, Paige et al. 1979, Kincade 1981a). In practice, stem cells are defined by their ability to reconstitute the hematopoietic system under appropriate conditions. The study of stem cells has been complemented by *in vivo* and *in vitro* assays.

In vivo assays test the ability of stem cells to repopulate lethally irradiated mice or mutant mice with hematopoietic deficiencies. The analyzed parameters include long term survival, hematopoietic regeneration and long-term reconstitution (Till and McCulloch 1980, Spangrude 1988, Fulop and Phillips 1989). The detection of stem cells in hematopoietic tissues has been achieved by a variety of methods which allow the identification of members of a single clone. These methods include chromosomal markers, allophenic mice and

chromosome integration sites of recombinant retroviruses (Abramson et al. 1977, Till and McCulloch 1980, Keller et al. 1985, Williams et al. 1984, Dick et al. 1985). The first *in vivo* repopulation assay was the spleen colony forming unit (CFU-S) technique performed by Till and McCulloch in the early 60's (Till and McCulloch 1961). The method consisted of the intravenous injection of bone marrow into irradiated mice. Within 7-12 days following the injection, some nodules were found in the spleen, each of them representing a clonal colony. The hemopoietic lineages included early granulocytic, macrophage, megakaryocytic and erythrocytic colonies, while later-appearing colonies were of mixed lineages. The injection of spleen colony cells into secondary irradiated recipients generated spleen colonies again (Barnes et al. 1968). These observations provided evidence of self-renewal, proliferation and differentiation. Work based on cell size by sedimentation velocity revealed a heterogeneity of spleen colonies in their reconstitution capacity (Worton et al. 1969). It also became clear that early CFU-S appearing around 8-10 days after cell transfer derive from more mature progenitors, and lack self-renewal capacity, in contrast to later-appearing CFU-S (11-14 days) which derive from more primitive progenitors, and have a limited reconstitution capacity in secondary transfers (Magli et al. 1982, Mulder at al. 1985, Visser et al. 1984). *In vitro* assays are based on a colony-forming assay, in which cells are cultured in soft agar over a feeder layer and conditioned medium. In this assay, a variety of colony-forming units can be identified making it possible to identify cell-cell interactions

and regulators for hemopoiesis. The development of long-term bone marrow cultures by Dexter permitted sustained *in vitro* hematopoiesis for months (Dexter et al. 1977). However, under these conditions lymphocyte production was not achieved. Whitlock and Witte modified the conditions to permit the selective growth of B lymphocytes (Whitlock and Witte 1982). Such long-term bone marrow cultures revealed a requirement of stromal cell interactions and colony stimulating factors for hemopoiesis. Early hemopoietic progenitors require a combination of cytokines including IL-1, IL-3, IL-6, CSF-1 and c-kit ligand. The requirements for stimulation factors vary among later committed progenitors (Kincade et al. 1988, Moore 1991). Notably, IL-7 is an essential factor for B lymphopoiesis. The *in vitro* and *in vivo* assays have permitted the elaboration of a hierarchy of hematopoietic precursors, from stem cells to fully differentiated terminal cells, in which the capacity for self renewal rapidly diminishes and dissappears with differentiation. Characterization and purification of stem cells have been difficult due to their low frequency (about 0.01%) in the hematopoietic tissues (Jordan et al 1990, Spangrude 1989). Stem cells are medium sized (8-10μm), have lymphoid morphology, slow sedimentation rate and light buoyant density. They are mainly in G_0, with only 15%-20% in S phase (Musaschi et al. 1991), and thus are 5-FU resistant (Mulder et al. 1985). They express in low amounts the glycoprotein Thy-1 (Muller-Sieburg et al.) and bind weakly the mitochondrial dye, rhodamine 123 (Bartoncello et al. 1985). They are Sca-1^+ (van de

Rijn et al. 1989), AA4.1$^+$ (marker use for fetal stem cell purificacion) (Jordan et al. 1990), and Lin$^-$ (B220$^-$, Mac-1$^-$, Gr$^-$, CD4$^-$, CD8$^-$) (Spangrude et al. 1988). Recently, Huang and Terstappen (1992) reported a CD34$^+$ HLA-DR$^-$ CD38$^-$ human fetal bone marrow cell able to give rise to both hemopoietic stem cells and stromal cells.

2. B cell development.

B lymphocytes are one of the main components of the immune system. Their primary function is the production of antibodies capable of binding to antigens potentially harmful.

B lymphocyte development in adult mice can be divided into two main phases, antigen-independent and antigen-dependent. The first phase includes the development of early precursors to form virgin sIgM$^+$ B cells, and occurs in the bone marrow. The second phase includes the differentiation from B lymphocytes to antibody-secreting plasma cells and memory cells, and takes place in the peripheral lymphoid organs (Osmond 1985).

During ontogeny, B lymphocytes (sIgM$^+$) appear at day 17 of mouse gestation (Raff et al. 1976). However, B cell precursors can be detected transiently at day 10 of gestation in placenta and embryonic blood, and at day 11 in fetal liver (Melchers and Abramczuc 1980, Paige et al. 1979). Small clusters of B220$^+$ cells can be seen in 12-day fetal liver, and 3-4 days later, sIgM$^+$ cells are detected in fetal liver, spleen and bone marrow (Paige et al. 1979, Kincade 1981). Postnatal spleen and liver produce a transient wave of B cell

precursors and B lymphocytes over the first 2-3 weeks of life. By this time the bone marrow has assumed the hematopoietic function (Melchers and Abramczuc 1980). The first cell to be committed solely to the B cell lineage has not been identified. In *in vitro* cultures, Cumano and Paige (1992) detected hemopoietic precursors from 11-12-day fetal liver able to give rise to B lymphocytes. These B cell precursors have the characteristic of bipotentiality, giving rise to both B cells and macrophages but to no other myeloid lineages (although the T cell capacity was not analyzed) (Cumano et al. 1992).

B cells in fetal and neonatal mice show immature features. The cells lack antigens on their surface and can easily be tolerized (Cambier et al. 1976, Metcalf and Klinman 1976). They show a preferential use of J_H proximal V_H genes (Wu 1990, Alt 1987), and lack N regions in IgH sequences (Feeney 1991). Newly produced B lymphocytes in the bone marrow can also be easily tolerized (Melcalf and Klinman 1977). They express sIgM in increasing density with time. Other markers, including Ia antigens, complement receptors and Fc receptors develop after the beginning of sIgM expression, a process that probably takes place partly within bone marrow sinusoids (Osmond and Batten 1984, Batten and Osmond 1984).

Virgin B lymphocytes leave the bone marrow via the marrow sinusoids and central vein, and reach the spleen where they mature fully by expressing IgD, becoming able to process and present antigens, and to respond to immunological challenge (Brahim and Osmond 1970, Yoshida and Osmond 1978, Osmond and Nossal 1974,

Jacobsen et al. 1990, Allman et al. 1992). Recognition of foreign antigens may activate B lymphocytes to proliferate and to differentiate into antibody-secreting plasma cells and memory B cells. Memory B cells represent a potentially long-lived pool of recirculating cells. A second encounter with the antigen triggers the proliferation and differentiation of memory B cells, giving rise to a secondary humoral immune response (Rajewsky et al. 1990, Rajewsky et al. 1987).

2.1. B cell production in the bone marrow.

The bone marrow is the primary organ in the production of B cell precursors in adult mice (Osmond and Everett 1964, Osmond and Nossal 1974). B lineage cells represent approximately 25-30% of total nucleated cells in adult bone marrow (Park and Osmond 1987). Their number is age dependent, peaking in postnatal life and declining with age (Miller and Osmond 1974). Aproximately 80% of bone marrow lymphocytes are small (less than 8μm in diameter), are in G_0 phase and have a high nuclear:cytoplasmic ratio. The remaining lymphoid cells are large and show proliferative activity (Miller and Osmond 1975). Experiments with ^{3}H-TdR continous infusion have revealed two distinct populations of small lymphocytes in the bone marrow, a major compartment of rapidly renewing cells and a minor recirculating pool of slowly renewing, long lived cells. The rapidly renewed population, approximately 90% of bone marrow small lymphocytes, has a renewal time of 3-4 days (half renewal time, 18h;

renewal rate, 3.9%/h) and derives from bone marrow precursor cells (Miller and Osmond 1975). A combination of ^{3}H-TdR labeling and surface IgM radiolabeling has revealed that approximately 50% of the bone marrow small lymphocytes are sIgM^{+} cells derived from bone marrow small sIgM^{-} lymphocytes, which in turn are renewed from proliferating precursors (Osmond and Nossal 1974, Landreth et al. 1981). The bone marrow production of B lymphocytes is approximately 10^{7} cells per day (Opstelten and Osmond 1983). If they succesfully complete the differentiation process, most of the newly-formed sIgM^{+} lymphocytes leave the bone marrow, and migrate to the spleen and lymph nodes.

2.2. B cell diversity.

B cell genesis generates a diversified repertoire of B cell specificities able to respond to a wide variety of antigens. This is achieved by immunoglobulin (Ig) diversification. The B cell antigen receptor is a glycoprotein consisting of the assembly of two identical polypeptide heavy (H) chains and two identical light (L) chains bound by disulfide bonds and non-covalent bonds. H and L chains are composed of a variable (V) region at the amino-terminal, each consisting of one folded domain of approximately 110 amino acid residues, and a constant (C) region at the carboxy-terminal end, consisting of one domain in the L chain and of three or four domains in the H chain. The constant region of the H chains (C_H) forms the Fc portion of the antibody, has the biological functions of fixing complement and

binding to Fc receptors, and it is the antibody anchoring site. The C_H region determines the different classes or isotypes of antibodies (IgM, IgD, IgG, IgE, and IgA). A B lymphocyte may "switch" isotypes but keep the same V region. The V_H region contains three complementarity determining regions (CDRs) intercalated into a framework of highly conserved amino acids. The CDRs are hypervariable regions, and form, with the corresponding CDRs of the V_L region, the antigen-binding surface of the antibody. There are two types of L chains, *kappa* and *lambda*. Either type can be associated with any of the different H isotypes. Antigen recognition by the variable domains of the Ig is achieved by an ordered set of Ig gene rearrangements which occur during the differentiation of precursor B cells. First, a diversity (D_H) segment is brought to a joining (J_H) segment. Then a variable (V_H) segment joins DJ, resulting in a functional VDJ-Cμ gene (Tonegawa 1983, Yancopoulus and Alt 1986). This is followed by rearrangements of *kappa* or *lambda* L chain genes in which a V_L segment is joined to a J_L segment to yield a functional L chain gene. The functional genes are transcribed into precursor mRNA, which in turn becomes mature mRNA after splicing. Mature mRNA is transcribed into polypeptide chains. The H and L polypeptide chains further assemble to produce a complete IgM molecule that is expressed on the surface membrane of a virgin B cell. The precise mechanisms of gene recombination are not well understood. Flanking each Ig gene there are conserved DNA recombinational signal sequences (RSS). The RSS are composed of

conserved heptamer and nonamer sequences separated by a nonconserved spacer of 12 or 23 nucleotide pairs. Appropriate joining occurs when a 12 spacer combines with a 23 spacer. The recombinational complex recognizes the RSS, produces endonucleolytic cuts at coding and recombination signal sequences and exonucleolytic degradation of coding sequences, and ligates the coding regions and RSS (Aguilera et al. 1987, Yancopoulos and Alt 1986). Terminal deoxynucleotidyl transferase (TdT) inserts nucleotides at the D_H-J_H and V_H-D_HJ_H junctions, contributing to antibody diversity (Alt and Baltimore 1983, Desiderio et al. 1984). The components of the recombinational enzymatic complex are not fully characterized. *RAG-1* and *RAG-2* gene products are involved either directly with recombinase activity, or as gene regulators or activators of recombinase proteins (Schatz et al. 1989, Oettinger et al. 1990). The lack of either *RAG-1* or *RAG-2* in mice produces a combined immunodeficiency due to the absence of VDJ rearrangements in T and B lymphocytes (Shinkai et al. 1992, Mombaerts et al. 1992).

Combinatorial diversification, resulting from all the possible combinations of V, (D) and J gene segments and the loss and gain of nucleotides during gene rearrangements, accounts for an estimated 10^8 possibilities. This number is further increased by somatic hypermutation in V region genes after antigenic stimulation (Rajewsky et al. 1987, Golub 1987); a process for selecting of high-affinity B-cell clones (Liu et al. 1989).

In order to ensure the monospecificity of the B cells, allelic exclusion occurs. Once a functional H chain gene rearrangement is produced, further rearrangements on the homologous chromosome with the H chain gene are inhibited. A similar effect occurs between L chain alleles, and between *kappa* and *lambda* (isotypic exclusion). The mechanisms of allelic exclusion are not fully understood, but may depend partially on a feedback regulation of gene rearrangement (Coleclough et al. 1981). Work with transgenic mice, in which H, L, or HL rearranged genes are introduced, has revealed that the presence of a functional transgene elicits allelic exclusion, although in an incomplete form (Neuberger et al. 1989, Weaver et al. 1985, Reichman-Fried et al. 1990, Ritchie et al. 1984). Expression of the membrane form of the μ chain in association with the Igβ-Igα heterodimer seems to be required in order to produce allelic exclusion (Manz et al. 1988, Iglesias et al. 1991). The products of $V_{pre\text{-}B}$ and *λ-5* genes (Kudo et al. 1987, Kudo and Melchers 1987), iota and omega respectively (Pillai and Baltimore 1987), which associate with μ chains forming a surrogate L chain, have been proposed to signal pre-B cells to stop further H chain rearrangements and to initiate L chain rearrangements (Tsubata and Reth 1990, Hollis et al. 1989).

2.3. B cell antigen receptor.

The sIgM molecules on mature B lymphocytes are non-covalently associated with a disulfide-linked heterodimer (Hombach et al. 1988,

Hombach et al. 1990, Campbell and Cambier 1990, Parkhouse 1990). The heterodimer consists of two glycoproteins of 34 KDa (Igα) and 39 KDa (Igβ), products of the *mb-1* and *B29* genes, respectively (Sakaguchi et al. 1988, Hermanson et al. 1988). Both genes are expressed in early B cell progenitors, prior to μ expression, while *B29* expression is turned down in plasma cells (Sakaguchi et al. 1988, Hermanson et al. 1988). Each IgH_C associates with one $\alpha-\beta$ heterodimer. In pre-B cells, the heterodimer associates with the surrogate light chain complex. Cross-linking of the B cell receptor produces autophosphorylation in Igα and Igβ, as well as activation of tyrosine kinases (Burkhardt et al. 1991), as in T lymphocytes after activation through the T-cell receptor (Veillette et al. 1991). However, the association of tyrosine kinases with the B cell receptor is less well characterized (Brunswick et al. 1992). Candidates are *c-lyn* (Yamanashi et al. 1990) and *c-blk* (Dymecki et al. 1989).

2.4. Cell surface molecules defining B cell differentiation.

CD45 (Leukocyte common antigen). CD45 defines a family of glycoproteins present on all nucleated hematopoietic cells. Its expression is controlled in a tissue-specific manner and the different forms are derived from alternative exon splicing of the same gene (Thomas and Lefrançois 1988). The higher molecular form (220 KDa, B220) is expressed by B cell progenitors as early as pro-B cells, before the expression of μ heavy chains (Kincade 1987, Park and Osmond 1987). The cytoplasmic domain of the CD45 molecule is a

tyrosine phosphatase (Ostergaard et al. 1989), implicated in T cell signalling in dephosphorylating the tyrosine kinase $p56^{lck}$ (Veillette and Davidson 1992). However, its role in Ig-mediated signals remains unclear.

BP-1/6C3. The antibodies, BP-1 (Cooper et al. 1986) and 6C3 (Whitlock et al. 1987), recognize a glycoprotein of 135-140 KDa expressed on B cell precursors (late pro-B cells and pre-B cells) but absent on mature B cells (Wu et al. 1990, Hardy et al. 1991). The antigen is also expressed on some stromal cell lines (Whitlock et al. 1987). BP-1/6C3 is a member of the metallopeptidase family of ectoenzymes, suggesting a possible role in hydrolyzing potential physiologically active peptide hormones (Wu et al. 1990).

Thy-1. Thy-1 is a 25-30 KDa glycoprotein bound to the membrane through a glycosyl-phosphoinositol-binding domain (Tse et al. 1985). The antigen is expressed on pluripotent hemopoietic stem cells, early B cell progenitors, T lymphocytes, early myelo- and erythropoietic progenitors, as well as certain non-hemopoietic cells. The intensity of expression varies. It is expressed in low amounts on mouse B cell progenitors. (Muller-Sieburg et al. 1986, Spangrude et al. 1988, Reichman-Fried et al. 1990). The expression of Thy-1 also differs between species. In the rat, Thy-1 is expressed strongly on all B lineage cells (Hermans et al. in press). Eμ-Thy-1.2 transgenic mice develop bone marrow and lymph node lymphoid hyperplasia,

suggesting a possible role in proliferation of early B cell precursors (Chen et al. 1987).

c-kit. The proto-oncogene *c-kit* encodes a transmembrane tyrosine kinase receptor (Chabott et al. 1988) expressed on hematopoietic progenitors (Ogawa et al. 1991). Mutations of the *c-kit* gene result in a variety of defects of hemopoietic stem cell, germ cell, and melanocyte development (Copeland et al. 1990, Hayashi et al. 1991). Upon interaction with its ligand, autophosphorylation of *c-kit*, as well as phosphorylation of other signal transducers, occurs (Miyazawa et al. 1991). The block of *c-kit* function by non-cytotoxic anti-*c-kit* mAb impairs the development of myeloid and erythroid progenitors, but not B lymphopoiesis (Ogawa 1991). The expression of *c-kit* on B cell progenitors and its role on B lymphopoiesis is described in Chapter 6.

CD5 (Ly-1). CD5 is a glycoprotein of 67 KD expressed on T cells and a minor B cell population (Herzenberg et al. 1986, Lanier et al. 1981). $CD5^+$ B cells in adult mice represent 20%-40% of peritoneal cells, but only 1%-2% of spleen cells, and they are even more rare in lymph nodes, Peyer's patches and bone marrow (Hayakawa et al. 1986). Reconstitution experiments demonstrate the capacity of liver, spleen and bone marrow of neonatal mice, and the failure of adult bone marrow cells to reconstitute $CD5^+$ B cells (Hayakawa et al. 1985). $Ly\text{-}1^+$ B cells appear to present a unique self-renewal capacity. Anti-IgM treatment eliminates permanently the population (Hayakawa et

al. 1986). CD5+ B cells differ in their distribution among different mouse strains. They are in low numbers in CBA/N and DBA/2H, in medium numbers in CBA, C3H and C57BL/10, high in BALB/c, and extremely high in Motheaten and NZB mice (Hayakawa et al. 1983). CD5+ B cells have been implicated in the production of natural polyreactive autoantibodies (Casali and Notkins 1989). CD5+ B cells differ from CD5- B cells in their failure to undergo somatic hypermutation after antigen encounter (Foster et al. 1988).

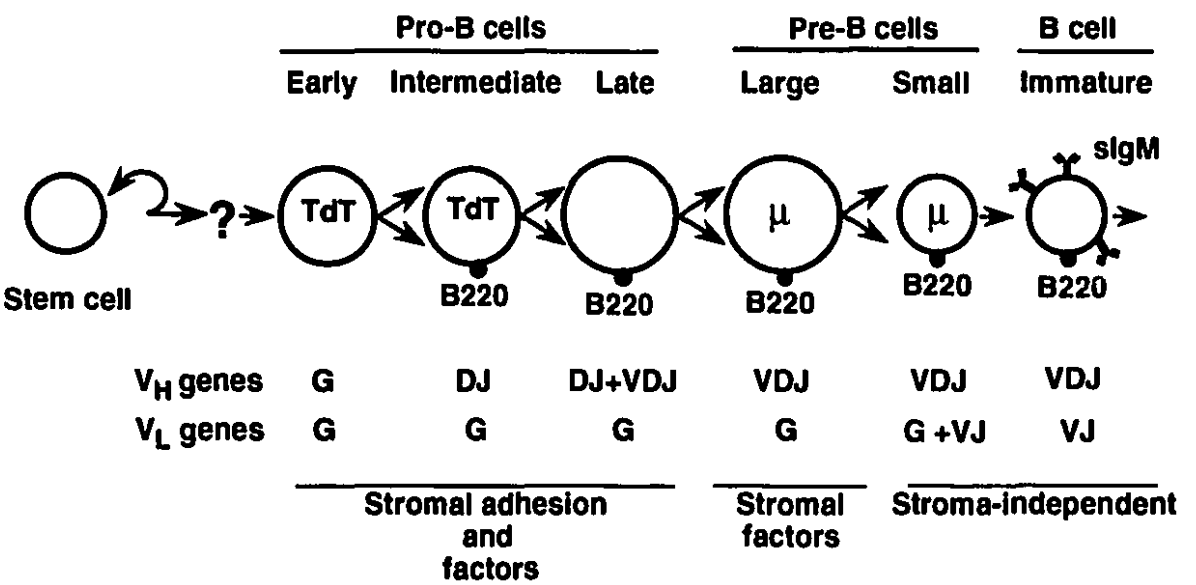
CD72 (Lyb-2). CD72 is a transmembrane type II protein of 40-45K. Its external domain is homologous to CD23 (von Hoegen et al. 1990). CD72 is expressed on B cell precursors and is lost at the antibody-producing stage (Tung 1986, von Hoegen et al. 1990). Anti-Lyb-2 antibodies induce B cell proliferation and differentiation, either alone or in combination with IL-4 (Snow et al. 1986, von Hoegen et al. 1990). Recently, CD72 was found to be the ligand for CD5 (Van de Velde et al. 1991).

3. B lymphopoiesis in the bone marrow.

3.1. Phenotypic differentiation of B cell precursors in the bone marrow.

Based on three cytological markers, a scheme of B cell differentiation in the mouse bone marrow has been proposed by Osmond et al.(Fig. 1).

Figure 1. A scheme of B lymphopoiesis in mouse bone marrow.


Pro-B cells
Pre-B cells
B cell
Early
Intermediate
Late
Large
Small
Immature
sIgM
?
TdT
TdT
μ
μ
Stem cell
B220
B220
B220
B220
B220
V_H genes
G
DJ
DJ+VDJ
VDJ
VDJ
VDJ
V_L genes
G
G
G
G
G +VJ
VJ
Stromal adhesion and factors
Stromal factors
Stroma-independent

The markers include 1) TdT, responsible for nucleotide insertions at gene segment junctions, and restricted to lymphocyte precursors (Park and Osmond 1989, Desiderio 1984), 2) the B220 (Ly-5, CD45) B-lineage associated glycoprotein (Kincade 1981a, Landreth et al. 1983, Coffman et al. 1983), and 3) μ heavy chains, expressed either as intracytoplasmic (cμ) or surface (sμ) molecules in sIgM (Opstelten and Osmond 1983). The use of these markers in double immunofluorescence labeling has resulted in the identification of six phenotypically distinct populations, and in the proposal of a scheme of differentiation for B cell precursors in the bone marrow. The scheme comprises three presumptive pro-B cell populations before μ expression: 1) Early pro-B cells; TdT^+B220^-, 2) Intermediate pro-B cells; TdT^+B220^+, and 3) Late pro-B cells; TdT^-B220^+ (Park and Osmond 1987). The next three populations, all of them expressing B220, include 1) large dividing pre-B cells, $c\mu^+s\mu^-$, 2) small non-dividing pre-B cells, $c\mu^+s\mu^-$, and 3) B lymphocytes, $s\mu^+$ (Landreth et al. 1981, Opstelten and Osmond 1983).

A combination of double immunofluorescence and stathmokinetic techniques has revealed that the population size and production rates of early progenitors increase progressively at successive differentiation stages. The cell size increases up to the late pro-B cell and large pre-B cell stage and decreases thereafter, with the production of the small non-dividing pre-B cells. The scheme provides for a sequence of at least 5-6 cell divisions, and a total

maturation time of 3.5 days from early pro-B cells to B lymphocytes. (Park and Osmond 1989, Opstelten and Osmond 1983).

3.1. Cell loss during B cell genesis.

The population dynamics of B cell precursors shows that in addition to much proliferative expansion, a substantial cell loss of approximately 75% occurs. Some loss may occur at any stage, but it is most evident at the pre-B cell stage (Deenan et al. 1990, Park and Osmond 1987, Opstelten and Osmond 1983, Landreth et al. 1981). The mechanisms initiating cell loss may include the development of either non-functional or autoreactive Ig genes (Coleclough 1983, Nemazee and Burki 1989, Murakami et al. 1992). Studies with severe combined immunodeficient (scid) mice, in which H chain gene rearrangements are defective (Bosma et al. 1983), have shown all B cell precursors are deleted at the late pro-B cell stage (Fulop and Phillips 1988, Osmond et al. 1992). Disruption of the μ membrane exons by gene targeting results in pre-B cell development arrest (Kitamura et al. 1991). Cell loss in the bone marrow can also result from deletion of autoreactive cells (clonal abortion). Nemazee and Burki (1989) demonstrated the elimination of autoreactive cells in transgenic mice expressing MHC I molecules with $H2^k$ haplotype in an $H2^k$ environment. Thus autoreactive cells may be deleted in the bone marrow following recognition of membrane-bound self antigen (Hartley et al. 1991). Transgenic mice expressing an anti-erythrocyte

autoantibody revealed *in vivo* deletion of autoreactive cells upon encounter with the self-antigen (Murakami et al. 1992).

3.2. Regulation of B lymphocyte genesis in bone marrow.

B cell genesis *in vivo* is a delicately controlled homeostatic process. Osmond et al. have shown that B cell genesis depends on a basal level of production, as well as a polyclonal expansion resulting from environmental stimulation (see below).

Experiments with anti-IgM treatment of mice from birth have revealed that B cell genesis is not dependent on a feedback control from peripheral B lymphocytes (Fulop et al. 1983, Opstelten and Osmond 1985). The finding that germ-free mice have a reduced bone marrow lymphocyte production suggested that environmental microbial stimulation may play a substantial role in determining the level of bone marrow lymphocyte production (Osmond 1986). Similarly, sheep RBC injection produced a transient increase in B cell production, a T cell-independent mechanism. Repeated sheep RBC stimulation produced a sustained increase in B cell genesis (Fulop and Osmond 1983, Fulop et al. 1986). Silica treatment or splenectomy abrogated these effects (Fulop and Osmond 1983, Pietrangeli et al. 1985), which could be transferred by the injection of spleen cells from stimulated donors into naive recipients (Pietrangeli and Osmond 1987). The results indicate a role of activated splenic macrophages in mediating the stimulatory effect.

Macrophages may contribute to B cell genesis by releasing soluble systemic factors. Such cytokines may have the capacity of controlling cell proliferation by altering the cell cycle, as well as regulating cell differentiation and maturation. Cytokines activate intracellular signals which may give rise to biochemical changes in targeted cells or may induce the release of secondary cytokines. The release of cytokines with local, paracrine or pleiotropic actions among other cell types is characteristic of macrophages.

Stimulated macrophages are one of the main sources of tumor necrosis factor (TNF). Its production is induced by IL-1, which in turn can be induced by TNF (Phillip and Epstein 1986). TNF has a broad variety of actions, including the stimulation of cell growth, induction of expression of adhesion molecules and histocompatibility molecules, synergy with several cytokines, and induction of apoptosis (Kehrl et al. 1987, Moore 1991). TNF enhances proliferation and differentiation of human B cells (Kehrl et al. 1987, Jelinek and Lipsky 1987). TNF can enhance radioprotection, as well as promote the proliferation and differentiation of granulocytic and monocytic lineages (Urbaschek et al. 1987).

IL-1 has many activities which overlap those of TNF in regulating early hemopoietic progenitors, growth activity, induction of expression of adhesion molecules, and release of secondary cytokines, IL-6 and IL-4, by stromal cells (Moore 1991, Dinarello 1987, King et al. 1988, Gimble et al. 1991). *In vitro*, IL-1 synergizes with other cytokines in promoting the proliferation of human B lymphocytes

(Bertoglio 1988, Jelinek and Lipsky 1987). It induces the synthesis of light chains and proliferation of a murine pre-B cell line (Giri et al. 1984, Billips at al. 1990). IL-1 has generally been regarded as suppressing B cell genesis. Recent *in vivo* experiments with continous infusion of small doses of IL-1, however, show an increase in the number and proliferative activity of pre-B cells, an effect thought to be mediated by stimulating bone marrow stromal cells to release short-range factors (Fauteux and Osmond, unpublished results).

In response to stimulation, macrophages produce both IL-1 and TNF (Dinarello 1992, Titus et al. 1991, Semenzato 1990). In chronic inflammatory granulomata, macrophages produce IL-1, TNF, both of which induce and maintain the granulomas, as well as IL-6 (Kindler 1989, Libert et al. 1990, Nordan and Potter 1986). IL-6 overlaps with IL-1 and TNF in its activities (Hirano et al 1986, Hirano 1992). The hemopoietic actions of IL-6 include stimulating the growth and differentiation of B and T lymphocytes and hemopoietic stem cells (Vink 1988, Vam Damme 1988, Moore 1991, Muench et al. 1992). IL-6 has been implicated in plasmacytoma genesis and myeloma growth (Nordan and Potter 1986, Kawano et al. 1988, Suematzu et al. 1989, Suetmatzu et al. 1992).

4. Bone marrow microenvironment and B cell genesis.

4.1. Bone marrow architecture.

The bone marrow is composed of two compartments, intravascular and extravascular. Hemopoiesis occurs in the extravascular compartment, where hemopoietic cells co-exist with stromal cells. Among these stromal cells are reticular cells, macrophages and adventitial reticular cells (Weiss 1984). The stromal cells are localized around the venous sinuses and near the endosteum. Their long processes are associated with hemopoietic cells. B cell progenitors start to differentiate in association with reticular cells near the endosteum, in discrete clusters (Jacobsen and Osmond 1990). With maturation, the cells would tend to move along the stromal processes towards the more centrally located sinuses. Newly-formed $sIgM^+$ B lymphocytes traverse the sinusoid wall and accumulate temporarily within the lumen of restricted segments of the sinusoids before their release into the blood (Hermans et al. 1989, Jacobsen et al. 1990). Aberrant B cells, undergoing a programmed cell death, are recognized and deleted by macrophages along the way (Osmond et al. 1992, Jacobsen and Osmond 1991).

4.2. Stromal-cell interactions and short-range mediators.

The development of the long term bone marrow culture system by Whitlock and Witte permitted the study of interactions between B cell precursors and bone marrow stromal cells (Whitlock and Witte 1982, Witte et al. 1987). B cell precursors depend for their survival

on stromal cells, an interaction which requires adhesion to stromal cells and stromal cell factors (Kincade et al. 1988, Kincade 1991). Adhesion molecules fall into three families: 1) Ig superfamily adhesion receptors which include dimeric forms, CD8, MHC-I and II, TCR components, and monomeric molecules, LFA-2 (CD2), LFA-3 (CD58), ICAM-1, ICAM-2, VCAM-1 and CD4; 2) the integrin family, heterodimers of non-covalently associated α and β subunits. Three integrin subgroups may be differentiated based on their β subunits: β-1 (CD29) represented by VLA-1,-2,-3,-4,-5,and -6; β-2 (CD18), LFA-1, Mac-1 and p150-95; and β-3 (CD61) including vitronectin receptor and platelet GP IIb/IIa. 3) the selectin family, which includes LAM-1 (Mel-14), ELAM-1 and CD62 (PADGEM, GMP-140) (Reviewed by Springer 1990). The roles of adhesion molecules are diverse. Some are used to promote cell-cell interactions, as in the case of LFA-1 which binds to ICAM-1 and ICAM-2, VLA-4 binding to VCAM-1, or CD2 to VLA-4. Other molecules are involved in cell-extracellular matrix interactions. Thus, VLA-4 binds to fibronectin, and VLA-2 binds to laminin and collagen. Adhesion molecules also work as signalling receptors (Schwartz 1992, Tanaka et al. 1993). The distribution of adhesion molecules is variable. Some members are widely expressed by different cell types, while others are restricted to particular cells. The β-2 subfamily is restricted to leukocytes (Springer 1990. Kincade et al. 1988). The disruption of cell-cell interactions between lymphocyte progenitors and stromal cells by enzymatic digestion or antibodies against adhesion molecules

can arrest the development of precursor B cells (Miyake et al. 1990a, Miyake et al. 1990b, Witte et al. 1987). Stromal cells produce many extracellular matrix components, including fibronectin, laminin, type IV collagen and glycosaminoglycans (Witte et al. 1987). Components of the cellular matrix have been implicated in cell adhesion, as well in binding short-range factors (Dorshkind 1990, Miyake et al 1990c, Bernardi et al. 1988, Miyake et al. 1991). Recently, the *in vivo* expression of a stromal cell marker localized to areas of contact between B cell precursors and stromal cells suggests that stromal cells may polarize surface antigens, forming special restricted niches for hemopoiesis (Jacobsen et al. 1992). Stromal cells produce short-range mediators, including IL-4, IL-7, and TGF-β, which affect B cells in culture (Kincade 1991). IL-7 has been shown *in vitro* to be the most relevant interleukin in the production of early B cell progenitors, probably before the rearrangements of light chains (Namen et al. 1988, Lee et al. 1989, Henderson et al. 1992). Recent studies *in vivo* show that continous infusion of IL-7 produces a sustained increase in the proliferation of both pro-B cells and pre-B cells in murine bone marrow (Valenzona and Osmond, unpublished results). IL-4 increases the *in vitro* survival of pre-B cells, and is a cofactor for pre-B cell differentiation (King et al. 1988, Simons et al. 1989, Billips et al. 1990).

5. B cell genesis and its implications in neoplasias.

The B cell lineage is prone to neoplastic changes. B cell tumors include a wide variety of phenotypes, from early precursors to late differentiated forms. The etiology of neoplasias involves a combination of more than one genetic accident (Marshall 1986, Croce 1987, Bishop 1987). Chromosomal abnormalities can be demonstrated in many B cell tumors, occurring alone or in combination with deletions, inversions, hyperdiploidy, hypodiploidy and pseudodiploidy. The associations of non-random chromosomal translocations with B and T cell leukemias and lymphomas are particularly intriguing. Many chromosomal translocations probably occur when precursor cells are actively rearranging antigen receptor genes. A role of recombinase enzymes in chromosomal translocations has been suggested (Haluska et al. 1987, Altiok et al. 1989). Oncogenes coincide with positions of specific translocation breakpoints (Bishop 1987), which may be directly implicated in dysregulating oncogene activity (Graves 1986, Felix 1987, Haluska 1987). Burkitt's lymphoma arises as a consequence of chromosomal translocations occuring in B cell progenitors, and is associated with endemic malaria, Epstein-Barr virus and AIDS (Klein and Klein 1985, Lenoir and Bornkamm 1987, Lombardi et al. 1987). All translocations in Burkitt's lymphoma involve a chromosome 8 breakpoint, q24, containing the *c-myc* oncogene. The other breakpoint includes one of the chromosomes containing Ig gene loci: heavy chain (14q32) in aproximately 75% of cases; λ chain (22q13) in

15%, and κ chain (2q12) in some 5%. In each case, *c-myc* lies adjacent to the Ig gene sequences (Kirsh et al. 1982, Yunis 1983). Murine plasmacytomas induced by mineral oil also contain a characteristic chromosomal translocation involving the IgH locus and *c-myc* (12;15) (Potter and Wax 1983, Piccoli et al. 1984). The *c-myc*/Ig translocations result in a constitutive expression of *c-myc* and cell cycle deregulation (ar-Rushdi et al. 1983, Nishikura et al. 1983, Croce 1987). Transgenic mice bearing a *c-myc* oncogene influenced by the IgH chain enhancer (Eμ-*myc* transgenic mice) develop B cell neoplasias after a prelymphomatous state (Langdon et al. 1986). The appearance of tumors is hastened in mice expressing two deregulated oncogenes, evidencing the cooperation between oncogenes in the development of neoplasias (Sinn et al. 1987, Vaux et al. 1988, Nunez et al. 1989, Strasser et al. 1990, McDonnell and Korsmeyer 1991). Dysregulations, possibly predisposing to oncogenesis, may result from perturbations at a number of different points in the chain of B cell regulatory events. However, the B cell precursors may be particularly susceptible to genetic accidents while proliferating at the time when Ig genes are being rearranged. If so, any condition associated with increased levels of B precursor cell proliferation might also carry an increased probability of producing genetic errors.

6. Purpose of the study and introduction to the experimental work.

The present series of studies has been designed to evaluate the effects of several mechanisms that may serve to regulate or to

perturb the proliferation and loss of precursor B cells in mouse bone marrow, and to consider the possible relevance of these effects to normal B cell genesis and oncogenesis. *In vivo* quantitation of phenotypically defined precursor B cells and of their population dynamics has been performed by double immunofluorescence labeling and mitotic arrest techniques.

The model of Osmond et al. proposes that B cell genesis in the bone marrow reflects two regulatory mechanisms; a basal production regulated by microenvironmental conditions, and a polyclonal amplification regulated by environmental stimuli, macrophage activation, and the systemic release of factors that may have modulatory effects on stromal cells (Fig. 2). From this model, two propositions can be made: 1) The *in vivo* clonal size and total number of B lymphocytes produced in the bone marrow can be influenced by external stimuli; 2) Conditions associated with external stimuli may produce sustained increases in proliferation of B lymphocyte progenitors in the bone marrow at the stage of Ig gene rearrangements, which in turn may increase the probability of genetic errors, dysregulation and initiation of neoplasia. In Chapters 3 and 4 we test this hypothesis that conditions associated with excessive non-specific macrophage activation may increase the proliferative activity of early B cell precursors in the bone marrow at a stage when Ig gene rearrangements occur, thus predisposing to B cell neoplasias. These studies comprise conditions of pristane-treatment and malaria-

infection, both of which are associated with chronic macrophage activation and with B cell neoplasias.

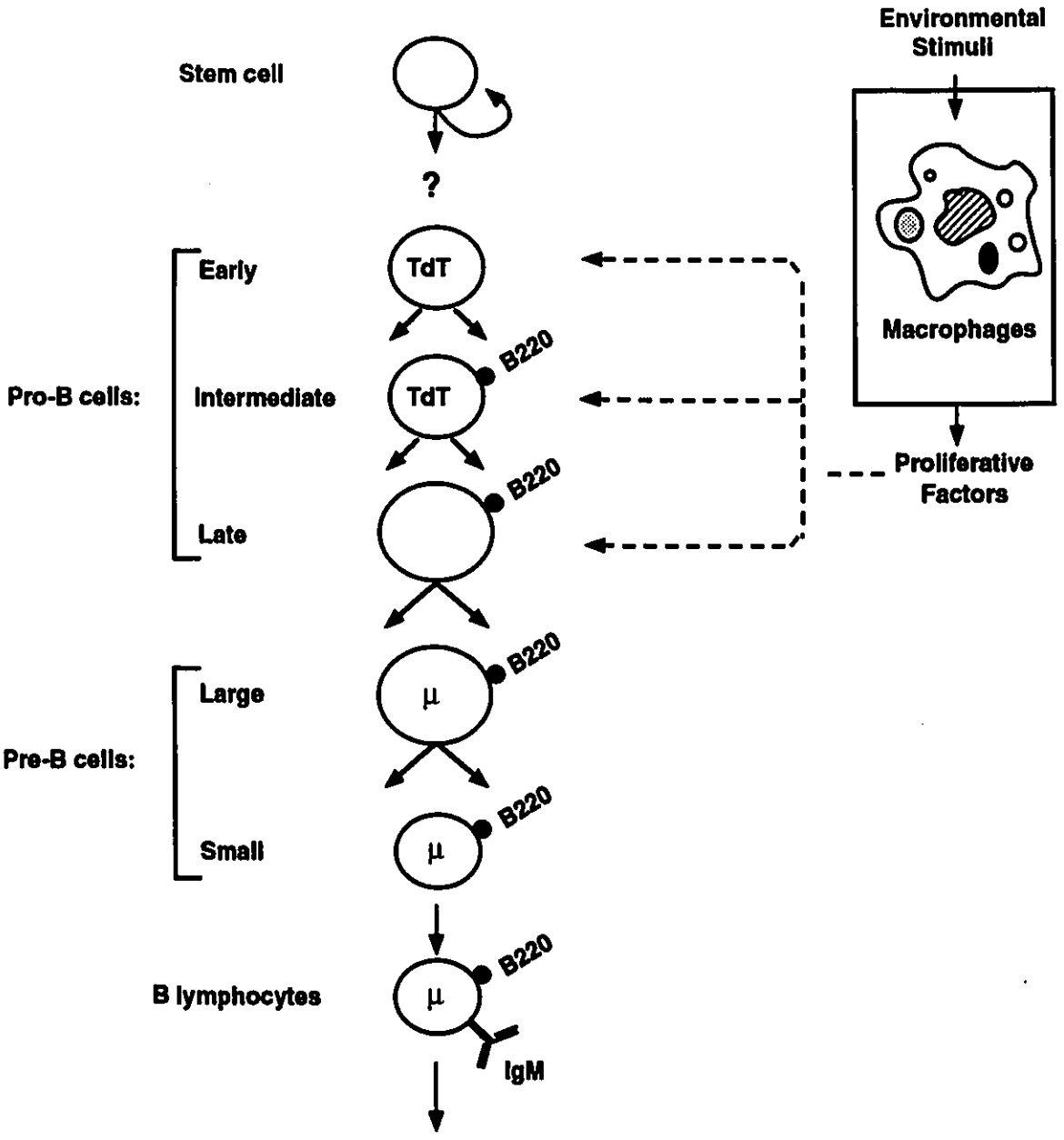
Dysregulations predisposing to oncogenesis may result from perturbations in a number of different regulatory events. In Chapter 5 we examine the cellular perturbations in the proliferative dynamics and microenvironmental associations of B cell precursors produced by dysregulation of the *c-myc* oncogene in Eμ-*myc* transgenic mice, all of which eventually sucumb to B cell tumors.

The hemopoietic cell lineages undergo processes of differentiation from stem cells and proliferative expansion. *In vivo*, B cell precursors pass continuously from one phenotypically defined compartment to the next. Cell growth factors and cell associations regulate the process of B cell differentiation and proliferation. However, the precise interactions and factors regulating early B precursor compartments remain largely unknown. Regulatory mechanisms operating during pro-B cell stages of development could have important implications for the normal clonal development of B cell lymphocytes as well as perturbations leading to B cell neoplasias and deficiencies. Chapter 6 deals with the stages in the development of B cell precursors at which the proto-oncogene *c-kit* is expressed under physiological conditions, examines their normal proliferative dynamics, and assesses the functional significance of *c-kit* activity on precursor B cell development, by using a neutralizing anti-*c-kit* antibody *in vivo*.

Dysregulation of B cell genesis is also produced by mutations which impair Ig gene rearrangement. In Chapter 7 we analyse the proliferative dynamics of B cell precursors in mice with the severe combined immunodeficiency mutation (scid) and the effects of introducing Ig heavy chain and light chain transgenes, thus evaluating the role of IgM expression in the differentiation and survival of precursor B cells in mouse bone marrow.

The rationale and objectives of each of these studies are presented more fully in the introductory sections of the appropriate Chapters.

Figure 2. Model of the influence on B cell genesis of macrophage-derived systemic cytokines.


Environmental Stimuli
Stem cell
?
Macrophages
Proliferative Factors
Pro-B cells:
Early
Intermediate
Late
TdT
TdT
B220
B220
B220
Pre-B cells:
Large
Small
μ
μ
B220
B220
B lymphocytes
μ
B220
IgM

CHAPTER 2

Materials and Methods

MATERIALS and METHODS

The following materials and methods are common to chapters 3, 4, 5, 6 and 7.

Antibodies.

Fluorescein isothiocyanate (FITC)- and tetramethyl rhodamine isothiocyanate (TRITC)-conjugated affinity-purified goat anti-mouse μ heavy chain (Kirkegaard and Perry Laboratories, and Southern Biotechnology Associates Inc., Birmingham, AL), were both used at 50μg protein/ml. Affinity-purified rat mAb 14.8 (Kincade et al 1981b) was used at 13μg protein/ml to bind to B220 surface glycoprotein. FITC-conjugated goat anti-rat IgG (Kirkegaard and Perry Laboratories) was used as the secondary antibody at 50μg protein/ml. mAb 14.8 was also used for direct immunofluorescence labeling after being FITC-conjugated and diluted 1:5. Rabbit anti-TdT (Supertechs Inc., Bethesda, MD) was used at 13μg protein/ml, and detected by the binding of TRITC-conjugated goat anti-rabbit IgG $F(ab')_2$ (Jackson Immunoresearch Laboratories Inc., Missisauga, ON) at 136μg protein/ml. All antibodies were diluted with PBS (pH 7.2) and centrifuged for 30min at 120,000g using an air-driven ultracentrifuge (Airfuge, Beckman Instruments, Palo Alto, CA) to remove aggregates just before use.

Metaphase arrest.

Mice were given vincristine sulfate (Sigma Chemical Co., St. Louis, MO) ip at a dose of 1mg/kg body weight to stop cells in metaphase 2h40min before sacrifice, a time interval which corresponds with the middle of the linear phase of accumulation of mitotic cells (Opstelten and Osmond 1983, Wright and Appleton 1980).

Bone marrow and spleen cell suspensions.

After killing mice by cervical dislocation, bone marrow was flushed from the femoral shafts using Eagles' MEM (pH 7.2) containing 10% (v/v) newborn calf serum (NCS) (Gibco Laboratories Life Technologies Inc., Grand Island, NY). Spleens were suspended by passing through a stainless steel sieve in ice-cold MEM-NCS. Particles were removed from bone marrow and spleen cell suspensions by sedimentation into 1ml NCS. Cells were centrifuged at 1100rpm for 7min at 4°C, resuspended in MEM-NCS and the number of nucleated cells was determined using an electronic particle counter (Coulter Electronics Ltd., Burlington, ON).

Immunofluorescence labeling.

Double labeling of surface and cytoplasmic μ chains.

Bone marrow and spleen cell suspensions (50μl; $40x10^6$ cells/ml) were incubated in microwell plates with an equal volume of anti-μ-FITC for 30min on ice to label surface μ chains (sμ). The cells were washed three times by centrifugation (1100rpm, 5min, 4°C) in PBS (pH 7.2) containing 2.5% NCS, and resuspended in 0.95ml of medium

containing 0.15M NaCl, 2.7mM EDTA (Fisher Scientific Co., Fairlawn, NJ) and 5% (wt/v) BSA (Boehringer Mannheim, Germany), pH 7.4. Samples of $4x10^5$ cells were cytocentrifuged at 1100rpm for 5min (Shandon Cytospin, Shandon Southern Instruments Inc., Sewickly, PA) onto glass slides previously coated by centrifugation (10min, 1500rpm) with 100µl of PBS/BSA (3% w/v) and quickly air-dried. The cells were fixed in 5% (v/v) glacial acetic acid in absolute ethanol (12min, on ice), hydrated in PBS, and incubated with 30µl of anti-µ-TRITC (30min, on ice) to label cytoplasmic µ chains (cµ), and washed.

