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A Characterization of Early B-Lymphopoiesis: The Differentiation of Lymphohemopoietic and Early Committed B-Lymphoid Progenitors

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A CHARACTERIZATION OF EARLY B-LYMPHOPOIESIS: THE DIFFERENTIATION OF LYMPHOHEMOPOIETIC AND EARLY COMMITTED B-LYMPHOID PROGENITORS

by

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TABLE OF CONTENTS

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ABSTRACT

TIMOTHY CHARLES BALL. Characterization of Early B-Lymphopoiesis: The Differentiation of Lymphohemopoietic and Early Committed B-Lymphoid Progenitors. (Under the direction of Dr. MAKIO OGAWA)

Studies of B-lymphopoiesis have long been hindered by the lack of assays capable of supporting the differentiation and identification of lymphohemopoietic and early committed B-lymphoid progenitors. The clonal identification of cell-cycle dormant lymphohemopoietic progenitors from mice treated with 5 flurouracil (5-FU) became possible with the development of the two-step assay for lymphohemopoietic progenitors. Studies presented in this dissertation are centered around elucidation of the processes of differentiation of lymphohemopoietic progenitors in an attempt to determine the sequence of events occurring in divergence of the B-lymphoid and myeloid lineages. To address this question a single-step assay capable of supporting the differentiation of proliferating lymphohemopoietic and early committed B-lymphoid progenitors was developed. This assay makes use of semisolid methylcellulose culture, in situ microscopic examination and flow cytometric analysis to identify progenitors with lymphohemopoietic and B-lymphoid potential. Using this assay the lymphohemopoietic progenitors present in normal mice were characterized, and of these, approximately 75% were found to be sensitive to the cytotoxic effects of 5-FU. The proliferation and commitment of primitive lymphohemopoietic progenitors was then examined, and found to proceed through 3 stages a lymphohemopoietic proliferative stage, a commitment stage and an early committed B-lymphoid proliferative stage. Finally using a modified version of the two-step assay, the

lineage potential of individual lineage negative Ly 6A/E (Sca-1) positive progenitors from normal mice were examined. Assuming the culture system is equally permissive for the differentiation of all myeloid lineages, the results support a model of random restriction of lineage potential with a late divergence of the B-lymphoid and the myeloid lineages, possibly resulting in the formation of a bipotential B-lymphoid/macrophage progenitor.

Chapter I

General Introduction

Timothy C. Ball

INTRODUCTION

Hemopoiesis is the process of the formation of the eight different types of mature blood cells from small a population of primitive stem cells. Hemopoietic stem cells are the most primitive of hemopoietic progenitor cells. They have the capability to give rise to all hemopoietic cells as well as having unlimited self-renewal capacity. Hemopoiesis has been subdivided into myelopoiesis, the production of myeloid lineage cells, and lymphopoiesis, the production of lymphoid lineage cells. The cells composing the myeloid lineage are monocytes/macrophages, megakaryocytes, erythrocytes and granulocytes. Three types of cells are classified as granulocytes based on their extremely well developed cytoplasmic granules: neutrophils, eosinophils and basophils. B- and T-lymphocytes comprise the lymphoid lineage. The designation of B- and T-lymphocytes was based on the recognition that the bursa of Fabricius, first described by Fabricius in 1621 (1), is the site of B-lymphopoiesis in birds as is best reviewed by Warner et al. (2). T-lymphopoiesis takes place in the thymus (3). Pluripotent cells are cells that have the capacity to generate the entire complement of myeloid and lymphoid lineage cells. Pluripotent stem cells reside in the bone marrow, and bone marrow is the normal site of both myelopoiesis and B-lymphopoiesis (4-7).

A brief discussion of the most important and commonly used terminology will now be presented in an attempt to ensure a clear understanding of the presented concepts, in addition, Table 1-1 has been provided as a handy reference for explanation of the terminology used throughout this text. As mentioned, a lymphohemopoietic progenitor is a progenitor that has the potential to give rise to cells of both the lymphoid and myeloid lineages. Since this study is limited to the B-lymphoid lineage, T-lymphoid potential, although important will not be considered. A progenitor is any cell that has proliferative

capacity, or the ability to give rise to a daughter cell. A terminally mature erythrocyte or neutrophil that no longer has the capacity to divide cannot be designated a progenitor. Progenitors can be additionally described as primitive or mature. These terms are used to describe the relative position of a progenitor in the hierarchy of differentiation. A restricted progenitor is a progenitor that is believed to be past the point of no return, or restricted to a certain fate. Thus a B-lymphoid restricted progenitor is one that is restricted to the production of B-lymphoid lineage cells, while a myeloid restricted progenitor can only give rise to myeloid lineage cells. The term stem cell is commonly used in referring to a cell with an extended capacity to self-renew as was done by Abramson (8). In my discussion, I will use the term stem cell very cautiously to denote only cells with unlimited capacity to self-renew and form mature cells of all hemopoietic lineages.

In 1956, Ford demonstrated donor bone marrow transplanted into an irradiated host reconstituted the hemopoietic system generating a chimera (9). However, it was not until 1977, that Abramson et al. unequivocally demonstrated the existence of a pluripotent hemopoietic stem cell capable of generating functional mature cells of both the myeloid and lymphoid lineages (8). This was achieved following the procedure of Wu et al. (10), generating chromosomal aberrations in stem cells with mild irradiation and reconstituting lethally irradiated mice with these mildly irradiated cells. The mice were then sacrificed and their bone marrow, spleen, lymph nodes and thymus were examined for the expression of functional mature B, T and myeloid lineage cells with identical chromosomal markers. Abramson injected irradiated donor marrow preparations into a total of 28 recipient mice and examined the resultant progeny 8 to 12 months after transplantation. A total of 10 mice were found to have high concentrations of marked cells. Of the 10 mice, 5 were found to have identically labeled cells in both the myeloid and the lymphoid lineages, while in three, only marked myeloid lineage cells could be identified and in the remaining 2 marked cells limited to the T-lymphoid lineage were found. Prior to designating the progenitors "restricted" they were transferred into secondary recipients to ensure that they continued to

demonstrate limited lineage expression. After ruling out artifacts such as stochastic variation and irradiation induced leukemia, restriction of lineage potential and aberration of pluripotent stem cells, the conclusion was drawn that they had isolated pluripotent, myeloid and T-lymphoid restricted stem cells. With their demonstration of pluripotent, myeloid and T-lymphoid restricted stem cells, a view of hemopoietic differentiation that envisioned an extremely early divergence of the lymphoid and the myeloid lineages was accepted, Figure 1-1. Since then, several investigators have corroborated the observations of Abramson, demonstrating the existence of pluripotent, myeloid and T-lymphoid restricted stem cells (11-13). In 1980, Harrison developed the competitive repopulation assay in which lethally irradiated mice are reconstituted with two different populations of marrow, a known competitor and an unknown donor population, that can be readily differentiated (14). Using simple statistical analyses of the resultant data and mathematical modeling, biological phenomenon regarding the repopulation of the hemopoietic system can be inferred. For example, by examining the correlation between T cell, B cell, granulocyte and platelet production by progenitors transplanted into recipient mice, inferences of the lineage potential of the progenitors is possible. In short, their analyses revealed no significant evidence of repopulation by lymphoid or myeloid restricted progenitors at any time after transplantation (15, 16). In all actuality, this assay may also produce more true to life results than other assay systems due to the reduction in purification trauma and environmental stress to which the progenitors are subjected prior to transplantation. Still, the conventional view of hemopoietic differentiation in which primitive lymphohemopoietic progenitors give rise to myeloid and lymphoid restricted progenitors holds.

In examining the process of B-lymphopoiesis, two investigators have developed models of B-lymphopoiesis that are contradictory to the conventional view of hemopoiesis. Davidson et al. in 1988 examined the relationships between B-lymphoid and macrophage differentiation using a transformed bone marrow derived cell line that coexpressed the Ly-1, B220 and Mac-1 antigens. B220 and Mac-1 are believed to be specific for cells of the

B-lymphoid and the macrophage lineages, respectively. They determined that individual cells of this cell line were capable of giving rise to Ly-1$^+$ B220$^+$ B cells and functional Ly-1$^+$ Mac-1$^+$ macrophages. These bipotential Ly-1$^+$, B220$^+$ and Mac-1$^+$ progenitors are believed to give rise to the Ly-1$^+$ subset of B cells which reside primarily in the pleural, pericardial and peritoneal cavities. With these observations they developed a model in which lymphohemopoietic progenitors give rise to bipotential B-lymphoid/macrophage progenitors which in turn generate functional Ly-1$^+$ B cells and macrophages (17).

Cumano et al. developed another model of B-lymphopoiesis after their identification of a population of bipotential B-lymphoid/macrophage progenitors in the livers of fetal mice that were a discreet population separate from the population of lymphohemopoietic stem cells (18). As in the model of Davidson et al. they also postulate the existence of a bipotential B-lymphoid macrophage progenitor. According to their model, there are seven phases of development through which a lymphohemopoietic progenitor passes in the generation of a functional immunoglobulin secreting plasma cell. In their model, phase I begins with a primitive lymphohemopoietic progenitor present in a developing fetus that gives rise to bipotential B-lymphoid/macrophage progenitors. In this phase the lymphohemopoietic progenitors can be identified by their expression of the AA4 antigen. In culture, these cells require the synergistic interaction of IL-11 in combination with c-kit ligand to initiate and support their proliferation and differentiation. Phase II of differentiation is composed of bipotential B-lymphoid/macrophage progenitors that express AA4 and Ly-6A antigens. These cells also proliferate and differentiate in the presence of IL-11 and SF. Phase II cells, then give rise to B-lymphoid restricted progenitors that are dependent on the synergistic interaction of SF and IL-7 to support continued proliferation and differentiation and macrophage progenitors. It is in Phase III, that the expression of the pan-B cell antigen, B220, occurs. As B-lymphoid progenitors enter phase IV, they loose their responsiveness to IL-7, and the transition into phase V is marked by the expression of surface IgM. The remaining phases of B-lymphopoiesis are of very late

stage B cell progenitors, and are beyond the scope of this discussion. Thus, although the conventional view of hemopoiesis, with its early divergence of the lymphoid and myeloid lineages is the most widely accepted model of hemopoiesis, models of B-lymphopoiesis incorporating a tangible bipotential B-lymphoid/macrophage progenitor also exist.

CHARACTERIZATION OF B-LYMPHOPOIESIS.

The progression of the characterization of B-lymphopoiesis from the early 1970's to present is shown in Figure 1-2. One of the more striking features of this figure is the advancement in the identification and characterization of progressively more primitive B-lymphoid progenitors over time. Initially using sedimentation velocity, Miller et al. (19) characterized the progenitor B (PB), B1 and B2 stages of B cell differentiation, all of which were positive for the expression of surface immunoglobulin. Hammerling et al. (20) examined the expression of the inducible immunoglobulin (Ig), immune response antigen (Ia) and C3 complement receptor (CR) surface antigens and further modeled B-lymphopoiesis by surface Ig^+ cells. Coffman et al. (21) and Kincade et al. (22) were able to advance the study B-lymphoid progenitors to the level of those progenitors that were negative for the expression of surface Ig by isolating monoclonal antibodies to B220. Finally, Hardy et al. (23) focused on characterizing the substages of B-lymphoid progenitors positive for the expression of B220. In this characterization, based on the expression, or lack thereof of, B220, CD43, HSA and BP-1 surface antigens, 5 distinct substages: A, B, C, C' and D were identified. By identifying such specific subpopulations, examination of the immunoglobulin gene rearrangement status resulted in a clear characterization of the progression of this process.

Surface Ig^+ progenitor B cells and their differentiation.

In 1972 Lafleur developed the first quantitative assay for B-cell precursors, the PB cell assay (24). The *in vitro* PB cell assay requires first, that populations of B cell progenitors be injected into lethally irradiated mice. Seven days after transplantation, the mice are immunized intraperitoneally with sheep erythrocytes. Eight days after immunization the mice are sacrificed and the number of plaque forming cells (PFC) per spleen is determined. The number of PFC per spleen is directly proportional to the number

of PB cells initially injected. Similarly, other populations of progenitors can be assayed for their potential to give rise to mature antigen responsive B-cells by modifying the duration of time between transplantation and antigenic challenge.

While studying the characteristics of PB cells, it was found that they were positive for the expression of surface immunoglobulin but were not responsive to antigenic challenge. Closer examination of the B cell progenitors revealed the presence of an intermediate B cell progenitor with a sedimentation velocity between that of the PB cells and mature B cells. These intermediate cells were designated B1 cells while mature B cells were designated B2 cells (25). These investigations led to the characterization of the progression of B-lymphopoiesis from a surface Ig^+ progenitor B (PB) cell to an antigen responsive B cell progenitor (B1) cell to a mature (B2) cell (26).

Expression of inducible surface antigens.

Hammerling analyzed the expression of 3 inducible surface antigens immunoglobulin (Ig), the antigen associated with the immune response gene (Ia) and the complement C3 receptor (CR) in an attempt to characterize B-lymphopoiesis (23). Bone marrow and spleen preparations were isolated and negative cytolytic selection to independently remove Ig^+ cells, Ia^+ cells and CR^+ cells was performed (24-27). After cytolysis of the Ig^+ cells, induction resulted only in the formation of Ig^+ cells. After cytolysis of Ia^+ cells, induction resulted in the formation of Ig^+ and Ia^+ cells. Finally, induction of the cells remaining after cytolysis of CR^+ cells resulted in the formation of Ig^+, Ia^+ and CR^+ cells. They therefore concluded, B-lymphopoiesis proceeds from the most primitive B cell progenitors that were Ig^-, Ia^-, CR^- cells to Ig^+, Ia^-, CR^- cells to Ig^+, Ia^+, CR^- cells to mature Ig^+,Ia^+,CR^+ cells.

Monoclonal antibodies and the characterization of B-lymphopoiesis.

In an attempt to dissect the early stages of B-lymphopoiesis, monoclonal antibodies were utilized to examine the expression of the Qa antigen by primitive hemopoietic and committed B-lymphoid progenitors (31-33). Qa was found to be expressed by pluripotent stem cells, granulocyte-macrophage progenitors and by B cells and their progenitors. Therefore, Qa was ineffective for use in enrichment or isolation of B-lymphoid precursors because of its nonspecific pattern of expression. The monoclonal antibody 19B5 (34), was found to bind hemopoietic cells in the marrow that were sensitive to infection by the Ableson murine leukemia virus. Paige et al. demonstrated that 19B5 was expressed by the earliest identifiable committed B-lymphoid progenitors, and was not expressed by multipotential stem cells (35). Later, monoclonal antibodies directed against the Lyb-1 and Lyb-2 antigens were found to be present on mature B cells and a small fraction of surface Ig negative cells present in bone marrow (36, 37). However, these antigens although expressed by B cells and their progenitors proved to have little usefulness due to their lack of specificity for populations of committed B-lymphoid progenitors.

In 1981 two monoclonal antibodies, 14.8 and RA3 3A1, were independently isolated and characterized by Kincade et al. (22) and Coffman et al., (21) respectively that have revolutionized the study of B-lymphopoiesis by surface Ig negative B-lymphoid progenitors. In the mid 1970's Trowbridge et al. characterized the expression of a surface glycoprotein with a molecular weight of approximately 220,000 daltons that was a member of the T200 family of lymphoid glycoproteins that appeared to be specifically expressed by cells of the B-lymphoid lineage (38, 39). The T200 family of glycoproteins is composed of 3 members with molecular weights of 170,000, 180,000 and 220,000 daltons (39). The expression of the 220,000 dalton protein was restricted to B cells and their progenitors, with the exception of a small population of activated T-lymphocytes present in enlarged lymph nodes (22, 41, 42). Coffman et al. proposed that this antigen be designated B220, denoting its molecular weight and its specificity to the B-lymphoid lineage (40). Further

characterization of the B-lymphoid progenitors positive for the expression of B220 revealed the earliest B-lymphoid progenitors expressing B220 were large Ig negative cells. Populations of large and small cytoplasmic μ chain positive pre-B cells and small B cells were positive for the expression of B220 (22).

