CONTROL OF THE LYMPHO-MYELOID POTENTIAL OF MURINE HEMATOPOIETIC STEM CELLS

by

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Abstract

Little is known about the mechanisms that regulate the maintenance and expression of the self-renewal and differentiation properties of hematopoietic stem cells (HSC) in vivo or in vitro. Murine HSC can be detected and quantitated by in vivo assays and there is evidence to suggest that many, if not all, HSC can be detected in vitro by their ability to sustain the long-term production of colony-forming cells in culture. However, at the time this thesis was initiated, reproducible and specific assays for the in vitro detection and manipulation of lympho-myeloid progenitors had not been developed. I have devised such an assay which relies on the joint expression of lymphoid and myeloid differentiative potentials in the same culture. My studies have shown that the cells detected by this assay, termed LTC-IC_{ML}, are closely related to totipotent cells with long-term competitive in vivo repopulating potential (CRU) by their similar resistance to killing by 5-fluorouracil and their >500-fold co-enrichment in a ~0.03% subpopulation of adult marrow cells.

The myeloid LTC-IC and LTC-IC_{ML} assays were used to determine whether the maintenance of long-term in vitro lympho-myeloid differentiation potential correlates with the persistence of in vivo multilineage reconstitution ability. We found that LTC-IC and LTC-IC_{ML} are both better maintained in culture than CRU, suggesting that the ability to generate clonogenic progenitors in vitro for ≥4 weeks and the ability to competitively reconstitute all lineages in myeloablated recipients can be differently regulated.

As a first step towards analyzing the mechanism(s) utilized by the stromal feeder layer in stimulating LTC-IC_{ML} in vitro, its replacement with two stromal cell-derived growth factors, interleukin (IL)-11 and the Flt3 ligand (FL) were investigated. A subpopulation of fetal liver cells highly enriched for CRU was used as a starting material since both IL-11 and FL had previously been reported to act on primitive fetal liver-derived hematopoietic cells. The results of these experiments show that both IL-11 and FL support the development and maturation of early B lineage cells but that their activities in this regard are not identical.

Table of contents

List of abbreviations

AGM	aorta-gonad-mesonephros
CAFC	cobblestone area-forming cell
CFC	colony-forming cell
CFU-S	colony-forming unit - spleen
CRU	competitive repopulating unit
CSF	colony-stimulating factor
ECM	extracellular matrix
EPO	erythropoietin
FL	Flk-2/Flt3 ligand
HSC	hematopoietic stem cell
IV	intravenous
IL-	interleukin-
LTC-IC	long-term culture-initiating cell
$LTC\text{-}IC_{ML}$	long-term culture-initiating cell (myeloid-lymphoid)
SF	Steel factor
YS	yolk sac

List of figures

List of tables

Je dédie cette thèse à ma mère

et à mes soeurs, Hélène, Huguette et Colette.

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Which brings me to Eibhlín, who taught me many useful skills and whose wit has provided many a good chuckle and to Cindy, Michelle, Balgarian, Candy, and Czara, who welcomed me into their home in the last throes of thesis writing. I am deeply grateful to you for your warmth and your friendship over the years.

I. Introduction

Most types of mature blood cells possess functional lifespans of only days. As a result, their numbers must be continually replenished from less mature precursors that have greater proliferative potential but are, nevertheless, limited to the generation of progeny belonging to only one or two lineages. Such cells are, in turn, derived from a hierarchy of progressively less developmentally restricted progenitors. The most primitive of these can give rise to all myeloid and lymphoid lineages and are additionally capable of producing functionally indistinguishable daughter cells through a process of self-renewal (Figure 1.1). The term "hematopoietic stem cell" (HSC) is used to refer to multipotent cells possessing such lympho-myeloid self-renewal ability which distinguishes them from the vast majority of progenitor cells.

Although HSC and progenitor cells can circulate (reviewed in (Metcalf and Moore 1971)), most normally reside in the bone marrow. The proliferation and differentiation of progenitors and the release of hematopoietic cells from the marrow are all believed to be regulated to some extent by fixed non-hematopoietic marrow elements, including endothelial, reticular, and fibroblastoid cells, through contact-dependent and independent mechanisms (reviewed in (Dorshkind 1990)). Daily demands for the production and export of blood cells under normal conditions and in response to common hematological stresses such as bleeding or infection, can be met at the level of proliferating marrow progenitor cells without detectable perturbations of the HSC pool which remains largely quiescent (Williams' Hematology, 5th ed.). HSC normally comprise <0.01% of the normal adult marrow cell population. This and the fact that HSC possess no distinguishing morphological features has made their direct study using conventional microscopy technically impractical. However, over 3 decades ago, it was found that these difficulties could be circumventedby stimulating individual progenitors to express their ability to give rise to morphologically and phenotypically recognizable progeny either in vivo or in vitro.

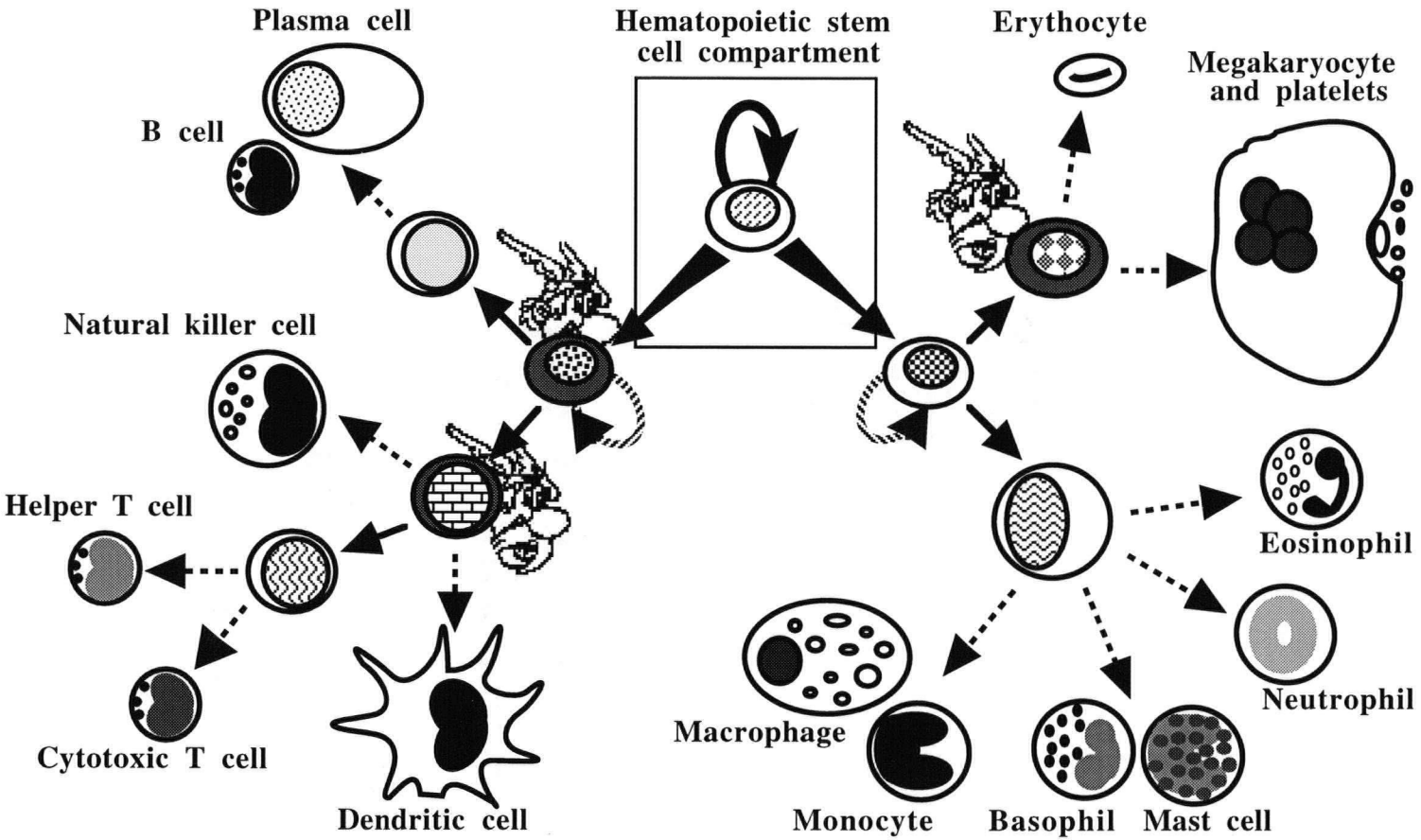


Figure 1.1 Diagrammatic model of the hierarchical structure of the hematopoietic system. The box outlines the stem cell compartment. The looped arrows represent self-renewal (hashed loops indicate more limited self-renewal potential). Cells marked with an Astérix identify stages of maturation that remain controversial or that have not been established. Dashed arrows indicate that known intermediates have been omitted for simplicity.

1.1 Hierarchical organization of hematopoietic cell types revealed by functional studies of single cells

The development of methods for revealing differences in the proliferative and differentiation potentials of separable populations of hematopoietic cells in vivo and in vitro has allowed the identification of developmental stages which precede the acquisition of recognizable lineage-specific morphological characteristics. However, this approach has also revealed considerable heterogeneity within and overlap between the earliest defined progenitor cell populations. Moreover, while the development of late precursors into end cells appears very tightly regulated in vivo, regulation of the numbers of hematopoietic cells at earlier stages appears to be less rigidly controlled and may also change during ontogeny.

1.1.1 Progenitor cell types defined by colony assays

Studies based on the formation of colonies yield three types of information about progenitor cells: (i) enumeration of colonies provides an estimate of the frequency of progenitors in a given test population; (ii) analysis of the cellular composition of clones can reveal the differentiative potential of the original clonogenic progenitor; and (iii) changes to the culture conditions can allow a definition of the responsiveness of different progenitor cell types to different factors. The use of colony assays to generate such information has allowed the identification and quantitation of cells at different stages of hematopoietic cell development as well as a description of the factors that can regulate their activities and the field of hematology owes much of its present understanding to the investigation of in vivo and in vitro colony-forming cells.

a) In vivo spleen colony-forming units (CFU-S)

The first quantitative colony assay for primitive hematopoietic cells was described in 1961 by Till and McCulloch (Till and McCulloch 1961). They found that a proportion of marrow cells injected intravenously (IV) into lethally irradiated recipients lodged in the spleen and proliferated there, forming discrete, macroscopic nodules within 7 to 12 days. These could be easily enumerated in situ and excised for further study. The single cell origin of the cells within individual nodules was established from analyses of spleen colonies obtained after injection of marrow cells from mice carrying radiation-induced chromosomal aberrations (Becker, et al 1963). Subsequent studies showed that the cells which give rise to individual spleen colonies (colony-forming unit-spleen, CFU-S) can vary markedly in their proliferative, differentiative, and self-renewal potential (Till, et al 1964; Wu, et al 1967; Worton, et al 1969; Magli, et al 1982; Priestley and Wolf 1985). Individual spleen colonies detectable within 7-9 days after injection of normal adult marrow cells usually contain a restricted spectrum of differentiated cells. They consist of either erythroid or granulocytic cells but rarely both (Curry and Trentin 1967; Magli, et al 1982), rare daughter CFU-S (Metcalf and Moore 1971) and very occasional lymphoid precursors (Lepault, et al 1993). Those subsequently detected on days 12-14 are often composed of multiple myeloid lineages and more frequently contain daughter CFU-S as well as B and T lymphocyte precursors (Siminovitch, et al 1963; Curry and Trentin 1967; Lepault, et al 1993). In addition, in the marrow of normal adult mice, a larger proportion of day 8 than day 12 CFU-S are actively cycling, as measured by bromodeoxyuridine incorporation (Hodgson and Bradley 1984; Pietrzyk, et al 1985). Day 8 CFU-S do not exclude the vital dye Rhodamine-123 to the same extent (Ploemacher and Brons 1989) and are more sensitive to killing by the cycle-active drug 5-fluorouracil (5-FU) (Hodgson and Bradley 1979). The fact that the majority of day 8 CFU-S can be physically separated from the bulk of day 12 CFU-S (Ploemacher and Brons 1989) in suspensions of normal adult mouse marrow cells also supports the concept that these cells represent two biologically distinct populations

and the greater differentiative and self-renewal potential of the latter would identify them as the more primitive.

Even within the subset of day 12 CFU-S, variable self-renewal and differentiative abilities are observed (Till, et al 1964; Wu, et al 1967). Two models, depicted in Figure 1.2, were initially proposed to account for this variability. The first, termed Hemopoiesis Engendered Randomly (HER), postulates that a single cell may either give rise to progeny like itself or be removed in some way and that these two events occur randomly and independently of the cell's previous history. Till et al (Till, et al 1964) found good agreement between the distribution of CFU-S they observed and a Monte-Carlo simulation of a simplified birth-and-death process with fixed birth and death probabilities and a fixed generation time. Two sample runs with the probabilities of birth and death set at 0.6 and 0.4, respectively, are shown in Figure 1.2. In this model, the fate of individual CFU-S is not tightly controlled but the behaviour of the population as a whole is regulated by mechanisms that establish the birth and death probabilities as well as the proportion of CFU-S that are cycling (Becker, et al 1963). A second model, appositely termed HIM (Hemopoietic Inductive Microenvironment), proposes that the behaviour of individual cells is determined by their particular microenvironmental interactions (Curry and Trentin 1967). In this case, pluripotent cells subjected to identical conditions would all respond in a very similar manner and give rise to essentially identical colonies. However, subsequent in vitro studies revealed that daughter pluripotent cell types are randomly distributed in macroscopic mixed myeloid-erythroid colonies (Humphries, et al 1981) and that pairs of clonogenic progenitors derived from individual, micromanipulated cells frequently express non-identical differentiative potential (reviewed in (Ogawa 1993)) despite essentially uniform culture conditions and the absence of the kind of heterogeneity inherent in a tissue microenvironment.

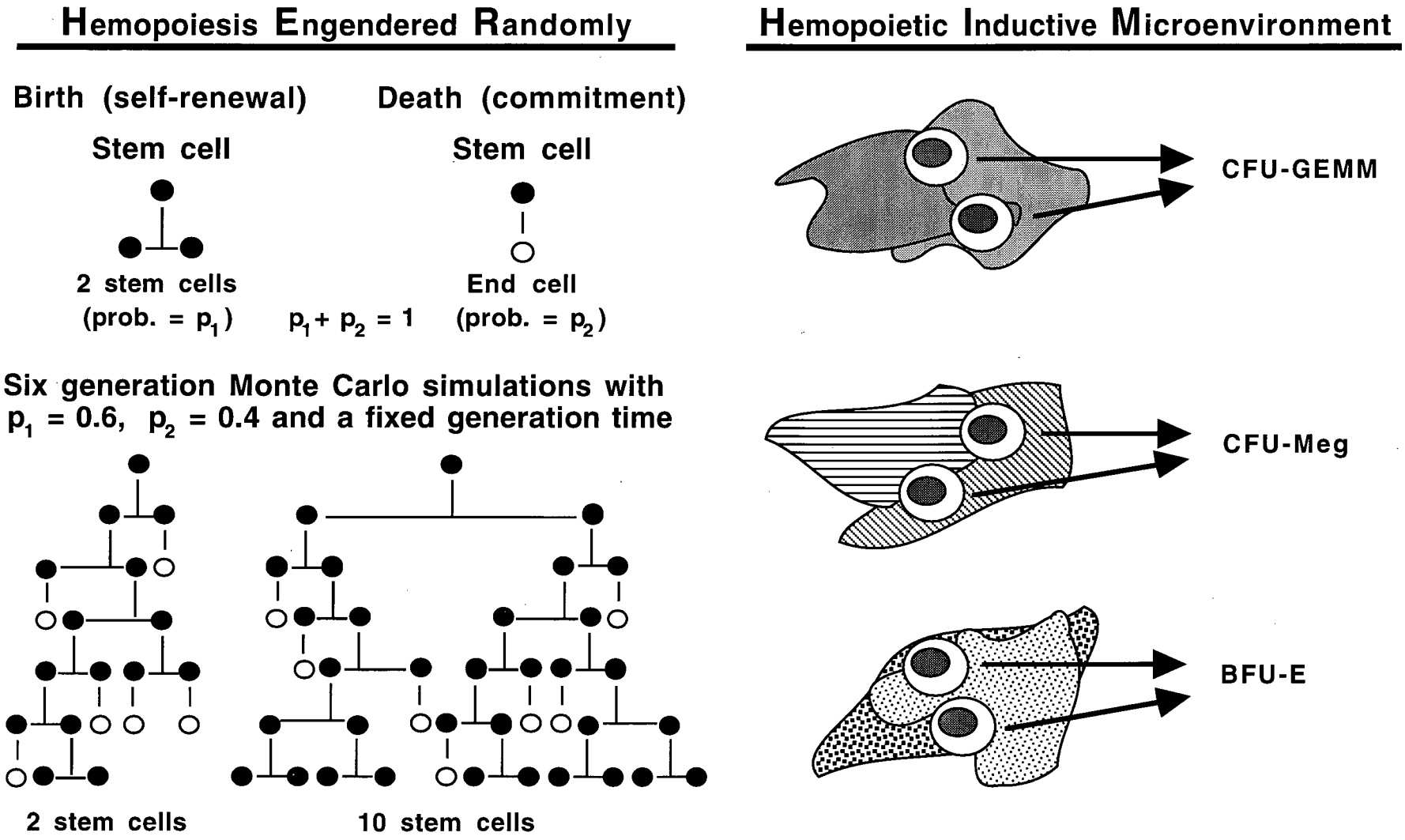


Figure 1.2 Models of stem cell commitment and self-renewal. Shown on the left are two possible runs of a Monte Carlo simulation of HSC self-renewal with the indicated probabilities of birth and death (adapted from Siminovitch, et al 1963). Illustrated on the right is the postulated directive effect of different microenvironments (depicted as flat, broad shapes) on the commitment of initially identical hematopoietic progenitor cells.

b) In vitro clonogenic progenitors

Identification of the precise molecular events that underlie early hematopoietic cell development requires procedures that allow the cells of interest to be detected and their initial responses to be examined. This need, plus the desire to investigate analogous primitive human hematopoietic progenitors provided a strong impetus to the identification of conditions that would allow the proliferative and differentiative potential of individual hematopoietic progenitor cells to be expressed and detected in vitro. There are two main components to the assays that were subsequently developed to achieve this goal: the use of a semi-solid matrix, such as agar, methylcellulose, or plasma clot serves to keep each clone physically distinct, and the establishment of an optimal concentration of growth stimulatory factors by providing a suitable underlying feeder cell layer or by incorporating appropriate culture supernatants or, more recently, recombinant growth factors into the culture medium. The first such assay described detected the growth of monocytic and granulocytic colonies in semi-solid agar cultures (Bradley and Metcalf 1966; Pluznik and Sachs 1965). Through extensions of this approach, committed progenitors for most of the myeloid and lymphoid lineages have now been identified (Figure 1.3, (Suda, et al 1989) and reviewed in (Dexter, et al 1984)). In general, the timing of appearance of mature cells in the colony can be used to discriminate lineage-restricted progenitors at different stages of differentiation. This is possible because the immediate progeny of more differentiated progenitors begin to undergo terminal maturation resulting in the rapid appearance of small colonies of mature cells. In contrast, the immediate progeny of more ancestral cell types apparently need to complete several additional cell cycles before any of the cells produced are able to initiate terminal maturation events. As a result, these progenitors can give rise to larger colonies in which evidence of mature forms appears after a greater delay. The majority of clonogenic cells give rise to only one or two types of mature progeny and will not be discussed further here.

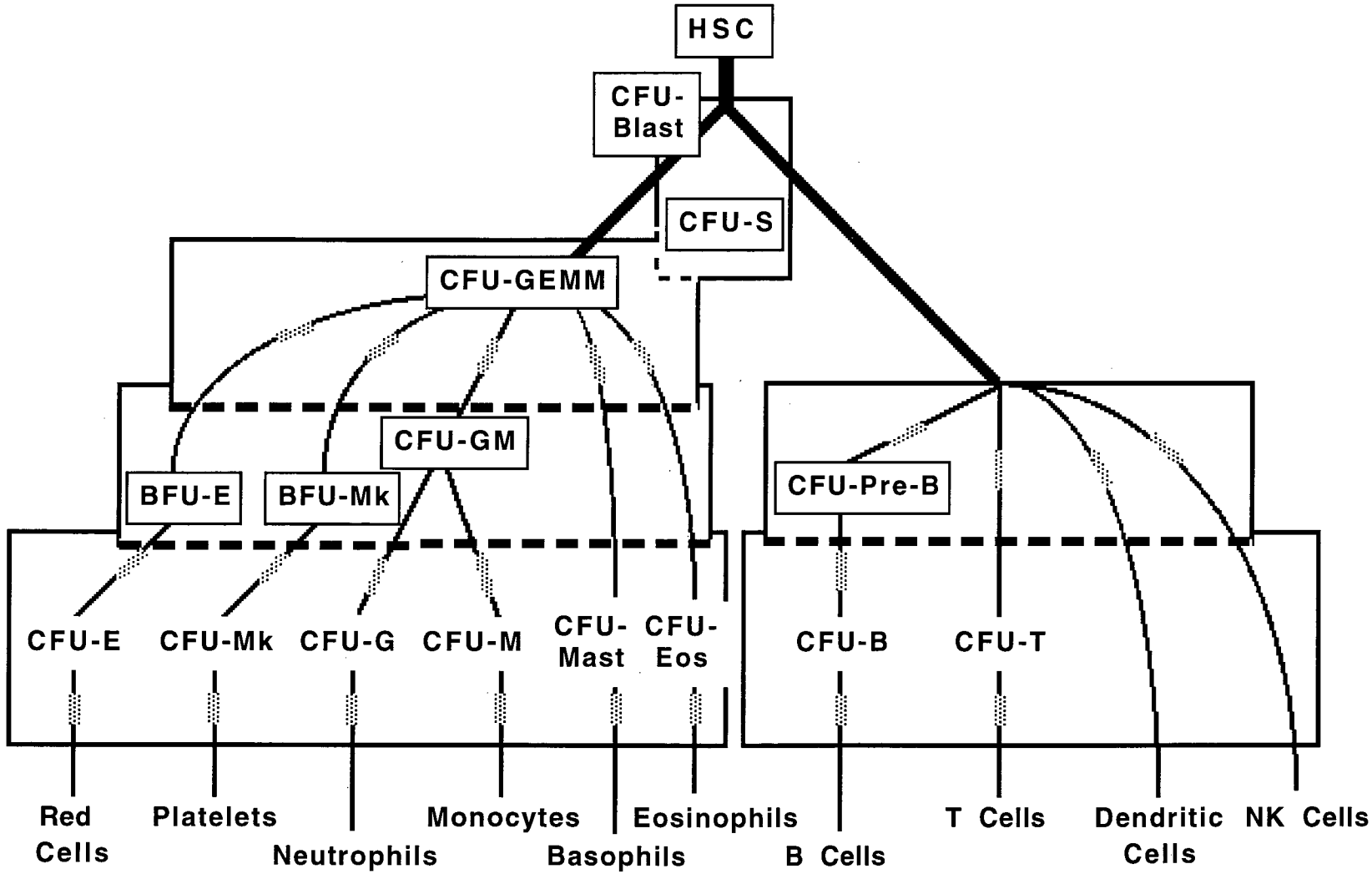


Figure 1.3 Progenitor cell types defined by colony assays.

Colony assays for murine and human multipotent myeloid progenitors have been available for almost two decades (Fauser and Messner 1979; Hara and Ogawa 1978; Johnson and Metcalf 1977). Soon after they were described, it was shown that there is some overlap between day 9 CFU-S in adult mouse marrow and progenitors detected in vitro by their ability to generate macroscopic multilineage colonies composed of granulocytic, erythroid, megakaryocytic, and monocytic cells (CFU-GEMM; Humphries, et al 1979b; Johnson 1980). Replating experiments have also shown that some of these multilineage colonies may contain daughter CFU-GEMM but that the majority of them display a rather limited ability to give rise to daughter CFU-GEMM in tertiary subcultures (Humphries, et al 1981). Such findings placed these multipotent progenitors in the upper reaches of the hematopoietic hierarchy and were soon followed by the description of a subset of murine (Nakahata and Ogawa 1982), and later human (Leary, et al 1984), pluripotent in vitro clonogenic progenitors with similar self-renewal capacity. These latter progenitors give rise to colonies that consist of 40-1000 cells with a blast morphology which may show no evidence of terminal maturation even after 16 days. Upon replating, 42 of 43 "blast" colonies analyzed in one report gave rise to CFU-GEMM with an average plating efficiency of 80% and, in 20 of 43 cases, secondary blast colonies were also observed (Nakahata and Ogawa 1982). Differentiation of blast cells into B and cytotoxic T lymphoid progenitors has also been demonstrated under appropriate in vitro culture conditions (Minato, et al 1988; Hirayama, et al 1992). In addition, it has been reported that IL-3-induced blast colonies derived from cells in the spleens of 5-FU-treated mice may contain day 8 and day 12 CFU-S, as well as cells capable of reconstituting both lymphoid and myeloid lineages for up to 10 weeks in irradiated recipients (Tsunoda, et al 1991).

A random distribution of derivative colony types was observed in single cell subcultures of dispersed primary blast colonies and of paired daughter cells replated by micromanipulation (Suda, et al 1983b; Suda, et al 1984). This further reinforced the concept that commitment and self-renewal still occur stochastically under a variety of growth stimulatory conditions. Two models of stochastic stem cell commitment have been suggested to

account for the lineage distributions observed (Figure 1.4, (Ogawa 1993)). The model shown on the left envisages the random, progressive restriction of differentiative potential and predicts the existence of oligopotential progenitors. The other accounts for multilineage colonies by allowing three types of stem cell divisions to occur stochastically: a duplication division generating two daughter HSC; a maintenance division giving rise to one stem cell and one random-lineage, monopotential progenitor cell; and a terminal division during which stem cell potential is lost and two monopotential progenitors are produced. However, as pointed out by Ogawa, the models shown in Figure 1.4 are based on studies of isolated progenitors in an artificial culture system and at least some of the variation in the colony types seen may be the result of localized cytokine interactions occurring during colony formation (Ogawa 1993).

After the identification of Steel factor (SF) and interleukin (IL)-7 as growth factors involved in the development of early B lymphoid cells (Lee, et al 1989; Suda, et al 1989; McNiece, et al 1991b), a second culture stage was added to the blast colony assay to evaluate their B lymphoid progenitor content. It was found that a considerable proportion (~40%) of primary blast colonies derived from highly purified marrow cells from 5-FU-treated mice contained cells that could give rise to both myeloid and lymphoid colonies when replated in the presence of SF and IL-7 (Hirayama, et al 1992). Quite recently, Ogawa and his colleagues have described a single stage assay containing SF, IL-7, erythropoietin (EPO), and IL-11 which allows an evaluation of both the myeloid and the lymphoid potentials of highly purified subpopulations of normal marrow cells (Ball, et al 1995). Although the data are limited at present, it appears that the myeloid potential of the colonies may be unaffected by the presence or absence of B lymphoid progenitors. Interestingly, no B lymphoid differentiation appears to occur when purified marrow cells from 5-FU-treated mice are plated in the same single stage assay, despite the fact that the colonies produced can readily give rise to B lymphoid colonies upon replating in cultures containing SF and IL-7. Ball et al suggest that the progenitors in normal marrow that could form primary lympho-myeloid colonies in the presence of IL-7 + SF

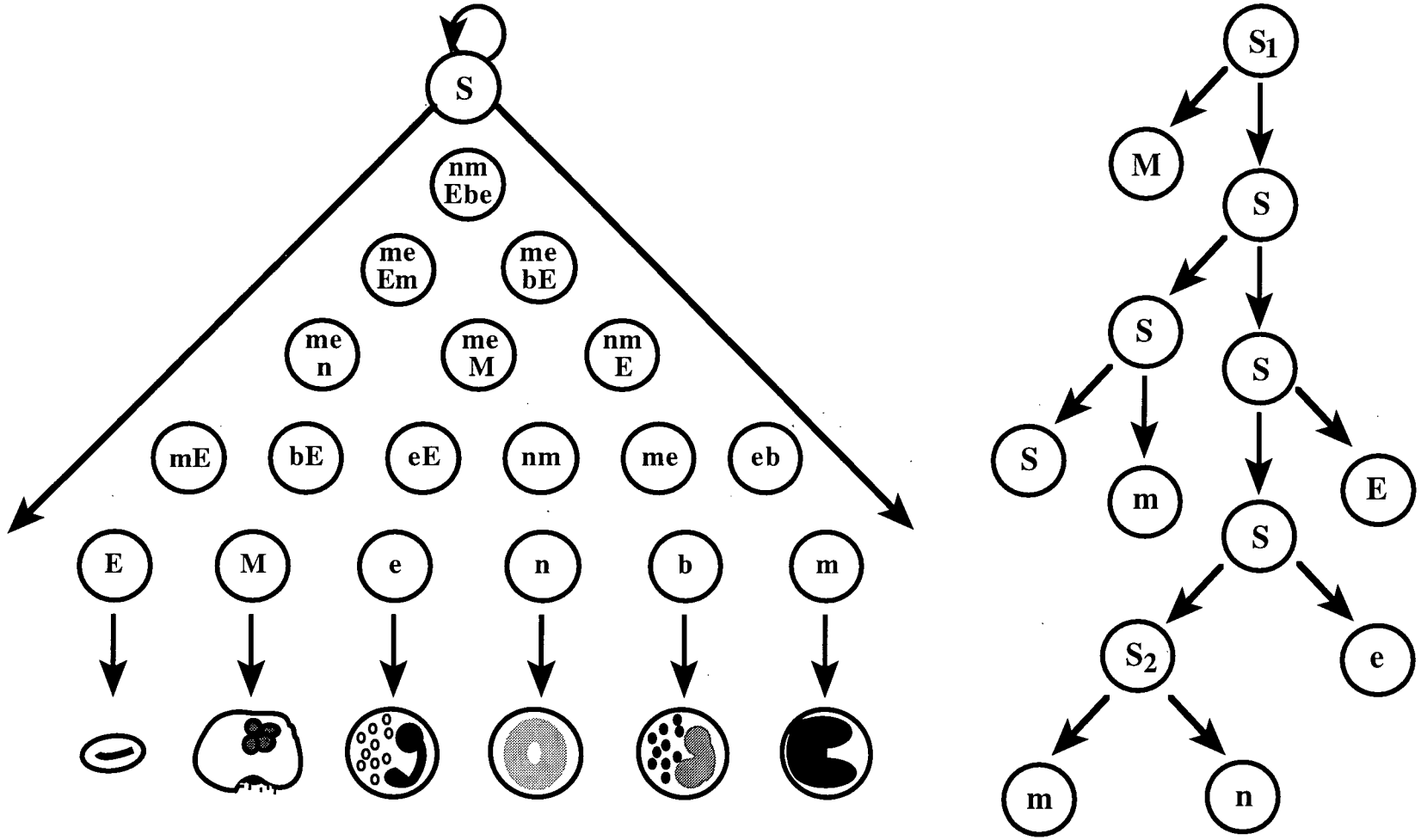


Figure 1.4 Models of the stochastic commitment of stem cells (S - stem cell, n - neutrophil, m - macrophage, e - eosinophil, b - basophil, M - megakaryocyte, E - erythrocyte). The model on the left proposes the progressive restriction in differentiation potential and concomitant generation of multi- and oligopotent progenitor cell types. The model on the right shows commitment to a single lineage with or without self-renewal. As shown, S_1 would give rise to a mixed myeloid colony with 2° blast colony-forming potential while S_2 would read out as a CFU-GM. (Adapted from Ogawa 1993)

+ IL-11 + Epo might have been actively proliferating cells that were thus sensitive to the cytotoxic action of 5-FU (Ball, et al 1995).

1.1.2 Primitive progenitors defined by assays of long-term proliferative potential

One of the defining characteristics of the HSC compartment is its ability to sustain blood cell production for the lifetime of an animal. Both in vivo and in vitro assays that use time to discriminate between cells capable of short-term as opposed to longer-term proliferation have been developed. As depicted in Figure 1.5, there is potentially considerable overlap in the cell populations detected by these different assays.

a) Long-term in vivo reconstituting cells

The most rigorous functional definition of a HSC is a cell that can provide the long-term reconstitution of all blood cell lineages in a recipient animal. In order to ensure the detection of graft-derived reconstitution, it is necessary to have some means of discriminating between hematopoietic cells of donor and host origin. Early experiments made use of cytogenetic markers, either naturally occurring, such as the T6 marker (Ford, et al 1966) or Y chromosome (Lamar 1984) or induced by prior sublethal irradiation of donor mice (Wu, et al 1967). More common now is the use of congenic mice which allow blood cell genotypes to be distinguished by electrophoresis of encoded Gpi-1^a/Gpi-1^b isoenzymes (Van Zant 1983), Hbb^d/Hbb^s hemoglobin species (Harrison 1980) or cell surface allo-antigens that are detectable by flow cytometry after labelling of the cells with appropriate antibodies, such as anti-Ly-5.1 and anti-Ly-5.2 (Spangrude and Scollay 1990)).

Figure 1.6 shows the general design of the long-term in vivo reconstitution assay used in this thesis. The cells of interest are injected IV, usually into supralethally irradiated recipients

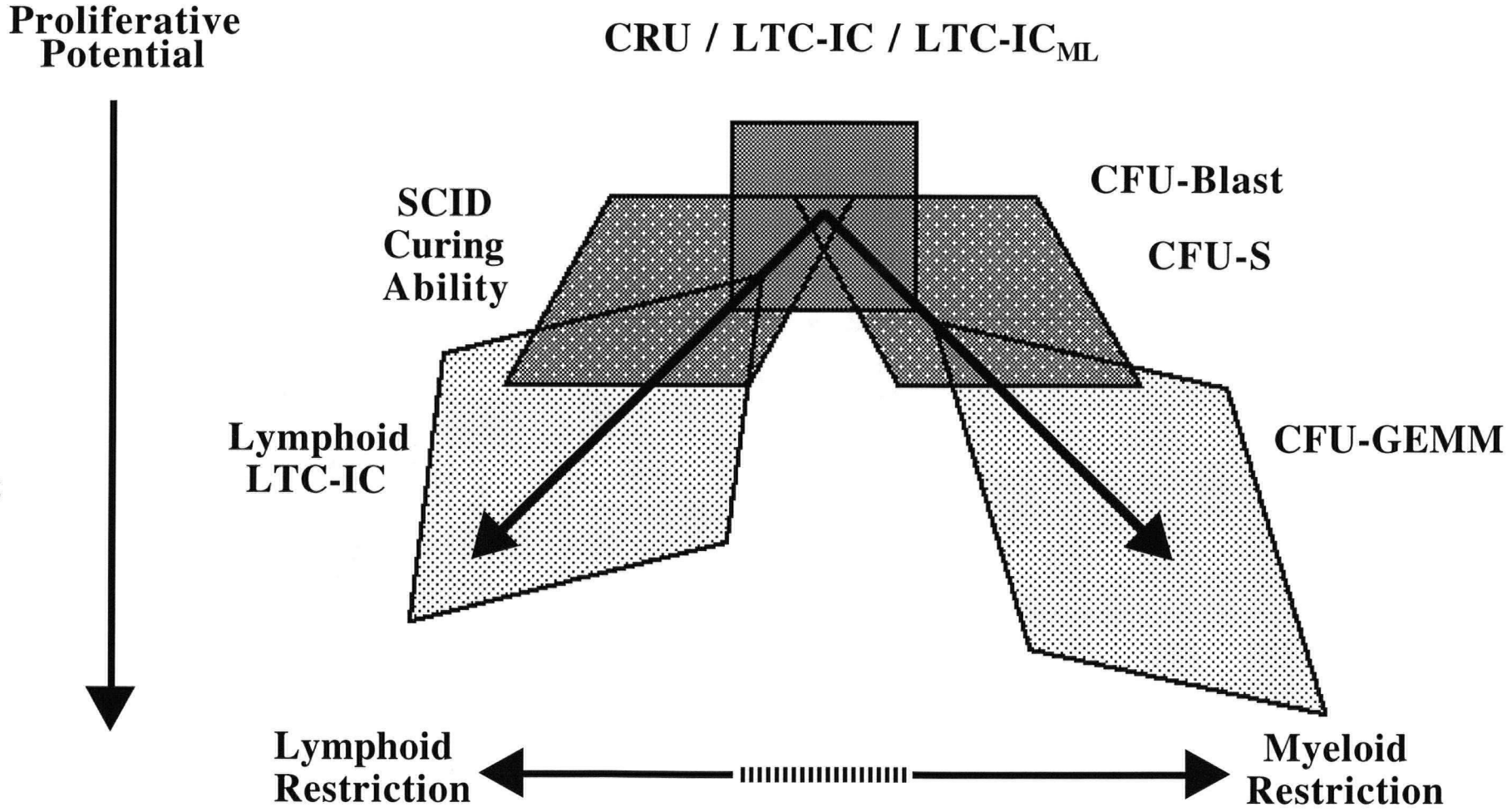


Figure 1.5 Progenitor cell types detected by long-term in vivo and in vitro assays.