Double labeling of B220 glycoprotein and µ chains.

Bone marrow and spleen cell suspensions (50µl; $40x10^6$ cells/ml) were incubated with an equal volume of mAb 14.8 (30min, on ice), washed, and exposed to FITC-conjugated goat anti-rat IgG (30min, on ice). The cells were washed, cytocentrifuged and fixed as above. Cytospot preparations were exposed to TRITC-anti-µ, as above, to label µ chains, including both cµ and sµ.

Double labeling of B220 glycoprotein and TdT.

After B220 labeling, as above, cytocentrifuge preparations were fixed in absolute methanol (30min, on ice), exposed to gradually reducing concentrations of methanol in PBS, incubated with normal goat serum, washed, and exposed to rabbit anti-TdT (overnight, room temperature) followed by TRITC-conjugated goat anti-rabbit IgG $F(ab')_2$ (30min, room temperature).

Immunofluorescence analysis.

Cytocentrifuged immunolabeled cells were mounted in 90% (v/v) glycerol (Fisher Scientific, Nepean, ON) in PBS (pH 8.0), containing 0.1% (w/v) para-phenylenediamine (Sigma Chemical Co., St Louis, MO), to reduce fading of the fluorescein during microscopy (Wright and Appleton 1980), and sealed with nail polish. The preparations were examined using a phase-contrast, epifluorescence microscope (Carl Zeiss of Canada, Don Mills, ON) equipped with a 100X neofluar PH2 oil immersion objective and an HBO 50 mercury lamp. Individual cells were scored for a) single labeling with either FITC alone ($B220^+\mu^-$) or TRITC alone ($c\mu^+s\mu^-$, TdT^+B220^-), b) double labeling with FITC plus TRITC (TdT^+B220^+, $c\mu^+s\mu^+$). Labeled cells were examined by phase contrast to detect cells in metaphase.

The incidence of TdT^+B220^- cells, TdT^+B220^+ cells, $B220^+\mu^-$ cells, $c\mu^+s\mu^-$ cells and $s\mu^+$ cells, as well as the proportion of cells in metaphase, were derived in each case by examining at least 2000 nucleated cells, including 100 cells of each phenotype. The incidence of labeled cells was multiplied by the total cellularity of the bone marrow and spleen suspensions to determine the absolute number of B lineage cells of each phenotype.

CHAPTER 3

Perturbation of B cell genesis in the bone marrow of pristane-treated mice: implications for plasmacytoma induction

SUMMARY

Previous work has demonstrated that the activation of splenic macrophages can stimulate primary B cell genesis in mouse bone marrow. We hypothesise that conditions of prolonged macrophage activation may predispose to oncogenesis in the B cell lineage by stimulating the proliferation of early precursor B cells, thus increasing the probability of genetic errors during Ig gene rearrangement. The present work has tested whether primary B cell genesis in the bone marrow is altered by intraperitoneal injection of pristane oil, a treatment which produces macrophage-rich granulomata in the peritoneal cavity and leads to plasmacytoma formation. Immunofluorescence labeling of intranuclear terminal deoxynucleotidyl transferase (TdT), cell surface B220 glycoprotein, cytoplasmic μ chains (cμ) and surface (sμ) chains together with mitotic arrest techniques, have been used to quantitate precursor B cell population dynamics. After a single intraperitoneal injection of 0.5ml pristane oil in BALB/cAn mice, a plasmacytoma-susceptible strain, the TdT^+ early and intermediate pro-B cells (TdT^+B220^-; TdT^+B220^+) showed sustained increases in population size and in the number of cells flowing through mitosis, returning towards normal values by 12-20 weeks. In contrast, the later precursor cell populations of late pro-B cells (TdT^-B220^+), pre-B cells ($c\mu^+s\mu^-$) and B cells ($s\mu^+$) were consistently depressed for 4-12 weeks. BALB/cAn mice treated in the drinking water with indomethacin, a

prostaglandin synthesis inhibitor, showed similar responses to pristane treatment. Pretreatment with silica particles, toxic to macrophages, prevented the proliferative response of TdT^+ pro-B cells to pristane injection. Injection of washed peritoneal cells from pristane-treated BALB/cAn mice produced some stimulation of pro-B cells in the bone marrow of normal recipient BALB/cAn mice. In DBA/2 mice, although resistant to pristane-induced plasmacytomagenesis, the responses of precursor B cells to pristane resembled those of BALB/cAn mice. The results reveal a prolonged proliferative stimulation of early precursor B cells, presumptively at the stage of V_H gene rearrangement, in the bone marrow of pristane-injected mice during the latent period before plasmacytoma development. This effect appears to be at least partially macrophage-mediated but not dependent on prostaglandin synthesis. The concurrent depression of late pro-B cell, pre-B cell and B cell populations suggests an exaggerated degree of cell loss, possibly reflecting an increased incidence of aberrant cells. The findings are consistent with the hypothesized causal link between high levels of macrophage activation and a bone marrow origin of B cell neoplasias. If so, strain-dependent resistance to pristane-induced plasmacytomagenesis appears to operate at a later stage in the oncogenic process.

INTRODUCTION

The B cell lineage is prone to neoplastic changes which can lead to a variety of phenotypically distinct tumors. Recent evidence suggests that B cell tumors develop as a result of a series of deregulatory steps which may start as mistakes in the rearrangement of immunoglobulin (Ig)[1] variable region (V) gene segments during the primary genesis of B cells in the bone marrow (1-11). The identity of susceptible precursor B cells and the factors which may initiate neoplastic change are unknown. Possible candidates are suggested by studies of primary B cell genesis in mice (12).

A sequence of proliferating precursor B cell stages in mouse bone marrow has been defined by immunofluorescence labeling of 3 molecular markers of B cell differentiation; terminal deoxynucleotidyl transferase (TdT), an intranuclear enzyme expressed during V_H gene rearrangement inserting nucleotide sequences at gene segment junctions (13,14), the B lineage-associated cell surface glycoprotein, B220 (15,16), and μ heavy chains of IgM, expressed either as free μ chains in the cytoplasm (cμ) or in IgM molecules at the cell surface. Three putative populations before expression of μ chains, termed early, intermediate and late pro-B cells (TdT^+B220^-, TdT^+B220^+, $B220^+\mu^-$), have been proposed to precede the development of pre-B cells ($c\mu^+s\mu^-$), which finally give rise to non-dividing B cells ($s\mu^+$) (21-24). A combination of phenotypic analysis and mitotic arrest techniques has been used to

quantitate both the number of cells in each population and the rate at which cells flow through proliferative cell cycle at each differentiation stage (17,20,21). A sequence of mitoses at each pro-B cell and pre-B cell stage leads to progressive expansion of the population of developing precursor B cells. This appears, however, to be accompanied by a considerable degree (75%) of cell deletion at the pre-B cell stage before the newly-formed virgin B cells are discharged from the bone marrow to enter the B cell populations in the peripheral immune system (17,21,24,25). Much cell loss may represent a culling of cells which had undergone defective V_H gene rearrangements (26).

The proliferative activity of precursor B cells in mouse bone marrow can be stimulated by the activation of distant macrophages in the spleen. A single intraperitoneal (ip) injection of sheep red blood cells (SRBC) is followed by a wave of increased proliferation of precursor B cells in the bone marrow (27,28), including TdT^+ pro-B cells undergoing V_H gene rearrangements (21). This effect on all stages of B cell genesis is abrogated completely by prior treatment with silica particles, an agent toxic to macrophages (29-32), and by splenectomy, indicating that it is mediated by activated macrophages located in the spleen (33-34). The stimulatory effect can be adoptively transferred by spleen cells from SRBC-injected donors to untreated hosts (35). Repeated injections of SRBC, simulating exposure to sustained environmental stimuli, produce expansion of

population sizes of precursor B cells in the bone marrow as well as an absolute increase in their proliferation, elevating the entire steady state of primary B cell genesis (36). These findings have led us to propose the hypothesis that pathological degrees of chronic macrophage activation may play an etiological role in initiating oncogenesis in the B cell lineage (12). A sustained increase in number and proliferative activity of pro-B cells, and thus in total V_H recombinatorial activity, would increase the probability of chromosome breaks and genetic aberrations, including chromosomal translocations, capable of initiating the multiple steps towards neoplasia.

A primary phase of macrophage activation is a prominent feature of pristane-induced murine plasmacytoma. The intraperitoneal injection of pristane oil (2,6,10,14-tetramethylpentadecane) in BALB/cAn mice is followed after a latent period of 120 days or more by the development of malignant plasmacytomas (37-41). The nonmetabolizable pristane oil evokes the formation on the peritoneum of chronic granulomatous tissue, rich in macrophages that have ingested pristane oil droplets and show evidence of activation (42). The plasmacytomas are characterized by a chromosomal translocation that juxtaposes the *c-myc* gene to one of the Ig-coding genes, resulting in constitutive expression of *c-myc* (43). The onset of plasmacytoma can be accelerated by repeated doses of pristane (38) or infection by Abelson murine leukemia virus

(A-MuLV) (39,41,43). The mechanism and initial target cell of pristane-induced plasmacytomagenesis have not been established. To determine whether pristane-induced granuloma formation and macrophage activation may exert an effect on the early stages of the B cell lineage we have now assayed precursor B cell populations and their proliferation in the bone marrow of pristane injected mice. Susceptible BALB/cAn mice have been examined at intervals up to 6 months after a single ip injection of 0.5ml pristane oil. The various precursor B cell populations have been quantitated by double immunofluorescence labeling and direct epifluorescence microscopy at a fixed interval (2h40min) after injecting vincristine sulfate to cause dividing cells to accumulate as they enter metaphase (17). Each differentiation stage has thus been analyzed for population size (number of cells/femur) and production rate (number of cells entering mitosis/unit time/femur) at various times after pristane injection. Treatment of BALB/cAn mice with indomethacin, a prostaglandin (PG) synthesis inhibitor (44), during the latent period of pristane-induced plasmacytoma development, greatly inhibits plasmacytoma formation without preventing the appearance of the characteristic granulomata (45). To determine whether indomethacin treatment might exert its protective effect by blocking pristane-induced changes in precursor B cell activity and whether such changes are PG-mediated, pristane-injected BALB/cAn mice have been given indomethacin continuously in the drinking water during

the analysis of bone marrow B lineage cells for periods up to 4 weeks. To examine whether pristane-induced effects on precursor B cells are mediated by activated macrophages, BALB/cAn mice have been given pristane ip after pretreatment with an injection of small particles of silica, toxic for macrophages. Previous work has shown that macrophage activation in the spleen following systemic administration of foreign materials is associated with a stimulation of precursor B cell proliferation in mouse bone marrow, apparently mediated by soluble factors (27,28,33-35). We have now used silica particles to ablate macrophages, as well as transfers of spleen cells and peritoneal exudate cells from pristane-injected donors, to examine the role of macrophages in producing the effects of pristane injection. Unlike BALB/cAn mice, some other inbred strains are genetically resistant to the induction of plasmacytomas by pristane (45). To examine whether genetic resistance may operate at the level of the early precursor B cells, these cells have been assayed for periods of up to 32 wk in the bone marrow of pristane-injected DBA/2 mice, a strain reported to be resistant to plasmacytoma induction (45). The work reveals that pristane injection exerts a stimulatory effect on the early and intermediate pro-B cells in the bone marrow, which maintain increased population sizes and proliferative activity for prolonged periods of time, together with a reduction in activity of more differentiated precursor B cells, suggesting the production of many aberrant cells. The work focuses

attention on the early stages of B cell genesis in the bone marrow as possible target cells for the initiation of genetic events leading to neoplasia.

MATERIALS and METHODS

Mice. BALB/cAn and DBA2 male mice were supplied by Dr. M. Potter (N.I.H. Bethesda, MD), and Dr. J. Wax (Hazelton Labs, MD). Mice were maintained in microisolators, and used at 6-10 wk.

Mineral Oil. Pristane (2,6,10,14-tetramethylpentadecane, Aldrich Chemical Co., Milwaukee, WI) was injected ip in a dose of 0.5ml (38).

Indomethacin. Mice were given indomethacin (Sigma Chemical Co., St. Louis, MO) or vehicle (0.2% ethanol) in the drinking water. Indomethacin was dissolved in absolute ethanol and diluted in tap water to achieve a final concentration of 10μg/ml (42,46). Water bottles were changed every 2 days. There was no observed difference in the amount of water consumed between groups of mice given indomethacin and groups given the vehicle alone.

Silica. Silica particles, 0.012μm in size (Sigma Chemical Co. St. Louis, MO) were suspended in sterile saline to a concentration of 10mg/ml and autoclaved. Mice were given 0.3ml of the suspension ip (33,34). Control mice received 0.3ml saline ip.

Peritoneal cell and spleen cell tranfers. Donor mice were injected ip with 0.5ml of pristane oil. Control animals received an ip injection of 0.5ml sterile saline. The animals were killed 15h later. The peritoneal cells were collected by peritoneal lavage (1ml saline was injected into the peritoneal cavity of dead mice, the abdomens were massaged, and the cells were aspirated using a syringe and 18G needle). The peritoneal cells were washed in PBS by centrifugation to removed any trace of oil, and were injected ip into naive syngeneic recipients, giving one donor's peritoneal cells per recipient. After collection of peritoneal cells, peritoneal cavities were opened and the spleens were removed. The spleens were rinsed thoroughly with PBS, chopped into pieces of approximately 2mm, suspended in 0.3ml of saline, and injected ip into naive recipients using a 18G needle (one donor's spleen equivalent per recipient). All procedures were carried out under sterile conditions.

The population sizes and production rates of the B cell precursors were determined using double immunofluorescence labeling and mitotic arrest techniques, as described in Chapter 2.

RESULTS

Precursor B cells in pristane-injected BALB/cAn mice.

A single ip injection of 0.5ml pristane oil produced marked perturbations in the populations of precursor B cells in the bone marrow of BALB/cAn mice (Table 1). Early and intermediate pro-B cells (TdT^+B220^- and TdT^+B220^+ cells, respectively) increased in number during the first 7-10d after pristane injection, remained elevated after 4 wk and returned towards normal values at 12-33 wk. In contrast, late pro-B cells ($B220^+\mu^-$), pre-B cells ($c\mu^+s\mu^-$) and B cells ($s\mu^+$) all declined sharply in numbers for 7-10d, remained depressed for 12 wk and recovered only partially at 24-33 wk. The number of $s\mu^+$ B cells in the spleen also tended to stay at subnormal or normal levels after a transient increase in the first 3-4d (Table 1). A measure of the rate of production of dividing precursor B cells at each phentoypic stage of differentiation was given by the number of cells per femur which had accumulated in metaphase during a standard time interval (2h40min) after injecting vincristine to stop cells in mitosis (Table 1). By this criterion, the production rates of early and intermediate pro-B cells increased rapidly to plateau values at 1-4 wk returning to normal or subnormal values by 12 wk. Late pro-B cells, on the other hand, after initially maintaining normal production rates for 7d, showed much reduced production at 4-12 wk, returning progressively to normal by the end of the 8 month experimental period.

Figure 1 compares the patterns of response in the various precursor B cell populations in the bone marrow by expressing the size of each cell population relative to its respective normal value. Early and intermediate pro-B cells showed sustained increases to about twice their normal population sizes, associated with 2.0-2.5Xnormal production rates. Late pro-B cells, pre-B cells and B cells in contrast, fell to 25-50% normal numbers for 12 wk. The production rate of late pro-B cells fell to about one quarter normal at 4-12 wk after initially maintaining normal values.

At particularly late intervals (6-8 months), studied in a second series of experiments, the production rates of pro-B cells again appeared to be elevated and the number of splenic B cells increased (Table 1).

Precursor B cells in pristane-injected BALB/cAn mice during tumor development.

Some BALB/cAn mice in the latter part of the experimental period (6-7 months after pristane administration) developed copious ascites fluid in which numerous plasma cells were detected in cytocentrifuged preparations stained with MacNeal's tetrachrome. The pattern of precursor B cell activity in the bone marrow of these mice with ascites, presumptive plasmacytomas, generally resembled that in mice having no evident ascites at similar post-pristane

intervals, though production of late pro-B and pre-B cells was more severely depressed (Table 2).

Precursor B cells in pristane-injected BALB/cAn mice treated with indomethacin.

Administration of 0.5ml pristane to control BALB/cAn mice given indomethacin vehicle (0.2% ethanol) in drinking water replicated the previously observed pattern of precursor B cell response in BALB/cAn bone marrow, TdT^+ pro-B cells expanded to 1.5-2.0 fold normal population sizes and 2.5-3.0 fold normal mitotic activity at 10-28d, while over the same time period the later differentiation stages were depressed to about half normal population sizes and 50-75% normal rates of mitoses (Table 3, Figs. 2,3). Treatment of a further group of pristane-injected BALB/cAn mice with indomethacin in the drinking water failed to modify any of these responses. Pristane injection again reproduced a pattern of stimulated TdT^+ cell activity and depression of later stages essentially identical to that seen in pristane-treated mice not treated with indomethacin (Table 3, Figs. 2,3). Splenic B cells showed a moderate reduction in numbers in both indomethacin-treated and untreated pristane-treated mice (Table 3).

Re-injection of pristane in BALB/c mice.

A second injection of pristane induces an increased frequency and acceleration in tumorigenesis in BALB/cAn mice (38). In order to

analyze the possible effects on B cell precursors in the bone marrow of a second injection of pristane, we injected 0.5ml pristane ip, followed by a second injection 6 wk later. The response to the second injection appeared to resemble that of the first injection but the values oscillated more widely (Table 4).

Effect of Silica Treatment on Precursor B Cells in Pristane-Injected BALB/cAn Mice.

Pretreatment of mice with an ip injection of silica particles 2h before pristane administration either prevented or substantially reduced the subsequent expansion of TdT^+ pro-B cell populations in the bone marrow, 14d and 28d later (Table 5). On the other hand, silica treatment exerted no modifying effect on the responses of pre-B cell and B cell populations. These were depressed to an equally severe extent in silica-treated and untreated pristane-injected mice.

Effect of Transfers of Peritoneal and Spleen Cells

To determine whether activated peritoneal and splenic macrophages have an effect on precursor B cell populations in the bone marrow, naive mice received, ip, peritoneal exudate or chopped spleens from either pristane-injected donors or saline-injected control mice. Bone marrow and spleen B cell populations were examined 3d and 7d after transfer (Table 6 and Fig. 4).

Peritoneal cell transfers resulted in increased pro-B cell numbers and proliferative activity at 3d, which had largely subsided by 7d. There

was little if any effect on the later differentiation B lineage populations. Spleen cell transfers appear to produce a marginal stimulatory effect on some pro-B cells at 3d and 7d.

Precursor B Cells in Pristane-Injected DBA/2 Mice.

Injection of 0.5ml pristane ip in DBA/2 mice produced a pattern of perturbation of precursor B cell populations resembling that in BALB/cAn mice (Table 7, Fig. 5). A generally lower number of cells per femur in DBA/2 mice reflected their smaller body size. Early and intermediate pro-B cells showed protracted increases to 1.5-3.5 fold normal numbers at 3-28d associated with 1.5-2.5 fold increases in rate of flow through mitosis, returning to normal values by 12 wk. Late pro-B cells, pre-B cells and B cells, on the other hand, all fell sharply to 25% normal population size by 4d and remained depressed until 12 wk, then increasing slowly towards normal values. B cells in the spleen increased transiently in number in the first wk and returned to normal values (Table 7). The number of late pro-B cells passing through mitosis fell to low levels from 3d to 12 wk, while the rate of pre-B cell mitosis fell less severely over the same time period (Fig. 5). In long-term experiments, as in BALB/cAn mice, an increased flow of precursor B cells through mitosis occurred in the bone marrow at 33 wk, associated with increased numbers of B cells in the spleen (Table 7).

DISCUSSION

In the present study we have found that a single ip injection of 0.5ml pristane oil produces marked central effects on primary B cell genesis in the bone marrow. The dynamics of production and differentiation of precursor B cell populations in the bone marrow are perturbed for protracted periods of time after pristane injection. This interval corresponds with the establishment of oil granulomata in the peritoneal cavity, preceding the development of plasmacytoma. The pristane-induced effects involve precursor B cells at all identifiable stages of phenotypic differentiation, including those in the process of rearranging Ig genes. Our findings raise the possibility that an initial pristane-induced, macrophage-mediated stimulation of precursor B cells may be responsible for initiating at an early stage of the B cell lineage in the bone marrow, the first of a series of transformational events, leading eventually to the appearance of neoplastic plasma cells.

Over a series of experiments, a characteristic pattern of response to pristane injection has consistently emerged. There are two main elements. First, the early and intermediate pro-B cell stages of differentiation show expansion of population size and increased levels of proliferative activity. These two cell populations, expressing intranuclear TdT, include precursor B cells undergoing V_H chain gene rearrangement. Second, in marked contrast, the precursor B cell populations at later stages of differentiation are all diminished in

population size and, in most cases, in mitotic activity. These apparently disparate changes in the earlier and later differentiation stages, respectively, take effect within the first few days of pristane injection and, once established, persist for many weeks or months. The reproducibility of this response has been established by observing essentially the same general features and timing in independent replicate experiments involving six separate series of mice, each followed for periods of 28-230 days after pristane injection. These experiments have used BALB/cAn strain mice, with and without treatment with indomethacin or silica and DBA/2 strain mice as detailed above.

The use of double immunofluorescence labeling and epifluorescence microscopy to quantitate the actual number of cells per femur at each phenotypic stage of differentiation, together with a mitotic arrest technique to indicate the number of cells flowing through mitosis with time in each phenotype compartment, has been used with consistent results in a series of studies to establish a dynamic model of B cell genesis in the bone marrow (17,20-24). In the normal kinetic steady state of B lymphopoiesis, each phenotypically distinct precursor cell population maintains a constantly reproducible size and mitotic production rate as a result of a dynamic balance between the rates at which cells "enter" and "leave" the phenotypic compartment (21,24,47). Successive cell compartments increase in population size as the outflow from one compartment is expanded by

cell division in the next. Changes in the cell dynamics in early compartments, though representing a small absolute number of cells, may potentially produce large changes in the final output of mature cells. In any given compartment, a doubling or halving of its characteristic population size and flow of cells through mitosis, as now seen in pristane-injected mice, represent substantial perturbations of normal cell dynamics.

The present findings suggest that mechanisms both of increased cell production and cell loss are implicated in the pattern of response of precursor B cells to pristane oil granuloma. The prolonged doubling in size of the TdT^+ pro-B cell compartments is accompanied by a comparable increase in the number of TdT^+ cells entering mitosis per unit time. This reveals a true stimulation of cell production and elevation of the kinetic steady state in these compartments, rather than a passive accumulation of cells. The resulting increased output of cells from the stimulated TdT^+ pro-B cells, is associated with a reduction in late pro-B cells, pre-B cells and B cells in pristane-injected mice. This strongly suggests a greater degree of cell loss than usual, allowing only a reduced fraction of the early and intermediate pro-B cells successfully to complete their differentiation. The marked reduction in the population size of late pro-B cells and their much reduced flow through mitosis, despite the increased cell production at the early and intermediate pro-B stages, suggests that much cell loss occurs at this stage. There is no evidence

to support other possible explanations, that the late pro-B cells may have emigrated from the bone marrow or switched to some other unrecognized phenotype. The pre-B cells, like late pro-B cells, are markedly reduced in total number in pristane-injected mice, but the number of pre-B cells passing through mitosis per unit time is less severely depressed. A considerable loss of pre-B cells appears to be a feature of B cell genesis under normal circumstances in mice (17,24,47). The present findings suggest that the pre-B cells in pristane-injected mice undergo an increased degree of cell loss, mainly at the post-mitotic stage. This is consistent with the accompanying fall in the number of B cells to 25-35% normal levels in the bone marrow. It is not excluded that there could also have been an acceleration of the maturation of pre-B cells to B cells and of the discharge of B cells from the bone marrow in response to unknown signals from the periphery. However, the failure of the splenic B cell population to show any sustained increase in numbers provides no support for this interpretation. The possible migration of newly-formed B cells into the pristane oil granuloma and peritoneal tissues is unknown. The present kinetic study thus demonstrates a true stimulation in the number of actively proliferating early and intermediate pro-B cells expressing TdT, and strongly suggests a substantial increase in the proportion of cells which subsequently abort at the late pro-B, pre-B and B cell stages. On the other hand, many cells do successfully develop into mature $sIgM^+$ B lymphocytes,

for dissemination to the peripheral B cell pool. After an early transient elevation, the B cells in the spleen were maintained at about 75% normal numbers throughout the development of pristane oil granuloma.

Pristane injection produces chronic granulomata, heavily infiltrated with macrophages, on the surface of the peritoneum (42). After ingesting droplets of pristane oil, the macrophages show evidence of activation, including the production of IL-6, a growth factor necessary for the initial growth of neoplastic plasmacytes, which precedes plasmacytoma formation (48). Previous studies have shown that macrophage activation in the spleen following systemic administration of sheep red blood cells (SRBC) or other foreign materials is associated with a stimulation of precursor B cell proliferation in mouse bone marrow, apparently mediated by soluble factors (27,28,33-35). Sustained macrophage activation produced by repeated administration of SRBC elevates the kinetic steady state of B cell genesis, expanding the populations of precursor B cells and increasing their production rates (36). The present preliminary experiments using silica particles to ablate macrophages and the transfers of peritoneal exudate cells and spleen cells from pristane-injected donors, suggest that the sustained stimulation of early and intermediate pro-B cells in the bone marrow of mice developing pristane oil granuloma is also macrophage-mediated.

Silica particles of the small uniform size and source used in the present work, are rapidly engulfed by and are directly toxic to macrophages (29-32). The cells are killed following the disruption of silica-containing phagolysosomes and the intracellular release of lysosomal enzymes (29). Further phagocytosis and macrophage-mediated immune responses are suppressed (30-32). The observed effect of pretreatment with silica particles in suppressing the stimulation of pro-B cells by pristane oil injection strongly suggests that the precursor B cell hyperactivity is the result of macrophage activation. Since silica particles may produce indirect functional effects on other cell populations, including lymphocytes, however, further studies will be desirable, using blocking agents (31) and alternative macrophage ablation techniques. Both peritoneal exudate cells and spleen cells from pristane-treated donors appear to be able to transfer a modest degree of stimulation of early precursor B cell populations to naive recipients, the peritoneal cells producing an earlier and more marked effect than the spleen cells.

The foregoing results reinforce the hypothesis that ip pristane acts indirectly on bone marrow B cell precursors via the activity of macrophages, many of which may be located in the peritoneal cavity. Which particular systemic secretion products of macrophages may be responsible for precursor B cell stimulation is unknown. Prostaglandin synthesis appears not to be involved, in view of the lack of effect of indomethacin treatment in the present work.

Current studies indicate that systemic administration of recombinant IL-1 in low doses can stimulate precursor B cells in the bone marrow whereas TNF is suppressive over a wide dose range (L. Fauteux, A. Folberg and D.G. Osmond, unpuplished data).

Silica administration does not substantially modify the depression of late pro-B cell, pre-B cell and B cell populations in the bone marrow, suggesting that these actions of pristane are largely independent of macrophages. The apparent duality of the pristane response, a silica-sensitive stimulation of early precursor B cells and silica-insensitive depression of late precursor B cells, raises the possibility that in addition to increased deletion of cells with defective genetic rearrangements resulting from the stimulation of early precursor B cells, some other mechanisms may contribute to the suppression of the later precursor B cells. The levels of steroids and of suppressive or chemotactic factors may be elevated as a result of the chronic inflammatory process. Again, however, indomethacin treatment indicates that this effect is not mediated by PGE synthesis.

The sustained stimulation of precursor B cells expressing TdT preceding the development of plasmacytomas in pristane-injected mice accords with the hypothesis that central events in B cell genesis in the bone marrow may be causally related to B cell oncogenesis. Pristane-induced murine plasmacytomas are consistently associated with deregulation of the *c-myc* oncogene resulting from a chromosomal translocation in which it is juxtaposed to the Ig H chain

locus (43). Such translocations may result from chromosome breaks occurring as genetic errors during the normal process of Ig V_H gene rearrangement (1-11). Pro-B cells expressing TdT would be particularly prone to such genetic errors (12). It has been proposed that the rate at which such errors may normally be expected among proliferating precursor B cells may constitute the major, if not the sole, initiating step in the etiology of childhood acute lymphoblastic leukemia in humans (9). Any circumstance which increases the number or rate of cell divisions among susceptible cells would increase the probability of chromosome breakage and translocations (4). Nucleotide sequence analysis and the use of a variety of clonal markers have indicated that translocations involving the heavy chain variable region and the *bcl-2* proto-oncogene can occur as an error in D-J rearrangement in the bone marrow in initiating the development of human nodular lymphoma (1,5,8). Thus, by elevating the number and proliferation of TdT^+ cells rearranging V_H chain genes the macrophage-mediated pristane effect would favor an increased incidence of defective B lineage cells. Many of these cells may abort, but others may survive and be selected into the peripheral B cell pool with a proliferative advantage so that with further deregulation, possibly associated with viral infection or with hypermutation and clonal expansion during secondary antigenic exposures, one of the progeny may in due course be driven to monoclonal neoplastic expansion after plasmacytic differentiation.

Other experimental models clearly show stimulated precursor B cell activity preceding B cell oncogenesis. Transgenic mice bearing a *c-myc* oncogene subjugated to the Ig heavy chain enhancer (Eμ) almost invariably develop a malignant pre-B/B lymphoma, preceded by a period of enhanced polyclonal or oligoclonal proliferation of pre-B cells in the bone marrow (4). We find that this preneoplastic phase in Eμ-*myc* transgenic mice is characterized by expansion of the populations of TdT^+ pro-B cells as well as pre-B cells together with evidence of increased cell loss at the pre-B cell stage (C. Sidman et al., in press). A *c-myc*/Ig translocation also characterizes human Burkitt's lymphoma associated with chronic malaria (2,6). In this condition, gross splenomegally is associated with a marked increase in the macrophage population, extensive phagocytosis of parasitized RBC, intense macrophage activation and release of diverse cytokines (49,50). In murine malaria produced by *Plasmodium yoelii* we have observed a central effect in the bone marrow closely resembling that in pristane-injected mice, stimulation of TdT^+ pro-B cells without a proportionate expansion of more differentiated precursor B cells (Chapter 4, this thesis, 51). These findings accord with the view that macrophage activation and polyclonal expansion of pro-B cell activity in the bone marrow could constitute a common mechanism whereby two such dissimilar agents as pristane oil and malaria parasites may predispose to a chromosomal translocation and neoplastic transformation in the B cell lineage.

Unlike BALB/cAn mice, other inbred strains, notably DBA/2, are reportedly resistant to the induction of plasmacytoma by pristane (45). The bone marrow response of pristane-injected DBA/2 mice, however, resembles that of BALB/cAn mice; early and intermediate TdT+ pro-B cell populations are expanded while later forms tend to be depressed. This finding raises the possibility that the genes responsible for strain restriction of plasmacytoma formation rather than acting to prevent an initiating oncogenic event in the bone marrow early in the B cell lineage, may operate at a later stage in tumor development. This would reflect strain differences in the level of natural cellular surveillance mechanisms capable of reorganizing and deleting neoplastic clones as they arise. Similarly, in BALB/cAn mice, the usual susceptibility to pristane-induced plasmacytoma induction can be prevented by administration of indomethacin in the drinking water (42). The mechanism of this effect is unknown. We now find that such indomethacin treatment does not modify the pristane-induced effects on precursor B cells in the bone marrow of BALB/cAn mice, suggesting that indomethacin also may act at a later stage in tumorigenesis. The reported finding that indomethacin therapy reduces plasmacytoma formation even if its onset is delayed for 60 days after pristane-injection (42) is consistent with a late or secondary action. Indomethacin itself is not toxic and does not prevent the formation of a peritoneal inflammatory exudate or peritoneal oil granulomatous tissue (42). Indomethacin has

suppressive activity against many tumors (46,52). This is attributed to its effect in inhibiting the synthesis of prostaglandins, especially PGE, which are produced in tumor-bearing hosts and suppress the antitumor effects of host natural killer (NK) cells, macrophages and cytotoxic lymphocytes (46). NK cell activity declines during the development of many experimental tumors (53) and is depressed during the preneoplastic phase in pristane-injected BALB/cAn mice (54). Transplanted plasmacytoma cells may grow equally well in pristane and pristane/indomethacin-treated mice (42). Nevertheless, indomethacin treatment of pristane-injected mice, by preventing the suppression of NK cells by PGE2 may expose developing clones of plasmacytoma cells to natural immunosurveillance mechanisms and deletion.

TABLE 1

B lineage cells in bone marrow and spleen of pristane-injected BALB/cAn mice[a]

Cell phenotype	0d[b]	2d	4d	7d	28d	82d	196d	230d
Cells/femur ($x10^5$)[c]								
TdT^+B220^-	1.0	1.2	1.8	1.9	1.8	1.3	0.8	1.0
TdT^+B220^+	0.9	1.8	2.0	1.7	1.9	0.9	2.4	1.9
$B220^+\mu^-$	18.0	12.8	8.8	10.2	9.4	2.5	7.1	5.4
$cu^+s\mu^-$	24.0	11.5	11.4	9.9	8.4	8.0	14.2	16.2
$s\mu^+$	24.3	15.7	14.3	6.0	6.3	7.0	15.5	14.0
Cells/spleen ($x10^6$)[c]								
$s\mu^+$	67.2	79.4	77.1	48.7	52.7	67.9	73.0	136.6
Cells in metaphase/femur ($x10^4$)[d]								
TdT^+B220^-	3.4	5.1	6.1	8.9	9.3	1.7	3.4	0.9
TdT^+B220^+	1.8	3.4	3.9	3.5	4.5	0.6	7.1	3.8
$B220^+\ \mu^-$	7.6	9.5	8.4	8.8	1.1	2.0	6.8	11.2
$cu^+s\mu^-$	19.2	12.8	15.9	15.0	15.7	6.0	18.5	14.0

a Values derived from a pool of three BALB/cAn mice (8 wk) given 0.5ml pristane ip

b Normal mice (0d) received no treatment

c Calculated from the incidence of cells/phenotype relative to all nucleated cells and the total cellularity of the bone marrow and spleen respectively

d Calculated from the total number of cells/femur/phenotype and the percentage of cells/phenotype in metaphase (metaphase index) 2h40min after injecting vincristine sulfate ip

TABLE 2

B lineage cells in bone marrow and spleen of pristane-injected BALB/cAn mice[a] during ascites development

Cell phenotype	Normal	Ascites	No ascites
Cells/femur ($x10^5$)[c]			
TdT^+B220^-	0.9	1.0±0.2	0.9±0.1
TdT^+B220^+	2.6	1.9±0.0	2.1±0.3
$B220^+\mu^-$	12.0	6.6±0.8	6.2±1.2
$cu^+s\mu^-$	27.0	12.0±2.8	15.2±1.4
$s\mu^+$	25.5	17.4±3.7	14.7±1.0
Cells/spleen ($x10^6$)[c]			
$s\mu^+$	73.0	118.0±36.0	105.0±45.0
Cells in metaphase/femur ($x10^4$)[d]			
TdT^+B220^-	1.2	3.0±1.0	2.0±1.0
TdT^+B220^+	1.6	3.0±1.5	5.0±2.0
$B220^+\ \mu^-$	12.0	5.0±1.0	9.0±3.0
$cu^+s\mu^-$	27.0	8.0±3.5	16.0±3.0

a Values derived from 4 ascitic BALB/cAn mice (1 mouse at 173 days and 3 mice at 192 days after injecting 0.5ml pristane ip) and two groups of 3 ascites-free BALB/cAn (196 and 230 days). [mean ± standard deviation]

b Calculated from the incidence of cells/phenotype relative to all nucleated cells and the total cellularity of the bone marrow and spleen respectively

c Calculated from the total number of cells/femur/phenotype and the percentage of cells/phenotype in metaphase (metaphase index) 2h40min after injecting vincristine sulfate ip

TABLE 3

B cells in spleen of pristane-injected BALB/cAn mice given indomethacin[a]

Treatment	Cells/spleen ($x10^6$)					
	0d	4d	7d	10d	14d	28d
Pristane alone[b]	95.0	96.2	91.2	88.8	90.3	64.9
Pristane + indomethacin[c]		99.8	91.0	86.8	85.8	72.9

a Each value was derived from cells pooled from groups of 3 mice

b 8-10 wk old BALB/cAn mice were given 0.5ml pristane ip

c 8-10 wk old BALB/cAn mice were given indomethacin continuously in drinking water (10µg/ml) after injecting 0.5ml pristane ip

TABLE 4

B lineage cells in bone marrow and spleen of BALB/cAn mice[a] given a second injection of pristane

Cell phenotype	0d[b]	3d[c]	7d	10d	15d	28d
Cells/femur ($x10^5$)[d]						
TdT^+B220^-	1.0	1.5	1.3	0.7	1.2	1.1
TdT^+B220^+	1.9	1.0	3.7	1.6	3.0	2.5
$B220^+\mu^-$	7.3	4.0	4.5	3.8	6.3	5.3
$cu^+s\mu^-$	23.8	12.6	15.7	10.0	15.2	17.1
$s\mu^+$	17.9	8.0	14.0	7.9	14.7	13.6
Cells/spleen ($x10^6$)[d]						
$s\mu^+$	56.0	94.4	108.0	62.0	88.4	84.6
$B220^+\mu^-$	8.0	13.7	15.7	8.9	12.6	11.5
Cells in metaphase/femur ($x10^4$)[e]						
TdT^+B220^-	0.8	0.8	1.8	0.2	1.2	0.4
TdT^+B220^+	1.5	0.7	3.0	1.8	2.7	1.0
$B220^+\ \mu^-$	10.9	5.6	5.9	6.0	5.0	6.4
$cu^+s\mu^-$	19.0	18.9	15.0	10.0	15.2	14.5

a Values derived from a pool of three BALB/cAn mice (8 wk)

b Normal mice received no treatment

c Days after a second ip injection of 0.5ml pristane given 41d after a first similar injection.

d Calculated from the incidence of cells/phenotype relative to all nucleated cells and the total cellularity of the bone marrow and spleen respectively

e Calculated from the incidence of cells/femur/phenotype and the percentage of cells/phenotype in metaphase (metaphase index) 2h40min after injecting vincristine sulfate ip

TABLE 5

Effect of silica treatment on B lineage cells in the bone marrow and spleen of pristane-injected BALB/cAn mice[a]

Treatment	Normal	14d after pristane		28d after pristane	
		No silica	Silica	No silica	Silica
Cells /femur ($x10^5$)[b]					
TdT+	3.9	4.8	3.6	8.8	2.7
$B220^+\mu^-$	8.8	26.0	13.0	13.0	5.8
$cu^+s\mu^-$	19.2	5.4	4.1	2.8	2.8
$s\mu^+$	17.8	2.3	1.5	7.1	4.9
Cells/spleen ($x10^6$)[c]					
$s\mu^+$	53.2	36.2	35.6	47.8	63.7

a 8-10 wk old BALB/cAn mice were injected with silica particles ip 2h before injecting 0.5ml pristane ip. Values were derived from cells pooled from groups of 3 silica treated mice and 3 untreated mice at each post-pristane interval and compared with cells from a group of 3 normal mice given neither silica nor pristane

c The absolute number of B lineage cells was calculated from their incidence relative to all nucleated cells and the total cellularity of the bone marrow and spleen respectively

TABLE 6

B lineage cells in bone marrow and spleen of BALB/cAn mice given peritoneal and spleen cells from pristane-treated mice[a]

		Peritoneal cells				Spleen cells			
		Saline[c]		Pristane[d]		Saline[c]		Pristane[d]	
Cell phenotype	0d[b]	3d	7d	3d	7d	3d	7d	3d	7d
Cells/femur ($x10^5$)[e]									
TdT^+B220^-	0.9	1.9	1.3	2.1	1.6	1.2	2.0	1.4	2.0
TdT^+B220^+	2.0	2.3	1.3	3.8	1.6	1.0	2.2	1.2	2.8
$B220^+\mu^-$	7.3	10.8	7.8	13.2	11.2	7.8	13.0	8.1	16.0
$cu^+s\mu^-$	23.8	20.6	24.2	20.9	23.8	13.9	30.7	17.6	26.5
$s\mu^+$	17.9	19.7	23.5	22.3	21.6	12.7	27.9	16.3	23.5
Cells/spleen ($x10^6$)[e]									
$s\mu^+$	56.0	62.4	79.9	86.7	69.9	72.2	101.2	55.0	99.0
$B220^+\mu^-$	8.0	11.9	19.1	18.1	14.0	13.4	18.0	10.0	17.6
Cells in metaphase/femur ($x10^4$)[f]									
TdT^+B220^-	0.7	2.6	0.8	2.6	1.5	1.0	2.0	1.1	2.9
TdT^+B220^+	1.8	2.5	1.2	5.9	1.3	0.7	2.9	1.0	3.4
$B220^+\mu^-$	10.9	21.6	10.1	44.0	13.4	9.8	28.2	12.6	39.9
$cu^+s\mu^-$	19.0	20.6	19.4	18.8	23.8	15.1	39.9	22.9	39.7

a Values derived from a pool of three BALB/c mice (8 wk)

b Normal mice received no treatment

c Control mice received peritoneal or spleen cells from mice injected with 0.5ml of saline i.p 15h prior to transfers

d Experimental mice received peritoneal or spleen cells from mice donors treated with 0.5ml of pristane ip 15h prior to transfers

e Calculated from the incidence of cells/phenotype relative to all nucleated cells and the total cellularity of the bone marrow and spleen respectively

f Calculated from the total number of cells/femur/phenotype and the percentage of cells/phenotype in metaphase (metaphase index) 2h40min after injecting vincristine sulfate ip

TABLE 7

B lineage cells in bone marrow and spleen of pristane-injected DBA/2 mice[a]

Cell phenotype	0d[b]	2d	7d	10d	28d	82d	196d	230d
Cells/femur ($x10^5$)[c]								
TdT^+B220^-	1.0	1.4	1.3	1.4	1.7	0.8	0.5	0.7
TdT^+B220^+	1.0	1.4	2.5	3.4	1.8	0.8	1.5	1.0
$B220^+\mu^-$	7.9	5.5	4.7	5.5	2.0	1.0	3.3	5.0
$cu^+s\mu^-$	10.8	10.0	4.3	7.3	3.6	6.0	6.9	9.9
$s\mu^+$	9.4	6.0	3.6	3.0	3.1	5.0	9.2	8.3
Cells/spleen ($x10^6$)[c]								
$s\mu^+$	31.5	57.6	34.7	27.0	43.0	32.8	51.0	90.2
Cells in metaphase/femur ($x10^4$)[d]								
TdT^+B220^-	3.0	3.9	4.6	1.6	7.7	1.5	0.6	1.4
TdT^+B220^+	1.9	2.3	4.3	2.7	2.6	1.3	1.8	2.0
$B220^+\ \mu^-$	7.8	3.8	3.1	0.6	0.7	1.3	2.5	11.3
$cu^+s\mu^-$	8.5	11.4	7.5	6.1	3.8	6.0	7.9	10.8

a Values derived from a pool of three DBA/2 mice (8 wk) given 0.5ml pristane ip at 7-8 wk of age

b Normal mice (0d) received no treatment

c Calculated from the incidence of cells/phenotype relative to all nucleated cells and the total cellularity of the bone marrow and spleen respectively

d Calculated from the total number of cells/femur/phenotype and the percentage of cells/phenotype in metaphase (metaphase index) 2h40min after injecting vincristine sulfate ip

Figure 1. B lineage cell populations in the bone marrow of BALB/cAn mice after ip pristane injection: total number of cells per femur (above); number of cells per femur in metaphase 2h40min after vincristine injection (below). Values are expressed as a percentage of normal values in groups of non-injected mice aged 9-10 wk (2-28d values) and 25-30 wk (83-230d values).

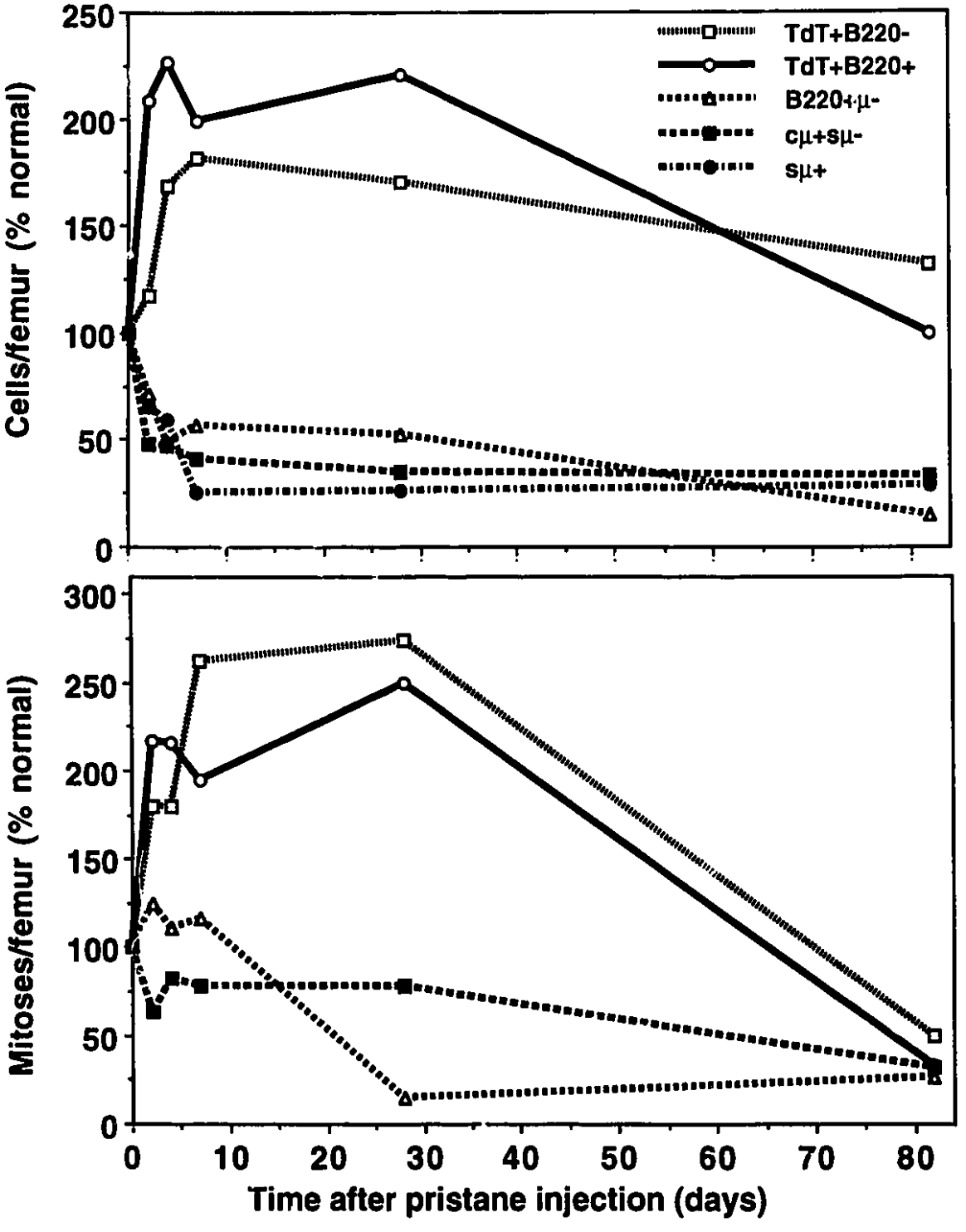

Cells/femur (% normal)
250
200
150
100
50
0
TdT+B220-
TdT+B220+
B220+μ-
cμ+sμ-
sμ+
Mitoses/femur (% normal)
300
250
200
150
100
50
0
0
10
20
30
40
50
60
70
80
Time after pristane injection (days)

Figure 2. Number of B lineage cells in the bone marrow of BALB/cAn mice given either ip pristane injection alone (above) or ip pristane injection followed by indomethacin treatment (below).