Having identified a B-lymphoid specific marker, B220, Kincade staged the expression of the antigens Qa, 19B5, 14.8 and surface Ig and characterized B-lymphopoiesis and the progression from primitive multipotential cells to mature B cells (43). Multipotential stem cells were characterized by their expression of Qa. The expression of 19B5 by Qa positive cells marked commitment to the B-lymphoid lineage. Eventually Qa^+ $19B5^+$ cells expressed B220. The B-lymphoid progenitors positive for the expression of B220 were subdivided into large Ig^- pre-B cells, large cytoplasmic μ^+ pre-B cells, small cytoplasmic μ^+ pre-B cells and small mature B cells (43).

Staging of $B220^+$ surface Ig^- B-lymphoid progenitors

Hardy et al. pursued further characterization of B-lymphopoiesis by making use of multicolor flow cytometry to isolate specific populations of surface Ig negative B-lymphoid progenitors, based on their differential expression of CD43, HSA , BP-1 (23, 44) and the pan-B cell antigen B220. Upon obtaining a population of $B220^+$ $CD43^+$ cells, they subdivided this population into 3 fractions: HSA^- $Bp\text{-}1^-$; HSA^+ $BP\text{-}1^-$ and HSA^+ $BP\text{-}1^+$. The remaining population of $B220^+$ $CD43^-$ cells were found to be HSA^+ and $BP\text{-}1^+$. By sorting, homogenous populations of each of the 4 fractions were obtained. Culture of these homogenous populations in the presence of the ST2 stromal cell line and IL-7, alone and in combination, revealed the progression of differentiation was most likely from $B220^+$ $CD43^+$ HSA^- $BP\text{-}1^-$ to $B220^+$ $CD43^+$ HSA^+ $BP\text{-}1^-$ to $B220^+$ $CD43^+$ HSA^+ $BP\text{-}1^+$ to $B220^+$ $CD43^+$ HSA^{+++} $BP\text{-}1^+$ ending with $B220^+$ $CD43^-$ HSA^+ $BP\text{-}1^+$, and these stages were designated A, B, C, C' and D respectively. With the isolation of such pure populations of B-lymphoid progenitors, the status of the Ig gene rearrangement of the

progenitors present in each stage was determined. These studies revealed that the Ig genes of cells in stage A were in germ line configuration, whereas 60% and 85% of the cells present in stages B and C exhibited D-JH rearrangement respectively. It was not until stage D that V rearrangements could be detected and 95% of these cells were positive for the D-JH rearrangement. These studies reveal that, with sufficient enrichment, studies of the molecular events of the various stages of B-lymphopoiesis can be examined.

IN VITRO B-LYMPHOPOIESIS.

To study the growth of pre-B cells infected with the Ableson murine leukemia virus *in vitro*, Whitlock and Witte modified the long term Dexter culture for myelopoiesis (45, 46). Their modifications included, increasing the culture temperature from 33 to 37 degrees Celsius and eliminating the addition of corticosteroids, known to stimulate granulopoiesis (47) while inhibiting several stages of B cell maturation (48). In their studies they observed the growth of B cells and their progenitors in the uninfected control cultures. Realizing the importance of this observation, they established a long term culture assay capable of supporting the differentiation of B cells and their progenitors. In 1982 Whitlock and Witte demonstrated that their modified Dexter cultures were capable of supporting extended *in vitro* B-lymphopoiesis for more than 1 year (49). Briefly, the entire marrow compartment of mice less than 4 weeks old was cultured at 37 degrees C in the presence of low concentrations of horse serum, 2 mercaptoethanol (ME) and the absence of corticosteroids. The cultured bone marrow cells proceeded through 4 distinct phases of growth: 1) the proliferation of adherent cells to confluency that ends with a decrease in the nonadherent cells; 2) the crisis phase, in which the nonadherent cells decline; 3) the proliferation of B-lymphoid progenitors; and 4) the rapid proliferation of sub populations of B-lineage cells (50).

Growth factor requirements of B-lymphopoiesis.

The differentiation of the B-lymphoid progenitors in the Whitlock-Witte culture system required close association and interaction between the stromal cell elements and the differentiating B-lymphoid progenitors. Stromal cell layers established from crude bone marrow preparations, as in the Whitlock-Witte system, are composed of adipocytes, fibroblasts, endothelial cells and other formed marrow elements. Therefore, to more completely define and understand the required interactions between individual types of

stromal cells and B-lymphoid progenitors, investigators isolated stromal cell lines that were capable of supporting B-lymphopoiesis or limited B-lymphopoiesis (51-65).

While in search of soluble mediators capable of supporting the differentiation of B-lymphoid progenitors, Namen et al. established the IxN/A6 cell line by transfecting bone marrow stromal cell layers with SV40 (66). IxN/A6 secreted a substance that was capable of supporting the differentiation of B-lymphoid progenitors isolated from Whitlock-Witte cultures. The factor secreted by IxN/A6, originally designated BP-1, synergistically enhanced the proliferation of B-lymphoid progenitors in serum-free cultures stimulated with LPS. Later, in 1988 Namen et al. cloned the gene of BP-1, purified the protein and designated the product interleukin (IL) -7 (67). Further characterization of the role of IL-7 in support of B-lymphopoiesis revealed that IL-7 was capable of supporting the proliferation of $B220^-$ and $B220^+$ progenitors isolated from long term Whitlock-Witte cultures.

As IL-7 was being characterized, the roles of the stromal cell clones ST2 (68) and PA6 (69) in B-lymphopoiesis were being examined. They demonstrated that ST2 was capable of supporting the complete differentiation of the B-lymphoid lineage. However, PA6 was unable to support B-lymphopoiesis by similar populations of B-lymphoid progenitors. In an attempt to determine if the PA6 stromal cell line was only permissive for the expression of the myeloid lineage, transfer experiments were conducted (70). These experiments revealed that PA6 was capable of supporting limited B-lymphopoiesis. Transfer of the arrested B-lymphoid progenitors to ST2 conditions permitted continued differentiation. Therefore, they concluded that B-lymphopoiesis required the presence of at least two major regulatory signals (70). Having been identified as a cytokine that supports B-lymphopoiesis by both $B220^-$ and $B220^+$ progenitors, it was logical to wonder if IL-7 was the regulatory signal that the PA6 cell line was lacking. Addition of IL-7 to PA6 cultures generated permissive conditions for B-lymphopoiesis, similar to the conditions of the ST2 cultures (68, 69). These observations revealed that the most primitive B-lymphoid

progenitors required the presence of the PA6 factor to proliferate and differentiate, then the combined interaction of PA6 factor and IL-7 was required, resulting in a progenitor that was capable of proliferation in the presence of IL-7 alone (71, 72).

In 1990 Zsebo et al. cloned the ligand for the murine c-kit tyrosine kinase receptor. This ligand/cytokine has been referred to as mast cell growth factor, stem cell growth factor, the ligand for c-kit and Steel factor (73). In this manuscript I will refer to this ligand as Steel factor (SF), as it is the factor that is deficient in the classically described Steel mutant mice (74). In 1992 McNiece et al. examined the role of SF in B-lymphopoiesis (75). Interestingly, SF was found to synergize with IL-7 in support of the formation of pre-B cell colonies by $B220^-$ B-lymphoid progenitors from normal murine bone marrow in agar culture.

The clonal identification of lymphohemopoietic progenitors from 5-FU treated mice.

Knowing that SF and IL-7 synergized in support of the proliferation and differentiation of $B220^-$ B-lymphoid progenitors in *in vitro* culture (75), Hirayama et al. in 1992 developed a culture assay capable of the clonal identification of lymphohemopoietic progenitors in the absence of stromal cell support (76). In this assay, primitive hemopoietic progenitors from mice treated with 5 flurouracil were enriched by a combination of density gradient centrifugation, negative immunomagnetic selection removing committed cells expressing Mac-1, Gr-1, TER119, L3T4, Lyt2, and B220 and fluorescence activated cell sorting (77). Cells positive for the expression of the Ly 6A/E (Sca-1) antigen (78) were sorted, and the cells present in this population were designated Lin^- $Sca\text{-}1^+$ cells. The enriched population of Lin^- $Sca\text{-}1^+$ cells were cultured in the presence of pokeweed mitogen spleen cell conditioned medium (PWM-SCM), SF, IL-7 and erythropoietin for 11 days. After 11 days of primary culture, the primary colonies were individually harvested, washed and divided into two aliquots, one for replating in myeloid suspension culture for identification of myeloid lineage expression and the other

for replating in B-lymphoid semisolid culture for identification of pre-B cell colonies. Two, 4 and 6 days after the initiation of the secondary culture, the cytology of the myeloid cells present in suspension culture was examined. The B-lymphoid cultures were examined for the presence of pre-B cell colonies after 12 days of secondary culture. Additional investigations revealed that the purified cytokines IL-11, IL-6 and G-CSF were capable of replacing PWM-SCM with no deleterious effects.

Having laid the foundation of the modeling, characterization and *in vitro* culture of B-lymphopoiesis, the investigations conducted in pursuit of the hypothesis: "Commitment to the individual myeloid and B-lymphoid lineages occurs randomly as a single event rather than as two events, commitment to myeloid and lymphoid lineage and later commitment of the myeloid and lymphoid restricted progenitors to the individual myeloid and lymphoid lineages." will be presented in the following chapters.

Table 1-1. Definitions of commonly used terms

B-lymphoid potential- gives rise to cells of the B-lymphoid lineage.
B-lymphoid progenitors- cells capable of giving rise to cells of the B-lymphoid lineage.
B-lymphopoiesis- the process of producing B-lymphoid lineage cells.
Bipotential B-lymphoid/macrophage progenitor- a cell capable of giving rise only to cells of the B-lymphoid and macrophage lineages.
Cell-cycle dormant- a progenitor that resides in the stage G_0 and is not engaged in replication stages G_1, S, G_2, or M of the cell cycle.
Clonal origin- derived from a single cell.
Colony forming unit- a cell capable of giving rise to a colony.
Colony forming unit spleen (CFU-S)- a hemopoietic progenitor capable of seeding in the spleen when transplanted into a lethally irradiated mouse, and forming a macroscopic hemopoietic colony in the spleen.
Day 8 CFU-S- progenitors forming hemopoietic colonies in the spleen of recipient mice 8 days after transplantation . A large majority of these progenitors are sensitive to the cytotoxic effects of the chemotherapeutic drug 5-FU, and are believed to be actively proliferating.
Day 12 CFU-S- progenitors forming hemopoietic colonies in the spleen of recipient mice 12 days after transplantation. These progenitors are resistant to the cytotoxic effects of 5-FU and are believed to be cell cycle dormant progenitors.
Commitment- the process of lineage restriction.
Committed- restricted to a certain pathway of differentiation.
Conventional view of hemopoietic differentiation- a view of hemopoietic differentiation that holds that hemopoietic stem cells reside in G_0, and when they begin the process of hemopoiesis, the myeloid and lymphoid lineage potentials quickly diverge giving rise to myeloid and lymphoid restricted progenitors with limited self-renewal capacity.
Differentiation- the process of becoming more mature.
Early acting triggering factor- a cytokine capable of triggering a cell cycle dormant hemopoietic progenitor to begin proliferating.
Enriched progenitors- a population of progenitors that contains a relatively high incidence of the progenitor of interest.
Flow cytometric analysis- examination of the antigen expression of a population of cells using fluorescent labeled antibodies and a flow cytometer.
Flow cytometric sorting- the isolation of a specific population of cells based on their size, internal structure and surface antigen expression using a fluorescence activated cell sorter (FACS).
Hemopoiesis- the process of generating blood/ hemopoietic cells of any or all lineages: neutrophils, macrophages, mast cells, erythrocytes, megakaryocytes, B-lymphoid and T-lymphoid cells.
Hemopoietic progenitor- a cell capable of giving rise to blood cells.
In-situ- refers to the presence of a hemopoietic colony in the medium in which it developed, i.e. methylcellulose.
Intermediate acting supportive factor- a cytokine which supports the survival and differentiation of primitive hemopoietic progenitors actively proliferating.
Late acting proliferative factor- a cytokine which supports the most terminal stages of differentiation of hemopoietic progenitors.
Lymphohemopoietic potential- the potential to give rise to cells of the myeloid and the lymphoid lineages.

Table 1-1. continued

Lymphohemopoietic progenitor- a cell capable of giving rise to cell of both the B-lymphoid and the myeloid lineages.
Lymphoid lineage- of or pertaining to B- and T lymphoid cells.
Lymphoid potential- having the ability to give rise to cells of both the B- and T-lymphoid lymphoid lineage
Lymphoid restricted progenitor- a progenitor with the capacity to give rise to cells of both the B- and T-lymphoid lineages.
Lymphomyeloid colony- a colony containing both B-lymphoid and myeloid lineage cells.
Lymphopoiesis- the segment of hemopoiesis pertaining to the production of lymphoid lineage cells.
Mature- a term that places the point of hemopoietic differentiation relatively closer to that of being terminally differentiated.
Micromanipulation- the process of the harvest and placement of an individual cell under direct microscopic visualization.
Myeloid lineage- of or pertaining to neutrophil, basophil, eosinophil, macrophage, monocyte, erythroid, megakaryocyte cells.
Myeloid potential- having the ability to give rise to cells of the myeloid lineage.
Myeloid restricted progenitor- a cell whose lineage expression is restricted to that of the myeloid lineage.
Myelopoiesis- the segment of hemopoiesis pertaining to the production of myeloid lineage cells.
Non-synchronized population-A population of progenitors that contains individual progenitors at varying stages of differentiation.
Pluripotent- having the ability to give rise to all blood cells: B-, T-lymphoid and myeloid lineage cells.
Pre-B cell- a B-lymphoid progenitor that has cytoplasmic immunoglobulin mu chains present in its cytoplasm and is positive for the expression of the B220 surface antigen.
Pre-B cell cluster- an identifiable aggregate of pre-B cells that is present in a lymphomyeloid colony.
Pre-B cell colony- a clonal colony of pre-B cells.
Primitive- a term that places the point of hemopoietic differentiation relatively closer to that of hemopoietic stem cells.
Primitive hemopoietic progenitors- progenitors that are formed by stem cells and remain very undifferentiated, having extensive proliferative and differentiative capacity but limited self-renewal capacity.
Pro-B cell- a B-lymphoid progenitor that gives rise to pre-B cells
Progenitor- a cell capable of giving rise to another cell.
Progeny- the resultant product of a cell division.
Proliferating- cells actively engaged in the cell cycle resulting in cell division.
Self-renewal- a cell division which gives rise to at least one daughter cell that is identical to the parent, associated with no differentiation.
Spleen colony- a colony of hemopoietic cells of clonal origin present in the spleen of a lethally irradiated mouse reconstituted with donor marrow.
Spleen colony assay- the first quantitative assay for the identification of primitive hemopoietic progenitors which requires the lethal irradiation of a recipient mouse followed by the transplantation of specific donor mouse hemopoietic progenitors, and the subsequent harvest and examination of the recipient mouse spleen for macroscopic hemopoietic colonies designated spleen colonies.