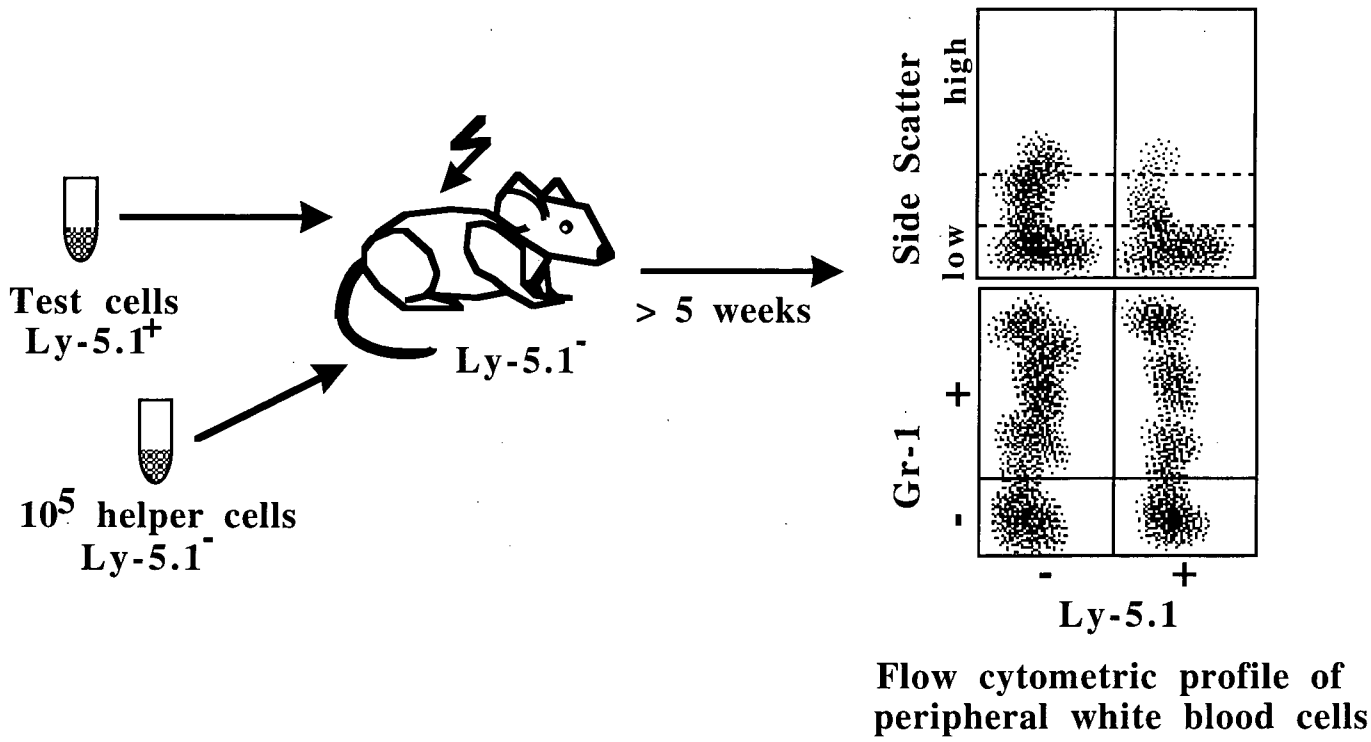


Figure 1.6 Competitive in vivo repopulation unit (CRU) assay. Test cells and helper cells are co-injected IV into irradiated animals. Test cells are distinguishable from helper and host blood cells by the presence (+) of the Ly-5.1 antigen on their cell surface. After > 5 weeks, small blood samples are taken from the recipient animals and examined for the presence of Ly-5.1 on myeloid (Gr-1$^+$) and lymphoid (low side scatter) cells. Recipients are considered to be reconstituted by test cells if there are Ly-5.1$^+$ lymphoid cells present and ≥ 5% of their Gr-1$^+$ cells are also Ly-5.1$^+$.

although, in theory at least, it should be possible to use any strategy that reduces the number or regenerative potential of the host HSC population. If the graft does not contain the cells necessary for short-term hematological rescue, the recipients will die of hematological failure, usually within 14-20 days. If the graft does not contain cells capable of long-term engraftment, only transient (1-2 months) reconstitution of blood cell production occurs. In cases where the long-term engraftment potential of small numbers of test cells is to be assessed, particularly where limiting dilution analysis techniques are being used to quantitate input HSC numbers, it may be necessary to co-inject an additional dose of cells to ensure the short-term survival of recipients. These so-called helper cells must be distinguishable from the test cells and, if necessary, from the hosts' own cells.

The size of the test cell and additional cell inocula and the time at which animals are analyzed both depend on the objective(s) of the experiment. The competitive repopulation assay first described by Harrison (Harrison 1980; Harrison, et al 1993) compares the relative ability of two populations to contribute to long-term hematopoietic reconstitution. In this case, the helper cell population usually consists of a fixed dose (usually $1\text{-}2x10^6$ cells) of fresh marrow which serves as the standard of repopulation potential against which the test population is measured. Harrison and his colleagues have shown that there is an inverse correlation between the number of HSC injected and the variance of the percentage repopulation observed in recipients which can be used to derive estimates of the HSC content of the test population (Harrison, et al 1993). However, because this method does not track individual HSC, it cannot distinguish between an increase in the proliferative capacity of HSC and an increase in the number of HSC present in the test population.

The competitive repopulation unit (CRU) assay described by Szilvassy et al, on the other hand, has been shown to specifically detect individual lympho-myeloid hematopoietic cells both by analysis of common reconstitution of lymphoid and myeloid tissues by limiting numbers of cells and by retroviral marking of individual HSC (Szilvassy, et al 1990; Fraser, et

al 1992). In this assay, limiting numbers of test cells are injected into groups of irradiated recipients together with ~10^5 normal marrow cells or helper cells compromised in their ability to competitively repopulate irradiated animals by serial transplantation (Szilvassy, et al 1990) which ensure the short-term survival of the recipients and provide a certain degree of competition. The frequency of CRU in the starting cell suspension can then be derived by means of limiting dilution statistics from the proportion of animals reconstituted at each dose of test cells used (Fazekas de St. Groth 1982).

These competitive repopulation strategies have been used to show that, overall, the HSC content of long-term cultures (LTC) is reduced by comparison to fresh marrow (Harrison, et al 1987; Fraser, et al 1990), although some HSC can proliferate in culture without losing their in vivo repopulating ability (Fraser, et al 1992). In addition, it has recently been shown that human marrow cell transplants can regenerate hematopoiesis in irradiated immunodeficient mice (Lapidot, et al 1992; Cashman, et al 1994) or immunodeficient mice carrying human organ transplants (Kyoizumi, et al 1992; Chen, et al 1994), thus potentially providing a basis for the future development of a CRU assay for human hematopoietic cells.

In vivo assays for primitive, lineage-restricted progenitors have also been described. Mice homozygous for the severe combined immunodeficiency (SCID) mutation lack functional B and T cells. Sublethally irradiated animals can be cured of this defect by a graft of normal or cultured marrow cells (Dorshkind, et al 1984; Dorshkind, et al 1986; Fulop and Phillips 1986). Since all subsequently detectable lymphocyte activity can be attributed to donor cells, this system provides a very sensitive read-out of lymphoid reconstitution potential. Myelopoiesis is, however, apparently normal is these animals (Bosma, et al 1983; Dorshkind, et al 1984) so that a lack of myeloid potential in the test population also needs to be demonstrated to establish the lymphoid restriction of the reconstituting cells in this system.

b) Long-term culture-initiating cells (LTC-IC)

The advantage of in vivo systems, even when perturbed by irradiation, drugs, or congenital hematological deficiencies, is that they provide information relevant to processes that occur in the intact animal. The corresponding disadvantage of such systems is that it is difficult to monitor, or selectively manipulate, the initial events that lead to hematopoietic reconstitution. In vitro systems which allow the generation of differentiating myeloid cells for several months (Dexter, et al 1977; Greenberger 1978) have provided the means to investigate the regulation and maintenance of hematopoietic progenitors and stem cells under conditions which reproduce many aspects of the marrow environment (Dexter, et al 1984; Eaves and Eaves 1988). In addition, these culture systems have served as the basis for the development of quantitative assays for very primitive hematopoietic cells.

Because both committed progenitor cells and their maturing progeny have finite proliferative potentials and the lifespans of their final products are relatively short, the presence of proliferating committed progenitors in cultures for extended periods of time implies their origin from a more primitive cell type in the starting inoculum. This reasoning led to the development of long-term culture assays for analogous primitive cells of human (Sutherland, et al 1990; Baum, et al 1992; Breems, et al 1994; Pettengell, et al 1994) and murine (Ploemacher, et al 1989) origin. Ploemacher et al monitored the appearance and disappearance of localized areas of proliferating hematopoietic cells, referred to as cobblestone areas because of their pavement-like appearance in the adherent layer of LTC over a period of 4 weeks (Ploemacher, et al 1989). They found a strong correlation between the frequency of such cobblestone areas (CA) detectable on day 28 and in vivo marrow repopulating cells capable of generating day 12 CFU-S and CFU-C in the marrow of irradiated recipients over a period of 12-13 days. Using populations that differed in their content of day 7-8 CFU-S, day 12 CFU-S and cells with marrow repopulating ability (MRA), they also showed that day 12 CFU-S frequencies correlated well with the presence cells detectable as CA (CAFC) on day 10 of LTC.

Furthermore, they showed that day 28 CAFC were largely separable from day 10 CAFC and day 12 CFU-S, thus validating this assay for the study of very primitive hematopoietic cells. By initiating micro-LTC with limiting numbers of cells, the frequency of CAFC in the starting population could also be determined (Ploemacher, et al 1991).

This methodology is similar to that independently developed for human cells where the presence of clonogenic cells in cultures initiated at limiting dilutions, rather than a visual end point, was used to derive the frequency and clonogenic output capacity of the initial hematopoietic precursors cells (Sutherland, et al 1990). Because the cells detected in this assay can give rise to clonogenic progeny in culture for ≥5-8 weeks, they were christened "long-term culture-initiating cells" (LTC-IC). Although in vivo assays are not, as yet, available to quantitate human HSC, grafts enriched for cells expressing CD34 can protect patients given myeloablative therapy against marrow failure (Berenson, et al 1990), suggesting that human in vivo repopulating cells are $CD34^+$. It has also been shown that the majority of LTC-IC can be recovered in the $CD34^+$ fraction of human bone marrow (Sutherland, et al 1989; Sutherland, et al 1990).

1.1.3 Early stages of B lymphoid development

The study of B and T cell development, unlike that of most of the other hematopoietic lineages, has relied more on the use of molecular and immunological techniques than on the detection of different types of progenitors by discriminating in vitro colony assays. Defined conditions that support the extensive proliferation of T cell progenitors, with or without their accompanying differentiation, have been difficult to establish and this situation may be further compounded by the fact that most of the cells produced during normal T cell development are eliminated by apoptotic mechanisms. On the other hand, specific cytokines that can support the formation of colonies by B cell progenitors (see above) have been identified. In addition,

conditions that allow their long-term propagation in vitro have been identified (Whitlock and Witte 1982). Nevertheless, early stages of B cell development have been defined most precisely in terms of changes in the expression of various cell surface markers and rearrangements in the immunoglobulin (Ig) genes that these cells undergo (reviewed in (Rolink and Melchers 1991)). Only relatively recently have responses to specific growth factors begun to be included as additional discriminating features of early stages of B cell development.

In the adult, the primitive B cell progenitor pool matures in the intersinusoidal spaces of the marrow in close contact with stromal cells in the absence of antigen stimulation (Jacobsen and Osmond 1990) and is culled of aberrant and self-reactive members before immature IgM^+ B cells enter the circulation (Deenan, et al 1990). As shown in Figure 1.7, the earliest B cell progenitors reported in adult mouse marrow are those that express neither the B220 (CD45RO) nor the Fall-3 antigens (Müller-Sieburg 1991). These early progenitors can give rise to B lymphocytes when co-cultured with an adherent stromal layer but, unlike Fall-3^+B220^- cells, cannot reconstitute all lineages in irradiated recipient animals. B220 is expressed on all subsequent stages of B cell development in combination with other antigens ((Coffman and Weissman 1981) and reviewed in (Rolink and Melchers 1993)). CD43 (leukosialin) is expressed on early progenitors of several lineages but its coexpression with B220 in the absence of heat stable antigen (HSA) marks an early pro-B stage in which rearrangement of the μ heavy chain diversity (D_H) and joining (J_H) regions is just beginning (Hardy, et al 1991). A variable (V_H) region is subsequently spliced to the DJ_H segment and then a constant (C_μ) region is added to produce the complete μ heavy chains ($VDJC_\mu$) detectable in the cytoplasm. Light chain gene rearrangement (commonly κ before λ) is generally initiated upon cytoplasmic μ heavy chain expression and, if both heavy and light chains are productively rearranged, cell surface expression of IgM occurs (reviewed in (Yancopoulos and Alt 1986)). The precise role of the so-called surrogate light chain, a complex of $V_{pre\text{-}B}$ and λ5 proteins, which has been found associated with μ heavy chain at the cell surface throughout early B cell development (Pillai and Baltimore 1987) is not yet completely clear. However, the analysis of λ5 knock-out

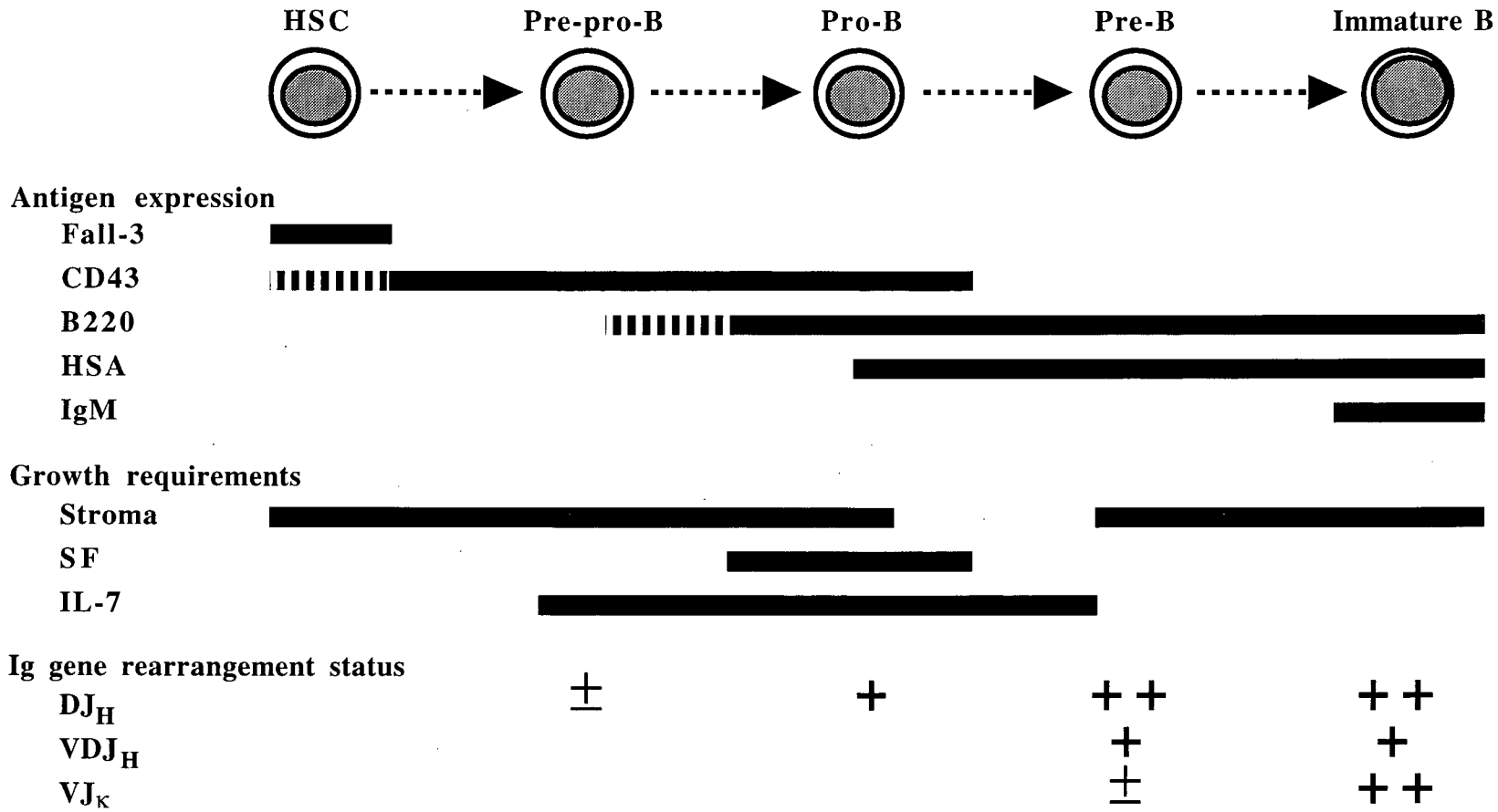


Figure 1.7 Summary of early stages of adult mouse B cell development. (Adapted from (Hardy, et al 1991) and (Rolink and Melchers 1993))

mice suggests that expression of the surrogate light chain is essential for the normal development of pre-B cells (Kitamura, et al 1992).

In parallel with these phenotypic and genomic changes, developing B lymphocytes undergo stage-dependent variations in their growth requirements. The transition to $B220^+$ pro-B cells is dependent on contact with an adherent stromal cell layer (Landreth and Dorshkind 1988; Sudo, et al 1989; Billips, et al 1992). Late pro-B cells can then proliferate in the absence of further contact with stromal cells, first in the presence of SF and IL-7 together (McNiece, et al 1991b; Billips, et al 1992) and then with IL-7 alone (Namen, et al 1988; Lee, et al 1989; Suda, et al 1989). Additional maturation again requires co-culture with a competent adherent cell layer (Cumano, et al 1990), although different signals appear to be involved. This is evidenced by the fact that, although fetal liver cells can progress from a $B220^-$ to a $B220^+$ pre-B cell stage in stromal cell-free cultures supplemented with IL-11 + SF + IL-7, the same conditions do not allow the further transition from a pre-B cell to a lipopolysaccharide-responsive B cell (Kee, et al 1994).

Many of these early stages of B cell development were identified in immortalized cell lines derived under lymphoid long-term culture conditions originally described by Whitlock and Witte (Whitlock and Witte 1982). However, these conditions do not maintain HSC (Kurland, et al 1984; Dorshkind, et al 1986). Myeloid LTC which support HSC maintenance (Fraser, et al 1990; Fraser, et al 1992) but not the growth of B or T lineage cells (Dexter and Spooncer 1978) can give rise to B lymphoid cells when they are switched to lymphoid LTC conditions (Denis and Witte 1986; Dorshkind 1986). In conjunction with the in vitro assays recently described for detecting lympho-myeloid progenitors in human fetal marrow (Baum, et al 1992), murine fetal liver (Cumano, et al 1992), and adult mouse marrow (Hirayama, et al 1992; Ball, et al 1995), we have hypothesized that this culture switching system may allow the process of B cell progenitor derivation from HSC to be examined in greater detail (see Thesis Objectives, Section 1.4, below).

1.1.4 Impact of cycling status and seeding efficiency on stem cell measurements

The seeding efficiency of CFU-S is measured by comparing the number of spleen colonies generated by a standard dose of cells with the number produced by a much larger dose of input cells recovered from the spleen 2-24 hours later (Siminovitch, et al 1963). Estimates of the seeding fraction, f, thus determined vary widely (3-25%, reviewed in (Metcalf and Moore 1971)). A number of factors have been shown to affect f value measurements. For example, CFU-S from marrow, spleen, fetal liver have different f values as do differentially cycling CFU-S (Lord, et al 1993). It has also been shown that there is a ~2-fold reduction in the detection of CFU-S as a result of antibody labelling of the injected cells (Szilvassy, et al 1989). However, populations of adult marrow cells can now be obtained by flow cytometry which are at least 10% pure CRU (Smith, et al 1991; Morrison and Weissman 1994). Thus for CRU, the plating efficiency must be at least 10%.

Even relatively modest differences in the self-renewal and death rates, cell cycle length or the proportion of the population proliferating can have a considerable impact on the rate at which mature cells are produced from HSC. This is illustrated in Figure 1.8 where theoretical growth curves were calculated for two populations (A and B) with slightly different kinetic parameters (A: % of population cycling = 25, % loss/division = 19, cell cycle length = 24 hrs; B: % cycling = 35, % loss = 20, cycle length = 22 hrs).

It has been shown that some types of primitive hematopoietic cells can enter and exit the cell cycle in long-term cultures upon the addition of various directly or indirectly acting factors (Eaves, et al 1991; Toksoz, et al 1992). However, not much else is known about the effects of different culture conditions on cell cycle kinetics nor on the seeding efficiency of HSC. Nor is it known how well currently defined culture conditions maintain the lympho-myeloid differentiation potential and self-renewal ability which are the defining attributes of HSC.

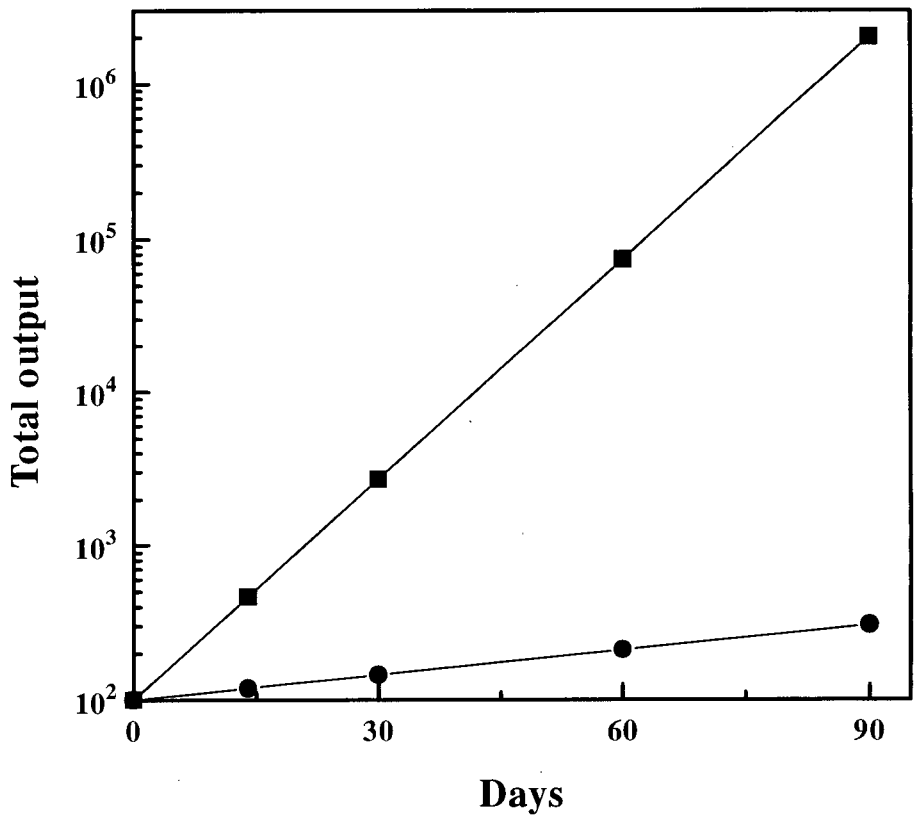


Figure 1.8 Impact of slight differences in cycle length, % population cycling, and % population dying/division. Line A (●) defined as cycle length = 24 hrs, % cycling = 25, % dying/division = 19. Line B (■) defined as cycle = 22 hrs, % cycling = 35, % dying = 20. Equation of the lines:

$$\text{total output} = \text{starting \#} \times ((1 + \%\ \text{cycling}) \times (1 - \%\ \text{dying}))^{(\text{days / cycle length})}$$

1.2 Regulation of early stages of hematopoiesis

The ordered development of mature blood cell populations from HSC must involve a series of control mechanisms that translate extracellular signals into the changing patterns of gene expression that ultimately define each of the various blood cell types. It is now well established that the survival, proliferation and maturation of different types of hematopoietic progenitors in vivo is regulated by their interactions with the soluble and membrane-bound products of a variety of cell types, including other blood cells, and with extracellular components of their environment. In the last 20 years, over 20 such mediators and their receptors have been identified and characterized at the molecular level. The cascade of intracellular events triggered by the interactions of hematopoietic cells with their molecular surroundings is now an area of intense investigation which is beginning to yield fascinating insights into the intricate network of molecules involved in transducing external signals to the nucleus.

1.2.1 Stromal cells

In normal adult animals, the marrow is the only site in which myelopoiesis, erythropoiesis, and lymphopoiesis proceed simultaneously. Moreover, although extramedullary hematopoiesis can occur transiently when progenitors are introduced IV, as is the case in experimental or clinical transplantation settings, long-term hematopoiesis becomes established only in the bone marrow. Thus local influences exerted by the marrow stroma appear critical for the support of sustained blood cell production (reviewed in (Dorshkind 1990; Lichtman 1984)).

Ultrastructural and radiolabelling studies have revealed newly produced and maturing blood cells of different lineages to be localized within the extravascular spaces between marrow sinuses in close association with fixed tissue elements, collectively referred to as stromal cells

(Weiss 1976; Tavassoli and Shaklai 1979; Lichtman 1981; Osmond, et al 1992). This population includes endothelial, reticular and fibroblastoid cells (Dorshkind 1990). Adipocytes are also present in the marrow but are not thought to represent a distinct population but rather to be derived from fibroblasts which have accumulated lipid (Lichtman 1981; Weiss 1976). Endothelial cells form a complete lining of the luminal side of the marrow sinuses and are, themselves, partially covered on their abluminal face by reticular cell processes (Lichtman 1984). Newly generated blood cells migrate through this barrier to enter the circulation. The volume of cytoplasm in reticular cells can be altered in response to hematological stress, thereby exposing more or less of the endothelial surface to migrating blood cells (Lichtman 1984). The control exerted by endothelial and reticular cells appears to go beyond the physical regulation of blood cell circulation, however. An indication of this is the fact that the stromal environments of the marrow and spleen can differentially support the development of different hematopoietic cell lineages. For example, in the mouse granulopoiesis predominates in the marrow while erythropoiesis is favoured in the spleen (Curry and Trentin 1967). Stromal cells may also alter the levels of growth factors and inhibitors present in the marrow cavity by influencing the influx or efflux of soluble mediators produced elsewhere (Shadduck, et al 1989), as well as by releasing such factors themselves. Stromal cells have been shown to produce a variety of growth regulators, either constitutively or in response to stimulation by growth factors or other agents (reviewed in (Coze 1994)).

Stromal cells of the fibroblast-adipocyte-endothelial lineages are not the only potential regulators of hematopoiesis within the marrow. The marrow microenvironment also contains numerous extracellular matrix (ECM) molecules, including fibronectin, hemonectin, various collagens, thrombospondin, laminin, and proteoglycans. It also, of course, includes the hematopoietic cells, themselves. Macrophages and lymphocytes, in particular, have the potential to exert regulatory influences through cell-cell interactions and the secretion of growth factors (reviewed in (Chabannon and Torok-Storb 1992)). The involvement of macrophages in early erythroid cell maturation is a case in point. In the marrow, developing erythroblasts are

found clustered around central macrophages which provide them with iron and release erythroid burst-promoting activities such as granulocyte-macrophage colony-stimulating factor (GM-CSF)). At a later stage, parasinal macrophages phagocytose erythroblast nuclei when they are expelled during red cell maturation (Lichtman 1984).

The examination in vitro of stromal cells and their interactions with hematopoietic cells has revealed that the extracellular matrix components produced in long-term cultures, and presumably in vivo, can influence the migration, proliferation, shape, and metabolic functions of developing blood cells in addition to providing a structural meshwork for cellular interactions (reviewed in (Dorshkind 1990; Verfaillie, et al 1994)). As shown in Figure 1.9, a number of specific receptor-ligand binding mechanisms by which hematopoietic cells and stromal cells can interact with each other and with the ECM have now been identified. These interactions can be regulated at several levels. In the case of SF and macrophage colony-stimulating factor (M-CSF) which are produced both in soluble and membrane-bound forms, stimulation of hematopoietic cells can occur without the need for stromal cells to secrete these factors (Stein, et al 1990; Toksoz, et al 1992). On another level of regulation, hematopoietic cells express a variety of adhesion receptors, such as integrins, CD44 and selectins, in a maturation stage and activation state-dependent manner (Clark, et al 1992; Hynes 1992). For example, reticulocytes lose their adherence to fibronectin as they mature into erythrocytes (Patel, et al 1985). In addition, interaction of at least some adhesion receptors with their ligands on stromal cells or in the ECM can also alter their own activation state (reviewed in (Hynes 1992)). Similarly, stromal cells can modify their pattern of interleukin mRNA synthesis in response to the growth factors that they, themselves, produce (Gutierrez-Ramos, et al 1992). The production of ECM components and the expression of adhesion receptors by stromal cells can be modulated by a variety of mediators. One example is the alteration by corticosteroids (Bentley, et al 1988) of the sulphation pattern of glycosaminoglycans and thus their ability to interact with other components of the microenvironment (Clark, et al 1992). Another is the ability of members of the transforming growth factor (TGF)-β family to regulate the synthesis

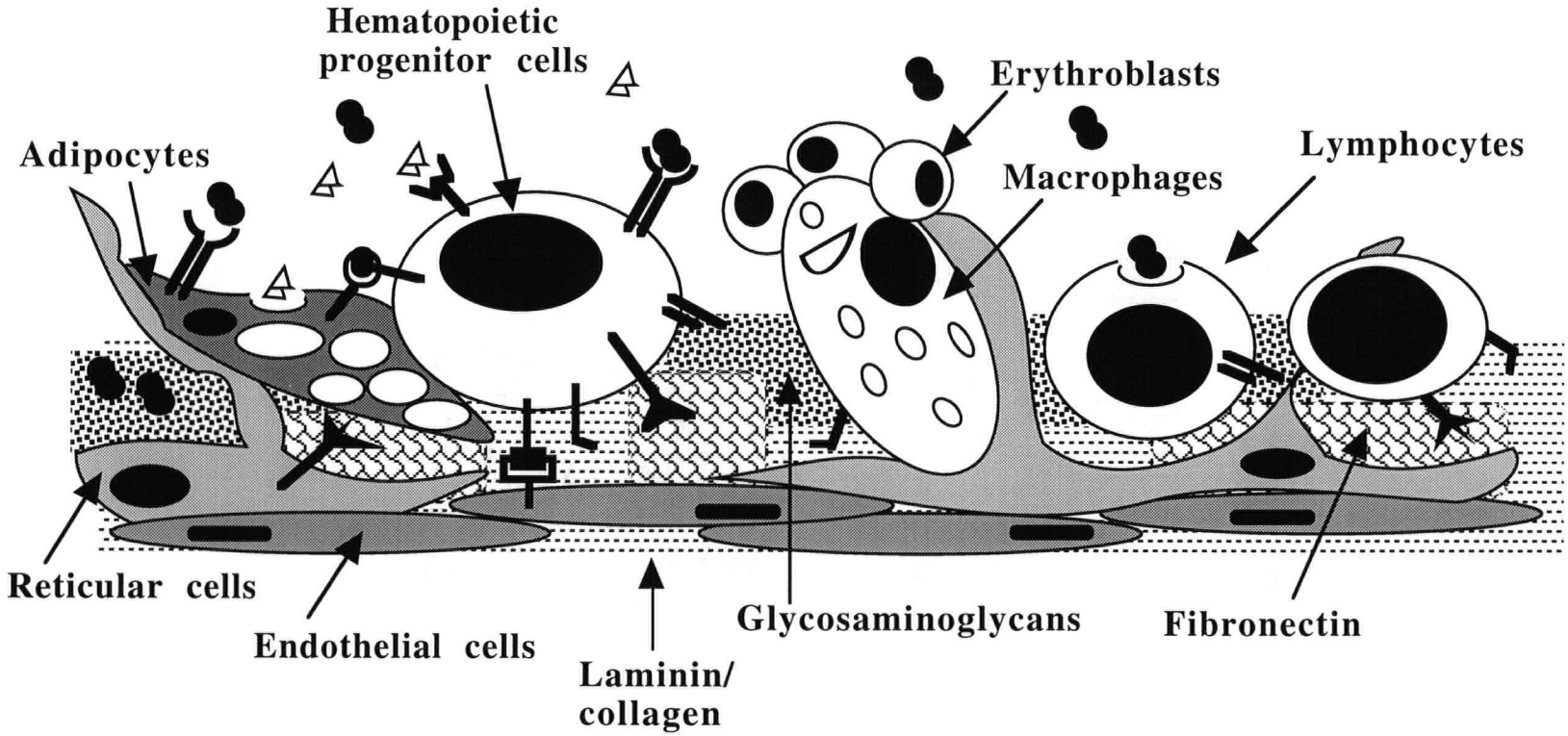


Figure 1.9 Elements of the marrow microenvironment.

of collagen and other ECM components (Sporn and Roberts 1989). Finally, glycosaminoglycans, particularly heparan sulphate, can sequester and effectively concentrate growth factors which regulate hematopoietic cell growth and migration as well as certain stromal cell functions. Examples of growth factors thus affected include IL-3 (Roberts, et al 1988), GM-CSF (Gordon, et al 1987), basic fibroblast growth factor (b-FGF) (Gospodarowicz and Cheng 1986; Rapraeger, et al 1991), and macrophage inflammatory protein (MIP)-1β (Tanaka, et al 1993). By this mechanism of local concentration, subliminal growth factor production by stromal cells might be able to exert a potent effect on hematopoietic target cells in their vicinity. This type of localized control of hematopoiesis was envisaged almost two decades ago (Schofield 1978) as an extension of the HIM model originally proposed by Curry and Trentin (Curry and Trentin 1967) (see section 1.1.1a, above).

1.2.2 Growth factors and inhibitors

The presence in culture supernatants conditioned by stromal cells of "activities" capable of regulating the proliferation and differentiation of hematopoietic cells eventually led to the identification and subsequent cloning of a large number of growth factors and inhibitors responsible for these activities. Hematopoietic colony-stimulating factors and interleukins are fairly small molecules with protein cores of 10-70 kilodaltons. They are generally soluble glycoproteins and are produced by a variety of cell types throughout the body (reviewed in (Coze 1994) and (Metcalf and Nicola 1995)). Sixty years after the discovery of erythropoietin in the serum of rabbits made anemic by bleeding, M-CSF, granulocyte (G)-CSF, GM-CSF, and multi-CSF (now known as IL-3) were purified and the types of colony growth they were originally found to support are reflected in their names. To date, more than 15 interleukins have been cloned, many of which have pleiotropic or overlapping activities, as illustrated in Table 1.1. The recent availability of biologically active recombinant growth factors, in combination with the use of hematopoietic cell lines and sorted cell populations has led to an explosion of

information about the biological effects of these factors. However, although more than 20 hematopoietic regulators have already been identified, there are still many "activities" that have not yet been assigned to any known cytokines. For example, no factor has yet been found that can replace the ability of S17 fibroblasts together with lipopolysaccharide to stimulate the maturation of pre-B cells into mature Ig-secreting lymphocytes (Kee, et al 1994).