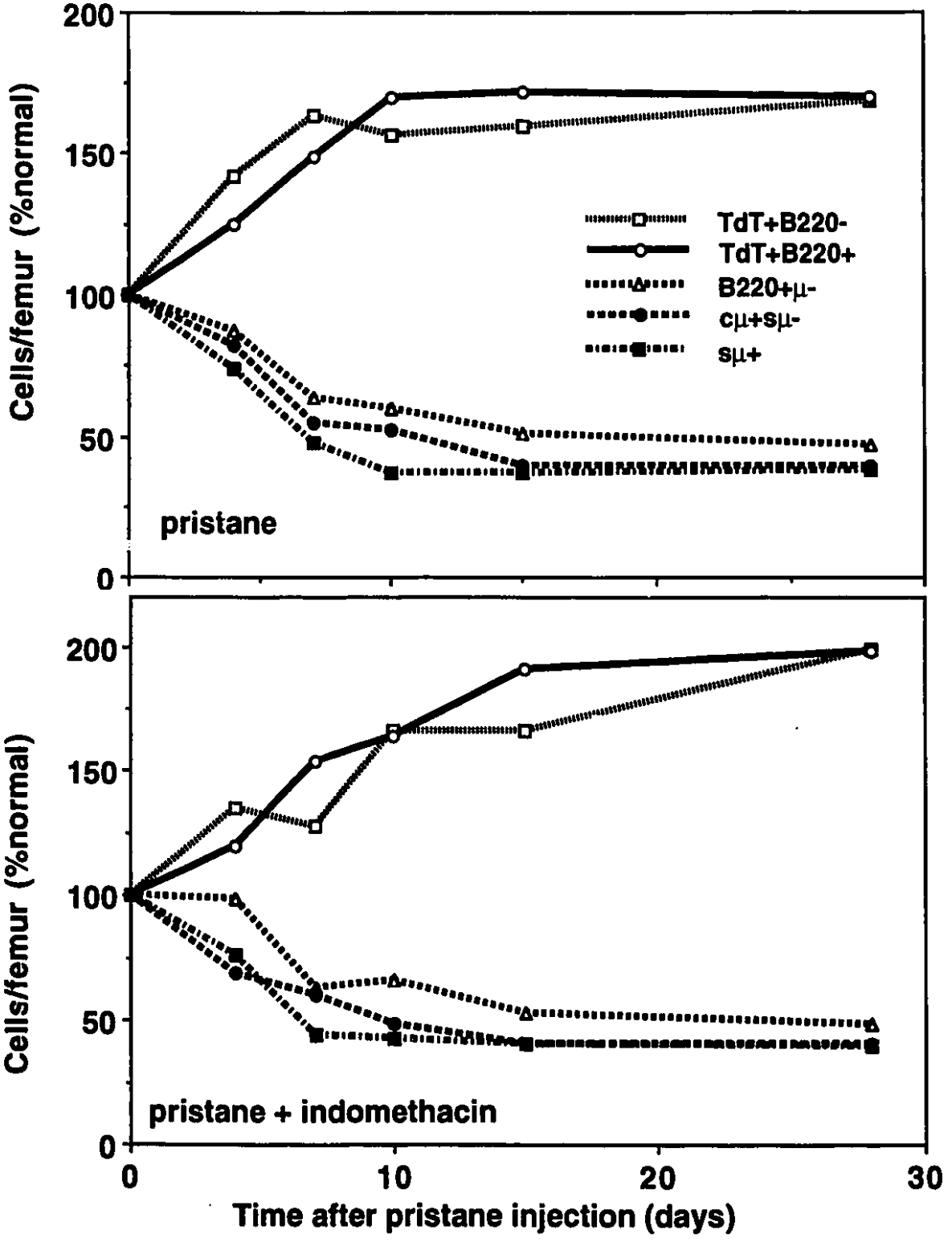

200
150
100
50
0
Cells/femur (%normal)
TdT+B220-
TdT+B220+
B220+μ-
cμ+sμ-
sμ+
pristane
pristane + indomethacin
0
10
20
30
Time after pristane injection (days)

Figure 3. Number of B lineage cells in post-vincristine metaphase arrest in the bone-marrow of BALB/cAn mice given either ip pristane injection alone (above) or ip pristane injection followed by indomethacin treatment (below).

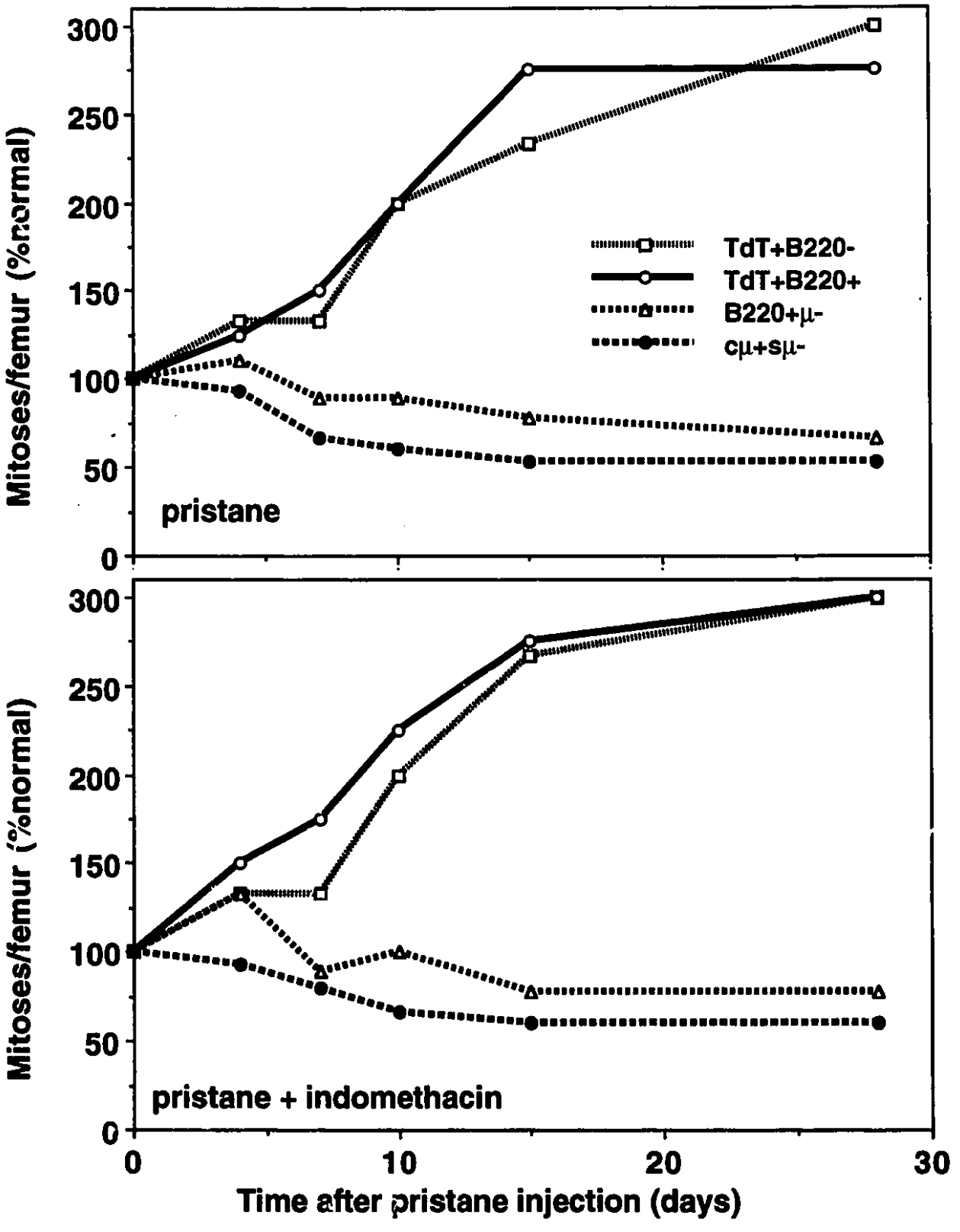

Mitoses/femur (%normal)
300
250
200
150
100
50
0
TdT+B220-
TdT+B220+
B220+μ-
cμ+sμ-
pristane
Mitoses/femur (%normal)
pristane + indomethacin
0
10
20
30
Time after pristane injection (days)

Figure 4. B lineage cell populations in the bone marrow of BALB/cAn mice that received peritoneal cells and spleen cells from donors injected with pristane 15h prior to the transfers and were analyzed 3 days and 7 days after transfers: total number of cells per femur (above); number of cells per femur in metaphase 2h40min after vincristine injection (below). Values are expressed as a percentage of control values (controls received cells from saline-injected donors).

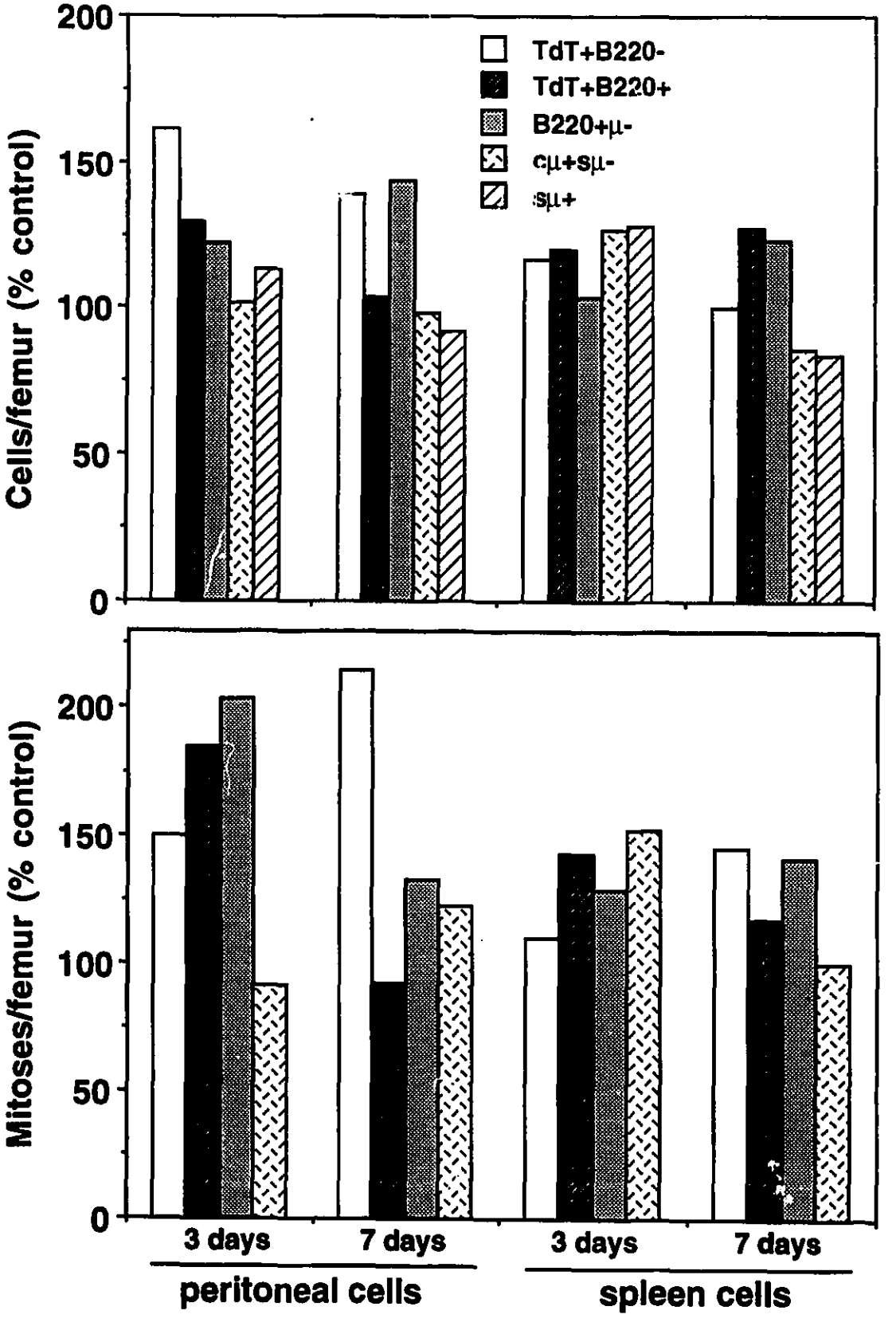
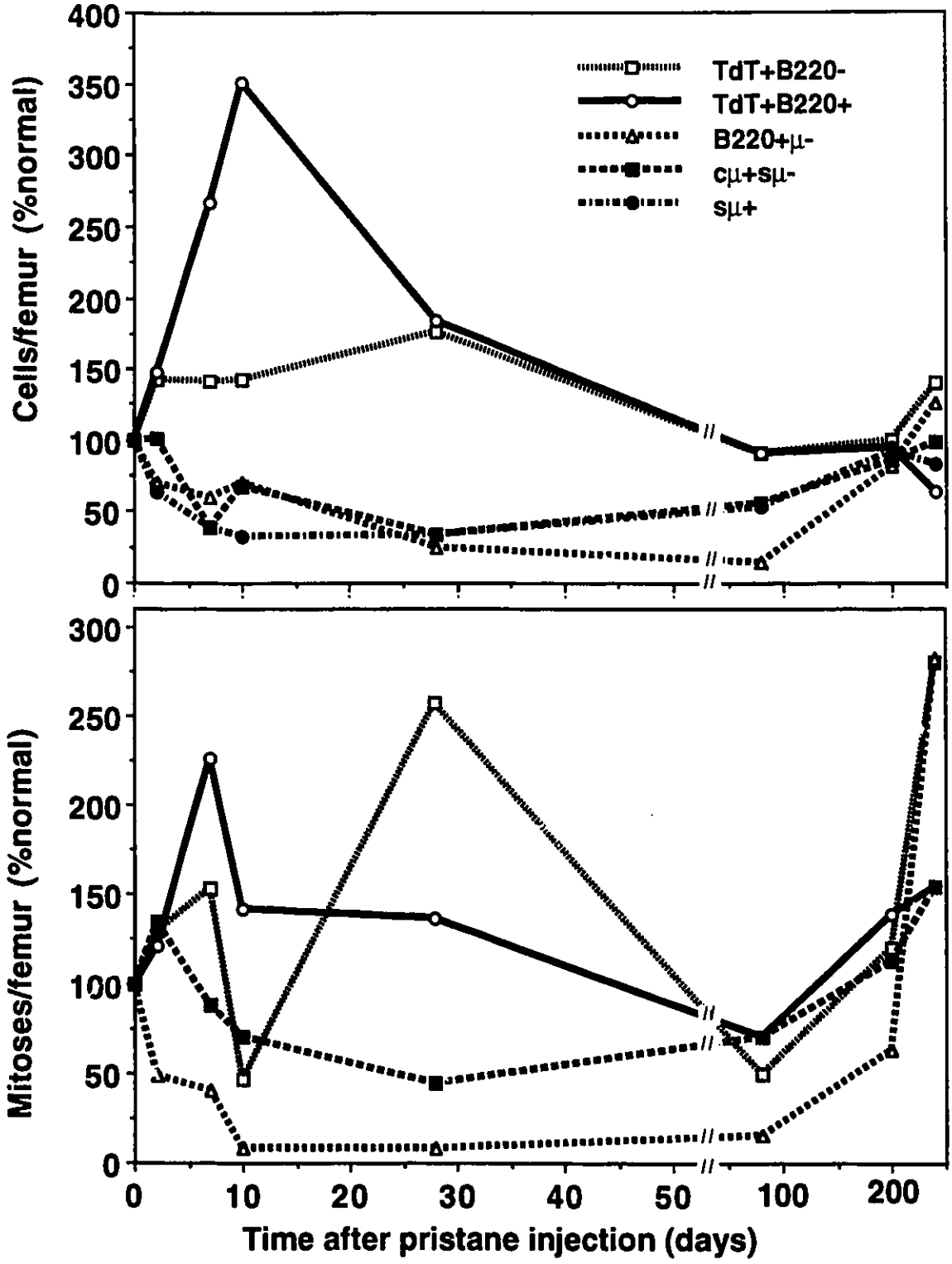

200
150
100
50
0
Cells/femur (% control)
TdT+B220-
TdT+B220+
B220+μ-
cμ+sμ-
sμ+
200
150
100
50
0
Mitoses/femur (% control)
3 days
7 days
3 days
7 days
peritoneal cells
spleen cells

Figure 5. B lineage cell populations in the bone marrow of DBA/2 mice after ip pristane injection: total number of cells per femur (above); number of cells per femur in metaphase 2h40min after vincristine injection (below). Values are expressed as a percentage of normal values in groups of non-injected mice aged 7 wk.


Cells/femur (%normal)
400
350
300
250
200
150
100
50
0
TdT+B220-
TdT+B220+
B220+μ-
cμ+sμ-
sμ+
Mitoses/femur (%normal)
300
250
200
150
100
50
0
0
10
20
30
40
50
100
200
Time after pristane injection (days)

REFERENCES

1. Tsujimoto, Y., J. Gorham, J. Cossman, E. Jaffee, and C.M. Croce. 1985. The t(14;18) chromosome translocations involved in B-cell neoplasms result from mistakes in VDJ joining. *Science* 229:1390.

2. Klein, G. and E. Klein. 1985. *Myc*/Ig juxtaposition by chromosomal translocations: some new insights, puzzles and paradoxes. *Immunol.Today* 6:208.

3. Greaves, M. F. 1986. Differentiation-linked leukemogenesis in lymphocytes. *Science* 234:697.

4. Langdon, W.Y., A.W. Harris, S. Cory, and J. M. Adams. 1986. The *c-myc* oncogene perturbs B lymphocyte development in Eμ-*myc* transgenic mice. *Cell.* 47:11.

5. Bakhsi, A., J.J. Wright, W. Graninger, M. Seto, J. Owens, J. Cossman, J.P. Jensen, P. Goldman, and S.J. Korsmeyer. 1987. Mechanism of the t(14;18) chromosomal translocation: Structural analysis of both derivative 14 and 18 reciprocal partners. *Proc.Natl.Acad.Sci.USA* 84:2396.

6. Lenoir, G.M., and G.W. Bornkum. 1987. Burkitt's lymphoma, a human cancer model for the study of the multistep model of cancer: proposal for a new scenario. *Adv.Viral Oncol.* 7:173.

7. Otsu, M., S. Katamine, M. Uno, M. Yamaki, Y. Ono, G. Klein, M.S. Sasaki, Y. Yaoita, and T. Honjo. 1987. Molecular characterization of novel reciprocal translocation t(6;14) in an Epstein-barr virus-transformed B-cell precursor. *Mol.Cell.Biol.* 7:708.

8. Bertoli, L.F., H. Kubagawa, G.V. Borzillo, P.D. Borrows, M.T. Schreeder, A.J. Carroll, and M.D. Cooper. 1988. Bone marrow origin of a B-cell lymphoma. *Blood* 72:94

9. Greaves, M.F. 1988. Speculations on the cause of childhood acute lymphoblastic leukemia. *Leukemia* 2:120.

10. Altiok, E., G. Klein, L. Zech, M. Uno, B.E. Henriksson, S. Battat, Y. Ono, and I. Ernberg. 1989. Epstein-Barr virus-transformed pro-B cells are prone to illegitimate recombination between the switch region of the μ chain gene and other chromosomes. *Proc.Natl.Acad.Sci.USA* 86:6333.

11. Nowell, P.C. 1990. What causes lymphocytic tumors? *Cancer Invest.* 8:49.

12. Osmond, D.G., Y.H. Park, and K. Jacobsen. 1988. B cell precursors in bone marrow: in vivo proliferation, localization, stimulation by activated macrophages and implications for oncogenesis. *Curr.Top.Microbiol.Immunol.* 141:2.

13. Desiderio, S.V., G.D. Yancopoulos, M. Paskind, E. Thomas, M.A. Boss, N. Landau, F.W. Alt, and D. Baltimore. 1984. Insertion of N regions into heavy-chain genes is correlated with expression of terminal deoxytransferase in B cells. *Nature* 311:752.

14. Kunkel, T.A., K.P. Gopinathan, D.K. Dube, E.T. Snow, and L.A. Loeb. 1986. Rearrangements of DNA mediated by terminal transferase. *Proc.Natl.Acad.Sci.USA* 83:1867.

15. Coffman, R.L. 1983. Surface antigen expression and immunoglobulin gene rearrangement during mouse pre-B cell development. *Immunol.Rev.* 69:5.

16. Kincade, P.W. 1987. Experimental models for understanding B lymphocyte formation. *Adv.Immunol.* 41:181.

17. Opstelten, D., and D.G. Osmond. 1983. Pre-B cells in mouse bone marrow: immunofluorescence stathmokinetic studies of the proliferation of cytoplasmic μ-chain-bearing cells in normal mice. *J.Immunol.* 131:2635.

18. Osmond, D.G., and J.J.T. Owen. 1984. Pre-B cells in bone marrow: size distribution profile, proliferative capacity and peanut agglutinin binding of cytoplasmic μ chain-bearing cell populations in normal and regenerating bone marrow. *Immunol.* 51:333.

19. Osmond, D.G., F. Melchers, and C.J. Paige. 1984. Pre-B cells in mouse bone marrow: in vitro maturation of peanut agglutinin binding B lymphocyte precursors separated from bone marrow by fluorescence-activated cell sorting. *J.Immunol.* 133:86.

20. Park, Y.H., and D.G. Osmond. 1987. Phenotype and proliferation of early B lymphocyte precursor cells in mouse bone marrow. *J.Exp.Med.* 165:444.

21. Park, Y.H., and D.G. Osmond. 1989. Dynamics of early B lymphocyte precursor cells in mouse bone marrow: proliferation of cells containing terminal deoxynucleotidyl transferase. *Eur.J.Immunol.* 19:2139.

22. Park, Y.H., and D.G. Osmond. 1989. Post irradiation regeneration of early B lymphocyte precursor cells in mouse bone marrow. *Immunol.* 66:343.

23 Osmond, D.G. 1986. Population dynamics of bone marrow B lymphocytes. *Immunol.Rev.* 93:103.

24. Osmond, D.G. 1990. B cell development in the bone marrow. *Sem.Immunol.* 2:173.

25. Deenen, G.J., I. Van Balen, and D. Opstelten. 1990. In rat B lymphocyte genesis sixty percent is lost from the bone marrow at the transition of nondividing pre-B cell to sIgM$^+$ B lymphocyte, the stage of Ig light chain gene expression. *Eur.J.Immunol.* 20:557.

26. Alt, F.W., T.K. Blackwell, and G.D. Yancopoulos. 1987. Development of the primary antibody repertoire. *Science* 238:1079.

27. Fulop, G.M., and D.G. Osmond. 1983. Regulation of bone marrow lymphocyte production. III. Increased production of B and non-B lymphocytes after administering systemic antigens. *Cell.Immunol.* 75:80.

28. Opstelten, D., and D.G. Osmond. 1985. Regulation of pre-B cell proliferation in bone marrow: immunofluorescence stathmokinetic studies of cytoplasmic μ chain-bearing cells in anti-IgM-treated mice, hematologically deficient mutant mice and mice given sheep red blood cells. *Eur.J.Immunol.* 15:599.

29. Allison, A.C., J.S. Harington, and M. Birbeck. 1966. An examination of the cytotoxic effects of silica on macrophages. *J.Exp.Med.* 124:141.

30. Levy, M.H. , and E.F. Wheelock. 1975. Effects of intravenous silica on immune and non-immune functions of the murine host. *J.Immunol.* 115:41.

31. Lotzova, E., and G. Cudkowicz. 1974. Abrogation of resistance to bone marrow grafts by silica particles. Prevention of the silica

effect by the macrophage stabilizer poly-2-vinylpyridine N-oxide. *J.Immunol.* 113:798.

32. Wirth, J.J., M.H. Levy, and E.F. Wheelock. 1976. Use of silica to identify host mechanisms involved in suppression of established friend virus leukemia. *J.Immunol.* 117:2124.

33. Fulop, G.M., and D.G. Osmond. 1983. Regulation of bone marrow lymphocyte production. IV. Cells mediating the stimulation of marrow lymphocyte production by sheep red blood cells: studies in anti-IgM-suppressed mice, athymic mice, and silica-treated mice. *Cell.Immunol.* 75:91.

34. Pietrangeli, C.E., and D.G. Osmond. 1985. Regulation of B-lymphocyte production in the bone marrow: role of macrophages and the spleen in mediating responses to exogenous agents. *Cell.Immunol.* 94:147.

35. Pietrangeli, C.E., and D.G. Osmond. 1987. Regulation of B-lymphocyte production in the bone marrow: mediation of the effects of exogenous stimulants by adoptively transferred spleen cells. *Cell.Immunol.* 107:348.

36. Fulop, G.M., Pietrangeli, C.E., and D.G. Osmond. 1986. Regulation of bone marrow lymphocyte production: IV. Altered kinetic steady state of lymphocyte production after chronic changes in exogenous stimuli. *Exp.Hematol.* 14:27.

37. Potter, M., and C. Boyce. 1962. Induction of plasma cell neoplasms in strain BALB/c mice with mineral oil and mineral oil adjuvants. *Nature* 193:1086.

38. Potter, M., and J.S. Wax. 1983. Peritoneal plasmacytomagenesis in mice: comparison of different pristane dose regimens. *J.Nat.Cancer Inst.* 71:391.

39. Potter, M., J.W. Hartley, J.S. Wax, and D. Gallahan. 1984. Effect of MuLV-related genes on plamacytomagenesis in BALB/c mice. *J.Exp.Med.* 160:435.

40. Potter, M., J.F. Mushinski, E.B. Mushinski, S. Brust, J.S. Wax, F. Wiener, M. Babonits, U.R. Rapp, and H.C. Morse, III. 1987. Avian *v-myc* replaces chromosomal translocation in murine plasmacytomagenesis. *Science* 235:787.

41. Silva, S., M. Babonits, F. Wiener, and G. Klein. 1989. Plasmacytoma induction in radiochimeras. *Int.J.Cancer* 44:337.

42. Potter, M., J.S. Wax, A.O. Anderson, and R.P. Nordan. 1985. Inhibition of plasmacytoma development in BALB/c mice by indomethacin. *J.Exp.Med.* 161:996.

43. Ohno, S., S. Migita, F. Wiener, M. Babonits, G. Klein, J.F. Mushinski, and M. Potter. 1984. Chromosomal translocations activating *myc* sequences and transduction of *v-abl* are critical events in the rapid induction of plasmacytomas by pristane and Abelson virus. *J.Exp.Med.* 159:1762.

44. Shen, T.Y. 1979. Prostaglandin synthetase inhibitors. In Antiinflammatory Drugs. J.R. Vane and S.H. Ferreira, editors. Springer-Verlag New York, Inc., New York. 305.

45. Potter, M. 1984. Genetics of susceptibility to plasmacytoma development in BALB/c mice. *Cancer Surv.* 3:247.

46. Parhar, R.S., and P.K. Lala. 1987. Amelioration of B16F10 melanoma lung metastasis in mice by a combination therapy with indomethacin and interleukin 2. *J.Exp.Med.* 165:14.

47. Kincade, P.W., G. Lee, T. Watanabe, L. Sun, and M.P. Scheid. 1981. Antigens displayed on murine B lymphocyte precursors. *J.Immunol.* 127:2262.

48. Nordan, R.P., P.G. Pumphrey, and S. Rudikoff. 1987. Purification and NH_2-terminal sequence of a plasmacytoma growth factor derived from the murine macrophage cell line P388D1. *J.Immunol.* 139:813.

49. Wyler, D.J. 1982. Malaria: host-pathogen biology. *Rev.Infect. Diseases* 4:785.

50. Wyler, D.J., J.J. Oppenheim, and L.C. Koontz. 1979. Influence of malaria infection on the elaboration of soluble mediators by adherent mononuclear cells *Infect.Immunol.* 24:151.

51. Osmond, D.G., Priddle, S. and S.A. Rico-Vargas. 1990. Proliferation of B cell precursors in bone marrow of pristane-conditioned and, malaria-infected mice: implications for B cell oncogenesis. *Curr.Topics Microbiol.Immunol.* 166:149.

52. Furuta, Y., E.R. Hall, S. Sanduja, T. Barkley, Jr., and L. Milas. 1988. Prostaglandin production by murine tumors as a predictor for therapeutic response to indomethacin. *Cancer Res.* 48:3002.

53. Lala, P.K., R.S. Parhar, and P. Singh. 1986. Indomethacin therapy abrogates the prostaglandin-mediated suppression of natural killer activity in tumor bearing mice and prevents tumor metastasis. *Cell.Immunol.* 99:108.

54. Freund, Y.R., and P.B. Blair. 1982. Depression of natural killer activity and mitogen responsiveness in mice treated with pristane. *J.Immunol.* 129:2826.

CHAPTER 4

Proliferation of B cell precursors in bone marrow of malaria-infected mice: implications for B cell neoplasias

SUMMARY

Malaria infection is associated in humans with the development of B cell neoplasias, but the mechanism of oncogenesis and the stage in B cell genesis at which the process may be initiated is controversial. We have found that mice infected with malaria (*Plasmodium yoelii*) show marked changes in the population size and proliferation rates of early precursor B cells in the bone marrow. Double immunofluorescence labeling, epifluorescence microscopy and mitotic arrest techniques have been used to quantitate the production of phenotypically defined populations of precursor B cells in the bone marrow. Malaria-infected mice showed a prolonged hepatosplenomegally, accompanied by a sustained proliferative stimulation of TdT^+ pro-B cell populations in the bone marrow. The effect, which was not restricted to one particular mouse strain, could be further prolonged by reinfection with malaria, and transferred to normal mice by injecting whole spleen cells and adherent spleen cell fractions from malaria-infected mice to non-infected recipients. However, stimulated pro-B cell activity produced no commensurate increase in the output of more differentiated precursor B cell populations. The results indicate that an increased production of early B precursors is followed by an increased rate of cell loss. These findings suggest that a stimulation of early precursor B cells at the stage of V_H Ig gene rearrangement in malaria-infected mice is associated with an increased genesis of aberrant cells with errors of

Ig gene rearrangements. The work accords with the hypothesis that conditions of chronically elevated macrophage activation can play a role in predisposing to dysregulation of primary B cell genesis and the initiation of neoplasia.

INTRODUCTION

The administration of various foreign agents to mice produces a wave of increased proliferation of precursor B cells in the bone marrow, an effect thought to be mediated by macrophages via the release of cytokines (1-5). Sustained macrophage stimulation results in a prolonged elevated kinetic state of B cell progenitor production (6). These findings raise the possibility of an etiological link between conditions of chronic macrophage stimulation and oncogenesis in the B cell lineage (7).

Bone marrow is the site of continuous primary genesis of B lymphocytes from B cell precursors that undergo a series of proliferative and differentiation stages (8-10). These stages have been defined by the use of three cellular markers: 1) TdT (11-12); 2) B220 glycoprotein, a B lineage-associated molecule (13); and 3) surface or cytoplasmic μ chains of IgM molecules (sμ and cμ, respectively). Double immunofluorescence labeling techniques have been used to define the various cell populations (9,14). By these criteria, six phenotypically distinct cell populations have been recognized, extending from the stage of V_H gene rearrangements to

the expression of surface IgM (Fig. 1). Three populations comprise putative early pro-B cells, before μ chain expression: early pro-B cells (TdT^+B220^-), intermediate pro-B cells (TdT^+B220^+) and late pro-B cells (TdT^-B220^+). B cell precursors then reach the pre-B cell ($B220^+c\mu^+s\mu^-$) stage when they begin to synthesize μ heavy chains and to rearrange the gene segments for the light chains of IgM. The pairing of heavy and light chains and the surface expression of IgM defines the final B lymphocyte stage ($B220^+s\mu^+$). The use of mitotic arrest techniques *in vivo* in combination with double immunofluorescence labeling has indicated that pro-B cells and large pre-B cells undergo a series of mitoses, while small pre-B cells mature into B lymphocytes without further division (9,10-14). The proliferative dynamics show a progressive increase in frequency, average cell size and production rate up to the pre-B cell stage. However, there is also a considerable cell loss of approximately 75%, mainly at the pre-B cell stage. This may largely represent the elimination of cells that have undergone defective V_H gene rearrangements (10,14-16).

Burkitt's lymphoma, a B cell neoplasia associated with endemic human malaria, is characterized by a chromosomal translocation juxtaposing the *c-myc* oncogene into the IgH chain locus (17,18). Such genetic errors may result from mistakes in Ig V_H gene rearrangements (19). In this respect, TdT^+ pro-B cells in the bone marrow could be susceptible populations. Any factor increasing their recombinational activity would increase the risk of genetic errors.

Malaria infection promotes a marked stimulation and proliferation of macrophages (20-22). The stimulated macrophages show an intense production of a variety of cytokines, including TNF and IL-1 (23-27). The phase of macrophage activation persists for a long period. We hypothesise that this effect could elevate the proliferative activity of B precursor cells rearranging Ig genes, predisposing to the initiation of B cell tumors which would become frankly neoplastic following further genetic accidents or other predisposing factors, including EBV infection in the case of human endemic malaria and Burkitt's lymphoma (18). The present work aims to determine whether malaria in mice has an effect on central B cell genesis that could be related to the initiation of B cell neoplasias.

MATERIALS and METHODS

Animals. C3H/HeJ mice were purchased from Jackson Laboratories (Bar Harbor, ME), BALB/c mice were purchased from Charles River Canada (St. Constant, PQ). Mice were maintained in microisolators, and used at 6-8 wk.

Parasites. Plasmodium yoelii non-lethal (17XNL) strain and *Plasmodium yoelii* lethal (17XL) strain were kindly provided by Dr. Hannah Shear (Department of Parasitology, New York University). Mice were injected ip with $2.5x10^5$-$10x10^7$ 17XNL-infected RBC in 0.2ml sterile saline. The lethal parasite strain dose was $0.5x10^6$

infected RBC. The degree of parasitemia (% infected erythrocytes) was followed using May-Grunwald-Giemsa stained blood smears.

Mineral Oil. Pristane (2,6,10,14-tetramethylpentadecane, Aldrich Chemical Co., Milwaukee, WI) was injected ip in a dose of 0.5ml (28).

Spleen cell transfers. Donor mice were injected with 10^6 non-lethal malaria-infected RBC. Control animals received an ip injection of 10^6 non-infected RBC. The animals were killed 2 wk after clearance of parasitemia. Under sterile conditions, the spleens were removed, pooled, and the total nucleated cells counted as described in Chapter 2. Each naive recipient mouse received an ip injection of $136x10^6$ spleen nucleated cells in 0.5ml sterile saline. The recipients of spleen cells from malaria-infected donors did not develop parasitemias.

Spleen adherent cell transfers. Donor mice were injected with $0.5x10^6$ non-lethal malaria-infected RBC. Control animals received an i.p. injection of $0.5x10^6$ RBC. When infected donor mice showed an 8% parasitemia, spleen cells were recovered and resuspended in MEM/NCS. Spleen cells were incubated in plastic Petri dishes (10^8 cells/5ml medium/dish) for 2h at 37°C in 5.6% $C0_2$. Non-adherent cells were removed with warm medium. Adherent cells were incubated with cold PBS for an additional 20min on ice. With vigorous pipetting, the adherent cells were washed off with cold PBS. The cells were washed, counted and their viability determined (95%). Recipients received an ip injection of $6x10^6$ nucleated cells/mouse in

0.2ml sterile saline. Controls received $4x10^6$ adherent cells. Experimental recipient mice did not develop parasitemia.

The population sizes and production rates of the B cell precursors were determined using double immunofluorescence labeling and mitotic arrest techniques, as described in Chapter 2.

RESULTS

Precursor B cells in malaria-infected mice.

Groups of 7wk old C3H/HeJ mice were given a single intraperitoneal injection of 10^7 mouse RBC infected with *P.yoelii* non-lethal strain (17XNL) and were examined at intervals up to 22 wk. Infected mice initially showed an increasing parasitemia (Fig.1), 30% RBC being infected at 2-3wk, but no parasites were detectable in the blood from 4wk onward. Spleen weight increased progressively during the parasitemia, reaching a tenfold enlargement at day 10. Thereafter, spleen weight declined but remained twice normal, even in the absence of blood parasites (Fig. 1). Liver weight increased to approximately twice normal levels from 10 days onwards (Fig. 1). The total cellularity of the spleen increased fivefold by 10d and remained approximately twice normal throughout the experimental period (Table 1). These findings demonstrate a chronic response to the single infection with malaria parasites.

The bone marrow was markedly perturbed during the malaria infection. Total bone marrow cellularity showed biphasic changes;

initially being normal or somewhat depressed at day 10 and then becoming elevated at 4-22 wk (Table 1). Early and intermediate pro-B cells (TdT^+B220^-, TdT^+B220^+) increased in numbers at day 7 after malaria infection, reaching a maximum at 4 wk (Table 2, Fig. 2). By 22 wk, early pro-B cells had returned to normal numbers, but intermediate pro-B cells remained elevated. Late pro-B cells ($B220^+\mu^-$) were initially depressed to half normal values at day 7, but they then increased to twice normal numbers by 4 wk. Later progenitors, pre-B cells ($c\mu^+s\mu^-$) and B lymphocytes ($s\mu^+$), were moderately increased at 4 wk. From day 65 onwards the number of pre-B cells and B lymphocytes remained near normal.

The proliferative activity of precursor B cells was elevated for prolonged periods of time. The metaphase index (Table 2, Fig. 2) for each phenotypic population was given by the number of cells per femur which had accumulated in metaphase during a standard time interval of 2h40min after injecting vincristine sulfate to stop cells in mitosis. This index of cell production for early progenitors increased to a maximum of more than 2.5 times normal for TdT^+ pro-B cells, and 3.5 normal for $B220^+\mu^-$ cells. Pre-B cell proliferation increased to 1.7 normal values. The populations returned towards normal values by the end of the experimental period.

Spleen $s\mu^+$ B cells (Table 2) increased in numbers to more than twice normal values, returning to normal by the end of the study. $B220^+\mu^-$ cells in the spleen increased initially during the

parasitemia, reaching 6-10 fold normal values at 10d-4 wk, and were still elevated at 22 wk (Table 2).

To examine the early events after a less severe malaria infection, further groups of C3H/HeJ mice were injected with $2.5x10^5$ mouse RBC infected with *P.yoelii* non-lethal strain (17XNL), and analyzed during the first 7 days after infection. Total bone marrow cellularity fluctuated, while spleen cell recovery increased progressively to nearly fivefold normal values at day 7 (Table 3). B cell precursors tended to oscillate (Table 4, Fig. 3). However, in general, the intermediate and late pro-B cell populations increased in cell numbers and proliferative activity while the remaining populations were at either normal or subnormal levels. B lymphocytes in the spleen increased to 300% normal numbers from 3 days to 7 days (Table 4).

Precursor B cells in BALB/c mice infected with malaria.

We tested the effects of malaria infection on BALB/c mice to determine whether the previous effects were restricted to the C3H/HeJ strain. Groups of BALB/c mice were injected ip with $0.5x10^6$ mouse RBC infected with *P. yoelii* 17XNL and examined after 2d,5d,8d,13d,36d, and 58d (Figs. 4 and 5). Parasitemia developed rapidly, reaching a maximum of 33% at 2-3 wk. The parasites were completely cleared from the blood by 4 wk. The liver and spleen weights increased to maximum values of 1.5X normal at day 8 and 11X normal at day 13, respectively, remaining elevated thereafter

(Fig. 4). Spleen cell recovery mirrored the changes in spleen weight, reaching a maximum of 9.5X normal values (Table 5).

All stages of B cell precursors in the bone marrow were perturbed (Table 6, Fig. 5). The TdT^+ pro-B cells increased in population size, and showed prolonged increases in proliferative activity throughout the experimental period. Late pro-B and pre-B cells were increased in numbers and proliferative activity initially but thereafter were depressed to near half values. By 58 days these populations had normalized.

Splenic $s\mu^+$ B lymphocytes increased in numbers to 4.6X normal at 13d, remaining 2X thereafter, while $B220^+\mu^-$ cells increased from three to more than tenfold (Table 6).

The BALB/c results generally accord with the previous study of non lethal malaria infection in C3H/HeJ mice, although there were some differences; a) the population size of TdT^+ cells in BALB/c mice was smaller but their proliferative rate was greater than in C3H/HeJ mice, b) later B cell progenitors were more reduced in numbers in BALB/c mice.

Precursor B cells in malaria re-infected BALB/c mice.

To analyze the possible implications of re-infection with malaria, a phenomenon that occurs repeatedly in humans in malaria endemic areas, groups of BALB/c mice were injected ip with $0.5x10^6$ mouse RBC infected with *P. yoelii* 17XNL. Eight wk later, four wk after clearance of parasitemia, groups of mice were re-injected with the

same dose of infected RBC and examined at further intervals of 2d,4d,9d,13d,18d,25d,33d, and 68d. The whole study thus extended over a period of 126d (Figs. 4 and 5).

After re-infection, the mice showed only a minor parasitemia (3%) which was rapidly cleared during the first week. The liver and spleen weights did not increase further, but remained approximately at the elevated levels persisting from the first infection (Fig. 4). Spleen cell recovery increased slightly after re-infection, while the bone marrow showed only a small increase of 113% at day 9 after re-infection, remaining near-normal thereafter (Table 6). The response of early precursor B cells to malaria re-infection generally resembled the response to the primary infection. TdT^+ pro-B cells, though fluctuating, again increased in population size and proliferative activity for at least 4 wk (Table 6, Figure 5). In contrast with the primary infection, however, the later precursor B cell populations were not depressed. B lymphocytes in the spleen remained at the high levels persisting from the primary infection and were still 2X normal values at the end of the study (Table 6).

Precursor B cells in mice infected with lethal malaria.

B cell precursors were examined at 2,4 and 7 days after initiating a lethal malaria infection by giving groups of BALB/c mice a single ip injection of $0.5x10^6$ mouse RBC infected with *P.yoelii* lethal strain (17XL), and examined after 2d,4d and 7d.

The mice showed a faster increase in parasitemia levels than in non-lethal malaria infection, 17% RBC being infected by day 7. Liver and spleen weights increased progressively. The spleen cellularity increased sharply at days 5-7, while the bone marrow cellularity decreased to 72% normal values by day 7 (not shown).

All B lineage populations in the bone marrow were altered (Table7, Fig. 6). Early pro-B cells were initially stimulated, showing a fivefold increase in their proliferative activity and some increase in population size by day 4, but the stimulation was no longer evident by 7 days. Intermediate pro-B cells were also initially stimulated to approximately double their proliferative rate at 2 days. In contrast, the number and proliferative activity of the late precursor B cell stages fell to approximately one third of initial values by 7 days.

Splenic B lymphocytes and $B220^{+}Ig^{-}$ cells increased threefold in numbers at 7 days (Table 7).

Spleen cell transfers from previously malaria-infected donors into normal mice.

To analyze the role of spleen cells including macrophages in the stimulatory effect of malaria on early B cell precursors, washed spleen cells from BALB/c mice previously infected with non-lethal malaria and 2 wk after clearance of the parasitemia, were transferred into groups of naive BALB/c recipients which were examined at intervals from 2 to 9 days thereafter.

No parasitemia developed in recipients. Spleen cellularity increased in both control and experimental recipient mice, (126% and 139% normal, respectively). The bone marrow cellularity increased initially (130%) in both control and experimental animals. Subsequently, the cellularity in control animals was near normal, while in experimentals it was slightly elevated (113%) (not shown).

Spleen cell transfers from previously malaria-infected donors produced an increase in population size and proliferative activity of TdT^+ early and intermediate pro-B cells, maximum at day 3, compared to controls (Table 8, Fig. 7) but produced little effect on later B cell progenitors.

Splenic B lymphocytes (Table 8) increased slightly in experimental mice compared to controls.

Spleen adherent cell transfers from malaria-infected mice into normal mice.

To test the role of macrophages in the positive stimulatory effect on early B cell precursors in malaria-infected mice, groups of naive BALB/c mice were given adherent spleen cells from malaria-infected donors and were examined at 36h,60h and 84h post-transfer.

Recipients of adherent cells from malaria-infected donors did not develop parasitemias. Spleen cellularity showed minor changes in control and experimental mice. Bone marrow cellularity was slightly increased in both control and experimental mice (110% and 120% normal values, respectively) (not shown).

Spleen adherent cell transfers from malaria-infected donors produced a stimulation of pro-B cells at 36h after transfers, while the pre-B cells and B lymphocytes did not differ from controls (Table 9, Fig. 8). At 60h the proliferative activity of early pro-B cells was still elevated, but later stages were at control levels or lower. By 84h the various B cell populations were near control numbers. Splenic B lymphocytes did not change from controls (Table 9).

Malaria re-infection and pristane-injection.

Pristane injection in normal mice produces an increase in population size and proliferation rates of early pro-B cells and intermediate pro-B cells, while the later progenitors are depressed (29). In order to examine whether the TdT^+ populations of malaria-infected mice remain responsive to other stimuli we re-infected BALB/c mice with *P.yoelii* 17XNL, as previously described, and then injected them ip with 0.5ml of pristane 1 wk after clearance of parasites. The proliferative activity of early pro-B cells was stimulated while the later progenitors were reduced in both population sizes and proliferative activities (Table 10, Fig. 9). Spleen B cells and $B220^+\mu^-$ cells increased twofold or more in number (Table 10).