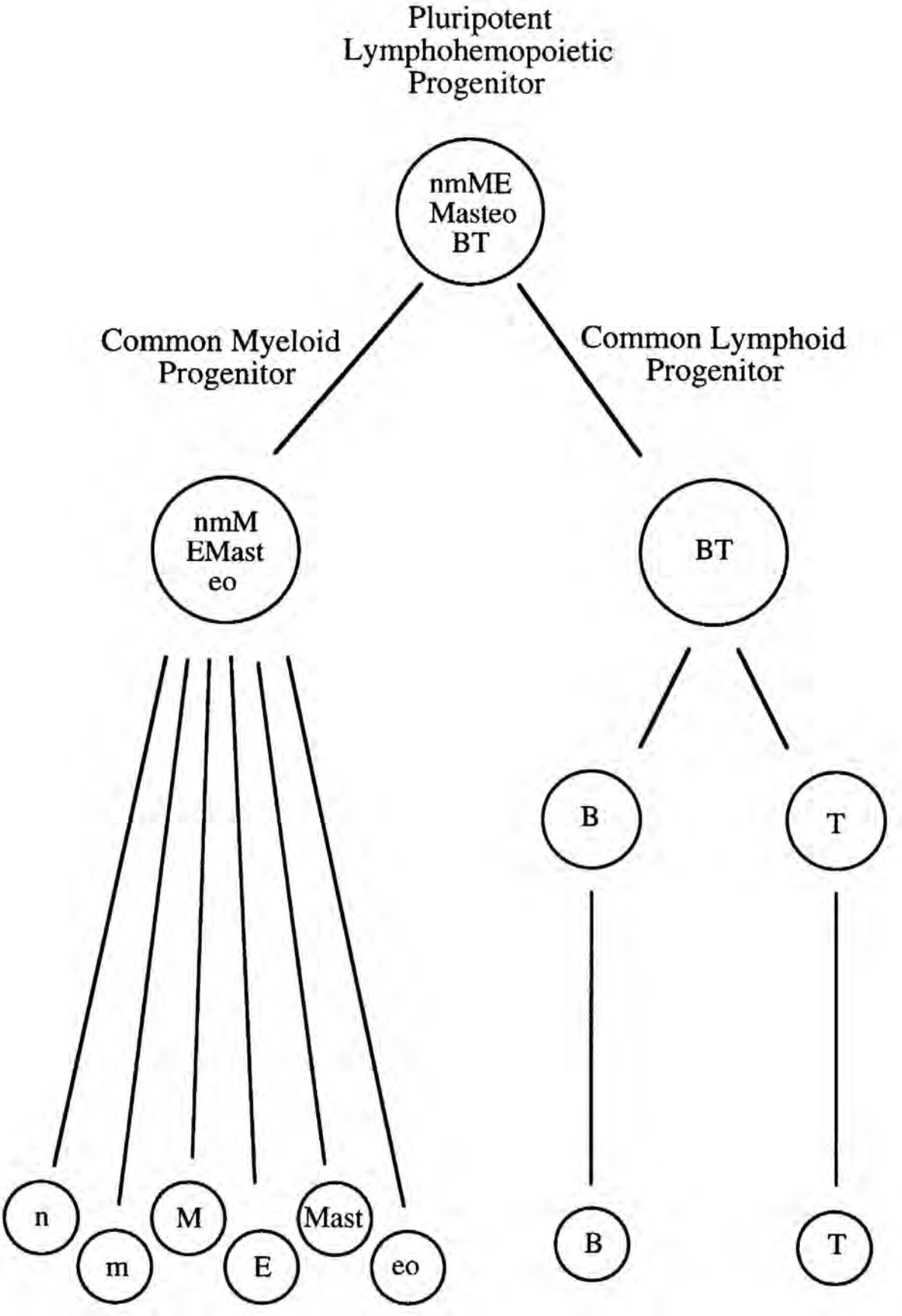
Table 1-1. continued

Steel Factor (SF)- a cytokine/ligand that is the ligand for the c-kit receptor. This cytokine in the past has been referred to as mast cell growth factor, stem cell factor and the ligand for the c-kit receptor.

Stem cells- the most primitive of hemopoietic progenitors with myeloid and lymphoid lineage potential and unlimited self-renewal potential.

Stochastic model of hemopoiesis- a model of hemopoiesis that holds the events governing hemopoietic cell commitment and proliferation are based in randomness, and that external factors are supportive and are not able to influence these processes.

Figure 1-1. The conventional view of hemopoietic differentiation. Only the most primitive hemopoietic progenitors are believed to retain lymphoid and myeloid potential. These progenitors are believed to give rise to common myeloid and common lymphoid progenitors. These common progenitors are believed to proliferate and result in the formation of the mature myeloid lineages: neutrophil, n; macrophage, m; erythroid, E; megakaryocyte, M; mast cell, Mast; eosinophil, eo and the lymphoid lineages B cells and T cells independently.


Pluripotent
Lymphohemopoietic
Progenitor
nmME
Masteo
BT
Common Myeloid
Progenitor
Common Lymphoid
Progenitor
nmM
EMast
eo
BT
B
T
n
m
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Mast
eo
B
T

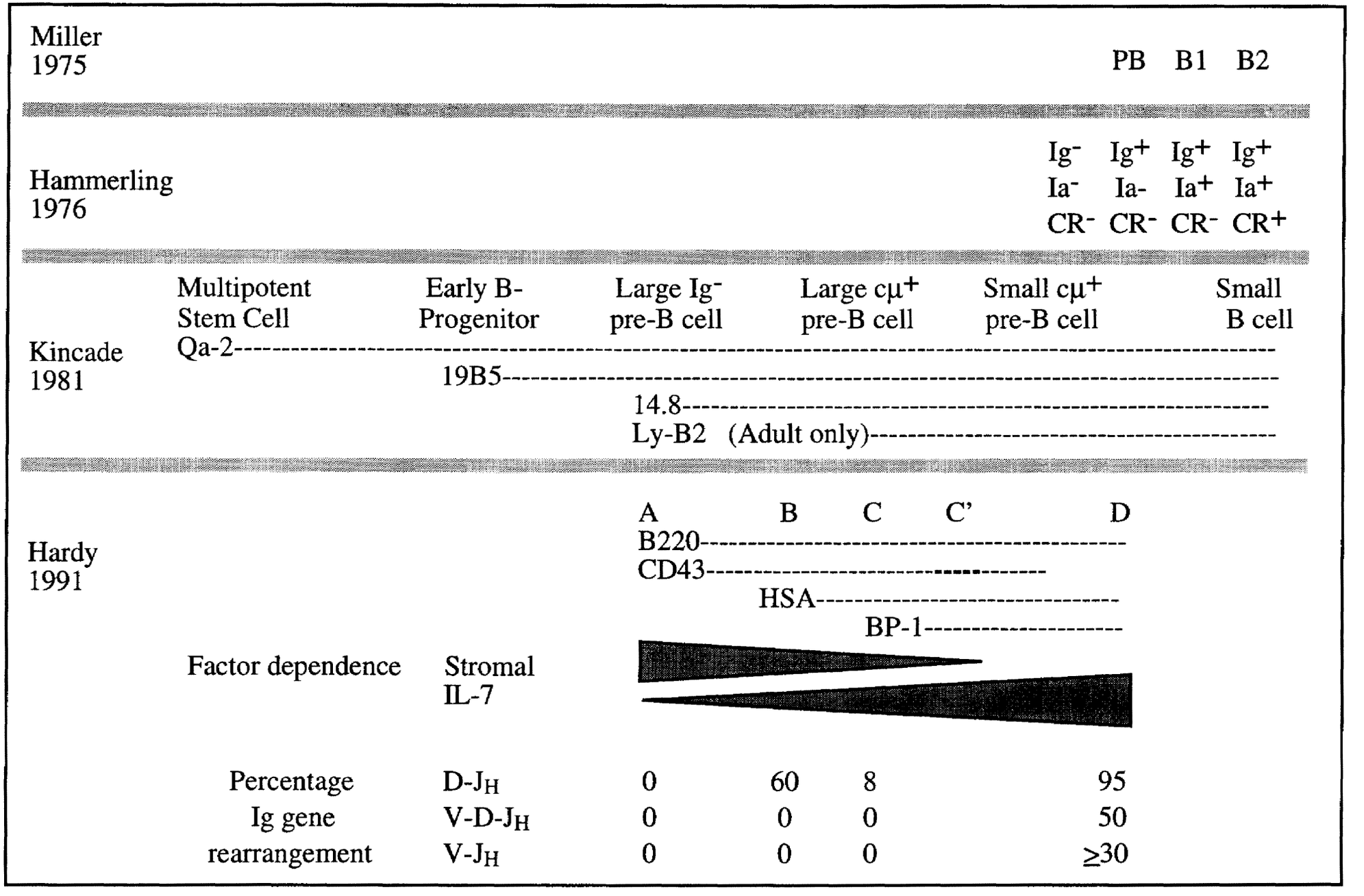
Figure 1-2. The progressive elucidation of the stages of B-lymphopoiesis by lymphohemopoietic and committed B cell progenitors.

In 1975 Miller et al. (16) using sedimentation velocity was able to identify 3 different populations of B cells and their progenitors. PB cells give rise to B1 cells that give rise to B2 cells, in each case these cells were identified as expressing surface Immunoglobulin. Miller, R. G. et al. Fed. Proc. 34; 145-150, 1975.

In 1976 Hammerling et al. (17) characterized B cells and their precursors based on antigen expression. Ig, surface immunoglobulin; Ia, immune response gene and CR, complement component, Hammerling, U. et al. Proc. Natl. Acad. Sci. USA. 73, 2008-2012, 1976.

In 1981 Kincade et al. (19) characterized B-lymphopoiesis using monclonal antibodies Qa, 19B5 and B-lymphoid specific 14.8 and the immunoglobulin expression of the progenitors at each stage, Kincade, P. W. et al. Adv. Immunol. 31; 177-245, 1981.

In 1991 Hardy et al. (20) characterized the stages of $B220^{+}$ sIg^{-} B-lymphoid progenitors using CD43, HSA and BP-1. Five stages of progenitors were identified based on thier antigen expression and the growth requirements and Ig gene rearrangement status was determined, Hardy, R. R. et al. J. Exp. Med. 173; 1213-1225, 1991.


Miller
1975
PB B1 B2
Hammerling
1976
Ig- Ig+ Ig+ Ig+
Ia- Ia- Ia+ Ia+
CR- CR- CR- CR+
Kincade
1981
Multipotent Stem Cell
Early B-Progenitor
Large Ig- pre-B cell
Large cμ+ pre-B cell
Small cμ+ pre-B cell
Small B cell
Qa-2
19B5
14.8
Ly-B2 (Adult only)
Hardy
1991
A B C C' D
B220
CD43
HSA
BP-1
Factor dependence
Stromal
IL-7
Percentage
Ig gene rearrangement
D-JH
V-D-JH
V-JH
0 60 8 95
0 0 0 50
0 0 0 ≥30

OUTLINE OF DISSERTATION

The work in this dissertation concentrates on the characterization of early B-lymphohemopoiesis by lymphohemopoietic and early committed B-lymphoid progenitors following the establishment of an *in vitro* culture assay capable of supporting the clonal differentiation of lymphohemopoietic progenitors isolated from 5-FU treated mice. The content of Chapter II was published as "Ball, T. C., F. Hirayama, and M. Ogawa. Lymphohematopoietic progenitors of normal mice. Blood. 85; 3086-3092, 1995." In this chapter the establishment of the single-step assay for the identification of murine lymphohemopoietic progenitors, and the characterization of the lymphohemopoietic progenitors from normal mice is described. The content of Chapter III was published as "Ball T. C., F. Hirayama, and M. Ogawa. Modulation of early B-lymphopoiesis by interleukin-3." Experimental Hematology. 24, 1225-1231, 1996. In this chapter 3 stages of early B-lymphopoiesis by lymphohemopoietic progenitors from 5-FU treated mice, and the effect of IL-3 exposure on progenitors in each of these stages is described. The content of Chapter IV remains unpublished. In this chapter I describe the expression of the B-lymphoid and the myeloid lineages by enriched progenitors isolated from normal mice is described, along with a modified version of a model of hemopoietic differentiation that includes a random restriction of lineage potentials with a late divergence of the B-lymphoid lineage from possibly a bipotential B-lymphoid/macrophage progenitor.

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Chapter II

Lymphohemopoietic Progenitors of Normal Mice

Timothy C. Ball, Fumiya Hirayama and Makio Ogawa

INTRODUCTION

A two-step methylcellulose culture assay for murine lymphohemopoietic progenitors capable of producing cells in B-cell and myeloid lineages was recently developed in Dr. Ogawa's laboratory (1). In this system, enriched bone marrow cells of 5-FU-treated mice were plated in the primary culture and the resulting colonies were analyzed in secondary cultures containing steel factor (SF, ligand for c-*kit*) and interleukin (IL)-7 for pre-B cell colony formation. SF plus IL-6, IL-11, granulocyte-colony stimulating factor (G-CSF) (7) or IL-12 (2) supported the differentiation and proliferation of B-cell progenitors in the primary colonies. IL-3 failed to support B-lymphopoiesis either alone or in combination with other factors. Furthermore, when added to permissive culture conditions, IL-3 and IL-1 independently inhibited the B-cell potential of the primary colonies (3). According to this culture assay, approximately 40% of the progenitors in the lineage-negative (Lin^-) Ly-6A/E $(Sca\text{-}1)^+$ population isolated from 5-FU-treated mice were lymphohemopoietic in nature (1).

The chemotherapeutic drug 5-FU kills actively proliferating cells while sparing cell cycle-dormant primitive hemopoietic progenitors. 5-FU has at least three mechanisms of action, the first and probably most important is the irreversible inhibition of thymidylate synthase which results in the depletion of thymidylate pools and the inhibition of DNA synthesis. The second and third mechanisms involve the direct incorporation into DNA and RNA, creating unstable molecules that lead to cell death as is discussed by Mini et al. (4). It is possible that some of the cell cycling, maturer progenitors present in normal mice may also possess lymphohemopoietic potential, and that the cytokine requirement of the cycling lymphohemopoietic progenitors may be different from that of the progenitors in 5-FU-treated mice. While the two-step culture assay may be suited for the synchronized

population of cell cycle-dormant progenitors in the marrow of 5-FU-treated mice, it may not be adequate for the progenitors present in normal mice since the latter are not synchronized and represent varying stages of development. Replating on a fixed day of incubation may significantly underestimate the number of progenitors. To address this problem, I have developed a single-step methylcellulose culture assay and examined the lymphohemopoietic progenitors in normal mice. My observations indicate that there are more lymphohemopoietic progenitors in the bone marrow of normal mice than in the marrow of 5-FU-treated mice.

MATERIALS AND METHODS

Growth factors.

Conditioned medium (CM) of Chinese hamster ovary (CHO) cells transfected with an expression plasmid containing murine SF cDNA was provided by Dr. D. Donaldson (Genetics Institute Inc., Cambridge , MA). Purified recombinant human IL-7 was provided by Dr. C. Faltynek (Sterling Drug Inc., Malvern, PA). Medium conditioned by CHO cells genetically engineered to produce murine IL-3 at a high titer (-70,000 U/ml) was a gift from Dr. T. Sudo (Biomaterial Research Institute, Yokohama, Japan). Purified bacterially-derived human G-CSF was provided by Dr. L. Sousa (Amgen, Thousand Oaks, CA). Purified recombinant human IL-11 was provided by Dr. P. Schendel and IL-12 by Dr. S. F. Wolf (Genetics Institute). Purified recombinant human IL-1α was provided by Dr. Y. Hirai, (Otsuka Pharmaceutical Co. Ltd., Tokushima, Japan). Purified human erythropoietin (Ep) was provided by the Genetics Institute Clinical Manufacturing Group. Serum-free conditioned medium from pokeweed mitogen-stimulated spleen cells (PWM-SCM) was prepared as described previously (5).