As is evident from Table 1.1, the same factor, e.g., SF or IL-6, can have very different effects on cells in different lineages, both within and outside the hematopoietic system. Others have different effects at different stages of maturation within the same lineage. For example, IL-1 stimulates B cell proliferation but inhibits pre-B cell growth. In addition, there is considerable functional overlap between many factors, i.e., different factors can exert very similar effects on a particular cell population. For example, IL-1, IL-4, and TGF-β all increase VCAM expression on endothelial cells while IL-3, G-CSF, and GM-CSF can all support the growth of granulocytic colonies. Of note as well is the requirement of highly purified primitive progenitor cells for the combined activity of several growth factors to promote their growth and differentiation maximally (Migliaccio, et al 1991; Li and Johnson 1992; Tsuji, et al 1992; Miura, et al 1993). Such a dependence on multiple activating stimuli may protect HSC and other immature progenitor cells from excessive turnover or amplification. The maximum proliferation of more mature progenitors like CFU-M, on the other hand, is possible in the presence of single factors such as M-CSF (Metcalf and Nicola 1995)).

Ogawa and his colleagues, amongst others, have extensively documented the different roles of various growth factors in supporting colony formation by highly purified mouse marrow cells. These studies have led them to group factors into three categories: late-acting lineage-specific factors such as EPO, M-CSF, and IL-5, intermediate-acting lineage-non-specific factors such as IL-4, IL-3, GM-CSF, and factors involved in activating or sustaining the cycling of previously dormant progenitors such as IL-6, IL-11, SF, and G-CSF. None of these categories are necessarily mutually exclusive. For example, G-CSF regulates the

Table 1.1 Illustrative list of the pleiotropic and overlapping activities of hematopoietic growth regulators.

Factor	Activities	References
Interleukin (IL)-1	- synergizes with TNF-α and IL-6 to activate B and T cells	Suda, et al 1989
	- inhibits pre-B colony formation	Hirayama, et al 1994a
	- induces GM-CSF, G-CSF and IL-6 production by stromal cells and macrophages	Yang, et al 1988
	- produces fever, sleepiness, anorexia, and other acute phase responses	Coze 1994
	- increases expression of VCAM on endothelial cells	Verfaillie, et al 1994
IL-3 (murine)	- inhibits pre-B colony formation	Hirayama, et al 1994a
	- induces IL-6 production by rhodamine bright hematopoietic progenitors	Schneider, et al 1991
	- increases production of IL-1, IL-6, TNF-α by macrophages	
	- stimulates formation of myeloid (single, mixed and multi-lineage) and blast colonies	
	- synergizes with erythropoietin (EPO) in erythroid burst formation	Metcalf and Nicola 1995
IL-4	- increases expression of VCAM on endothelial cells	Verfaillie, et al 1994
	- induces immunoglobulin (Ig) class switch to IgE and IgG1	Paul 1991
	- stimulates proliferation of B cells and T and mast cell lines	Zlotnik, et al 1987
	- enhances antigen presentation by macrophages	
	- supports proliferation of primitive hematopoietic progenitors in combination with IL-11, IL-6, or G-CSF	Hirayama, et al 1994b
IL-5	- stimulates eosinophilic and basophilic progenitor growth and increases chemotaxis	Coze 1994
	- {B and T cell activities}	
IL-6	- stimulates hematopoiesis in embryonic stem cells in vitro	Biesecker & Emerson 1993
	- induces high rates of Ig secretion in activated B cells	Hirano, et al 1985
	- regulates release of hepatic acute phase proteins	Gauldie, et al 1987
	- synergizes with other growth factors to induce proliferation of hematopoietic progenitor cells	Ogawa 1993
	- supports megakaryocytopoiesis in combination with IL-3	Han and Caen 1994
IL-7	- stimulates proliferation of early B cell progenitors	Namen, et al 1988
	- stimulates proliferation of PMA-primed T cells	Chazen, et al 1989
	- induces secretion of IL-6 by macrophages	Alderson, et al 1991

IL-11	- supports megakaryocytopoiesis in combination with IL-3	
	- stimulates T cell-dependent development of Ig-producing B cells	Paul, et al 1990
	- enhances IL-3 dependent colony formation	Musashi, et al 1991
	- inhibits adipogenesis in fibroblasts	Coze 1994
	- supports development of fetal liver B lymphoid progenitors in combination with IL-7 and SF	Kee, et al 1994
Granulocyte colony stimulating factor (G-CSF)	- supports formation of granulocytic colonies	Metcalf and Nicola 1983
	- synergizes with IL-3 in support of multilineage colony formation	Ikebuchi, et al 1988
	- inhibits B lymphopoiesis in vivo and in vitro	Lee, et al 1993
GM-CSF	- at low concentrations, stimulates granulocytic (G), macrophage (M) and GM colony formation	
	- at higher concentrations, also stimulates eosinophil, megakaryocytic and increased GM colony formation	Metcalf and Nicola 1995
	- in combination with EPO, stimulates immature BFU-E precursors	Inaba, et al 1992
	- stimulates dendritic cell formation from marrow precursors	Bussolino, et al 1989
	- stimulates endothelial cell proliferation	
Steel Factor (SF)	- in combination with other growth factors supports proliferation of both immature and lineage-restricted lymphoid and myeloid progenitor cells	Martin, et al 1990
	- induces hypoplastic marrow failure when administered in vivo before 5-fluorouracil (5-FU)	Molineux, et al 1994
	- involved in migration and proliferation of embryonic stem cell	
	- required for development of primordial germ cells and melanocytes	Coze 1994
Flk-2/Flt3 ligand	- supports proliferation of B cell progenitors in synergy with IL-7 and SF	
	- synergizes with IL-6, IL-11, or G-CSF in supporting proliferation of lymphomyeloid progenitors in culture	Hirayama, et al 1995
	- in combination with IL-6, expands CFU-S numbers in cultures of purified cells	Hudak, et al 1995
Transforming growth factor (TGF)-β	- increases expression of VCAM on endothelial cells	Verfaillie, et al 1994
	- inhibits proliferation of epithelial and hematopoietic cells	
	- downregulates production of IL-1 and other inflammatory cytokines	McCarthy 1994
	- increases IL-6 production by monocytes	Turner, et al 1990
	- stimulates growth of mesenchymal cells	Moses and Yang 1990
	- inhibits IL-7 stimulation of B lymphopoiesis	Lee, et al 1989

proliferation and maturation of neutrophil progenitors (Metcalf and Nicola 1983) but can also act with SF in supporting blast colony formation (Hirayama, et al 1992). The earliest acting growth factors can be further subdivided on the basis of their functional similarities. Effective stimulation of primitive progenitors occurs when cytokines from at least two of the subgroups interact with each other (Ogawa 1993). Another way of thinking of growth factor interactions (Metcalf 1993) distinguishes the synergy between cytokines such as SF + GM-CSF that results in an increased cell production by responsive progenitors (McNiece, et al 1991a) and the recruitment of progenitors by factors such as SF + Epo that increases the number responding cells (Miller, et al In Press). The exposure of hematopoietic progenitor cells, whatever their developmental stage, to multiple cytokines could thus result in their enhanced recruitment and proliferation.

Overlap in the ability of different hematopoietic growth factors to support the proliferation and differentiation of primitive progenitors in vitro would predict that the loss of any single factor might have little impact on the generation in vivo of early hematopoietic progenitors while potentially eliminating a subset of mature end cells. This has proven to be the case in several strains of mice now known to be naturally deficient in the production of a particular growth factor or its receptor such as Steel factor (Sl), c-kit (W), or M-CSF (op/op) mutants. In addition, a large array of mice rendered deficient by targeted gene disruption in embryonic stem cells, like the knock-outs for IL-7, IL-2 and the IL-7 receptor have shown a similar picture. An illustrative list of the phenotypes of such animals is shown in Table 1.2. Although the reduction in mature end cells can be quite dramatic when a growth factor or its receptor is absent such as lethal anemias in some Sl and W mutants and B and T cell deficiencies in IL-7 and IL-7 receptor knock-outs, to date no single or double growth factor or growth factor receptor knock-out has resulted in the complete abolition of a particular lineage, indicating that considerable redundancy exists in vivo even for so-called lineage-specific regulators.

Table 1.2 Representative list of mice deficient for hematopoietic regulatory molecules.

Deficiency	Phenotype	References
Growth factors and GF receptors		
Steel factor (Sl mutant)	Sl/Sl embryonic lethal, Sl/Sl^{d} viable; severe macrocytic anemia leading to death late in gestation; lack of hair pigmentation; mast cell deficiency and sterility in viable animals; defective hematopoietic microenvironment (can be cured by bone implants but not marrow grafts)	Russell 1979; Bernstein, et al 1991
Steel factor receptor, c-kit (W mutant)	W/W embryonic lethal, W/W^{v} viable; similar to Sl mutants but defect in hematopoietic stem cells (i.e. can be cured by normal marrow graft)	Russell 1979; Bernstein, et al 1991
Flk-2/Flt3 (knock-out)	Viable; slight deficiencies in primitive B lymphoid progenitors; slightly deficient reconstitution upon transplantation	Mackarehtschian, et al 1995
FL and c-kit (FL knock-out x W/W^{v})	Reduced viability and postnatal lethality; severely reduced myeloid and lymphoid cellularity	Mackarehtschian, et al 1995
M-CSF (op mutant)	op/op viable; toothless; hypocellular marrow due to bone overgrowth; lack of osteoclasts; decreased numbers of some macrophage populations; phenotype ameliorates with age	Yoshida, et al 1990; Wiktor-Jedrzejczak, et al 1992
GM-CSF (knock-out)	Viable and fertile; no major perturbation of hematopoiesis but all mice develop lung infections with infiltrating B cells and macrophages	Stanley, et al 1994
GM-CSF and M-CSF (GM-CSF knock-out x op mutant)	Combination of op/op and GM-CSF knock-out phenotypes but more severe lung disease; numerous functional macrophages present	Lieschke, et al 1994
common β chain for IL-3*, GM-CSF, IL-5 receptors (knock-out)	Lymphocytic infiltration of the lungs; low numbers of eosinophils; marrow cells unresponsive to GM-CSF or IL-5 but response to IL-3 normal	Nishinakamura, et al 1995
IL-2 (knock-out)	Viable; changes in isotype levels of serum Ig; reduced natural killer cell activity; delayed T helper cell responses	Schorle, et al 1991; Kundig, et al 1993

IL-4 (knock-out)	Viable; normal B and T cell development; strongly reduced serum levels of IgG1 and IgE; no IgE detectable upon nematode infection	Kuhn, et al 1991
IL-7 (knock-out)	Viable; block in pro-B to pre-B transition in marrow; 20-fold decrease in thymic cellularity but normal distribution of CD4 and CD8; splenic B and T cell numbers decreased; immature B cells in the spleen; remaining splenic lymhocytes normally responsive to mitogens	von Freeden-Jeffry, et al 1995
IL-7 receptor (knock-out)	Similar to IL-7 knock-out; deficient thymocyte expansion preceding T cell receptor gene rearrangement	Peschon, et al 1994
common γ chain for high affinity binding of IL-2, IL-4, and IL-7 receptors, and possibly IL-9 and IL-13 receptors (human defect)	X-linked severe combined immunodeficiency; absent or profoundly reduced numbers of T cells; diminished Ig production and responsiveness to immunization by B cells	Leonard, et al 1994
Intracellular signal transducers and transcription factors (TF)		
$p56^{lck}$: lymphoid-specific tyrosine kinase (knock-out)	Viable; pronounce thymic atrophy; dramatic reduction in $CD4^+CD8^+$ thymocyte population; no single-positive thymocytes detectable and no peripheral T cells present	Molina, et al 1992
Ikaros: lymphoid-specific TF (knock-out)	Viable but fail to thrive; no B, T, dendritic, or natural killer cells; earliest lymphoid progenitors absent; peripheral lymphoid organs absent or rudimentary; normal myeloid and erythroid development	Georgopoulos, et al 1994
Pax5 (BSAP): B cell-specific TF (knock-out)	Pax5 -/- viable but runted, surviving animals fertile; abnormal posterior midbrain patterning; complete block in B cell development at pro-B stage	Urbánek, et al 1994
GATA-1 TF (embryonic stem cell chimera)	No mature GATA-1$^-$ erythroid cells in chimeric mice although GATA-1$^-$ embryonic stem cells contributed to all other tissues tested, including leucocytes	Pevny, et al 1991
GATA-2 TF (knock-out)	GATA-2 -/- embryonic lethal; severe anemia; 100-fold reduction in yolk sac of all hematopoietic precursors	Tsai, et al 1994

* The IL-3 receptor in mice (but not humans) has an additional β subunit not shared with the IL-5 or GM-CSF receptors.

The regulation of hematopoiesis also involves mechanisms by which proliferative responses can be specifically curtailed to facilitate the restoration of homeostasis after illness or injury. The positive and negative signals required to ensure a physiologically appropriate balance may operate at many levels. For example, as an initial, self-limiting response to M-CSF stimulation, monocytes and macrophages rapidly downregulate their surface expression of M-CSF receptors (Metcalf and Nicola 1995). B and T cells, for their part, can secrete TGF-β, the most potent known suppressant of lymphocyte proliferation (Sporn and Roberts 1989). At a different level, transmodulation of the responsiveness of hematopoietic cells to GM-CSF, IL-3 and IL-5 can occur due to the competitive usage by their respective receptors of a common β subunit necessary for high affinity ligand binding (Ihle, et al 1994). Finally, several negative regulators, termed chemokines, have been identified which act predominantly to inhibit the proliferation of very primitive hematopoietic cells. The interactions of TGF-β and IL-1 provide a paradigm for the dynamic and antagonistic control of hematopoiesis. IL-1 is a pro-inflammatory molecule involved in the activation of immune functions, the stimulation of cytokine production, including G-CSF, GM-CSF, M-CSF and IL-3, by a variety of cells, and the increased expression of growth factor receptors and cell surface adhesion molecules (Coze 1994). TGF-β, on the other hand, inhibits each of these activities and, in addition, stimulates the secretion and inhibits the cell surface expression of the IL-1 receptor (Ruscetti, et al 1992). Of particular interest is the ability of hematopoietic inhibitors such as TGF-β and MIP-1α to maintain primitive progenitors in a non-cycling state, thereby protecting them from the deleterious effects of chemotherapeutic agents designed to kill proliferating tumour cells (reviewed in (Moore 1991)). TGF-β and MIP-1α have both been shown to prevent the re-entry of immature progenitors into S-phase in cultures upon stimulation by either a half medium exchange or the addition of IL-1 (Cashman, et al 1990) and significantly reduce the generation of CFU-GEMM by highly purified $CD34^+$ $CD45RA^{low}$ $CD71^{low}$ primitive human cord blood progenitor cells (Mayani, et al 1995).

1.2.3 Receptor signalling and transcription factors

The receptors for the CSFs and most of the interleukins thus far cloned have now also been identified. The majority of the receptors are members of the hemopoietin receptor superfamily which is characterized by the distinctive arrangement in the extracellular domain of 4 conserved cysteine residues and the presence, usually proximal to the outer cell membrane, of a conserved Trp-Ser-X-Trp-Ser motif (where X can be any amino acid). The hemopoietin receptors do not possess intrinsic kinase activity but are believed to activate associated members of the Jak, Src, Fes, and Abl families of tyrosine kinases upon ligand binding. In many cases, the specific, ligand-binding components of these receptors associate with promiscuous subunits which confer high affinity binding such as the common β subunit of IL-3, IL-5, and GM-CSF or signal transducing capabilities as is the case with the gp130 molecule shared by IL-6, LIF, and IL-11 (Ihle, et al 1994; Kishimoto, et al 1994). Another family of growth factor receptors includes the M-CSF receptor c-fms, c-kit, flk-2/flt3 and the platelet-derived growth factor receptor (PDGFR). The defining features of this subfamily of tyrosine kinase receptors include the presence of an intracellular kinase domain split by a non-catalytic insert of 60-100 amino acids and five extracellular immunoglobulin-like domains (Yarden, et al 1987). It is believed that these receptors can directly deliver intracellular signals upon ligand binding (Ullrich and Schlessinger 1990).

In general, the binding of hematopoietic growth factors to their receptors, whether hemopoietin receptors or receptor tyrosine kinases, induces homo- or heterodimerization of receptor components which then initiate downstream signalling pathways via a host of intracytoplasmic intermediates, including various kinases and phosphatases, as well as their respective substrates. Ultimately, the transmission of these signals to the nucleus can alter the transcriptional regulation of cell type-specific genes (Ihle, et al 1994; Kishimoto, et al 1994). Since cells from different lineages express different arrays of cellular products, it is likely that the production of many of these molecules might controlled at a transcriptional level during

differentiation. Indeed, number of transcription factors involved regulating in the development of particular hematopoietic cell lineages have recently been identified. As illustrated in Figure 1.10, and like the hematopoietic growth factors, these transcription factors can be classified according to their pattern of expression and activity (Ness and Engel 1994). For example, disruption of the GATA-2 gene results in a marked reduction of all hematopoietic precursors (Tsai, et al 1994). Some transcription factors, like c-Myb, are expressed only in immature cells but their expression is not lineage-restricted (Ness and Engel 1994). The Ikaros transcription factors, on the other hand, are required for the development of all lymphoid but not myeloid lineages (Georgopoulos, et al 1994), while SCL/tal-1 is mainly expressed in myeloid and erythroid progenitors (Bockamp, et al 1994; Orkin 1995). Interestingly, PU.1 appears to cross the lymphoid-myeloid divide since embryos deficient for this transcription factor have normal numbers of erythroid and megakaryocytic progenitors but are defective in their development of monocyte, granulocyte, B cell and T cell progenitors (Scott, et al 1994). Pax5 and GATA-1 are necessary for the development of single-lineage progenitors within the B and erythroid lineages, respectively (Urbánek, et al 1994; Weiss and Orkin 1995), although GATA-1 is also expressed in mast cells and megakaryocytes (Ness and Engel 1994). Other transcription factors, such as MZF1, NFAT, and Oct-2, are expressed in more mature cell types of a single lineage (neutrophils, T cells and B cells, respectively) (Ness and Engel 1994). Although the transcription factors discussed here are shown in Figure 1.10 as acting only within the hematopoietic system, many of them are also expressed in unrelated tissues in which they play roles in the differentiation of non-hematopoietic cells (Ness and Engel 1994; Orkin 1995). For example, Pax5 is involved in midbrain patterning (Urbánek, et al 1994) and GATA-1 is expressed in Sertoli cells in the testes (Yomogida, et al 1994). The specificity of transcription factors, like that of growth factors and inhibitors, is very much a function of the context in which they act.

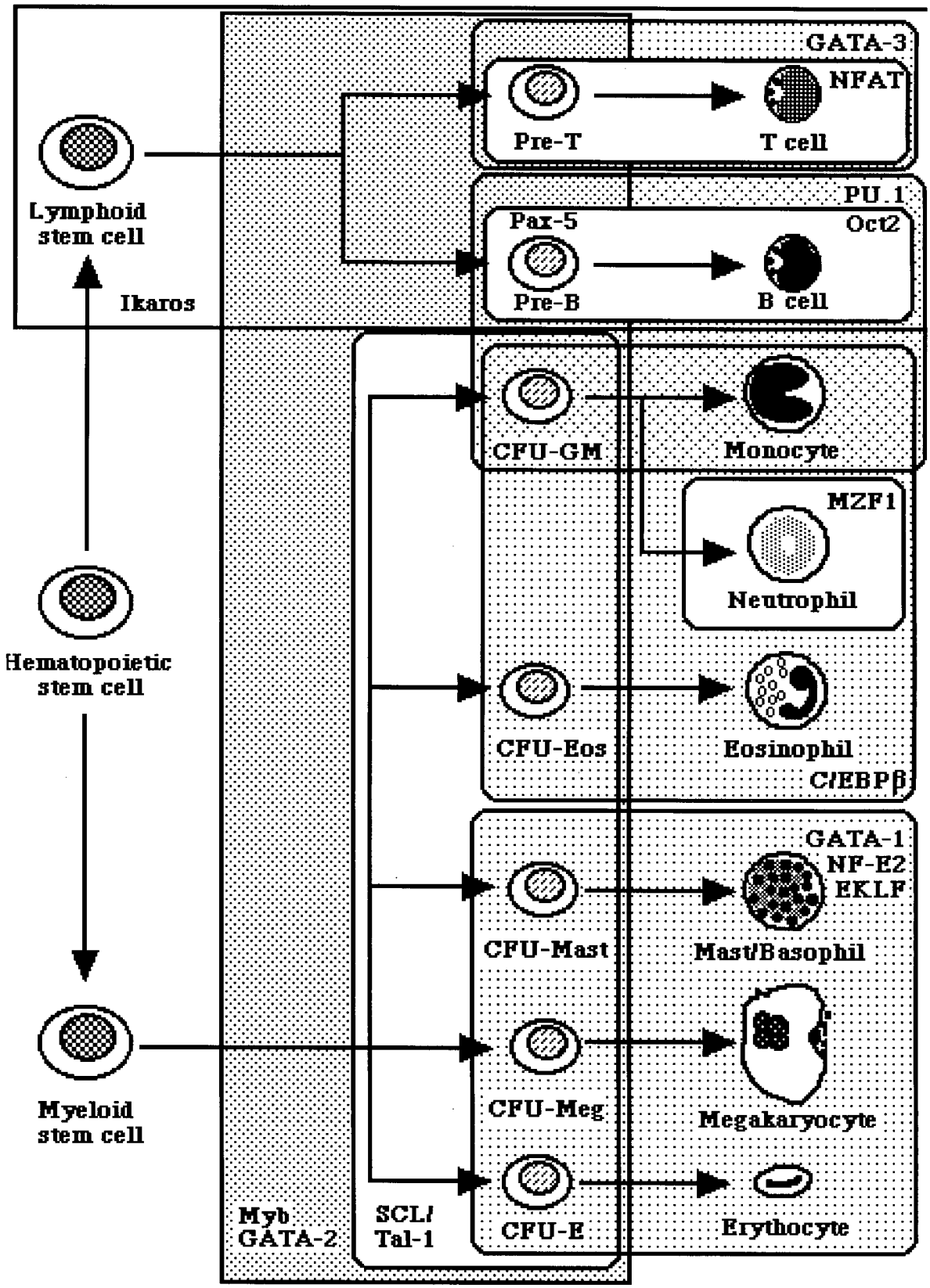


Figure 1.10 Transcription factors involved in hematopoietic cell development. (Adapted from Ness 1994)

1.3 Ontogeny of the murine hematopoietic system

In the developing embryo, the first morphologically recognizable hematopoietic cells appear 7.5-8 days postcoitus as erythropoietic islands generally confined to the yolk sac (YS) mesoderm ((Theiler 1989) and reviewed in (Metcalf and Moore 1971)). In vitro colony-forming cells (CFC) can be isolated from the same presomitic and 1-4 somite stage (Metcalf and Moore 1971). Beginning at the 10- to 12-somite stage (8.5 days postcoitus), B lymphoid precursors can be isolated from the body, YS (Cumano, et al 1993), and paraaortic splanchnopleura (Godin, et al 1993). At this stage, the YS is also a source of CFU-S (Metcalf and Moore 1971). By day 9, the heart begins to beat and blood circulation in the embryo is initiated. CFC are then found in similar numbers in the YS and in the circulating blood. In birds and amphibians, grafting experiments have shown that, after an initial transitory wave of YS hematopoiesis, definitive blood cell production is derived from stem cells in the mesodermal region surrounding the aorta (Dieterlen-Lièvre and Martin 1981; Turpen, et al 1981; Maeno, et al 1985; Cormier and Dieterlen-Lièvre 1988). In mice, HSC capable of long-term reconstitution of all blood lineages have been detected by day 10 (31- to 40-somite stage) in the corresponding aorta-gonad-mesonephros (AGM) region (Medvinsky, et al 1993) and are also detectable in the YS and fetal liver by day 11 (Metcalf and Moore 1971; Medvinsky, et al 1993). Whether adult HSC first originate in the YS and subsequently migrate to the AGM region and fetal liver or whether HSC from the AGM region seed the other two organs is currently an area of active investigation. Blood cell production in the YS and AGM region then wanes as the fetal liver becomes the primary site of embryonic and early postnatal hematopoiesis. The fetal bone marrow is colonized by blood stem cells 3-4 days before birth and gradually replaces the liver as the predominant hematopoietic organ by 2 weeks postpartum and thereafter remains the principal site of normal hematopoietic cell development (Metcalf and Moore 1971).

1.3.1 Comparison of hematopoietic stem cells from fetal and adult tissues

The exact relationship between fetal and adult HSC is still unclear. As mentioned above, there are cells present in the embryo as early as 10 days postcoitus that are capable of sustaining long-term hematopoiesis after transplantation into irradiated adult mice. It is not known, however, whether these same cells or those present in the marrow of adult mice are able to recapitulate the development of hematopoietic cells that occurs in the fetus from day 10 onward. Although HSC derived from fetal and adult sources are similar in possessing lympho-myeloid potential and an extensive ability to maintain this property through subsequent cell divisions, they have been found to behave differently in several respects.

Physically, ontologically earlier progenitors are larger and less dense (Rebel, et al In Press). Unlike the vast majority adult marrow HSC, they express the cell surface antigen AA4.1 and, by 14 days postcoitus, virtually all express some level of Mac-1, an antigen normally expressed by myelo-monocytic cells in adult mice (Rebel, et al In Press). A larger proportion of fetal HSC than adult marrow HSC also appear to be actively proliferating (Fleming, et al 1993). Fetal HSC also reconstitute primary and secondary transplant recipients to a much greater degree than do their adult marrow counterparts (Rebel, et al In Press) but the extent to which this may be due to an increased proportion of proliferating HSC, a decreased loss of HSC to death or differentiation, or an increased self-renewal rate is not clear at present.

The erythroid, B and T cell differentiative potentials expressed by fetal and adult HSC are also somewhat different. Studies in sheep have shown that early fetal HSC give rise to erythrocytes expressing fetal $\alpha_2\gamma_2$ hemoglobin whereas erythrocytes derived from HSC later in development produce adult $\alpha_2\beta_2$ hemoglobin (Wood and Weatherall 1973; Wood, et al 1976; Bunch, et al 1981; Wood, et al 1985). It has also been shown that a developmental switch occurs in the subsets of T and B cells produced by fetal and adult HSC (Ikuta, et al 1990; Kantor, et al 1992). An apparently B cell-macrophage-restricted progenitor has been identified

in fetal liver but an equivalent cell in adult marrow has not been detected (Cumano, et al 1992). Thus the concept of an HSC capable of perpetuating itself without change from the time of its original generation in the fetus to the end of adult life is now being questioned. It seems likely that in mammals, as in amphibians and birds, a transient wave of hematopoiesis occurs in the fetus which is then replaced by a "definitive" stage of hematopoiesis later in gestation (Dieterlen-Lièvre 1975; Dieterlen-Lièvre and Martin 1981; Turpen, et al 1981; Maeno, et al 1985).

1.4 Thesis objectives

As described in the preceding sections, it is now well established that mammalian hematopoiesis originates throughout adult life from a self-maintaining population of cells with the capacity to generate multiple types of lymphoid and myeloid blood cells. Moreover, in vivo studies have shown that many of these have enormous proliferative capacity. Such cells in the mouse can be detected and quantitated by appropriate in vivo assays. However, at the time this thesis was initiated, reproducible and specific assays for their detection in vitro had not been developed.

The overall goal of this thesis was to obtain information about the regulation of totipotent hematopoietic stem cell differentiation and self-renewal using in vitro approaches on the assumption that this might facilitate the analysis and manipulation of these processes. As a first objective, I therefore sought to explore the possibility of modifying the LTC-IC assay to detect the cells of interest. The first chapter describes the features of the assay that was devised for this purpose, its validation as a quantitative and specific method for measuring primitive cells with lympho-myeloid potential and the frequency of the cells it detects in various cell suspensions. Comparison of these values with LTC-IC, CRU and other types of later hematopoietic progenitors was then pursued to establish the interrelationship of these operationally defined cell types. These studies are described in Chapter III and have been published (Lemieux, et al 1995).

The key finding from this part of my work was that the LTC-IC and LTC-IC_{ML} assays appear to measure the same cells in adult mouse marrow as are detected in vivo as CRU, albeit with apparently different efficiencies. My next objective was to analyze the maintenance of LTC-IC and LTC-IC_{ML} populations in vitro under various LTC conditions since it had previously been shown that some CRU can undergo self-renewal divisions under myeloid LTC conditions. In clinical and research settings where hematopoietic cells are kept in culture for

some length of time, for example during marrow purging or gene transfer, it is crucial to know how the reconstituting cells are affected by such ex vivo manipulations. It was, therefore, of interest to determine whether the persistence of in vitro lympho-myeloid potential as measured by the LTC-IC_{ML} assay is predictive of in vivo HSC maintenance (as measured by the CRU assay. To do this, marrow cells from normal and myeloablated mice were cultured under various conditions and for variable periods in order to document the in vitro maintenance of in vivo repopulating cells and in vitro lympho-myeloid progenitors. However, because the frequency of cells detected using the LTC-IC_{ML} assay was found to be lower than anticipated, another series of experiments were first undertaken to see if further modifications to the LTC-IC_{ML} assay might increase its sensitivity. These studies and the results obtained are presented in Chapter IV.

The final objective was to investigate whether certain specific growth factors could replace the role of the feeders at very early stages of hematopoietic cell differentiation as a first step towards elucidating the mechanisms by which the functions of LTC-IC_{ML} might be regulated. Here the approach taken was to initiate cultures with a rare, purified subpopulation of fetal liver cells known to be highly enriched in its content of CRU (Rebel, et al In Press). These cells were then cultured for 1-2 weeks in the absence of stromal cells but in the presence of various combinations of Flt3 ligand, IL-7, SF and IL-11. These growth factors were selected for these studies because each had been shown to be produced by stromal fibroblasts and to have activity in stimulating early B lineage progenitors. At the end of the culture period, the types of cells present were analyzed. The results of these experiments are described in Chapter V.

II. Materials and methods

2.1 Animals

Eight- to 16-week old (C57BL/6J x C3H/HeJ)F_1 (B6C3F_1) and (C57BL/6 Pep3b x C3H/HeJ)F_1 (PepC3F_1) mice bred and maintained in the animal facility of the B.C. Cancer Research Centre (Vancouver, B.C.) were used in all experiments. Parental strain breeders were originally obtained from the Jackson Laboratories (Bar Harbor, ME). B6C3F_1 mice are homozygous for the Ly-5.2 allele. PepC3F_1 are Ly-5.1/Ly-5.2 heterozygotes.

2.2 Preparation of marrow cell suspensions

A 21-gauge needle was used to flush marrow cells from femurs and tibias into cold Alpha medium containing 2% fetal calf serum (FCS) (StemCell Technologies Inc., Vancouver, B.C.). Single-cell suspensions were then obtained by repeated gentle aspiration through the needle. For studies of in vivo effects of 5-fluorouracil (5-FU), mice were injected IV with 150 mg/kg body weight of sterile 5-FU (Hoffman La Roche, Basel) 2 days prior to harvesting the marrow. For antibody labelling experiments, cells were collected in ice-cold Hank's balanced salt solution (StemCell) containing 2% FCS (HF), incubated for 30 minutes on ice in ammonium chloride to lyze red blood cells, centrifuged and gently resuspended in HF.

2.3 Antibodies and growth factors

Recombinant murine Steel Factor, recombinant human IL-7, and recombinant human Flt3 ligand were generous gifts from Immunex (Seattle, WA). Human IL-11 and human IL-6

purified from supernatants of COS cells transiently expressing the appropriate cDNAs were prepared and titered for bioactivity in the Terry Fox Laboratory (Vancouver, BC).

The monoclonal antibodies used for flow cytometric analyses and sorting were E13-161.7 (anti-Sca-1), S7 (anti-CD43), the lineage markers RA3-6B2 (anti-B220), RB6-8C5 (anti-Gr-1), 53-7.3 (anti-Ly-1), M1/70 (anti-Mac-1), and Ter119 (an erythroid lineage marker (Ikuta, et al 1990)). Hybridomas producing monoclonal anti-Sca-1, anti-B220, and anti-Gr-1 antibodies were provided by Dr. G. Spangrude (Rocky Mountain Laboratory, Hamilton, MT). Dr. Spangrude also provided the hybridoma producing the anti-Ly-5.1 antibody (A20-1.7) used to detect donor-derived cells in CRU assays (see below). Ter119 was a gift from Dr. T. Kina (Kyoto University, Sakyo-ku, Kyoto). The hybridomas 53-7.3 and M1/70 were purchased from the American Type Culture Collection (ATCC, Rockville, MD). Biotinylated S7 was purchased from Pharmingen (San Diego, CA). Dr. S. Szilvassy (Systemix, Palo Alto, CA) provided the hybridoma which produces the anti-IgG Fc receptor antibody 2.4G2 used to block non-specific staining. Anti-B220, anti-Gr-1, anti-Mac-1, anti-Ly-1, Ter119 were labelled with fluorescein isothiocyanate (FITC) and anti-Sca-1 was labelled with cyanine-5-succinimidyl ester under the direction of Dr. P. Lansdorp (Terry Fox Laboratory) from whom they were obtained. Biotinylated wheat germ agglutinin (WGA) was purchased from Vector Laboratories Inc. (Burlingame, CA), streptavidin conjugated to phycoerythrin (SAPE) and allophycocyanin (APC) from Cedarlane Laboratories Ltd. (Hornby, ON), and propidium iodide (PI) from Sigma Chemical Co. (St. Louis, MO).

2.4 Cell purification

Adult PepC3F$_1$ marrow cells were incubated for 30 minutes with the following FITC-labelled lineage-specific (Lin) antibodies: anti-B220, anti-Ly-1, anti-Gr-1, and anti-Mac-1. After 2 washes with HF to remove unbound antibody, the cells were incubated for 25 minutes with BioMag® sheep anti-fluorescein IgG magnetic beads (Advanced Magnetics, Cambridge,

MA) and Lin$^+$ cells depleted by magnetic separation according to the manufacturer's directions. After 2 more washes with HF, the cells were incubated for 30 minutes with biotinylated wheat-germ agglutinin (WGA) and again washed twice. The cells were then stained with an antibody cocktail containing the same FITC-labelled anti-Lin antibodies as before, Cy-5-labelled anti-Sca-1, and SAPE. The cells were then washed two final times, with PI added to the last wash to allow the detection of nonviable (PI$^+$) cells. An aliquot of stained but unsorted cells was set aside for use in the determination of enrichment and recovery values. Cells were sorted on a FACStar$^+$ (Becton Dickinson & Co., San Jose, CA) equipped with 5 W argon and 30 mW helium neon lasers. Viable (PI$^-$) cells were gated to retain the population of Sca-1$^+$ cells with low forward and orthogonal light scattering properties. From this population, the Lin$^-$ WGA$^+$ fraction was collected under sterile conditions into 50% HF. Use of the same controls and gate settings as previously described (Rebel, et al 1994) allowed the reproducible isolation of the 0.02% of total nucleated PepC3F1 marrow cells that have a Sca-1$^+$ Lin$^-$ WGA$^+$ phenotype.

The protocol used for sorting Pep3b fetal liver cells was very similar (Rebel, et al In Press). Viable Sca-1$^+$ cells with somewhat higher forward and side scatter properties than adult marrow cells were selected only if they were also B220$^-$ Ly-1$^-$ Ter119$^-$ Gr-1$^-$. The anti-Mac-1 antibody was excluded from this lineage cocktail since almost all day 14.5 fetal liver cells express the Mac-1 antigen (Rebel, et al In Press). In addition, no selection was made on the basis of WGA binding.

2.5 In vivo assays

2.5.1 Colony-forming unit-spleen (CFU-S)

The day 12 CFU-S content of cell suspensions was determined as originally described (Till and McCulloch 1961). Briefly, appropriate dilutions of the cells to be tested (e.g., 4-8x10^4 normal marrow cells or 5-8x10^5 marrow cells from 5-FU treated mice) were injected

into syngeneic recipients that had been irradiated with 950 cGy given in a single dose at ~100 cGy/min from a ^{137}Cs γ-ray source (Atomic Energy of Canada, Chalk River, Ont.). The spleens were excised 12 days later, fixed in Telleyesniczky's solution and macroscopic surface colonies counted.