DISCUSSION

The present work reveals that malaria infection has marked effects on the central genesis of B cells in mouse bone marrow. Previous

work in this laboratory has suggested that precursor B cells, including those undergoing V_H chain Ig gene rearrangements, can be stimulated by a variety of external environmental agents, an effect which is mediated by macrophages located in the spleen (1-6). Malaria infection produces an intense recruitment and expansion of macrophage populations which phagocytose parasitized RBC and show evidence of sustained stimulation, including greatly augmented production of a variety of cytokines (23-27,30). Substantial hepatosplenomegally persists for long periods of time. The protracted changes now seen in the genesis of B cell precursors in the bone marrow reflect the prolonged nature of the disease processes. The possibility arises that the increase in the proliferation rates now observed in early B progenitors, an event probably mediated by stimulated macrophages, could initiate transformational changes in populations prone to genetic errors.

Using a combination of double immunofluorescence and stathmokinetic techniques we have previously analyzed the frequency, population size and proliferative properties of each precursor B cell stage under normal *in vivo* conditions (9,14,15). In the present study, we find that the earliest defined stages of B cell genesis, pro-B cells expressing TdT, show a sustained increase in number of cells entering mitosis per unit time during non-lethal malaria infection of C3H/HeJ mice. On the other hand, the later B cell progenitors and B cells do not show a corresponding overexpansion in the bone marrow. The marked increase in B lymphocytes in the

spleen during malaria infection would appear to be due to a polyclonal activation by malaria parasites, rather than an increased output of newly-formed B cells from the bone marrow.

The bone marrow response observed in BALB/c mice infected with non-lethal malaria resembles that of C3H/HeJ infected mice, expansion of TdT^+ populations without a corresponding increase of later stages. Thus, the effects of *P.yoelii* infection on bone marrow B cell precursors are not restricted to a particular mouse strain.

In areas where human malaria is endemic, re-infection is a common feature. Our results in mice suggest that, under these circumstances, the previously described effects of malaria on B cell populations in the bone marrow can be made to persist for prolonged periods of time.

P.yoelii exists in two strains, lethal and non-lethal. We find that lethal malaria infection produces only a transient stimulation of TdT cells, followed by a generalized depression of pre-B and B cells in the bone marrow. The observed effect may reflect the severity of the clinical course and the stress of lethal infection in mice which die soon after infection.

The transfer of washed spleen cells and of adherent spleen cell fractions from malaria infected mice to normal mice produces a wave of proliferative stimulation of TdT^+ pro-B cells in the recipients. This provides strong, though not conclusive, evidence that the bone marrow effects of malaria infection, like those following SRBC

administration (2-6) are mediated by the activity of splenic macrophages.

The findings suggest that for prolonged periods of time during non-lethal malaria infection an elevated cell production at the pro-B cell stage is followed by an increased degree of cell loss, permitting only a fraction of cells to continue their differentiation. The cell loss may be viewed as a selection stage, most commonly eliminating cells that have undergone defective or possibly autoreactive rearrangements of Ig genes (15). However, in malaria-infected mice, many viable B lymphocytes are still produced. This raises the question of whether these cells may carry genetic errors that, although non lethal to the cell, could be augmented by further transformational changes occurring during hypermutational events in secondary B cell activation in the spleen leading ultimately to malignancy.

Two phenotypically distinct B cell neoplasias, Burkitt's lymphoma associated with human malaria and murine plasmacytoma, both show a similar characteristic *c-myc*/Ig loci juxtaposition (18,33,34). In murine plasmacytoma, as in malaria, there is a sustained macrophage stimulation. Pristane-injected mice develop granulomatous tissue rich in macrophages that have ingested oil droplets (28,35) a process leading to the release of a variety cytokines (36-38). In pristane-injected mice, we have observed an effect in the bone marrow that closely resembles the pattern in malaria-infected mice, an increase in population size and proliferative rate of early and intermediate pro-B cells with a discrepancy in numbers in the more differentiated

stages. The sustained increase in the level of proliferative activity of the TdT^+ pro-B cell precursors in each case could predispose to genetic errors including chromosomal translocations which could represent the initiating events leading to neoplasia. Together with previous work, the present results suggest that pathologically elevated levels of macrophage activation may have a central effect on B cell genesis in the bone marrow and thus play a role in predisposing to B cell neoplasias.

TABLE 1

Total nucleated cells in bone marrow and spleen of C3H/HeJ[a] mice during infection with *Plasmodium yoelii* 17XNL

	Number of cells at post-infection intervals of:						
Total nucleated cells	0d[b]	3d	7d	10d	29d	65d	158d
Cells/femur ($x10^5$)	137	142	154	124	259	177	191
Cells/spleen ($x10^6$)	150	192	617	775	616	326	255

a Each value was derived from cells pooled from groups of 3 mice given 10^6-10^7 *Plasmodium yoelii* 17XNL-infected RBC at 7 wk of age

b Normal mice (0d) received no infected RBC

TABLE 2

Number of B lineage cells in bone marrow of C3H/HeJ[a] mice during infection with *Plasmodium yoelii* 17XNL

Cell phenotype	0d[b]	Number of cells at post-infection intervals of: 3d	7d	10d	29d	65d	158d
Cells/femur ($x10^5$)[c]							
TdT^+B220^-	1.1	1.2	1.6	2.6	2.6	1.5	0.8
TdT^+B220^+	1.2	1.2	1.5	2.3	4.4	2.9	2.5
$B220^+\mu^-$	6.0	7.1	3.1	7.2	14.0	9.0	7.8
$cu^+s\mu^-$	15.0	16.8	17.2	12.5	19.9	19.8	11.5
$s\mu^+$	11.0	12.5	18.0	11.7	22.0	12.6	8.6
Cells/spleen ($x10^6$)[c]							
$s\mu^+$	75.0	94.7	223.9	117.8	217.0	147.0	94.4
$B220^+\mu^-$	6.8	7.7	17.8	40.0	64.8	11.0	17.9
Cells in metaphase/femur ($x10^4$)[d]							
TdT^+	4.8	6.0	4.7	12.7	10.5	8.8	5.2
$B220^+\mu^-$	6.0	13.5	7.1	9.6	21.8	11.9	6.4
$cu^+s\mu^-$	15.5	23.4	31.0	26.3	29.2	30.0	25.8

a Each value was derived from cells pooled from groups of 3 mice given 10^6-10^7 *Plasmodium yoelii* 17XNL-infected RBC at 7 wk of age

b Normal mice (0d) received no infected RBC

c Incidence relative to all nucleated cells of the bone marrow and spleen

d Calculated from the total number of cells/femur/phenotype and the percentage of cells/phenotype in metaphase (metaphase index) 2h40min after injecting vincristine sulfate ip

TABLE 3

Total nucleated cells in bone marrow and spleen of C3H/HeJ[a] mice during infection with *Plasmodium yoelii* 17XNL

	Number of cells at post-infection intervals of:						
Total nucleated cells	0d[b]	1d	2d	3d	5d	6d	7d
Cells/femur ($x10^5$)	137.0	120.0	164.2	181.0	120.0	115.0	153.0
Cells/spleen ($x10^6$)	65.0	71.6	88.4	93.8	126.6	189.6	302.0

[a] Each value was derived from cells pooled from groups of 3 mice given $2.5x10^5$ *Plasmodium yoelii* 17XNL-infected RBC at 6 wk of age

[b] Normal mice (0d) received no infected RBC

TABLE 4

B lineage cells in bone marrow of C3H/HeJ[a] mice during infection with *Plasmodium yoelii* 17XNL

	Number of cells at post-infection intervals of:						
Cell phenotype	0d[b]	1d	2d	3d	5d	6d	7d
Cells/femur ($x10^5$)[c]							
TdT^+B220^-	1.1	0.7	1.5	1.5	0.6	0.6	1.3
TdT^+B220^+	1.2	3.7	4.7	2.8	3.0	2.7	3.9
$B220^+\mu^-$	6.0	7.2	13.2	12.7	9.6	11.5	9.7
$cu^+s\mu^-$	15.0	8.8	20.0	11.0	21.0	11.0	13.8
$s\mu^+$	11.0	7.2	12.9	12.7	7.2	18.4	12.2
Cells/spleen ($x10^6$)[c]							
$s\mu^+$	32.5	35.0	42.0	39.0	52.0	61.0	91.0
Cells in metaphase/femur ($x10^4$)[d]							
TdT^+	4.6	5.3	3.7	3.2	5.8	2.9	5.8
TdT^+B220^-	2.2	0.9	0.9	1.0	0.8	0.7	0.5
TdT^+B220^+	2.4	4.5	2.8	2.2	4.9	2.2	5.4
$B220^+\mu^-$	6.0	7.2	11.0	17.0	5.8	4.6	7.8
$cu^+s\mu^-$	15.5	10.5	22.0	5.5	8.6	5.5	9.1

a Each value was derived from cells pooled from groups of 3 mice given $2.5x10^5$ *Plasmodium yoelii* 17XNL-infected RBC at 6wk of age

b Normal mice (0d) received no infected RBC

c Calculated from the incidence of cells of each phenotype relative to all nucleated cells and the total cellularity of the bone marrow and spleen

d Calculated from the total number of cells/femur/phenotype and the percentage of cells/phenotype in metaphase (metaphase index) 2h40min after injecting vincristine sulfate ip

TABLE 5

Total nucleated cells in bone marrow and spleen of BALB/c[a] mice during infection and re-infection with *Plasmodium yoelii* 17XNL

Total nucleated cells	Number of cells after infection							Number of cells after re-infection							
	0d[b]	2d	5d	8d	13d	36d	58d	0d[b]	2d	4d	9d	13d	18d	25d	33d	68d
Cells/femur ($x10^5$)	182	198	159	169	180	194	201	182	220	205	227	206	170	209	217	190
Cells/spleen ($x10^6$)	137	136	364	400	1300	351	346	162	390	430	380	443	390	352	390	365

a Each value was derived from cells pooled from groups of 3 mice given $0.5x10^6$ *Plasmodium yoelii* 17XNL-infected RBC at 6 wk of age; the mice were re-infected 2wk after clearance of parasitemia with $0.5x10^6$ PY17XNL-infected RBC

b Normal mice (0d) received no infected RBC

TABLE 6

B lineage cell populations in bone marrow of BALB/c[a] mice during infection and re-infection with *Plasmodium yoelii* 17XNL

	Number of cells after infection							Number of cells after re-infection							
Cell phenotype	0d[b]	2d	5d	8d	13d	36d	58d	0d[b]	2d	4d	9d	13d	18d	25d	33d	68d
Cells/femur ($x10^5$)[c]																
TdT^+B220^-	1.0	0.8	1.1	1.7	1.1	1.8	1.3	1.0	1.0	0.8	0.8	0.4	1.2	0.9	1.7	1.6
TdT^+B220^+	1.9	2.4	2.4	2.0	1.1	2.4	2.7	1.9	3.9	2.2	4.3	2.6	2.8	4.3	2.8	2.1
$B220^+\mu^-$	8.0	13.8	4.9	6.0	5.4	5.8	6.0	7.3	7.7	7.2	8.0	8.2	6.8	11.6	8.7	6.5
$c\mu^+s\mu^-$	19.0	29.0	17.9	9.2	9.2	17.5	19.0	23.7	20.0	20.5	30.0	22.7	17.9	24.7	19.5	19.0
$s\mu^+$	16.0	19.8	16.9	10.1	21.6	17.5	16.0	17.8	24.0	19.3	28.0	21.6	17.0	22.2	23.0	17.7
Cells/spleen ($x10^6$)[c]																
$s\mu^+$	61.7	57.2	109.2	104.0	286.2	106.0	138.0	68.0	148.4	193.7	148.4	184.6	136.6	140.6	140.0	156.0
$B220^+\mu^-$	7.4	8.6	25.5	20.8	96.3	19.7	30.0	9.7	11.7	36.2	26.2	30.1	19.9	19.0	28.8	21.5
Cells in metaphase/femur ($x10^4$)[d]																
TdT^+B220^-	0.8	2.2	1.9	3.9	2.1	3.4	1.0	0.8	0.9	0.4	0.5	0.7	1.6	1.0	2.0	2.6
TdT^+B220^+	2.1	2.4	2.7	3.4	2.2	4.8	2.5	1.5	4.4	1.4	3.9	2.6	3.6	4.7	2.8	2.3
$B220^+\mu^-$	11.2	15.2	6.0	4.2	6.5	11.6	7.8	11.0	8.3	9.4	8.3	15.6	8.4	10.4	13.4	5.9
$c\mu^+s\mu^-$	17.1	29.0	21.5	6.4	6.0	8.8	17.1	20.0	16.6	16.4	18.0	11.4	17.9	22.2	17.4	15.8

a Each value was derived from cells pooled from groups of 3 mice given $0.5x10^6$ *Plasmodium yoelii* 17XNL-infected RBC at 6 wk of age; the mice were re-infected two wk after clearance of parasitemia with $0.5x10^6$ PY17XNL-infected RBC

b Normal mice (0d) received no infected RBC

c Calculated from the incidence of cells of each phenotype relative to all nucleated cells and the total cellularity of the bone marrow and spleen

d Calculated from the total number of cells of each phenotype and the percentage of cells of each phenotype in metaphase (metaphase index) 2h40min after injecting vincristine sulfate ip

TABLE 7

B lineage cells in bone marrow of BALB/c[a] mice during infection with *Plasmodium yoelii* 17XL

Cell phenotype	Cells at post-infection intervals of: 0d[b]	2d	4d	7d
Cells/femur(x10^5)[c]				
TdT^+B220^-	1.0	1.1	1.4	0.9
TdT^+B220^+	1.9	2.0	1.0	1.6
$B220^+\mu^-$	8.0	6.8	8.5	5.0
$cu^+s\mu^-$	20.0	17.1	11.8	6.6
$s\mu^+$	16.4	12.0	10.0	5.2
Cells/spleen (x10^6)[c]				
$s\mu^+$	61.7	54.0	71.0	169.0
$B220^+\mu^-$	7.4	5.0	8.5	23.0
Cells in metaphase/femur(x10^4)[d]				
TdT^+B220^-	0.8	3.0	4.2	1.3
TdT^+B220^+	2.1	4.0	1.4	1.0
$B220^+\mu^-$	11.2	6.8	9.8	6.3
$cu^+s\mu^-$	17.1	12.5	11.0	5.3

a Each value was derived from cells pooled from groups of 3 mice given 2.5x10^5 *Plasmodium yoelii* 17XL-infected RBC at 7 wk of age

b Normal mice (0d) received no infected RBC

c Calculated from the incidence of cells of each phenotype relative to all nucleated cells and the total cellularity of the bone marrow and spleen

d Calculated from the total number of cells/femur/phenotype and the percentage of cells/phenotype in metaphase (metaphase index) 2h40min after injecting vincristine sulfate ip

TABLE 8

B cell populations in BM of BALB/c[a] mice transferred with spleen cells from malaria-infected donors

Cell phenotype	0d[c]	Control mice[b] 2d	3d	4d	5d	7d	9d	Experimental mice 2d	3d	4d	5d	7d	9d
Cells/femur ($x10^5$)[d]													
TdT^+B220^-	1.0	0.7	0.9	1.1	1.0	0.8	0.6	1.2	1.3	0.9	1.3	1.4	1.0
TdT^+B220^+	1.9	1.9	1.7	3.5	3.0	2.4	2.2	3.1	3.9	4.0	4.0	3.0	3.1
$B220^+\mu^-$	7.3	12.0	9.4	12.9	11.6	11.7	8.3	8.9	11.2	15.4	8.8	10.8	7.9
$c\mu^+s\mu^-$	23.8	34.6	44.8	49.0	33.6	28.9	28.7	39.0	46.1	41.4	45.0	32.5	29.0
$s\mu^+$	17.9	28.0	40.0	45.8	34.9	29.3	23.9	32.6	41.2	39.0	33.6	34.0	26.9
Cells/spleen ($x10^6$)[d]													
$s\mu^+$	56.0	78.0	63.0	93.4	83.0	82.8	74.8	65.6	73.2	108.7	91.0	61.9	73.1
$B220^+\mu^-$	8.0	12.0	10.8	19.0	13.8	9.5	11.4	9.3	10.8	24.8	19.0	11.5	13.9
Cells in metaphase/femur ($x10^4$)[e]													
TdT^+B220^-	0.8	1.0	0.6	1.1	0.4	1.3	0.3	2.5	1.3	0.6	1.4	1.2	2.2
TdT^+B220^+	1.5	1.5	1.2	2.4	2.0	1.6	1.4	2.3	3.2	4.0	2.4	3.3	4.4
$B220^+\mu^-$	10.9	12.0	10.3	15.5	11.6	9.4	9.1	11.6	7.8	10.8	10.0	13.0	10.4
$c\mu^+s\mu^-$	19.0	20.8	11.8	29.0	13.4	15.6	27.3	31.2	23.0	30.6	18.0	17.5	20.3

[a] Each value was derived from cells pooled from groups of 3 mice (7wk) given $136x10^6$ spleen nucleated cells from donors infected with $1x10^6$ *Plasmodium yoelii* 17XNL-infected RBC. The transfers were done 2wk after clearance of parasitemia

[b] Control mice received $130x10^6$ syngenic spleen nucleated cells from normal donors

[c] Normal mice (0d) received no cells

[d] Calculated from the incidence of cells of each phenotype relative to all nucleated cells and the total cellularity of the bone marrow and spleen

[e] Calculated from the total number of cells of each phenotype and the percentage of cells of each phenotype in metaphase (metaphase index) 2h40min after injecting vincristine sulfate ip

TABLE 9

B lineage cell populations in BM of BALB/c[a] mice transferred with spleen adherent cells from malaria-infected donors

		Control mice[b]			Experimental mice		
Cell phenotype	0h[c]	36h	60h	84h	36h	60h	84h
Cells/femur($x10^5$)[d]							
TdT^+B220^-	1.0	0.8	0.6	0.6	1.0	0.7	0.7
TdT^+B220^+	1.9	2.1	2.9	2.7	3.1	2.9	2.8
$B220^+\mu^-$	7.3	10.6	9.4	8.2	11.0	9.0	8.6
$c\mu^+s\mu^-$	23.8	21.4	35.7	28.0	24.0	24.3	31.0
$s\mu^+$	17.9	16.3	25.4	23.4	16.4	12.0	23.7
Cells/spleen($x10^6$)[d]							
$s\mu^+$	56.0	65.5	55.0	56.3	60.5	60.0	69.0
$B220^+\mu^-$	8.0	8.6	6.0	6.5	7.2	6.0	8.0
Cells in metaphase/femur($x10^4$)[e]							
TdT^+B220^-	2.3	3.1	2.6	4.2	4.5	3.7	3.2
TdT^+B220^+	0.8	0.6	0.3	0.7	0.9	0.8	0.6
$B220^+\mu^-$	10.9	7.4	9.4	9.0	13.2	9.0	11.2
$c\mu^+s\mu^-$	19.0	19.3	17.6	19.6	20.0	14.6	26.0

[a] Values derived from cells pooled from groups of 3 mice (7wk) given $6x10^6$ spleen adherent cells from malaria-infected donors.
[b] Control mice received $4x10^6$ syngenic spleen adherent cells from normal donors
[c] Normal mice (0d) received no cells
[d] Calculated from the incidence of cells/phenotype relative to all nucleated cells and the total bone marrow and spleen cellularity
[e] Calculated from the total number of cells/femur/phenotype and the percentage of cells/phenotype in metaphase (metaphase index) 2h40min after injecting vincristine sulfate ip

TABLE 10

B lineage cell populations in bone marrow of BALB/c[a] mice infected with malaria followed by an ip injection of pristane

Cell phenotype	0d[b]	2 d	4 d	8 d
Cells/femur ($x10^5$)[c]				
TdT^+B220^-	1.0	1.7	1.3	2.1
TdT^+B220^+	1.9	1.7	1.3	2.1
$B220^+\mu^-$	7.3	5.5	4.0	4.0
$c\mu^+s\mu^-$	23.8	11.2	10.0	10.6
$s\mu^+$	17.9	18.3	10.0	10.6
Cells/spleen ($x10^6$)[c]				
$s\mu^+$	68.0	94.0	153.9	115.7
$B220^+\mu^-$	8.0	18.8	19.8	23.1
Cells in metaphase/femur ($x10^4$)[d]				
TdT^+B220^-	0.8	3.7	0.8	1.3
TdT^+B220^+	1.5	4.0	1.0	1.0
$B220^+\mu^-$	10.9	8.3	2.8	3.2
$c\mu^+s\mu^-$	19.0	7.2	10.0	5.4

a Each value was derived from cells pooled from groups of 3 mice given 0.5^6 *P.yoelii* 17XNL-infected RBC at 6 wk of age; the mice were re-infected two wk after clearance of parasitemia with 0.5^6 infected RBC. After clearance of parasitemia the mice were injected ip with 0.5ml of pristane

b Normal mice (0d) received no infected RBC

c Calculated from the incidence of cells/phenotype relative to all nucleated cells and the total bone marrow and spleen cellularity

d Calculated from the number of cells/phenotype and the percentage of cells of each phenotype in metaphase (metaphase index) 2h40min after injecting vincristine sulfate ip

Figure 1. Parasitemia and weights of the spleen and liver in C3H/HeJ mice infected with 10^7 malaria infected mouse RBC.

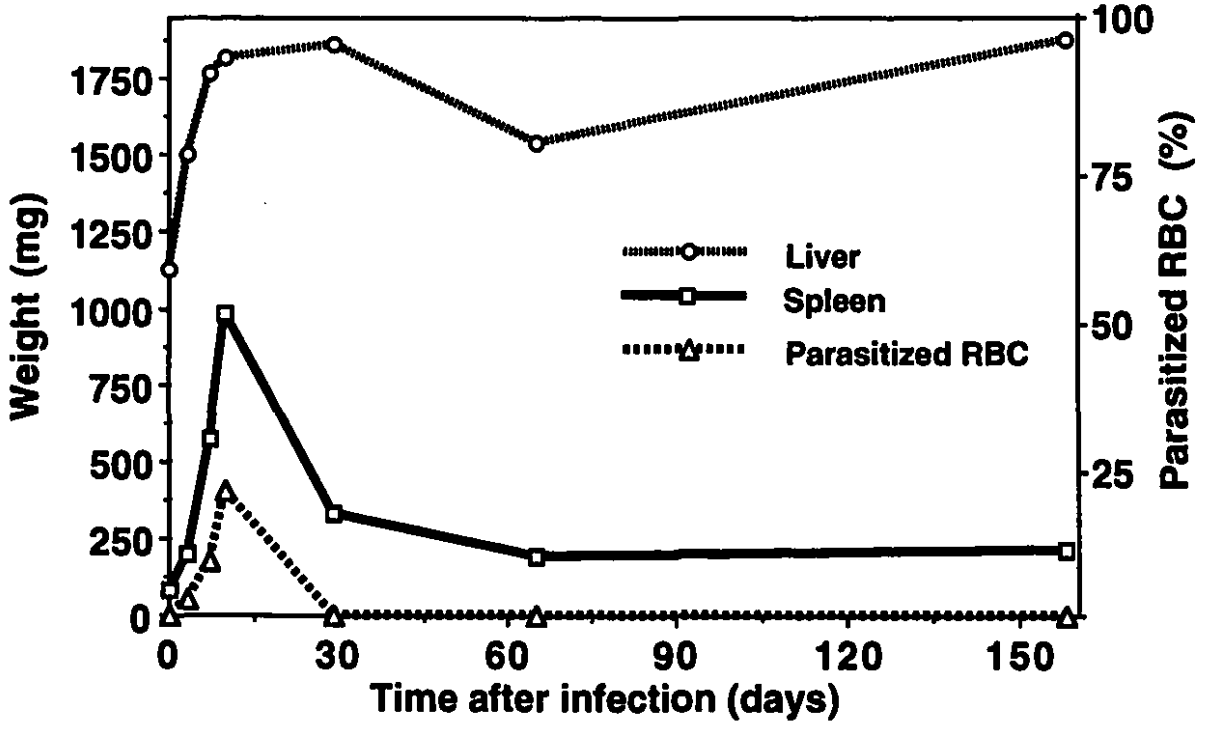

Weight (mg)
1750
1500
1250
1000
750
500
250
0
0
30
60
90
120
150
Time after infection (days)
100
75
50
25
Parasitized RBC (%)
Liver
Spleen
Parasitized RBC

Figure 2. B lineage cell populations in the bone marrow of C3H/HeJ mice injected with 10^7 malaria-infected mouse RBC. Values are expressed as a percentage of normal values of groups of non-infected mice: cells per femur (above); cells in metaphase per femur, 2h40min after vincristine injection (below).

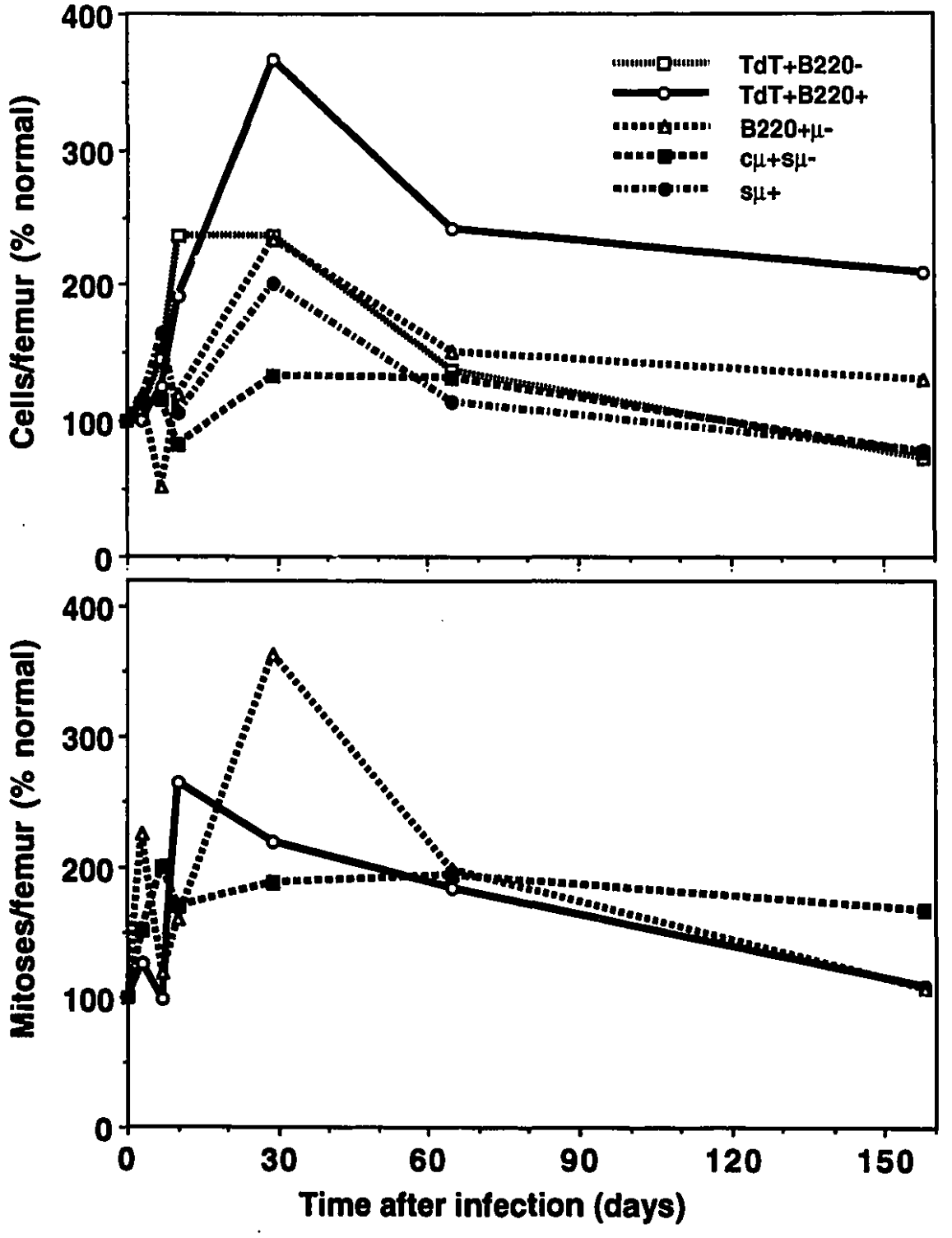

400
300
200
100
0
Cells/femur (% normal)
TdT+B220-
TdT+B220+
B220+μ-
cμ+sμ-
sμ+
400
300
200
100
0
Mitoses/femur (% normal)
0
30
60
90
120
150
Time after infection (days)

Figure 3. B lineage cell populations in the bone marrow of C3H/HeJ mice injected with $2.5x10^5$ malaria-infected mouse RBC. Values are expressed as a percentage of normal values of groups of non-infected mice: cells per femur (above); cells in metaphase per femur, 2h40min after vincristine injection (below).

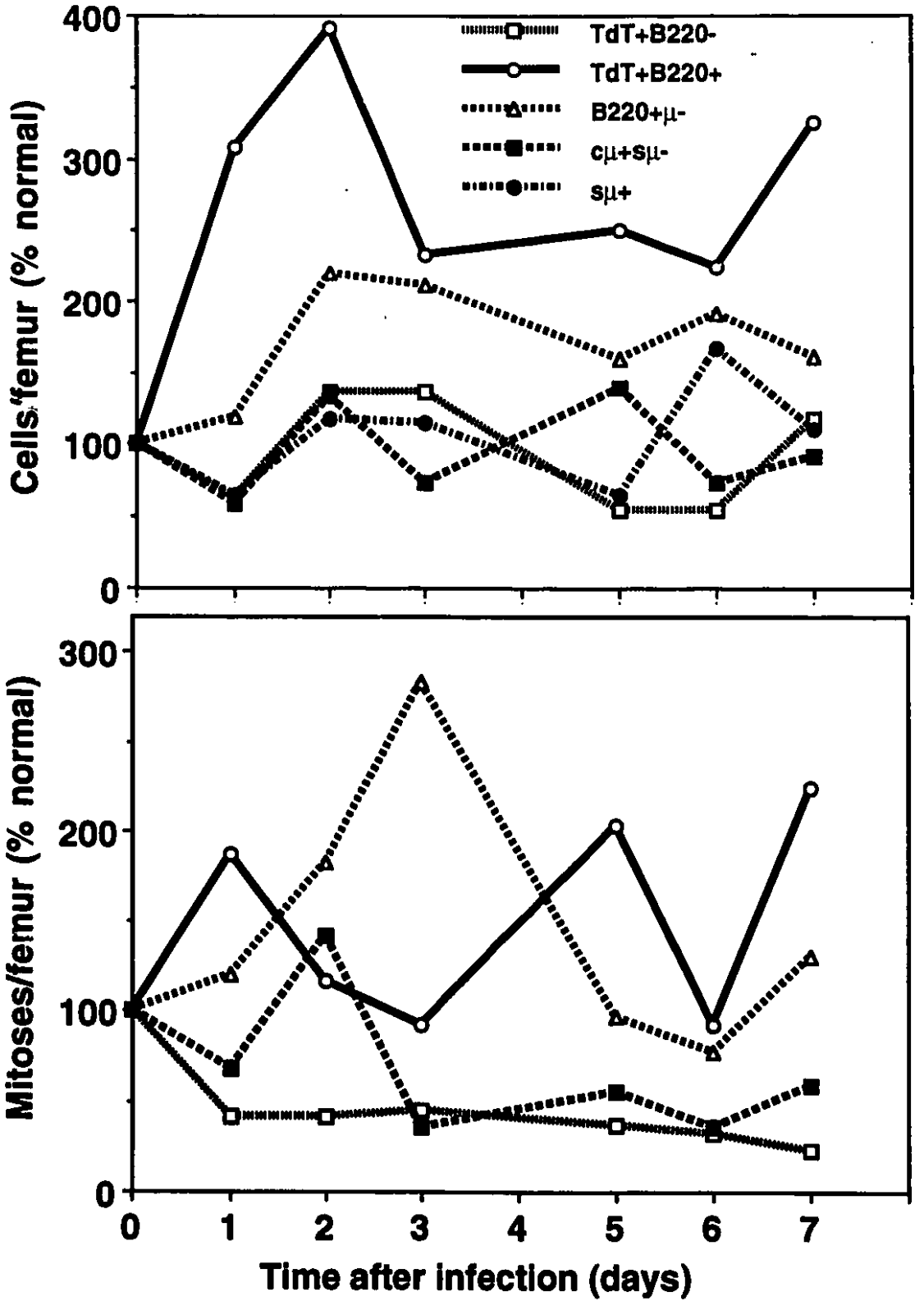

400
300
200
100
0
Cells/femur (% normal)
TdT+B220-
TdT+B220+
B220+μ-
cμ+sμ-
sμ+
300
200
100
0
Mitoses/femur (% normal)
0
1
2
3
4
5
6
7
Time after infection (days)

Figure 4. Parasitemia and weights of the spleen and liver in BALB/c mice during repeated injection of $0.5x10^6$ malaria-infected mouse RBC. Mice were infected at 0 days and again at 58 days (arrow).

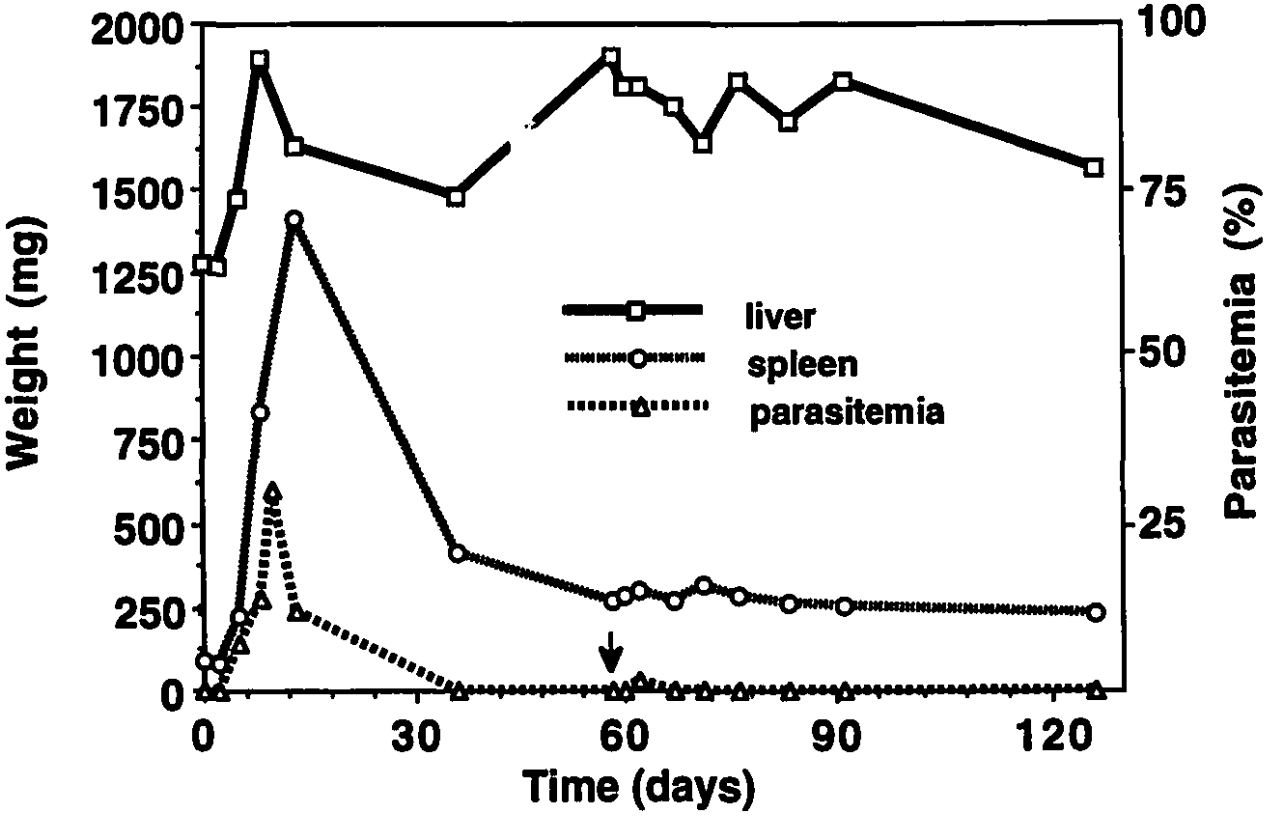

2000
1750
1500
1250
1000
750
500
250
0
Weight (mg)
100
75
50
25
Parasitemia (%)
liver
spleen
parasitemia
0
30
60
90
120
Time (days)

Figure 5. B lineage cell populations in the bone marrow of BALB/c during repeated injection of $0.5x10^6$ malaria-infected mouse RBC. The mice were infected at 0 days and again at 58 days. Values are expressed as a percentage of normal values of groups of non-infected mice: cells per femur (above); cells in metaphase per femur, 2h40min after vincristine injection (below).

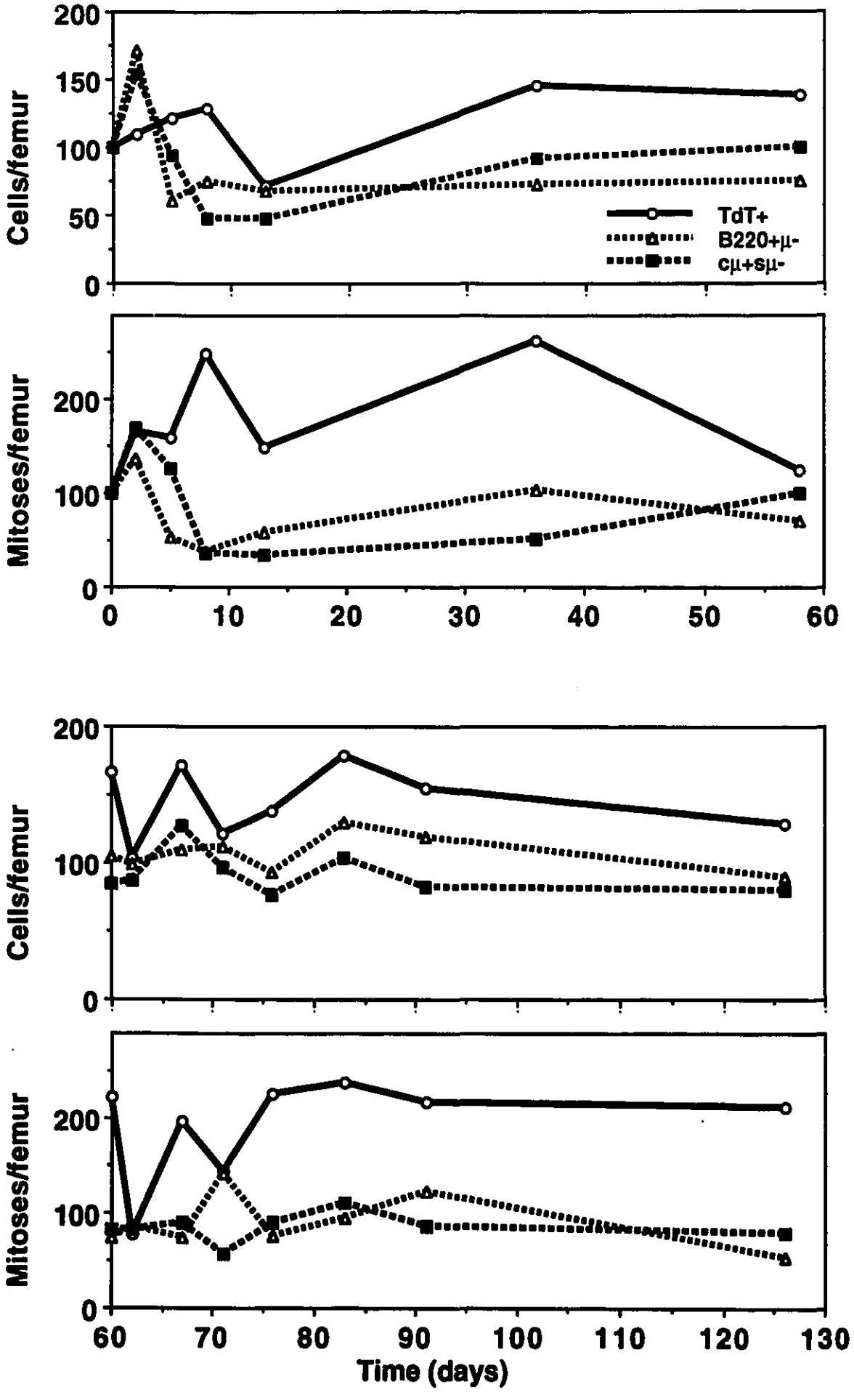

Cells/femur
200
150
100
50
0
TdT+
B220+μ-
cμ+sμ-
Mitoses/femur
200
100
0
0
10
20
30
40
50
60
Cells/femur
200
100
0
Mitoses/femur
200
100
0
60
70
80
90
100
110
120
130
Time (days)

Figure 6. B lineage cell populations in the bone marrow of BALB/c mice injected with $0.5x10^6$ mouse RBC infected with malaria parasites (lethal strain). Number of cells per femur (above); number of cells in metaphase per femur, 2h40min after vincristine injection (below).

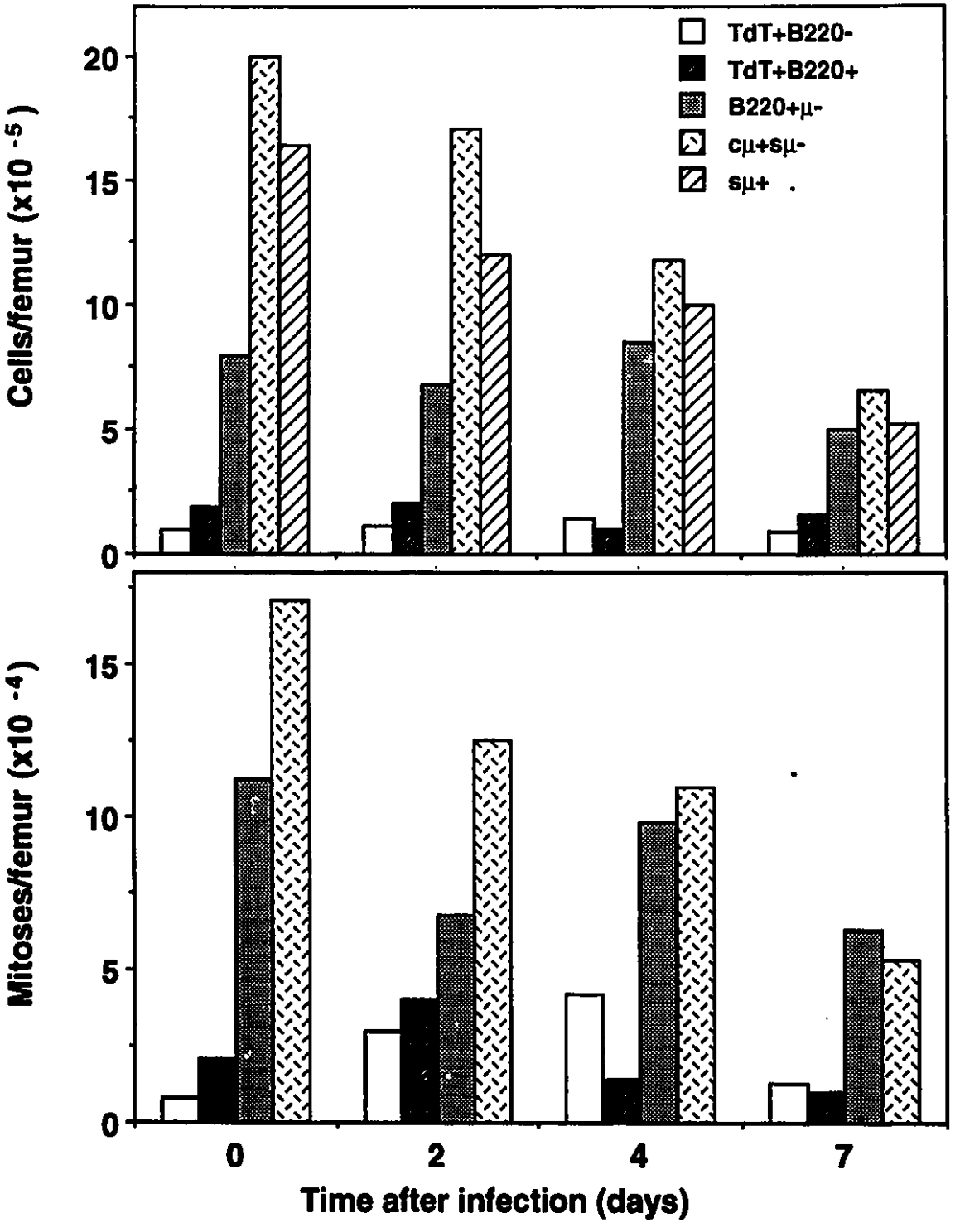

TdT+B220-
TdT+B220+
B220+μ-
cμ+sμ-
sμ+
Cells/femur (x10 -5)
20
15
10
5
0
Mitoses/femur (x10 -4)
15
10
5
0
0
2
4
7
Time after infection (days)

Figure 7. B lineage cell populations in the bone marrow of BALB/c mice that received spleen cells from previously malaria-infected donors. Values are expressed as a percentage of control values of groups that received spleen cells from normal mice: cells per femur (above); cells in metaphase per femur, 2h40min after vincristine injection (below).