Monoclonal antibodies (mAbs).

Hybridoma D7 (anti-Ly-6A.2/E.1, rat IgG 2a) was kindly provided by Dr. Paul Kincade of Oklahoma Medical Research Foundation (Oklahoma City, OK). Hybridoma 14.8 (anti-B220, rat IgG2b), and M1/70.15.11.5.HL (anti-Mac-1, rat IgG2b) were obtained from the American Type Culture Collection (Rockville, MD). These antibodies were purified from the culture supernatants by affinity chromatography on a Protein G Sepharose 4 Fast Flow column (Pharmacia LKB Biotechnology, Piscataway, NJ). TER119 (anti-erythrocyte mAb) was provided by Dr. T. Kina (Department of Immunology, Chest Disease Research Institute, Kyoto University, Kyoto Japan). Biotin-conjugated RB6-8C5 (anti-Gr-1, rat IgG2b) was purchased from PharMingen (San Diego,

CA). Affinity-purified FITC-labeled goat-anti-rat IgG was purchased from Kirkegaard & Perry Laboratories Inc. (Gaithersburg, MD). Anti-Mac-1 and TER119 were conjugated in our laboratory with biotin purchased from Vector Laboratories (Burlingame, CA). Rat IgG was purchased from Zymed Laboratories (San Francisco, CA). Biotin-conjugated rat IgG2b was purchased from Caltag Laboratories (San Francisco, CA). Streptavidin-conjugated phycoerythrin (PE) was purchased from Jackson Laboratories (West Grove, PA). Propidium iodide (PI) was purchased from Sigma Chemical Company (St. Louis, MO) and used at a final concentration of 10 ug/ml.

Purification of progenitor cells.

Bone marrow cells were harvested from the femora and tibiae of 10 to 16 week-old normal BDF1 mice (Charles River, Raleigh, NC) and from mice 2 days after i.v. injection of 5-FU (Adria Laboratories, Columbus OH) at 150 mg/kg (body weight). Cells were enriched by a combination of density gradient separation, depletion of lineage marker positive cells and positive sorting for the presence of the Ly-6A/E antigen (6) as described previously (7).

Methylcellulose culture of enriched progenitors from normal mice.

Fifty to 60 enriched normal marrow cells were plated in a 35 mm suspension culture dish (Becton Dickinson Labware, Lincoln Park, NJ) containing α-medium (ICN, Irvine, CA), 1.2% (1500 centipoises) methylcellulose (Shinetsu Chemical Co., Tokyo, Japan), 30% fetal calf serum (Intergen, Purchase, NY), 1% deionized fraction V bovine serum albumin (BSA, Sigma Chemical Co.), 1 x10^{-4}M mercaptoethanol (ME, Eastman Organic Chemicals, Rochester, NY), 2 U/ml Ep and designated combinations of growth factors. Dishes were incubated at 37 degrees C in a humidified atmosphere flushed with 5% CO_2. In some experiments, enriched progenitors were individually plated by micromanipulation in methylcellulose cultures as described previously (8). Unless specified

otherwise, the concentrations of cytokines used here are as follows: SF CHO-CM, 5 U/ml; IL-1α, 10 ng/ml; IL-3 CHO-CM, 200 U/ml; IL-7, 5 ng/ml; IL-11, 100 ng/ml; IL-12, 10 ng/ml; Ep, 2 U/ml; G-CSF, 1 ng/ml and PWM-SCM, 5%.

Flow cytometric analysis of the B-cell potentials of individual colonies.

On day 16 and 17 of culture, after in situ examination of the types of colonies on an inverted microscope, all colonies except small granulocyte-macrophage (GM) and macrophage (M) colonies were harvested for analysis of $B220^+$ cells. Bone marrow cells of a normal mouse were used as control cells. Samples were first incubated with rat-anti-mouse B220 and then with goat-anti-rat IgG-FITC. Polyclonal rat IgG was added to block the free binding sites of the goat-anti-rat IgG-FITC molecules. The samples were then incubated with a cocktail of biotin-conjugated rat-anti-mouse mAb's consisting of anti-Mac-1, anti-Gr-1, and TER119 followed by streptavidin-PE. Finally, the samples were incubated with propidium iodide (PI) at a concentration of 10 ug/ml. The cells from individual colonies were analyzed by flow cytometry using a FACStar Plus flow cytometer (Becton Dickinson, San Jose, CA) for the presence of $B220^+$, Mac-1^-, Gr-1^-, $TER119^-$ and PI^- cells.

Two-step methylcellulose culture assay for lymphohemopoietic progenitors.

Enriched marrow cells from 5-FU-treated mice were assayed for lymphohemopoietic progenitors by using the two-step culture assay as described previously (7). Briefly, cells were plated in methylcellulose culture containing SF, IL-7, Ep and IL-11 for 11 days, at which time individual colonies were lifted from the medium with a micropipet. The colonies were washed and divided into two aliquots; two-thirds were stained with a polychrome stain to examine myeloid cell morphology and one-third was replated in methylcellulose culture containing SF and IL-7. Twelve days after replating, the lymphoid cultures were examined for the presence of pre-B cell colonies (1,

9). Enriched progenitors from normal mice were cultured in the presence of identical cytokine mixtures. However, the optimal day for replating of the primary colonies to observe maximal B-lymphoid expression was determined to be day 8.

Analysis of myeloid lineage potentials of lymphohemopoietic progenitors.

Enriched progenitors from normal mice were individually micromanipulated into methylcellulose medium containing SF, IL-7, Ep and IL-11, and primary colonies were allowed to develop. On day 11 of culture, one half of each colony present was lifted from the medium and was transferred into 200 ul of myeloid specific culture medium containing α-medium, 30% FCS, 1% BSA, 1 x 10^{-4} M 2ME, 5% PWM-SCM, 200 U/ml IL-3 and 2 U/ml Ep in a 96 well flat bottom tissue culture plate (Costar, Cambridge, MA). On day 4 of secondary culture myeloid lineage expression was examined with May-Grunwald Giemsa staining of cytospin preparations. The portion of each colony remaining in the initial semisolid culture was harvested on day 19 of culture and analyzed by flow cytometry to identify $B220^+$, $Mac\text{-}1^-$, $Gr\text{-}1^-$, $TER119^-$ and PI^- cells.

RESULTS

Identification of lymphohemopoietic colonies by flow cytometry.

When the enriched bone marrow cells from normal mice were cultured in the presence of SF, IL-7, Ep and IL-11 for 16 days, some lymphomyeloid colonies were easily identifiable in situ by their characteristic appearance. These colonies had the appearance of multilineage myeloid colonies revealing red color of hemoglobin while also presenting a few clumps of small round cells characteristic of pre-B cell colonies. A photomicrograph of a representative colony is presented in Fig. 2-1. In addition, there were pure pre-B cell colonies and clusters with the morphology described previously (1, 9) and large multilineage myeloid colonies without an apparent B-cell component. However, the majority of the growth seen in culture consisted of small GM and M colonies containing fewer than 2,000 cells. In order to more accurately identify the colonies expressing a B-lymphoid component, I carried out flow cytometric analysis of individual colonies for identification of $B220^+$ cells.

First, I established the sensitivity and specificity of the assay using a mixing experiment. In this experiment, 10^5 cells from myeloid colonies known to lack pre-B cells and varying numbers of pre-B cells from pooled pre-B cell colonies were combined and subjected to flow cytometric analysis. The former population was obtained by culture of enriched post-5-FU marrow cells for 14 days in the presence of SF, IL-7, Ep, IL-11 and IL-3 (3) and the latter from day 11 primary colonies derived from enriched post-5-FU marrow cells recultured in the presence of SF and IL-7 (1, 9). Using LYSIS II software, forward and side scatter gates were set to include cells present in the lymphoid window (10) and fluorescence gates were activated to include $B220^+$, Mac-1^-, Gr-1^-, TER119^- and PI^- cells. Routinely 4,000 events were collected for examination of individual colonies. The results of this experiment are presented in Fig. 2-2A. As can be seen, the number of $B220^+$ events is directly proportional to the percentage of pre-B cells present in an individual

sample. Furthermore, I determined the threshold sensitivity, the minimum percentage of pre-B cells that must be present in an individual colony to be identified to be 5%. In activation of the lymphoid and fluorescence windows, acquired debris and PI positive events are excluded, accounting for the discrepancy between the number of events acquired and the number of events reported in the 100% pre-B cell sample of Fig. 2-2A. The flow cytometric analyses of the B-Mix colony seen in Fig. 2-1. are shown in Fig. 2-2B. The colony was lifted from the medium and divided into two aliquots. One was stained with the myeloid mAb cocktail and rat IgG (C) and the other stained with the myeloid mAb cocktail and the anti-B220 mAb (E). As can readily be seen, the colony contained many B220+ pre-B cells.

Comparison of in situ morphological identification and analysis by use of flow cytometry of individual colonies soon led to the conclusion that the latter method, flow cytometry, was superior to the former, morphology, in identification of the B-lymphoid component. Results of three such comparisons are shown in Table 2-1. There was excellent concordance between in situ microscopic examination and flow cytometric analysis in the identification of pure pre-B cell colonies and B-Mix colonies, multilineage myeloid colonies revealing typical pre-B cell colonies and clusters. However, a significant number of apparent myeloid-restricted multilineage colonies (Mix colonies) revealed the presence of a B-cell component upon flow cytometric analysis. As stated in the Materials and Methods section, the GM and M colonies contained fewer than 2,000 cells and could not be individually analyzed by flow cytometry. When all 77 of the GM and M colonies in experiment 1, Table 2-1 were pooled and examined for B220+ cells by flow cytometry, no pre-B cells were identified. Based on these observations, I established a single stage culture for the identification of lymphohemopoietic colonies from normal mice in which all colonies except small GM and M colonies were analyzed for B220+ cells by flow cytometry.

Clonality of the lymphomyeloid colonies identified by the single-step assay.

To establish single cell origin of the lymphomyeloid colonies, I carried out micromanipulation of individual progenitor cells into semisolid culture. In experiment 1 of Table 2-2, a total of 90 Lin^- $Sca\text{-}1^+$ cells were micromanipulated into cultures containing SF, IL-7 and Ep. Twenty-two colonies developed by day 16 of culture revealing a 24% plating efficiency. Seventeen of the colonies were small GM and M colonies and were not analyzable by flow cytometry, while 5 of the colonies were harvested for analysis. One of the colonies was a lymphomyeloid colony and 4 were pre-B cell colonies. In experiments 2 and 3 a total of 98 Lin^- $Sca\text{-}1^+$ cells were micromanipulated into cultures containing SF, IL-7, Ep and IL-11. The plating efficiency was 32% and a total of seven lymphomyeloid colonies were identified. These results clearly established the clonal origin of the lymphomyeloid colonies.

Cytokine requirements of lymphohemopoietic progenitors.

I next examined the minimal cytokine combinations required for formation of lymphomyeloid colonies from enriched normal bone marrow cells. The results of this examination are shown in Table 2-3. The combination of SF and Ep supported the development of only myeloid colonies, a majority of which were small GM and M colonies. IL-7 plus Ep supported formation of few GM and M colonies but failed to support formation of pre-B cell colonies. The combination of SF, IL-7 and Ep supported formation of not only GM and M colonies, but also pre-B cell colonies and lymphomyeloid colonies. The numbers of GM and M colonies supported by the combination of SF, IL-7 and Ep were similar to those supported by SF plus Ep.

Effects of Early-Acting Cytokines.

Earlier, several cytokines were identified which appear to act synergistically to shorten the G_0 period of primitive progenitors and to support the proliferation of

lymphohemopoietic progenitors of 5-FU-treated mice (11). I tested these early-acting cytokines for possible synergistic effects on colony formation by normal marrow cells supported by SF, IL-7, plus Ep. The results are presented in Table 2-4, experiment 1. Again, the combination of SF, IL-7 and Ep supported lymphomyeloid and lymphoid colony formation from normal marrow cells. Addition of IL-11, G-CSF or IL-12 to the combination of SF, IL-7 and Ep greatly increased the numbers of all types of colonies primarily lymphomyeloid, myeloid and small GM and M colonies. IL-12 exhibited the least activity and the colonies developing in the presence of IL-12 were smaller than the colonies developing in the presence of either IL-11 or G-CSF as reported previously with post 5-FU marrow cells (2).

It has been reported IL-3 and IL-1α independently act as potent negative regulators of the early stages of B-lymphopoiesis using enriched marrow cells of 5-FU-treated mice (3). In the next experiment I studied the effects of these cytokines on lymphomyeloid colony formation from normal marrow cells. Results of two separate experiments are presented in experiments 2 and 3 of Table 2-4. As can readily be seen, IL-3 and IL-1α independently suppressed the development of lymphomyeloid colonies and totally abrogated formation of pre-B cell colonies.

Incidence of lymphohemopoietic progenitors in normal and 5-FU treated mice.

It was of interest to compare the incidence of lymphohemopoietic progenitors present in normal mice with that in mice injected with 5-FU. To obtain a more valid result, the single-step and the two-step assays were compared using progenitors from normal marrow. It was however, impossible to assay progenitors from 5-FU-treated mice using the single-step assay because of the proliferative capacity of the cells and the kinetics of B-lymphoid expression. Approximately 21 days of culture were required before any B-lymphoid lineage expression was detectable by FACS analysis at which time a majority of the colonies had diameters greater than 2 mm and were rapidly degenerating. Marrow cells

were harvested from two femora and two tibiae of each animal. The means and standard deviations of four individual experiments conducted in each group are presented in Table 2-5. The numbers of lymphohemopoietic progenitors in the two femora and two tibiae of a normal mouse estimated by the single-step and the two-step assay were 3,400 ± 600 and 4,700 ± 2,200, respectively and did not differ significantly. In contrast, the total number of lymphohemopoietic progenitors in a 5-FU-treated mouse identifiable by the two-step assay, was approximately one-fourth that of a normal mouse.

Further, the single-step and the two-step assays identified 5 ± 2 and 5 ± 1 percent of the colonies from a normal mouse as B-lymphoid, respectively, whereas no B-lymphoid colonies were identified from the progenitors from 5-FU-treated mice.

Examination of the myeloid lineage potentials of individual progenitors of normal mice.

A total of 49 Lin^- $Sca\text{-}1^+$ cells isolated from normal mice were individually micromanipulated into methylcellulose cultures containing SF, IL-7, Ep and IL-11. Fifteen of the 49 cells gave rise to colonies on day 11 of culture, 2 of which were degenerated and not suitable to evaluate. Four of the primary colonies gave rise to cells of both the myeloid and the B-lymphoid lineages. Results of cytochemical analyses of the cells are presented in Table 2-6. While the number of colonies examined is small, there appears to be no significant difference in the myeloid potentials of colonies regardless of the presence or absence of B-lymphoid potential.

DISCUSSION

The development of an assay capable of the clonal identification of lymphohemopoietic progenitors under defined culture conditions has proven useful in the study of the mechanisms of early B- lymphopoiesis. Already, this assay has led to characterization of the cytokine requirements of early B-lymphopoiesis *in vitro* and identification of possible negative modulatory roles of the cytokines IL-1α and IL-3 on early B-lymphopoiesis (2-3). I have now modified the two-step culture assay for the progenitors in 5-FU-treated mice to develop a single-step clonal culture assay capable of supporting the differentiation and proliferation of lymphohemopoietic progenitors of normal mice.