2.5.2 Competitive repopulation unit (CRU) assay

A modification of the limiting dilution assay for CRU described by Szilvassy et al (Szilvassy, et al 1990; Szilvassy and Cory 1993) was used. Briefly, decreasing numbers of test cells of PepC3F_1 origin were injected together with 10^5 unseparated fresh adult marrow cells from B6C3F_1 mice into B6C3F_1 recipients that had been irradiated with 950 cGy (as for CFU-S assays). After ≥5 weeks, a small blood sample was taken from the tail of each recipient and the nucleated cells then stained with FITC-labelled anti-Ly-5.1 and biotinylated anti-Gr-1 plus SAPE. Animals showing Ly-5.1$^+$ staining of both lymphoid and myeloid cells in the circulation, as determined by their light scattering profiles (lymphoid window) and anti-Gr-1 staining (>5% of Gr-1$^+$ [myeloid] cells also Ly-5.1$^+$), were considered to be repopulated by CRU present in the injected test cells. CRU frequencies were calculated by limiting dilution analysis using the maximum likelihood solution (Fazekas de St. Groth 1982).

2.6 In vitro assays

2.6.1 Clonogenic cell assays

To assay for erythroid (BFU-E), mixed granulocyte/macrophage (CFU-GM), and multi-lineage (CFU-GEMM) clonogenic progenitors, cells were suspended in Alpha medium containing 0.8% methylcellulose, 30% FCS, 1% bovine serum albumin, 0.1 mM ß-mercaptoethanol (ß-ME), 3 U/ml human erythropoietin, and 2% pokeweed mitogen-stimulated

mouse spleen cell conditioned medium (StemCell) and 1 ml aliquots plated in 35 mm petri dishes (StemCell). Colonies were scored in situ after 14 days of incubation at 37°C in a humidified atmosphere of 5% CO_2 in air, using criteria established and used in this laboratory for many years (Humphries, et al 1979a).

A modification of a previously described CFU-pre-B assay was used to detect clonogenic pre-B cell progenitors (Suda, et al 1989). Briefly, cells were plated in 1 ml cultures of Alpha medium containing 0.8% methylcellulose, 30% of a selected batch of FCS, 0.1 mM ß-ME (StemCell) plus 0.2 ng/ml recombinant human interleukin-7 (IL-7, Immunex, Seattle, WA) and, for cultured cells, 5 ng/ml recombinant murine Steel Factor (SF, Immunex). The predominant colony type arising from fresh marrow cells plated in IL-7 alone for 7 days at 37°C in 5% CO_2 in air possessed the distinctive morphology of B lymphoid colonies (Lee, et al 1989; Suda, et al 1989; McNiece, et al 1991b) and >90% of the cells from individually analyzed colonies were $B220^+$ by flow cytometry. In contrast, cells harvested from "switched" long-term cultures (see below) frequently gave rise to some monocytic and granulocytic colonies when the CFU-pre-B assays contained SF as well as IL-7. In addition, there were other colonies produced in these assays that did not have a typical pre-B colony morphology but were found to contain cells expressing B220 and having a typical lymphoid morphology upon staining with May-Grünwald-Giemsa. These colonies often contained two distinct $B220^+$ populations, one much brighter than the other, possibly indicating a mixture of pro- ($B220^+$) and pre-B ($B220^{++}$) cells. In cases where it was difficult to make a lineage assignment based solely on colony morphology, the colonies were individually plucked and either stained with May-Grünwald-Giemsa for morphologic analysis or pooled by colony type for analysis of B220 expression by flow cytometry. Preliminary experiments confirmed (Lee, et al 1989; McNiece, et al 1991b) that the number of CFU-pre-B detected in fresh marrow in the presence of IL-7 alone or in cultured cell preparations in the presence of SF plus IL-7 was a linear function of the input cell concentration (data not shown).

2.6.2 Myeloid long-term culture-initiating cell (LTC-IC) assay

Test cells were cultured in Alpha medium supplemented with 12.5% FCS, 12.5% horse serum, 0.1 mM ß-ME, 2 mM L-glutamine, 0.2 mM i-inositol, and 20 µM folic acid (MyeloCult, StemCell) to which 10^{-6} M freshly dissolved hydrocortisone sodium hemisuccinate (Sigma) was added shortly prior to use. Cultures were initiated by seeding the test cells onto irradiated (1500 cGy), adherent marrow feeder layers established 10-14 days earlier in the same medium from an inoculum of 1-1.5 x 10^6 normal mouse marrow cells per cm^2 of tissue culture surface. The cultures were then maintained at 33°C with weekly half medium changes and concomitant removal of half of the non-adherent cells. After 4 weeks, each culture was harvested separately, corresponding adherent and non-adherent fractions pooled, and then each combined suspension assayed for BFU-E, CFU-GM, and CFU-GEMM. Using the same statistical methods as for calculating CRU frequencies, the frequency of LTC-IC in the test population was determined from the proportion of negative cultures (i.e., those containing none of these myeloid clonogenic cells) at each input cell dose tested.

2.6.3 Lympho-myeloid long-term culture-initiating cell (LTC-IC_{ML}) assay

To detect and quantitate those LTC-IC that can give rise to lymphoid as well as myeloid clonogenic cells in vitro, a two-stage culture system was used. The rationale for the details of this procedure and the experiments validating it are presented in the Chapter III. Test cells were initially seeded onto pre-established irradiated marrow feeders in the same way as for the LTC-IC assay described above and carried in the same manner for the first 4 weeks. Cultures set up for limiting dilution assays were seeded into either 96-well plates (Nunclon, Nunc A/S, Denmark or Falcon, Becton Dickinson Labware, Lincoln Park, N.J.) or 24-well plates (Falcon or Corning, Corning Glass Works, Corning, N.Y.), depending on the number of cells seeded per well (up to 10^5 cells per well in 96-well plates and up to 10^6 cells per well in 24-well

plates). Larger cultures were generally set up in either 35 mm tissue culture dishes (Corning) (up to $2x10^6$ cells per dish) or T25 tissue culture flasks (Falcon or Corning) (up to $8x10^6$ cells per flask). At the end of the 4 week myeloid culture period, all of the medium and non-adherent cells were removed, the adherent layer was washed twice with warm RPMI 1640 (StemCell) to minimize the amount of residual horse serum and hydrocortisone (since both of these inhibit murine B lymphopoiesis (Whitlock and Witte 1982)), and the volume of medium originally removed was replaced with RPMI 1640 supplemented with 5% FCS and 50 μM ß-ME (LymphoCult, StemCell). The cultures were then placed at 37°C (Whitlock and Witte 1982) for a further week. At the end of this time, each culture was harvested individually, trypsinized adherent cells pooled with their corresponding non-adherent cells, and half of the combined cells were assayed for myeloid clonogenic cells (BFU-E, CFU-GM, CFU-GEMM) and the other half for CFU-pre-B. LTC-IC_{ML} frequencies in the original test cell suspension were calculated as described above for CRU and LTC-IC from the proportion of negative cultures (defined in this case as those that did not contain *both* myeloid *and* lymphoid clonogenic cells) at each input cell dose tested.

2.6.4 Stromal cell-free culture of fetal liver cells

Fetal liver cells were cultured at 37°C in Alpha-MEM supplemented with 200 mM glutamine, 10^{-4} M β-mercaptoethanol and, unless otherwise indicated, 30% of a selected fetal calf serum (StemCell) in 35-mm (StemCell) or 60-mm (Falcon) petri dishes. After 1 week, half of the spent medium was removed, centrifuged and the pelleted cells resuspended in the same volume of fresh medium and returned to their original cultures. In some experiments, the cells were cultured in Iscove's medium and the FCS was replaced with a serum substitute which consisted 200 μg/ml transferrin, 10 μg/ml insulin, 1% bovine serum albumin, 0.07% sodium bicarbonate (StemCell), and 40 μg/ml human low density lipoproteins (Sigma). Appropriate growth factors were added as described in the text at the initiation of the cultures and again after 1 week as part of the half medium exchange.

III. Characterization and purification of a primitive hematopoietic cell type in adult mouse marrow capable of lympho-myeloid differentiation in long-term marrow "switch" cultures

3.1 Summary

In this chapter, I describe a modification of the assay for long-term culture-initiating cells (LTC-IC) that allows a subset of murine LTC-IC, designated as LTC-IC_{ML}, to express both their myeloid (M) and lymphoid (L) differentiative potentials in vitro. The modified assay involves culturing test cells at limiting dilutions on irradiated mouse marrow feeder layers for an initial 4 weeks under conditions that support myelopoiesis and then for an additional week under conditions permissive for B lymphopoiesis. All of the clonogenic pre-B progenitors (CFU-pre-B) detected in such post-switch LTC appear to be the progeny of uncommitted cells present in the original cell suspension since exposure of lymphoid-restricted progenitors to myeloid LTC conditions for ≥7 days was found to irreversibly terminate CFU-pre-B production and, in cultures initiated with limiting input cells numbers such that no progenitors of any type detected in >70% of cultures one week after the switch, the presence of CFU-pre-B was tightly associated with the presence of myeloid clonogenic cells, regardless of the purity of the input population. Limiting dilution analysis of the proportion of negative cultures measured for different numbers of input cells showed the frequency of LTC-IC_{ML} in normal adult mouse marrow to be 1 per $5x10^5$ cells with an enrichment of ~500-fold in the Sca-1^+ Lin^- WGA^+ fraction as also found for competitive in vivo repopulating units (CRU) and conventionally defined LTC-IC. LTC-IC_{ML} also exhibited the same resistance to treatment in vivo with 5-fluorouracil as CRU and LTC-IC, thereby distinguishing these three populations from the great majority of both in vitro clonogenic cells and day 12 CFU-S. The ability to quantitate cells with dual lymphoid and myeloid differentiation potentials in vitro, without the need for their prior purification, should facilitate studies of totipotent hematopoietic stem cell regulation.

3.2 Introduction

The existence in adult mouse marrow of hematopoietic cells with the potential to generate both lymphoid and myeloid progeny for extended periods of time in vivo is well established (Wu, et al 1968; Abramson, et al 1977; Dick, et al 1985; Lemischka, et al 1986). More recently, in vivo methods for quantitating these cells have been developed (Harrison 1980; Hodgson, et al 1982; Szilvassy, et al 1990). These methods have allowed significant strides to be made in their purification, characterization and partial separation from cells with short-term in vivo repopulating ability (CFU-S) (Visser and van Bekkum 1990; Spangrude, et al 1991), although the proportion of day 12 CFU-S that may also be long-term repopulating lympho-myeloid cells remains controversial (Spangrude, et al 1988; Ploemacher and Brons 1989; Ploemacher, et al 1989; Jones, et al 1990). In vitro methodologies for demonstrating the lympho-myeloid differentiation of single progenitor cells in highly purified populations of both fetal (Cumano, et al 1992) and adult (Hirayama, et al 1992; Ball, et al 1995) murine origin have also been described recently. The latter has been useful not only for establishing the phenotype of cells in the adult that have lympho-myeloid potential but also for identifying specific growth factor combinations that allow these differentiative potentials to be expressed (Ogawa 1993). However, because the assay procedure requires the initial isolation of a highly purified population, its utility to address many questions is limited both by practical considerations and the reliance on the questionable assumption that any particular pattern of surface marker expression will be invariably associated with the defining differentiative or proliferative properties of the most primitive hematopoietic cell types (Rebel, et al 1994).

An alternative approach to detecting very primitive hematopoietic cells in both unseparated and purified mouse marrow makes use of the long-term culture (LTC) system first described by Dexter et al (Dexter, et al 1977).This culture system has been shown to reproduce many features of the environment within the marrow in vivo and to support for several weeks the maintenance and self-renewal of cells with in vivo lympho-myeloid repopulating ability

(Fraser, et al 1990; Fraser, et al 1992). The frequency in adult mouse marrow of cells that form "cobblestone areas" of proliferating cells visible in the adherent layer of LTC after 4-5 weeks (cobblestone area-forming cells, CAFC) correlates well with the frequency of cells with in vivo long-term repopulating ability (LTRA), both before and after their extensive purification (Down and Ploemacher 1993). In addition, some cultures with cobblestone areas also contain cells that can reconstitute lymphoid and myeloid lineages upon their transplantation into irradiated recipient mice (Ploemacher, et al 1991). Thus CAFC and in vivo repopulating cells have been considered to be overlapping, if not identical, populations. Direct examination of the lymphopoietic potential of murine CAFC has been precluded by the fact that the conditions used for their detection are not permissive for lymphopoiesis (Dexter and Spooncer 1978). However, it has previously been shown that B lymphoid cell development may be observed when established LTC are "switched" to conditions originally described by Whitlock and Witte (Whitlock and Witte 1982) that do support the long-term in vitro production of murine B lineage cells (Denis and Witte 1986; Dorshkind 1986). We now show that extension of the conventional limiting dilution CAFC assay with a one week culture period under Whitlock-Witte conditions after an initial 4 weeks under standard myeloid conditions and the use of both a myeloid and a pre-B clonogenic cell read-out (depicted in Figure 3.1) allows the quantitation in vitro of a subpopulation of LTC-initiating cells (LTC-IC) that have demonstrable myeloid *and* lymphoid potential (LTC-IC_{ML}). These cells also share other distinguishing properties of long-term in vivo repopulating cells. The LTC-IC_{ML} assay is applicable to unseparated as well as highly purified starting populations and should therefore complement and extend existing methodologies for investigating the molecular basis and control of hematopoietic stem cell self-renewal and lineage determination.

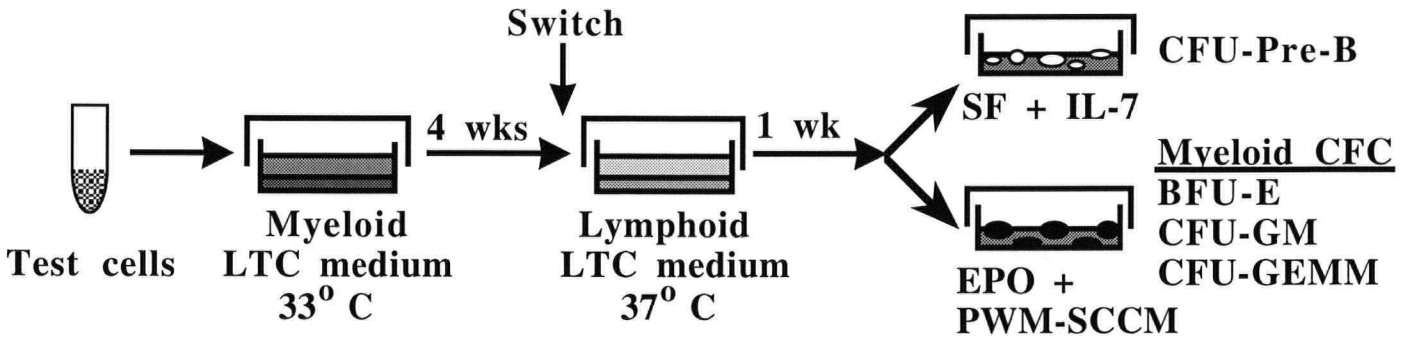


Figure 3.1 Diagram of the methodology used to assay LTC-IC_{ML}. Test cells are seeded onto preestablished irradiated marrow feeder layers in myeloid LTC medium and maintained for 4 weeks with weekly half medium changes. The cultures are then "switched" by removing and discarding all of the medium and nonadherent cells, washing twice with warm RPMI, and replacing the original volume with lymphoid LTC medium. Switched cultures are then maintained for a further week before being harvested and the pooled adherent and nonadherent cells assayed for their content of CFU-pre-B and myeloid CFC (BFU-E + CFU–GM + CFU-GEMM).

3.3 Results

3.3.1 Similarities of LTC-IC and CRU

Although the results of human LTC-IC (Sutherland, et al 1990) and murine CAFC studies suggested that a murine LTC-IC assay would detect very primitive cells in adult mouse marrow, it had not yet been formally shown that this was the case. We therefore compared the frequency and phenotype of LTC-IC as defined here using a 4 week myeloid clonogenic cell output end point with various other progenitor cell types detectable in normal adult mouse marrow, including CRU, as shown in Table 3.1. The frequencies of LTC-IC and CRU were calculated by limiting dilution analysis as described in the Methods. As can be seen, the frequency, and hence the number, of CRU in normal marrow was found to be the same as that measured previously (Szilvassy, et al 1990; Rebel, et al 1994) and is approximately twice that of LTC-IC but only approximately 1/100th that of either the pre-B lymphoid or total myeloid clonogenic progenitors detectable in vitro. In addition, the small subpopulation of Sca-1$^+$Lin$^-$WGA$^+$ cells in normal adult mouse marrow that was previously shown to be highly enriched in CRU (Jurecic, et al 1993; Rebel, et al 1994) was found to be similarly enriched approximately 700-fold in LTC-IC and with a similar recovery, with simultaneous elimination >90% of the in vitro clonogenic progenitors. Interestingly, although CFU-G, CFU-M, and CFU-GM comprised most of the clonogenic cells detected in the 4 week harvests of these LTC-IC assays, 3 of the 9 cultures seeded with input cell numbers that resulted in <30% of positive assays at week 4 so that the probability of >1 LTC-IC per culture was <15% also contained one or more CFU-GEMM. As expected for the clonogenic cell output from individual progenitors (Till, et al 1964; Humphries, et al 1981; Ogawa 1993), the total clonogenic cell content of each of these 9 cultures varied markedly, ranging from 1 to 356 granulocyte and/or macrophage progenitors (median=20) with up to 38 CFU-GEMM (median<1).

Table 3.1 Comparison of the relative enrichments and recoveries of various functionally defined primitive hematopoietic cells in the Sca-1$^+$ Lin$^-$ WGA$^+$ fraction of normal adult mouse marrow cells.

Progenitor Type	1/Frequency		Enrichment (fold)	Recovery (%)
	Unseparated cells	Sca-1$^+$ Lin$^-$ WGA$^+$ cells		
CRU	(1.4 ± 0.1) x 10^4 (3)	30 ± 2 (2)	510 ± 8 (2)	13.0 ± 1 (2)
LTC-IC	(3.0 ± 0.8) x 10^4 (4)	45 (1)	690 (1)	15 (1)
LTC-IC$_{ML}$	(47 ± 6) x 10^4 (8)	700 ± 200 (2)	540 ± 40 (2)	7.0 ± 0.1 (2)
CFU-pre-B	420 (1)	>140 (1)	<3 (1)	< 0.1 (1)
Myeloid CFC	120 ± 5 (2)	5 ± 1 (2)	24 ± 5 (2)	0.7 ± 0.2 (2)

Values shown represent the mean ± SEM from (n) experiments. Recovery and enrichment values were calculated from experiments where the same cells were assessed both before and after their purification.

Table 3.2 shows a comparison of the effect 150 mg/kg body weight of 5-FU given in vivo on the marrow content of CRU and LTC-IC 2 days later. In this case, frequency measurements were multiplied by the total femoral cellularity to obtain the absolute population size estimates shown. CRU and LTC-IC populations both showed 3 to 4-fold decreases after 5-FU treatment. In contrast, parallel measurements of the number of in vitro clonogenic myeloid and pre-B progenitors as well as day 12 CFU-S in the same marrow cell suspensions showed these populations to be markedly reduced by ≥80-fold, as previously reported (Hodgson and Bradley 1979; Suda, et al 1983a; Vetvicka, et al 1986). Thus by their frequency, surface antigen expression, and sensitivity to 5-FU in vivo, LTC-IC defined by a 4-week myeloid clonogenic cell end point were indistinguishable from CRU and both were separable from the majority of cells detectable by short-term colony assays, including day 12 CFU-S.

Data from these studies were also used to analyze the relationship between the number of cells seeded into LTC-IC assay cultures and the average myeloid clonogenic cell output measured 4 weeks later. As shown in Figure 3.2, the clonogenic cell content of 4 week-old myeloid LTC was found to be directly proportional to the input cell number over a wide range of values. The slope of the lines fitted to the data did not significantly differ from linearity in LTC-IC assays of unseparated marrow from normal ($p=0.59$) or 5-FU treated ($p=0.48$) mice nor for those initiated with 25-100 Sca-1$^+$Lin$^-$WGA$^+$ cells ($p=0.36$). These results validate the use of graded doses of input marrow cells and limiting dilution analysis to quantitate the LTC-IC content of both unseparated marrow cells suspensions and derivative fractions that may be variably enriched in their LTC-IC content.

Table 3.2 Comparison of the effect of 5-FU in vivo on different types of hematopoietic progenitor populations.

Progenitor type	No. of progenitors / femur				Fold decrease
	Normal mice		2 days post 5-FU		
CFU-pre-B	$(2.0 \pm 0.38) \times 10^4$	(8)	<0.36 *	(6)	$>5.5 \times 10^4$
CFC	$(6.8 \pm 0.55) \times 10^4$	(5)	550 ± 200	(5)	120
Day 12 CFU-S	$(3.2 \pm 0.59) \times 10^3$	(4)	40 ± 14	(3)	80
LTC-IC	640 ± 140	(4)	200 ± 120	(2)	3
$LTC\text{-}IC_{ML}$	37 ± 4	(8)	13 ± 3	(5)	3
CRU	1100 ± 190	(3)	290 ± 150	(2)	4

Values shown represent the mean ± SEM from (n) experiments.

* No CFU-pre-B colonies have been detected in $>10^7$ cells assayed to date.

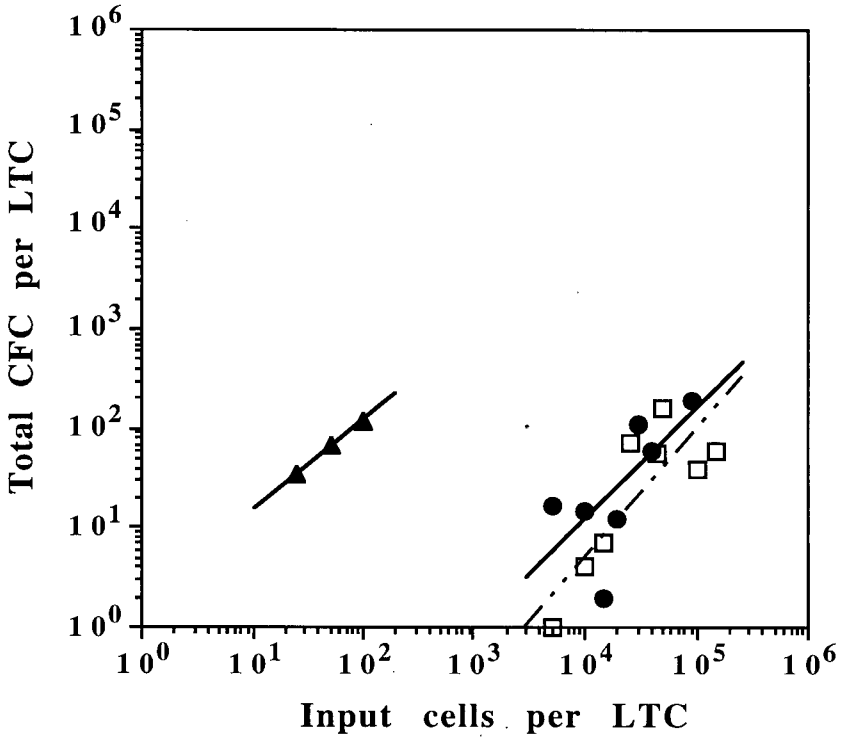


Figure 3.2 Linear relationship between the concentration of input cells seeded into LTC-IC assay cultures and the number of myeloid clonogenic cells measured after maintenance of the cultures for 4 weeks under myeloid LTC conditions. ▲ = Sca-1$^+$Lin$^-$WGA$^+$ cells [slope = 0.88, 95% C.I. = 0.02 to 1.7], ● = unseparated normal marrow [solid line, slope = 1.3, 95% C.I. = 0.14 to 2.4], □ = marrow from 5-FU treated mice [dashed line, slope = 1.3, 95% C.I. = 0.29 to 2.3].

3.3.2 Development of an assay for lympho-myeloid LTC-IC (LTC-IC_{ML})

The LTC-IC assay described above clearly provides a means of quantitating a population of primitive cells that share a number of features of cells with in vivo lympho-myeloid differentiation potential. Nevertheless, the culture conditions used do not allow detection of the lymphoid potential that some LTC-IC might be expected to possess. To overcome this limitation, we took advantage of the observation that when murine myeloid LTC are "switched" to conditions that are permissive for B lymphopoiesis, production of B lineage cells can sometimes be initiated (Denis and Witte 1986; Dorshkind 1986). We hypothesized that if cultures were switched after an initial 4 week period under myeloid LTC conditions, in order that all myeloid clonogenic cells detected could be assumed to have arisen from LTC-IC, it might be possible to identify a time when the production of CFU-pre-B was well under way before the production of myeloid clonogenic cells had markedly decreased. To investigate this possibility, we measured the rate of appearance of CFU-pre-B and the concomitant disappearance of myeloid progenitors in LTC-IC assay cultures that, instead of being harvested at 4 weeks, were switched to Whitlock-Witte conditions for variable periods of time and then harvested and assayed as described in the Methods. Figure 3.3 shows the combined results of 3 such experiments. As expected, no CFU-pre-B were present after 4 weeks under myeloid LTC conditions nor for the first 4 days after the switch to lymphoid LTC conditions. Within 7 days, however, CFU-pre-B had become readily detectable and over the course of the next 7 days their numbers continued to increase rapidly before levelling off. Concomitantly, the initially large number of myeloid clonogenic cells present in 4 week-old myeloid LTC declined progressively with <1% of the input number or <0.1% of the number detectable at the time of the switch still present by the third week post-switch. Based on the initial findings from these experiments, subsequent studies focussed on an analysis of cultures harvested one week after the switch (shown schematically in Figure 3.1) at a time when CFU-pre-B, although not yet present at maximal levels, are nevertheless readily detectable.

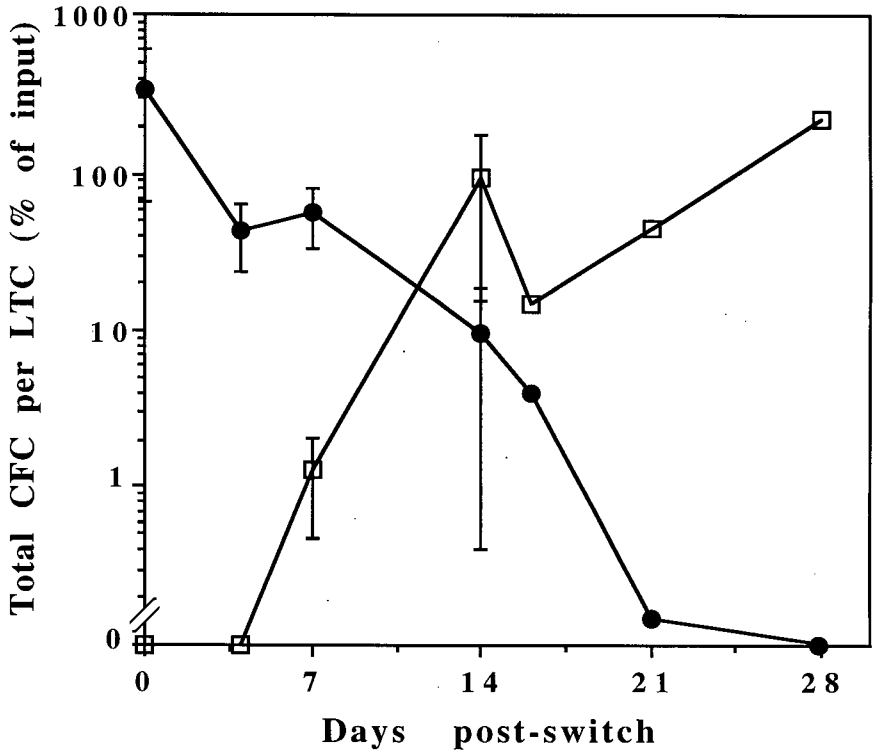


Figure 3.3 Kinetics of the appearance of CFU-pre-B (□) and the concomitant rate of disappearance of myeloid clonogenic cells (●) in LTC maintained for an initial 4 week period under myeloid LTC conditions and then switched to lymphoid LTC conditions. Bulk myeloid LTC were initiated with $\geq 2x10^6$ normal marrow cells in either 35 mm or 60 mm tissue culture dishes containing preestablished irradiated mouse marrow adherent layers. Day 0 refers to data for cultures maintained under standard myeloid LTC conditions for 4 weeks at 33°C. The remaining cultures were switched to lymphoid LTC conditions and then harvested at the times shown for assessment of both types of clonogenic cells (CFC). In these experiments, CFU-pre-B colony assays contained IL-7 but no SF to reduce the background of myeloid colonies that are stimulated when SF is present. Values shown are the mean ± SEM for data from 3 experiments. To allow data from different experiments to be combined, clonogenic cell yields have been expressed as a percentage of the number measured for the original marrow input of that experiment.

3.3.3 Validation of the LTC-IC_{ML} assay

To evaluate the usefulness of the LTC-IC_{ML} assay as a method for quantitating cells in variously manipulated test cell suspensions, the relationship between the number of cells initially seeded into each assay culture and the number of clonogenic myeloid and lymphoid cells detectable 5 weeks later was assessed. As shown in Figure 3.4, the output of CFU-pre-B increased linearly with increasing input cell concentrations ($p=0.91$). As expected from Figure 3.2, the number of myeloid clonogenic cells measured simultaneously also increased as a function of the input cell number (data not shown). However, the majority of these would have been derived from myeloid-restricted LTC-IC (see below) and hence a meaningful assessment of the relationship between input cell dose and output of LTC-IC_{ML}-derived myeloid clonogenic cells could not be made.

Use of the design shown in Figure 3.1, even as a limiting dilution procedure, to detect an input cell with dual lymphoid and myeloid potential assumes that none of the CFU-pre-B detected one week after switching culture conditions would be derived from a lymphoid-restricted progenitor present at the initiation of the culture. The consistent absence of CFU-pre-B in 4 week-old cultures at the time of the switch (Figure 3.3, above) provides some support for this assumption. However, it would not exclude the potential persistence of a corticosteroid/horse serum-resistant, lymphoid-restricted CFU-pre-B progenitor. To address such a possibility, three additional types of analyses were undertaken. In the first, the distribution of CFU-pre-B and myeloid clonogenic cells was examined in cultures initiated with limiting numbers of input cells. For this analysis, we evaluated only those cultures where the input was such that $\leq 30\%$ of the cultures assessed at the end of the 5 week period were found to contain any type of clonogenic cell, either lymphoid or myeloid. In such cases, it is unlikely ($p<0.15$) that more than one cell with the capacity to generate either lymphoid or myeloid clonogenic progeny detectable 5 weeks later would have been seeded into a single culture. Eleven of the 98 such cultures analyzed (Table 3.3) contained CFU-pre-B. In 10 of these,

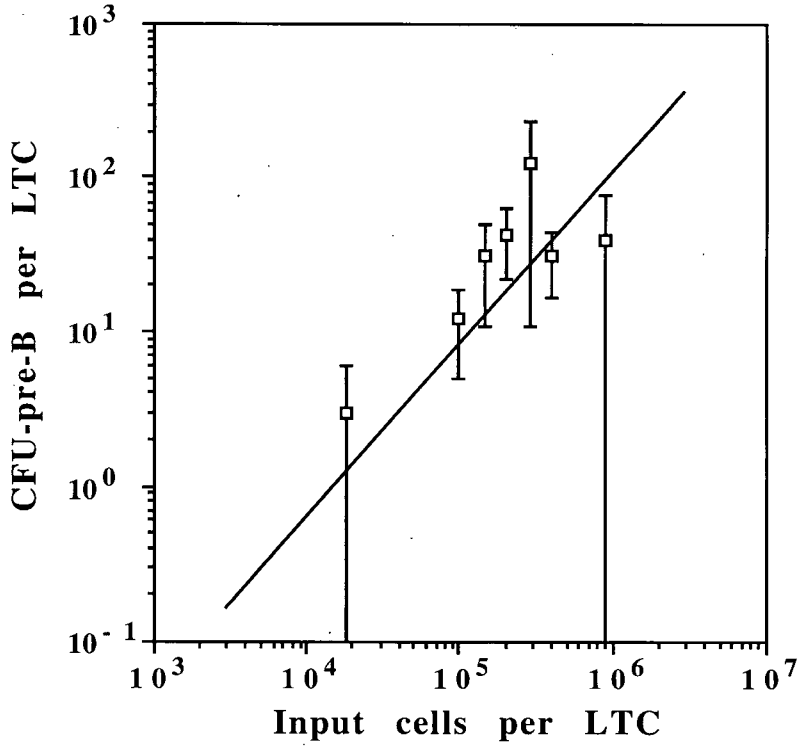


Figure 3.4 Linear relationship between the number of CFU-pre-B detected in LTC-IC_{ML} assay cultures and the concentration of normal, unseparated marrow cells initially seeded (slope = 1.1, 95% C.I. = -0.49 to 2.6). Values shown represent the mean ± SEM of data pooled from 3 limiting dilution experiments.

Table 3.3 The presence of myeloid and lymphoid progenitors in cultures initiated at limiting input dilutions is not due to the coincident plating of lineage-restricted LTC-IC.

	Frequency of Positive Cultures *			
Myeloid CFC-positive (M^+)	22/98	(22%)		
CFU-pre-B-positive (L^+)	11/98	(11%)		
Observed M^+L^+	10/98	(10%)	10/22	(45%) M^+ are also L^+
			10/11	(91%) L^+ are also M^+
Predicted M^+L^+ due to	2/98	(2%)	2/22	(11%) M^+ would be L^+
coincidence			2/11	(22%) L^+ would be M^+

*Since LTC-IC are more frequent than LTC-IC_{ML}, only input numbers such that ≤30% of LTC contained any type of clonogenic cell after 5 weeks were considered in this analysis since <15% of such cultures are expected to contain more than 1 responding cell.

myeloid clonogenic cells were also present; i.e., significantly more often than the 2 of 11 expected if the presence of myeloid clonogenic cells and CFU-pre-B in the same culture were due to the coincident input of two independently segregating progenitor cell types occurring at the observed frequencies of 22% myeloid-positive and 11% lymphoid-positive cultures ($p<0.0001$, Fisher's Exact Test). These 11 cultures were also useful for defining the variability in clonogenic cell output characteristic of individual LTC-IC_{ML}. The number of CFU-pre-B present amongst the 5 week progeny of single LTC-IC_{ML} ranged from 2 to 146 (median=55) and the corresponding number of myeloid clonogenic cell progeny ranged from 3 to 138 (median=9). The latter were comprised mainly of CFU-M and CFU-GM but in one of the 11 cultures, included 6 CFU-GEMM.

The second approach we used to investigate the single cell origin of mixed lymphoid-myeloid cultures in the LTC-IC_{ML} assay was to evaluate the distribution of progenitor types in cultures initiated with subpopulations of normal mouse marrow. The Sca-1$^-$ fraction was found to be depleted of LTC-IC_{ML} ~5-fold and these cells enriched ~40-fold in the Sca-1$^+$ fraction. Further selection of the ~0.02% of total BM in the Sca-1$^+$Lin$^-$WGA$^+$ subfraction did not allow the lymphoid and myeloid clonogenic cell-producing activities of whole marrow to be separated and both types of progenitors could be found together in cultures initiated with as few as 35 Sca-1$^+$ Lin$^-$ WGA$^+$ cells. As shown in Table 3.1, this fraction was found to be enriched ~500-fold for LTC-IC_{ML} and similarly enriched for CRU and LTC-IC and with approximately the same overall recovery (7%).