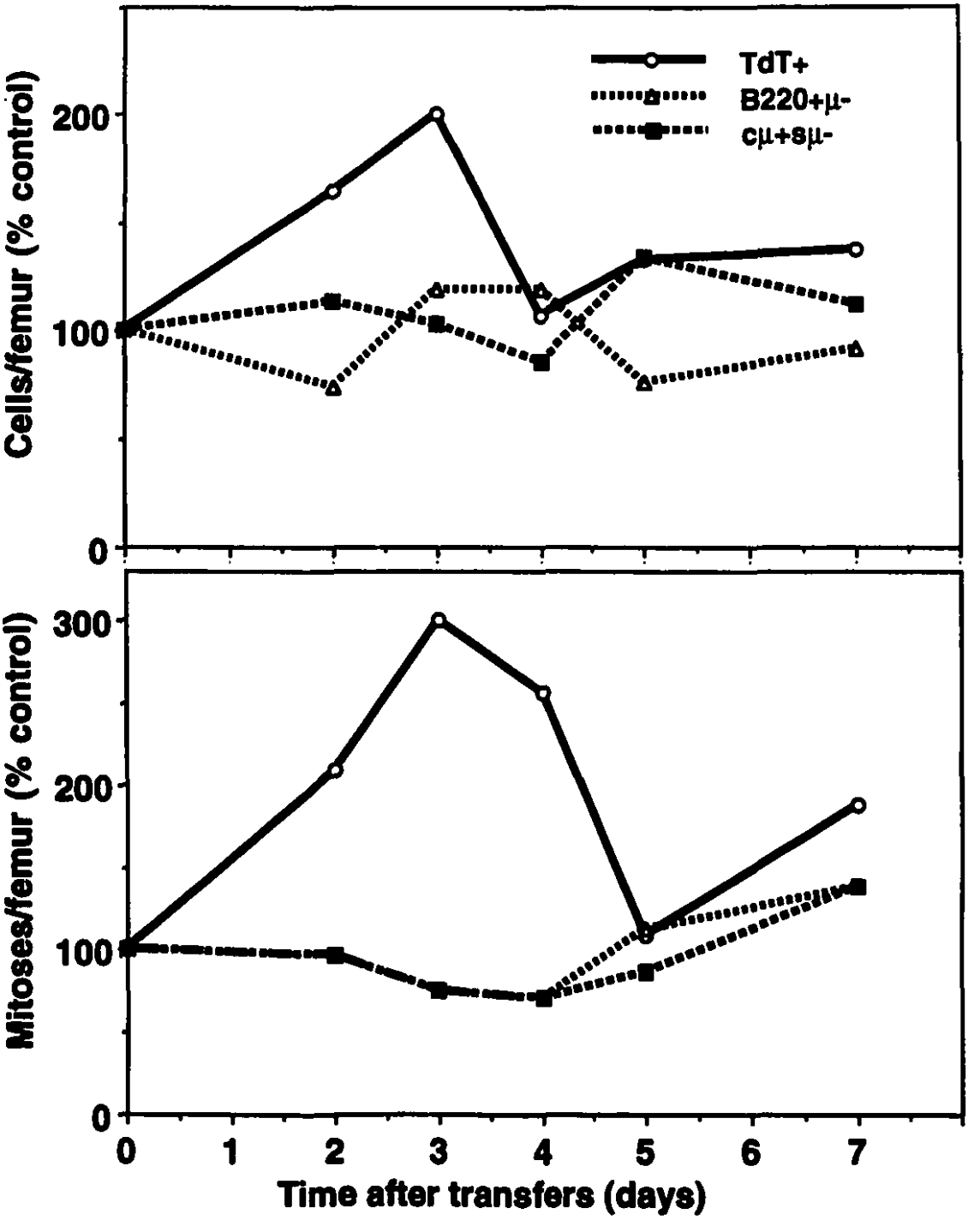

TdT+
B220+μ-
cμ+sμ-
Cells/femur (% control)
200
100
0
Mitoses/femur (% control)
300
200
100
0
0
1
2
3
4
5
6
7
Time after transfers (days)

Figure 8. B lineage cell populations in the bone marrow of BALB/c mice that received spleen adherent cells from malaria-infected donors. Values are expressed as a percentage of control values of groups that received spleen adherent cells from normal mice: cells per femur (above); cells in metaphase per femur, 2h40min after vincristine injection (below).

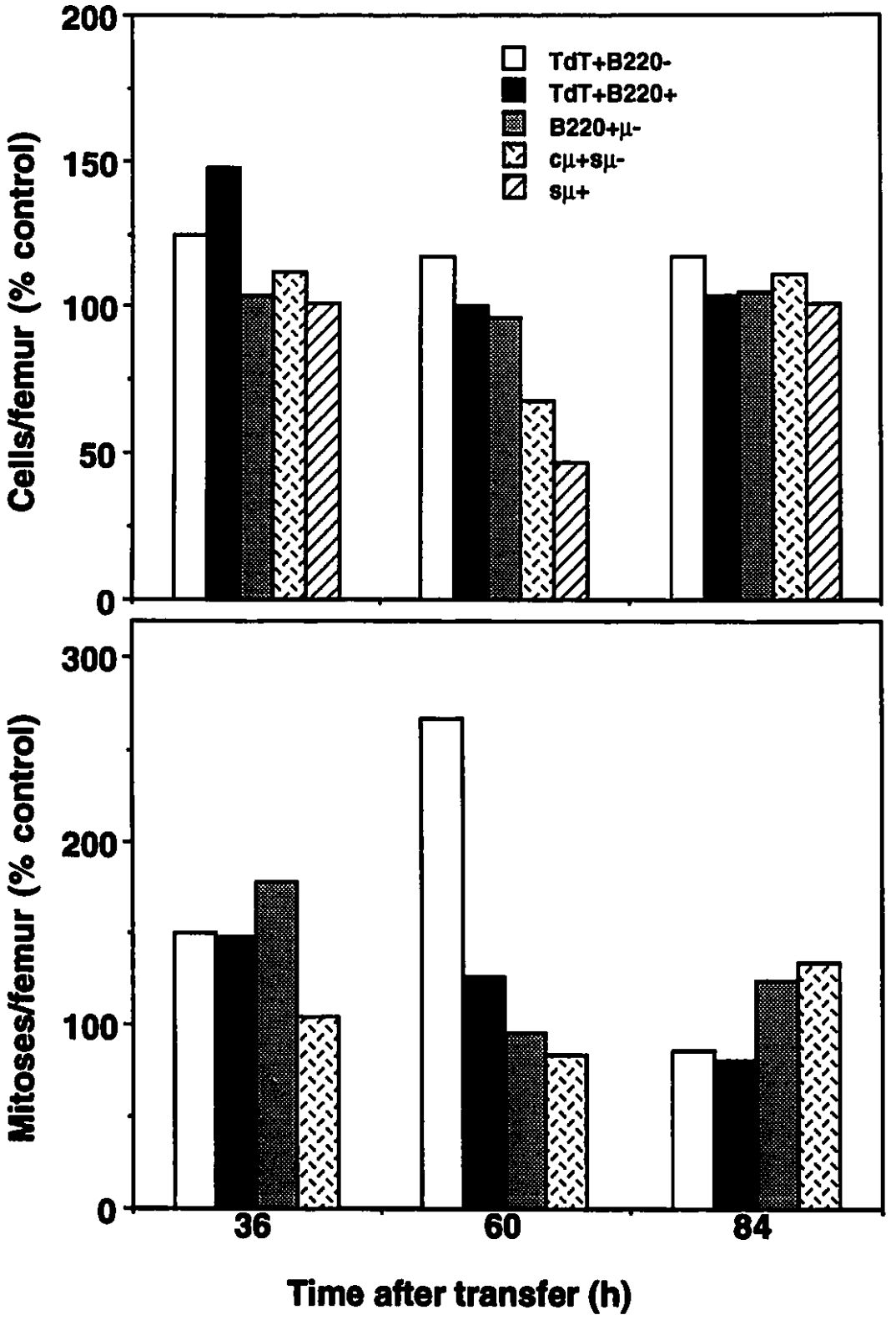
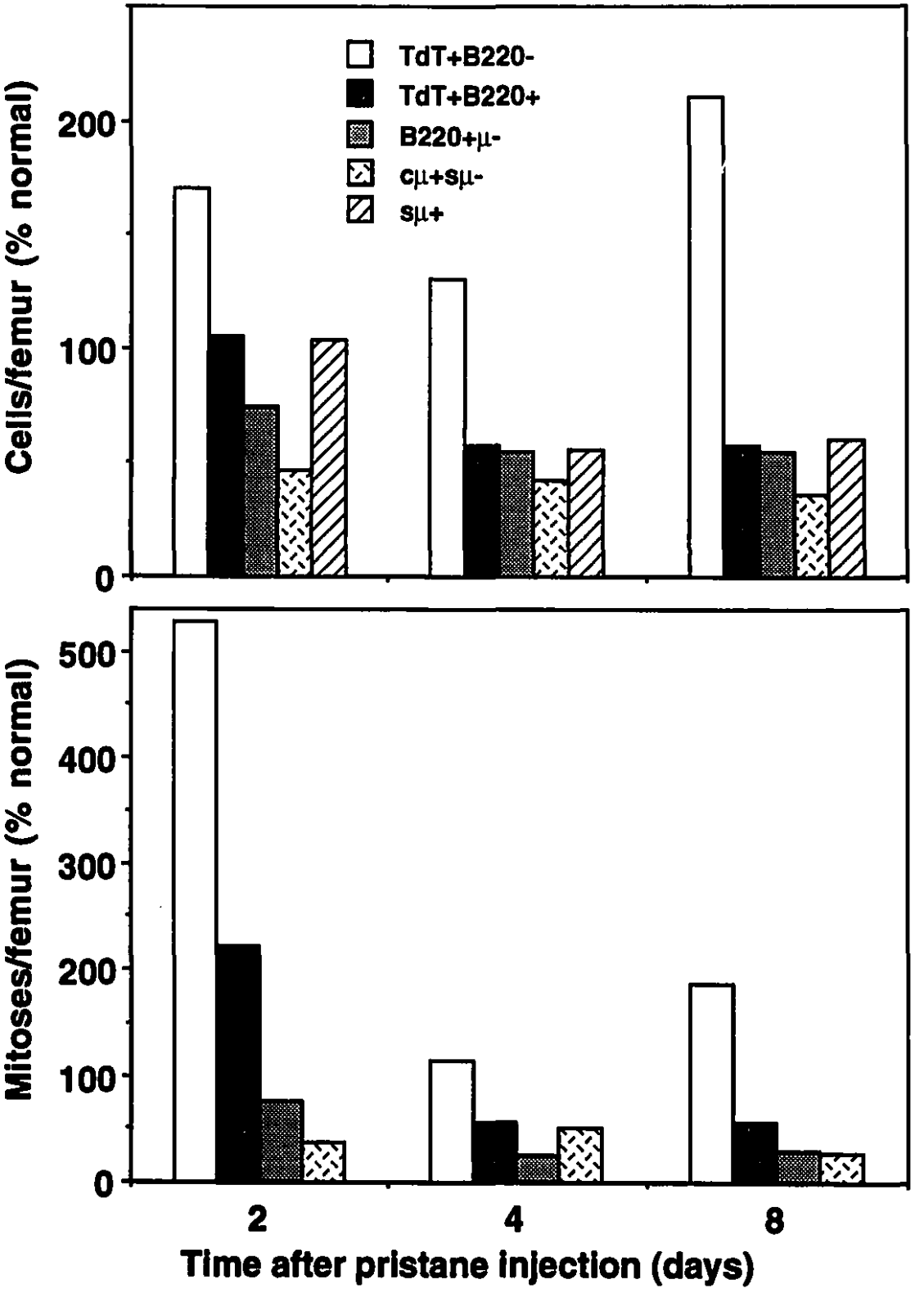

200
150
100
50
0
Cells/femur (% control)
TdT+B220-
TdT+B220+
B220+μ-
cμ+sμ-
sμ+
300
200
100
0
Mitoses/femur (% control)
36
60
84
Time after transfer (h)

Figure 9. B lineage cell populations in the bone marrow of BALB/c mice that were infected with malaria, re-infected, and then pristane-injected. Values are expressed as a percentage of normal values of groups of normal mice: cells per femur (above); cells in metaphase per femur, 2h40min after vincristine injection (below).


TdT+B220-
TdT+B220+
B220+μ-
cμ+sμ-
sμ+
Cells/femur (% normal)
200
100
0
Mitoses/femur (% normal)
500
400
300
200
100
0
2
4
8
Time after pristane injection (days)

REFERENCES

1. Fulop, G.M., and D.G. Osmond. 1983. Regulation of bone marrow lymphocyte production. III. Increased production of B and non-B lymphocytes after administering systemic antigens. *Cell.Immunol.* 75:80.

2. Opstelten, D., and D.G. Osmond. 1985. Regulation of pre-B cell proliferation in bone marrow: immunofluorescence stathmokinetic studies of cytoplasmic μ chain-bearing cells in anti-IgM-treated mice, hematologically deficient mutant mice and mice given sheep red blood cells. *Eur.J.Immunol.* 15:599.

3. Fulop, G.M., and D.G. Osmond. 1983. Regulation of bone marrow lymphocyte production. IV. Cells mediating the stimulation of marrow lymphocyte production by sheep red blood cells: studies in anti-IgM-suppressed mice, athymic mice, and silica-treated mice. *Cell.Immunol.* 75:91.

4. Pietrangeli, C.E., and D.G. Osmond. 1985. Regulation of B-lymphocyte production in the bone marrow: role of macrophages and the spleen in mediating responses to exogenous agents. *Cell.Immunol.* 94:147.

5. Pietrangeli, C.E., and D.G. Osmond. 1987. Regulation of B-lymphocyte production in the bone marrow: mediation of the effects of exogenous stimulants by adoptively transferred spleen cells. *Cell.Immunol.* 107:348.

6. Fulop, G.M., C.E. Pietrangeli, and D.G. Osmond. 1986. Regulation of bone marrow lymphocyte production: IV. Altered kinetic steady state of lymphocyte production after chronic changes in exogenous stimuli. *Exp.Hematol.* 14:27.

7. Osmond, D.G., Y.H. Park, and K. Jacobsen. 1988. B cell precursors in bone marrow: in vivo proliferation, localization, stimulation by activated macrophages and implications for oncogenesis. *Curr.Top.Microbiol.Immunol.* 141:2.

8. Osmond, D.G., and Y.H. Park. 1987. B Lymphocyte progenitors in mouse bone marrow. *Int.Rev.Immunol.* 2:241.

9. Park, Y.H., and D.G. Osmond. 1987. Phenotype and proliferation of early B lymphocyte precursor cells in mouse bone marrow. *J.Exp.Med.* 165:444.

10. Park, Y.H., and D.G. Osmond. 1989. Dynamics of early Blymphocyte precursor cells in mouse bone marrow proliferation of cells containing terminal deoxynucleotidyl transferase. *Eur.J.Immunol.* 19:2139.

11. Desiderio, S.V., G.D. Yancopoulos, M. Paskind, E. Thomas, M.A. Boss, N. Landau, F.W. Alt, and D. Baltimore. 1984. Insertion of N regions into heavy-chain genes is correlated with expression of terminal deoxyltransferase in B cells. *Nature* 311:752.

12. Kunkel, T.A., K.P. Gopinathan, D.K. Dube, E.T. Snow, and L.A. Loeb. 1986. Rearrangements of DNA mediated by terminal transferase. *Proc.Natl.Acad.Sci.USA* 83:1867.

13. Kincade, P.W. 1987. Experimental models for understanding B lymphocyte formation. *Adv.Immunol.* 41:181.

14. Opstelten, D., and D. G. Osmond. 1983. Pre-B cells in mouse bone marrow: immunofluorescence stathmokinetic studies of the proliferation of cytoplasmic μ-chain-bearing cells in normal mice. *J.Immunol.* 131:2635.

15. Osmond, D.G. 1990. B cell development in the bone marrow. *Semin.Inmmunol.* 2:173.

16. Deenen, G.J., I. Van Balen, and D. Opstelten. 1990. In rat B lymphocyte genesis sixty percent is lost from the bone marrow at the transition of nondividing pre-B cell to sIgM+ B lymphocyte, the stage of Ig light chain gene expression. *Eur.J.Immunol.* 20:557.

17. Klein, G., and E. Klein. 1986. Conditioned tumorigenicity of activated oncogenes. *Cancer Res.* 46:3211.

18. Lenoir, G.M., and G.W. Bornkamm. 1987. Burkitt's lymphoma, a human cancer model for the study of the multistep model of cancer: proposal for a new scenario. *Adv.Viral Oncol.* 7:173.

19. Tonewaga, S. 1983. Somatic generation of antibody diversity. *Nature* 302:575.

20. Freeman, R.R., and C.R. Parish. 1978. Spleen cell changes during fatal and self-limiting malarial infections of mice. *Immunology* 35:479.

21. Weiss, L., U. Gedulgig, and W. Weidanz. 1986. Mechanisms of splenic control of murine malaria: reticular cell activation and the development of a blood-spleen barrier. *Am.J.Anat.* 176:251.

22. Weiss, L. 1990. The spleen in malaria: the role of barrier cells. *Immunol.Letters* 25:165.

23. Shear, H.L., C. Ng, and Y. Zhao. 1990. Cytokine production in lethal and non-lethal murine malaria. *Immunol.Letters* 25:165.

24. Wyler, D.J. 1982. Malaria: host-pathogen biology. *Rev.Infect. Diseases* 4:785.

25. Wyler, D.J., J.J. Oppenheim, and L.C. Koontz. 1979. Influence of malaria infection on the elaboration of soluble mediators by adherent mononuclear cells *Infect.Immunol.* 24:151.

26. Dinarello, C.A. 1992. Role of interleukin-1 in infectious diseases. *Immunol.Rev.* 127:119.

27. Titus, R.G., B. Sherry, and A. Cerami. 1991. The involvement of TNF, IL-1 and IL-6 in the immune response to protozoan parasites. *Immunol.Today* 12:A13.

28. Potter, M., and J.S. Wax. 1983. Peritoneal plasmacytomagenesis in mice: comparison of different pristane dose regimens. *J.Nat.Cancer Inst.* 71:391.

29. Osmond, D.G., S. Priddle, and S.A. Rico-Vargas. 1990. Proliferation of B cell precursors in bone marrow of pristane-conditioned and, malaria-infected mice: implications for B cell oncogenesis. *Curr.Topics Microbiol.Immunol.* 166:149.

30. Semenzato, I. 1990. Tumor necrosis factor: a cytokine with multiple biological activities. *Br.J.Cancer* 61:354.

31. Park, Y.H., and D.G. Osmond. 1989. Post irradiation regeneration of early B lymphocyte precursor cells in mouse bone marrow. *Immunol.* 66:343.

32. Dorshkind, K., G.M. Keller, R.A. Phillips, R.G. Miller, G.C. Bosma, M. O'Toole, and M.J. Bosma. 1984. Functional status of cells from lymphoid and myeloid tissues in mice with severe combined immunodeficiency disease. *J.Immunol.* 132:1804.

33. Ohno, S., S. Migita, F. Wiener, M. Babonits, G. Klein, J.F. Mushinski, and M. Potter. 1984. Chromosomal translocations activating *myc* sequences and transduction of *v-abl* are critical

events in the rapid induction of plasmacytomas by pristane and Abelson virus. *J.Exp.Med.* 159:1762.

34. Klein, G., and E. Klein. 1985. *Myc*/Ig juxtaposition by chromosomal translocations: some new insights, puzzles and paradoxes. *Immunol.Today* 6:208.

35. Potter, M., and C. Boyce. 1962. Induction of plasma cell neoplasms in strain BALB/c mice with mineral oil and mineral oil adjuvants. *Nature* 193:1086.

36. Nordan, R.P., P.G. Pumphrey, and S. Rudikoff. 1987. Purification and NH_2-terminal sequence of a plasmacytoma growth factor derived from the murine macrophage cell line P388D1. *J.Immunol.* 139:813.

37. Kindler, V., A-P. Sappino, G.E. Grau, P-F. Piguet, and P. Vassalli. 1989. The inducing role of tumor necrosis factor in the development of bactericidal granulomas during BCG infection. *Cell* 56:731.

38. Libert, C., Brouckaert, P., Shaw, A., and W. Fiers. 1990. Induction of interleukin 6 by human and murine recombinant interleukin 1 in mice. *Eur.J.Immunol.* 20:691-694.

CHAPTER 5

Bone marrow B cell populations during tumorigenesis in Eμ-*myc* transgenic mice

ABSTRACT

Transgenic mice bearing the *c-myc* proto-oncogene under control of the immunoglobulin heavy chain (IgH) enhancer (Eμ-*myc* mice) undergo a reproducible series of developmental stages and die from malignancies of the B lymphocyte lineage. Three different stages are detected by peripheral blood analysis: an initial phase characterized by the presence in the peripheral blood of many large proliferating B lineage cells; a middle phase of varying time among affected mice, in which proliferating B lymphocytes disappear from the blood, and a final period characterized by the reappearance of proliferating B lineage cells and development of malignant clones. To investigate the cellular events underlying tumorigenesis in this model, we have quantitated B cell precursors and production rates produced by *c-myc* deregulation during the three stages. The findings revealed that during the pretumorous phase, the B cell precursors in the bone marrow, including pro-B cells undergoing IgH gene recombinant activity, were increased in population size and mitotic activity, while the B lymphocytes were actually reduced in numbers. The results suggest that constitutive *c-myc* expression in B cell precursors produces two opposing effects, an increase in proliferation of B cell precursors and a negative cell selection. The final emergence of a tumor clone in Eμ-*myc* mice may represent the development of a

dysregulated cell able to resist normal mechanisms of cell death or to evade negative selection.

INTRODUCTION

Precursor B cells are derived from stem cells through a series of proliferation and differentiation events before giving rise to mature B lymphocytes, a process under a precise homeostatic control (Osmond 1990). The use of three markers (μ heavy chains, B220 glycoprotein and TdT), in combination with double immunofluorescence labeling and stathmokinetic techniques permitted the identification of six phenotypically distinct B cell populations in the mouse bone marrow: 1) presumptive pro-B cells, at the stage of heavy chain gene rearrangement; early, intermediate and late pro-B cells (TdT^+B220^-, TdT^+B220^+, $B220^+\mu^-$, respectively); 2) pre-B cells ($c\mu^+s\mu^-B220^+$), including large dividing and small non-dividing cells, and 3) B lymphocytes expressing sμ and B220 (Park and Osmond 1987). The proliferation dynamics of B precursor cells show a progressive increase in numbers, cell size and production rate among succesive populations up to the large pre-B cell stage. However, during the clonal expansion there is a cell selection evidenced by a marked cell loss which accounts for 75% of B cell precursors (Opstelten and Osmond 1983, Deenan at al. 1990). Cell loss may represent the elimination of aberrant cells with defective

immunoglobulin gene rearrengements, autoreactivity and potentially oncogenic deregulation. Recent studies have revealed the presence of B lineage cells undergoing apoptosis, potentially aberrant cells, being recognized and eliminated by resident macrophages (Jacobsen and Osmond 1990, 1991, Osmond et al 1992).

The proto-oncogene *c-myc* is involved in normal growth regulation. The *c-myc* protein product acts as a transcriptional activation factor required for cell proliferation in the transition from G_0 to G_1, and from G_1 to S phase (Heikkila et al. 1987, Amati et al. 1992, Kretzner et al. 1992). The expression of *c-myc* gene is controlled at various stages of the cell cycle. Different modes of deregulation have been proposed for the *c-myc* gene: point mutations, deletions, amplification, insertional mutagenesis and through translocations (Klein and Klein 1985, Lenoir and Bornkamm 1987, Dufort et al. in press). Under these conditions, the gene product may be affected in different ways; a hyperactive product in causing an uncontrolled proliferative state which may be the first step towards neoplastic transformation (Klein and Klein 1985, Lenoir and Bornkamm 1987).

Mice transgenic for a *c-myc* gene driven by the IgH enhancer (Eμ-myc) are a unique experimental model for lymphomagenesis. In these circumstances the constitutive expresion of *c-myc* gene by the precursor B cell populations maintains the cells in a continuous proliferative cell cycle. The final event is the development of B cell neoplasias and the death of the animal (Langdon et al. 1986, Harris et

al. 1988). By peripheral blood lymphocyte proliferation analysis, three different periods can be identified in Eμ-*myc* transgenic mice: an initial period characterized by B lymphoid proliferation in the peripheral blood, a middle period in which peripheral lymphocyte proliferation is absent, and a final period in which peripheral lymphocyte proliferation returns, reflecting a malignant tumor clone (Sidman et al. 1988). The period of time before the appearance of the malignancy indicates that additional events occur in the tumor clones before their full tumor transformation. To investigate the cellular events leading up to oncogenesis, we have investigated the proliferative dynamics of B cell precursors in the bone marrow of Eμ-*myc* transgenic mice.

MATERIALS and METHODS

Mice. The mice used were congenic C57BL/6JSmn (B6) and C57BL/6JSmn-Eμ-*myc*. The latter were derived from the original segregating (B6xSJL)FE2-Eμ-*myc* population (Adams et al. 1985). The different phases of the tumorigenic process in Eμ-*myc* transgenic mice were defined by forward and side scatter analysis of peripheral blood lymphocytes (Sidman et al. 1988). The mice were kindly provided by Dr. Charles L. Sidman, (Dept. of Molecular Genetics, Biochemistry and Microbiology, University of Cincinnati College of Medicine).

The population sizes and production rates of the various B cell precursors were determined using double immunofluorescence labeling and mitotic arrest techniques as decribed in Chapter 2.

RESULTS

Precursor B cells were examined in bone marrow and spleen suspensions from C57BL/6JSmn-Eμ-*myc* mice identified as being in the three phases, early, middle or late, in their characteristic tumorigenic progression, by flow cytometry of blood lymphocytes, compared with cells of congenic C57BL/6JSmn mice.

Cellularity of bone marrow and spleen. The absolute numbers of nucleated cells in the bone marrow were increased in the middle (180%) and final (140%) phases compared to control values (Table 1). Spleen cellularity was increased in all three phases compared to control values. The increase was more marked in the early (550%) and final (275%) phases, than in the middle phase (175%) (Table 1).

Precursor B-cell populations in the bone marrow of Eμ-myc transgenic mice. The population sizes of early (TdT^+B220^-), intermediate (TdT^+B220^+) and late ($B220^+\mu^-$) pro-B cells; pre-B cells ($c\mu^+s\mu^-$); and B lymphocytes, as well as the production rate of proliferating pro-B and pre-B cells were determined using double immunofluorescence labeling and mitotic arrest techniques, as

described. Early pro-B cells and intermediate pro-B cells showed no evident alteration in population size at any stage of the Eμ-*myc* tumorigenic progression, whereas late pro-B cells were markedly elevated in the middle-phase (438%) and final phase (250%) of Eμ-myc mice. Pre-B cells were increased in both early and intermediate phase Eμ-*myc* animals (120% and 153% respectively), but were reduced to less than half (41%) control values in the final phase. In contrast, the B lymphocyte population size was markedly reduced during the early and middle phases (37% and 65%), finally increasing to 4.5fold control values in animals with frank B cell tumors (Table 2 and Fig. 1)

To determine whether these population size changes were due to alterations in cell generation rates, the number of cells entering mitosis per unit time was measured. The major alterations in B lineage cell kinetics were marked increases in the generation rates of intermediate pro-B cells (382%), late pro-B cells (267%) and pre-B cells (303%) in the bone marrow of Eμ-*myc* mice during the middle phase (Table 2 and Fig. 1). In the early and late phases of Eμ-*myc* mice the numbers of cells passing through mitosis in the various compartments were not significantly different from controls.

Population size of B cells in the spleen of Eμ-myc mice. The number of mature B lymphocytes in the spleen of Eμ-*myc* mice during the intermediate phase resembled that of control mice, but was elevated

during both early (206%) and late (179%) phases compared to control values (Fig. 3).

DISCUSSION

B cell genesis in the bone marrow occurs in a process strictly regulated by microenvironmental interrelationships and systemic growth factors (Osmond 1990, Kincade et al. 1988). Deregulations in this delicate process may lead to the development of tumors. Transgenic mice carrying a *c-myc* oncogene driven by the immunoglobulin heavy chain enhnacer die of tumors of the B lineage phenotype after a varying period of time (Adams et al. 1985, Harris et al. 1988). The lag before the appearance of the malignancy indicates that *c-myc* by itself is not enough to produce tumors, and that additional events occur before full transformation and the emergence of a tumor clone.

Three stages can be identified by peripheral blood analysis in Eμ-*myc* transgenic mice; an initial stage where peripheral B lineage cells proliferate; a middle stage, of weeks or months, when proliferating B lymphocytes disappear from the blood; and a final period where peripheral lymphocyte proliferation returns, reflecting a malignant tumor clone (Sidman et al 1988). In the present study, analysis of B cell precursors in the bone marrow during the different peripheral blood lymphocyte periods shows striking phenomena. Especially

important is the middle period when there is no active peripheral lymphoid proliferation. The bone marrow shows an increase in population size and proliferative activity among the earlier B cell precursors, without an expected increased in B lymphocytes. These are actually reduced in numbers in the bone marrow, and are similar to control values in the spleen. This is in contrast to the final period when population size and proliferative rate of earlier B cell precursors are diminished, but the numbers of B lymphocytes are increased in the bone marrow as well as in the spleen.

From these observations, two critical changes seem to occur during the prelymphomatous phase in Eμ-*myc* mice. First, there is an early polyclonal proliferative period in which proliferating B cells are prominent in the blood, and the pre-B cells in the bone marrow show an increased proliferative rate and population size. This is followed by a disappearance of proliferating B lineage cells in the peripheral blood, but an increase in the bone marrow population sizes and proliferative rates among the early, intermediate and late pro-B cells, and pre-B cells. However, B lymphocytes are greatly reduced in the bone marrow during the prelymphomatous phase, while after an increase in the early polyclonal proliferative period the spleen B lymphocyte numbers become near normal. Thus, in the pretumorous phase, there is a marked increase in numbers and rates of generation of precursor B cells, yet the number of mature B lymphocytes is actually less than normal. This suggests that there is a stimulation of

precursor cell proliferation but that this is followed by an increased fraction of cell loss.

Cell loss is a characteristic of B cell genesis in the bone marrow (Park and Osmond 1989), and is thought to reflect the elimination of apoptotic aberrant cells by local resident macrophages (Jacobsen and Osmond 1990, 1991, Osmond et al. 1992). Electron microscopy at the prelymphomatous phase suggests that greatly increased numbers of B220+ B lineage cells in the bone marrow are undergoing apoptosis, and are rapidly recognized and ingested by resident macrophages (Sidman et al. 1993, Jacobsen et al. unpublished data). The evidence suggests that the egress of B cell progenitors from the bone marrow to the periphery is normally controlled. Aberrant dysregulated cells are signalled to undergo a programmed cell death, being rapidly recognized and eliminated by macrophages. It may be postulated that the final emergence of a tumor clone is a consequence of further genetic accidents in the rapidly proliferating precursor B cell pool, resulting in a capacity to evade the normal bone marrow screening mechanisms and to become disseminated.

Two other pathological conditions resemble the effects of the Eμ-*myc* transgene in mice; Burkitt's lymphoma, associated with malaria, and murine plasmacytoma. Both entities are characterized by an Ig/*myc* translocation and prolonged macrophage activation (Klein and Klein 1985, Lenoir and Bornkamm 1987, Haluska et al. 1987, Potter et al. 1987, Ohno et al. 1989). *Plasmodium yoelii* infection and a single

intraperitoneal pristane injection produce a sustained stimulation of early B cell precursors in the bone marrow of mice without a proportionate expansion at later stages (this thesis, Chapters 3 and 4). The stimulation of precursor B cells rearranging Ig heavy chain genes in these conditions, as in Eμ-*myc* mice, may increase the probability of genetic accidents involving cooperative oncogenes, which may predispose the cells to neoplastic transformation (Vaux et al. 1988, Bissonette et al. 1992, Fanidi et al. 1992), possibly by the acquisition of survival mechanisms to block the cell death pathway and/or to avoid macrophage recognition and deletion.

TABLE I

Total nucleated cells in bone marrow and spleen of Eμ-*myc* B6 transgenic mice[a]

	Control	Early	Middle	Late
Cells/femur ($x10^5$)	159	139	287	225
Cells/spleen ($x10^6$)	120	660	210	330

[a] Each value was derived from a pool of three mice

TABLE 2

Number of B lineage cells in bone marrow of Eμ-*myc* B6 transgenic mice[a]

Cell phenotype	Control	Early	Middle	Late
Cells/femur ($x10^5$)[b]				
TdT^+B220^-	0.6	0.4	0.4	0.6
TdT^+B220^+	3.0	1.1	1.7	1.1
$B220^+\mu^-$	8.0	10.0	35.0	20.0
$cu^+s\mu^-$	51.0	61.2	78.0	21.1
$s\mu^+$	22.3	7.0	14.6	99.0
Bone marrow cells in metaphase/femur ($x10^4$)[c]				
TdT^+B220^-	0.2	0.2	0.2	0.4
TdT^+B220^+	0.9	0.2	4.2	0.5
$B220^+\mu^-$	15.0	12.3	40.0	10.2
$cu^+s\mu^-$	18.0	26.0	54.6	14.0

a Each value was derived from a pool of three mice

b Calculated from the incidence of cells of each phenotype relative to all nucleated cells and the total cellularity of the bone marrow

c Calculated from the total number of cells/femur/phenotype and the percentage of cells/phenotype in metaphase (metaphase index) 2h40min after injecting vincristine sulfate ip

Figure 1. Total nucleated cells in the bone marrow and spleen of Eμ-*myc* mice in the three phases, early, middle or late of their characteristic tumorigenic progression, and of congenic B6 mice. Values derived from cells pooled from groups of three mice.

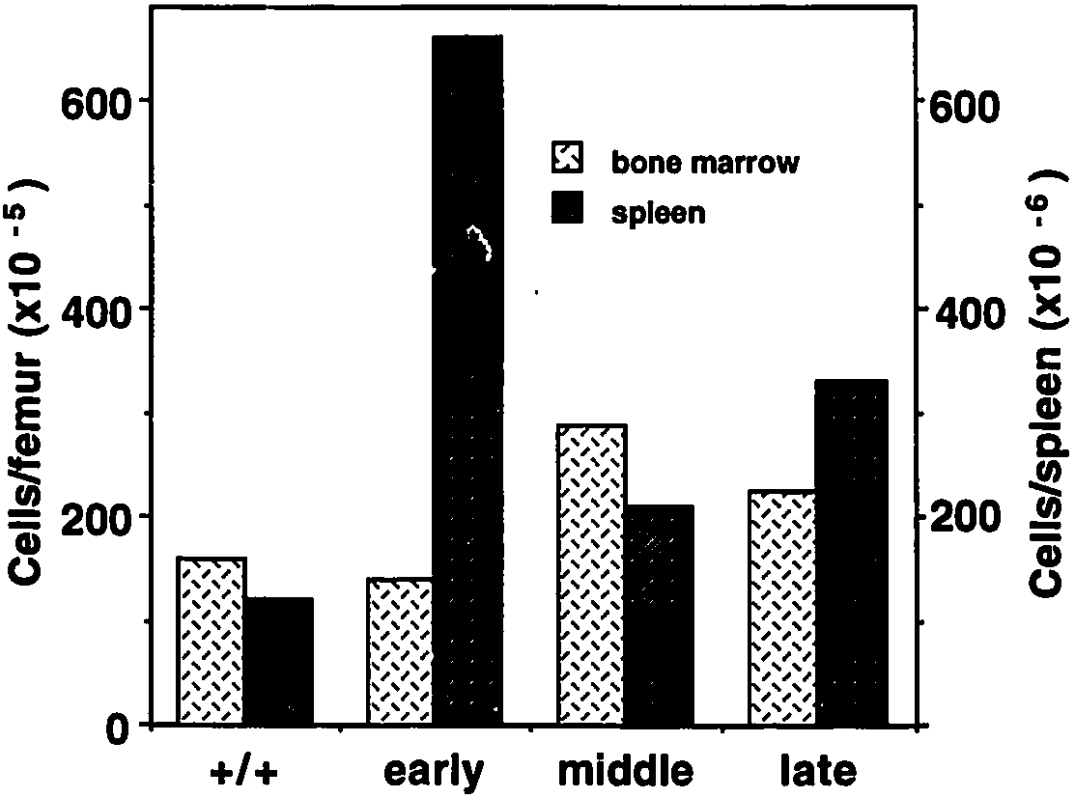

600
400
200
0
Cells/femur (x10 -5)
600
400
200
Cells/spleen (x10 -6)
bone marrow
spleen
+/+
early
middle
late

Figure 2. B lineage cells in the bone marrow of Eμ-*myc* mice in the three phases of tumorigenic progression, and of congenic B6 mice, indicating the actual number of cells per femoral shaft (above) and the number of cells entering mitosis per unit time (2h40min) (below). Values derived from cells pooled from groups of three mice.

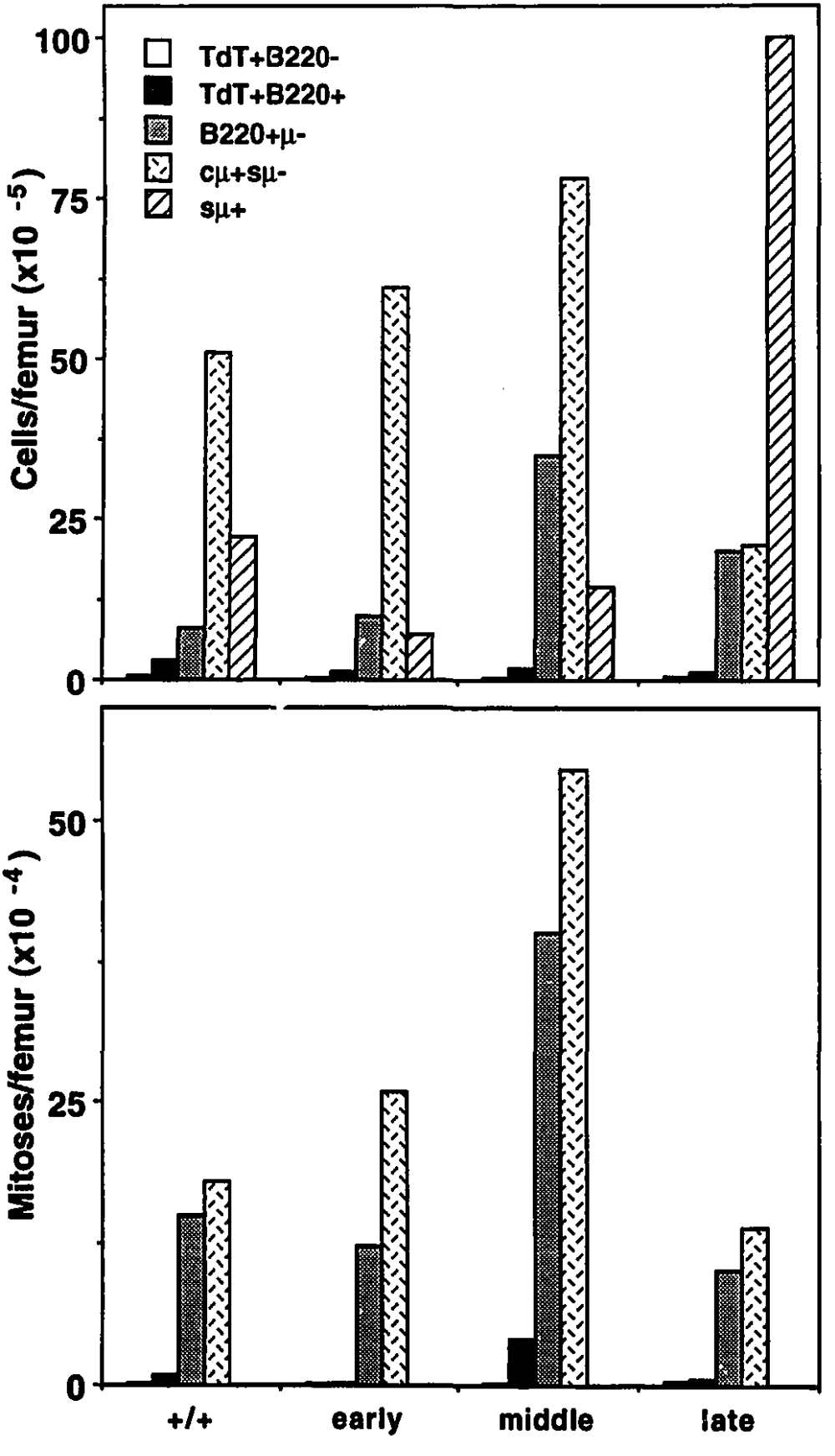
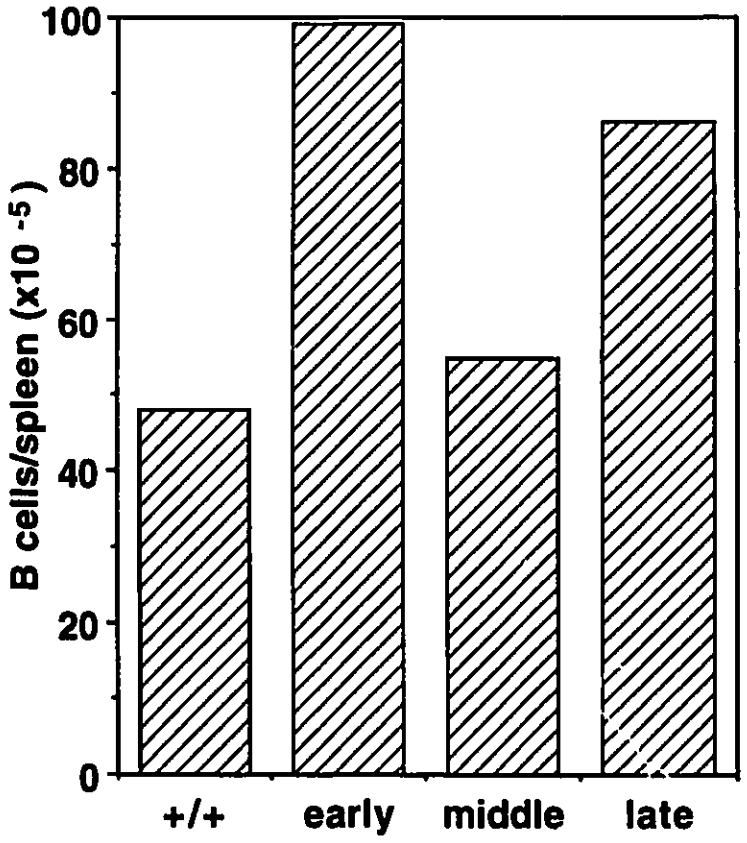

TdT+B220-
TdT+B220+
B220+μ-
cμ+sμ-
sμ+
Cells/femur (x10 -5)
100
75
50
25
0
Mitoses/femur (x10 -4)
50
25
0
+/+
early
middle
late

Figure 3. Number of B cells ($s\mu^+$) in the spleen of Eμ-*myc* mice in the three phases of tumorigenic progression, and of congenic B6 mice. Values derived from cells pooled from groups of three mice.


100
80
60
40
20
0
B cells/spleen (x10 -5)
+/+
early
middle
late

REFERENCES

Adams, J.M., Harris, A.W., Pinkert, C.A., Corcoran, L.M., Alexander, W.S., Cory, S., Palmitier, R.D., Brinster, R.L. (1985): The *c-myc* gene driven by immunoglobulin enhancers induces lymphoid malignancy in transgenic mice. Nature 318:533-538

Amati, B., Dalton, S., Brooks, M.W., Littlewood, T.D., Evan, G.I., Land, H. (1992): Transcriptional activation by the human c-Myc oncoprotein in yeast requires interaction with Max. Nature 359:423-426

Bissonette, R.P., Echeverri, F., Mahboubi, A., Green, D.R. (1992): Apoptotic cell death induced by *c-myc* is inhibited by *bcl-2*. Nature 359:552-554

Dufort, D., Drolet, M., Nepveu, A.: A protein binding site from the murine *c-myc* promoter contributes to transcriptional block. Oncogene (in press)

Fanidi, A., Harrington, E.A., Evan, G.I. (1992): Cooperative interaction between *c-myc* and *bcl-2* proto-oncogenes. Nature 359:554-556

Harris, A.W., Pinkert, C.A., Crawford, M., Langdon, W.Y., Brinster, R.L., Adams, J.M. (1988): The Eμ-*myc* transgenic mouse: a model for high-incidence spontaneous lymphoma and leukemia of early B cells. J.Exp.Med. 167:353-371

Haluska, F.G., Tsujimoto, Y., Croce, C.M. (1987): The t(8;14) chromosome translocation of the Burkitt's lymphoma cell line Daudi occurred during immunoglobulin gene rearrangement and involved the heavy chain diversity region. Proc.Natl.Acad.Sci.USA 84:6835-6839

Jacobsen, K., Osmond, D.G., (1990): Microenvironmental organization and stromal cell associations of B lymphocyte precursor cells in mouse bone marrow. Eur.J.Immunol. 20:2395-2404

Jacobsen, K., Osmond, D.G. (1991): Cell deletion in B-cell genesis and the role of bone marrow macrophages. In lymphatic tissues and the in vivo immune response. Ed. Imhof, B.A., Akini, S.-B., and Ezine, S. pp 343-353

Kincade, P.W., Lee, G., Pietrangeli, C.E., Hayashi, S-I., Gimble, J.M. (1988): Cells and molecules that regulate B lymphopoiesis in bone marrow. Ann.Rev.Immunol. 7:111-143

Klein, G., Klein, E. (1985): *Myc*/Ig juxtaposition by chromosomal translocations: some new insights, puzzles and paradoxes. Immunol.Today 6:208-215

Kretzner, L., Blackwood, E.M., Eisenman, R.N. (1992): Myc and Max proteins possess distinct transcriptional activities. Nature 359:426-428

Langdon, W.Y., Harris, A.W., Cory, S., Adams, J.M. (1986): The *c-myc* oncogene perturbs B lymphocyte development in Eμ-*myc* transgenic mice. Cell 47:11-18

Lenoir, G.M., Bornkamm, G.W. (1987): Burkitt's lymphoma, a human cancer model for the study of the multistep development of cancer: proposal for a new scenario. Adv. in Viral Oncology 7:173-206

Ohno, S., Babonits, M., Wiener, F., Spiro, J., Klein, G. (1979): Non-random chromosome changes involving the Ig gene-carrying chromosomes 12 and 6 in pristane-induced mouse plasmacytomas. Cell 18:1001-1007

Opstelten, D., Osmond, D.G. (1985): Regulation of pre-B cell proliferation in bone marrow: immunofluorescence stathmokinetic studies of cytoplasmic μ chain-bearing cells in anti-IgM-treated mice, hematologically deficient mutant mice and mice given sheep red blood cells. Eur.J.Immunol. 15:599-605

Osmond, D.G., Kim, N., Manoukian, R., Phillips, R.A., Rico-Vargas, S.A., Jacobsen, K. (1992): Dynamics and localization of early B-lymphocyte precursor cells (pro-B cells) in the bone marrow of scid mice. Blood 79:1695-1703

Potter, M., Mushinski, J.F., E.B., Brust, S., Wax, J.S., Wiener, F., Babonits, M., Rapp, U.R., Morse, H.C. (1987): Avian *v-myc* replaces chromoso- mal translocation in murine plasmacytomagenesis. Science 235:787-789

Park, Y.H., Osmond, D.G. (1987): Phenotype and proliferation of early B lymphocyte precursor cells in mouse bone marrow. J.Exp.Med. 165:444-458

Sidman, C.L., Marshall, J.D., Harris, A.W. (1988): Genetic studies on Eμ-*myc* transgenic mice. Curr.Top.Microbiol.Immunol. 141:94-99

Vaux, D.L., Cory, S., Adams, J.M. (1988): *Bcl-2* gene promotes haemopoietic cell survival and cooperates with *c-myc* to immortalize pre-B cells. Nature 335:440-442

CHAPTER 6

c-kit expression by B cell precursors in mouse bone marrow: stimulation of B cell genesis by *in vivo* treatment with anti-*c-kit* antibody

SUMMARY

To examine the possible *in vivo* role of the *c-kit* receptor in B lymphopoiesis we have evaluated the proliferative dynamics of precursor B cell populations expressing *c-kit* in mouse bone marrow and the effects of administering a neutralizing anti-*c-kit* mAb, ACK2. Double immunofluorescence labeling, epifluorescence microscopy and mitotic arrest techniques have been used to examine bone marrow cells from 23-27g BALB/c mice. Almost one half of all TdT^+ cells and one quarter of all $B220^+$ B lineage cells coexpressed *c-kit*, mainly at low intensities, and were actively proliferating *in vivo*. The incidence of TdT^+*c-kit*$^+$ cells was 0.6% and these were mainly large cells (9-15μm); TdT^+*c-kit*$^-$ cells were usually smaller (5-8μm). $B220^+$*c-kit*$^+$ cells represented 4.3±0.2% of all bone marrow cells. Other *c-kit*$^+$ cells, lacking B cell markers, totalled 3.6±0.1% of bone marrow cells, expressed *c-kit* in high intensities and had an exceptionally rapid turnover rate. Mice given five injections of 1mg mAb ACK2 on alternate days and examined on the ninth day showed reduced bone marrow cellularity, with an almost complete absence of erythroid and granulocytic cells. In contrast, B lymphopoiesis was markedly stimulated. B lineage cells formed 80% of bone marrow cells. The populations of intermediate pro-B cells (TdT^+B220^+), late pro-B cells ($B220^+\mu^-$) and pre-B cells ($c\mu^+s\mu^-$) were all increased 2-4 fold in cell number and production rate. B lymphocytes were also increased in both the bone marrow and spleen. The results demonstrate that

many early precursor B cells in mouse bone marrow constitutively express *c-kit* receptor. The failure of ACK2 treatment to block B lymphopoiesis suggests, however, that *c-kit* receptor function is not essential for precursor B cell development and that the *in vivo* role of *c-kit* in normal B cell genesis can be replaced by alternative signalling systems. It is postulated that the stimulation of B cell genesis induced by ACK2 may reflect a perturbation in the interlineage competition for microenvironmental factors which underlies the normal balance between B lymphopoiesis and other hemopoietic lineages *in vivo*.