My studies resulted in the identification of a significant number of progenitors capable of giving rise to both B-lymphoid and myeloid lineages. My calculation indicates that approximately 4 times more lymphohemopoietic progenitors are present in the bone marrow of a normal mouse than in a 5-FU-treated mouse. Recently, Lepault et al. (12) examined the T- and B-lymphoid potential of spleen-colony forming units (CFU-S), and observed that the majority of day-8 CFU-S and almost all of day-12 CFU-S have T- and B-lymphoid potentials. My observations in culture corroborate those of Lepault et al. *in vivo* and suggest that many lymphohemopoietic progenitors are present in the normal mice, and that a significant population of the progenitors is sensitive to the cytotoxic effects of 5-FU.

For the most part, the responses of the majority of lymphohemopoietic progenitors isolated from normal mice to synergistic cytokines (IL-11, IL-12 and G-CSF) and inhibitory cytokines (IL-1α and IL-3) are similar to those isolated from 5-FU treated mice. However, a small number of progenitors of normal mice required only SF, IL-7 and Ep to form lymphomyeloid colonies. The lack of this requirement for a additional synergistic factors by some of the lymphohemopoietic progenitors of normal mice may suggest these progenitors are actively proliferating. The single-step assay for lymphohemopoietic

progenitors of normal mice will be useful in the study of commitment of lymphohemopoietic progenitors.

Table 2-1. Comparison of in situ Colony Identification with Detection by Flow Cytometry

	Number of Colonies			
	B-Mix	Pre-B cell	Mix	GM/M
Exp 1	18 (18)	11 (11)	27 (3)	77
Exp 2	10 (10)	11 (11)	16 (1)	32
Exp 3	19 (18)	8 (7)	23 (2)	31

The numbers indicate colonies diagnosed in situ on an inverted microscope. The numbers in the bracket indicate colonies revealing $B220^+$ cells by flow cytometry. $Lin^- Sca\text{-}1^+$ cells of normal mice were cultured in the presence of SF, IL-7, Ep and IL-11. B-Mix, multilineage colonies revealing B-lymphoid clusters in situ; Pre-B Cell, Pre-B cell colonies and clusters; Mix, multilineage myeloid colonies ostensibly lacking B-lymphoid element.

Table 2-2. Clonal Origin of the Lymphohemopoietic Colonies of Normal Mice

			Number of Colonies				
			Analyzed by Flow Cytometry				
Exp	Growth factors	Number of cells plated	Lymphomyeloid	Lymphoid	Myeloid	Small GM/M	Total
1	SF, IL-7, Ep	90	1	4	0	17	22
2	SF, IL-7, Ep, IL-11	49	3	1	2	10	16
3	SF, IL-7, Ep, IL-11	49	4	1	3	7	15

Colonies were examined on day 16 or 17 of culture.

Table 2-3. Minimum Cytokine Requirements of Lymphohemopoietic Progenitors

	Colonies/ 2 dishes				
	Analyzed by Flow Cytometry			Small	
Growth factors	Lymphomyeloid	Lymphoid	Myeloid	GM/M	Total
None	0	0	0	0	0
Ep	0	0	0	0	0
IL-7, Ep	0	0	0	3	3
SF, Ep	0	0	4	24	28
SF, IL-7, Ep	4	8	2	26	40

Lin^- $Sca\text{-}1^+$ cells of normal mice were plated in cultures at a concentration of 60 cells per dish. Colonies were diagnosed in situ and analyzed by flow cytometry on day 16 or 17 of culture.

Table 2-4. Effects of Early-Acting Cytokines on Lymphohemopoietic Colony Formation

	Number of Colonies/6 dishes				
	Analyzed by Flow Cytometry				
Growth Factors	Lymphomyeloid	Lymphoid	Myeloid	Small GM/M	Total
		Experiment 1			
SF, IL-7, Ep	1	4	12	20	37
SF, IL-7, Ep, IL-11	17	4	40	81	142
SF, IL-7, Ep, G-CSF	6	9	42	92	149
SF, IL-7, Ep, IL-12	8	7	41	62	118
		Experiment 2			
SF, IL-7, Ep, IL-11	13	9	61	104	187
SF, IL-7, Ep, IL-11, IL-3	3	0	76	99	178
		Experiment 3			
SF, IL-7, Ep, IL-11	13	4	19	35	71
SF, IL-7, Ep, IL-11, IL-1α	0	0	9	69	78

Lin^- $Sca\text{-}1^+$ cells were cultured at a concentration of 50 cells per dish. Six dishes were examined on day 16 or 17 of culture.

Table 2-5. Comparison of the Incidences of Lymphohemopoietic Progenitors Present in Two Femora and Two Tibiae of a Normal Mouse and a Mouse Treated with 5-FU

	Normal mouse		5-FU-treated mouse
	single-step assay	two-step assay	two-step assay
Nucleated bone marrow cells	$(4.6 \pm 0.5) \times 10^7$	$(4.3 \pm 0.6) \times 10^7$	$(1.4 \pm 0.6) \times 10^7$
$Lin^- Sca\text{-}1^+$ cells	$(1.4 \pm 0.4) \times 10^5$	$(8.7 \pm 2.9) \times 10^4$	$(2.9 \pm 2.0) \times 10^4$
Colony forming efficiency	$40 \pm 18\%$	$31 \pm 7\ \%$	$41 \pm 6\%$
Percent of lymphomyeloid colonies	$12 \pm 5\%$	$18 \pm 10\%$	$38 \pm 6\%$
Estimated number of lymphohemopoietic progenitors	$3{,}400 \pm 600$	$4{,}700 \pm 2{,}200$	$1{,}100 \pm 600$

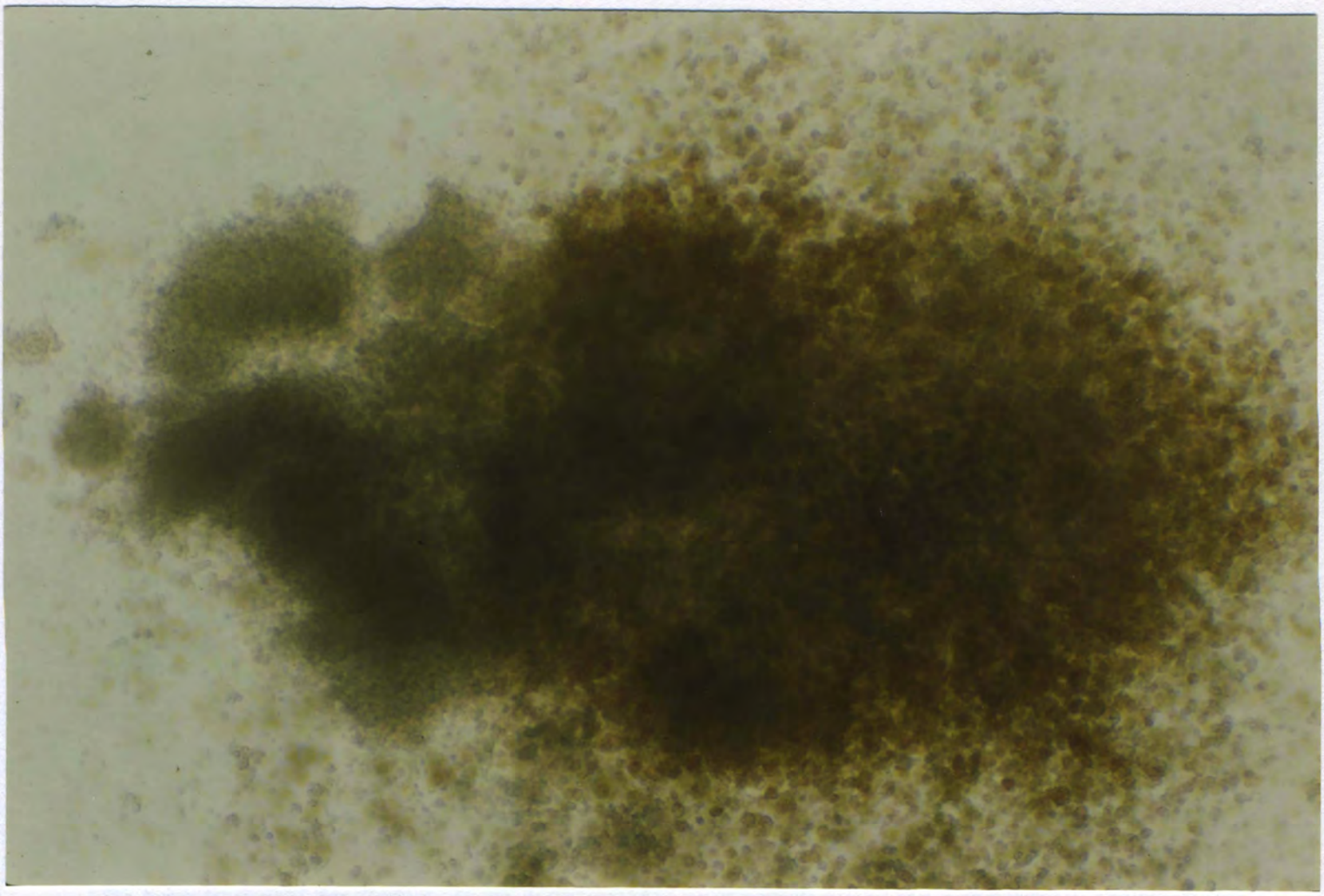
$Lin^- Sca\text{-}1^+$ progenitors were cultured in the presence of IL-11, SF, IL-7 and Ep. Primary colonies from 5-FU-treated and normal mice were replated on day 11 and day 8 of primary culture respectively. Means and standard deviations of four experiments for each group.

Table 2-6. The Myeloid Lineage Potential of the Progenitors

Colony #	Myeloid lineage expression	B220$^+$ cells
1	nmEMastMe	+
2	nmE	+
3	nm	+
4	nm	+
5	m	-
6	nmMast	-
7	nmEMast	-
8	nm	-
9	nm	-
10	nm	-
11	nm	-
12	nm	-
13	None	+

Initial methylcellulose cultures were established with SF, IL-7, Ep and IL-11. One half of each colony was lifted from semisolid culture on day 11 and was placed in liquid culture containing IL-3, PWM-SCM and Ep. Myeloid lineage expression was examined on day 4 of secondary culture, and the remaining portion of each colony was harvested and analyzed by flow cytometry on day 19 of culture. Abbreviations: n, neutrophil; m, macrophage; E, erythrocyte; Mast, mast cell; M, megakaryocyte; e, eosinophil.

Figure 2-1. (A) A representative B-Mix colony containing B-lymphoid and myeloid components. This colony was derived from Lin$^-$ Sca-1$^+$ cells of normal mice grown in the presence of SF, IL-7, Ep and IL-11 for 16 days. (B) Magnification of a portion of the same colony revealing (1) typical B-lymphoid clusters and (2) erythroid components.



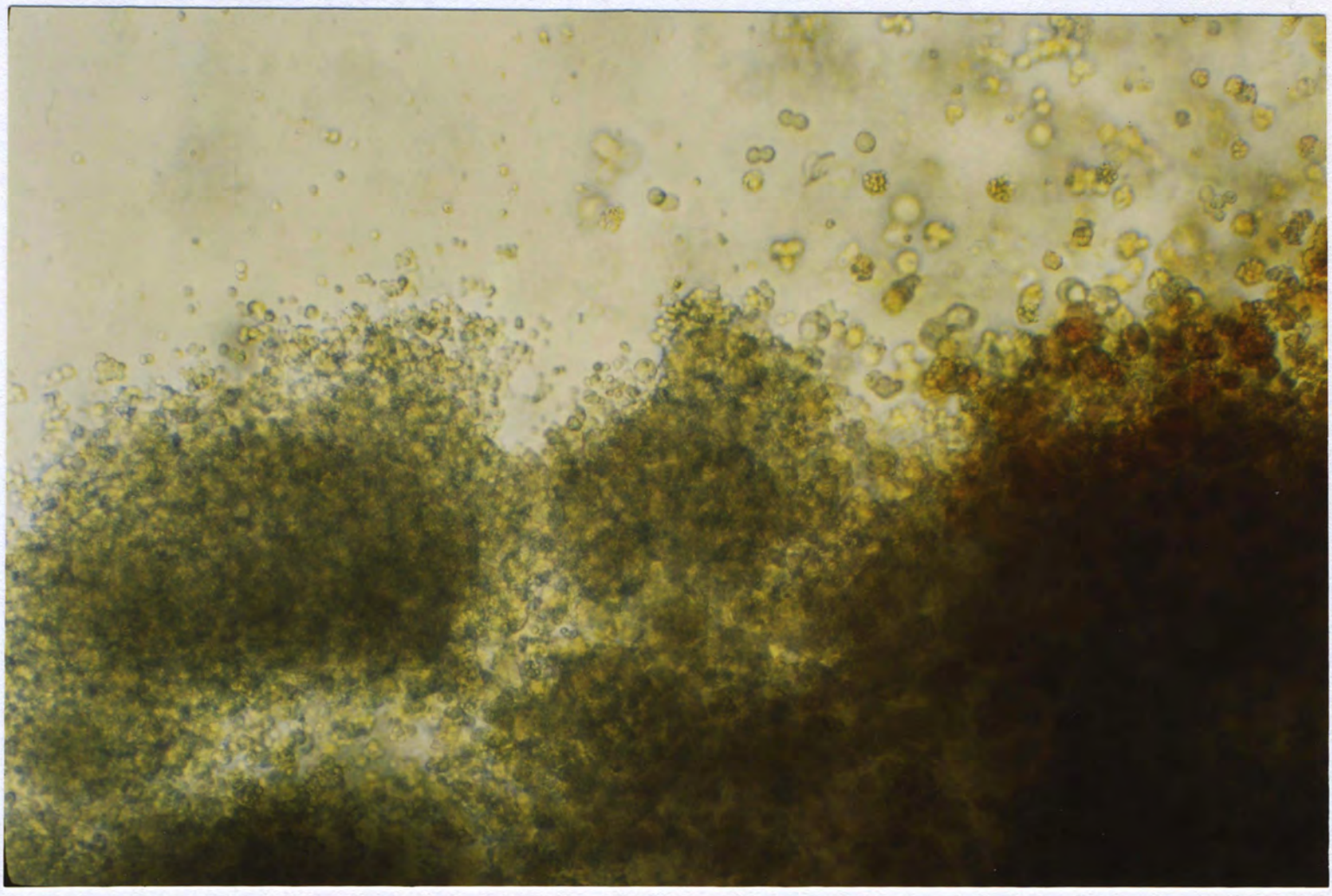
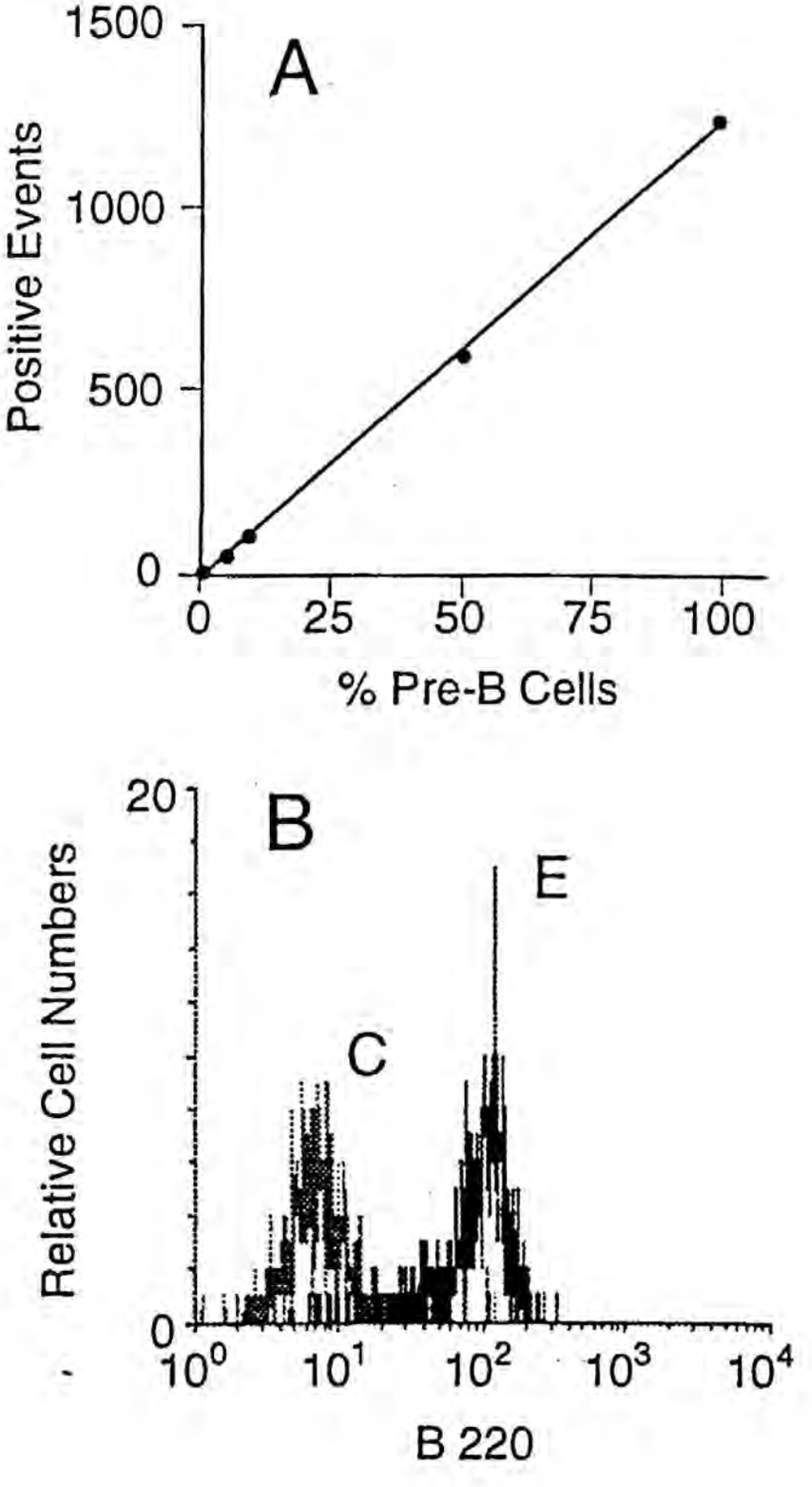