A third set of experiments was set up to address the possibility that there might exist a very early (pre-CFU-pre-B) but lymphoid-restricted progenitor that could persist throughout the initial 4 weeks of myeloid LTC but not yield detectable progeny CFU-pre-B until after the switch to conditions permissive for their survival. The possible existence of such a cell has been inferred from the finding that cells from 6 to 20 week-old lymphoid LTC can upon adoptive transfer into immunocompromised mice reconstitute and sustain polyclonal B and T

lymphopoiesis for up to 10-12 weeks (Kurland, et al 1984; Dorshkind, et al 1986), even though neither CFU-S nor marrow repopulating cells could be detected after 6 weeks in lymphoid LTC (Kurland, et al 1984) (see also Figure 3.3). We therefore initiated a series of cultures with normal marrow and maintained them for 8 weeks at 37°C as described by Whitlock and Witte (Whitlock and Witte 1982). At the end of this initial culture period, duplicate cultures were harvested and their CFU-pre-B content measured (Table 3.4). Half of the remaining flasks were then switched to myeloid LTC conditions by removing and discarding all of the culture medium and nonadherent cells, washing the adherent layer twice with warm RPMI and then replacing the original volume with myeloid LTC medium and incubating the cultures at 33°C. The remaining flasks were mock switched by performing the same manipulations but replacing the original volume of lymphoid LTC medium with an equal amount of fresh lymphoid LTC medium and returning the cultures to 37°C. After a further 7 days, all but the control cultures were switched to lymphoid LTC conditions and then maintained for a final 4 weeks without further change. Control cultures were maintained throughout under lymphoid LTC conditions although a mock switch was performed in parallel with the switched cultures. All cultures were then harvested and assayed individually for CFU-pre-B. As shown in Table 3.4, production of CFU-pre-B could not be reinitiated in lymphoid cultures that had been exposed to myeloid LTC conditions for 7 days in spite of the continued output of CFU-pre-B in control LTC maintained uninterrupted under lymphoid conditions, suggesting that myeloid LTC conditions are highly and rapidly toxic to the earliest reported lymphoid progenitor types.

3.3.4 Sensitivity of LTC-IC_{ML} to 5-FU in vivo

Given the greater resistance of CRU and LTC-IC populations, as compared to CFU-S and in vitro clonogenic pre-B and myeloid progenitors, to the effectss of a single injection of 150 mg/kg body weight of 5-FU, it was of interest to examine the same cell suspensions for

Table 3.4 Disappearance of lymphoid-restricted progenitors from 8 week-old lymphoid LTC after a 7 day period of culture under myeloid LTC conditions.

	Days under myeloid LTC conditions	Days post switch to lymphoid LTC conditions	CFU-pre-B per LTC
Experiment #1	0	0	1690 *
	7	0	nd
	0	28	24600
	7	28	0
Experiment #2	0	0	8160 *
	7	0	nd
	0	28	62500
	7	28	0

In these experiments, $8x10^6$ normal marrow cells were seeded onto preestablished feeders in T25 tissue culture flasks and maintained for 8 weeks under lymphoid LTC conditions with standard bi-weekly medium exchanges in order to provide a test population of lymphoid-restricted progenitor cells. Some of the cultures were harvested at this time and assayed for their CFU-pre-B content. Others were then mock switched (i.e., two washes with RPMI before replacing the culture medium with fresh lymphoid LTC medium) and carried for a further 4 weeks as before. The last group of cultures were switched to myeloid LTC conditions (i.e., myeloid LTC culture medium, 33°C) after 2 washes with RPMI and carried for 1 week under these conditions before being switched back to lymphoid LTC conditions. At each time point, duplicate T25 flasks were assayed for CFU-pre-B colony assays containing IL-7 + SF, except where noted.

* CFU-pre-B colony assays contained only IL-7.

nd = not done.

the effect of this treatment on LTC-IC_{ML}. Accordingly, as for the other types of progenitors measured, LTC-IC_{ML} numbers were determined by limiting dilution analysis and population sizes calculated before and after 5-FU treatment. The results are presented in Table 3.2. It can be seen that the 5-FU treatment reduced the femoral content of LTC-IC_{ML} ~3-fold. This is similar to the effect of 5-FU on both CRU and LTC-IC and is in marked contrast to the ≥80-fold reduction observed for both myeloid and lymphoid- restricted progenitors detected in short-term in vitro clonogenic assays as well as for day 12 CFU-S.

3.4 Discussion

Much attention is currently focussed on the identification and characterization of molecular events that bring about changes in the totipotency of hematopoietic stem cells, including those that remain functionally intact throughout normal adult life. Such studies require biological assays that permit the full differentiative potential of these cells to be displayed and that also allow their discrimination from progeny with more restricted properties. The approach that appears to satisfy these requirements best involves the transplantation of test cells into a congenic host that has been sufficiently perturbed hematologically to provide a suitable environment for any injected hematopoietic stem cell to generate identifiable progeny belonging to all of the various hematopoietic lineages (Keller, et al 1985; Fraser, et al 1990; Szilvassy, et al 1990; Smith, et al 1991). While this approach has been useful in establishing that many CFU-S are not totipotent long-term repopulating cells (reviewed in (Visser and van Bekkum 1990)), it does not easily lend itself to investigations of the stage at which any particular differentiative function of a manipulated or altered stem cell may become manifest. Moreover, the extent and nature of the stimulation provided by the microenvironment of the injected mouse is difficult to assess or modify.

An alternative approach is to try to define in vitro conditions and end points that allow the specific detection of totipotent hematopoietic cells. Recently, conditions have been described for the in vitro detection of murine cells with B lymphoid as well as varying degrees of myeloid differentiation potential. However, the applicability of these procedures is restricted to either fetal test cells (Baum, et al 1992; Cumano, et al 1992) or highly purified adult marrow populations (Hirayama, et al 1992; Ball et al, 1995). In this report, we have shown how the principles of the LTC-IC assay, previously developed for both human (Sutherland, et al 1990) and murine (Ploemacher, et al 1989) myeloid progenitors, can be used to overcome the inherent limitations of all of the approaches reported to date. First, the LTC-IC_{ML} assay procedure we have described is performed entirely in vitro. Second, it is both quantitative and specific for cells with lympho-myeloid differentiation potential. Finally, it can be applied to unseparated as well as highly purified populations of adult mouse bone marrow.

Several lines of investigation were pursued to establish the specificity of the LTC-IC_{ML} assay since previous work by Dorshkind (Dorshkind 1986) and Denis and Witte (Denis and Witte 1986), on which the LTC-IC_{ML} assay is based, had not excluded the important possibility that a lymphoid-restricted progenitor might persist under Dexter culture conditions and then subsequently generate CFU-pre-B when the cultures were switched to more permissive conditions for this latter cell type. Analysis of the clonogenic cell content of LTC-IC_{ML} assay cultures initiated with ≤ 1 clonogenic precursor per culture ($p>0.85$) showed that the presence of CFU-pre-B and myeloid clonogenic cells 5 weeks later were not independent events ($p>0.9999$). Second, even in highly purified cell suspensions, cells able to produce CFU-pre-B in the LTC-IC_{ML} assay could not be separated from cells able to generate myeloid progeny. Third, although cells from lymphoid cultures have previously been shown to reconstitute polyclonal B lymphopoiesis but not myelopoiesis upon adoptive transfer into immunodeficient mice (Kurland, et al 1984; Dorshkind, et al 1986), we were unable to demonstrate the persistence of either CFU-pre-B or their putative lymphoid-restricted precursors after even a single week of exposure to myeloid LTC conditions. Taken together,

these results provide strong evidence that the LTC-IC_{ML} assay detects individual cells with lympho-myeloid potential.

Some differences were found, however, in the frequencies of cells detected as CRU, LTC-IC and LTC-IC_{ML}, particularly the latter. One possible explanation for these discrepancies is that each assay detects a distinct population. Another possibility is that the end point used in each case measures biologically identical or highly overlapping populations but with different efficiencies. The 4 day lag time that we observed before the appearance of CFU-pre-B in post-switch LTC-IC_{ML} assay cultures (Figure 3.3) and the failure to detect the persistence of any earlier type of B lineage restricted precursor (Table 3.4) suggests that the CFU-pre-B detected in post-switch cultures may have to differentiate from those LTC-IC_{ML} still present after a 4 week period of culture under myeloid culture conditions. Previous studies have shown that CRU decline approximately 7-fold during that time (Fraser, et al 1992). Thus the design of the LTC-IC_{ML} assay may include a corresponding inherent decrease in plating efficiency.

We have also demonstrated that the myeloid clonogenic progenitor output of the LTC-IC assay and the CFU-pre-B output of the LTC-IC_{ML} assay are linearly related to the concentration of LTC-IC and LTC-IC_{ML}, respectively, in the starting cell suspension. Moreover, this was true for a wide range of input cell numbers, both for unseparated and highly purified test cell populations although, not surprisingly, some inhibition was apparent at the highest starting cell concentrations. Such a linear input-output relationship validates the use of limiting dilution statistics to derive absolute frequencies of the cell type being assayed, as long as excessive input cell concentrations are avoided.

In the present study, we exploited this fact to carry out a comparison of the numbers and properties of cells defined as myeloid and lympho-myeloid LTC-IC and CRU. All three methods were found to detect cells with the same rare surface antigen phenotype and high resistance to in vivo administration of 5-FU, the latter presumably reflecting a similarly slow

rate of turnover of these cells in the normal adult mouse. Importantly, these characteristics of LTC-IC, LTC-IC_{ML} and CRU were not shared by the >90% of progenitors detectable in short-term clonogenic assays, including day 12 CFU-S (Table 3.2). While it has been clear for some time that not all day 12 CFU-S are detectable as CRU (Ploemacher and Brons 1989), the data in Table 3.2 provide the first quantitative evidence that some CRU and LTC-IC are not likely to be detectable as day 12 CFU-S, since 2 days after in vivo exposure of mice to 5-FU the number of day 12 CFU-S present in the femur was <20% of the number of CRU or LTC-IC.

In summary, we have devised a new in vitro method for identifying and quantitating lympho-myeloid cells (LTC-IC_{ML}) in adult mouse bone marrow which relies on the joint expression of these differentiative potentials in the same culture. The molecular signals responsible for stimulating the various differentiation events that LTC-IC_{ML} must undergo to be detected remain undefined although, based on what has been learned for human LTC-IC (Baum, et al 1992; Sutherland, et al 1993) and murine and human CAFC (Neben, et al 1993; Breems, et al 1994), we presume that these cells are activated by factors produced by fibroblasts in the irradiated adherent marrow feeder layers. Both LTC-IC and LTC-IC_{ML} appear to be closely related to totipotent murine cells with long-term in vivo repopulating potential (CRU). The LTC-IC and LTC-IC_{ML} assays may, therefore, serve as useful surrogate procedures for investigating factors that affect CRU function and maintenance in culture. The present studies also support the likelihood that the human LTC-IC assay may detect cells with similar properties including long-term in vivo repopulating potential.

IV. Characterization of properties that allow stromal cell-responsive lympho-myeloid cells (LTC-IC_{ML}) to be detected in vitro and their distinction from in vivo repopulating ability

4.1 Summary

In Chapter III (also (Lemieux, et al 1995)), a method was described for detecting the ~10% of long-term culture-initiating cells (LTC-IC) present in normal adult mouse marrow that can express their dual lymphoid and myeloid differentiation potential in vitro. Also, a close relationship of these cells, referred to as LTC-IC_{ML}, to those with long-term in vivo lympho-myeloid repopulating potential, referred to as competitive repopulating units (CRU) was established from experiments demonstrating their co-purification, in spite of a 15-30-fold difference in their apparent frequency. To examine further the relationship between cells detectable by these two assays, a series of additional studies were undertaken. These have shown that two modifications to the procedure can increase the output of CFU-pre-B in the LTC-IC_{ML} assay ~2-fold. The first is to prolong the second phase of the assay when the cells are maintained under lymphoid conditions from 7 to 10 days. The second is to include another lymphoid cell stimulating factor, Flt-3 ligand (FL), in the CFU-pre-B assay, itself. However, neither of these manipulations increased the frequency of input cells ultimately detectable as LTC-IC_{ML}. Reduction of the initial, myeloid LTC phase of the LTC-IC_{ML} assay from 4 wks to 1 wk revealed a previously undescribed, 5-FU and steroid-resistant primitive B lineage progenitor cell type present in marrow at a frequency comparable to that of myeloid-restricted progenitors and higher than that of LTC-IC_{ML}. The ability of these primitive B lineage precursors to persist in myeloid LTC for at least 1 wk, although not for 4 wks, as shown in Chapter III, precludes the adoption of this shortened protocol for the routine measurement of individual stroma-responsive cells with lympho-myeloid differentiation potential. The use of S17 fibroblasts instead of mouse LTC adherent feeder layers also failed to increase the sensitivity of the LTC-IC_{ML} assay, although this did improve CRU maintenance, as reported

previously (Wineman, et al 1993). Interestingly, both LTC-IC and LTC-IC_{ML} numbers were better maintained in myeloid LTC (4 ± 1 -fold and 1.5 ± 0.5 -fold decrease, respectively, in the first 3-4 wks, n=2) than CRU (10 ± 2 -fold decrease in the same period, n=3). Thus different mechanisms appear to allow lympho-myeloid cells to express their differentiation potentialities in long-term cultures and after transplantation into myeloablated recipients. These findings build on previous observations indicating a discrepancy in the importance of SF in these two situations and support a model of HSC regulation in which retention of totipotentiality and maintenance of responsiveness to specific regulators are not tightly linked.

4.2 Introduction

Blood cell production originates throughout adult life from a small population of hematopoietic stem cells characterized by an enormous proliferative capacity and an ability to generate both lymphoid and myeloid progeny. Such cells have been known to exist in the marrow of both adult mice and humans for many years (Wu, et al 1968; Abramson, et al 1977; Turhan, et al 1989). However, quantitative methods that allow their definitive and specific distinction from other types of primitive cells with more restricted proliferative or differentiation properties have only recently been devised (Ploemacher, et al 1989; Sutherland, et al 1990; Szilvassy, et al 1990). For measurements of absolute frequencies of murine hematopoietic stem cells, an in vivo limiting dilution assay for competitive repopulating units (CRU) (Szilvassy, et al 1990) offers the most direct approach and has been pivotal in allowing the identification of various factors that may support (Fraser, et al 1992) or alter stem cell renewal (Sauvageau, et al 1995). Nevertheless, in vivo systems do not lend themselves as readily to an analysis of the earliest stages of stem cell commitment as would suitable in vitro alternatives. Assays based on the co-culture of primitive hematopoietic cells with stromal fibroblasts under conditions permissive for long-term myelopoiesis and stem cell maintenance have been described for both human (Sutherland, et al 1989; Sutherland, et al 1990; Breems, et

al 1994) and murine (Ploemacher, et al 1989; Lemieux, et al 1995) systems. The end point in these assays is the detection in culture of colony-forming progeny for periods exceeding the life span of intermediate progenitors, i.e., ≥4 weeks for murine cells (Ploemacher, et al 1989; Lemieux, et al 1995) and ≥5-6 weeks for human cells (Sutherland, et al 1990; Baum, et al 1992; Breems, et al 1994). The use of graded doses of input cells in these assays then allows the derivation of progenitor frequencies by limiting dilution analysis.

The so-called long-term culture-initiating cells (LTC-IC) thus detected share numerous features with CRU and, in adult mouse bone marrow, are co-purified in fractions enriched ≥500-fold for CRU (Lemieux, et al 1995). This would lead one to anticipate that most LTC-IC might also have lymphopoietic potential as was, in fact, suggested by experiments with human fetal marrow (Baum, et al 1992). However, corticosteroids and horse serum, which are key components of the growth medium used in LTC-IC assays (Dexter, et al 1977; Greenberger 1978) are highly toxic for murine lymphoid cells (Whitlock and Witte 1982), thus requiring a two step procedure to detect murine LTC-IC with lympho-myeloid potential. Based on the observations of Dorshkind (Dorshkind 1986) and Denis and Witte (Denis and Witte 1986) that B lymphoid cell production could be initiated when established murine myeloid LTC were "switched" to steroid/horse serum-free medium, we tested the feasibility of superimposing this modification on the standard limiting dilution murine LTC-IC assay. Evaluation of the myeloid and pre-B clonogenic cell content of cultures maintained for an initial 4 weeks under myeloid LTC conditions and then for a further week under conditions permissive for pre-B cell production (Whitlock and Witte 1982) revealed the existence of a subset of LTC-IC, termed LTC-IC_{ML}, with both myeloid and lymphoid potential (Lemieux, et al 1995). However, the frequency of LTC-IC_{ML} was consistently ~15-fold lower than LTC-IC and ~30-fold lower than CRU, regardless of the purity of the cell suspension assayed.

In this chapter, the results of experiments designed to explain this discrepancy by testing various strategies that might either improve the detection of B lymphopoietic ability or

reduce the potential loss of lympho-myeloid cells during the first 4 weeks of culture under myeloid LTC conditions are described. Although the results obtained have not allowed the sensitivity of the LTC-IC_{ML} assay to be significantly increased, they have revealed an unexpected disparity in the rate of decline of cells detectable as CRU and LTC-IC/LTC-IC_{ML} when these are maintained under LTC conditions. In addition, they have allowed the identification of a previously unrecognized lymphoid-restricted, steroid-resistant progenitor cell type different from cells capable of producing CFU-pre-B for ≥8 weeks under lymphoid LTC conditions.

4.3 Results

4.3.1 Effect of prolonging the lymphoid phase of the LTC-IC_{ML} assay and using FL in CFU-pre-B assays on LTC-IC_{ML} plating efficiency and CFU-pre-B generation

Previous time course studies of bulk cultures of adult mouse marrow switched to lymphoid conditions after an initial 4 week period under myeloid LTC conditions had shown that CFU-pre-B numbers continued to increase rapidly (~30-fold) between the first and second weeks after the switch and that myeloid CFC remained at >10% of input for up to 10 days (Lemieux, et al 1995) (see also Figure 4.3 below). We therefore compared the effect of assessing CFU-pre-B and myeloid CFC on the 10^{th} rather than the 7^{th} day after the switch in cultures initiated with limiting numbers of marrow cells from normal or 5-FU-treated mice. In the experiments with normal marrow, we also tested the effect of supplementing the CFU-pre-B assays with 100 ng/ml of FL since this factor has recently been shown to support early stages of lymphoid cell differentiation (Hannum, et al 1994; Hirayama, et al 1995;

Mackarehtschian, et al 1995). Figure 4.1 shows the frequency distributions of myeloid and lymphoid CFC numbers per LTC-IC_{ML} assay culture obtained for each of the conditions compared. The average number of clonogenic myeloid and CFU-pre-B progenitors detected in the positive cultures is shown in Table 4.1. It can be seen that extension of the lymphoid culture period from 7 to 10 days significantly increased the number of CFU-pre-B detected in some LTC-IC_{ML} assay cultures and hence also increased their average number per culture. CFU-pre-B detection was further enhanced up to 3-fold when FL was incorporated into the methylcellulose medium used in the CFU-pre-B assays. Nevertheless, neither of these modifications significantly changed the proportion of cultures in which at least one CFU-pre-B could be detected, regardless of the origin of the input cells. Moreover, extension of the period of culture under lymphoid conditions from 7 to 10 days did cause a slight decrease not only in the number of myeloid CFC per culture but also in the proportion of cultures in which myeloid CFC were detected. This effect was particularly pronounced in assays of 5-FU marrow where the proportion of cultures in which no myeloid CFC were detected increased from 4% to 50%. This suggests that there may be subtle differences or changes in the behaviour of the ~30% of marrow LTC-IC_{ML} that survive 5-FU treatment. Overall, the frequency of cultures containing both lymphoid and myeloid CFC did not change as a result of changing the duration of the lymphoid culture phase of the LTC-IC_{ML} assay (Table 4.1).

Figure 4.1 Distribution of clonogenic cells in 5 week-old LTC-IC_{ML} switched to lymphoid LTC conditions for either a "standard" 7 day (panels A and B) or "extended" 10 day (panels C and D) period prior to harvesting and assessment. Multiple replicate cultures were seeded with $5x10^4$ marrow cells from normal or 5-FU-treated mice and each culture then assayed individually to determine its content of CFU-pre-B (A, C) and myeloid CFC (B, D).

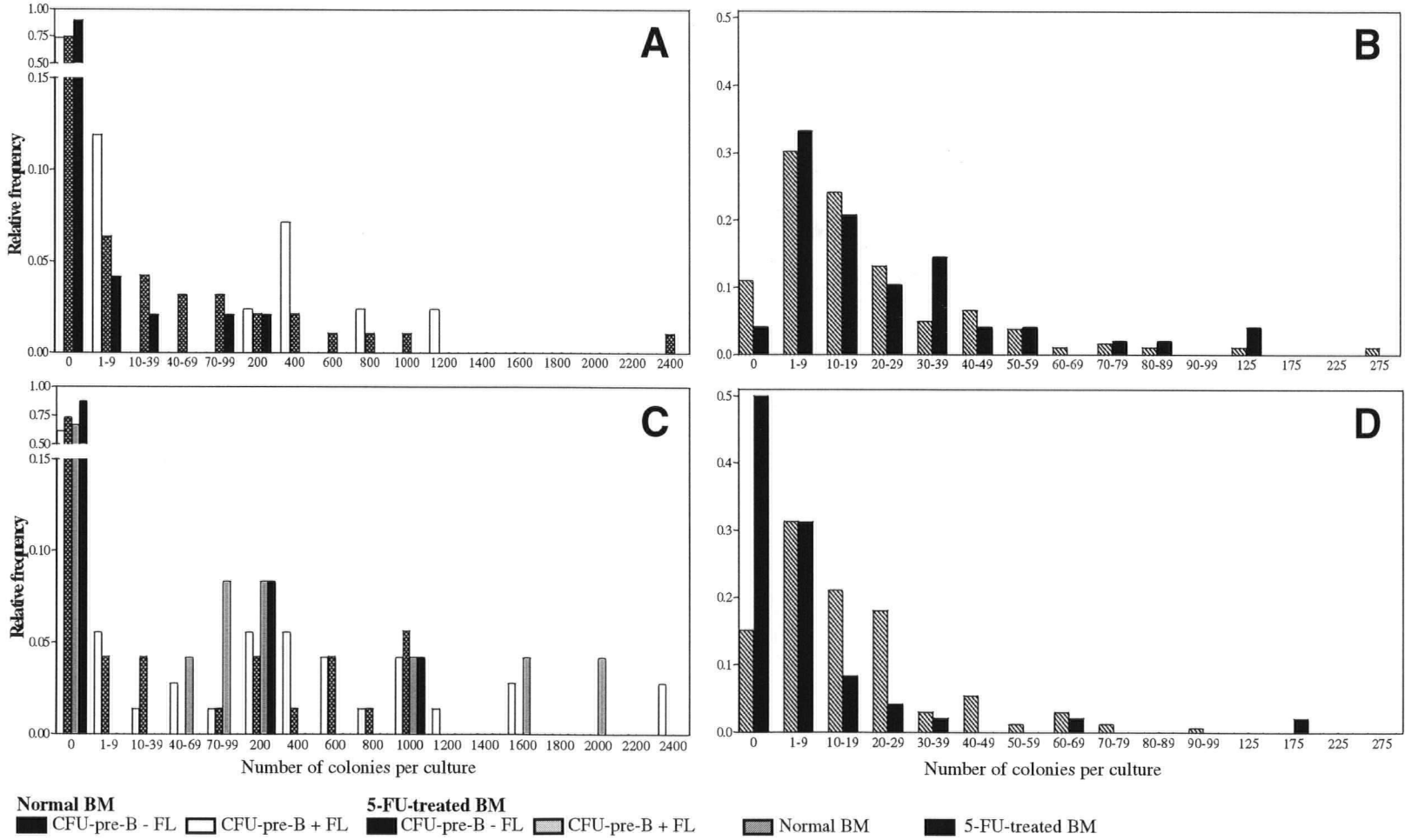

A
B
C
D
Relative frequency
Relative frequency
0 1-9 10-39 40-69 70-99 200 400 600 800 1000 1200 1400 1600 1800 2000 2200 2400
0 1-9 10-19 20-29 30-39 40-49 50-59 60-69 70-79 80-89 90-99 125 175 225 275
Number of colonies per culture
Number of colonies per culture
Normal BM
CFU-pre-B - FL
CFU-pre-B + FL
5-FU-treated BM
CFU-pre-B - FL
CFU-pre-B + FL
Normal BM
5-FU-treated BM

Table 4.1 Descriptive statistics of LTC-IC_{ML} assay cultures initiated with $5x10^4$ marrow cells and maintained for 4 weeks under myeloid LTC conditions before being switched for the indicated period to lymphoid LTC conditions.

	7 day switch			10 day switch		
	CFU-pre-B - FL	CFU-pre-B + FL	Myeloid CFC	CFU-pre-B - FL	CFU-pre-B + FL	Myeloid CFC
Normal marrow						
average	69	82	20	108	242	16
median	0	0	12	0	0	10
std dev	287	236	30	265	525	18
SEM	30	36	3	32	62	2
n	94	42	136	71	72	143
5-FU marrow						
average	5		23	67	222	9
median	0		13	0	0	1
std dev	22		28	230	544	24
SEM	3		4	47	111	3
n	48		48	24	24	48

Results pooled from 3 independent experiments each for normal and 5-FU marrow.
n = number of cultures assayed

4.3.2 Effect of substituting S17 cells as feeders in the LTC-IC_{ML} assay

Since others had shown that S17 cells could support the reappearance of B lineage cells in bulk cultures (Collins and Dorshkind 1987) and also maintain long-term in vivo repopulating cells at close to input numbers for up to 3 weeks which is better than with irradiated marrow adherent layers (Wineman, et al 1993), it was of interest to determine whether S17 cells might also be superior to normal marrow feeders for the detection of LTC-IC_{ML}. However, when this was tested, it was found that the use of S17 cells as feeders gave the same LTC-IC_{ML} frequencies for both normal and 5-FU marrow cell suspensions as when feeders established from fresh marrow were used (Table 4.2). Moreover, as shown in Table 4.3, the lack of an increase in LTC-IC_{ML} plating efficiency on S17 feeders could not be attributed to a decline of their capacity to maintain CRU numbers as compared to either irradiated marrow feeders or a combination of SF (100ng/ml) and IL-6 (10 ng/ml) (Miura, et al 1993; Rebel, et al 1994).

These results suggest that although CRU and LTC-IC_{ML} in normal adult marrow may be the same cells, some of the attributes responsible for their detection in these two assays may be different and hence differently regulated. Such a concept already exists in the human system, where SF has been shown to be unnecessary for human LTC-IC maintenance or detection under LTC conditions, even though SF alone can maintain LTC-IC and, in combination with other factors, can effectively substitute for a suitable fibroblast-containing feeder in their detection (Sutherland, et al 1993). Furthermore, recent experiments with murine cells have confirmed that SF is not necessary for LTC-IC detection (Dougherty 1995) but that the in vivo detection of CRU in adult mouse marrow is dependent on SF stimulation (Miller, et al In Press). To test this hypothesis further, a series of additional experiments were performed to determine whether the number of cells detectable as CRU, LTC-IC, LTC-IC_{ML}, and day 12 CFU-S would change relative to one another when normal marrow cells were maintained in culture under different conditions. The results of these experiments are described below.

Table 4.2 Ability of S17 cells to substitute for primary marrow feeder layers in the standard LTC-IC_{ML} assay protocol.

	Normal marrow		5-FU marrow	
Feeder layer	Per femur *	Per 10^5 †	Per femur	Per 10^5
Primary marrow	40 (26 - 65)	0.23 ± 0.04	12 (6 - 24)	0.22 ± 0.06
S17 stromal cell line	48 (36 - 64)	0.30 ± 0.07	18 (15 - 22)	0.32 ± 0.05

* Number of LTC-IC_{ML} per femur (range)

† Number of LTC-IC_{ML} per 10^5 cells ± SEM

Table 4.3 Ability of S17 cells to support CRU in cultures of unseparated mouse marrow cells maintained for 1 week in myeloid LTC medium in comparison to cultures containing pre-established irradiated primary marrow feeders and stromal cell-free suspension cultures supplemented with SF and IL-6.

		Marrow feeder	S17 cell line	Suspension culture + IL-6 + SF *
Experiment	#1	33	117	7
	#2	31	30	4
	#3	15	27	7
Average ± S.D.		26 ± 10	58 ± 51	6 ± 2

Duplicate or triplicate T25 flasks with the indicated feeders or growth factors were seeded with $3x10^6$ total normal marrow cells. The flasks for each group were harvested 1 week later and their pooled adherent and non-adherent cells assessed for the presence of CRU as described in the Methods.

* Myeloid LTC medium was supplemented with IL-6 (10 ng/ml) and SF (50 ng/ml) at the initiation of the cultures.

4.3.3 Differential maintenance in vitro of various functional attributes of primitive hematopoietic cells

As can be seen in Figure 4.2, the number of CRU in unseparated suspensions of normal adult marrow decreased ~10-fold over the course of 3-4 weeks when the cells were kept under myeloid LTC conditions and a further 10-fold over the next 3-4 weeks (data not shown). This rate of decline in repopulating potential is consistent with previous findings (Fraser, et al 1992; van der Sluijs, et al 1993). Interestingly, in the same experiments CFU-S, LTC-IC and LTC-IC_{ML} were all found to decline less rapidly (i.e., by 2, 4 and 1.5-fold, respectively, after 3-4 weeks). Maintenance of the same input cells on the same feeders but in medium permissive for pre-B cell production consistently provided poorer support for each of these cell types, which all decreased to $\leq$3% of input levels by 3-4 weeks.

4.3.4 Kinetics of CFU-pre-B disappearance under myeloid LTC conditions and their rate of reappearance after switching to lymphoid LTC conditions

In the previous chapter, we showed that cells capable of maintaining long-term B lymphopoiesis in culture were highly sensitive to and hence rapidly killed by exposure to corticosteroids and possibly other factors present in horse serum. This suggested that all of the CFU-pre-B detected in the LTC-IC_{ML} assay might have to be produced by lympho-myeloid progenitors still present at the time of the switch to lymphoid LTC conditions. Since such cells clearly decline several fold during the initial 4 weeks of culture under myeloid LTC conditions (Figure 4.2), it seemed likely that an increased number of LTC-IC_{ML} would be detected by switching to lymphoid conditions sooner. To determine how early this might be done, fresh marrow cells were cultured for an initial 4 or 7 days under myeloid LTC conditions and the cultures were then switched to lymphoid LTC conditions for a further 7 to 21 days. At the time of the switch, and at weekly intervals thereafter, duplicate cultures were harvested and assayed

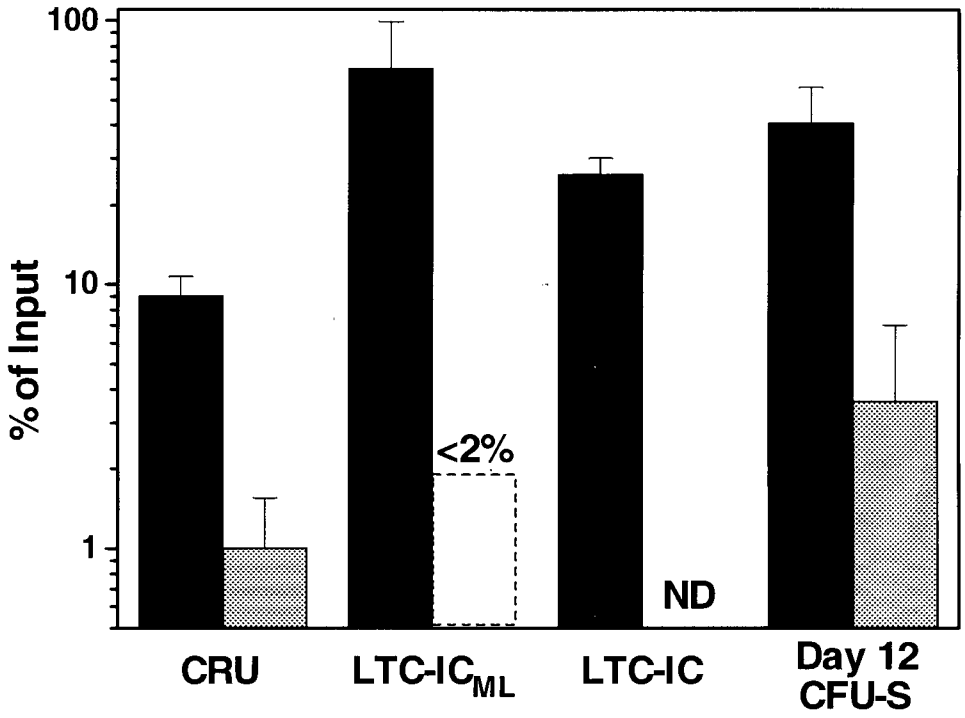
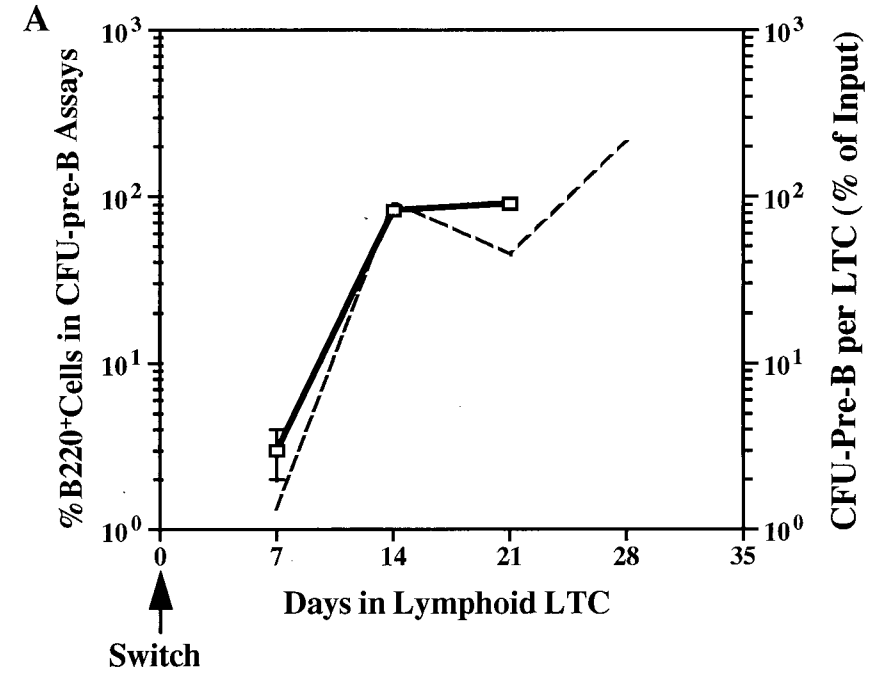
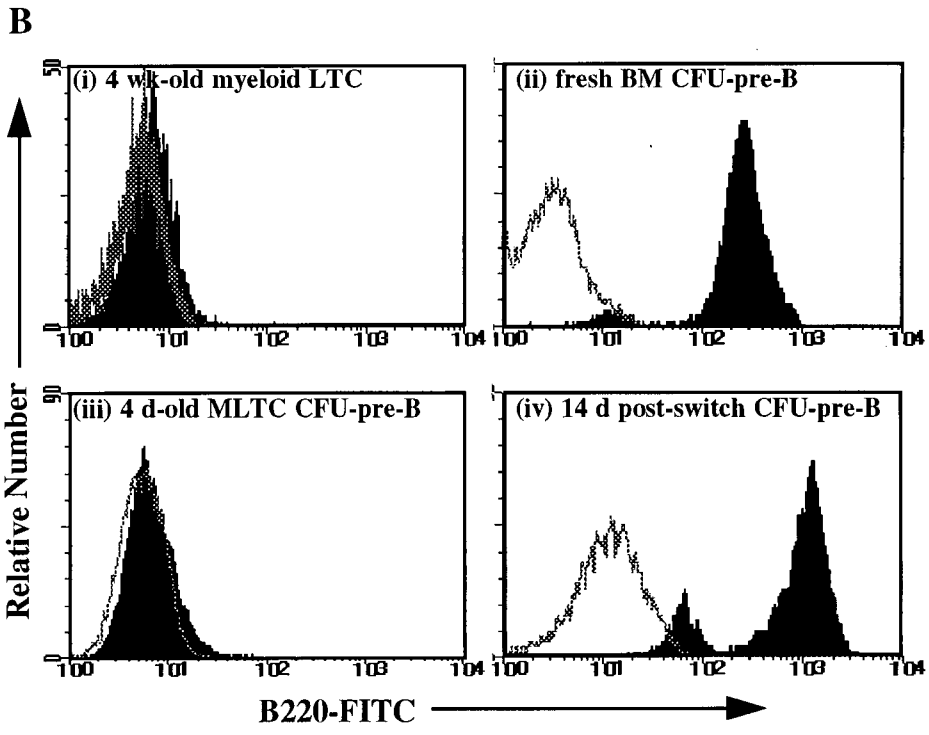


Figure 4.2 Relative maintenance of early hematopoietic progenitors for 3-4 weeks under myeloid (■) or lymphoid (▨) LTC conditions.

for CFU-pre-B. The presence of lymphoid cells in the resulting colonies was verified by recovering all the cells from the methylcellulose assay cultures and examining their surface expression of B220 by flow cytometry. The lower panel of Figure 4.3 shows representative profiles of the cells recovered from one such experiment. We had previously shown that well established, 8 week-old lymphoid LTC permanently lose the ability to generate CFU-pre-B within 7 days of being switched to myeloid LTC conditions (Lemieux, et al 1995). The results shown in the upper panel of Figure 4.3 indicate that all CFU-pre-B disappear within 4 days of their being placed under myeloid LTC conditions (day 0 on the graph) and then reappear with exactly the same kinetics as previously shown for marrow cells maintained for ≥4 weeks under myeloid LTC conditions (Dorshkind 1986; Denis and Witte 1986; Lemieux, et al 1995).