INTRODUCTION

In mammalian bone marrow a variety of hemopoietic cell lineages undergo processes of differentiation from stem cells and proliferative expansion under local microenvironmental control. In doing so, the cells can be considered to pass through a series of developmental compartments defined by sequential changes in their phenotypic properties and control mechanisms. Cells in an early differentiation compartment of the B lymphocyte lineage before the expression of the μ heavy chain of IgM (pro-B cells), transiently express terminal deoxynucleotidyl transferase (TdT), an intranuclear enzyme which inserts nucleotides at VDJ joining sites during rearrangement of VDJ segments of IgM heavy chain genes (1,2). The B220 glycoprotein also first becomes expressed at the cell surface during this stage and

remains associated with the B lymphocyte lineage thereafter (1,3). Cells then enter the pre-B cell compartment when they begin to synthesize μ heavy chains and to rearrange the gene segments for the light chains of IgM. The pairing of heavy and light chains and the surface expression of IgM defines the final B lymphocyte compartment. Pro-B cells and large pre-B cells undergo a series of mitoses, while small pre-B cells mature into B lymphocytes without further division (1-3). Differentiating B lineage cells *in vivo* remain intimately associated with stromal reticular cells until the stage of terminal maturation (4-6). B cell genesis requires direct interactions with stromal cells and the release of stromal cell growth factors, notably IL-7 (7,8). Systemic pleiotropic factors may also act on B cell genesis *in vivo*, probably by stimulating stromal cells to release specific signals for precursor B cell development (6,9). However, the local molecular interactions which regulate the proliferation and differentiation of early precursor B cell compartments remain largely unknown. Control mechanisms operating during the pro-B cell stages of development would have important implications for the normal clonal development of B lymphocytes and for perturbations leading to B cell neoplasias and deficiencies.

The proto-oncogene *c-kit*, localized within the dominant white spotting (W) locus in mice, encodes a transmembrane tyrosine kinase receptor molecule (10). Mutations at the W locus affecting various domains of the *c-kit* receptor, result in defective development of hemopoietic stem cells, germ cells and melanocytes (11). The ligand

for the *c-kit* receptor (kit ligand, stem cell factor, mast cell growth factor), which has been cloned and mapped to the Steel (Sl) locus, exists in both soluble and membrane forms (12-15). The latter is expressed on certain bone marrow stromal cells, probably being implicated in cell-cell interactions promoting cellular adhesion and migration (16,17). Interaction of *c-kit* receptor with its ligand induces autophosphorylation of *c-kit*, phosphorylation of second messenger signal transducers involved in cell proliferation (18,19), and, possibly, the induction of cytokine expression (20). The *c-kit* receptor is expressed on a variety of primitive hemopoietic precursor cells (21-23) whose proliferation can be stimulated by kit ligand acting synergistically with other cytokines (24-27). Studies blocking *c-kit* function by a non-cytotoxic anti-*c-kit* mAb (ACK2) have indicated that *c-kit* plays an essential role in the *in vivo* development of myeloid and erythroid progenitors (21). The question remains whether *c-kit* expression may also be involved in regulating B lymphopoiesis. *In vitro* studies have detected *c-kit* expression by certain cultured precursor B cells and cell lines characterized by early stages of Ig gene rearrangement (24,28). Kit ligand augments the stimulatory effect of IL-7 on precursor B cells *in vitro*, an effect which is inhibited by mAb ACK2 (28). *In vivo*, on the other hand, the expression of *c-kit* by B lineage cells has been equivocal and mAb ACK2 has appeared not to impair B cell development (21,22).

The present work was designed to determine at which differentiation stages B cell progenitors may express *c-kit* under physiological

conditions *in vivo*, to examine the proliferative dynamics of these cells, and to assess the *in vivo* functional significance of *c-kit* activity on precursor B cell development. First, we have used double immunofluorescence and stathmokinetic techniques to quantitate *c-kit*-bearing cells and their proliferative activity in precursor B cell compartments of mouse bone marrow. Secondly, we have examined the effects of the anti-*c-kit* mAb ACK2 *in vivo* on the number and proliferative activity of cells in each phenotypically defined compartment of precursor B cell development. Our results demonstrate that many actively proliferating early precursor B cells express *c-kit in vivo*. Interestingly, however, treatment with a neutralizing anti-*c-kit* antibody, which suppresses erythropoiesis and granulopoiesis, actually stimulates B cell genesis. Our findings suggest that *c-kit* receptor function is not essential for the development of precursor B cells *in vivo*, and raise questions concerning the role of interlineage relationships in the regulation of lymphopoiesis and hemopoiesis in the bone marrow.

MATERIALS and METHODS

Animals. BALB/c mice were purchased from Charles River Canada (St. Constant, PQ) and maintained in microisolators. Mice were analyzed individually.

The population sizes and production rates of the different B cells precursors were determined using double immunofluorescence

labeling and mitotic arrest techniques as described in Chapter 2, including the following steps for *c-kit* labeling and anti-*c-kit* treatment *in vivo*.

Antibodies. Ammonium sulfate-precipitated rat anti-mouse *c-kit* monoclonal antibody (mAb), ACK-2, (Dr. S-I. Nishikawa, Kumamoto University, Japan) was used at a concentration of 17µg protein/ml as a primary immunolabeling agent. Tetramethyl rhodamine isothiocyanate (TRITC)- and fluorescein isothiocyanate (FITC)-conjugated goat anti-rat IgG (Heavy and Light chains) (Kirkegaard and Perry Laboratories, Gaithersburg, MD), were used as secondary antibodies at 17µg and 50µg protein/ml, respectively. mAb 14.8 was also used for direct immunofluorescence labeling after being FITC-conjugated and diluted 1:5.

Anti-c-kit treatment in vivo. Mice were injected i.v. with 1mg of anti-*c-kit* mAb ACK2 in 0.1ml sterile isotonic chloride (0.9%) on alternate days. Control mice were similarly injected with 0.1ml of sterile isotonic sodium chloride (0.9%). Each mouse was given 5 injections and sacrificed on day 9, one day after the final injection.

Labeling of c-kit. Bone marrow cells (50µl; $40x10^6$cells/ml) were incubated in microwell plates with an equal volume of mAb ACK2 for 30min on ice. The cells were washed, resuspended in 50µl MEM-NCS and incubated with either TRITC- or FITC-conjugated goat anti-rat IgG (30min, on ice). The cells were cytocentrifuged and fixed in

absolute methanol (30min, on ice) and hydrated in gradually reducing concentrations of methanol in PBS.

Double labeling of c-kit and TdT. After *c-kit* labeling, cytocentrifugation and fixation, as described above, cell preparations were incubated with normal goat serum, washed, and then exposed to rabbit anti-TdT (overnight, room temperature) followed by TRITC-conjugated goat anti-rabbit IgG $F(ab')_2$ (30min, room temperature).

Double labeling of c-kit and B220 glycoprotein. After *c-kit* labeling, cell suspensions were incubated with FITC-conjugated mAb 14.8 (30min, on ice), cytocentrifuged and fixed in absolute methanol (30min, on ice).

The incidence of *c-kit*$^+$cells, *c-kit*$^+$B220$^+$cells, *c-kit*$^+$TdT$^+$cells, TdT$^+$B220$^-$ cells, TdT$^+$B220$^+$cells, B220$^+\mu^-$ cells, cμ^+sμ^- cells and sμ^+ cells, as well as the proportion of cells in metaphase, were examined as described in Chapter 2.

RESULTS

c-kit$^+$ *Cells in Mouse Bone Marrow.*

Cells expressing *c-kit*, as detected by the surface binding of mAb ACK2, totalled 6.6±0.2% of all cells in bone marrow cell suspensions. Approximately one third of the *c-kit*$^+$ cells showed bright labeling, while the remainder were *c-kit* dull (Table 1). A repeat experiment

confirmed these values (not shown). The *c-kit*$^+$ cells had undifferentiated blastoid and lymphoid morphology.

Expression of c-kit and TdT. Almost half of the total TdT$^+$ cells in the bone marrow coexpressed *c-kit*, mainly at dull intensity (Table 1). These TdT$^+$ cells were mainly large cells (9.0-15.0μm nuclear diameter), while the TdT$^+$ cells lacking *c-kit* were usually smaller (5.0-8.0μm nuclear diameter).

Expression of c-kit and B220 glycoprotein. Nearly one quarter of total B220$^+$ cells coexpressed *c-kit*, accounting for more than one half of the total *c-kit*$^+$ cells (Table 2). The total incidence of *c-kit*$^+$ cells in these experiments, using a TRITC-conjugated secondary antibody to detect the binding of mAb ACK2, was consistently slightly higher than that previously observed using an FITC-conjugated secondary antibody. The coexpression of *c-kit*, B220 and TdT is summarised in Fig.1.

Expression of c-kit and μ chains. No cells bearing either cytoplasmic or surface μ heavy chains were found to coexpress *c-kit*.

Proliferative dynamics of c-kit$^+$ *cells.*

The flow of cells through mitosis was indicated by the proportion of cells of each phenotype that had accumulated in metaphase during a standard interval of time (2h40min) after injecting vincristine sulfate to stop dividing cells in mitosis. This treatment did not appreciably perturb the population size of *c-kit*$^+$, TdT$^+$ or B220$^+$ cells (Tables 1,2).

All populations of *c-kit*$^+$ cells were in active cell cycle but their rate of entry into mitosis differed markedly depending upon whether or not they coexpressed B cell lineage markers. The *c-kit*$^+$ cells that coexpressed either TdT or B220 glycoprotein showed similar, moderately large accumulations of mitotic cells (13-15%), whereas the proportion of *c-kit*$^+$ cells lacking B lineage markers (B220) which entered mitosis in the same period of time was considerably greater (36%) (Tables 1,2; Fig.1).

Effects of in vivo Anti-c-kit (mAb ACK2) Treatment on B Cell Precursors in Bone Marrow.

To examine the functional significance of *c-kit* expression by B cell precursors, BALB/c mice were injected i.v. with purified mAb ACK2 on alternate days for 5 injections (21). Control mice received parallel injections of sterile saline. On day 9, bone marrow cells were analysed for the population size and proliferative activity of B cell progenitors. A third group of normal mice received no treatment.

Cellularity of bone marrow and spleen in ACK2-treated mice. The total number of nucleated cells in the bone marrow was reduced by 40% in anti-*c-kit*-treated mice compared with the saline-treated mice. In contrast, the spleen cellularity in anti-*c-kit*-treated mice was increased by 25% (Fig. 2). Values for the saline-treated controls were intermediate between those of untreated mice and ACK2-treated mice.

Population sizes of precursor B cells in bone marrow of ACK2-treated mice. The reduced cellularity in the bone marrow of ACK2-treated mice was associated with an almost complete absence of erythroid precursors and greatly reduced incidences of myeloid (granulocytic, monocytic) cells, as evaluated in Giemsa-stained cytocentrifuged preparations. Most of the residual cells exhibited lymphoid morphology.

In sharp contrast to erythroid and myeloid cells, the incidence of B lineage cells, as detected by immunofluorescence labeling, was greatly increased to form the predominant cell population in the bone marrow of anti-*c-kit*-treated mice. The total incidence of B lineage cells in the bone marrow increased from 28-33% in normal and saline-injected mice to 80% after anti-*c-kit* treatment (Table 3). In addition to their elevated incidence, the actual number of B lineage cells per femur was substantially increased in ACK2-treated mice (Fig.3). All precursor B cell populations, apart from early pro-B cells, were expanded. The intermediate pro-B cells (TdT^+B220^+), late pro-B cells ($B220^+\mu^-$) and pre-B cells ($c\mu^+s\mu^-$) approximately doubled in absolute numbers compared with control animals. The mature $s\mu^+$ B cells also increased in numbers. The values in saline-treated control mice closely resembled those in normal mice (Fig.3).

Proliferative dynamics of precursor B cells in the bone marrow of ACK2-treated mice. Pro-B and pre-B cells entered mitosis in increased numbers in ACK2-treated mice compared with saline-

treated or normal mice. The actual number of intermediate pro-B cells (TdT^+B220^+), late pro-B cells ($B220^+\mu^-$) and pre-B cells ($c\mu^+s\mu^-$) entering mitosis per unit time showed absolute increases of 2-4 times the normal values (Fig.3). These findings thus indicate a true increase in the levels of precursor B cell proliferation after anti-c-kit antibody treatment.

c-kit$^+$ *cells in bone marrow of ACK2-treated mice.* ACK2 injections in doses of 1mg/mouse have been shown to saturate the ACK2 epitope on the surface of hemopoietic cells while leaving other *c-kit* epitopes exposed and the cells intact (21, S-I. Nishikawa, data not shown). The present work confirmed that *c-kit*$^+$ cells survive the ACK2 treatment, using an indirect labeling technique with a secondary antibody which would stain all cells which had previously bound ACK2 (Table 4).

Population size of B cells in the spleen of ACK2-treated mice. B cells ($s\mu^+$) were substantially increased in numbers in the spleen of ACK2-treated animals compared with saline-injected controls and normal mice (Fig.4). The number of $B220^+\mu^-$ cells was also increased.

DISCUSSION

The present study shows that the *c-kit* tyrosine kinase receptor is constitutively expressed by many early precursor B cells in mouse

bone marrow. Yet, paradoxically, *in vivo* treatment with a neutralizing anti-*c-kit* antibody, ACK2, which suppresses other forms of hemopoiesis, actually stimulates the proliferation of precursor B cells and the production of B lymphocytes. The findings raise questions concerning the role of the *c-kit* receptor in the interaction of precursor B cells and other lineages with stromal cells *in vivo*.

The total incidence of *c-kit*$^+$ cells in mouse bone marrow now detected by immunofluorescence labeling with mAb ACK2 directed against the extramembrane domain of the *c-kit* receptor accords well with the higher values of previous studies (21-23). B220$^+$ lineage cells in mouse bone marrow have previously been reported by flow cytometry either not to coexpress *c-kit* (22) or to do so only in low numbers (21). Epifluorescence microscopy of individual cells now demonstrates, however, that more than one half of the *c-kit*$^+$ cells coexpress surface B220 glycoprotein, representing about one fifth of the total B220$^+$ cells. Furthermore, *c-kit* is coexpressed by one half of the TdT$^+$ cells, a compartment whose *c-kit* status has not been previously reported. The findings indicate that *c-kit* is normally expressed in vivo by many pro-B cells during the phase of Ig heavy chain gene rearrangement but not by pre-B cells and subsequent stages after μ chain synthesis. This is consistent with *in vitro* functional studies indicating that B lineage clones which express *c-kit* receptor (28) and bone marrow cells which respond to synergistic stimulation by kit ligand and IL-7 (24) are characterized by D-J_H rearrangements, ie. are pro-B cells, whereas cultured pre-B cells in

which κ light chains are rearranged but not expressed have no transcripts for *c-kit* (32).

Pro-B cells which express the *c-kit* receptor are normally in active cell cycle *in vivo*. Their rates of entry into mitosis resemble those previously noted for TdT$^+$ and B220$^+\mu^-$ pro-B cells in general (2,3). In contrast, *c-kit*$^+$ cells which do not not coexpress B lineage markers appear to be kinetically distinct, entering mitosis at considerably higher rates. Accurate measurements of cell turnover can be made only from the linear accumulation of mitoses at multiple intervals after administering vincristine (1,2,30,31). However, assuming an initial lag period of 1-1.5h before vincristine becomes effective in arresting pro-B cells in metaphase (1,2), the present findings suggest a turnover time in the order of 8-12h for the *c-kit*$^+$B220$^+$ cell compartment but only 3.2-4.6h for the *c-kit*$^+$B220$^-$ population. The *c-kit*$^+$TdT$^+$ cells tend to be the largest cells of the TdT$^+$ population, all of which are in cell cycle (2). Possibly, *c-kit* expression may be cell cycle-related, becoming detectable during the later phases of the cell cycle. If so, the cell cycle duration would actually be longer than the apparent average cell cycle times indicated by the above turnover values.

Bone marrow cells express *c-kit* receptor in a range of intensities. The relatively low level of *c-kit* expression by B lineage precursors in the present work accords with previous observations in both mouse bone marrow (21) and pro-B cell clones *in vitro* (28). The *c-kit*$^+$ cells which do not coexpress B lineage markers are thus distinct by two

criteria, high proliferative activity and intense *c-kit* expression. These properties and their lymphoblastoid morphology are compatible with reports that the population of *c-kit*^{bright} cells in mouse bone marrow include primitive hemopoietic progenitors of the erythroid and myeloid lineages (22,21). The possible functional relationships between *c-kit*^{bright} progenitor cells and B-lineage committed cells remain to be established.

The physiological role of *c-kit* expression in precursor B cell development has been unclear. Based on the effects of kit ligand in enhancing responses to regulatory factors *in vitro*, a stimulatory role has been proposed. Kit ligand potentiates the effects of IL-7 in stimulating the proliferative responses of cultured precursor B cells showing D-J_H rearrangements (32) and B220 expression (24), and in promoting the formation of pre-B cell colonies (33,34). The well-documented synergistic effects of kit ligand on the stimulation of primitive hemopoietic precursor cells *in vitro* by a variety of cytokines (IL-1, IL-3, IL-6, EPO, GM-CSF) illustrate that a multiplicity of factors may regulate early precursor cell development in the bone marrow (25,26,27,35,36). The anti-*c-kit* antibody, mAb ACK2, provides a tool to examine the role of *c-kit* receptor expression by antagonizing *c-kit* function, without injuring the target cell (21,37). mAb ACK2 inhibits the stimulatory effects of kit ligand and IL-7 on early thymocytes (38), as well as arresting embryonic melanocyte development (37) and adult spermatogenesis (39) *in vivo*. mAb ACK2 also reduces the proliferation and growth rate of pro-B clones

cultured on stromal cells with IL-7 (28), reinforcing the notion that *c-kit* may serve a proliferative role in B cell genesis.

In view of the *c-kit* expression by early B-lineage cells together with the reported effects of kit ligand stimulation and *c-kit* receptor blockade, it is at first sight surprising that *in vivo* treatment with mAb ACK2 not only fails to depress B cell genesis in the bone marrow but actually stimulates precursor B cell production. Ogawa et al. reported that ACK2-treatment in mice suppressed erythropoiesis and myelopoiesis but the incidences of $B220^+$ cells and IL-7-responsive cells among residual bone marrow cells were higher than normal (21). The present study confirms these findings and further extends them by showing that precursor B cell populations in ACK2-treated mice are expanded in terms of actual numbers of cells per femur as well as incidence, that this expansion affects successive precursor compartments of intermediate pro-B cells, late pro-B cells, pre-B cells and B lymphocytes, and that in each of the proliferative precursor cell compartments an increased number of cells flows through mitosis per unit time. The results demonstrate that anti-*c-kit* antibody treatment *in vivo* produces a true increase in precursor B cell production from the initiation of Ig heavy chain gene rearrangement onwards, resulting in an expansion of the peripheral B cell pool.

The failure of mAb ACK2 treatment to block the proliferation of precursor B cells suggests, contrary to views based on *in vitro* studies, that *c-kit*-mediated signals are not essential for precursor B

cell development *in vivo*. This conclusion is consistent with findings that pre-B cell proliferation remains active in W/W^v and Sl/Sl^d mutant mice (40), and that substantial proliferation and growth of *c-kit*$^+$ precursor B cell clones can still persist in the presence of ACK2 *in vitro* (28). The reports of either a depressant effect of ACK2 or a stimulatory effect of kit ligand on precursor B cell proliferation *in vitro* have been based on IL-7-dependent systems (24,28,34). *In vivo*, however, defects of *c-kit* can apparently be compensated by the activity of alternative IL-7 potentiating signalling systems which may include Insulin-Like Growth Factor I (41).

The expression of the *c-kit* receptor may not necessarily correlate with its function. Mouse oocytes express *c-kit* strongly but ACK2 administration produces no evident defect in oocyte activation or ovulation, even though the same treatment depletes spermatogonia (39). Both *c-kit* and kit ligand are expressed in the brain, lungs, and placenta, tissues which are not apparently defective in W/W or Sl/Sl mutant mice (42). Thus, the lack of any depressant effect of ACK2 on early precursor B cells *in vivo* could indicate that they express the *c-kit* receptor in a nonfunctional form possibly in an alternatively spliced sequence (11). Possibly, however, the *c-kit* receptor may function *in vivo* to mediate effects other than proliferative stimulation (17,43,44). Interactions between *c-kit* and kit ligand could be involved in the microenvironmental localization, stromal cell adhesion and migration of early precursor B cells within the bone marrow (5).

The intriguing observation remains that ACK2 treatment actually stimulates precursor B cell proliferation and B lymphocyte genesis *in vivo*. Conversely, treatment of mice with kit ligand (stem cell factor) increases the number of erythroid and myeloid cells but markedly depresses the lymphoid cells in the bone marrow (S.C. Miller, personal communication). The mechanisms of these effects remain speculative. ACK2-binding might be postulated to deliver a positive signal to *c-kit*$^+$ precursor B cells. ACK2-binding does not functionally mimic the binding of kit ligand to *c-kit* (21), however, and in no other cell system has ACK2 been reported to have such a stimulatory effect. Alternatively, the effects of ACK2 treatment may be of an indirect nature. B lymphopoiesis occurs *in vivo* alongside erythropoiesis and granulopoiesis, precursor cells of each lineage being commonly associated with individual stromal reticular cells (45, and Jacobsen et al. unpublished observations). An altered activity in one lineage may indirectly favour or compromise the activity of others, as in the phenomenon of interlineage competition. Administration of recombinant G-CSF in mice stimulates granulopoieis in the bone marrow but simultaneously depresses erythropoiesis (46,47) and B lymphopoiesis (48). The latter cells rapidly recover and overshoot after G-CSF administration stops, showing that precursor B cell function had not been compromised. Erythropoietin administration *in vivo* accelerates erythropoiesis but neutrophil production is depressed (49). In human cyclic neutropenia, pre-B cells in the bone marrow fluctuate in inverse

relationship to the neutrophil cycle (50). Granulocyte precursors and lymphoid cells undergo reciprocal fluctuations in the bone marrow during post-irradiation regeneration (51,52). Administration of ACK2, by inhibiting erythropoiesis and granulopoiesis, may permit enhanced access of precursor B cells to stromal growth factors or survival factors, normally available in limiting quantities, thus indirectly stimulating B lymphopoiesis. The interlineage effects of anti-*c-kit* antibody treatment provide a model for further studies of the balance of microenvironmental factors which regulate lympho-hemopoiesis in the bone marrow.

TABLE 1

Incidence and metaphase index of *c-kit*$^+$ and TdT$^+$ cells in bone marrow[a]

Cell phenotype	Incidence (%)[b] Normal mice	Incidence (%)[b] Vincristine-treated mice	Metaphase Index (%)[c]
c-kit$^+$	6.6 ± 0.5	7.0 ± 0.3	25.8
c-kit$^+$ bright[d]	2.0 ± 0.2		
c-kit$^+$ dull[e]	4.7 ± 0.3		
TdT$^+$	1.4 ± 0.2	1.8 ± 0.2	13.3
TdT$^+$*c-kit*$^-$	0.7 ± 0.1	1.1 ± 0.1	14.0
TdT$^+$*c-kit*$^+$	0.6 ± 0.0	0.8 ± 0.0	13.0
TdT$^+$*c-kit*$^+$ bright[d]	0.1 ± 0.0		
TdT$^+$*c-kit*$^+$ dull[e]	0.5 ± 0.0		

a Each value was derived from three BALB/c mice (23-27g body weight) analyzed separately (mean±SE)

b Incidence relative to all nucleated cells of the bone marrow

c Percentage of cells of each phenotype in metaphase 2h40min after injecting vincristine sulfate ip

d Intense punctate labeling (>20 fluorescent spots/cell)

e Light punctate labeling (<20 fluorescent spots/cell)

TABLE 2

Incidence and metaphase index of *c-kit*$^+$ and B220$^+$ cells in bone marrow[a]

Cell phenotype	Incidence (%)[b] Normal mice	Incidence (%)[b] Vincristine-treated mice	Metaphase Index (%)[c]
c-kit$^+$	7.9 ± 0.3	7.4 ± 0.5	25.8
c-kit$^+$B220$^-$	3.6 ± 0.1	3.1 ± 0.3	36.4
B220$^+$	19.1 ± 0.3	18.1 ± 1.3	
B220$^+$*c-kit*$^-$	14.8 ± 0.2	13.8 ± 0.7	
B220$^+$*c-kit*$^+$	4.3 ± 0.2	4.4 ± 0.4	14.5

a Each value was derived from three BALB/c mice (23-27g body weight) analyzed separately (mean±SE)

b Incidence relative to all nucleated cells of the bone marrow

c Percentage of cells of each phenotype in metaphase 2h40min after injecting vincristine sulfate ip

TABLE 3

Incidence of B lineage cells in bone marrow and spleen of ACK2-treated BALB/c mice[a]

Cell phenotype	Normal[b]	Saline-treated[c]	ACK2-treated[d]
Bone marrow (%)[e]			
TdT^+B220^-	0.5	0.3	0.4
TdT^+B220^+	1.1	1.1	3.1
$B220^+\mu^-$	4.0	6.4	16.1
$c\mu^+s\mu^-$	13.0	11.3	37.2
$s\mu^+$	9.8	13.6	22.7
Total B lineage cells	28.4	32.7	79.5
Spleen (%)[e]			
$s\mu^+$	44.0	48.4	54.7
$B220^+\mu^-$	7.0	9.4	11.4

a Each value was derived from three BALB/c mice (23-27g body weight) analyzed separately (mean)
b Normal mice received no treatment
c Control mice received saline injections
d ACK2-treated mice received 5 iv injections of 1 mg ACK2 in saline on alternate days and were examined at 9d
e Incidence relative to all nucleated cells of the bone marrow and spleen

TABLE 4

c-kit+ cells in bone marrow of ACK2-treated BALB/c mice[a]

c-kit+	Normal[b]	Saline-treated[c]	ACK2-treated[d]
Incidence (%)[e]	6.6 ± 0.5	6.5 ± 0.9	7.4 ± 0.4
Cells/femur ($x10^5$)[f]	14.7 ± 0.4	11.7 ± 0.5	8.8 ± 0.7

a Values were derived from three normal BALB/c mice and two saline-injected and ACK-2-treated BALB/c mice(23-27g body weight) analyzed separately (mean±SE)

b Normal mice received no treatment

c Control mice received saline injections

d ACK2-treated mice received 5 iv injections of 1mg ACK2 in saline on alternate days and were examined at 9d

e Incidence relative to all nucleated cells of the bone marrow

f Calculated from the total number of cells/femur of each phenotype

Figure 1. Incidence and mitotic activity of total *c-kit*$^+$ cells and of *c-kit*$^+$ cells coexpressing either B220 glycoprotein or TdT in normal mouse bone marrow.

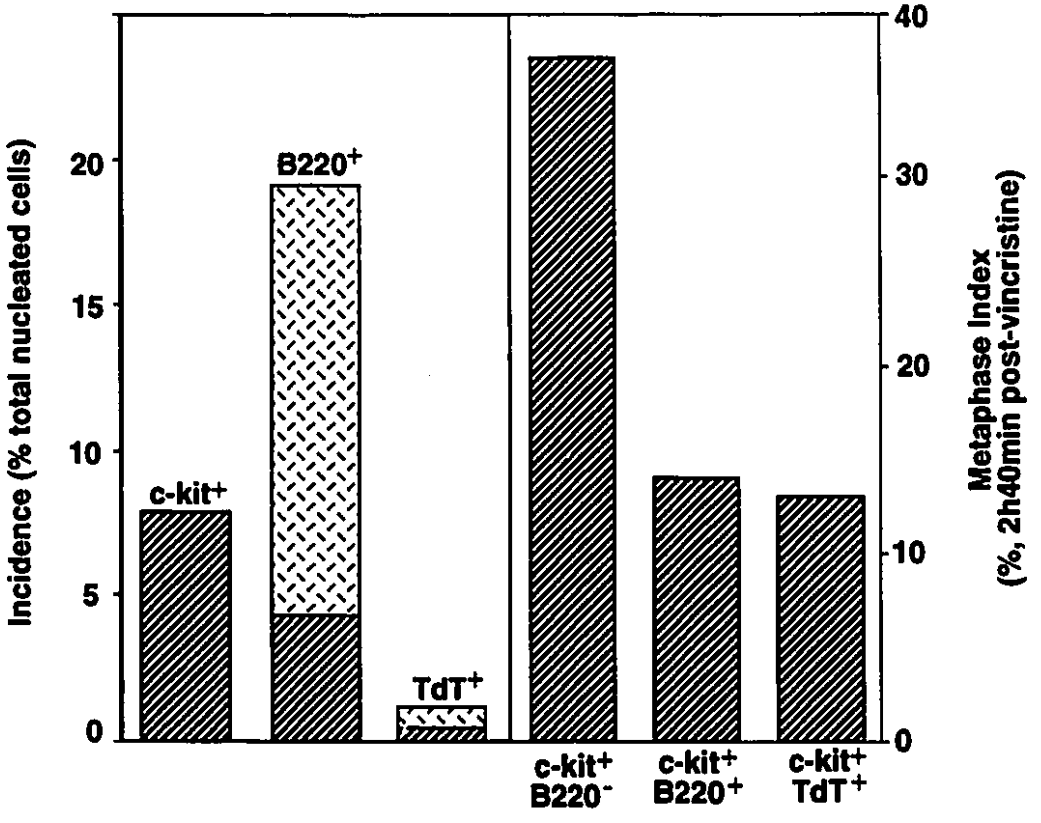

Incidence (% total nucleated cells)
0
5
10
15
20
B220+
c-kit+
TdT+
Metaphase Index
(%, 2h40min post-vincristine)
0
10
20
30
40
c-kit+
B220-
c-kit+
B220+
c-kit+
TdT+

Figure 2. Total nucleated cells in the bone marrow and spleen of normal mice, saline-injected control mice and mice treated with anti-*c-kit* antibody (mAb ACK2).

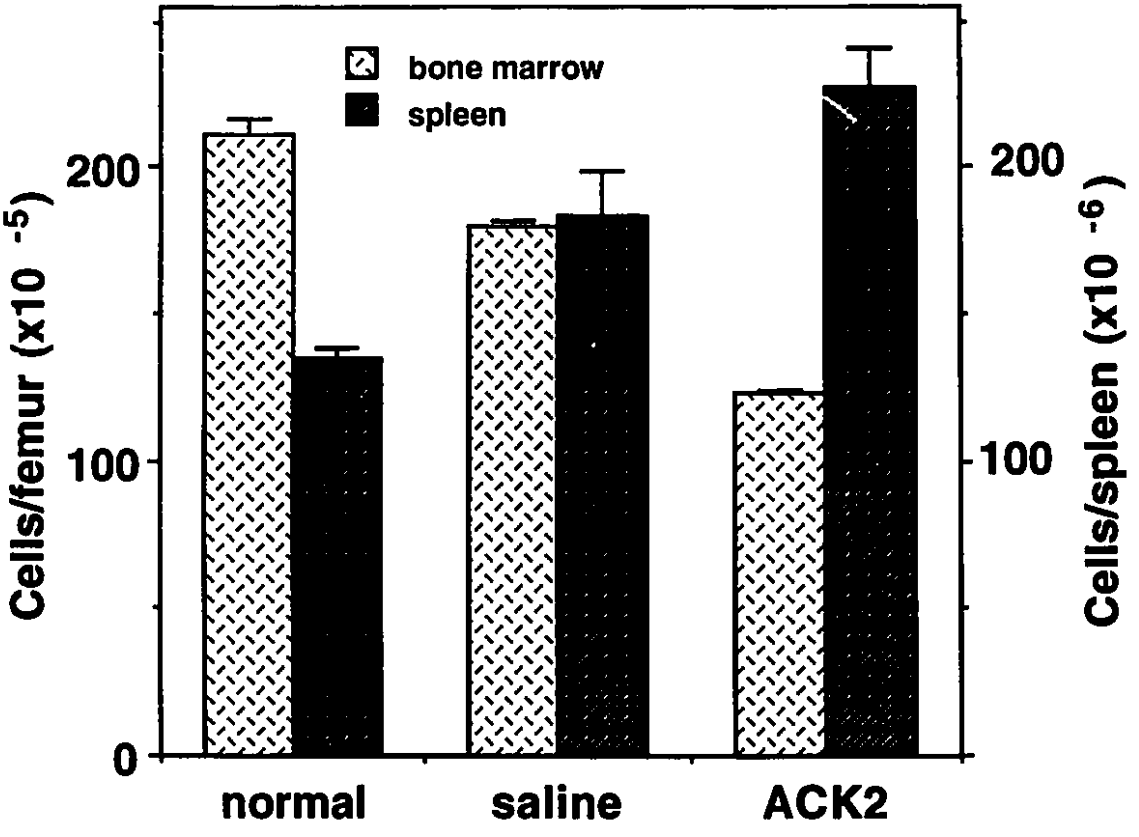

bone marrow
spleen
200
100
0
Cells/femur (x10 -5)
200
100
Cells/spleen (x10 -6)
normal
saline
ACK2

Figure 3. B lineage cells in the bone marrow of normal mice, saline-injected control mice and mice treated with anti-*c-kit* antibody (mAb ACK2), indicating the number of cells per femoral shaft (above) and the number of cells entering mitosis per unit time (2h40min) (below).

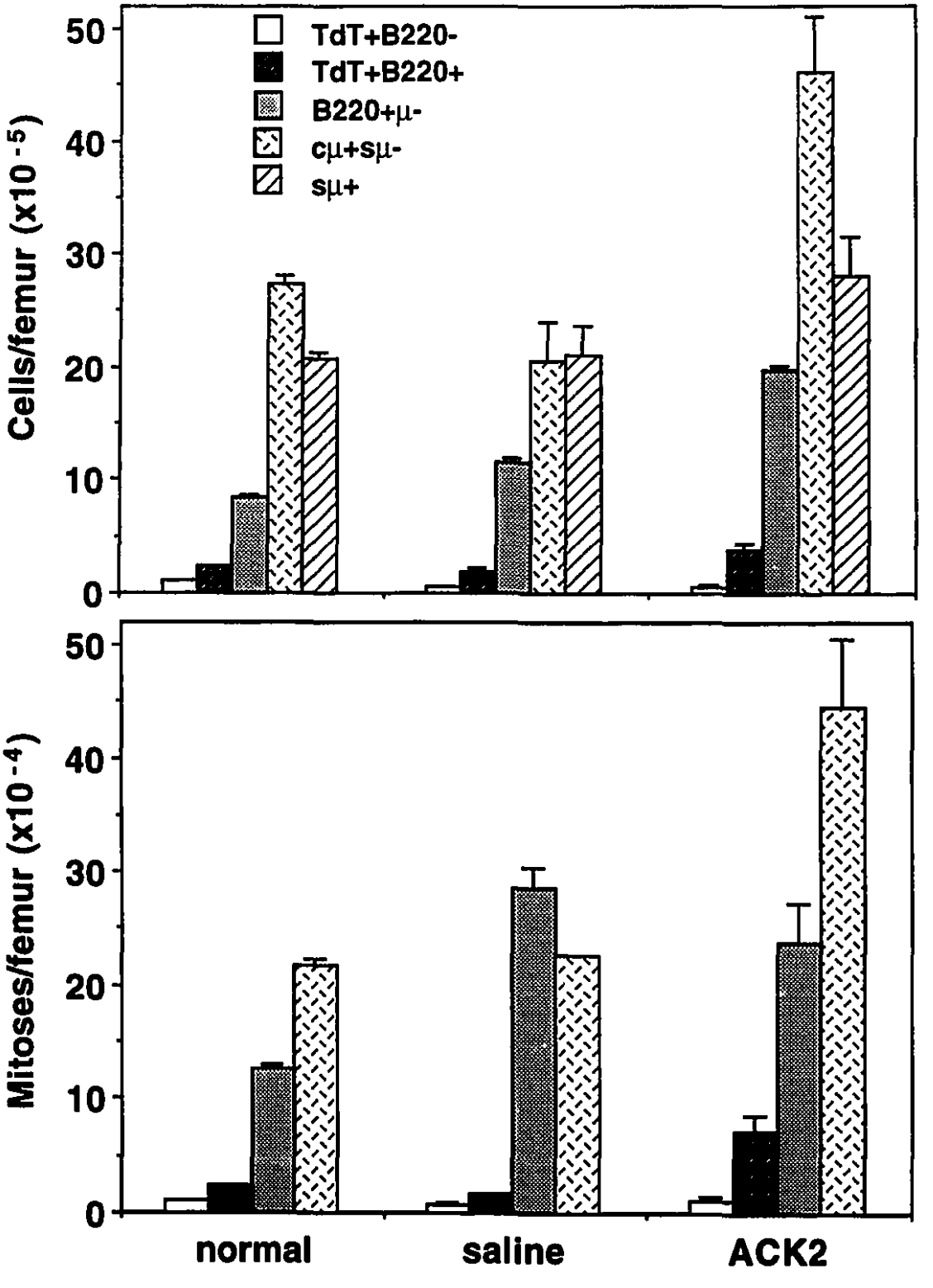
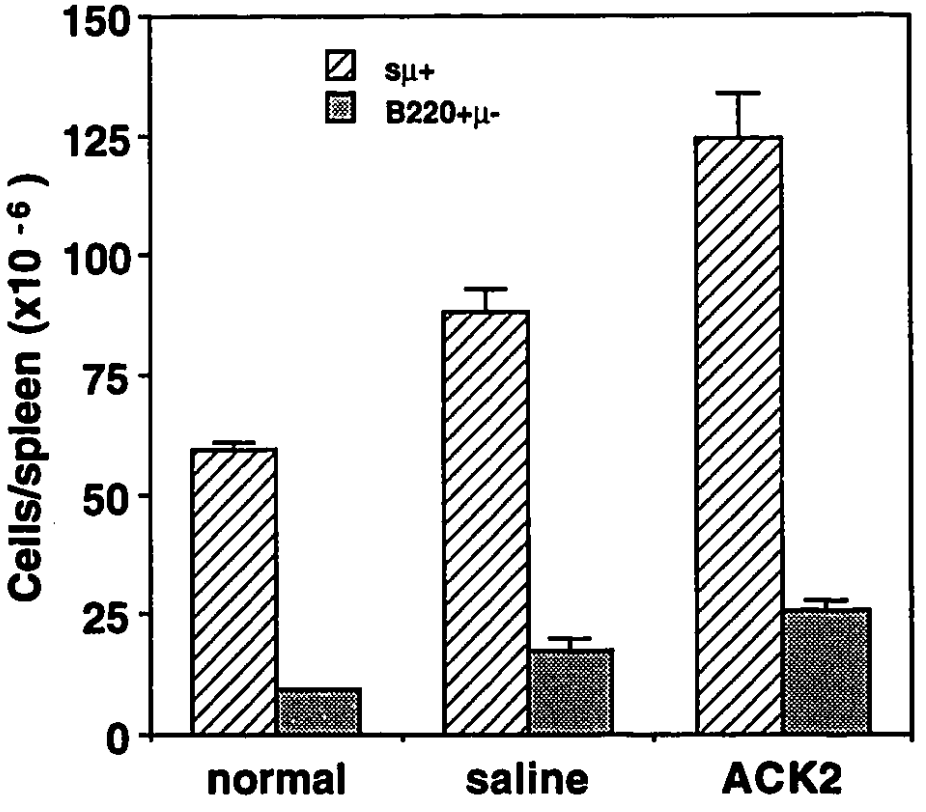

TdT+B220-
TdT+B220+
B220+μ-
cμ+sμ-
sμ+
Cells/femur (x10⁻⁵)
Mitoses/femur (x10⁻⁴)
0
10
20
30
40
50
normal
saline
ACK2

Figure 4. Number of B cells ($s\mu^+$) and $B220^+\mu^-$ cells in the spleen of normal mice, saline-injected control mice and mice treated with ant-*c-kit* antibody (mAb ACK2).


150
125
100
75
50
25
0
Cells/spleen (x10 -6)
sμ+
B220+μ-
normal
saline
ACK2

REFERENCES

1. Park, Y. H., and D.G. Osmond. 1987. Phenotype and proliferation of early B lymphocyte precursor cells in mouse bone marrow. *J. Exp. Med.* 165:444.

2. Park, Y.H., and D.G. Osmond. 1989. Dynamics of early Blymphocyte precursor cells in mouse bone marrow proliferation of cells containing terminal deoxynucleotidyl transferase. *Eur. J. Immunol.* 19:2139.

3. Osmond, D.G., and Y.H. Park. 1987. B Lymphocyte progenitors in mouse bone marrow. *Int. Rev. Immunol.* 2:241.

4. Jacobsen, K., J. Tepper, and D.G. Osmond. 1990. Early B lymphocyte precursor cells in mouse bone marrow: subosteal localization of $B220^+$ cells during post-irradiation regeneration. *Exp. Hematol.* 18:304.

5. Jacobsen, K. and D.G. Osmond. 1990. Microenvironmental organization and stromal cell association of B lymphocyte precursor cells in mouse bone marrow. *Eur. J. Immunol.* 20:2395.

6. Osmond, D.G. 1990. B cell development in the bone marrow. *Semin. Inmmunol.* 2:173.

7. Kincade, P.W., G. Lee, C.E. Pietrangeli, S. Hayashi, and J.M. Gimble. 1989. Cells and molecules that regulate B lymphopoiesis in bone marrow. *Ann.Rev. Immunol.* 7:111.

8. Kunisada, T., M. Ogawa, S-I. Hayashi, T. Era, and S-I. Nishikawa. 1992. Interleukin-7 and B lymphopoiesis. *In* Interleukins:

Molecular Biology and Immunology. Chem.Immunol. T. Kishimoto, editor. Karger, Basel. Vol 51, pp205-235.

9. Osmond, D.G. 1990. Proliferation kinetics and the life span of B cells in central and peripheral lymphoid organs. *Curr. Opinion Immunol.* 3:179.

10. Chabot, B., D.A. Stephenson, M.V. Chapman, P. Besmer, and A.Bernstein. 1988. The proto-oncogene *c-kit* encoding a transmembrane tyrosine kinase receptor maps to the mouse W locus. *Nature* 335:88.

11. Hayashi, S-I, K. Kunisada, M. Ogawa, K. Yamaguchi, and S-I. Nishikawa. 1991. Exon skipping by mutation of an authentic splice site of *c-kit* gene in W/W mouse. *Nucleic Acids Res.*19:1267.

12. Copeland, N.G., D.J. Gilbert, B.C. Cho, P.J. Donovan, N.A. Jenkins, D. Cosman, D. Anderson, S.D. Lyman, and D.E. Williams. 1990. Mast cell growth factor maps near the steel locus on mouse chromosome 10 and is deleted in a number of steel alleles. *Cell* 63:175.

13. Zsebo, K.M., J. Wypych, I.K. McNiece, H.S. Lu, K.A. Smith, S.B. Karkare, R.K. Sachdev, V.N. Yushenkoff, N.C. Birkett, L.R. Williams, V.N. Satyagal, W. Tung, R.A. Bosselman, E.A. Mendiaz, and K.E. Langley. 1990. Identification, purification, and biological characterization of hematopoietic stem cell factor from buffalo rat liver-conditioned medium. *Cell* 63:195.

14. Williams, D.E., J. Eisenman, A. Baird, C. Rauch, K. Van Ness, C.J. March, L.S. Park, U. Martin, D.Y. Mochizuki, H.S. Boswell, G.S. Burgess, D. Cosman, and S.D. Lyman. 1990. Identification of a ligand for the *c-kit* proto-oncogene. *Cell* 63: 167.

15. Huang, E., K. Nocka, D.R. Beier, T-Y. Chu, J. Buck, H-W Lahm, D. ellner, P. Leder, and P. Besmer. 1990. The hematopoietic growth factor KL is encoded by the Sl locus and is the ligand of the *c-kit* receptor, the gene product of the W locus. *Cell* 63:225.

16. Flanagan, J.G., D.C. Chan, and P. Leder. 1991. Transmembrane form of the *c-kit* ligand growth factor is determined by alternative splicing and is missing in the Sl^d mutant. *Cell* 64:1025.

17. Adachi, S., Y. Ebi, S-H. Nishikawa, S-I. Hayashi, M. Yamazaki, T. Kasugai, T. Yamamura, S. Nomura, and Y. Kitamura. 1992. Necessity of extracellular domain of W (*c-kit*) receptor for attachment of murine cultured mast cells to fibroblasts. *Blood* 79:650.

18. Ullrich, A. and J. Schlessinger. 1990. Signal transduction by receptors with tyrosine kinase activity. *Cell* 61:203.

19. Miyazawa, K., P.C. Hendrie, C. Mantel, K. Wood, L.K. Ashman, and H. E. Broxmeyer. 1991. Comparative analysis of signaling between mast cell growth factor (*c-kit* ligand) and granulocyte-macrophage colony stimulating factor in a human factor-dependent myeloid cell line involves phosphorilation of *raf-1*, GTPase-activating protein and mitogen-activated protein kinase. *Exp. Hematol.* 19:1110.