Figure 2-2. Detection of B220+ pre-B cells by flow cytometric analysis. (A) Establishment of the specificity of the flow cytometric analysis to detect pre-B cells present at varying percentages in standardized samples. The discrepancy between the number of events acquired and the number of events reported in the 100% pre-B cell sample was caused by exclusion of debris by forward scatter gating and dead cells by PI staining. (B) Fluorescence histograms of (C) isotype control and (E) analysis of the B-Mix colony shown in Fig. 1.


A
1500
1000
500
0
Positive Events
0
25
50
75
100
% Pre-B Cells
B
20
0
Relative Cell Numbers
C
E
10^0
10^1
10^2
10^3
10^4
B 220

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Chapter III

Modulation of Early B-Lymphopoiesis by IL-3

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INTRODUCTION

IL-3 is a glycoprotein with an approximate weight between 22 and 32 kDa depending on the degree of N-linked glycosylation. Its three dimentional structure is believed to have 5 alpha helical domains with one disulfide bond stabilizing the structure. The IL-3 receptor is a heterodimeric receptor composed of a unique alpha subunit and a beta subunit that is common to the GM-CSF and IL-5 receptors. Signal transduction requires the binding of IL-3 by the alpha subunit and the subsequent formation of a disulfide bond between the alpha and beta subunits (1, 4). In addition to the common beta subunit, murine cells have an IL-3 specific beta subunit which is functional but to a lesser degree than the common beta subunit (5). Formation of a functional signal transducing IL-3 receptor complex results in the rapid phosphorylation of tyrosine, serine and threonine residues (6-12); however, the receptor has no intrinsic kinase activity (7). In defining the segments of the beta subunit that are essential for signal transduction and activation of second messenger cascades, investigators have made use of specific deletion mutants. Kinoshita et al. examined the supression of apoptotic death of IL-3 dependent murine pro-B and myeloid progenitor cell lines. Their studies revealed that proliferation and prevention of apoptosis are mediated by different second messenger cascades which are activated by distinct segments of the beta subunit. Truncation of the C-terminal cytoplasmic domain of the beta subunit revealed that the membrane proximal region was responsible for the activation of *c-myc* and the prevention of apoptosis, while the distal region was responsible for the activation of p21*ras* cascade and the stimulation of proliferation (13). P21*ras* activates phosphitidyl choline specific phospholipase C, which in-turn increases the concentration of diacylglycerol, activating protein kinase C (PKC). PKC phosphorylates *raf-1* which causes the phosphorylation of MAP kinase and the induction of the

transcription factors *c-fos/c-jun* (14-16). Sato et al. created truncation mutants of the human common beta subunit. Deletion of amino acids 626 - 881 resulted in no activation of the p21*ras* cascade and no induction of the *c-fos/c-jun* transcription factors or p70 S6 kinase, a tyrosine kinase responsible for the induction of the ribosomal protein S6. Deletion of amino acids 455 - 517 resulted in no activation *c-myc* or pim-1 a cooperative second messenger expressed only in hematopoietic cells (6,17). The membrane proximal region is also associated with activation of the Janus kinase (Jak)-2, and signal transducer and activator of transcription (STAT)-5, pathway in addition to induction of *c-myc* and pim-1 (18-20). On phosphorylation, the STAT proteins dimerize and bind to specific response elements in the promoter regions of target genes and modulate their transcription. Two classes of STAT binding sites, the interferon stimulated response element and the interferon gamma activation site (GAS), have to date been identified (19). Recently, Nagata et al. discovered that IL-3 activates not only STAT5, but also Tyk2, STAT1 and STAT3 in several IL-3 dependent murine cell lines (20).

In our laboratory, Hirayama et al. recently developed a two-step clonal culture assay capable of supporting the differentiation of lymphohemopoietic progenitors along both the B-cell and the myeloid lineages under stromal cell-free conditions, and this assay was used to identify IL-3 as a negative modulator of B-lymphopoiesis (21, 22). Subsequently, a single-step assay was developed to identify and characterize the non-synchronized population of lymphohemopoietic progenitors present in normal mice (23). In this assay, enriched marrow cells from normal mice were plated in semisolid culture containing combinations of cytokines similar to those in the primary culture of the two-step assay. After 16 to 17 days of culture, the dishes were examined with an inverted microscope, and all colonies except small granulocyte-macrophage (GM) and macrophage (M) colonies were harvested for flow cytometric analysis of $B220^+$ pre-B cells. I identified a population of lymphohemopoietic progenitors capable of generating lymphomyeloid colonies, colonies containing both B-lymphoid and myeloid lineage cells, in SF, IL-7 and

Ep alone. However, the majority of lymphohemopoietic progenitors present in normal mice require IL-11 in addition to SF, IL-7 and Ep to support colony formation. Approximately 5% of the colony-forming Lin^- $Sca\text{-}1^+$ progenitors from normal mice gave rise to lymphoid restricted pre-B cell colonies. These progenitors were capable of colony formation in the presence of SF, IL-7 and Ep, and the addition of a synergistic triggering factor did not significantly increase colony formation by these progenitors. Finally, I determined that treatment with 5-FU decreased the number of lymphohemopoietic progenitors to approximately one-fourth the number present in a normal mouse while eliminating the population of progenitors that give rise to the B-lymphoid restricted colonies. Since the single-step assay is capable of identifying actively proliferating Lin^- $Sca\text{-}1^+$ lymphohemopoietic and committed B-lymphoid progenitors, it provided an excellent method for the study of the earliest processes of B-lymphopoiesis. I have utilized the single-step assay to examine the proliferation of lymphohemopoietic progenitors and their commitment to the B-lymphoid lineage, and to characterize the effects of IL-3 on proliferating lymphohemopoietic and early committed B-lymphoid progenitors.

MATERIALS AND METHODS

Growth factors.

Purified recombinant murine SF (the ligand for c-kit) was provided by Genetics Institute Inc. (Cambridge, MA). Purified recombinant human IL-7 was provided by Dr. C. Faltynek (Sterling Drug Inc., Malvern, PA). Medium conditioned by CHO cells genetically engineered to produce murine IL-3 at a high titer (-70,000 U/ml) was a gift from Dr. T. Sudo (Biomaterial Research Institute, Yokohama, Japan). Purified recombinant human IL-11 was provided by Dr. P. Schendel (Genetics Institute). Purified human Ep was provided by the Genetics Institute Clinical Manufacturing Group.

Monoclonal antibodies (mAbs).

See Materials and Methods of Chapter 2.

Purification of progenitor cells.

See Materials and Methods of Chapter 2.

Culture of progenitors in liquid or semisolid conditions.

Fifty enriched cells were plated in either 1 ml of liquid in one well of a 24 well flat bottom tissue culture plate (Becton Dickinson Labware, Lincoln Park, NJ) or 1 ml of semisolid methylcellulose culture in a 35 mm suspension culture dish (Becton Dickinson Labware, Lincoln Park, NJ). The materials and methods are discussed in Chapter 2. The concentrations of cytokines used in culture are as follows: SF, 100 ng/ml; IL-3 CHO-CM, 200 U/ml; IL-7, 5 ng/ml; IL-11, 100 ng/ml and Ep, 2 U/ml.

Two-step methylcellulose culture assay for lymphohemopoietic progenitors.

See Materials and Methods of Chapter 2. In experiments requiring cultured pre-B cells, pre-B cell colonies were harvested and then subjected to flow cytometric cell sorting for $B220^+$ and PI^- cells present in the lymphoid window (24).

The single-step assay for the identification of lymphohemopoietic progenitors present in normal mice.

See Chapter 2.

Identification of lymphohemopoietic progenitors present in primary suspension cultures.

Enriched progenitors from 5-FU-treated mice were plated in suspension culture containing IL-11, SF, IL-7 and Ep at a concentration of 50 cells per ml. At various time points the total nucleated cell count was determined and the cells were washed 3 times with α-MEM/10% FCS. A portion of the cells were replated in 1 ml of semisolid culture containing IL-11, SF, IL-7 and Ep at a concentration maximizing the number of colonies per dish without sacrificing clonality. The secondary colonies present in culture were then examined with an inverted microscope from 12 to 16 days after replating, depending on the duration of the primary culture, and all colonies except small GM and M colonies were harvested for FACS analysis.

Exposure of proliferating progenitors to IL-3.

Fifty enriched progenitors were plated in 1 ml of suspension culture containing SF, IL-11, IL-7 and Ep. On the designated day, IL-3 was added to each culture for the appropriate exposure period. At the conclusion of the exposure period to IL-3, the IL-3 was removed by washing the cells 3 times with 10 ml of α-MEM/10% FCS. After washing, the cells were recultured in 1ml of suspension culture containing SF, IL-11, IL-7 and Ep. After 11 days of total primary suspension culture, as in the two-step assay for

lymphohemopoietic progenitors, the pre-B cell colony forming ability was assessed by preparing 6 dishes of semisolid media containing SF and IL-7 for each exposure group and culturing 1/200th of the cells present in each condition per dish. The presence or absence of pre-B cell colonies was noted 12 days after initiation of the lymphoid cultures.

RESULTS

Commitment of primitive lymphohemopoietic progenitors to the B-lymphoid lineage.

Since the single-step assay is capable of supporting and identifying colony formation by actively proliferating lymphohemopoietic and committed B220 negative B-lymphoid progenitors, progenitors that give rise to lymphomyeloid colonies and B-lymphoid colonies, I utilized the single-step assay to examine the proliferation of lymphohemopoietic progenitors and their commitment to the B-lymphoid lineage. Fifty enriched progenitors were cultured per well in 1 ml of suspension culture containing IL-11, SF, IL-7 and Ep for replating on days 4, 5, 6, 7, 9 and 11 of primary culture. On the designated day of replating, the total nucleated cell count (TNCC) was determined, and a portion of the cells present in primary culture were replated into methylcellulose culture containing a combination of cytokines identical to the primary culture, IL-11, SF, IL-7 and Ep. After 12 to 16 days in culture, approximately 21 days after the initiation of the primary suspension culture, in situ examination of the colonies present in secondary culture was performed. All colonies except small GM and M colonies less than 2,000 cells were harvested for FACS analysis. After in situ examination for myeloid lineage expression and FACS analysis for the presence of $B220^+$ pre-B cells, colonies in secondary culture were designated Lymphomyeloid, B-lymphoid, Myeloid, or GM and M. The results of these replating experiments are presented in Table 3-1. Of the colonies appearing in secondary cultures initiated by cells replated on days 4, 5 and 6, only lymphomyeloid, myeloid and GM and M colonies were identified. Interestingly, on day 7 of culture, of the colonies expressing the B-lymphoid lineage, 60% were identified as lymphomyeloid while 40% were identified as being B-lymphoid. In the cultures initiated with cells replated on days 9 and 11, only B-lymphoid and GM and M colonies appeared in secondary culture. These results suggest that on days 0 through 6 the lymphohemopoietic progenitors proliferate. On or about day 7, the lymphohemopoeitic progenitors begin to commit to the B-lymphoid

lineage, generating B-lymphoid restricted progenitors and finally by day 9 of culture, the commitment to the B-lymphoid lineage appears to be complete and the proliferation of committed B-lymphoid progenitors takes place. However, it could be argued that the appearance of B-lymphoid restricted progenitors on day 7 of culture could be attributed to a small population of committed B-lymphoid progenitors that require 7 days of culture to expand to detectable levels. To dispel this argument, we attempted to determine that there were no committed B-lymphoid progenitors in the initial population and to examine the kinetics of commitment of individual enriched marrow cells. In a series of 7 experiments 209 enriched progenitors from normal mice were cultured in the presence of IL-11, SF, IL-7 and Ep for a minimum of 16 days. Of these progenitors, all expressed cells of the myeloid lineage and no committed B-lymphoid progenitors were identified, data not shown. The kinetics of commitment of indivdiual progenitors was examined by plating enriched progenitors in semisolid culture containing IL-11, SF, IL-7 and Ep for 3, 4, 6 and 11 days, at which time primary colonies were harvested and were replated in semisolid culture containing identical cytokines as the primary culture. Indivdiual primary colonies that were replated on days 3 and 4 of primary culture and expressed the B-lymphoid lineage gave rise only to lymphomyeloid colonies in secondary culture. Of the primary colonies replated on day 6 which expressed B-lymphoid potential, two-thirds gave rise only to lymphomyeloid colonies while the remaining third gave rise to both lymphomyeloid and B-lymphoid colonies in secondary culture. Finally, of the individual colonies replated on day 11 of primary culture which expressed the B-lymphoid lineage, only B-lymphoid colonies were identified in secondary cultures, data not shown. These observations indicate that enriched progenitors from mice treated with 5-FU, when cultured in permissive culture conditions first proliferate for approximately 6 days prior to commitment to the B-lymphoid lineage on or about day 7.

Timed exposure to IL-3.