Figure 4.3 (A) Kinetics of reappearance of $B220^+$ lymphoid cells in short-term myeloid cultures after being switched to lymphoid culture conditions. Normal marrow cells (10^6 per 35 mm tissue culture dish) were maintained in myeloid cultures for 4 (□) or 7 days (not shown since indistinguishable from 4 days) and then switched (day 0 on the X-axis) to lymphoid LTC conditions for a further 7 to 21 days. At each time point, duplicate cultures were harvested and plated individually in 10 ml of CFU-pre-B assay mixture per 60 mm non-tissue culture dish. Seven days later, the entire contents of each assay dish was evaluated for the presence of $B220^+$ cells as described in the Methods. The dashed line portrays the findings reported previously (Lemieux, et al 1995) for CFU-pre-B numbers in cultures maintained under myeloid LTC for 4 weeks prior to switching to lymphoid LTC conditions. (B) B220 staining profiles of cells from 4 week-old (i) myeloid LTC and cells from CFU-pre-B assays of either fresh marrow (ii) or cells from 4 day-old myeloid LTC before (iii) and after (iv) switching to lymphoid culture conditions for 14 days. Unstained controls are shown as outlines.


A
%B220+Cells in CFU-pre-B Assays
CFU-Pre-B per LTC (% of Input)
Days in Lymphoid LTC
Switch


B
(i) 4 wk-old myeloid LTC
(ii) fresh BM CFU-pre-B
(iii) 4 d-old MLTC CFU-pre-B
(iv) 14 d post-switch CFU-pre-B
Relative Number
B220-FITC

4.3.5 Evidence for a novel corticosteroid-resistant, lymphoid-restricted progenitor cell type

We then tested the possibility of using a 7 day rather than a 4 week initial myeloid culture period and a 10 rather than 7 day secondary phase under lymphoid culture conditions for the quantitation of LTC-IC_{ML} by limiting dilution analysis. The results of 2 experiments with marrow cells from normal mice and 4 experiments with marrow from 5-FU-treated mice are shown in Table 4.4. For normal marrow, not all of the rare wells that contained both CFU-pre-B and myeloid CFC could be explained by coincident plating of a lymphoid and a myeloid-restricted precursor in the original input population ($p = 0.033$, Fisher's Exact test). In the case of 5-FU marrow, the number of wells containing both lymphoid and myeloid clonogenic cells was too small to exclude the possibility of coincidence ($p = 0.16$). The presence of cultures containing only CFU-pre-B at a frequency equal to that of cultures containing only myeloid CFC was unanticipated since all cultures initiated with limiting numbers of adult marrow cells that contained CFU-pre-B were also found to contain myeloid CFC in standard 5 week LTC-IC_{ML} assays (Lemieux, et al 1995). Moreover, the fact that cultures containing only CFU-pre-B were seen in the present experiments using the shortened LTC-IC_{ML} assay protocol, regardless of whether the 5000 input cells were from normal or 5-FU-treated mice indicates that the lymphoid-restricted progenitors thus detectable are, like CRU and LTC-IC, relatively resistant to 5-FU. To our knowledge, the identification in normal adult mouse marrow of a lymphoid-restricted, 5-FU-resistant population of progenitors that can survive under myeloid LTC conditions for 7 days, but not 4 weeks, has not previously been reported. In the context of the present study, the existence of such cells detracts significantly from the advantages of using a shorter LTC-IC_{ML}. Even though LTC-IC_{ML} plating efficiency could be enhanced, their distinction from lymphoid and myeloid-restricted progenitors can only be made when very low input cell numbers are used such that ≤10-30% of cultures contain clonogenic cells of any type. The calculated LTC-IC_{ML} frequency for normal marrow derived from the percentage of negative cultures, adjusted to exclude those expected to be due to coincidence, is 1 in 1.7×10^5

Table 4.4 Frequencies of myeloid and lymphoid progenitors in cultures maintained for 1 week under myeloid LTC conditions and then for 10 days under lymphoid LTC conditions: Evidence for a 5-FU and steroid-resistant, lymphoid-restricted precursor of CFU-pre-B.

Clonogenic cells present	Normal BM		5-FU BM	
None	120/171	(70%)	90/112	(80%)
Myeloid	29/171	(17%)	19/112	(17%)
Lymphoid	32/171	(19%)	5/112	(4%)
Both myeloid and lymphoid	10/171	(6%)	2/112	(2%)
Expected coincidence of lymphoid and myeloid	5/171	(3%)	0.8/112	(0.7%)

BM cells were seeded onto pre-established, irradiated marrow feeders at 5000 cells/well in 96-well plates and maintained under myeloid LTC conditions for 7 days. These micro-LTC were then switched to lymphoid LTC conditions for 10 days before being harvested and assayed for their content of myeloid CFC and CFU-pre-B. Results for normal and 5-FU-treated marrow have been pooled from 2 and 4 experiments, respectively. For normal marrow, the calculated frequency of cultures expected to contain both myeloid and lymphoid clonogenic cells due to coincidence is significantly lower than the observed values ($p = 0.03$). For 5-FU marrow, coincidence could not be excluded ($p = 0.16$). The frequency of LTC-IC_{ML} calculated from the corrected values are 1 in 1.7×10^5 normal marrow cells and 1 in 5.6×10^5 5-FU-treated marrow cells.

cells. This is ~3 times higher than the value obtained using the original 5 week assay (1 in $4.3x10^5$, Table 4.2, see also Chapter III) and only 3-fold lower than the frequency of CRU in 1 week-old myeloid LTC (1 in $5.4x10^4$ cells). Such findings are consistent with the hypothesis that the lower frequency of LTC-IC_{ML} measured in the 5 week assay is largely explained by their decline during the first 4 weeks of the assay. On the other hand, the frequency of LTC-IC_{ML} calculated for 5-FU marrow in the shortened (17 day) LTC-IC_{ML} assay was 1 in $5.6x10^5$ cells which is not significantly different from the frequency of 1 in $4.5x10^5$ cells obtained in the 5 week LTC-IC_{ML} assay (Table 4.2) and is ~20-fold lower than the frequency of CRU in freshly isolated 5-FU marrow. These data suggest that the LTC-IC_{ML} that are sensitive to 5-FU are also poorly maintained in culture. It also appears that the LTC-IC_{ML} which survive 5-FU treatment decline more rapidly in culture.

4.4 Discussion

Much effort has gone into the development of assays which specifically detect the ability of individual hematopoietic cells to give rise to both lymphoid and myeloid progeny for extended periods of time. The first quantitative assay for very primitive progenitors, the CFU-S assay (Till and McCulloch 1961), has now been shown to detect some cells with lympho-myeloid potential (Lepault, et al 1993), although it is clearly not specific for such cells (Visser and van Bekkum 1990). The CRU assay has this specificity (Fraser, et al 1990; Szilvassy, et al 1990; Szilvassy and Cory 1993; Rebel, et al 1994) but does not lend itself to the direct investigation of mechanisms mediating self-renewal or commitment events in recently stimulated cells. Methods for detecting the lympho-myeloid potential of primitive murine hematopoietic progenitors stimulated to form colonies in methylcellulose cultures have also been described very recently (Hirayama, et al 1992; Ball, et al 1995), but they require highly purified cell suspensions as starting material. LTC-based assays, such as those that measure cobblestone areas (Ploemacher, et al 1989; Breems, et al 1994) and LTC-IC (Sutherland, et al

1990; Lemieux, et al 1995) are more generally applicable but only the LTC-IC_{ML} assay directly monitors lymphoid as well as myeloid potential. Unfortunately, the very low frequency of cells detected by the originally described LTC-IC_{ML} assay, as compared to LTC-IC and CRU, also constrain its applicability.

In the present study, we found that the numbers of cells detectable as LTC-IC and LTC-IC_{ML} were both better maintained over a period of 3-4 weeks in myeloid LTC than the number of cells detectable as CRU. The differential maintenance of these properties in the LTC system suggests that the ability to generate both myeloid and B lymphoid clonogenic progenitors for extended periods in vitro in response to stromal cells and the ability to reconstitute myeloablated recipients in response to signals produced in such animals involve different regulatory mechanisms. This extends previous findings from studies with W-mutant mice which showed that close to normal numbers of LTC-IC/LTC-IC_{ML} are present in adult W^{41}/W^{41} mice whereas CRU numbers appear markedly reduced to ~20-fold lower levels than normal (see Appendix Tables 2 and 3). Thus SF appears to be crucial for the detection of CRU in vivo but not for their in vivo generation nor for the detection in vitro of LTC-IC or LTC-IC_{ML}.

Previous studies have suggested that lymphoid LTC conditions support lymphopoiesis exclusively (reviewed by Dorshkind and Witte, (Dorshkind and Witte 1987)). However, these studies were not sufficient to exclude the possibility that cells with lympho-myeloid potential might persist in these cultures and be able to subsequently produce myeloid progeny under appropriate conditions. Our data show that CRU numbers decline 100-fold during the first 3-4 weeks of lymphoid culture and, as anticipated, are undetectable by 6-8 weeks. Myeloid LTC, on the other hand, have been shown to contain readily detectable numbers of in vivo lympho-myeloid repopulating cells for at least 5 weeks (Fraser, et al 1990). It has also been reported that myeloid LTC selectively maintain high levels of transplantable, lymphoid-restricted stem cells capable of curing severe combined immunodeficiency (SCID) mice of their B and T cell

deficiency (Fulop and Phillips 1989). However, experiments using retrovirally-marked cells from myeloid LTC to reconstitute lethally irradiated $B6C3F_1$ mice did not reveal a preponderance of lymphoid-restricted reconstitution (Fraser, et al 1990) although examples of such cells were seen. In vitro, we have shown that the ability to generate CFU-pre-B in switched 4 week-old myeloid cultures is tightly coupled to an ability to also produce myeloid CFC (Lemieux, et al 1995). However, in the present studies, a lymphoid-restricted cell type which persists for ≥ 7 days in myeloid LTC was identified. This lymphoid precursor is thus distinguishable from and probably more primitive than any stage of B lymphoid development described to date, including CFU-B, Abelson murine leukemia virus targets, CFU-pre-B, and cells capable of sustained CFU-pre-B production in lymphoid LTC, all of which disappear within 4-7 days in myeloid LTC (Rosenberg and Baltimore 1976; Phillips, et al 1984; Lemieux, et al 1995). Whether this cell might also possess T lymphopoietic potential is not yet known.

A differential loss under the same culture conditions of the ability to be detected in vivo as CRU or in vitro as LTC-IC/LTC-IC_{ML}, which we presume characterizes a single cell type generated in vivo, presumably reflects corresponding differences in the types of exogenous factors that can regulate the expression of these functions. The LTC system, in which cytokine production by feeders can be manipulated by genetic (Zipori and Lee 1988; Corey, et al 1990; Hogge, et al 1991; Otsuka, et al 1991; Sutherland, et al 1991) or immunological strategies (Cashman, et al 1990), offers an attractive model for identifying the molecular nature of these differences. Similarly, this model provides a unique opportunity for further examination of the mechanisms responsible for the differential maintenance of responsiveness to regulatory factors.

V. FL and IL-11 differ in their ability to support the stromal cell-independent development and expansion of $B220^+$ lymphocytes from purified $B220^-$ fetal liver progenitors

5.1 Summary

In the previous chapter, we found that the presence of FL in CFU-pre-B methylcellulose cultures performed at the end of standard 5 week LTC-IC_{ML} assays of adult marrow cells did not enhance the detection of LTC-IC_{ML} although its addition did slightly increase the average number of CFU-pre-B produced per culture. The results of previous chapters suggest that CFU-pre-B can develop from lympho-myeloid progenitors present in myeloid LTCs, presumably in response to changes in the production of stromal cell-derived factors resulting from the switch from myeloid to lymphoid culture conditions. In the present chapter, experiments were undertaken to determine whether FL and IL-11, both of which are normally produced by stromal cells (Paul, et al 1990; Hannum, et al 1994), might mimic the ability of these cells to support the differentiation of B lineage cells from very primitive precursors of CFU-pre-B in vitro. Sca-1^+ lineage-depleted ($B220^-$) cells were isolated from day 14.5 murine fetal livers to obtain a test population that was highly enriched in HSC as measured by both CRU and LTC-IC assays (Rebel, et al In Press; C. Miller, personal communication). In addition, similarly enriched fetal liver cell populations had been shown to proliferate in response to FL and IL-11 (Lyman, et al 1993; Hannum, et al 1994; Kee, et al 1994). These cells were incubated for 1-2 weeks in stromal cell-free suspension cultures supplemented with all possible combinations of FL, IL-11, SF and IL-7 since previous studies had shown that SF and IL-7 can stimulate the proliferation of early $B220^+$ lymphocytes but cannot, either alone or together, support the development of such cells from $B220^-$ precursors. The results of these experiments indicated that $B220^+$ cells could be detected by day 7 in cultures containing FL, SF and IL-7 and, by day 14, the total number of cells was expanded up to 6000-fold. Eighty to 100% of the cells produced under these conditions were $B220^+$ and

~50% of these exhibited a pro-B phenotype ($B220^+CD43^+$). In serum-free cultures, ~10% of the cells were Mac-1^+ or Gr-1^+ but in serum-containing cultures, the production of myeloid cells appeared to be greatly reduced. In contrast, fewer $B220^+$ cells were produced, both relatively and absolutely when IL-11 was substituted for FL. In addition, ~10-30X more myeloid cells were generated in cultures containing IL-11, SF and IL-7 than with FL, SF and IL-7, both in the presence and in the absence of FCS. Thus although both FL and IL-11 can support the differentiation of primitive $B220^-$ progenitors and the amplification of their $B220^+$ progeny, their activities do not appear to be identical.

5.2 Introduction

The commitment of HSC to the B lymphoid developmental pathway occurs continuously throughout adult life in the sinusoidal spaces of the bone marrow (Hermans, et al 1989; Jacobsen and Osmond 1990). The molecular mechanisms that support this process, however, remain poorly described. IL-7 can stimulate the development of pre-B cells in vitro (Namen, et al 1988; Lee, et al 1989; Suda, et al 1989; Williams, et al 1990) and it appears to be a limiting regulator of pre-B cell generation in vivo (Peschon, et al 1994; von Freeden-Jeffry, et al 1995). However, IL-7 is probably not necessary for the generation or maintenance of cells with lympho-myeloid differentiation potential. For example, in the absence of IL-7, SF in combination with IL-11, IL-6 or G-CSF can support the production in vitro of clonal cultures of blast cells that can give rise to secondary colonies of pre-B cells upon replating into a culture medium that contains SF and IL-7 (Hirayama, et al 1992). The combination of SF, IL-7 and IL-11 has also been recently identified as sufficient to support the development of B lineage cells from lympho-myeloid progenitors purified from day 12 fetal livers (Kee, et al 1994). However, the fact that W mice deficient for the SF receptor have normal numbers of CFU-pre-B, pre-B and B cells (Mekori and Phillips 1969; Landreth, et al 1984; Miller, et al In Press)

argues that SF is also not a limiting regulator of pre-B cell generation in vivo, or its effects can be compensated for by the activities of other factors.

A cytokine which might be anticipated to possess such activity is the recently cloned ligand for the Flt3 tyrosine kinase receptor (FL) (Lyman, et al 1993; Hannum, et al 1994). Murine Flt3 was cloned from fetal liver cells highly enriched for HSC. It belongs to a subfamily of cell surface receptors characterized by their split intracytoplasmic tyrosine kinase domains. Other receptors in this family include c-kit (the receptor for SF), c-fms (the receptor for M-CSF) and the platelet-derived growth factor receptor (Matthews, et al 1991; Rosnet, et al 1991). Mice that are deficient for Flt3 by gene knock-out exhibit some deficiencies in their B and T lymphocyte progenitors which became more marked after their transplantation into irradiated recipients or when combined with a W/W^v genotype (Mackarehtschian, et al 1995). FL, like SF, is widely expressed in murine and human tissues and is produced by most stromal cell lines thus far examined (Hannum, et al 1994).

In this report it is shown that FL in combination with SF and IL-7 can support the development of $B220^+$ lymphocytes in suspension cultures of Sca-1^+B220^- day 14.5 fetal liver cells. Furthermore, as anticipated from previous studies of W/W^v mice, the ability to respond to SF in vitro was not absolutely required for the production of either $B220^+$ or myeloid (Mac-1^+ or Gr-1^+) cells nor did its addition increase the production of either of these cell types in cultures supplemented with IL-7, IL-11 and FL. We also show that FL does not promote the production of myeloid cells from these same primitive progenitor cells as effectively as IL-11. Thus, although the activities of these two stromal cell-derived factors overlap they do not appear to be identical.

5.3 Results

A population of hematopoietic cells depleted of myelo-monocytic (Gr-1$^+$), erythroid (Ter119$^+$), T lymphoid (Ly-1$^+$) and B lymphoid (B220$^+$) cells (so-called Lin$^-$ cells) and enriched for very early progenitor cells (Sca-1$^+$) was isolated from day 14.5 fetal liver cell suspensions and cultured as described in the Methods. The rationale for using this population as the starting material was three-fold: (i) it contains few, if any, B lymphoid-restricted progenitors; (ii) it is highly enriched for cells with unrestricted differentiative potential; and (iii) by enriching for early progenitors, cultures could be seeded with very small numbers of cells, thereby minimizing potential cell density-related effects, at least initially.

5.3.1 FL synergizes with IL-7 and SF in the production of B220$^+$ lymphocytes from B220$^-$ progenitors

As shown in Table 5.1, the number of cells produced per 100 Sca-1$^+$Lin$^-$ fetal liver cells seeded 14 days earlier in stromal cell-free but FCS-containing liquid cultures supplemented with IL-7, SF and FL was at least 60-fold greater than the combined output of any pairwise combination of these factors. None of the cultures supplemented with a single factor contained any detectable viable cells after 7 days. Flow cytometric analyses of cultures containing IL-7+FL revealed mainly dead cells (93% PI$^+$) and only ~35% of the viable cells expressed the B220 antigen. The ability of cells to proliferate in the presence of IL-7 and FL was variable and may be a function of the precise gestational age of the fetuses used in different experiments. IL-7+SF+FL-supplemented cultures, on the other hand, consistently contained large numbers of B220$^+$ cells and only very low numbers of cells expressing the myelomonocytic markers Mac-1 (CD11b) or Gr-1 (Figures 5.1 and 5.2 (panel A)). To further characterize them, the cells produced in these cultures we additionally stained with an anti-CD43 antibody (S7) used by Hardy and his colleagues to subdivide early stages of B cell development (Hardy, et al 1991).As shown in Figures 5.2 (panel C) and 5.3, approximately

Table 5.1 Cell production from 100 Sca-1$^+$Lin$^-$ cells incubated for 14 days in stromal cell-free suspension cultures in medium containing FCS and the growth factors indicated.

Growth factors added	Total cells	B220$^+$	(Mac-1/Gr-1)$^+$
IL-7+SF	85 ± 85	na	na
SF+FL	0 ± 0	na	na
IL-7+FL	(4.3 ± 3.6) x 10^3	5.6 x 10^2 †	na
IL-7+SF+FL	(2.7 ± 1.0) x 10^5	(2.3 ±1.0) x 10^5	(6.3 ± 3.2) x 10^2
SF+IL-11	(1.2 ± 0.0) x 10^4	- *	+ *
IL-7+IL-11	0 ± 0	na	na
IL-7+SF+IL-11	(5.2 ± 2.7) x 10^4	(1.1 ± 0.3) x 10^4	(1.9 ± 1.1) x 10^4
FL+IL-11	(7.4 ± 5.9) x 10^4	-	+
IL-7+FL+IL-11	(1.5 ± 0.4) x 10^6	(1.2 ± 0.4) x 10^6	(2.0 ± 0.7) x 10^5

† Only 1 experiment assessible by FACS

* Presence or absence on May-Grünwald-Giemsa-stained cytospins. Lymphocytes are quite fragile and may be suboptimally detected on cytospins.

na= not assessed

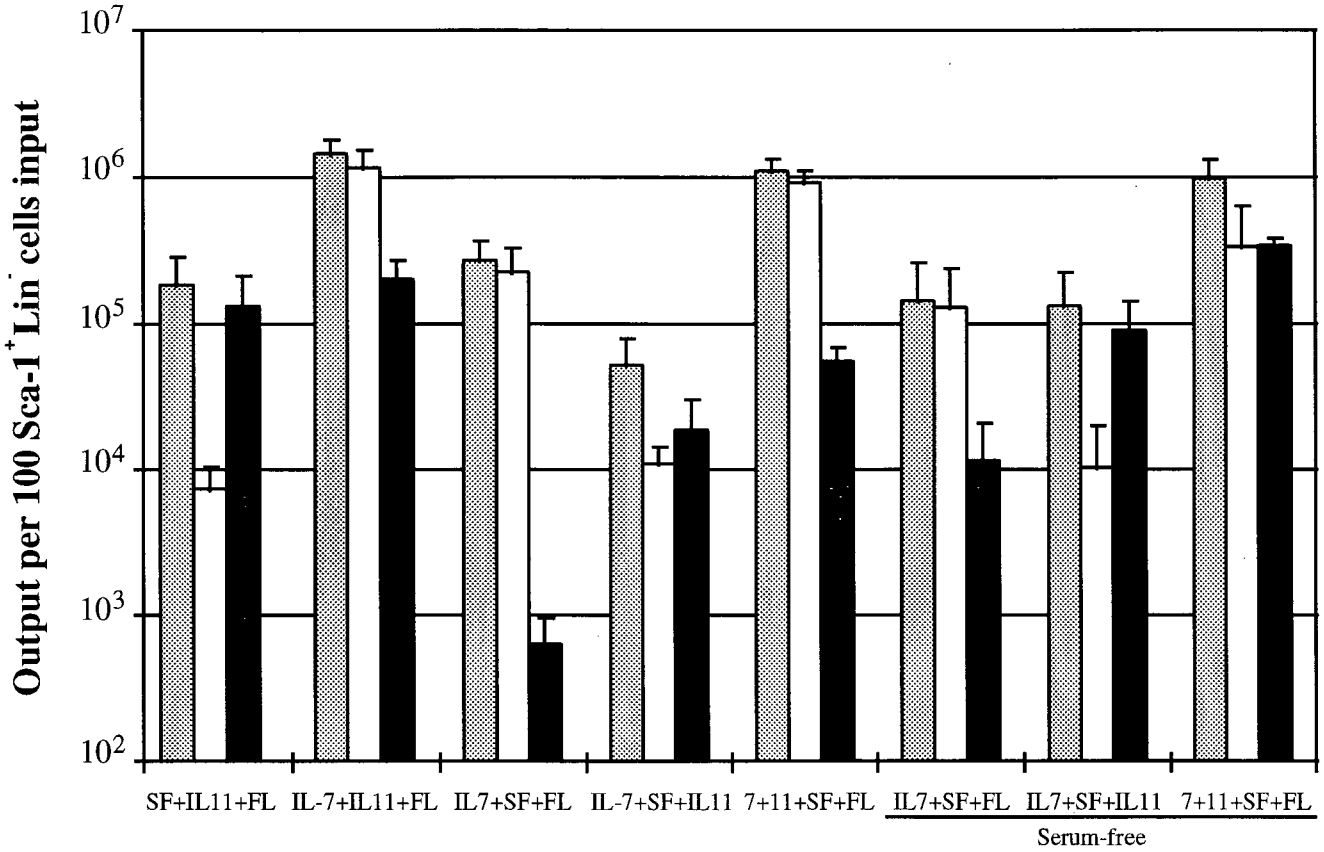


Figure 5.1 Total (▨), B220⁺ (□), and (Mac-1 or Gr-1)⁺ (■) cell output per 100 Sca-1⁺ Lin⁻ day 14.5 fetal liver cells maintained for 14 days in suspension cultures supplemented with the growth factors indicated.

50% were less mature, $CD43^+B220^+$ pro-B cells and 50% were more mature, $CD43^-B220^+$ pre-B cells.

5.3.2 FL synergizes with IL-7 and IL-11 to support the production of $B220^+$ lymphocytes from $B220^-$ progenitors

As can be seen from Figure 5.1, the largest numbers of $B220^+$ cells were produced in 14 day-old cultures containing IL-7, FL and IL-11. The addition of SF to this combination did not further enhance the production of these cells despite its demonstrated ability to synergize with both IL-7+FL and IL-7+IL-11 in the generation of $B220^+$ cells (Table 5.1). In fact, substituting SF for any of the other three factors resulted in an overall reduction in the number of $B220^+$ cells produced. This effect was particularly marked when IL-7 (130-fold) or FL (100-fold) was omitted, suggesting an essential role for both FL and IL-7 in the optimal production and amplification of $B220^+$ cells from a $Sca\text{-}1^+Lin^-$ ($B220^-$) phenotype. Interestingly, as shown in Figure 5.3, the proportion of $B220^+CD43^-$ (pre-B) cells relative to $B220^+CD43^+$ (pro-B) cells produced in the absence of either SF or IL-7 was increased relative to cultures in which both of these factors were present from 1:1 to 10:1 and 5:1, respectively. This may be related to the fact that early pre-B cell lines and clones are reportedly induced to undergo V_H to DJ_H and light chain locus rearrangements upon withdrawal of IL-7 or stromal cell support (Rolink, et al 1991).

5.3.3 Evidence that the activities of FL and IL-11 in myelo- and lymphopoiesis are overlapping but not identical

It is also apparent from Figure 5.1 that FL is a more potent factor than IL-11 in supporting the production of $B220^+$ lymphocytes from $Sca\text{-}1^+B220^-$ cells and that the combined presence of FL and IL-11 in cultures that also contained IL-7+SF had a synergistic

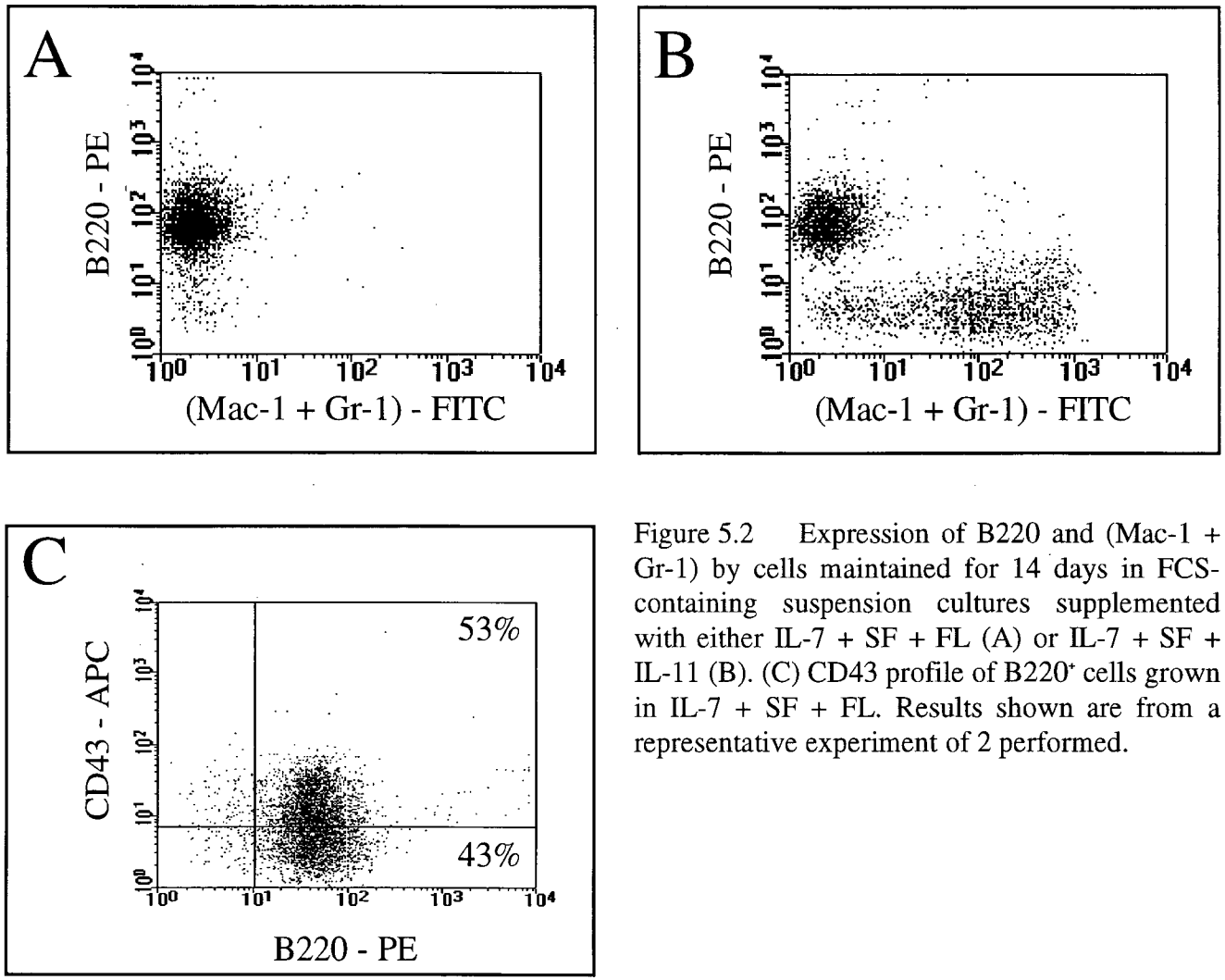


Figure 5.2 Expression of B220 and (Mac-1 + Gr-1) by cells maintained for 14 days in FCS-containing suspension cultures supplemented with either IL-7 + SF + FL (A) or IL-7 + SF + IL-11 (B). (C) CD43 profile of $B220^+$ cells grown in IL-7 + SF + FL. Results shown are from a representative experiment of 2 performed.

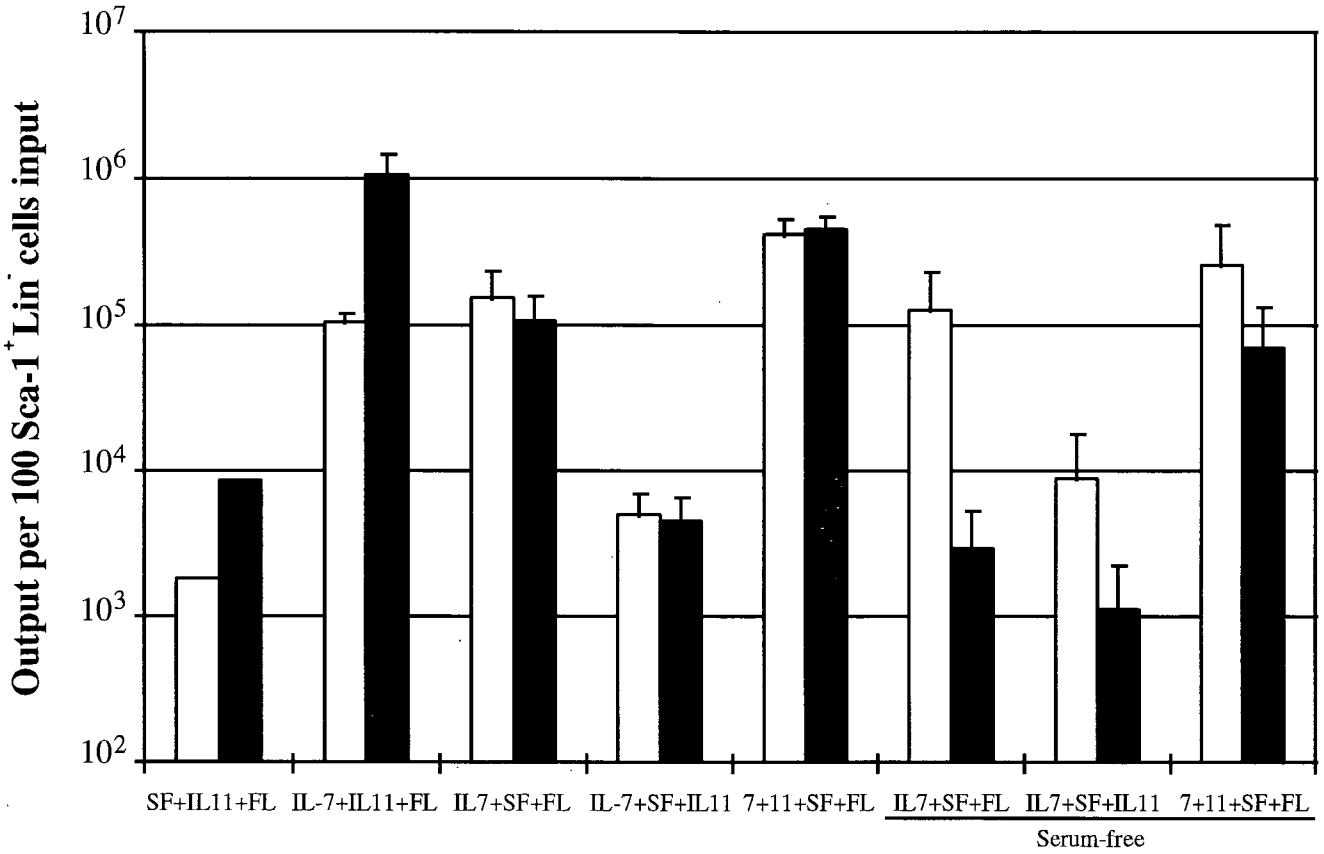


Figure 5.3 CD43 profile (□ = $CD43^+$; ■ = $CD43^-$) of $B220^+$ cells produced in suspension cultures supplemented with the growth factors indicated.

effect on this process, giving a 4-fold increase in $B220^+$ cells in the 4 factor combination over the sum of those obtained with IL-7+SF+FL and IL-7+SF+IL-11. FL was also found to have a greater synergizing effect with IL-7 on $B220^+$ cell production (35-fold more $B220^+$ cells with IL-7+SF+IL-11+FL than with SF+IL-11+FL and SF+IL-11+IL-7 combined) than did IL-11 (only 4-fold more $B220^+$ cells with all 4 factors than the sum of SF+FL+IL-11 and FL+SF+IL-7 combined). Furthermore, as can be seen in panels A and C of Figure 5.2, very few Mac-1^+ or Gr-1^+ cells and approximately equal numbers of $B220^+$ pro- and pre-B cells were produced in bulk cultures containing IL-7+SF+FL-supplemented medium. In contrast, in cultures containing IL-7+SF+IL-11 (panel B), a substantial population of myeloid cells (~30% of the total) were produced in addition to $B220^+$ cells. The unique ability of IL-11 to stimulate the extensive production of myeloid cells from Sca-1^+Lin$^-$ precursors is also documented in Figure 5.1. Interestingly, the presence of FCS appeared to have a strongly inhibitory effect on myelopoiesis in these cultures. Thus the number of myeloid cells produced in the absence of IL-11 was ~18-fold higher in serum-free cultures and even in the presence of IL-11 was 6-fold higher. In spite of this effect of FCS on the generation of myeloid cells, the overall number of $B220^+$ cells produced in the absence of IL-11 was not significantly affected by the use of a defined serum substitute although the relative proportion of $B220^+CD43^+$ to $B220^+CD43^-$ cells was altered (Figure 5.3).