20. Avraham, H., E. Vannier, S. Cowley, S. Jiang, S. Chi, C.A. Dinarello, K.M. Zsebo, and J.E. Groopman. 1992. Effects of the stem cell factor, *c-kit* ligand, on human megakaryocytic cells. *Blood* 79:365.

21. Ogawa, O., Y. Matsuzaki, S. Nishikawa, S-I. Hayashi, T. Kunisada, T. Sudo, T. Kina, H. Nakauchi, and S.I. Nishikawa. 1991.

Expression and function of *c-kit* in hemopoietic progenitor cells. *J. Exp. Med.* 174:63.

22. Okada, S., H. Nakauchi, K. Nagayoshi, S. Nishikawa, S-I. Nishikawa, Y. Miura and T. Suda. 1991. Enrichment and characterization of murine hematopoietic stem cells that express *c-kit* molecule. *Blood* 78:1706.

23. Papayannopoulou, T., M. Brice, V. Broudy, and K.M. Zsebo. 1991. Isolation of *c-kit* receptor-expressing cells from bone marrow, peripheral blood, and fetal liver: Functional properties and composite antigenic profile. *Blood* 78:1403.

24. Billips, L.G., D. Petitte, K. Dorshkind, R. Narayanan, C-P. Chiu, and K.S. Landreth. 1992. Differential roles of stromal cells, interleukin-7 and kit-ligand in the regulation of B lymphopoiesis. *Blood* 79:1185.

25. Bodine, D.M., D. Orlic, N.C. Birkett, N.E. Seidel, and K.M. Zsebo. 1992. Stem cell factor increases colony-forming unit-spleen number in vitro in synergy with interleukin-6, and in vivo in Sl/Sl^d mice as a single factor. *Blood* 73:913.

26. Pietsch, T., U. Kyas, U. Steffens, E. Yakisan, M.R. Hadam, W-D. Ludwig, K.M. Zsebo, and K. Welte. 1992. Effects of human stem cell factor (*c-kit* ligand) on proliferation of myeloid leukemia cells: heterogeneity in response and synergy with other hematopoietic growth factors. *Blood* 80:1199.

27. Muench, M.O., J.G. Schneider, and M.A.S. Moore. 1992. Interactions among colony-stimulating factors, IL-1, IL-6, and kit-ligand in the regulation of primitive murine hematopoietic cells. *Exp. Hematol.* 20:339.

28. Rolink, A., M. Streb, S-I. Nishikawa, and F. Melchers. 1991. The *c-kit*-encoded tyrosine kinase regulates the proliferation of early pre-B cells. *Eur. J. Immunol.* 21:2609.

29. Kincade, P.W, G. Lee, T. Watanabe, L. Sun, and M. P. Scheid. 1981. Antigens displayed on murine B lymphocyte precursors. *J. Immunol.* 127:2262.

30. Opstelten, D., and D. G. Osmond. 1983. Pre-B cells in mouse bone marrow: immunofluorescence stathmokinetic studies of the proliferation of cytoplasmic μ-chain-bearing cells in normal mice. *J. Immunol.* 131:2635.

31. Wright, N. A., and D.R. Appleton. 1980. The metaphase arrest technique. A critical review. *Cell tiss. Kinet.* 13:643.

32. Henderson, A.J., R. Narayanan, L. Collins, and K. Dorshkind. 1992. Status of kL chain gene rearrangements and *c-kit* and IL-7 receptor expression in stromal cell-dependent pre-B cells. *J. Immunol.* 149:1973.

33. Martin, F.H., S.V. Suggs, K.E. Langley, H.S. Lu, J. Ting, K.H. Okino, C.F. Morris, I.K. McNiece, F.W. Jacobsen, E.A. Mendiaz, N.C. Birkett, K.A. Smith, M.J. Johnson, V.P. Parker, J.C. Flores, A.C. Patel, E.F. Fisher, H.O. Erjavec, C.J. Herera, J. Wypych, R.K. Sachdev, J.A. Pope, I. Leslie, D. Wen, C-H. Lin, R.L. Cupples, and K.M. Zsebo. 1990. Primary structure and functional expression of rat and human stem cell factor DNAs. *Cell* 63:203.

34. McNiece, I.A., K.E. Langley, and K.M. Zsebo. 1991. The role of recombinant stem cell factor in early B cell development. *J. Immunol.* 146: 3785.

35. Leary, A.G., H.Q., Zeng, S.C., Clark, and M. Ogawa. 1992. Growth factor requirements for survival in G_0 and entry into the cell

cycle of primitive human hemopoietic progenitors. *Proc.Natl. Acad. Sci. USA.* 89:4013.

36. Shull, R.M., S.V., Suggs, K.E., Langley, K.H. Okino, F.W. Jacobsen, and F.H. Martin. 1992. Canine stem cell factor (*c-kit* ligand) supports the survival of hemopoietic progenitors in long-term canine marrow culture. *Exp. Hematol.* 20:1118.

37. Nishikawa, S., M. Kusakabe, K. Yoshinaga, M. Ogawa, S-I. Hayashi, T. Kunisada, T. Era, T. Sakakura, and S-I. Nishikawa. 1991. In utero manipulation of coat color formation by a monoclonal anti-*c-kit* antibody: two distinct waves of *c-kit*-dependency during melanocyte development. *EMBO J.* 10:2111.

38. Godfrey, D.I., Zlotnik, A., and T. Suda. 1992. Phenotypic and functional chraracterization of *c-kit* expression during intrathymic T cell development. *J. Immunol.* 149:2281.

39. Yoshinaga, K., S. Nishikawa, M. Ogawa, S. Hayashi, T. Kunisada, T. Fujimoto, and S-I. Nishikawa. Role of c-kit in mouse spermatogenesis: identification of spermatogonia as a specific site of *c-kit* expression and function. *Development* (in press).

40. Opstelten, D., and D.G. Osmond. 1985. Regulation of pre-B cell proliferation in bone marrow: immunofluorescence stathmokinetic studies of cytoplasmic μ chain-bearing cells in anti-IgM-treated mice, hematologically deficient mutant mice and mice given sheep red blood cells. *J. Immunol.* 15:599.

41. Dorshkind, K., N. Ramaswamy, and K.S. Landreth. 1993. Identification of stromal cell derived soluble mediators that regulate primary B lymphopoiesis. *J.Cell Biochem.* (in press)

42. Matsui, Y., K.M. Zsebo, and L.M. Hogan. 1990. Embryonic expression of a haematopoietic growth factor encoded by the Sl locus and the ligand for *c-kit*. *Nature* 347:667.

43. Bischop, S.C., and C.A. Dahinden. 1992. *c-kit* ligand: a unique potentiator of mediator release by human lung mast cells. *J. Exp. Med.* 175:237.

44. Weshil, B.K., M. Tsai, E.N. Geissler, K.M. Zsebo, and S.J. Galli. 1992. The rat *c-kit* ligand, stem cell factor, induces *c-kit* receptor-dependent mouse mast cell activation in vivo. Evidence that signaling through the *c-kit* receptor can induce expression of cellular function. *J. Exp. Med.* 175:245.

45. Jacobsen, J., K. Miyake, P.W. Kincade, and D.G. Osmond. 1992. Highly restricted expression of a stromal cell determinant in mouse bone marrow in vivo. *J. Exp. Med.* 176:927.

46. Podja, Z., G. Molineux, and T.M. Dexter. 1990. Hemopoietic effects of short-term in vivo treatment of mice with various doses of rhG-CSF. *Exp. Hematol.* 18:27.

47. de Haan, G, M. Loeffler, and W. Nijhof. 1992. Long-term recombinant human granulocyte colony-stimulating factor (rhG-CSF) treatment severely depresses murine marrow erythropoiesis without causing an anemia. *Exp. Hematol.* 20:600.

48. Dorshkind, K. 1991. In vivo administration of recombinant granulocyte-macrophage colony-stimulating factor results in a reversible inhibition of primary B lymphopoiesis. *J. Immunol.* 146:4204.

49. Christensen, R.D., K.W. Liechty, J.M. Koenig, K.R. Schibler, and R.K. Ohls. 1991. Administration of erythropoietin to newborn

rats results in diminished neutrophil production. *Blood* 78:1241.

50. Engelhard, D., K.S. Landreth, N. Kapoor, P.W. Kincade, L.E. DeBault, A. Theodore, and R.A. Good. 1983. Cycling of peripheral blood and marrow lymphocytes in cyclic neutropenia. *Proc. Natl. Acad. Sci. USA* 80:5734.

51. Harris, P.F. (1956). Quantitative examination of bone marrow in guinea-pigs after gamma irradiation. *Brit. med. J.* ii:1032.

52. Osmond, D.G., P.J. Roylance, W.R. Lee, T.G. Ramsell, and J.M. Yoffey. 1966. The effect of unilateral limb shielding on the haemopoietic response of the guinea-pig to gamma irradiation (150r.). *Brit. J. Haemat.* 12:365.

CHAPTER 7

Production and loss of B lymphocyte precursor cells in the bone marrow of Ig-transgenic-scid mice

ABSTRACT

To examine the role of IgM gene expression in the proliferation and survival of precursor B cells, we have quantitated B cell genesis in the bone marrow of mutant scid (severe combined immunodeficiency) mice, unable to rearrange Ig genes, and scid mice bearing transgenes for λ chains, μ chains and μ+λ chains of IgM. Immunolabeling and epifluorescence microscopy have been used to define precursor B cells by expression of TdT, B220 glycoprotein, and cytoplasmic or surface μ chains (cμ,sμ), noting cells in mitosis after giving vincristine. The number and proliferative activity of early and intermediate pro-B cells (TdT^+B220^-; TdT^+B220^+) were normal in all mouse groups. Scid and λ-transgenic-scid mice produced some late pro-B cells ($TdT^-B220^+\mu^-$), but no pre-B ($c\mu^+s\mu^-$) or B ($s\mu^+$) cells. μ-transgenic-scid mice produced more late pro-B cells and many pre-B cells, but no B cells. μλ-transgenic-scid mice produced variable numbers of pre-B and B cells. The results demonstrate that B lineage cells begin to differentiate normally regardless of their capacity to express Ig genes, but then they all abort if unable to express IgM, the stage of cell loss depending upon the stage of Ig defect. The findings provide models for further studies of the mechanisms of B cell death and selection in the bone marrow.

INTRODUCTION

Mice homozygous for the severe combined immunodeficiency (scid) autosomal mutation are devoid of mature B and T lymphocytes as a result of defective antigen receptor gene recombination (Bosma et al. 1983, Schuler et al 1986, Lieber et al. 1988, Malynn et al. 1988, Okazaki et al. 1988). However, scid mouse bone marrow contains certain early B cell precursors (Fulop et al. 1988, Osmond et al. 1992). Studies of cell population dynamics in scid bone marrow show normal numbers and proliferation of early and intermediate pro-B cells, while late pro-B cells are depleted and no pre-B cells or B lymphocytes are detected. Thus, after beginning to develop with normal population dynamics, the developing B lineage cells, being unable to rearrange μ chain genes, all appear to abort during the late pro-B cell stage (Osmond et al. 1992). In the present study, we have examined the role of IgM expression in the proliferation and survival of B cell precursors in scid mice bearing transgenes for λ chains, μ chains and μ+λ chains of IgM.

MATERIALS and METHODS

Mice. μ-transgenic mice were the M54 transgenic line carrying a functional μ heavy-chain transgene that was derived from the hybridoma 17.2.25 as described by Grosschedl et al. (1984). λ-transgenic mice carrying a functional light-chain transgene also derived from the 17.2.25 hybridoma were prepared by F.W. Alt et al.

(Columbia University, New York, NY) (unpublished). μλ-transgenic mice were derived by R.A. Phillips (Ontario Cancer Institute, Toronto, ON) by crossing the μ- and λ–transgenic mice. Ig-transgenic-scid mice were derived by R.A. Phillips, by crossing the transgenic mice with C.B-17/Icr scid mice (Bosma et al. 1983). All animals were kindly provided by R.A. Phillips. Mice were used when 18-32 weeks old.

The population sizes and production rates of the different B cell precursors were determined using double immunofluorescence labeling and mitotic arrest techniques, as described in Chapter 2. Mice were analyzed individually.

RESULTS

Total cell recovery in bone marrow and spleen of Ig-transgenic-scid mice. Total nucleated cells in bone marrow of scid mice were approximately 60% of those recovered for normal mice. The Ig-transgenic-scid mice showed similar or somewhat higher values (Table 1, Fig. 1). The numbers of spleen nucleated cells were subnormal in scid mice (approximately 70% normal), and even lower in the various Ig-transgenic-scid mice (Table 1, Fig. 1).

B cell precursors in bone marrow of Ig-transgenic-scid mice. All three populations of pro-B cells were detected in the bone marrow of scid mice, λ-transgenic-scid mice, μ-transgenic-scid mice and λμ-

transgenic-scid mice. The incidence and number of early pro-B cells (TdT^+B220^-) and intermediate pro-B cells (TdT^+B220^+) in scid mice resemble normal values (Tables 2 and 3, Fig. 2). $B220^+\mu^-$ cells were subnormal in incidence and absolute numbers (Tables 2 and 3). By subtraction, the number of late pro-B cells was near one third of normal numbers. These values observed for B cell precursors in the bone marrow of scid mice are similar to previously reported values in scid mice (Osmond et al. 1992). No difference in population size of early and intermediate pro-B cells was found between the three groups of Ig-transgenic-scid mice and scid mice (Tables 2 and 3, Fig. 2). In λ-transgenic-scid mice, the number of $B220^+\mu^-$ cells was similar to that in scid mice. In contrast, in μ-transgenic-scid mice and μλ-transgenic-scid mice the $B220^+\mu^-$ cells were considerably more numerous than in scid mice (Table 3, Fig. 2). Pre-B cells ($c\mu^+s\mu^-$) and B lymphocytes ($s\mu^+$) were completely absent in both scid and λ-transgenic-scid mice (Table 2, Fig. 2). μ-transgenic-scid mice showed considerable numbers of pre-B cells but no B cells (Tables 2 and 3, Fig. 2). μλ-transgenic-scid mice had low numbers of both pre-B cells and B lymphocytes (Tables 2 and 3, Fig. 2).

One μλ-transgenic-scid mouse behaved differently from the others, having near normal percentages of late pro-B cells, pre-B cells and B lymphocytes in the bone marrow and B lymphocytes in the spleen. These values are not included in the tables and figures.

Proliferative kinetics of B cell precursors in bone marrow of Ig-transgenic-scid mice. Early, intermediate and late pro-B cells in scid mouse bone marrow were in active mitotic cell cycle. The numbers of TdT^+B220^- cells and TdT^+B220^+ cells which accumulated in metaphase within a standard time interval after administering vincristine sulfate were similar in scid, λ-transgenic-scid, μ-transgenic-scid, and λμ-transgenic-scid mice, (Tables 2 and 3, Fig. 2), and were similar to normal values (not shown). In contrast, the number of mitotic $B220^+\mu^-$ cells, similar in all four experimental groups of animals (Fig. 2), was only one fifth of normal values (not shown). The numbers of pre-B cells passing through mitosis in μ-transgenic-scid mice and μλ-transgenic-scid mice represented only 25% and 5% of the respective values previously reported for normal mice (Tables 2 and 3, Fig. 2).

Cell populations in the spleen of Ig-transgenic-scid mice. No $s\mu^+$ cells were observed in scid, λ-transgenic-scid or μ-transgenic-scid mouse spleens, while $s\mu^+$ B lymphocytes were present in low numbers, (5.8%; $3.4x10^5$), in μλ-transgenic-scid mice (Tables 2 and 3, Fig. 3).

DISCUSSION

B cell genesis in mouse bone marrow is a highly regulated process influenced by genetic, microenvironmental and systemic regulatory

factors (Kincade et al. 1987). During this process, two important events occur; a clonal expansion among early B cell precursors followed by a marked cell loss (75%) (Park and Osmond 1989, Osmond 1990). In the present study we have examined the role of IgM expression in the proliferation, differentiation and survival of precursor B cells. We have compared scid mutant mice, which are virtually unable to rearrange endogenous Ig genes successfully, and scid mice bearing transgenes for λ chains, μ chains and μ+λ chains. The results demonstrate that B lineage cells are able to begin to differentiate normally, regardless of their capacity to express Ig genes, but in the absence of IgM expression they all abort. This occurs at various stages of differentiation, depending upon the stage of the defect in the Ig gene rearrangement.

Scid mice show the presence of the three pro-B cell populations; early, intermediate and late pro-B cells. Whereas the first two populations are present in previously described normal numbers, the late pro-B cells are decreased in numbers. The pro-B cell populations are all active in cell cycle. They are not, therefore, in a state of static arrest, but are moving along the differentiation pathway. No pre-B cells or B lymphocytes are identified. This confirms the previous findings of a complete cell abortion and deletion at the late pro-B cell stage in the abscence of successful IgH chain gene rearrangement (Osmond et al. 1992).

In mice transgenic for Ig genes, the expression of endogenous Ig genes is impaired by inhibition of gene rearrangement but the

transgene is expressed (Weaver et al. 1985, Ritchie at al. 1984, Neuberger et al. 1989). Mice used in the present study were all tested by analysis of tail DNA samples (conducted by Dr. F. Alt, Howard Hughes Med. Inst., Boston, MA) to confirm that they expressed the transgenes. .

The introduction of a functional λ transgene into scid mice does not restore the normal flow of differentiation, nor promote leakiness from late pro-B cells to pre-B cells and B lymphocytes. The λ-transgenic-scid mice in this respect behave as scid mice. Thus, in the absence of μ heavy chain gene rearrangements, the precursor B cell aborts at an early stage, before any substantial light chain gene rearrangements would normally be activated.

The μ-transgenic-scid mice produce higher numbers of late pro-B cells than the scid mice as well as substantial, though subnormal numbers, of pre-B cells. The total number of pre-B cells is 60% normal and the numbers passing through mitosis per unit time are 25%-30% normal. Thus, the functionally rearranged μ transgene apparently provides the necessary signal to rescue cells up to the pre-B cell stage. The disproportionately low numbers of pre-B cells passing through mitosis suggest that many of the pre-B cells are in the postmitotic stage of development when light chain gene rearrangements would normally be occurring. However, in the absence of a functional light chain and of the ability to formulate a complete Ig molecule, all cells abort at the pre-B cell stage.

A complete differentiation of precursor B cells into B lymphocytes is achieved in μλ-transgenic-scid mice. Surprisingly, while the population sizes and proliferation rates of the pro-B cells are similar to those in μ-transgenic-scid mice, the numbers of pre-B cells in the μλ-transgenic-scid animals are substantially less than μ-transgenic-scid mice, and the number of B lymphocytes in the bone marrow only reaches one tenth normal values. B cells also appear in the spleen of μλ-transgenic-scid animals, but their numbers are only 5% of normal values. Therefore, μλ-transgenes in scid mice, permitting the expression of complete IgM molecules, can rescue B cells, but they do not fully reconstitute the bone marrow and peripheral B lymphocyte pool. As indicated, one of the μλ-transgenic-scid animals in the present work behaved differently from the others, with high numbers of pre-B cells and B cells, as well as splenic B lymphocytes. Such a heterogeneity in the number of peripheral B cells and immunoglobulin levels has been found occasionally in μλ-transgenic-scid mice (R.A. Phillips, personal communication). The responsible factors remain to be identified.

B lymphocyte monospecificity is normally ensured by allelic exclusion. The production of a functional heavy chain gene rearrangement normally inhibits further gene rearrangements on the homologous chromosome. A similar event occurs in light chain formation and between the *kappa* and *lambda* chain genes (isotypic exclusion). Studies with transgenic mice show that allelic exclusion may depend partially on a feedback gene rearrangement regulation

(Weaver et al. 1985, Ritchie et al. 1985, Neuberger et al. 1989). Possibly, the subnormal numbers of pre-B cells and B cells in μ and μλ-transgenic-scid mice reflect an unsuccessful attempt to rearrange endogenous Ig genes, despite the presence of a functionally rearranged transgene, with consequent cell elimination (Reichman-Fried et al. 1990). The suppression of endogenous Ig genes may fail because the transgene is not expressed early enough or at high enough levels to block endogenous recombination.

The presence of relatively low numbers of B lymphocytes in the spleen of μλ-transgenic-scid animals may reflect an inability of these virgin cell clones to expand or to be selected into a long-lived peripheral B lymphocyte pool. Newly-formed B lymphocytes from the bone marrow need to mature for a further 1-4 day period before becoming fully immunologically responsive (Osmond 1986). Their total life span is usually programmed to be short if they do not become activated in the peripheral tissues (Osmond 1993). In normal conditions, the diverse specificities of newly-generated B cells ensure that some will encounter their specific antigen, and subsequently enter the activated state which leads to longevity among some of their progeny and expansion of the peripheral B cell pool. Since the B cell progenitors in μλ-transgenic-scid mice all contain the same rearranged transgenes, they will bear the same specificity. As a result, most of the newly-formed B cells will presumably soon die in the peripheral lymphoid tissues due to the

low probability of encountering antigen which matches their specificity, as well as the lack of T cell help.

A substantial degree of cell loss is a characteristic of B cell genesis (Opstelten and Osmond 1985, Park and Osmond 1989, Deenen et al. 1990). Aberrant Ig gene recombination is thought to be a major cause of cell elimination (Alt et al. 1987, Osmond 1990). One mechanism of cell death is by apoptosis, characterized morphologicaly by cell size reduction and nuclear condensation, followed by cellular and nuclear fragmentation (Wyllie et al. 1980). *In situ* electron microscopy has revealed B lineage cells undergoing apoptosis in scid mice and being eliminated by resident macrophages (Osmond et al. 1992).

The findings are generally consistent with those of Reichman-Fried et al (1990), who demonstrated, using another transgene construct, that the μ transgene increased the number of $B220^{+}sIgM^{-}$ precursor cells in the bone marrow of scid mice but did not produce $B220^{+}sIgM^{+}$ B lymphocytes, while μκ transgenes permitted the development of subnormal numbers of both $B220^{+}sIgM^{-}$ precursor cells and $B220^{+}sIgM^{+}$ B lymphocytes. In addition, the present work provides an analysis of the successive phenotypic populations of precursor B cells and their proliferative dynamics in Ig-transgenic-scid mice, demonstrating, as in scid mice, that the cells are not in a state of developmental arrest but continue to enter the B cell lineage and to differentiate and proliferate actively until they abort, thus allowing the stage of abortion to be identified and correlated with the state of

Ig gene rearrangement. One substantial difference in findings is the virtual absence of peripheral B lymphocytes previously reported in the spleen and lymph nodes, even in μκ-transgenic-scid mice. The reason for this discrepancy in results remains to be established.

The expression of Ig genes is clearly of central importance in the development of precursor B cells in the bone marrow. Successful Ig rearrangements are essential for cell survival. The present work stresses the probability that much of the cell loss normally seen in B cell genesis may occur as a result of ineffective Ig gene rearrangements. Since the loss is normally most evident at the pre-B cell stage the aberrant process would appear to involve mainly the later stages of H chain gene rearrangement (V_H-D_HJ_H joining) or the rearrangement of L chain genes and the pairing of H and L chains. The normal process of cell abortion and deletion thus provides an effective mechanism to prevent useless or potentially harmful B cells reaching the peripheral immune system. Whether this represents a process of positive or negative selection, remains to be seen. Possibly, all precursor B cells may be programmed to die unless positively rescued by survival signals, including the successful completion of Ig gene rearrangements, leading to the expression of genes blocking the cell death programme (eg. *bcl-2*). Alternatively, otherwise viable precursor cells may be negatively selected by death signals, including unsuccessful Ig gene rearrangement, which initiate a cell death programme. Continuing work on *in situ* organization of B lineage cells in bone marrow of Ig-transgenic-scid mice is being

conducted to examine the role of apoptotic cell death and macrophage-mediated deletion.

The present study has established scid and Ig-transgenic-scid mice as useful models for further studies on the production of genetically aberrant B cell precursors, as well as the culling mechanisms which either select them out or permit them to enter the peripheral B cell pool.

TABLE 1

Total nucleated cells in bone marrow and spleen of scid/Ig-transgenic mice[a]

	scid	scid/λ	scid/μ	scid/μλ
Bone marrow cells ($x10^5$)				
	105.6 ± 15.2	128.2 ± 6.7	142.3 ± 21.9	112.5 ± 9.5
Spleen cells ($x10^6$)				
	98.5 ± 25.7	61.4 ± 2.0	63.7 ± 10.0	59.1 ± 1.0

[a] Each value was derived from three Ig-transgenic-scid mice (18-32 wk) analyzed separately (mean± standard error)

TABLE 2

Incidence and metaphase index of B lineage cells in bone marrow and spleen of Ig-transgenic-scid mice[a]

Cell phenotype	scid	scid/λ	scid/μ	scid/μλ
Bone marrow (%)[b]				
TdT^+B220^-	0.7 ± 0.1	0.8 ± 0.1	0.6 ± 0.0	0.7 ± 0.0
TdT^+B220^+	2.6 ± 0.1	2.7 ± 0.1	2.7 ± 0.2	2.9 ± 0.1
$B220^+\mu^-$	3.9 ± 0.2	4.2 ± 0.1	4.8 ± 0.1	5.1 ± 0.4
$c\mu^+s\mu^-$	0.0	0.1 ± 0.1	11.1 ± 0.4	3.2 ± 0.0
$s\mu^+$	0.0	0.0	0.0	2.3 ± 0.0
Spleen (%)[b]				
$s\mu^+$	0.0	0.0	0.0	5.8 ± 0.3
$B220^+\mu^-$	0.5 ± 0.1	0.5 ± 0.0	1.3 ± 0.1	1.2 ± 0.1
Bone marrow cells in metaphase (%)[c]				
TdT^+B220^-	6.3 ± 0.7	6.6 ± 0.9	7.3 ± 0.7	6.5 ± 0.5
TdT^+B220^+	8.6 ± 0.3	9.0 ± 0.6	10.0 ± 0.0	9.5 ± 1.5
$B220^+\mu^-$	10.0 ± 0.6	8.3 ± 0.3	8.6 ± 0.9	9.5 ± 0.5
$c\mu^+s\mu^-$	0.0	0.0	3.3 ± 0.9	2.5 ± 0.5

a Each value was derived from three Ig-transgenic-scid mice (18-32 wk) analyzed separately (mean± standard error)

b Incidence relative to all nucleated cells of the bone marrow and spleen

c The percentage of cells of each phenotype in metaphase (metaphase index) 2h40min after injecting vincristine sulfate ip

TABLE 3

Number of B lineage cells in bone marrow and spleen of Ig-transgenic-scid mice[a]

Cell phenotype	scid	scid/λ	scid/μ	scid/μλ
Bone marrow cells ($x10^5$)[b]				
TdT^+B220^-	0.8 ± 0.2	1.1 ± 0.1	0.8 ± 0.1	0.8 ± 0.0
TdT^+B220^+	2.7 ± 0.3	3.5 ± 0.3	3.8 ± 0.4	3.3 ± 0.3
$B220^+\mu^-$	4.4 ± 0.6	5.7 ± 0.5	6.2 ± 1.2	5.6 ± 0.7
$c\mu^+s\mu^-$	0.0	0.0	16.0 ± 3.1	3.6 ± 0.2
$s\mu^+$	0.0	0.0	0.0	2.5 ± 0.1
Spleen cells ($x10^6$)[b]				
$s\mu^+$	0.0	0.0	0.0	3.4 ± 0.2
$B220^+\mu^-$	0.5 ± 0.1	0.2 ± 0.0	0.8 ± 0.2	0.7 ± 0.0
Bone marrow cells in metaphase ($x10^4$)[c]				
TdT^+B220^-	0.4 ± 0.1	0.7 ± 0.1	0.6 ± 0.0	0.5 ± 0.0
TdT^+B220^+	2.3 ± 0.3	3.1 ± 0.4	3.7 ± 0.4	3.2 ± 0.0
$B220^+\mu^-$	4.3 ± 0.7	4.7 ± 0.3	5.1 ± 0.5	5.3 ± 0.8
$c\mu^+s\mu^-$	0.0	0.0	5.8 ± 2.6	0.9 ± 0.1

a Each value was derived from three Ig-transgenic-scid mice (18-32 wk) analyzed separately (mean± standard error)

b Calculated from the incidence of cells of each phenotype relative to all nucleated cells and the total cellularity of the bone marrow and spleen respectively

c Calculated from the number of cells of each phenotype and the percentage of cells of each phenotype in metaphase (metaphase index) 2h40min after injecting vincristine sulfate ip

Figure 1. Total number of nucleated cells in scid, λ-transgenic-scid, μ-transgenic-scid, and μλ-transgenic-scid mouse bone marrow and spleen.

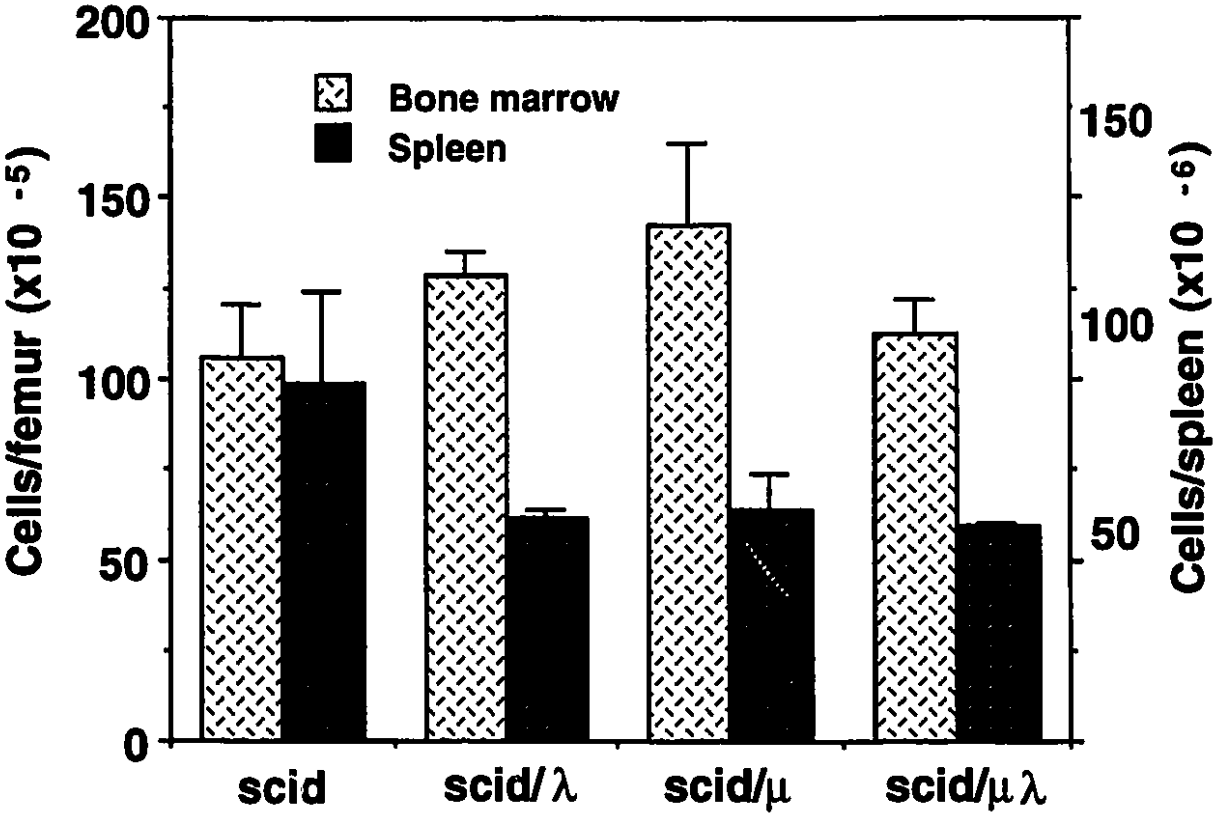

200
150
100
50
0
Cells/femur (x10 -5)
Bone marrow
Spleen
150
100
50
Cells/spleen (x10 -6)
scid
scid/λ
scid/μ
scid/μλ

Figure 2. Number of B lineage cells (above) of various phenotypes in femoral bone marrow of scid, λ-transgenic-scid, μ-transgenic-scid, and μλ-transgenic-scid mice. Number of B lineage cells of various phenotypes in mitosis (below) of scid, λ-transgenic-scid, μ-transgenic-scid, and μλ-transgenic-scid mice, sampled 2h40min after vincristine sulfate administration.

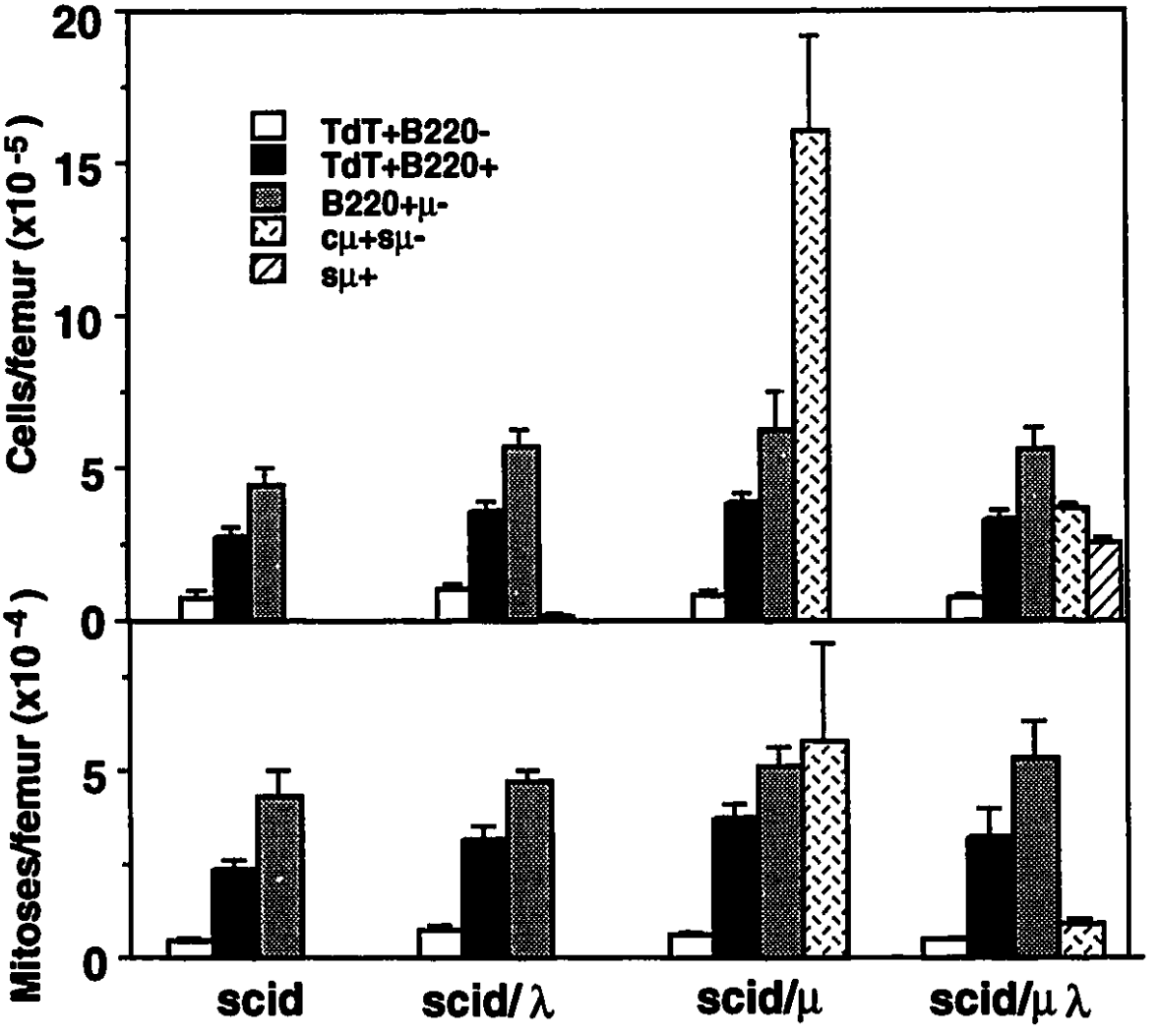
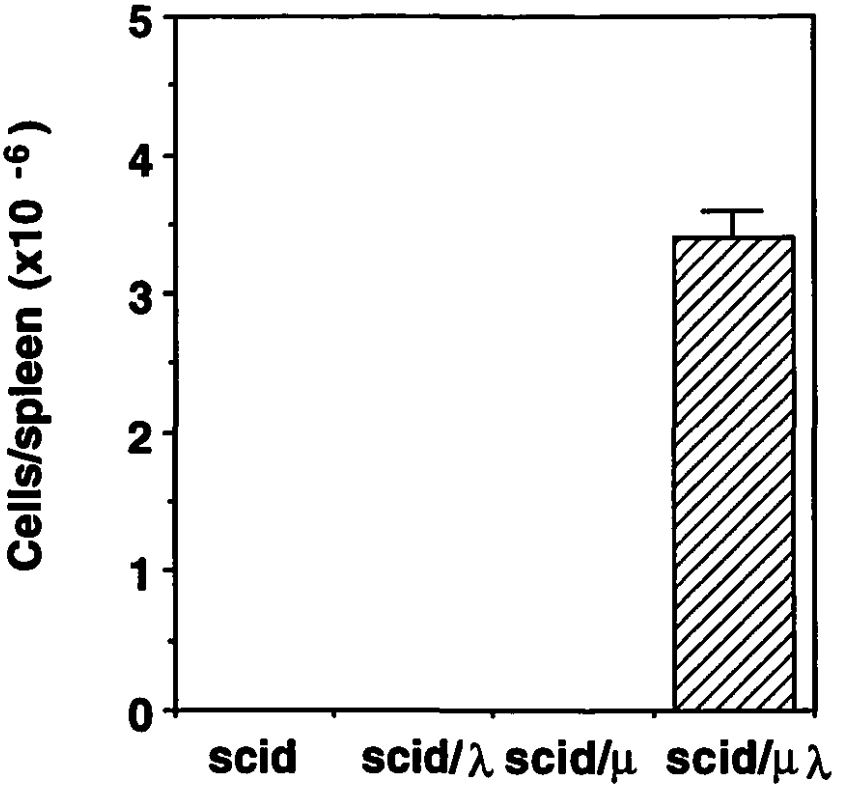

20
15
10
5
0
Cells/femur (x10 -5)
TdT+B220-
TdT+B220+
B220+μ-
cμ+sμ-
sμ+
5
0
Mitoses/femur (x10 -4)
scid
scid/λ
scid/μ
scid/μ λ

Figure 3. Number of B lymphocytes in spleen of scid, λ-transgenic-scid, μ-transgenic-scid, and μλ-transgenic-scid mice.


Cells/spleen (x10 -6)
5
4
3
2
1
0
scid
scid/λ
scid/μ
scid/μ λ

REFERENCES

Alt, F.W., Blackwell, T.K., Yancopoulos, G.D. (1987): Development of the primary antibody repertoire. Science 238:1079-1087

Bosma, G.C., Custer, R.P., Bosma, M.J. (1983): A severe combined immunodeficiency mutation in the mouse. Nature 301:527-530

Deenen, G.J., Van Balen, I., Opstelten, D. (1990): In rat B lymphocyte genesis sixty percent is lost from the bone marrow at the transition of non-dividing pre-B cell to sIgM$^+$ B lymphocytes, the stage of Ig light chain gene expression. Eur.J.Immunol. 20:557

Fulop, G.M., Phillips, R.A. (1988): Early B-cell precursors in scid mice: normal numbers of cells transformable with Abelson Murine Leukemia Virus (A-MuLV). Cell.Immunol. 113:192-201

Grosschedl, R., Weaver, D., Baltimore, D., Constantini, F. (1984): Introduction of a μ immunoglobulin gene into the mouse germ line: specific expression in lymphoid cells and synthesis of functional antibody. Cell 38:647-658

Kincade, P.W. (1987): Experimental models for understanding B lymphocyte formation. Adv.Immunol. 41:181-267

Lieber, M.R., Hesse, J.E., Lewis, S., Bosma, G.C., Rosenberg, N., Mizuchi, K., Bosma, M.J., Gellert, M. (1988): The defect in murine severe combined immune deficiency: joining of signal sequences but not coding segments in V(D)J recombination. Cell 55:7-16

Malynn, B.A., Blackwell, T.K., Fulop, G.M., Rathbun, G.A., Furley, A.J.W., Ferrier, P., Heinke, L.B., Phillips, R.A., Yancopoulus, G.D., Alt, F.W. (1988): The scid defect affects the final step of the immunoglobulin VDJ recombinase mechanism. Cell 54:453-460

Nemazee, D.A., Burki, K. (1989): Clonal deletion of B lymphocytes in a transgenic mouse bearing anti-MHC class I antibody genes. Nature 337:562-566

Neuberger, M.S., Caskey, H.M., Peterson, S., Williams, G.T., Surani, M.A. (1989): Isotype exclusion and transgene down-regulation in immunoglobulin-l transgenic mice. Nature 338:350-352

Okazaki, K., Nishikawa, S-I., Sakano, H. (1988): Aberrant immunoglobulin gene rearrangement in scid mouse bone marrow cells. J.Immuno. 141:1348-1352

Opstelten, D., Osmond, D.G. (1985): Regulation of pre-B cell proliferation in bone marrow: immunofluorescence stathmokinetic studies of cytoplasmic μ chain-bearing cells in anti-IgM-treated mice, hematologically deficient mutant mice and mice given sheep red blood cells. Eur.J.Immunol. 15:599-605

Osmond, D.G. (1986): Population dynamics of bone marrow B lymphocytes. Immunol.Rev. 93:104-124

Osmond, D.G. (1990): B cell development in the bone marrow. Semin.Immunol. 2:173-180

Osmond, D.G., Kim, N., Manoukian, R., Phillips, R.A., Rico-Vargas, S.A., Jacobsen, K. (1992): Dynamics and localization of early B-lymphocyte precursor cells (pro-B cells) in the bone marrow of scid mice. Blood 79:1695-1703

Osmond, D.G. (1993): The turnover of B-cell populations. Immunol.Today 14:34-37

Park, Y-H., Osmond, D.G. (1989): Dynamics of early B-lymphocyte precursor cells in mouse bone marrow: proliferation of cells

containing terminal deoxynucleotidyl transferase. Eur.J.Immunol. 19:2139-2144

Reichman-Fried, M., Hardy, R.R., Bosma, M.J. (1990): Development of B-lineage cells in the bone marrow of scid/scid mice following the introduction of functionally rearranged immunoglobulin transgenes Proc.Natl.Acad.Sci.USA. 87:2730-2734

Ritchie, K.A., Brinster, R.L., Storb, U. (1984): Allelic exclusion and immunoglobulin gene rearrangement in κ transgenic mice. Nature 312:517-520

Schuler, W., Weiler, I.J., Schuler, A., Phillips, R.A., Rosenberg, N., Mak, T., Kearney, J.F., Perry, R.P., Bosma, M.J. (1986): Rearrangement of antigen receptor genes is defective in mice with severe combined immune deficiency. Cell 46:963-972

Weaver, D., Costantini, F., Imanishi-Kari, T., Baltimore, D. (1985): A transgenic immunoglobulin μ gene prevents rearrangements of endogenous genes. Cell 42:117-127

Wyllie, A.H., Kerr, J.F.R., Currie, A.R. (1980): Cell death: the significance of apoptosis. Int.Rev.Cytol. 68:251

CHAPTER 8

General conclusion

GENERAL CONCLUSION

8.1 General Summary.

Mammalian bone marrow is the site of production of B lymphocytes which are derived by differentiation and proliferation from hemopoietic stem cells. Previous immunofluorescence and stathmokinetic techniques have permitted the study of the frequency, size distribution and proliferative properties of successive populations of B cell precursors *in vivo*. Light and electron microscope radioautographic studies *in situ* have revealed special microenvironmental conditions associated with B cell genesis in the bone marrow. *In vitro* cultures have demonstrated the essential regulatory role of stromal cells and their short-range growth factors in B cell genesis, while *in vivo* studies have demonstrated polyclonal amplification as a result of external environmental stimuli and macrophage activation.

The B cell lineage is prone to neoplasias, the etiology of which remains unclear. It is evident, however, that neoplasias are the result of a combination of more than one dysregulatory genetic accident, and that early B cell precursors are extremely vulnerable to such genetic aberrations due to their proliferative and Ig recombinant activities.

The administration of foreign agents to mice produces an increase in population size and mitotic activity among the various B cell

precursors in the bone marrow, including those at the stage of intense Ig gene rearrangements, effects that are macrophage-mediated.

The present series of studies has been designed to evaluate the effects of several mechanisms that may serve to regulate or to perturb the proliferation and loss of precursor B cells in mouse bone marrow, and to consider the possible relevance of these effects to normal B cell genesis and oncogenesis.

Injection of pristane mineral oil ip produces plasmacytomas in susceptible BALB/cAn mouse strain, after a prolonged pretumorous period associated with intense macrophage activation in peritoneal granulomata. The present work shows that a single ip injection of pristane in BALB/cAn mice produces a marked perturbation of B cell precursor dynamics in the bone marrow, lasting for months. The effect produces a dichotomy between the early and intermediate pro-B cells, which increase to more than twice their normal population sizes and mitotic rates, and the later precursors, late pro-B cells, pre-B cells and B lymphocytes, which decline to half normal numbers or less during the same period. The findings show a previously unsuspected central effect of pristane treatment on B cell precursors in the bone marrow. The chronic increase in the kinetic steady state of TDT^+ cells undergoing V_H gene rearrangements (ie early and intermediate pro-B cells) is in agreement with our proposed hypothesis, that chronic macrophage activation could

perturb primary B cell genesis and, in doing so, could predispose to the initiation of B lineage neoplasias. The depression of later precursors suggests that the enhanced proliferative activity at the stages of VDJ heavy chain gene rearrangements is followed by an increased degree of cell loss at the late pro-B cell stage. It may be speculated that the chronic intense stimulation of TdT^+ cells favours an increased incidence of aberrant Ig gene rearrangements, many of which are lethal to the cells, which are rapidly eliminated. Some of the surviving cells, however, could carry non-lethal genetic errors that may constitute the first of a series of oncogenic events. The similarity in findings in the bone marrow of BALB/cAn mice treated with indomethacin and in the plasmacytoma-resistant DBA/2 strain suggests that indomethacin and genetic strain-dependent factors do not prevent plasmacytoma induction by modifying the central effects of pristane treatment on precursor B cells in the bone marrow, but may act peripherally, possibly by enhancing tumor surveillance mechanisms. A role of macrophages in the stimulatory effect of pristane treatment on TdT^+ progenitors is suggested by the ability of silica treatment to diminish this effect, as well as by the ability of peritoneal cells and spleen adherent cells to partially to transfer the effect into naive recipients.