Knowing that IL-3 exerts a negative modulatory effect on early B-lymphopoiesis by progenitors isolated from both 5-FU treated (21, 22) and normal mice (23), and having demonstrated that early B-lymphopoiesis by progenitors from 5-FU treated mice apparently proceeds through somewhat discrete stages, I attempted to discern upon which stage of early B-lymphopoiesis IL-3 exerts its negative modulatory effect. In order to do this, IL-3 was added for 48 or 72 hour intervals to permissive suspension culture conditions containing IL-11, SF, IL-7 and Ep. Fifty enriched progenitors were cultured in suspension culture containing SF, IL-11, IL-7 and Ep for a total of 11 days and were then recultured into lymphoid specific semisolid culture containing SF and IL-7 to assess the B-lymphoid potential. IL-3 was added on the designated day and removed by washing as shown in Figure 3-1. Enriched progenitors cultured in the absence of IL-3 for 11 days gave rise to 72±7 pre-B cell colonies per dish in secondary culture. Interestingly, exposure of proliferating lymphohemopoeitic progenitors to IL-3 on days 4 to 6 of culture increased the number of pre-B cell colonies appearing in culture to 125±14 per dish ($p>0.001$) in secondary culture. This observation was similar to our previously published observation in which 48 hour exposure to IL-3 from day 0 to day 2 increased the number of pre-B cell colonies appearing in secondary culture approximately 100% above that of control (22). In an additional experiment, proliferating progenitors exposed to IL-3 for 48 hours gave rise to 49±8 pre-B cell colonies per dish, 50% higher than the control group. Despite the positive effect of IL-3 on proliferating lymphohemopoietic progenitors, exposure of committed B-lymphoid progenitors to IL-3 beyond day 6 of culture, abrogated B-cell potential in secondary culture. These results indicate that 48 hour interval exposure of proliferating lymphohemopoietic progenitors to IL-3 accentuates the formation of lymphohemopoietic progenitors, while 48 hour exposure of early committed B-lymphoid progenitors to IL-3 abrogates B-cell potential.

Proliferation of lymphohemopoeitic progenitors in the presence of IL-3.

Having demonstrated that lymphohemopoietic progenitors exist in primary culture for approximately 6 days, and having shown that interval exposure from day 4 to day 6 accentuates the expression of the B-lymphoid lineage in secondary culture, I was quite puzzled by the previously reported observation, that continuous exposure of proliferating progenitors to IL-3 for 4 or more days inhibited the expression of the B-lymphoid lineage in secondary culture. Therefore, I examined the kinetics of the proliferation of lymphohemopoietic progenitors in the absence and in the continuous presence of IL-3. In this experiment, 50 enriched progenitors from mice treated with 5-FU were cultured in suspension culture containing IL-11, SF, IL-7, Ep and IL-3 for 84, 90, 96, 102, and 108 hours. At each time point the TNCC was determined, the cells were thoroughly washed and a portion of the washed cells were replated in the conditions of the single step-assay. Secondary cultures were examined after 16 days for the presence of lymphomyeloid and B-lymphoid colonies. In control cultures, 50 cells were cultured in the presence of IL-11, SF, IL-7 and Ep, and at 96, 120, 144, 168 and 216 hours the TNCC was determined and a portion of the cells were replated in the conditions of the single-step assay. The total number of lymphohemopoietic progenitors present in culture was calculated and the proliferation of lymphohemopoietic progenitors in the presence and in the absence of IL-3 is diagrammed in Figure 3-2. The maximum proliferation of lymphohemopoietic progenitors in the absence of IL-3 occurred at 144 hours, and by 216 hours no lymphohemopoietic progenitors were identified. However, in the presence of IL-3 the degree and the duration of proliferation of the lymphohemopoietic progenitors was altered. In the presence of IL-3 the maximum proliferation of lymphohemopoietic progenitors occurred at 96 hours, 48 hours earlier than in the absence of IL-3, and the maximum amount of proliferation of lymphohemopoietic progenitors in the presence of IL-3 was approximately 10 fold lower than in the absence of IL-3. Interestingly at 96 hours, more lymphohemopoietic progenitors were identified in the cultures containing IL-3 than were

identified in the cultures in which IL-3 was absent, supporting the conclusion that IL-3 accentuates the proliferation of lymphohemopoietic progenitors. Additionally, no restricted B-lymphoid colonies were identified in the cultures containing IL-3 as expected. Thus, short interval exposure of proliferating lymphohemopoietic progenitors to IL-3 appears to accentuate their proliferation while long interval exposure to IL-3 appears to shorten the duration of the proliferation of lymphohemopoietic progenitors.

Pre-B cell formation in the presence of IL-3.

Since very early committed B-lymphoid progenitors, those present in culture from day 6 to day 11, are very sensitive to the inhibitory effects of IL-3, I attempted to determine the point at which the committed B-lymphoid progenitors became non-sensitive to the toxic effects of IL-3. I cultured enriched progenitors from mice treated with 5-FU in semisolid culture containing IL-11, SF, IL-7 and Ep for 14 days, at which time twenty primary colonies were harvested, pooled and sorted by flow cytometry for Mac-1$^-$, Gr-1$^-$, TER119$^-$ and PI$^-$ cells. The sorted cells were then cultured at a concentration of $5x10^4$ cells/ml in a secondary suspension culture containing SF, IL-11, IL-7 and Ep in the presence and absence of IL-3 for 10 days. The cultures were expanded as necessary and were analyzed by flow cytometry for the presence of B220$^+$ pre-B cells. The dot plot analyses presented in Figures 3-3 A and B are representative of cells after culture in the absence and the presence of IL-3 respectively. Only PI$^-$ cells present in the lymphoid window (25) were included in these analyses. As can be seen, a distinct population of B220$^+$ pre-B cells developed in both the absence and presence of IL-3. Calculation of the total number of pre-B cells present in the cultures with and without IL-3 was $(2.3 \pm 0.5) \times 10^5$ and $(3.5 \pm 3.3) \times 10^5$ respectively. Therefore, by day 14 of culture the committed B-cell progenitors are no longer sensitive to the cytotoxic effects of IL-3, and IL-3 has little or no negative effect upon their proliferation.

Pre-B cell proliferation in the presence of IL-3.

A pure population of cultured pre-B cells was obtained by sorting pooled pre-B cell colonies for $B220^+$ and PI^- cells by flow cytometry. These sorted cells were then cultured in triplicate suspension cultures at a concentration of $5x10^4$ cells per ml. The cell number was then determined 3, 4 and 6 days after initiation of the culture, and all cultures were expanded on day 4 of culture. As can be seen in Figure 3-4, IL-3 had no effect, positive or negative, on the proliferation of cultured pre-B cells.

DISCUSSION

It appears that the primitive lymphohemopoietic progenitors from mice treated with 5-FU proliferate for 6 to 7 days prior to their commitment to the B-lymphoid lineage. B-lymphoid committed progenitors can first be identified on day 6 or 7 of primary culture in conjunction with lymphohemopoietic progenitors, and beyond day 9 of culture, no lymphohemopoietic progenitors, only committed B-lymphoid progenitors can be identified. Although it is possible that the pooled progenitors used in a majority of these studies are contaminated by committed B-lymphoid progenitors, the observed proliferation and disappearance of the lymphohemopoietic progenitors cannot be disputed, and it is probable that the lymphohemopoietic progenitors generate the majority of the committed B-lymphoid progenitors observed. Therefore, early B-lymphopoiesis by this population of lymphohemopoietic progenitors appears to proceed through 3 stages: a lymphohemopoietic proliferative stage; a commitment stage and an early committed B-lymphoid proliferative stage that ends in the production of $B220^+$ pre-B cells.

It has been previously reported that IL-3 appears to have a negative modulatory role on the process of early B-lymphopoiesis by progenitors isolated from 5-FU treated and normal mice (21-23). Having identified three stages of early B-lymphopoiesis by lymphohemopoietic progenitors the stage in which IL-3 exerts its negative modulatory effect was determined. Similar to the previously published results, 48 hour exposure of proliferating lymphohemopoietic progenitors, between day 4 and 6 of culture, increased the number of pre-B cell colonies appearing in secondary culture approximately 50 to 70%. However, when proliferating committed B-lymphoid progenitors, present in culture from day 7 to day 11, were exposed to IL-3 the B-lymphoid potential was abrogated, and on day 14 and beyond IL-3 exerted no negative effect on committed B-lymphoid progenitors. These observations suggest the early committed B-lymphoid proliferative stage, the stage marked by the proliferation of committed B-lymphoid progenitors ending in the formation

of $B220^+$ pre-B cells, could be further subdivided based on the inhibition or lack thereof by IL-3.

Hardy et al. has established a series of stages of B-lymphoid differentiation by progenitors expressing B220 (25). These stages have been designated A, B, C, C' and D. Briefly, the most primitive identifiable committed B-lymphoid progenitors express B220 and CD43 and are characteristic of the progenitors present in stage A, while stage D is composed of $B220^+$, $CD43^-$, HSA^+, $BP\text{-}1^+$ and cytoplasmic μ chain positive pre-B cells (25-27). Previously, several investigators have established IL-3 dependent B-lymphoid progenitor, pro- and pre-B cell lines, thus implicating IL-3 as a very important regulator of B-lymphopoiesis (28-32). However, in each case these cell lines were at least positive for the expression of B220 and several of them were pre-B cell lines. We have demonstrated that IL-3 has no negative effect upon the proliferation of the committed B-lymphoid progenitors present on or after day 14 of culture, and it is quite possible that the IL-3 dependent cell lines reside in a similar stage of differentiation as the progenitors present in culture on day 14 and later.

Interestingly, when lymphohemopoietic progenitors were cultured in the presence of IL-3, their progeny could be identified in culture for a maximum of 102 hours. The proliferation of lymphohemopoietic progenitors reached a maximum at 96 hours, approximately 48 hours prior to the maximum in the absence of IL-3. Additionally, the maximum number of lymphohemopoietic progenitors present at 96 hours, although at that time point was greater than the control, was approximately 10 fold less than the maximum number of lymphohemopoietic progenitors generated in the absence of IL-3. The observation that short exposure of proliferating lymphohemopoietic progenitors to IL-3 enhances the proliferation of lymphohemopoietic progenitors and that constant exposure decreases both the duration and the degree of proliferation of lymphohemopoietic progenitors indicates that IL-3 has a very complex mechanism of action. If IL-3 functions by stimulating proliferation and differentiation while sacrificing the self-renewal, then short

exposure of proliferating progenitors to IL-3 may increase proliferation without significantly altering self-renewal while, exposure for longer periods may have a negative effect on self-renewal in an attempt to generate larger numbers of more mature hemopoietic cells. This would account for the observation that short exposure increases the production of lymphohemopoietic progenitors while longer exposure increases the rate of differentiation. Similar results have been obtained in my examination of the effect of IL-3 on the maintenance of long term reconstitution potential of primitive hemopoietic progenitors in that self-renewal potential is more rapidly lost by progenitors cultured in the presence of IL-3 when compared with progenitors cultured in the absence of IL-3 (33).

Unfortunately, because of the inability to significantly purify the early committed B-lymphoid progenitors, I am unable to determine whether IL-3 directly inhibits committed B-lymphoid progenitors. Since IL-3 is a very strong stimulus for the proliferation of myeloid lineage cells, it is possible that the observed cytotoxic effect is an indirect effect mediated by other cells present in the cultures. Regardless of the exact mechanism, be it direct or indirect, IL-3 appears to function as a negative modulator of the proliferation of the earliest committed $B220^-$ and $B220^+$ B-lymphoid progenitors. In culture, my results corroborate the previous *in vivo* findings of Cockayne et al., implicating IL-3 as a negative modulator of B-lymphopoiesis (34). In their studies, they developed transgenic mice expressing antisense IL-3 RNA, essentially blocking IL-3 production in these mice. Their mice developed either a lymphoproliferative syndrome or neurologic dysfunction. Of interest to me is the lymphoproliferative syndrome which is marked by an aggressive proliferation of $B220^+/sIgM^-$ B-lineage blasts throughout the mice. Together these results suggest that IL-3 is a very important negative effector of B-lymphopoiesis, dampening the proliferation of the earliest B-lymphoid committed progenitors.

Finally the apparent capacity of IL-3 to shorten the duration and the degree of self-renewal of lymphohemopoietic progenitors suggests that great caution should be exercised

in using IL-3 to expand lymphohemopoietic progenitors *in vitro* as it may induce their differentiation.

Table 3-1. Kinetics of Lymphohemopoietic Progenitor Proliferation and Commitment

Day of suspension culture	TNCC	No. of cells replated/dish in secondary culture	Analyzed				
			Lymphomyeloid	B-lymphoid	Myeloid	GM&M	Total
4	$5x10^3$	50	2±1	0	3±3	24±5	27±5
5	$1x10^4$	50	1±1	0	1±1	18±4	19±3
6	$1.4x10^4$	100	2±2	0	2±1	21±2	24±3
7	$1.7x10^5$	200	6±2	4±2	0±1	103±17	113±17
9	$1.1x10^6$	500	0	2±1	0±0	15±4	18±4
11	$2.8x10^6$	1/200th	0	74±7	0	ND	ND

Fifty post 5-FU Lin^- $Sca\text{-}1^+$ progenitors were cultured in suspension culture in the presence of SF, IL-11, IL-7 and Ep. On the designated day of suspension culture the total nucleated cell count (TNCC) was determined and a sample of the cultured cells was plated in semisolid culture containing SF, IL-11, IL-7 and Ep. After 16 to 17 days of secondary culture the dishes were examined. All colonies except small GM and M colonies were harvested for flow cytometric analysis. Data represent mean and standard deviation of 10 culture dishes.

Figure 3-1. Interval exposure of proliferating progenitors to IL-3. Fifty enriched progenitors were cultured in primary suspension culture containing IL-11, SF, IL-7 and Ep. IL-3 was added to the cultures for the designated exposure period and was removed by washing. After washing, the cells were resuspended in the original culture conditions. On day 11 1/200th of the cells present were replated in semisolid lymphoid suspension culture containing SF and IL-7 to assess the B-lymphoid potential. Pre-B cell colonies number represents the mean and standard deviation of 6 dishes, $p < 0.001$.

IL-3 Exposure	Day of primary culture								Pre-B cell colonies no.
	4	5	6	7	8	9	10	11	
None									72±7
Day 4-6									125±14
Day 4-7									1±1
Day 5-7									0±0
Day 7-9									0±0
Day 9-11									1±1

Figure 3-2. Proliferation of lymphohemopoietic progenitors in the presence of IL-3. Fifty enriched progenitors were cultured in suspension culture containing IL-11, SF, IL-7 and Ep in the presence or absence of IL-3. The lymphohemopoietic progenitors present in cultures containing IL-3 were examined at 84, 90, 96, 102 and 108 hours, while progenitors cultured in the absence of IL-3 were examined at 96, 120, 144, 168 and 216 hours. The number of progenitors identified at 108 hours in the presence of IL-3 and at 216 hours in the absence of IL-3 was 0.