5.4 Discussion

Previous studies of purified Sca-1^+Lin$^-$ cells isolated from the marrow normal adult mice growing in methylcellulose cultures have shown that ~5% of these cells can give rise to colonies containing a mixture of B and myeloid lineage cells when exposed to the combination of IL-7, SF, IL-11 and erythropoietin (Ball, et al 1995). FL, in synergy with IL-6, IL-11 or G-CSF, has also been shown to support the proliferation of lympho-myeloid progenitors and,

alone or in combination with SF or IL-7, of B cell progenitors present in this Sca-1$^+$Lin$^-$ population (Hirayama, et al 1995). The results presented here and those of others (Kee, et al 1994) indicate that these factors are also involved in the earliest stages of fetal B cell development. However, although the activities of IL-11, SF and FL overlap significantly, they also show differential effectiveness in promoting the production of cells at different stages of myeloid and lymphoid development. A proposed model of how this may occur is shown in Figure 5.4. This model incorporates the principle that additive or synergistic interactions of factors which individually have modest effects on cells of a particular developmental pathway can partially or completely compensate for the absence of a more dominant factor. In support of this model, the results obtained in the present studies show FL to be a very potent but not exclusive stimulator of B220$^+$ cell production from B220$^-$ progenitors. Thus, this process can also occur, albeit much less efficiently, in its absence if an appropriate combination of other factors is present (e.g., IL-7+SF+IL-11). The relatively mild impairment of lymphoid and stem cell development in Flt3 knock-out mice and the more severe hematological defects that have been identified in W/W^v Flt3 -/- animals (Mackarehtschian, et al 1995) are also consistent with such a model.

According to such a model of primitive hematopoietic cell regulation, under conditions where positively-acting growth factors are limiting, the presence of negatively-acting factors can exert marked effects. In the experiments described here, FL became as effective as IL-11 in stimulating the generation of myeloid cells from Sca-1$^+$Lin$^-$ precursors in the presence of SF+IL-7 when the FCS was replaced with BSA, transferrin, insulin and lipids. This suggests that some component of the FCS used in these experiments was inhibitory for early stages of myelopoietic cell development but that this could be partially offset by the presence of IL-11 but not FL.

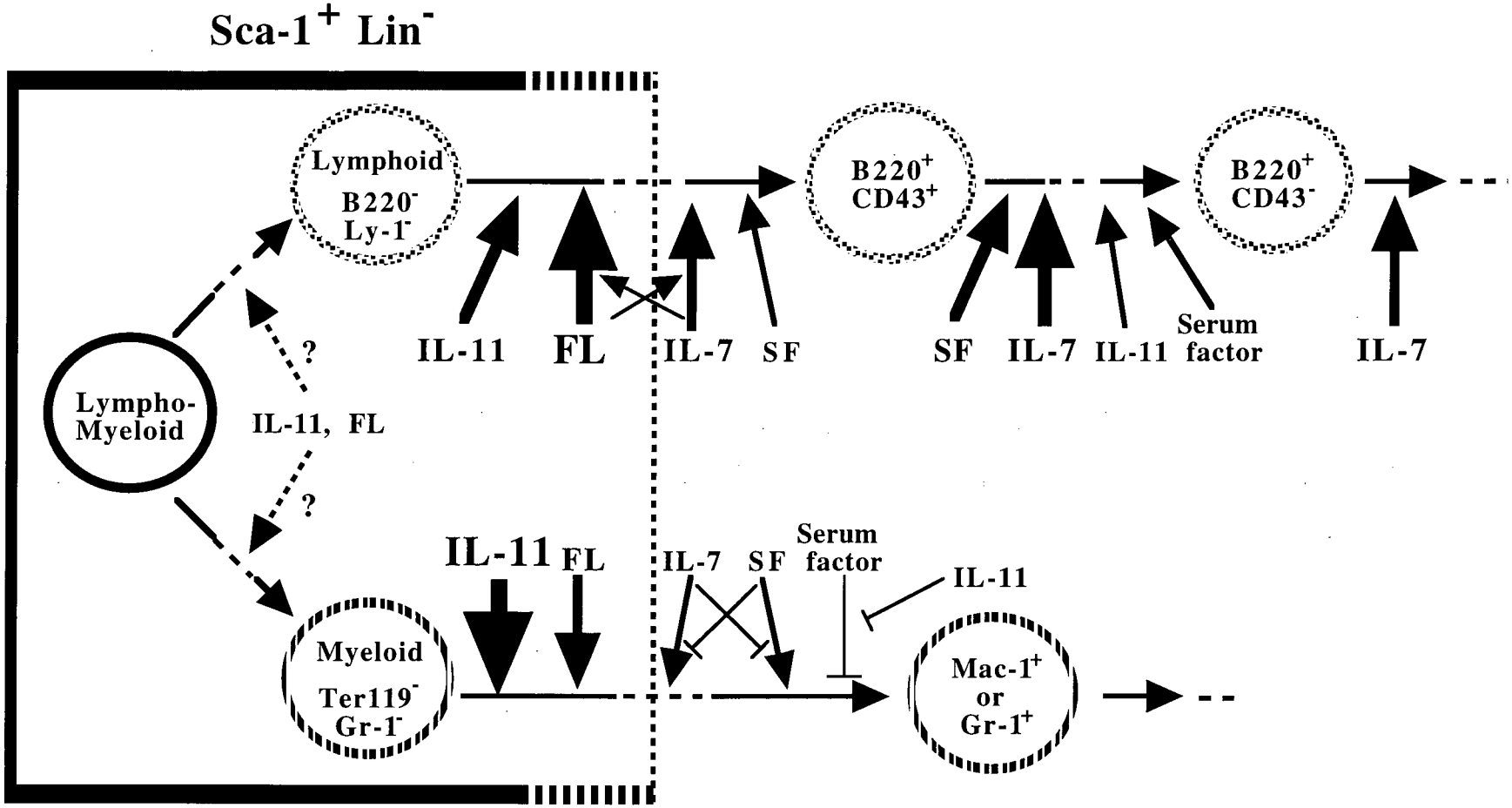


Figure 5.4 Proposed model of the interactions of IL-7, IL-11, SF and FL effects in promoting the development of early B lymphoid and myeloid cells from pluripotent progenitors. The size of the arrows and characters represents the relative effect of each factor on the transition indicated. Crossed arrows indicate synergy and crossed mallets represent inhibition.

In summary, both IL-11 and FL, in combination with IL-7 and SF, were found to support the development of $B220^+$ lymphocytes from highly purified populations of primitive, $B220^-$ hematopoietic progenitor cells obtained from mouse fetal liver and cultured in the absence of a stromal cell feeder layer. Since both these factors have previously been found to support the development of B lymphoid cells (Figure 5.1 and (Kee, et al 1994; Musashi, et al 1991; Ball, et al 1995; Hirayama, et al 1995)) they might also allow the development of B lineage cells from HSC produced or persisting in LTC to proceed in fully defined culture media. However, since these studies have all made use of highly purified starting cell populations, it remains to be seen whether these factors will be sufficient to obtain similar results from more heterogeneous cell suspensions before and after their manipulation in LTC.

VI. Conclusions

As a first step towards studying HSC regulation, a new in vitro method for specifically identifying and quantitating lympho-myeloid cells in adult mouse bone marrow was developed. This assay relies on the joint expression of the myeloid and lymphoid differentiative potentials of individual LTC-IC maintained for 4 weeks under myeloid LTC conditions before being switched to lymphoid conditions. Analysis of the clonogenic cell content of LTC-IC_{ML} assay cultures initiated at limiting dilutions showed that the presence of CFU-pre-B was associated with that of myeloid CFC significantly more often than could be explained by the random co-plating of two populations of lymphoid and myeloid-restricted progenitors. Furthermore, cells able to produce CFU-pre-B in the LTC-IC_{ML} assay could not be separated from cells with myeloid differentiation potential, even in cultures initiated with as few as 35 cells from suspensions that were enriched more than 500-fold in their content of LTC-IC_{ML}. It was also found that neither CFU-pre-B nor lymphoid long-term culture-initiating cells were detectable after as little as one week of their exposure to myeloid LTC conditions. Taken together, these results provide strong evidence that the LTC-IC_{ML} assay detects individual cells with lympho-myeloid potential. It was also shown that the CFU-pre-B output in LTC-IC_{ML} assay cultures was linearly related to the number of input cells initially seeded, whether they were derived from total normal marrow, highly purified normal marrow, and marrow exposed to 5-FU in vivo. The CFC output of standard "myeloid" LTC-IC assays was also shown to increase as a linear function of the input cell dose.

The results presented in this thesis also indicate that LTC-IC and LTC-IC_{ML} are closely related to each other and to totipotent murine cells with long-term in vivo repopulating potential. This conclusion is based on the finding that all three assays detect cells in adult mouse marrow that have the same very rare Sca-1^+ Lin^- WGA^+ phenotype and also exhibit a similar resistance to 5-FU. In both mice and humans, a significant proportion of marrow cells

detected as LTC-IC show a low retention of the vital dye Rhodamine-123 and are relatively resistant to various chemotherapeutic drugs (Ploemacher and Brons 1989; Sutherland, et al 1989; Sutherland, et al 1990). Like their murine counterparts, human LTC-IC are enriched in those subpopulations that are most enriched for primitive progenitor cells (Berenson, et al 1990; Sutherland, et al 1990). In addition, the same stromal cell lines can be used in assays to detect either human or murine LTC-IC and in neither case is the presence of or responsiveness to SF required (Kodama, et al 1992; Sutherland, et al 1993; Miller, et al In Press). These findings support the likelihood that the human LTC-IC assay may detect cells with similar properties including long-term in vivo repopulating potential. Recent reports indicating that individual $CD34^+Thy\text{-}1^+$ fetal human marrow cells can give rise to both $CD15^+33^+$ myeloid and $CD10^+19^+$ lymphoid cells in long-term cultures further reinforces this possibility (Baum, et al 1992).

Although they are closely related to both CRU and LTC-IC, LTC-IC_{ML} are detected at 15-30-fold lower frequencies. This appears to be, in part, a consequence of the design of the assay, itself. The experiments detailed in Chapter IV show that the CFU-pre-B detected in standard LTC-IC_{ML} assays after they have been switched to conditions that support pre-B lymphopoiesis have to differentiate from those LTC-IC_{ML} still present after the initial 4 week period under myeloid culture conditions. Neither the addition of FL to the CFU-pre-B methylcellulose assays nor the extension of the lymphoid culture period significantly increased the detection of LTC-IC_{ML}. Since it was found that CRU numbers decline approximately 10-fold within 3-4 weeks in myeloid LTC (Fraser, et al 1992; Chapter IV), experiments were undertaken to investigate the possibility of improving the plating efficiency of LTC-IC_{ML} by shortening the initial myeloid culture period. Since the experiments described in Chapter III has suggested that the earliest known B lymphoid restricted progenitors disappeared from such cultures within one week, reduction of the initial myeloid culture period to 1 week was evaluated. However, shortening the LTC-IC_{ML} assay to a 1 week period under myeloid LTC conditions followed by 10 days under lymphoid LTC conditions revealed the existence of a

previously undescribed 5-FU and steroid/horse serum-resistant CFU-pre-B progenitor cell type at sufficiently high frequencies to render this approach less attractive for the routine measurement of LTC-IC_{ML}. Nevertheless, the prediction that much of the discrepancy in CRU and LTC-IC_{ML} frequencies could be attributed to the imperfect maintenance in vitro of lympho-myeloid progenitors was confirmed by these experiments. The frequency of LTC-IC_{ML} detectable in normal marrow was ~3 times greater when the initial myeloid LTC period was shortened from 4 weeks to 1 week, only 3-fold lower than the frequency of CRU in 1 week-old myeloid LTC, although still ~10 times lower than the frequency of CRU in freshly isolated marrow.

It seems likely that a more complete understanding of the cytokines able to stimulate or inhibit HSC proliferation and differentiation will allow more efficient LTC-IC_{ML} detection protocols to be developed. Conversely, any factor(s) that can significantly increase the detection of LTC-IC_{ML} might do so by enhancing the maintenance or proliferation of HSC. Feeders that either overexpress (Zipori and Lee 1988; Corey, et al 1990; Hogge, et al 1991; Otsuka, et al 1991; Sutherland, et al 1991) or do not express (Kodama, et al 1992; Sutherland, et al 1993) known factors have been used in the past to examine the mechanisms by which primitive hematopoietic progenitors are driven to proliferate and differentiate in vitro. Similarly, exogenous addition of neutralizing antibodies to specific growth factors or inhibitors could also be used to investigate these processes (Zipori and Lee 1988; Cashman, et al 1990). In addition, novel regulators of HSC functions might be identified by screening cloned stromal cell lines for their ability to increase the plating efficiency LTC-IC_{ML} or their individual clonogenic cell output.

My investigations into the reasons for the discrepancy in CRU, LTC-IC and LTC-IC_{ML} frequencies also revealed that both LTC-IC and LTC-IC_{ML} are better maintained for 3-4 weeks in myeloid LTC than CRU. The long-term ability to generate both myeloid and B lymphoid clonogenic progenitors in vitro and the ability to fully reconstitute a myeloablated recipient thus

appear to be subject to different regulatory mechanisms. In addition, HSC surviving 5-FU treatment appear to be subtly different from the bulk of HSC in their in vitro responses. Furthermore, although marrow from 5-FU-treated mice can reconstitute irradiated recipients as effectively and competitively as normal marrow (Lerner and Harrison 1990), their ability to repopulate non-ablated animals is significantly less (Stewart, et al 1993; Ramshaw, et al 1995). Since 5-FU is commonly used to eliminate the majority of cycling progenitors in the marrow as well as to bring quiescent HSC into cycle to facilitate gene transfer, it may be of some importance to clarify these differences and to determine their potential impact on the ability of transfected HSC to maintain normal long-term hematopoiesis in vivo.

The molecular signals responsible for stimulating the various differentiation events that CRU and LTC-IC_{ML} must undergo to be detected remain undefined. Recent studies in which I was involved (Miller, et al In Press, see Appendix 1) suggest that SF plays a more essential role in the recruitment of CRU during hematological reconstitution after bone marrow transplantation than in their detection in the LTC system when stimulated by fibroblasts. Thus other factor(s) appear to be able to compensate for the absence of SF in the retention of lympho-myeloid differentiation potential. Since both IL-11 and FL act on very primitive hematopoietic progenitors, it would be interesting to see whether their removal from LTC might affect the capacity of HSC to express either their lymphoid or their myeloid potential under these conditions.

IL-11 and FL are also of interest because of their overlapping but non-identical roles in supporting the development of $B220^+$ lymphocytes from fetal liver-derived $B220^-$ progenitors in the absence of a stromal cell feeder layer. Although the precise cellular target of each factor is not yet clear, preliminary studies with single cell cultures suggest that IL-11 may act to preferentially expand an earlier progenitor cell type than FL. It is conceivable that HSC present in LTC might also express their B lymphoid potential in the presence of IL-11, FL, SF

and IL-7 under fully defined culture conditions. Indeed, it may well be that these factors may be differentially expressed under myeloid and lymphoid LTC conditions.

The present studies have also identified a novel lymphoid-restricted cell type which persists for ≥7 days in myeloid LTC and thus, unfortunately, precludes the shortening of the initial myeloid culture period in LTC-IC_{ML} assays. This lymphoid precursor is probably more primitive than any stage of B lymphoid development described to date. Whether this cell also possesses unexpressed T lymphopoietic potential is unknown at present. Unlike the B lymphoid progenitors capable of proliferating in the short-term (2 weeks) lymphoid culture assay originally described by Müller-Sieburg et al (Müller-Sieburg, et al 1986), these steroid-resistant CFU-pre-B precursors are resistant to 5-FU treatment. It would therefore be of interest to know whether these novel B lineage progenitor cells can also be separated from HSC by a lack of expression of the Fall-3 antigen (Müller-Sieburg 1991).

The ability of HSC to differentiate along T cell lineages has to date remained poorly quantitated because of the need for thymocytes to be positively selected in the presence of thymic stromal cells in order to remain viable (reviewed in (von Boehmer 1994)). One way to circumvent this process would be to use thymic organ cultures as a third read-out in an LTC-IC_{ML}-like assay. This option is not particularly appealing for practical reasons. It is already very time-consuming to divide individual micro-cultures into myeloid CFC and CFU-pre-B assays. The inclusion of a third read-out would make the system even more unwieldy. However, a potentially more practical approach might be to use hematopoietic cells from mice in which the control of programmed cell death has been genetically altered, possibly by overexpression in developing thymocytes of the bcl-2 gene (Sentman, et al 1991; Strasser, et al 1991; Siegel, et al 1992). Failure to be positively selected might not, in this case, result in thymocyte death (Sentman, et al 1991; Siegel, et al 1992; Strasser, et al 1994; Tao, et al 1994) and might allow the detection of T lineage cells in the presence of appropriate feeder layers or growth factors. Indeed, immortalized thymic stromal cells might be found that could, either

alone or together with marrow fibroblasts, provide the necessary factors to stimulate the full development of all hematopoietic lineages under appropriate culture conditions.

In conclusion, the present studies have established the feasibility of specifically detecting individual hematopoietic cells capable of giving rise to both myeloid and lymphoid progeny in vitro. Cells in adult mouse bone marrow that can exhibit such wide in vitro differentiation potentials can be identified as a subset of LTC-IC and are closely related to and probably overlap extensively with long-term in vivo reconstituting cells generated in vivo during normal development. However, in spite of evidence that the capacity to be detected in LTC-IC/LTC-IC_{ML} and CRU assays are co-ordinately maintained during normal development, they become dissociated when the cells are held in vitro under LTC conditions. Thus HSC appear to respond to multiple, but not entirely overlapping, factors in their detection as LTC-IC_{ML} or CRU and the responsiveness to these factors may be differentially maintained in LTC.

Identification of the molecular properties that maintain HSC functions remains one of the central issues in hematopoietic cell research. What genes regulate hematopoietic stem cell behaviour, what changes initiate lineage commitment, how is the balance between commitment to different lineages regulated, how is this related to the progressive limitation of proliferative potential, and what role do external factors play in regulating these processes are all questions which cannot yet even be formulated in molecular terms. It is thus of interest to speculate as to how the key findings of this thesis might facilitate future progress in resolving these basic questions in HSC biology. One lead is the observed difference in CRU and LTC-IC/LTC-IC_{ML} maintenance in LTC. The combined use of CRU and LTC-IC_{ML} assays might therefore be useful in screening for specific factors which may affect the ability of HSC to retain their differentiative and proliferative capacity in spite of loss of CRU activity or, conversely, which would recapture the latter. Another important finding of these studies was the overlapping but not identical effects of FL and IL-11 seen on cells undergoing the earliest stages of lineage commitment. One explanation of the results of these studies is that FL and IL-11 might, in fact,

be capable of influencing the developmental fate of HSC. Identification of differences in the intermediates active in the signalling pathways of these factors might thus provide an approach to delineating which genes might be involved in these processes. Alternatively, it may be of interest to determine whether HSC interactions with FL or IL-11 could differentially alter the expression of known hematopoietic transcription regulators, such as the GATAs or Ikaros family members.

As described in Appendix 1, very few HSC from W^{41}/W^{41} mutant mice are detected in the CRU assay. Nevertheless, their presence at near normal numbers can be inferred from their assessment in the LTC-IC_{ML} assay which also requires the expression of lympho-myeloid potential and the generation of sizeable daughter clones. This suggests that there may be at least two pathways which connect at some level within the cell to stimulate HSC differentiation. The use of the LTC-IC_{ML} assay, in conjunction with the CRU assay, might therefore provide the basis of a screening strategy for the identification of specific factors or intracellular mediators which effect this response. Finally, the availability of the LTC-IC_{ML} assay may also make it possible to examine the role of molecules not directly involved in the regulation or expression of the proliferative or differentiation potentials of HSC but which may, nevertheless, be essential for their in vivo function. The loss of homing receptors, for example, might result in reduced detectability of HSC in vivo without altering LTC-IC_{ML} detection. The combined used of CRU and LTC-IC_{ML} assays might then provide a powerful approach to identifying such molecules or pathways they activate. Thus the combined use of quantitative in vivo and in vitro assays may offer a variety of new strategies for characterizing, at the molecular level, changes in the inherent capabilities of HSC and allowing these to be distinguished from mechanisms that alter their ability to realize their full range of functional activities in vivo.

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Appendix 1 - Studies of W-mutant mice provide evidence for alternate mechanisms capable of activating hematopoietic stem cells

STUDIES OF W-MUTANT MICE PROVIDE EVIDENCE FOR ALTERNATE MECHANISMS CAPABLE OF ACTIVATING HEMATOPOIETIC STEM CELLS

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Abstract

Previous studies have suggested that Steel Factor (SF) can influence the behaviour of many types of hematopoietic progenitor cells both in vivo and in vitro, although whether these may include the most primitive populations of totipotent repopulating cells remains controversial. To approach this question, we measured the number of Sca-1$^+$Lin$^-$WGA$^+$ cells, the number of cells with demonstrable myeloid (LTC-IC) or both myeloid and lymphoid (LTC-IC$_{ML}$) potential in 4 to 5 week-old long-term cultures containing irradiated primary marrow feeder layers, as well as the number of multi-lineage long-term in vivo repopulating cells (CRU) present in the marrow of W^{42}/+ or W^{41}/W^{41} mice as compared to +/+ controls. There was no significant effect of either of these W mutations on the number of Sca-1$^+$ Lin$^-$ WGA$^+$ cells and, in W^{41}/W^{41} mice, neither LTC-IC nor LTC-IC$_{ML}$ populations appeared to be affected. On the other hand, although W^{41}/W^{41} and W^{42}/+ cells could both be detected in the in vivo CRU assay, their numbers were markedly reduced (17- and 7-fold, respectively) in spite of the fact that both of these W-mutant genotypes contained near normal numbers of Day 9 and Day 12 CFU-S. In vitro quantitation of erythroid (BFU-E), granulopoietic (CFU-GM), multi-lineage (CFU-GEMM) and pre-B clonogenic progenitors (CFU-pre-B) also revealed no differences in the numbers (or proliferative potential) of any of these cells when W^{41}/W^{41} or W^{42}/+ and normal mice were compared, although Day 3 BFU-E from both types of W-mutant mice showed no response to the typical enhancing effect exerted by SF on their +/+ counterparts. Taken together these findings are consistent with the view that SF activation of c-kit receptor-induced signalling events is not a rate-limiting mechanism controlling red blood cell production during normal development until hematopoietic cells differentiate beyond the Day 3 BFU-E stage. Nevertheless normal hematopoietic stem cells do appear to be responsive to SF since their W-mutant counterparts display a disadvantage in the vivo setting which is exaggerated under conditions of hematopoietic regeneration. On the other hand, alternative mechanisms also appear to contribute to the regulation of hematopoietic stem cell numbers in vivo and to their detection as LTC-IC in vitro.

Introduction

Steel Factor (SF), also called mast cell growth factor, stem cell factor, and kit-ligand, is a pleiotropic cytokine [1]. Within the hematopoietic system, many types of primitive hematopoietic cells both before and after their apparent commitment to a single pathway of differentiation have been shown to express receptors for SF and also appear to be responsive to ligand binding [2-5]. In vitro, SF has been shown to act on hematopoietic cells primarily as a synergistic factor in concert with a variety of other factors including interleukin-3 (IL-3), IL-7, granulocyte-macrophage colony-stimulating factor (GM-CSF), G-CSF and erythropoietin (Ep) according to the target cell stimulated [6-10]. However, for most of these progenitors, interaction with SF does not appear to be absolutely required for their continuing viability or for their ability to continue to proliferate and differentiate. In contrast, studies of the effect of suppressing SF receptor-mediated signalling in vivo, either by administration of an antibody that blocks SF binding to its receptor [2], or by analysis of Sl or W mutant mice (two loci where the genes for SF and the receptor for SF, also known as c-kit, are found) [11] have provided evidence that SF is a physiological regulator of certain aspects of hematopoiesis in vivo [12,13]. In particular, the latter studies have demonstrated a requirement for SF in mice to maintain an adequate output of mature red cells and tissue mast cells throughout adult life. The accompanying production in vivo of earlier progenitor types appears to be less affected when these are quantitated by in vitro assays that do not use SF [14,15]. On the other hand, interference with SF-receptor interactions does markedly delay or reduce regeneration of these same types of hematopoietic progenitors in irradiated, marrow transplanted mice [16]. Conversely, normal cells can competitively repopulate even unperturbed W-mutant mice [17,18]. These latter findings suggest that SF may also serve as a physiological regulator of the earliest stages of hematopoiesis in vivo but in a fashion that may not appear limiting except under conditions of high demand.

Such considerations refocus interest on the question of the potential role of SF in regulating the recruitment and self-maintenance of the most primitive types of totipotent hematopoietic cells both during normal life and under conditions of post-transplant recovery. To approach these questions, we have first compared the progenitor content of W^{41}/W^{41} and $W^{42}/+$ mice using a variety of in vitro and in vivo end points to detect the presence of primitive hematopoietic cells. In addition, we investigated the ability of sublethally irradiated W^{41}/W^{41} and $W^{42}/+$ mice to serve as recipients in a CFU-S assay using +/+ cells. The W^{41} and W^{42} mutations are both point mutations that occur within the tyrosine kinase domain of the c-kit coding sequence and result in amino acid substitutions that demonstrably reduce the kinase activity of the SF receptor [19,20]. Our findings pinpoint the Day 3 BFU-E stage of erythropoietic cell development as one that is very sensitive to perturbation of SF-receptor interactions in vitro. The generation during ontogeny of more primitive populations of cells, including those with lympho-myeloid potential, was not found to be SF-dependent although evidence for a role of SF in their preferential recruitment in vivo was obtained.

Methods and Materials

Animals. C57Bl/6J (Ly-5.2), congenic C57Bl/6J:Pep 3b (Ly-5.1), congenic C57Bl/6J-W^{41}/W^{41} (Ly-5.2) and C57Bl/6J-$W^{42}/+$ (Ly-5.2) breeders, obtained originally from Dr. L. Schultz and Dr. J. Barker at the Jackson Laboratories (Bar Harbour, MA) were bred and maintained in the animal facility of the British Columbia Cancer Research Centre. All animals were housed in micro-isolator units and provided with sterilized food and water. For irradiated mice, the water was also acidified with HCl to a pH of 3.

Cell Preparation. Marrow cells were obtained by flushing femoral shafts with Alpha MEM containing 2% fetal calf serum (2% FCS, StemCell Technologies, Inc., [STI]

Vancouver, B.C.) using a 21 gauge needle attached to a 3cc syringe. The cells were then further suspended by gentle aspiration once or twice through the same needle.

Phenotype Studies. Marrow cells from W^{41}/W^{41}, $W^{42}/+$ and C57Bl/6J mice were analyzed for their content of cells with the following phenotype: low forward and orthogonal light scatter properties, absence of several hematopoietic lineage markers (Lin), presence of the Ly6 antigen (Sca-1), and ability to bind wheat germ agglutinin (WGA), using a FACStar$^+$ (Becton Dickinson & Co. San Jose, CA) equipped with 5-W argon and 30 mW helium neon lasers. Antibodies against mature hematopoietic cell markers (Lin) included anti-Mac-1 (M1/70: myelomonocytic), anti-GR-1 (RB6-8C5: granulocytic), anti-B220 (RA3-6B2: lymphocytes) and anti-Ly-1 (53-7.3: T lymphocytes). Specific details of the origin and use of these immunochemical reagents, staining and FACS analysis procedures were as described in detail elsewhere [21].

Isolation of Sca-1$^+$Lin$^-$WGA$^+$ Cells. Marrow cells from W^{41}/W^{41} mice were incubated for 35 min on ice with 3 μg/ml of Fc receptor blocking antibody (2.4G2) followed by the addition of the fluorescein isothiocyanate (FITC) - labelled Lin antibodies described above. After two washes with Hanks containing 2% FCS, cells were incubated for 35 min on ice with BioMag" Sheep anti-FITC IgG magnetic beads (Advanced Magnetics, Cambridge, MA). Lin$^+$ cells were depleted from the cell suspension by magnetic separation and the remaining cells were then treated with biotinylated WGA for 35 min on ice followed by 2 washes. The cells were then incubated with FITC-labelled Lin antibodies, anti-Sca-1-Cyanine 5 succinimidyl ester (Cy5) and streptavidin-conjugated phycoerythrin (PE) for 35 min on ice. Propidium iodide (PI) was included in the final wash and Sca-1$^+$ Lin$^-$ WGA$^+$ cells isolated using a FACStar$^+$ [21].

Assay for Spleen Colony-forming Units (CFU-S). Recipients were irradiated with ^{137}Cs γ-rays (at 110 cGy/min) in a single dose as indicated in each experiment and then

injected intravenously with $1x10^5$ bone marrow cells each. Animals were sacrificed 9 or 12 days later and their spleens either placed in fixative for macroscopic colony counts [22] or dissected individually for DNA extraction and Southern analysis.

Southern Analysis. Tissue samples were dispersed and washed in phosphate buffered saline. One volume of packed cells was then incubated with ten volumes of proteinase K solution (1 mg/ml proteinase K, 10 mM Tris-pH 7.4, 10 mM ethylenediamine tetraacetic acid [EDTA], 150 mM sodium chloride and 0.4% sodium dodecyl sulfate [SDS]) for an initial 15 min at 65°C and then overnight with gentle shaking at 37°C. The solution was extracted multiple times using phenol:chloroform (1:1) and nucleic acids were precipitated from the aqueous phase using a 1/10 volume of 3 M sodium acetate (pH=5.2) and 2.5 volumes of ethanol. The pellet was washed twice in 70% ethanol and resuspended in TE (10 mM Tris, 1mM EDTA, pH=8.0). DNA was digested with EcoR1 or BamH1 (Gibco BRL, Gaithersburg, MD) at 2-3 U/μg of genomic DNA for 8-16 hrs at 37°C. Control samples containing 1% to 50% male DNA and test samples were electrophoresed through a 0.8% agarose gel and transferred to nylon membranes (Zeta-Probe; Bio-Rad, Mississauga, Ont., Canada). The blots were then probed with a Y chromosome-specific fragment labelled to a high specific activity using ^{32}P and a random-primer kit (Gibco BRL). The filters were washed to a final stringency of 0.2% SSC (150 mM NaCl, 15 mM sodium citrate, pH=7.0), 0.1% SDS, and 0.1% sodium pyrophosphate at 62°C, and autoradiography performed using Kodak XAR-5 film. The M34 plasmid containing a 2.3 kb Y chromosome-specific segment was kindly provided by Dr. Singh [23].

Assay for Competitive Repopulating Units (CRU). Groups of 6 to 8 C57Bl/6J (Ly5.2) or C57Bl/6J: Pep 3b (Ly-5.1) mice were irradiated with a single dose of 950 cGy (at 110 cGy/min) from a ^{137}Cs γ-ray source and then injected intravenously with varying numbers of W-mutant test cells together with $\leq 10^5$ normal marrow cells or $\leq 2 \times 10^5$ "compromised" marrow cells with a normal content of CFU-S but a reduced content of CRU obtained from

donors that had previously been reconstituted with marrow cells from a first marrow transplant as previously described [24]). In both cases these latter cells were of the same genotype as the recipients. CRU assay recipients were evaluated for evidence of lympho-myeloid repopulation of test cell origin ≥ 8 weeks following transplantation. In some experiments, male test cells were injected into female C57Bl/6J recipients in which case only those whose marrow and thymus contained ≥ 5% male DNA were scored as positive. In other experiments, the test cells were injected into C57Bl/6J:Pep 3b recipients, in which case test cell-derived lymphoid and myeloid progeny were detected by FACS analysis after staining of blood samples with FITC-labelled anti-Ly-5.2 antibody clone ALI4A2 [25]. The presence of ≥ 1% Ly-5.2$^+$ cells including both lymphoid and myeloid elements, as defined by their distinct forward and orthogonal light scattering characteristics, was then used to identify recipients that had been injected with at least 1 CRU from the test cell population. CRU frequencies were calculated by the method of maximum likelihood from the proportion of negative mice obtained in each group [26].

Methylcellulose Assays for Colony-Forming Cells. To quantitate Day 3 BFU-E, marrow cells were suspended in a semi-solid mixture of Alpha medium containing 0.8% methylcellulose, 30% FCS, 1% deionized bovine serum albumin, 2mM glutamine, 0.1mM 2-mercaptoethanol (2-ME), and human erythropoietin (Ep) (at the concentration indicated) (STI) with or without 50 ng/ml purified recombinant murine SF (Immunex, Seattle, WA). Duplicate or triplicate 1.1 ml volumes containing 8 x 10^4 marrow cells were then plated in 35 mm petri dishes (STI) and incubated for 3 days at 37°C in a humidified atmosphere of 5% CO_2 in air. Erythroid colonies containing ≥ 3 clusters of ≥ 8 erythroblasts each were scored directly in situ as previously described [27]. For quantitation of more primitive BFU-E, as well as CFU-GM and CFU-GEMM, 3 x 10^4 cells/ml were cultured in the same methylcellulose medium described above supplemented 3U/ml Ep and 10 ng/ml each of purified recombinant murine IL-3 and human IL-6 (and when indicated, 50 ng/ml of SF). Murine IL-3 and human IL-6 were purified from the supernatants of COS cells transiently transfected with the

appropriate murine and human cDNAs. Cultures were then incubated under the same conditions as for Day 3 BFU-E but colonies were not scored until after 14 days using the same criteria for distinguishing large (but not macroscopic) erythroid colonies (from BFU-E), colonies consisting of ≥ 20 granulocytes and/or macrophages (from CFU-GM) and macroscopically visible colonies containing both of these lineages as well as large megakaryocytes (from CFU-GEMM) [28]. CFU-pre-B were assessed by plating cells in a semi-solid Alpha medium containing 0.8% methylcellulose, 30% of a selected batch of FCS, 2mM glutamine and 0.1 mM 2-ME (STI) plus 0.2 ng/ml human IL-7 (Immunex). Morphologically distinctive colonies of B-lineage cells were scored in these cultures after 1 week of incubation under the same conditions of temperature, CO_2 and humidity. To optimize the detection of CFU-pre-B in the LTC-IC_{ML} assays (see below), the CFU-pre-B methylcellulose assay medium was further supplemented with 5 ng/ml of murine SF.

Limiting Dilution Assays to Quantitate Long-term Culture-Initiating Cells (LTC-IC). Marrow cells were suspended in myeloid long-term culture medium (Alpha medium containing 12.5% FCS, 12.5% horse serum, 0.2 mM 2-ME, 0.2 mM i-inositol, 2 μM folic acid and an additional 2mM L-glutamine, [STI] to which freshly dissolved hydrocortisone sodium hemisuccinate [Sigma Chemicals, St. Louis, MO] was added just prior to use to give a final concentration of 10^{-6} M). 150 μL aliquots of cells at varying concentrations in this medium were then placed into individual wells of 96-well microtiter plates each of which contained a previously established, irradiated (1500 cGy) primary mouse marrow feeder layer [29]. These cultures were then maintained for 4 weeks at 33°C with weekly removal of half of the nonadherent cells and replacement of half of the medium. At the end of this period, all of the cells present in each well (including the adherent as well as the nonadherent cells) were harvested, combined and assayed for the presence of BFU-E, CFU-GM and CFU-GEMM. LTC-IC frequencies were then calculated by the method of maximum likelihood from the proportion of negative wells (wells containing no detectable myeloid clonogenic progenitors) measured for each concentration of cells tested [26].

To detect and quantitate a subset of LTC-IC with the ability to give rise to lymphoid (CFU-pre-B) as well as myeloid clonogenic cells in vitro (referred to as LTC-IC_{ML}), a modified version of the "standard" LTC-IC assay procedure was used. Details of the design and validation of the LTC-IC_{ML} methodology have been described elsewhere [30]. Briefly, cells were cultured for the first 4 weeks in the same way as for standard LTC-IC assays, but then, instead of harvesting the cells, all of the medium and nonadherent cells were removed, the adherent layer was washed twice with pre-warmed RPMI 1640 (STI) and fresh lymphoid long-term culture medium (RPMI 1640, 5% FCS, 50 μM 2-ME) then added. These cultures were then transferred to 37°C and one week later the entire culture was harvested and divided into two equal portions. One half was plated individually into methylcellulose assays to detect the presence of BFU-E, CFU-GM and CFU-GEMM and the other half into CFU-pre-B colony assays. The LTC-IC_{ML} frequency in the original test suspension was calculated exactly as for LTC-IC by relating the proportion of negative wells to the number of cells assayed per well; however in this case, a well was considered negative if it did not contain both myeloid and lymphoid clonogenic cells.