We have shown that mice infected with the malaria parasite, *Plasmodium yoelii*, develop a rapid parasitemia that is completely cleared by week 4. This is accompanied by hepatosplenomegally and

intense macrophage activation. B cell genesis in the bone marrow also shows much disturbance. The pro-B cells increase rapidly in population size and proliferation rates to 2-3 fold normal levels which are maintained for prolonged periods of time. In contrast, later progenitors do not show a corresponding stimulatory effect. The study demonstrates a previously unsuspected effect of malaria infection on the early stages of the B cell lineage in the bone marrow. Pro-B cells in the process of rearranging V_H genes undergo prolonged proliferative stimulation. The fact that this is not accompanied by a commensurate increase in the production of late pro-B, pre-B cells and B lymphocytes, suggests that pro-B cell proliferation is followed by extensive cell loss.

The close similarities in the pattern of bone marrow B lineage responses to pristane and malaria are remarkable, given the wide biological and physical differences between the two agents. Both conditions have in common, however, a period of intense macrophage activation before the production of *myc*/Ig translocation and B cell neoplasia. Our findings are consistent with the hypothesis that pro-B cell stimulation by macrophage-derived products, promoting the genesis of aberrant Ig gene recombinations, represents a common oncogenesis-initiating mechanism.

Eμ-*myc* transgenic mice develop B lineage cell tumors after a lag period. During this preneoplastic stage we find that both the TdT^+ pro-B cells as well as the pre-B cells expand in population size and

proliferative activity, without, however, a commensurate increase in B lymphocytes in either the bone marrow or peripheral immune system. The findings suggest that an elevated level of pro-B cell proliferation, in this case driven by *c-myc* protein expression, is followed by increased cell loss at the pre-B cell stage. The latter could reflect either the dual effect of the *c-myc* gene itself in maintaining cells in mitotic cycle and initiating programmed cell death, or the effect of secondary genetic aberrations. The findings generally resemble those of the pristane and malaria models, an increase in early B cell progenitor activity occurring without a subsequent expansion of later progenitors. In Eμ-*myc* transgenic mice, the expanded pro-B cell activity may increase the chances of secondary genetic errors in addition to the *myc* transgene, eventually producing a dysregulated cell capable of evading the cell death/selection mechanisms and initiating a tumor clone.

Mice affected by the scid mutation have profound defects in B lymphocyte development, due to aberrant VDJ recombination. In scid mice, we find that early pro-B cells and intermediate pro-B cells have normal population sizes and proliferation rates, whereas late pro-B cells are reduced in numbers and mitotic activity to one third normal values, while both pre-B cells and B lymphocytes are absent. Despite defective VDJ recombinase activity, precursor B cells still progress through mitotic cell cycle and are not in a state of static arrest. The aberrant cells can progress only to the beginning of the

late pro-B cell stage, however, before apparently being rapidly deleted. The findings have established the point at which the scid B lineage aborts. The introduction of a functional rearranged μ transgene rescues the cells up to the pre-B cell stage, while a rearranged μλ transgene permits full differentiation to B lymphocytes. However, the rescue is incomplete and unable fully to reconstitute the normal bone marrow and peripheral pools. Possibly, many unsuccessful endogenous rearrangements may be produced, leading to a subsequent cell deletion. The study has stressed the importance of successful Ig gene rearrangement for cell survival and has established the scid and Ig-transgenic-scid mice as useful models for further studies of the production of genetically aberrant precursor B cells as well as the culling mechanisms which either select them out or permit them to enter the peripheral B cell pool.

The hypothesis that conditions involving pronounced macrophage activation can be associated with prolonged stimulation of pro-B cell proliferation in the bone marrow has been strengthened. To this has been added findings which suggest that stimulated pro-B cell proliferation is not necessarily followed by an increased B cell output, but rather by an apparently increased degree of cell loss (pristane; malaria; Eμ-*myc*; Ig-trangenic-scid). Normal B lymphopoiesis shows a high rate of cell loss, largely attributed to aberrant cells with defective V_H gene recombination. In view of this and the observed deletion of cells known to be either aberrant or dysregulated (Eμ-

myc; Ig-transgenic-scid), it can be proposed that the additional stimulation of pro-B cell proliferative activity mediated by macrophage activation or the *c-myc* transgene increases the frequency of genetic mistakes during VDJ recombination. Not all these genetic errors, however, would necessarily be lethal to the cell but could in some cases constitute the first of a series of oncogenic deregulatory steps.

The findings suggest that two mechanisms may be equally important both in determining the normal output of B cells and in the initiation of B cell neoplasias in the bone marrow: 1) mechanisms which increase the rate at which pro-B cells are produced and thus the rate of formation of genetically aberrant cells, 2) mechanisms which cause aberrant cells to abort or to be selected out in the bone marrow. A stimulation of the first mechanism, a lack of efficiency or evasion of the second, and a combination of both these effects could increase the risk of disseminating potentially dysregulated B cells.

REFERENCES

Abramson, S., Miller, R.G., Phillips, R.A. (1977): The identification in adult bone marrow of pluripotential and restricted stem cells of the myeloid and lymphoid systems. J.Exp.Med. 145:1567-1579

Aguilera, R.J., Akira, S., Okazaki, K., Sakano, H. (1987): A pre-B cell nuclear protein that specifically interacts with the immunoglobulin V-J recombination sequences. Cell 51:909-917

Allman, D.M., Ferguson, S.E., Cancro, M.P. (1992): Peripheral B cell maturation. I. Immature peripheral B cells in adults are heat-stable antigen^{hi} and exhibit unique signalling characteristics. J.Immunol. 149:2533-2540

Alt, F.W, Baltimore, D. (1983): Joining of immunoglobulin heavy chain gene segments: implications from a chromosome evidence of three D-J_H fusions. Proc.Natl.Acad.Sci.USA 79:4118-4122

Alt, F.W., Blackwell, T.K., Yancopoulos, G.D. (1987): Development of the primary antibody repertoire. Science 238:1079-1087

Altiok, E., Klein, G., Zech, L., Uno, M., Henriksson, B.E., Battat, S., Ono, Y., Ernberg, I. (1989): Epstein-Barr virus-transformed pro-B cells are prone to illegitimate recombination between the switch region of the μ chain gene and other chromosomes. Proc.Natl.Acad.Sci.USA 86: 6333-6337

ar-Rushdi, A., Nishikura, K., Erikson, J., Watt, R., Rovera, G., Croce, C.M. (1983): Differential expression of the translocated and untranslocated *c-myc* oncogene in Burkitt's lymphoma. Science 222:390-393

Barnes, W.H., Evans, E.P., Ford, C.E., West, B.J. (1968): Spleen colonies in mice: karyotypic evidence of multiple colonies from single cells. Nature 219:518-520

Bartoncello, I., Hodgson, G.S., Bradley, T.R., Hunter, S.D., Barber, L. (1985): Multiparameter analysis of transplantable hemopoietic stem cells: I. The separation and enrichment of stem cells homing to marrow and spleen on the basis of rhodamine-123 fluorescence. Exp.Hematol. 13:999-1006

Batten, S.J., Osmond, D.G. (1984): The localization of B lymphocytes in mouse bone marrow: radioautographic studies after in vivo perfusion of radiolabeled anti-IgM antibody. J.Immunol.Meth. 72:381-399

Bernardi, P., Patel, V.P., Lodish, H.F. (1987): Lymphoid precursor cells adhere to two different sites on fibronectin. J.Cell Biol. 105:489-498

Bertoglio, J.H. (1988): B-cell-derived human interleukin 1. Crit.Rev.Immunol. 8:299-313

Billips, L.G., Petite, D., Landreth, K.S. (1990): Bone marrow stromal cell regulation of B lymphopoiesis: interleukin-1 and IL-4 regulate stromal cell support of pre-B cell production in vitro. Blood 75:611-619

Bishop, J.M. (1987): The molecular genetics of cancer. Science 235:305-311

Bosma, G.C., Custer, R.P., Bosma, M.J. (1983): A severe combined immunodeficiency mutation in the mouse. Nature 301:527-530

Brahim, F., Osmond, D.G. (1970): Migration of bone marrow lymphocytes demonstrated by selective bone marrow labeling with Thymidine-H^3. Anat.Rec. 168:139-160

Brunswick, M., Burkhardt, A., Finkelman, F., Bolen, J., Mond, J.J. (1992): Comparison of tyrosine kinase activation by mitogenic and nonmitogenic anti-IgD antibodies. J.Immunol. 149:2249-2254

Burkhardt, A.L., Brunswick, M., Bolen, J.B., Mond, J.J. (1991): Anti-immunoglobulin stimulation of B lymphocytes activates src-related protein-tyrosine kinases. Proc.Natl.Acad.Sci.USA 88:7410-7414

Burstein, H.J., Tepper, R.I., Leder, P., Abbas, A.K. (1991): Humoral immune functions in IL-4 transgenic mice. J.Immunol. 147:2950-2956

Casali, P., Notkins, A.L. (1989): $CD5^+$ B lymphocytes, polyreactive antibodies and the human B-cell repertoire. Immunol.Today 10:364-368

Cambier, J.C., Kettman, J.R., Vitetta, E.S., Uhr, J.W. (1976): Differential susceptibility of neonatal and adult murine spleen cells to in vitro induction of B-cell tolerance. J.Exp.Med. 144:293-297

Campbell, K.S., Cambier, J.C. (1990): B lymphocyte antigen receptor (mIg) is non-covalently associated with a disulfide linked, inducibly phosphorylated glycoprotein complex. EMBO J. 9:441-448

Chabott, B., Stephenson, D.A., Chapman, M.V., Besmer, Bernstein, P.A. (1988): The proto-oncogene *c-kit* encoding a transmembrane tyrosine kinase receptor maps to the mouse W locus. Nature 335:88-89

Chen, S., Botteri, F., van der Putten, H., Landel, C.P., Evans, G.A. (1987): A lymphoproliferative abnormality associated with inappropriate expression of the Thy-1 antigen in transgenic mice. Cell 51:7-19

Coffman, R.L., Weissman, I.L. (1981): A monoclonal antibody that recognizes B cells and B cell precursors in mice. J.Exp.Med. 153:269-279

Coleclough, C. (1983): Chance, necessity and antibody gene dynamics. Nature 303:23-26

Cooper, M.D., Mulvaney, D., Coutinho, A., Cazenave, P-A. (1986): A novel cell surface molecule on early B-lineage cells. Nature 321:616-618

Copeland, N.G., Gilbert, D.J., Cho, B.C., Donovan, P.J., Jenkins, N.A., Cosman, D., Anderson, D., Lyman, S.D., Williams, D.E. (1990): Mast cell growth factor maps near the steel locus on mouse chromosome 10 and is deleted in a number of steel alleles. Cell 63:175-183

Croce, C.M. (1987): Role of chromosome translocations in human neoplasia. Cell 49:155-156

Cumano, A., Paige, C.J. (1992): Enrichment and characterization of uncommitted B-cell precursors from fetal liver at day 12 of gestation. EMBO J. 11:593-601

Cumano, A., Paige, C.J., Iscove, N.N., Brady, G. (1992): Bipotential precursors of B cells and macrophages in murine fetal liver. Nature 356:612-615

Desiderio, S.V., Yancopoulos, G.D., Paskind, M., Thomas, E., Boss, M.A., Landau, N., Alt, F.W., Baltimore, D. (1984): Insertion of N regions into heavy chain genes is correlated with expression of terminal deoxytransferase in B cells. Nature 311:752-755.

Dexter, T.D., Allen, T.D., Lajtha, L.G. (1977): Conditions controlling the proliferation of haemopoietic stem cells in vitro. J.Cell Physiol. 91:335-344

Dick, J.E., Magli, M.C., Huszar, D., Phillips, R.A., Bernstein, A. (1985): Introduction of a selectable gene into stem cells capable of long-term reconstitution of the hemopoietic system of W/W^v mice. Cell 42:71-79

Dinarello, C.A. (1987): The biology of interleukin 1 and comparison to tumor necrosis factor. Immunol.Letters 16:227-232

Dinarello, C.A. (1992): Role of interleukin-1 in infectious diseases. Immunol.Rev. 127:119-146

Dorshkind, K. (1990): Regulation of hemopoiesis by bone marrow stromal cells and their products. Annu.Rev.Immunol. 8:111-137

Dymecki, S.M., Niederhuber, J.E., Desiderio, S.V. (1989): Specific expression of a tyrosine kinase gene, *blk*, in B lymphoid cells. Science 247:332-336

Feeney, A.J. (1991): Lack of N regions in fetal and neonatal mouse immunoglobulin V-D-J junctional sequences. J.Exp.Med. 172:1377-1390

Felix, C.A., Wright, J.J., Poplack, D.G., Reaman, G.H., Cole, D., Goldman, P., Korsmeyer, S.J. (1987): T cell receptor α-, β-, and γ-genes in T cell

and pre-B cell acute lymphoblastic leukemia. J.Clin.Invest. 80:545-556

Forster, I., Gu, H., Rajewsky, K. (1988): Germline antibody V regions as determinants of clonal persistence and malignant growth in the B cell compartment. EMBO J. 7:3693-3703

Fulop, G., Gordon, J., Osmond, D.G. (1983): Regulation of lymphocyte production in the bone marrow. I. Turnover of small lymphocytes in mice depleted of B lymphocytes by treatment with anti-IgM antibodies. J.Immunol. 130:644-648

Fulop, G., Osmond D.G. (1983): Regulation of bone marrow lymphocyte production. IV. Cells mediating the stimulation of marrow lymphocyte production by sheep red blood cells: studies in anti-IgM-suppressed mice, athymic mice, and silica-treated mice. Cell.Immunol. 75:91-102

Fulop, G., Pietrangeli, C.E., Osmond D.G. (1986): Regulation of bone marrow lymphocyte production. IV. Altered kinetic steady state of lymphocyte production after chronic changes in exogenous stimuli. Exp.Hematol. 14:27-34

Fulop, G.M., Phillips, R.A. (1988): Early B-cell precursors in scid mice: normal numbers of cells transformable with Abelson Murine Leukemia Virus (A-MuLV). Cell.Immunol. 113:192-201

Fulop, G.M. and Phillips, R.A. (1989): Use of scid mice to identify and quantitate lymphoid-restricted stem cells in long-term bone marrow cultures. Blood 74:1537-1544

Gimble, J.M., Hudson, J., Henthorn, J., Hua, X., Burstein, S.A. (1991): Regulation of interleukin 6 expression in murine marrow stromal cells. Exp.Hematol. 19:1055-1060

Giri, J.G., Kincade, P.W., Mizel, S.B. (1984): Interleukin 1-mediated induction of κ-light chain synthesis and surface immunoglobulin expression on pre-B cells. J.Immunol. 132:223-228

Golub, E.S. (1987): Somatic mutation: diversity and regulation of the immune repertoire. Cell 48:723-724

Greaves, M.F., Chan, L.C. (1986): Is spontaneous mutation the major 'cause' of childhood acute lymphoblastic leukaemia?. Brit.H.Haemat. 64:1-13

Haluska, F.G., Tsujimoto, Y., Croce, C.M. (1987): The t(8;14) chromosome translocation of the Burkitt's lymphoma cell line Daudi occurred during immunoglobulin gene rearrangement and involved the heavy chain diversity region. Proc.Natl.Acad.Sci.USA 84:6835-6839

Hardy, R.R., Carmack, C.E., Shinton, S.A., Kemp, J.D., Hayakawa, K. (1991): Resolution and characterization of pro-B and pre-pro-B cell stages in normal mouse bone marrow. J.Exp.Med. 173:1213-1225

Hartley, S.B., Crosbie, J., Brink, R., Kantor, A.B., Basten, A., Goodnow, C.C. (1991): Elimination from peripheral lymphoid tissues of self-reactive B lymphocytes recognizing membrane-bound antigens. Nature 353:765-769

Hayakawa, K., Hardy, R.R., Parks, D.R., Herzenberg, L.A. (1983): The "Ly-1-B" cell subpopulation in normal immunodefective, and autoimmune mice. J.Exp.Med. 157:202-218

Hayakawa, K., Hardy, R.R., Herzenberg, L.A., Herzenberg, L.A. (1985): Progenitors for Ly-1 B cells are distinct from progenitors for other B cells. J.Exp.Med. 161:1554-1568

Hayakawa, K., Hardy, R.R., Stall, A.M., Herzenberg, L.A., Herzenberg, L.A. (1986): Immunoglobulin-bearing B cells reconstitute and maintain the murine Ly-1 B cell lineage. Eur.J.Immunol. 16:1313-1316

Hayashi, S-I, Kunisada, K., Ogawa, M., Yamaguchi, K., Nishikawa, S-I. (1991): Exon skipping by mutation of an authentic splice site of *c-kit* gene in W/W mouse. Nucleic Acids Res. 19:1267-1271

Henderson, A.J., Narayanan, R., Collins, L. Dorshkind, K. (1992): Status of κL chain gene rearrangements and *c-kit* and IL-7 receptor expression in stromal cell-dependent pre-B cells. J.Immunol. 149:1973-1979

Hermans, M.H.A., Hartsuiker, H., Opstelten, D. (1989): An in situ study of B-lymphopoiesis in rat bone marrow. Topographical arrangement of terminal deoxynucleotidyl transferase-positive cells and pre-B cells. J.Immunol. 142:67-73

Hermans, M.H.A., Deenen, G-J., Kroese, F., Opstelten, D. (1993): A new phosphatidylinositol-linked molecule identified on early B lymphoid cells in rat bone marrow (in press).

Hermanson, G.G., Eisenberg, D., Kincade, P.W., Wall, R. (1988): *B29*: A member of the immunoglobulin gene superfamily exclusively expressed on B-lineage cells. Proc.Natl.Acad.Sci.USA 85:6890-6894

Herzenberg, L.A., Stall, A.M., Lalor, P.A., Sidman, C., Moore, W.A., Parks, D.R., Herzenberg, L.A. (1986): The Ly-1 B cell lineage. Immunol.Rev. 93:81-102

Hirano, T., Yasukawa, K., Harada, H., Taga, T., Watanabe, Y., Matsuda, T., Kashiwamura, S., Nakajima, K., Koyama, K., Iwamatsu, A., Tsunasawa, S., Sakiyama, F., Matsui, H., Takahara, Y., Taniguchi, T., Kishimoto, T. (1986): Complementary DNA for a novel human interleukin (BSF-2) that induces B lymphocytes to produce immunoglobulin. Nature 324:73-76

Hirano, T. (1992): Interleukin-6 and its relation to inflammation and disease. Clin.Immunol. & Immunopathology S60-S65

Hollis, G.F., Evans, R.J., Stafford-Hollis, J., Korsmeyer, S.J., McKearn, J.P. (1989): Immunoglobulin λ light-chain-related genes 14.1 and 16.1 are expressed in pre-B cells and may encode the human immunoglobulin ω light-chain protein. Proc.Natl.Acad.Sci.USA 86:5552-5556

Hombach, J., Leclecq. L., Radbruch, A., Rajewsky, K., Reth, M. (1988): A novel 34-Kd protein co-isolated with the IgM molecule in surface IgM-expressing cells. EMBO J. 7:3451-3456

Hombach, J., Tsubata, T., Leclecq, L., Stappert, H., Reth, M. (1990): Molecular components of the B-cell antigen receptor complex of the IgM class. Nature 343:760-762

Huang, S. and Terstappen, L.W.M.M. (1992): Formation of haematopoietic microenvironment and haematopoietic stem cells from single human bone marrow stem cells. Nature 360:745-749

Iglesias, A., Kopf, M., Williams, G.S., Buhler, B., Kohler, G. (1991): Molecular requirements for the μ-induced light chain gene rearrangements in pre-B cells. EMBO J. 10:2147-2156

Jacobsen, K., Tepper, J., Osmond, D.G. (1990): Early B-lymphocyte precursor cells in mouse bone marrow: sublethal localization of B220+ cells during postirradiation regeneration. Exp.Haematol. 18:304-310

Jacobsen, K., Osmond, D.G., (1990): Microenvironmental organization and stromal cell associations of B lymphocyte precursor cells in mouse bone marrow. Eur.J.Immunol. 20:2395-2404

Jacobsen, K., Osmond, D.G. (1991): Cell deletion in B-cell genesis and the role of bone marrow macrophages. In lymphatic tissues and the in vivo immune response. Ed. Imhof, B.A., Akini, S.-B., Ezine, S., pp 343-353

Jacobsen, K., Miyake, K., Kincade, P.W., Osmond, D.G. (1992): Highly restricted expression of a stromal cell determinant in mouse bone marrow. J.Exp.Med. 176:927-935

Jelinek, D.F., Lipsky, P.E. (1987): Enhancement of human B cell proliferation and differentiation by tumor necrosis factor-α and interleukin 1. J.Immunol. 139:2970-2976

Jordan, C.T., McKearn, J.P., Lemischka, I.R. (1990): Cellular and developmental properties of fetal hemopoietic stem cells. Cell 61:953-963

Kawano, M., Hirano, T., Matsuda, T., Taga, T., Horii, Y., Iwato, K., Asaoku, H., Tang, B., Tanabe, O., Tanaka, H., Kuramoto, A., Kishimoto, T. (1988): Autocrine generation and requirement of BSF-2/IL-6 for human multiple myelomas. Nature 332:83-85

Kehrl, J.H., Miller, A., Fauci, A.S. (1987): Effect of tumor necrosis factor α on mitogen-activated human B cells. J.Exp.Med. 166:786-791

Kehrl, J.H., Alvarez-Mon, M., Delsing, G.A., Fauci, A.S. (1987): Lymphotoxin is an important T cell-derived growth factor for human B cells. Science 238:1144-1146

Keller, G., Paige, C., Gilboa, E., Wagner, E.F. (1985): Expression of a foreign gene in myeloid and lymphoid cells derived from multipotent haematopoietic precursors. Nature 318:149-154

Kincade, P.W. (1981a): Formation of B Lymphocytes in Fetal and Adult Life. Adv.Immunol. 31:177-245

Kincade, P.W, Lee, G., Watanabe, T., Sun, L., Scheid, M.P. (1981b): Antigens displayed on murine B lymphocyte precursors. J.Immunol. 127:2262-2268.

Kincade, P.W. (1987): Experimental models for understanding B lymphocyte formation. Adv.Immunol. 41:181-267

Kincade, P.W., Lee, G., Pietrangeli, C.E., Hayashi, S-I., Gimble, J.M. (1988): Cells and molecules that regulate B lymphopoiesis in bone marrow. Ann.Rev.Immunol. 7:111-143

Kincade, P.W. (1991): Molecular interactions between stromal cells and B lymphocyte precursors. Sem.Immunol. 3:379-390

Kindler, V., Sappino, A-P., Grau, G.E., Piguet, P-F., Vassalli, P. (1989): The inducing role of tumor necrosis factor in the development of bactericidal granulomas during BCG infection. Cell 56:731-740

King, A.G., Wierda, D., Landreth, K.S. (1988): Bone marrow stromal cell regulation of B-lymphopoiesis. I. The role of macrophages, IL-1, and IL-4 in pre-B cell maturation. J.Immunol. 141:2016-2026

Kirsh, I.R., Morton, C.C., Nakahara, K., Leder, P. (1982): Human immunoglobulin heavy chain genes map to a region of translocations in malignant B lymphocytes. Science 216:301-303

Kitamura, D., Roes, J., Kuhn, R., Rajewsky, K. (1991): A B cell-deficient mouse by targeted disruption of the immunoglobulin μ chain gene. Nature 350:423-426

Klein, G., Klein, E. (1985): *Myc*/Ig juxtaposition by chromosomal translocations: some new insights, puzzles and paradoxes. Immunol.Today 6:208-215

Kudo, A., Sakaguchi, N., Melchers, F. (1987): Organization of the murine Ig-related *λ5* gene transcribed selectively in pre-B lymphocytes. EMBO J. 6:103-107

Kudo, A., Melchers, F. (1987): A second gene, $V_{pre\text{-}B}$ in the *λ5* locus of the mouse, which appears to be selectively expressed in pre-B lymphocytes. EMBO J. 6:2267-2272

Landreth, K.S., Rosse, C., Clagett, J. (1981): Myelogenous production and maturation of B lymphocytes in the mouse. J.Immunol. 127:2027-2034

Landreth, K.S., Kincade, P.W., Lee, G., Medlock, E.S. (1983): Phenotypic and functional characterization of murine B lymphocyte precursors isolated from fetal and adult tissues. J.Immunol. 131:572-580

Langdon, W.Y., Harris, A.W., Cory, S., Adams, J.M. (1986): The *c-myc* oncogene perturbs B lymphocyte development in Eμ-*myc* transgenic mice. Cell 47:11-18

Lanier, L.L., Warner, N.L., Ledbetter, J.A., Herzerberg, L.A. (1981): Quantitative immunofluorescent analysis of surface phenotypes of murine B cell lymphomas and plasmacytomas with monoclonal antibodies. J.Immunol 127:1691-1697

Lee, G., Namen, A.E., Gillis, S., Ellingsworth, L.R., Kincade, P.W. (1989): Normal B cell precursors responsive to recombinant murine IL-7 and inhibition of IL-7 activity by tansforming growth factor-β. J.Immunol. 142:3875-3883

Lenoir, G.M., Bornkamm, G.W. (1987): Burkitt's lymphoma, a human cancer model for the study of the multistep development of cancer: proposal for a new scenario. Adv. in Viral Oncology 7:173-206

Libert, C., Brouckaert, P., Shaw, A., Fiers, W. (1990): Induction of interleukin 6 by human and murine recombinant interleukin 1 in mice. Eur.J.Immunol. 20:691-694

Liu, Y-J., Joshua, D.E., Williams, G.T., Smith, C.A., Gordon, J., MacLennan, I.C.M. (1989): Mechanism of antigen-driven selection in germinal centers. Nature 342:929-931

Lombardi, L., Newcomb, E.W., Dalla-Favera, R. (1987): Pathogenesis of Burkitt's lymphoma: expression of an activated *c-myc* oncogene causes the tumorigenic conversion of EBV-infected human B lymphoblast. Cell 49:161-170

Manz, J., Denis, K., Witte, O., Brinster, R., Storb, U. (1988): Feedback inhibition of immunoglobulin gene rearrangement by membrane μ, but not by secreted μ heavy chains. J.Exp.Med. 168:1363-1381

Marshall, C.J. (1986): Oncogenes. J.Cell Sci. 4:417-430

McDonnell, T.J., Korsmeyer, S.J. (1991): Progression from lymphoid hyperplasia to high-grade malignant lymphoma in mice transgenic for the t(14;18). Nature 349:254-256

Melchers, F., Abramzuck, J. (1980): Murine embryonic blood between day 10 and 13 of gestation as a source of immature precursor B cells. Eur.J.Immunol. 10:763-767

Metcalf, D., Moore, M.A.S. (1971): Haemopoietic cells. In: Frontiers of Biology, Vol. 24, Neuberger A, Tatum E.L. eds. North Holland, Amsterdam

Metcalf, E.J., Kinman, N.R. (1976): In vitro tolerance induction of neonatal murine B cells. J.Exp.Med. 143:1327-1340

Metcalf, E.J., Kinman, N.R. (1977): In vitro tolerance induction of bone marrow cells: a marker for B cell maturation. J.Immunol. 118:2111-2115

Miller, S.C., Osmond, D.G. (1974): Quantitative changes with age in bone marrow cell populations of C3H mice. Exp.Hematol. 2:227-236

Miller, S.C., Osmond, D.G. (1975): Lymphocyte populations in mouse bone marrow: quantitative kinetic studies in young, pubertal and adult C3H mice. Cell Tiss.Kinet. 8:97-110

Miyake, K., Medina, K.L., Hayashi, S-I., Ono, S., Hamaoka, T., Kincade, P.W. (1990a): Monoclonal antibodies to Pgp-1/CD44 block lympho-hemopoiesis in long-term bone marrow. J.Exp.Med. 171:477-488

Miyake, K., Medina, K.L., Hishihara, K., Kimamoto, M., Auerbach, P., Kincade, P.W. (1990b): A VCAM-like adhesion molecule on murine bone marrow stromal cells mediates binding of lymphocyte precursors in culture. J.Cell Biol. 114:557-565

Miyake, K., Underhill, C.B., Lesley, J., Kincade, P.W. (1990c): Hyaluronate can function as a cell adhesion molecule and CD44 participates in hyaluronate recognition. J.Exp.Med. 172:69-75

Miyake, K., Weissman, I.L., Greenberger, J.S., Kincade, P.W. (1991): Evidence for a role of the integrin VLA-4 in lympho-hemopoiesis. J.Exp.Med. 173:599-607

Miyazawa, K., Hendrie, P.C., Mantel, C., Wood, K., Ashman, L.K., Broxmeyer, H.E. (1991): Comparative analysis of signaling between mast cell growth factor (*c-kit* ligand) and granulocyte-macrophage colony stimulating factor in a human factor-dependent myeloid cell line involves phosphorilation of *raf-1*, GTPase-activating protein and mitogen-activated protein kinase. Exp.Hematol. 19:1110-1123

Mombaerts, P., Iacomini, J., Johnson, R.S., Herrup, K., Tonegawa, S., Papaioannous, V.E. (1992): *RAG-1* deficient mice have no mature B and T lymphocytes. Cell 68:869-877

Moore, M.A.S. (1991): Clinical implications of positive and negative hematopoietic stem cell regulators. Blood 78:1-19

Muench, M.O., Schneider, J.G., Moore, M.A.S. (1992): Interactions among colony-stimulating factors, IL-1β, IL-6, and kit ligand in the

regulation of primitive murine hemopoietic cells. Exp.Hematol. 20:339-349

Mulder, A.H., Visser, J.W.M., van den Engh, G.J. (1985): Thymus regeneration by bone marrow cell suspensions differing in the potential to form early and late spleen colonies. Exp.Hematol. 13:768-775

Muller-Sieburg, C.E., Whitlock, C.A., Weissman, I.L. (1986): Isolation of two early B lymphocyte progenitors from mouse marrow: a committed pre-pre-B cell and a clonogenic Thy-1^{lo} haemopoietic stem cell. Cell 44:653-662

Murakami., M., Tsubata, T., Okamoto, M., Shimizu, A., Kumagai, S., Imura, H., Honjo, T. (1992): Antigen-induced apoptotic death of Ly-1 B cells responsible for autoimmune disease in transgenic mice. Nature 357:77-80

Musaschi,T., Yang, Y-C., Paul, S.R., Clark, S.C., Sudo, T., Ogawa, M. (1991): Direct and synergistic effects of interleukin 11 on murine hemopoiesis in culture. Proc.Natl.Acad.Sci.USA. 88:765-769

Namen, A.E., Lupton, S., Hjerrild, K., Wignall, J., Mochizuki, D.Y., Schmierer, A., Mosley, B., March, C.J., Urdal, D., Gillis, S., Cosman, D., Goodwin, R.G. (1988): Stimulation of B cell progenitors by cloned murine interleukin-7. Nature 333:571-573

Nemazee, D.A., Burki, K. (1989): Clonal deletion of B lymphocytes in a transgenic mouse bearing anti-MHC class I antibody genes. Nature 337:562-566

Neuberger, M.S., Caskey, H.M., Peterson, S., Williams, G.T., Surani, M.A. (1989): Isotype exclusion and transgene down-regulation in immunoglobulin-1 transgenic mice. Nature 338:350-352

Nishikura, K., ar-Rushdi, A., Erikson, J., Watt, R., Rovera, G., Croce, C.M. (1983): Differential expression of the normal and of the translocated human *c-myc* oncogenes in B cells. Proc.Natl.Acad.Sci.USA 80:4822-4826

Noelle, R.P., Krammer, J., Ohara, J., Uhr, J.W., Vitetta, E.S. (1984): Increased expression of Ia antigens on resting B cells: an additional role for B-cell growth factor. Proc.Natl.Acad.Sci.USA 81:6149-6153

Nordan, R.P., Potter, M. (1986): A macrophage-derived factor required by plasmacytomas for survival and proliferation in vitro. Science 233:566-569

Nunez, G., Seto, M., Seremetis, S., Ferrero, D., Grignani, F., Korsmeyer, S.J., Dalla-Favera, R. (1989): Growth- and tumor-promoting effects of deregulated *bcl-2* in human B-lymphoblastoid cells. Proc.Natl.Acad.Sci.USA 86:4589-4593

Oettinger, M.A., Schatz, D.G., Gorka, D.G., Baltimore, D. (1990): *RAG-1* and *RAG-2*, adjacent genes that synergistically activate V(D)J recombination. Science 248:1517-1523

Ogawa, O., Matsuzaki, Y., Nishikawa, S., Hayashi, S.I., Kunisada, T. Sudo, T., Kina, T., Nakauchi, T., Nishikawa, S-I. (1991): Expression and function of *c-kit* in hemopoietic progenitor cells. J.Exp.Med. 174:63-71

Opstelten, D., Osmond, D.G. (1983): Pre-B cells in mouse bone marrow: immunofluorescence stathmokinetic studies of the

proliferation of cytoplasmic μ-chain-bearing cells in normal mice. J.Immunol. 131:2635-2640

Opstelten, D., Osmond, D.G. (1985): Regulation of pre-B cell proliferation in bone marrow: immunofluorescence stathmokinetic studies of cytoplasmic μ chain-bearing cells in anti-IgM-treated mice, hematologically deficient mutant mice and mice given sheep red blood cells. Eur.J.Immunol. 15:599-605

Osmond, D.G., Everett, N.B. (1964): Radioautographic studies of bone marrow lymphocytes in vivo and in diffusion chamber cultures. Blood 23:1-17

Osmond, D.G., Nossal, G.J.V. (1974). Differentiation of lymphocytes in mouse bone marrow. II. Kinetics of maturation and renewal of antiglobulin-binding cells studied by double labeling. Cell.Immunol. 13:132-145

Osmond, D.G., Batten, S.J. (1984): Genesis of B lymphocytes in the bone marrow: extravascular and intravascular localization of surface IgM-bearing cells in mouse bone detected by electron microscope radioautography after in vivo perfusion of I^{125} anti-IgM antibody. Am.J.Anat. 170:349-365

Osmond, D.G. (1985): The ontogeny and organization of the lymphoid system. J.Inv.Dermatol. 85:1s-9s

Osmond, D.G., Kim, N., Manoukian, R., Phillips, R.A., Rico-Vargas, S.A., Jacobsen, K. (1992): Dynamics and localization of early B-lymphocyte precursor cells (pro-B cells) in the bone marrow of scid mice. Blood 79:1695-1703

Ostergaard, H.L., Shackelford, D.A., Hurley, T.R., Johnson, P., Hyman, R., Sefton, B.M., Trowbridge, I.S. (1989): Expression of CD45 alters phosphorilation of the *lck*-encoded tyrosine protein kinase in murine lymphoma T cell lines. Proc.Natl.Acad.Sci.USA 86:8959-8963

Park, Y.H., Osmond, D.G. (1987): Phenotype and proliferation of early B lymphocyte precursor cells in mouse bone marrow. J.Exp.Med. 165:444-458

Park, Y-H., Osmond, D.G. (1989): Dynamics of early B-lymphocyte precursor cells in mouse bone marrow: proliferation of cells containing terminal deoxynucleotidyl transferase. Eur.J.Immunol. 19:2139-2144

Parkhouse, R.M.E. (1990): Three B-cell surface molecules associating with membrane immunoglobulin. Immunol. 69:298-302

Paige, C.J., Kincade, P.W, Moore, M.A.S. and Lee, G. (1979): The fate of fetal and adult B-cell progenitors grafted into immunodeficient CBA/N mice. J.Exp.Med. 150:548-563

Phillip, R., Epstein, L.B. (1986): Tumor necrosis factor as immunomodulator and mediator of monocyte cytotoxicity induced by itself, γ-interferon and interleukin-1. Nature 323:86-89

Piccoli, S.P., Caimi, P.G., Cole, M.D. (1984): A conserved sequence at *c-myc* oncogene chromosomal translocation breakpoints in plasmacytomas. Nature 310:327-330

Pietrangeli, C.E., Osmond D.G. (1985): Regulation of B-lymphocyte production in the bone marrow: role of macrophages and the spleen in mediating responses to exogenous agents. Cell.Immunol. 94:147-158

Pietrangeli, C.E., Osmond D.G. (1987): Regulation of B-lymphocyte production in the bone marrow: mediation of the effects of exogenous stimulants by adoptively transferred spleen cells. Cell.Immunol. 107:348-357

Pillai, S., Baltimore, D. (1987): Formation of disulphide-linked $\mu 2\omega 2$ tetramers in pre-B cells by the 18K ω-immunoglobulin light chain. Nature 329:172-174

Potter, M., Wax, J.S. (1983): Peritoneal plasmacytomagenesis in mice: comparison of different pristane dose regimens. JNCI 71:391-395

Raff, M.C., Megson, M., Owen, J.J.T., Cooper, M.D. (1976): Early production of intracellular IgM by B-lymphocyte precursors in mice. Nature 259:224-226

Rajewsky, K., Forster, I., Cumano, A. (1987): Evolutionary and somatic selection of the antibody repertoire in the mouse. Science 238:1088-1094

Rajewsky, K., Gu, H., Vieira, P., Forster, I. (1990): Growth and selection of B cells in vivo. Cold Spring Harbor Symp.Quant.Biol. 54:1-29

Reichman-Fried, M., Hardy, R.R., Bosma, M.J. (1990): Development of B-lineage cells in the bone marrow of scid/scid mice following the introduction of functionally rearranged immunoglobulin transgenes Proc.Natl.Acad.Sci.USA. 87:2730-2734

Ritchie, K.A., Brinster, R.L., and Storb, U. (1984): Allelic exclusion and immunoglobulin gene rearrangement in κ transgenic mice. Nature 312:517-520

Sakaguchi, N., Kashiwamura, S., Kimoto, M., Thalmann, P., Melchers, F. (1988): B lymphocyte restricted expression of *mb-1*, a gene with CD3-like structural properties. EMBO J. 7: 3457-3464

Schatz, D.G., Oettinger, M.A., Baltimore, D. (1989): The V(D)J recombination activating gene, *RAG-1*. Cell 59:1035-1048

Schwartz, M.A. (1992): Transmembrane signalling by integrins. TIBS 2:304-308

Semenzato, I. (1990): Tumor necrosis factor: a cytokine with multiple biological activities. Br.J.Cancer 61:354-361

Shinkai, Y., Rathbun, G., Lam, K-P., Oltz, E.M., Stewart, V., Mendelsohn, M., Charron, J., Datta, M., Young, F., Stall, A.M., Alt, F.W. (1992): *RAG-2* deficient mice lack mature lymphocytes owing to inability to initiate V(D)J rearrangement. Cell 68:855-867

Simons, A., Zharhary, D. (1989): The role of IL-4 in the generation of B lymphocytes in the bone marrow. J.Immunol. 143:2540-2545

Sinn, E., Muller, W., Pattengale, P., Tepler, I., Wallace, R., Leder, P. (1987): Coexpression of MMTV/*v-ras* and MMTV/*c-myc* genes in transgenic mice: synergistic action of oncogenes in vivo. Cell 49:465-475

Snow, E.C., Mond, J.J., Subbarao, B. (1986): Enhancement by monoclonal anti-Lyb-2 antibody of antigen-specific B lymphocyte expansion stimulated by TNP-ficoll and T lymphocyte-derived factors. J.Immunol. 137:1793-1796

Spangrude, G.J., Heimfeld, S. and Weissman, I.L. (1988): Purification and characterization of mouse hematopoietic stem cells. Science 241:58-62

Spangrude, G.J. (1989): Enrichment of murine haemopoietic stem cells: diverging roads. Immunol.Today 10:344-350

Springer, T.A. (1990): Adhesion receptors of the immune system. Nature 346:425-434

Strasser, A., Harris, A.W., Bath, M.L., Cory, S. (1990): Novel primitive lymphoid tumours induced in transgenic mice by cooperation between *myc* and *bcl-2*. Nature 348:331-333

Suematsu, S., Matsuda, Aosaka, K., Nakano, N., Ohno, S., Miyazaki, J-I., Yamamura, K-I., Hirano, T., Kishimoto, T. (1989): IgG1 plasmacytosis in interleukin 6 transgenic mice. Proc.Natl.Acad.Sci.USA 86:7547-7551

Suematsu, S., Matsusaka, T., Matsuda, T., Ohno, S., Miyazaki, J-I., Yamamura, K-I., Hirano, T., Kishimoto, T. (1992): Generation of plasmacytomas with the chromosomal translocation t(12;15) in interleukin 6 transgenic mice. Proc.Natl.Acad.Sci.USA 89:232-235

Tanaka, Y., Adams, D.H., Hubscher, S., Hirano, H., Siebenlist, U., Shaw, S. (1993): T-cell adhesion induced by proteoglycan-immobilized cytokine MIP-1β. Nature 361:79-82

Thomas, M.L., Lefrançois, L. (1988): Differential expression of the leucocyte-common antigen family. Immunol.Today 9:320-325

Till, J.E. and McCulloch, E.A. (1980): Hemopoietic stem cell differentiation. Biochem.Biophys.Acta 605:431-459

Till, J.E., McCulloch, E.A. (1961): A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. Rad.Res. 14:213-222

Titus, R.G., Sherry, B., Cerami, A. (1991): The involvement of TNF, IL-1 and IL-6 in the immune response to protozoan parasites. Immunol.Today 12:A13-A16

Tonegawa, S. (1983): Somatic generation of antibody diversity. Nature 302:575-581

Tse, A.G.D., Barclay, A.N., Watta, A., Williams, A.F. (1985): A glycophospholipid tail at the carboxyl terminus of the Thy-1 glycoprotein of neurons and thymocytes. Science 230:1003-1005

Tsubata, T., Reth, M. (1990): The products of pre-B cell-specific genes ($\lambda 5$ *and* $V_{pre\text{-}B}$) and the immunoglobulin μ chain form a complex that is transported onto the cell surface. J.Exp.Med. 172:973-976

Tung, J-S., Shen, F-W., LaRegina, V., Boyse, E.A. (1986): Antigenic complexity and protein-structural polymorphism in the Lyb-2 system. Immunogenetics 23:208-210

Urbaschek, R., Mannel, D.N., Urbaschek, B. (1987): Tumor necrosis factor induced stimulation of granulopoiesis and radioprotection. Lymphokine Res. Summer, 6 (3):179-186

van Damme, J., van Beeumen, J., Decock, B., van Snick. J., de Ley, M., Billiau, A. (1988): Separation and comparison of two monokines with lymphocyte activating factor activity: IL-1β and hybridoma

growth factor (HGF). Identification of leukocyte-derived HGF as IL-6. J.Immunol. 140:1534-1541

van de Rijn, M., Heimfeld, S., Spangrude, G.J., Weissman, I.L. (1989): Mouse hematopoietic stem-cell antigen Sca-1 is a member of the Ly-6 antigen family. Proc.Natl.Acad.Sci.USA. 86:4634-4638

Van de Velde, I., von Hoegen, I., Luo, W., Parnes, J.R., Thielemans, K. (1991): The B-cell surface protein CD72/Lyb-2 is the ligand for CD5. Nature 351:662-665

Vaux, D.L., Cory, S., Adams, J.M. (1988): *Bcl-2* gene promotes haemopoietic cell survival and cooperates with *c-myc* to immortalize pre-B cells. Nature 335:440-442

Veillette, A., Abraham, N., Caron, L., Davidson, D. (1992a): The lymphocyte-specific tyrosine protein kinase $p56^{lck}$. Sem.Immunol. 3:143-152

Veillette, A., Davidson, D. (1992b): Src-related protein tyrosine kinases and T-cell receptor signalling. TIG 8:61-65

Vink, A., Coulie, P.G., Wauters, P., Nordan, R.P., Van Snick, J. (1988): B cell growth and differentiation activity of interleukin-HP1 and related murine plasmacytoma growth factors. Synergy with interleukin 1. Eur.J.Immunol. 18:607-612

Visser, J.W.M., Bauman, J.G.J., Mulder, A.H., Eliason, J.F., DeLeeuw, A.M. (1984): Isolation of murine pluripotent haemopoietic stem cells. J.Exp.Med. 159:1576-1590

von Hoegen, I., Nakayama, E., Parnes, J.R. (1990): Identification of a human protein homologous to the mouse Lyb-2 B cell differentiation

antigen and sequence of the corresponding cDNA. J.Immunol. 144:4870-4877

Weaver, D., Costantini, F., Imanishi-Kari, T., Baltimore, D. (1985): A transgenic immunoglobulin μ gene prevents rearrangements of endogenous genes. Cell 42:117-127

Weiss, L., Sakai, I. (1984): The hematopoietic stroma. Am.J.Anat. 170:447-463

Whitlock, C.A., Witte, O.N. (1982): Long-term culture of B lymphocytes and their precursors from murine bone marrow. Proc.Natl.Acad.Sci.USA. 79:3608-3612

Whitlock, C.A., Tidmarsh, G.F., Muller-Sieburg, C., Weissman, I.L. (1987): Bone marrow stromal cell lines with lymphopoietic activity express high levels of a pre-B neoplasia-associated molecule. Cell 48:1009-1021

Williams, D.A., Lemischka, I.R., Nathan, D.G., Mulliger, R.C. (1984): Introduction of new genetic material into pluripotent haematopoietic stem cells of the mouse. Nature 310:476-480

Williams, P. (1991): Interleukin-4: a prototypic immunoregulatory lymphokine. Blood 77:1859

Witte, P.L., Robinson, M., Henley, A., Low, M.G., Stiers, D.L., Perkins, S., Fleishman, R.A., Kincade, P.W. (1987): Relationships between B-lineage lymphocytes and stromal cells in long-term bone marrow cultures. Eur.J.Immunol. 17:1473-1484

Worton, R.G., McCulloch, E.A., Till, J.E. (1969): Physical separation of hemopoietic stem cells differing in their capacity for self-renewal. J.Exp.Med. 130:91-103

Wright, N. A., and Appleton, D.R. (1980): The metaphase arrest technique. A critical review. Cell tiss.Kinet. 13:643.

Wu, G.E., Atkinson, M.J., Ramsden, D.A., Paige, C.J. (1990): VH gene repertoire. Sem.Immunol. 2:207-216

Wu, Q., Lahti, M.J., Air, G.M., Burrows, P.D., Cooper, M.D. (1990): Molecular cloning of the murine BP-1/6C3 antigen: A member of the zinc-dependent metallopeptidase family. Proc.Natl.Acad.Sci.USA 87: 993-997

Yamanashi, Y., Kakiuchi, T, Mizuguchi, J., Yamamoto, T., Toyoshima, K. (1990): Association of B cell antigen receptor with protein tyrosine kinase Lyn. Science 251:192-194

Yancopoulos, G.D., Alt, F.W. (1986): Regulation of the assembly and expression of variable-region genes. Ann.Rev.Immunol. 4:339-368

Yoshida, Y., Osmond, D.G. (1978): Homing of bone marrow lymphoid cells. Transplantation 25:246-251

Yunis, J.J. (1983): The chromosomal basis of human neoplasia. Science 221:227-236