Results

W^{41}/W^{41} and $W^{42}/+$ Mutations Affect the Responsiveness of Day 3 BFU-E to SF but not to Ep. Both W^{41}/W^{41} and $W^{42}/+$ mice are viable into adulthood and exhibit a similar degree of anemia [13]. In vitro, SF is well known for its ability to enhance the Ep-dependent formation of large erythroid and multi-lineage colonies detectable after 1 to 2 weeks of incubation [31]. However, in spite of the known marked decrease (10 to 100-fold) in Ep responsiveness that accompanies the differentiation of normal Day 3 BFU-E into CFU-E in vivo, an analysis of the potential effect of SF on murine Day 3 BFU-E, an immediate precursor of the cells referred to as CFU-E, has not been reported. Therefore, we first examined the possibility that SF might influence the Ep-dependent proliferation and maturation of normal

Day 3 BFU-E in vitro. The results shown in Figure 1A indicate that exogenously provided SF enhanced the number of Day 3 BFU-E detectable in assays of +/+ marrow cells at every Ep concentration tested (up to 3 U/ml), although the extent of this enhancing effect was also Ep dose-dependent (decreasing from ~10-fold at 0.03 U/ml of Ep to ~ 2-fold at 3 U/ml). As expected, no such effect was evident in corresponding Day 3 BFU-E assays of marrow cells obtained from either W^{41}/W^{41} or $W^{42}/+$ mice (Figure 1B and 1C) although the innate Ep sensitivity of the Day 3 BFU-E (assessed in the absence of added SF) from both normal and W-mutant sources appeared indistinguishable (Figure 2). Since no plateau was achieved in these dose response studies, it is not possible to accurately estimate the total Day 3 BFU-E population in any of these mice. Nevertheless, from the data obtained at 3 U/ml of Ep in the assays ± SF, any differences in frequency would appear to be ≤ 2 fold. Since the femoral cellularity of all strains was also found to be similar (data not shown) the total Day 3 BFU-E populations in all 3 strains can be assumed to be similar.

Normal Numbers of CFU-S and In Vitro Clonogenic Progenitors in W^{41}/W^{41} and $W^{42}/+$ Mice. Previous studies of W^{41}/W^{41} mice have reported that they contain very few cells capable of producing macroscopically visible spleen colonies within 9 days after transplantation into lethally irradiated +/+ recipients [18,32]. Upon repeating these experiments with cells of either W^{41}/W^{41} or $W^{42}/+$ origin, we found that readily detectable, macroscopic colonies (albeit somewhat smaller than those produced by +/+ cells) were visible both 9 and 12 days after injection of lethally irradiated +/+ mice with cells of either W-mutant genotype. The colonies present at both time points were further shown to be exclusively of donor (i.e., male and hence W-mutant) origin by Southern analysis of DNA from individually removed colonies using a Y chromosome-specific probe [23] (data not shown). Quantitation of the frequency of these W-mutant CFU-S and hence their population size (number per femur) showed their numbers to be only slightly reduced (~ 2-fold) relative to +/+ or $W^{41}/+$ mice assessed in the same experiments (Table 1).

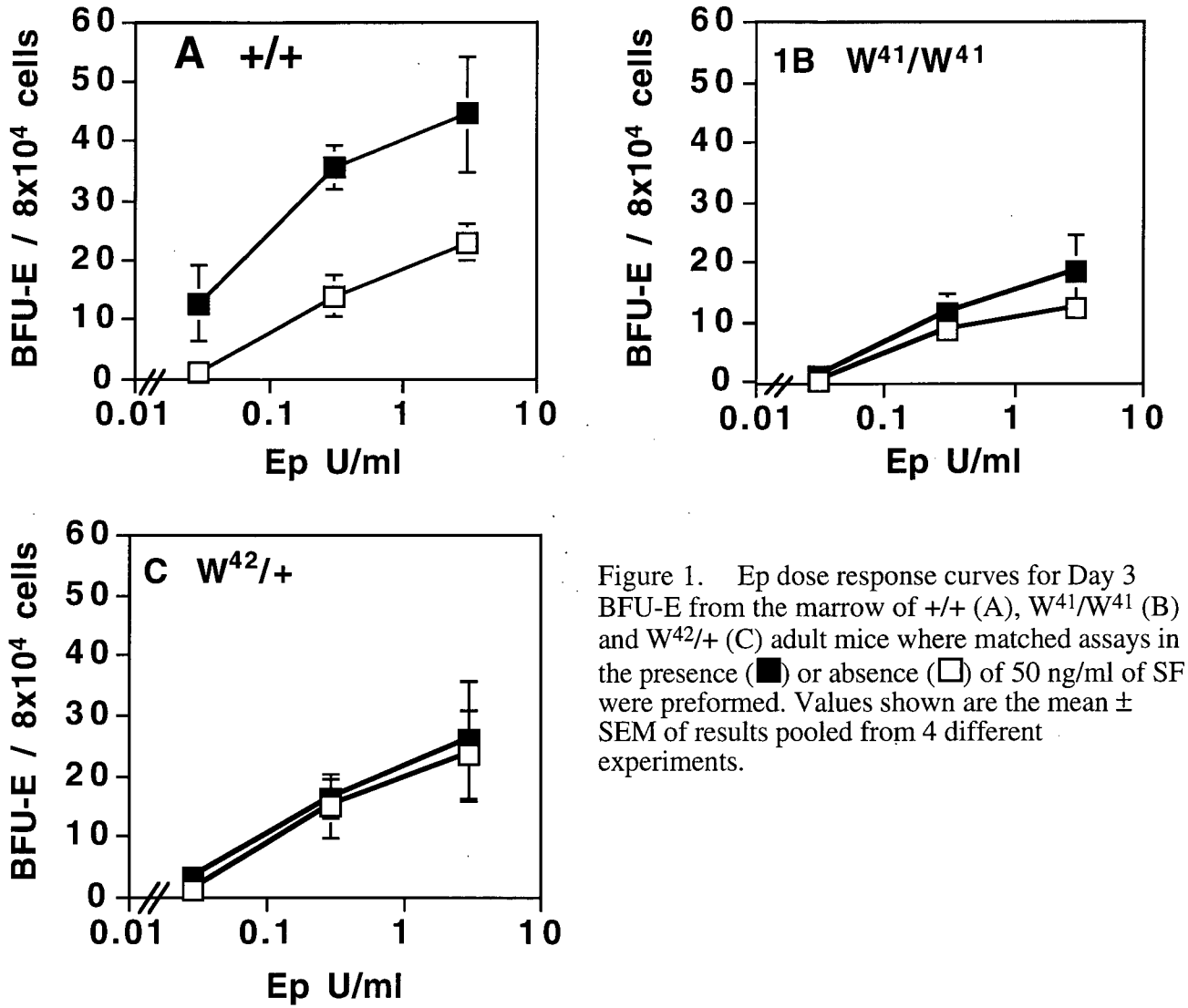


Figure 1. Ep dose response curves for Day 3 BFU-E from the marrow of +/+ (A), W^{41}/W^{41} (B) and $W^{42}/+$ (C) adult mice where matched assays in the presence (■) or absence (□) of 50 ng/ml of SF were preformed. Values shown are the mean ± SEM of results pooled from 4 different experiments.

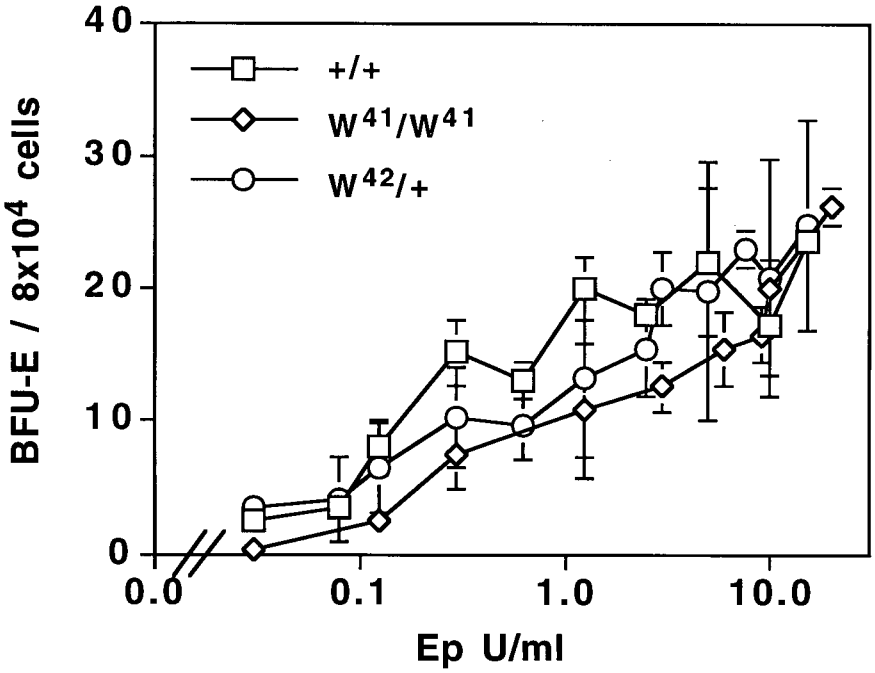


Figure 2. Full Ep dose response curves for Day 3 BFU-E from the marrow of +/+ (□), W^{41}/W^{41} (○) and $W^{42}/+$ (◇) mice.

TABLE 1 RESULTS OF CFU-S DETERMINATIONS ON MARROW CELLS FROM +/+, $W^{41}/+$, W^{41}/W^{41} AND $W^{42}/+$ MICE

Genotype of Injected Cells	CFU-S per 10^5 Cells		CFU-S per Femur	
	Day 9	Day 12	Day 9	Day 12
+/+	22 ± 1 (10)	21 ± 1 (15)	3400 ± 200	3200 ± 200
$W^{41}/+$	17 ± 2 (3)	21 ± 2 (3)	2600 ± 200	3100 ± 300
W^{41}/W^{41}	12 ± 2 (6)	12 ± 1 (10)	1600 ± 200	1600 ± 100
$W^{42}/+$	10 ± 1 (4)	12 ± 1 (8)	1300 ± 200	1600 ± 200

10^5 cells of each genotype were injected into +/+ recipients previously irradiated with 900 cGy and the number of macroscopic spleen colonies present 9 and 12 days later then counted. Values shown represent the mean ± SEM of counts from (n) recipients pooled from 1-3 experiments.

A similar lack of effect of these W mutations on the progenitors of mixed colonies that are able to achieve a macroscopic size after 14 days of growth in vitro (referred to here as CFU-GEMM) was also observed. The results of 2 experiments in which colony formation was stimulated by a combination of IL-3, IL-6 and Ep are shown in Figure 3. Under these culture conditions, the addition of SF did not further enhance colony formation by normal CFU-GEMM (or BFU-E or CFU-GM) and neither the plating efficiency nor the size of colonies obtained from their W-mutant counterparts was affected by added SF (data not shown). W^{41}/W^{41} mice were additionally examined for evidence of an effect of this c-kit mutation on the in vivo generation of CFU-pre-B and $B220^+$ B cell populations. However, as for all of the myeloid clonogenic cell types studied, the total number of CFU pre-B per femur (Figure 3) and $B220^+$ cells (W^{41}/W^{41}: 4.3 x 10^6/femur, +/+: 4.7 x 10^6/femur) were comparable to those measured in +/+ controls.

Taken together these results suggest that signalling through the c-kit receptor does not play a limiting role in regulating the generation (or maintenance) in vivo of a variety of early myeloid and lymphoid progenitor cell types during normal adult life. Similarly, expression of their differentiative and proliferative potential in vitro does not appear to require efficient SF stimulation.

Analysis of W-mutant Cells Defined by Different End Points Associated with Normal Hematopoietic Stem Cell Activity. As a first approach to evaluating the hematopoietic stem cell compartment present in adult W^{41}/W^{41} and $W^{42}/+$ mice, we tested marrow cells from both genotypes for the presence of CRU using a quantitative limiting dilution assay as described in the Methods. The results are shown in Table 2. It can be seen that both W^{41}/W^{41} and $W^{42}/+$ CRU could be detected although their frequencies (and hence total numbers per femur) relative to congenic C57Bl/6J- +/+ controls (1 per 10^4 +/+ marrow cells, or ~ 10^3 CRU per +/+ femur [21]) were markedly reduced (~ 17- and 7-fold, respectively).

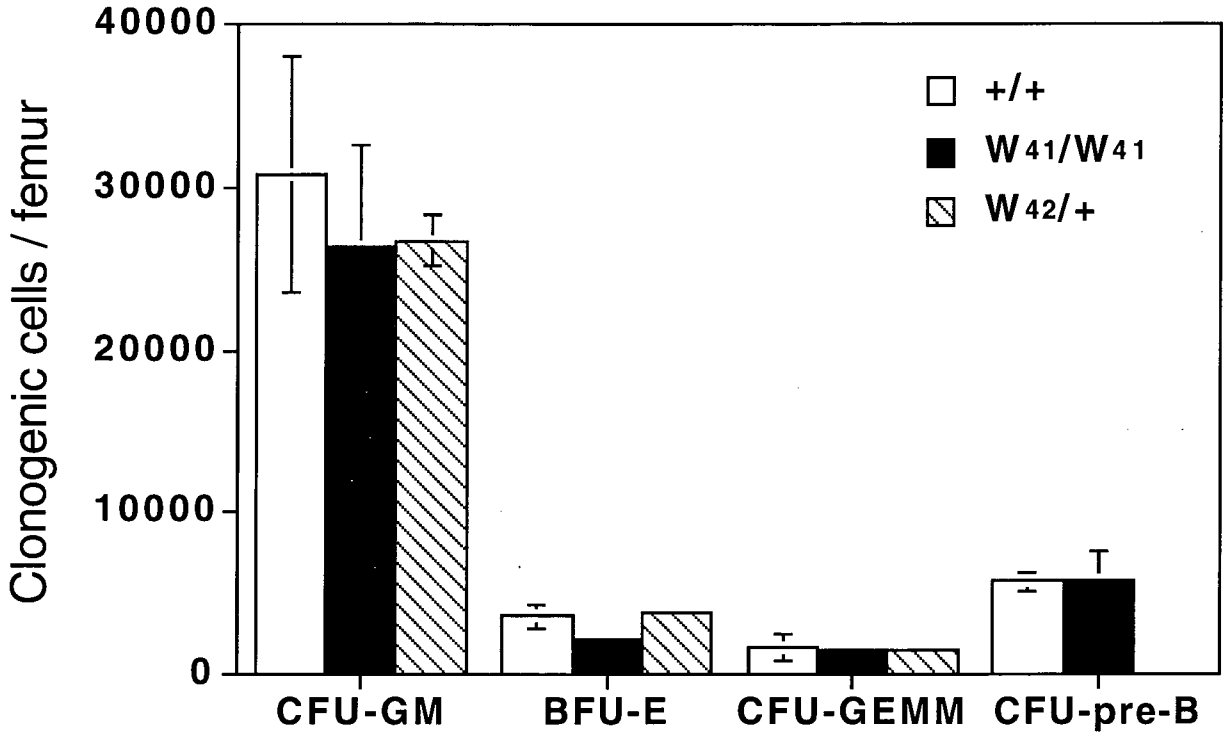


Figure 3. Comparison of the numbers of total CFU-GM, BFU-E, CFU-GEMM and CFU-preB present per femur in +/+, W^{41}/W^{41} and $W^{42}/+$ mice. Values shown represent the mean ± SEM of data from 2 experiments in which colonies were counted in 14 day-old methylcellulose cultures containing IL-3, IL-6, and Ep (myeloid colonies) or 7 day-old methylcellulose cultures containing IL-7 (colonies of pre-B cells) as described in the Methods.

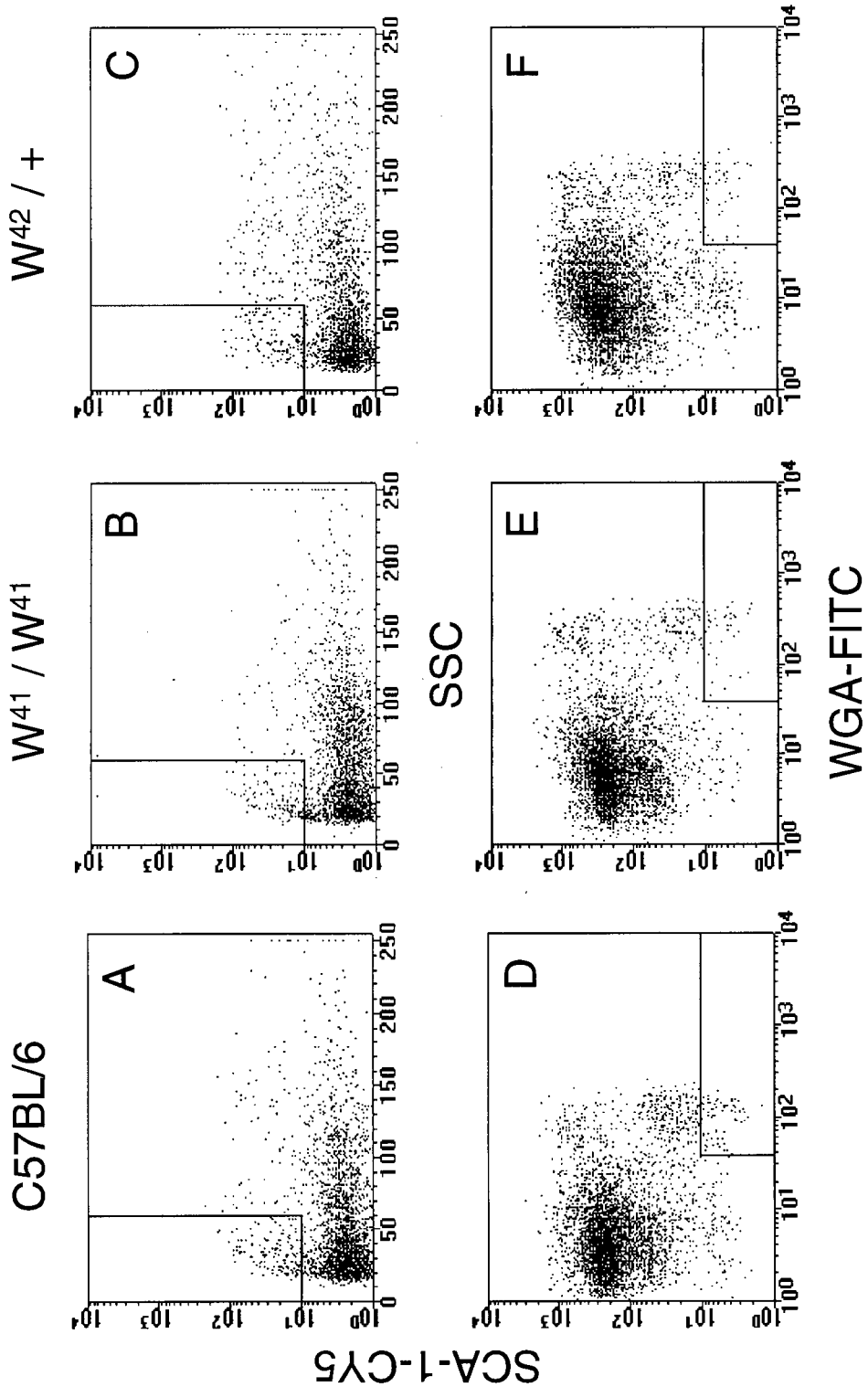
TABLE 2 RESULTS OF CRU ASSAYS PERFORMED ON MARROW CELLS FROM W^{41}/W^{41} AND $W^{42}/+$ MICE

Genotype of Test Cells	No. of Test Cells Injected	Cells Coinjected as a Helper/ Competitor Population	No. of Positive/Total Recipients	(Frequency of CRU x 10^3)$^{-1}$	Total CRU Per Femur
W^{41}/W^{41}	4×10^5	none	6/6		
(unseparated)	2×10^5	none	6/6		
	2×10^5	2×10^5 compromised	4/7	220	50-80
	2×10^5	1×10^5 normal BM	1/6	[170-270]	
	2×10^5	5×10^5 normal BM	2/7		
	2×10^5	2×10^4 normal BM	5/6		
W^{41}/W^{41}	600	1×10^5 normal BM	1/6	2.2	
(Sca-1^+Lin$^-$ WGA^+)	200	1×10^5 normal BM	1/6	[1.1-4.5]	
$W^{42}/+$	2×10^5	2×10^5 compromised	14/15		
(unseparated)	6×10^4	2×10^5 compromised	2/4		
	1.5×10^4	2×10^5 compromised	1/5	88	120-190
	2×10^5	2×10^4 normal BM	5/6	[70-110]	
	2×10^5	5×10^4 normal BM	8/8		
	6×10^4	6×10^4 normal BM	1/5		
+/+ unseparated				13	680 - 2100
				[7.4 - 23]	
+/+ Sca^+ Lin^- WGA^+				.036	
				[.023-.057]	

CRU frequencies were calculated from all data regardless of the type or number of other cells injected since for the variations used these did not appear to significantly affect the values obtained. Derived CRU frequency and total values are shown together with the range [ ] corresponding to ± SEM. Values shown for control (+/+) mice are from (21).

The CRU assay may be considered as the most stringent procedure for quantitating hematopoietic stem cells since it depends on the expression of the properties of pluri-potentiality and extensive and sustained proliferative activity in vivo [24]. However, the detection of CRU also depends on the continuing amplification of their differentiating progeny as well as the retention of other characteristics that may be of particular importance to hematopoietic reconstitution in the myeloablated mouse. It was therefore of interest to evaluate additional indicators of very primitive cell populations in these comparisons of W-mutant and normal mice. One such approach would be to focus on an assessment of Sca-1$^+$ Lin$^-$ WGA$^+$ marrow cell population since in normal animals these constitute a very small proportion (.035 ± 0.02%) of the total marrow population and are enriched for CRU [21]. Examination by FACS analysis of the distribution of these markers on marrow cells from W^{41}/W^{41}, $W^{42}/+$ and +/+ mice revealed the presence of very similar populations in all cases (representative FACS profiles are shown in Figure 4). Analysis of the data obtained from 5 mice of each genotype showed that the total proportion and hence number of W-mutant cells of the Sca-1$^+$ Lin$^-$ WGA$^+$ phenotype (per femur) were not significantly different from normal (Figure 4). Nevertheless, when this subpopulation was isolated from W^{41}/W^{41} marrow by FACS and then assayed for CRU, the frequency of CRU was again found to be markedly reduced and to approximately the same extent as the original population of unseparated W^{41}/W^{41} cells (Table 2: i.e., 50-fold relative to the CRU content of the same phenotypically defined subpopulation of +/+ cells).

Figure 4. FACS profile of +/+ (A and D), W^{41}/W^{41} (B and E) and $W^{42}/+$ (C and F) viable marrow cells analyzed for their expression of Sca-1 versus their orthogonal light scattering characteristics (A to C) and for cells within the gated rectangles shown in A to C then further analyzed for expression of Lin markers and binding of WGA (D to F). The percentage of Sca-1$^+$Lin$^-$WGA$^+$ cells in each starting population was calculated from the number of cells present in the gated rectangles shown in D to F (Lin$^-$WGA$^+$) as a proportion of Sca-1$^+$ cells with low forward and side scatter (SSC) properties. Percent values shown represent mean ± SEM from 5 separate determinations in each case. The range () of numbers of Sca-1$^+$Lin$^-$ WGA$^+$ cells per femur is shown.


C57BL/6
W41 / W41
W42 / +
A
B
C
D
E
F
SCA-1-CY5
SSC
WGA-FITC

The second approach used to evaluate very primitive hematopoietic cells in W^{41}/W^{41} mice was their ability to be detected as LTC-IC. As described elsewhere, we have shown that LTC-IC from normal mice co-purify with CRU in the Sca-1^+ Lin^- WGA^+ fraction of adult marrow cells, thus distinguishing both of these cell types from most cells that are detectable as Day 12 CFU-S [30]. In addition, we evaluated W-mutant marrow cells in a modified version of the LTC-IC procedure that allows the dual lymphopoietic and myelopoietic potential of at least some LTC-IC to be expressed in vitro (hence the term $LTC\text{-}IC_{ML}$ to specifically designate this subset of LTC-IC). The results of limiting dilution assays to quantitate both of these cell types in the marrow of W^{41}/W^{41} mice are shown in Table 3. Both the frequency (and hence the absolute number) of marrow LTC-IC assessed by either of these end points were slightly decreased (3- to 4- fold) relative to +/+ controls, but not nearly to the same extent as the decrease apparent when the CRU assay was used (Table 2).

Sublethally Irradiated W-mutant Mice Support Spleen Colony Formation by CFU-S of +/+ Origin. It has been previously demonstrated that the hematopoietic system of untreated W/W^v mice can be largely replaced by injected +/+ cells although the rate and extent to which this occurs in different lineages varies [33,34]. Interestingly, this finding has also been found to hold true for W^{41}/W^{41} [18] mice even though the W^{41} mutation has only minimal effects on the intrinsic ability of cells to form spleen colonies (Table 1) in contrast to cells from W/W^v mice which do not form visible (macroscopic) spleen colonies [32,35]. It was therefore of interest to determine whether either W^{41}/W^{41} or $W^{42}/+$ mice in the absence of myeloablative treatment could serve as recipients in a standard CFU-S assay of injected +/+ cells. As shown in Table 4, when otherwise untreated, W^{41}/W^{41}, or $W^{42}/+$ mice were injected with 10^5 +/+ marrow cells, no discrete colonies were discernable on the surface of the spleen of any recipient genotype either 9 or 12 days later, although most of the spleens were enlarged and had a mottled appearance. However, when W^{41}/W^{41} or $W^{42}/+$ mice were given 330 cGy of irradiation prior to the injection of 10^5 +/+ cells, colonies of equivalent size and number to those seen in +/+ and $W^{41}/+$ animals given 900 cGy were observed. This was in marked

TABLE 3 RESULTS OF LTC-IC AND LTC-IC_{ML} DETERMINATIONS ON MARROW CELLS FROM W^{41}/W^{41} AND +/+ MICE

Assay	Genotype	No. of Exp'ts	(Frequency x 10^3)$^{-1}$	Total Per Femur
LTC-IC	+/+	4	18 (15 - 21)	860 (730 - 1000)
	W^{41}/W^{41}	4	52 (42 - 64)	250 (200 - 300)
LTC-IC_{ML}	+/+	2	400 (300 - 500)	40 (30 - 45)
	W^{41}/W^{41}	2	1600 (1200 - 2000)	8 (6 - 11)

Values shown are the mean and the range defined by ± SEM calculated from data pooled from 2 to 4 limiting dilution experiments as described in the Methods.

TABLE 4 EFFECT OF RADIATION DOSE ON THE ABILITY OF +/+, $W^{41}/+$, W^{41}/W^{41} AND $W^{42}/+$ RECIPIENTS TO STIMULATE MACROSCOPIC SPLEEN COLONY FORMATION BY +/+ MARROW CELLS

Genotype of Recipient	Radiation Dose to Recipients (cGy)	No. of Spleen Colonies Seen	
		Day 9	Day 12
+/+	330	nd	0 (7)
+/+	900	22 ± 1 (10)	21 ± 1 (15)
$W^{41}/+$	330	nd	0.3 ± 0.2 (6)
$W^{41}/+$	900	16 ± 1.5 (6)	16 ± 1 (11)
W^{41}/W^{41}	0	0 (4)	0.3 ± 0.3 (4)
W^{41}/W^{41}	330	17 ± 1 (5)	25 ± 1 (6)
$W^{42}/+$	0	0 (4)	0.3 ± 0.3 (4)
$W^{42}/+$	330	18 ± 2 (5)	23 ± 2 (10)

Values shown represent the mean ± SEM of counts obtained from (n) recipients in 1 to 3 experiments in which each recipient was injected with 10^5 C57Bl/6J marrow cells and then sacrificed 9 or 12 days later.

nd = not done.

contrast to either +/+ or W^{41}/+ recipients given 330 cGy in which macroscopic spleen colonies were not detectable. Therefore, although untreated W^{41}/W^{41} and W^{42}/+ animals do not support the generation of macroscopically visible spleen colonies from intravenously injected cells, they will do so after irradiation with a significantly lower dose than congenic +/+ recipients. In the absence of any donor cells, 500 cGy was the minimum radiation dose required to cause >10% lethality (within 30 days) in W^{41}/W^{41} and W^{42}/+ mice, respectively whereas the corresponding dose for +/+ (C57Bl/6J) mice was 750 cGy.

Discussion

Parameters Inherent in the Assessment of Progenitors from W-mutant Mice. The present studies were undertaken to investigate the relative importance of SF as a physiological regulator of hematopoiesis in vivo at different stages of progenitor development and maturation. To approach this question we focussed our studies on W^{41}/W^{41}, and in some cases W^{42}/+ mutant mice, since both of these genotypes survive to adulthood and are fertile (thus making their propagation and investigation easier than other severely affected W-mutant genotypes such as W/W^{v}) but, nevertheless, are also anemic and can be competitively repopulated by injected +/+ cells [18]. Moreover, both the W^{41} and W^{42} mutations have been characterized as single base pair changes that result in a reduction in the kinase activity of the activated SF receptor encoded by the W (c-kit) gene [19,20]. In spite of their anemia, neither show detectable changes in the cellularity of their marrow suggesting that other gross perturbations of hematopoiesis might not be expected. On the other hand, compensatory mechanisms may allow normal blood cell counts to be maintained even in the face of marked perturbations of earlier compartments. Therefore we included in our analyses the use of a multiplicity of procedures and end points to directly measure cells at different stages of hematopoietic differentiation from the earliest known totipotent lympho-myeloid progenitors to those at intermediate to late stages on the erythroid, granulopoietic and B-cell pathways.

Nevertheless, in evaluating any given cell type it was important to anticipate the possibility that its detection might be dependent on SF stimulation even though other regulators able to maintain that stage of hematopoiesis might be operative in the normal adult.

The Role of SF in Regulating Erythropoiesis In Vivo. Our findings did not reveal a significant decrease in the number of cells present in W^{41}/W^{41} or $W^{42}/+$ mice at any stage of erythropoietic progenitor differentiation down to the most mature BFU-E, consistent with previous findings for W/W^{v} mice [14]. Previous studies have shown that primitive BFU-E in normal mice are responsive to SF [10,31] but the present findings are the first to demonstrate an effect of SF on the ability of such late murine erythroid progenitors as Day 3 BFU-E to proliferate and differentiate further in vitro. This effect was less marked when the concentration of Ep was very high (although even at 3 U/ml of Ep an enhancing effect of SF could still be seen). Interestingly, W-mutant strains that are anemic have been found to have markedly elevated levels of circulating erythropoietin [36] and are characteristically hypo-responsive to additional erythropoietin administration [37]. This is what might be predicted from the in vitro findings for Day 3 BFU-E described here which demonstrate an ability of increased Ep concentrations to largely, but not completely, compensate for a defective SF/c-kit signalling mechanism.

The Role of SF in Regulating Stem Cell Recruitment Post BMT. A physiological role of SF in regulating the hematopoietic stem cell population has been less well defined. Expression of c-kit on cells with totipotent (lympho-myeloid) differentiation capacity demonstrable both in vivo [38-40] and in vitro [41] has been reported. However, in vitro SF alone has not been shown to promote the self-renewal or even the proliferation of these cells, although it has been postulated that SF may act as a survival factor [42,43]. On the other hand, Ikuta et al have shown the generation of near normal numbers of CFU-S and Sca-1^{+} Lin^{-} Thy-1^{low} cells (which include all transplantable stem cells in the adult animal) during fetal development in Sl/Sl mice [38]. Since these mice are genetically unable to produce SF, these

findings preclude SF as a unique stimulator of hematopoietic stem cell proliferation and/or viability in vivo. Analysis of the role of SF in vivo in adults has been more difficult to study since elimination of this pathway results in embryonic lethality due to the severity of the anemia caused. Nevertheless, in other severe W-mutant genotypes that are viable (e.g., W/W^v) little perturbation of hematopoiesis, other than inadequacies in the generation of red cells and tissue mast cells, is observed. In addition, it has been known for some time that near normal numbers of multipotent myeloid progenitors (CFU-GEMM) are present in such mice from in vitro studies that utilized stimulatory factors we now appreciate can effectively substitute for SF-containing growth factor cocktails (e.g., see Figure 3). However, these multipotent cells also probably overlap little with stringently defined hematopoietic stem cell populations such as those detectable as CRU [25]. These findings therefore still fail to address the question of the in vivo role of SF at the level of the most primitive cells that sustain hematopoiesis in adulthood.

To obtain more direct information on this question, we used a variety of strategies to assess the presence and functional integrity of stem cells from W^{41}/W^{41} and $W^{42}/^{+}$ mice. These revealed a striking disparity between the number of W-mutant cells that could be detected in a standard in vivo CRU assay (Table 2) and the number that could be detected by their possession of a phenotype characteristic of normal CRU ($Sca\text{-}1^{+}$ Lin^{-} WGA^{+}, Figure 4), or by their ability to generate lymphoid and/or myeloid clonogenic progeny after 4-5 weeks of culture in the presence of marrow stromal feeder layers (Table 3), a property that appears to be shared by at least some long-term in vivo repopulating cells [30]. The simplest interpretation of these data is that all of these assays detect cells with the same extensive proliferative and differentiative potentialities but are variably dependent on the integrity of a SF/c-kit signalling mechanism. Thus, stimulation by SF would be implicated as a major mechanism used to promote the rapid generation of all hematopoietic lineages in the myeloablated recipient but would not be anticipated to be part of the mechanism by which stromal support of primitive cells (LTC-IC) is necessarily achieved in the long-term culture system. Interestingly, we have

recently shown this to be the case for human [44] LTC-IC which can be cultured on Sl/Sl fibroblasts as well as on their +/+ counterparts. Similarly, addition to long-term cultures of an antibody to c-kit that blocks SF binding did not alter the maintenance of long-term repopulating cells although later stages of myelopoiesis were depressed [45] [46]. Nevertheless, in the case of human LTC-IC, SF could substitute for the role of feeders in supporting the maintenance of these primitive cells, albeit not their generation of myeloid clonogenic progeny [44].

The Role of SF in Regulating Stem Cell Numbers in the Absence of Myeloablation. What about the role of SF in regulating hematopoietic stem cell activation in the non-ablated adult? If our assumptions about the identity of CRU and LTC-IC are correct, then the demonstration of near normal LTC-IC (and LTC-IC_{ML}) numbers in adult W^{41}/W^{41} mice would suggest that SF may not be a major in vivo regulator of these cells at any stage of ontogeny. However, the fact that a small proportion of the total marrow content of +/+ mice can repopulate all lineages of untreated W^{41}/W^{41} mice, although at variable rates in each lineage, makes it seem more likely that SF normally does have at least a modulating role. Such a model is further supported by the studies reported here showing that W^{41}/W^{41} and $W^{42}/+$ mice will both stimulate the formation of macroscopic spleen colonies by injected +/+ cells after receiving relatively low (nonlethal) doses of irradiation (330 cGy).

The apparently increasing effect of a defective SF receptor on stem cells situated in an environment of increasingly damaged hematopoiesis suggests either that SF is upregulated under such circumstances or, alternatively, that other factors that normally counteract or substitute for the stimulatory effects of SF are down regulated. At present, very little is known about the identity of factors that can act directly on CRU or LTC-IC to regulate their proliferative state. However, it seems likely from studies of closely related populations that a number of cytokines will have redundant actions on these cells [47], many of which are known to be produced by murine stromal cells [48]. The fact that a defective stem cell response to SF can have any detectable physiological consequence would therefore lend support to a minimal

model of normal hematopoietic cell regulation in which the developing progenitors are typically exposed to limiting concentrations of multiple stimulators which alone would be insufficient to maintain an adequate output of mature blood cells.

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