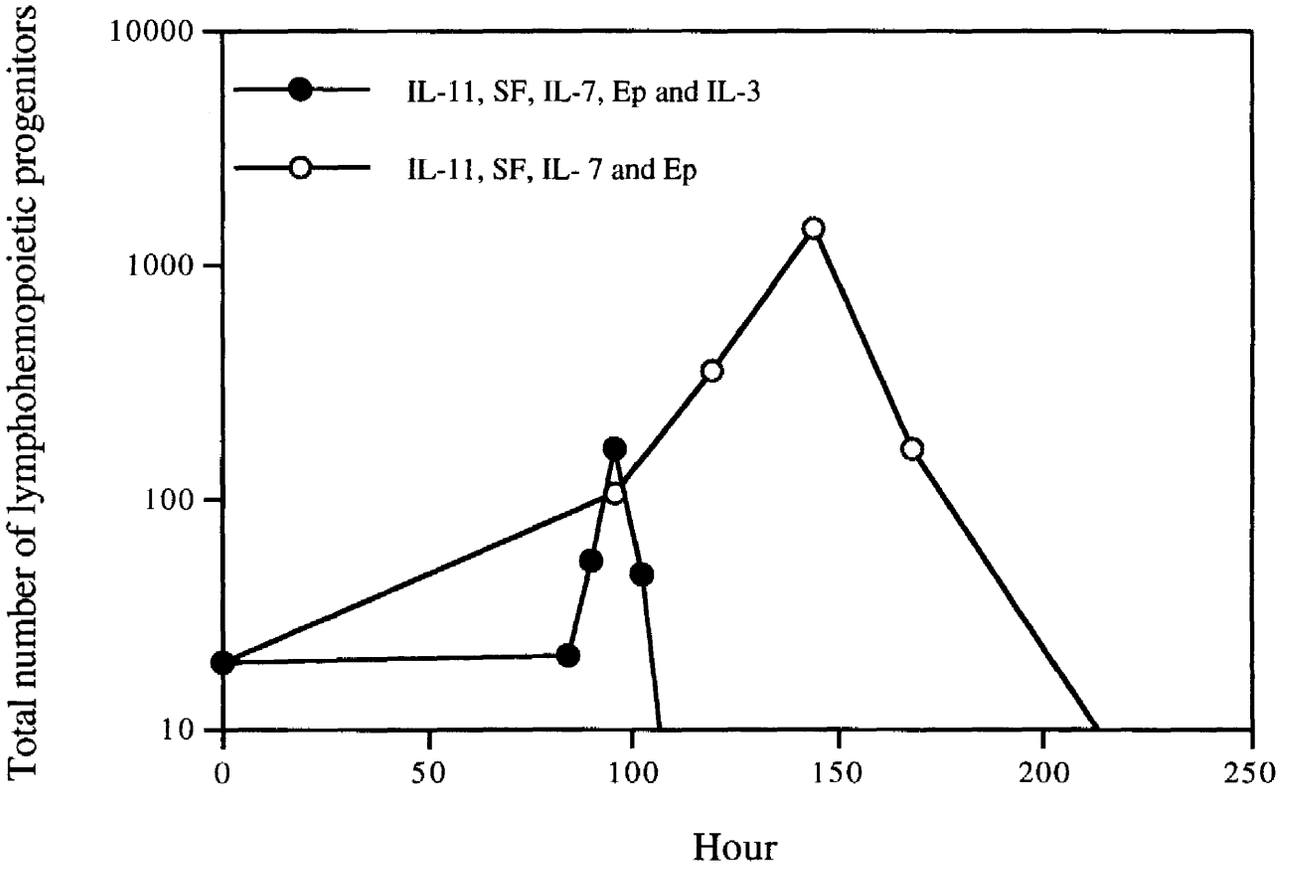

Total number of lymphohemopoietic progenitors
10000
1000
100
10
IL-11, SF, IL-7, Ep and IL-3
IL-11, SF, IL- 7 and Ep
0
50
100
150
200
250
Hour

Figure 3-3. Generation of $B220^+$ pre-B cells in the presence and absence of IL-3. Two color analysis of day 14 $Mac\text{-}1^-$, $Gr\text{-}1^-$, $TER119^-$ and PI^- colony cells after 9 days of suspension culture in SF, IL-11, IL-7 and Ep (A) and SF, IL-11, IL-7, Ep and IL-3 (B). The analysis includes only PI^- cells present in the lymphoid window.

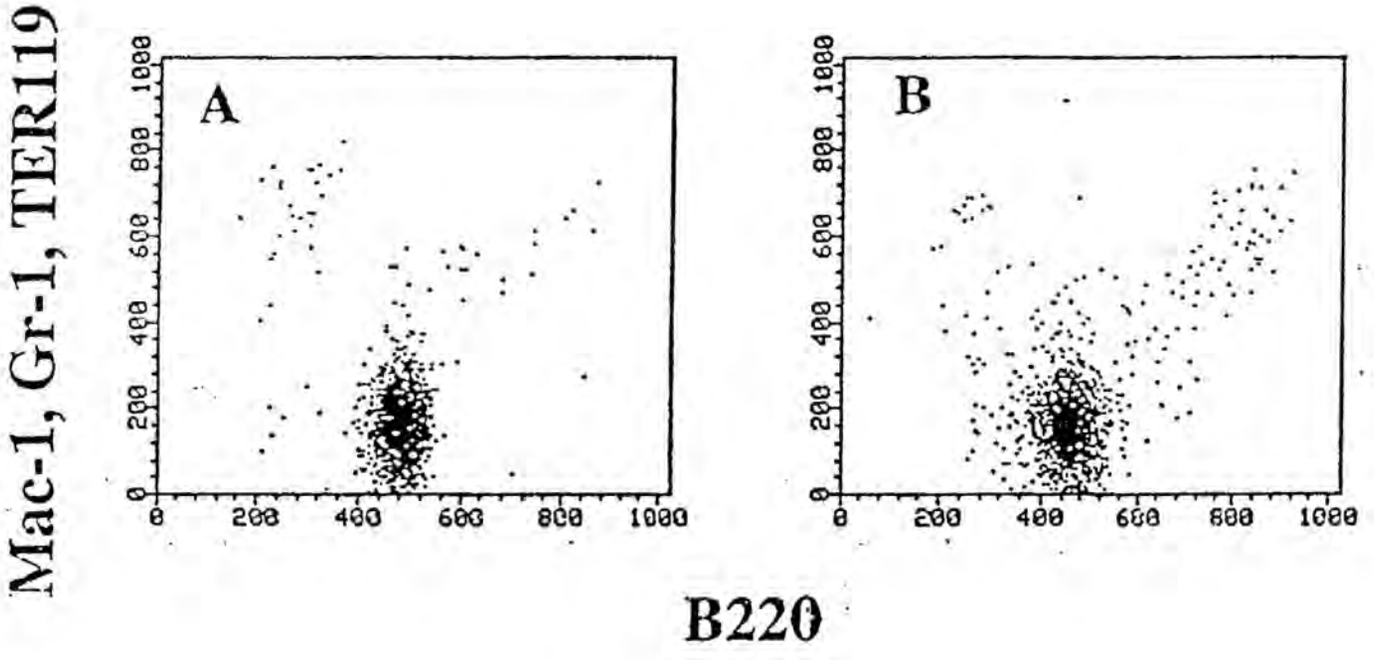
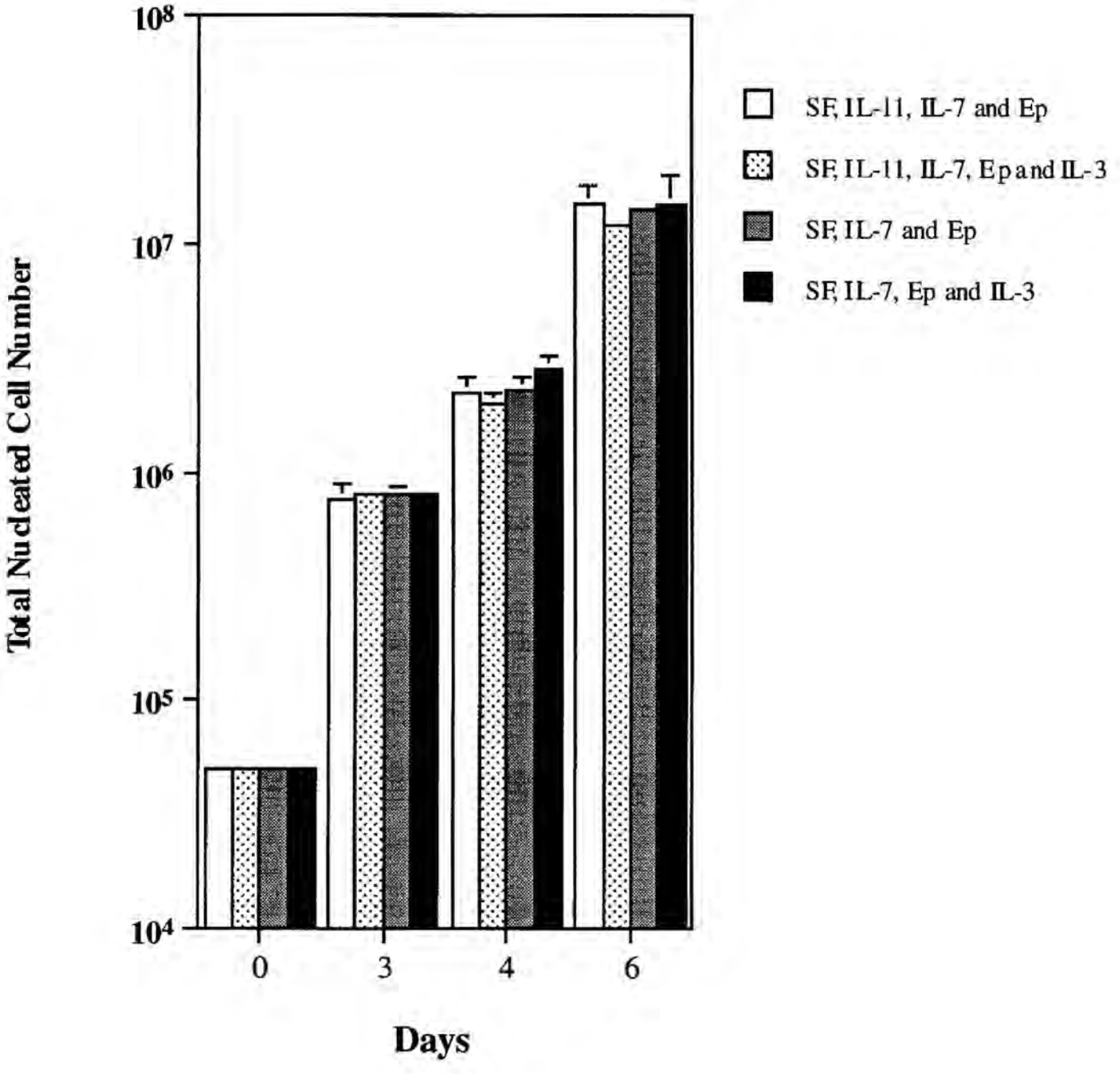

Mac-1, Gr-1, TER119
A
B
0
200
400
600
800
1000
B220

Figure 3-4. Proliferation of pre-B cells in the presence and absence of IL-3. $B220^+$ pre-B cells were sorted from pooled cultured pre-B cell colonies and were cultured in suspension culture. The data represent mean and standard deviation of triplicate cultures.


Total Nucleated Cell Number
10^8
10^7
10^6
10^5
10^4
0
3
4
6
Days
SF, IL-11, IL-7 and Ep
SF, IL-11, IL-7, Ep and IL-3
SF, IL-7 and Ep
SF, IL-7, Ep and IL-3

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Chapter IV

Late Divergence of B-Lymphoid and Myeloid Lineages: Evidence Supporting a Model of Random Restriction of Lineage Potential, and the Possible Existence of a Bipotential B-Lymphoid/Macrophage Progenitor

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INTRODUCTION

The most widely accepted view of the differentiation of primitive cell-cycle dormant lymphohemopoietic progenitors holds that only the most primitive progenitors have lymphohemopoietic potential. As these progenitors begin to proliferate, it is believed that the divergence of the B-lymphoid and the myeloid lineage potentials is occurs, forming lymphoid and myeloid restricted progenitors. These lymphoid and myeloid restricted progenitors give rise to cells of the lymphoid and myeloid lineages, as is shown in Figure 1-1. Only recently with the development of the two-step and the single-step assays for lymphohemopoietic progenitors, has it been possible to examine the B-lymphoid and the myeloid potential of primitive hemopoietic progenitors (1, 2). Prior to this, examination of the lineage potential of individual primitive hemopoietic progenitors was restricted primarily to the myeloid lineage. Till et al. developed the first quantitative assay for primitive hemopoietic progenitors while investigating the reconstitution of lethally irradiated mice with donor marrow. Macroscopic nodules of proliferating hemopoietic cells were noted to be present in the spleens of the irradiated recipient mice 7 to 14 days after transplantation. These nodules were called spleen colonies, and the progenitors giving rise to these colonies were designated colony forming unit-spleen (CFU-S) (3). These progenitors were found to have high proliferative capacity and differentiative capacity along with self-renewal capacity, thus fulfilling the three criteria of stem cell as defined by Till (4). In 1979 Hodgson et al. identified two populations of CFU-S which formed spleen colonies that were macroscopically identifiable 10 or 13 days after transplantation (5). They also found that mice treated with the chemotherapeutic drug 5-FU (6) retained only the population of CFU-S which formed spleen colonies on day 13. In 1982 Magli et al. examined the appearance and disappearance of spleen colonies over time, and their results corroborated

the observations of Hodgson et al. suggesting two distinct populations of CFU-S; a more mature population giving rise to spleen colonies present on day 8 and a more primitive population giving rise to spleen colonies present on day 12 after transplantation (7). Throughout these investigations, only myeloid lineage cells, macrophages, granulocytes, megakaryocytes and erythroid progenitors were found to be present in spleen colonies (1,2). The lack of lymphoid differentiation in such an important lymphoid organ as the spleen suggested that these cells were myeloid restricted stem cells with self-renewal capacity. As late as 1979, definitive demonstration of B-lymphoid expression by CFU-S had not been produced (8-10). It was not until 1984 that individual day 12 CFU-S were demonstrated to have the ability to give rise to cells of both B- and T-lymphoid lineages upon reinjection into irradiated mice (11). Lepault recently corroborated these results and further established that 100% of day 12 CFU-S and many day 8 CFU-S have both B- and T-lymphoid potential (12).

Results obtained in the characterization of the lymphohemopoietic progenitors present in normal mice and in the study of the proliferation and commitment of primitive lymphohemopoietic progenitors (2, 13) suggest that the divergence of the B-lymphoid and myeloid lineages does not occur as hypothesized in the conventional view of hemopoietic differentiation. Therefore, to further examine the mechanism of lineage divergence, I used the two-step assay and a modified version of the two-step assay to examine the lineage expression of Lin^- $Sca\text{-}1^+$ progenitors from 5-FU treated and normal mice. Assuming that the culture systems are equally permissive for the expression of all myeloid lineages, the large degree of heterogeneous expression of the myeloid lineage by individual progenitors is consistent with previous observations and supports a stochastic model of lineage commitment (14). Of the lymphohemopoietic progenitors from normal mice, the majority gave rise to only 1 or 2 myeloid lineages in addition to the B-lymphoid lineage, and it was these progenitors that were most sensitive to the cytotoxic effects of 5-FU. No significant positive relationship between the number of myeloid lineages expressed and the

coexpression of the B-lymphoid lineage was identified. The conventional view of hemopoietic differentiation in which the divergence of lymphoid and myeloid lineages occurs very early would predict such a positive relationship, since only the most primitive progenitors would retain both lymphoid and myeloid potential. Assuming the culture system is equally permissive for the differentiation of all myeloid lineages, these results support a model of random restriction of lineage potential with a late divergence of the B-lymphoid and the myeloid lineages, possibly resulting in the formation of a bipotential B-lymphoid/macrophage progenitor.

MATERIALS AND METHODS

Growth factors.

Conditioned medium (CM) of Chinese hamster ovary (CHO) cells that had been transfected with an expression plasmid containing murine SF cDNA was provided by (Genetics Institute Inc., Cambridge , MA). Purified recombinant human IL-7 was provided by Dr. C. Faltynek (Sterling Drug Inc., Malvern, PA). Medium conditioned by CHO cells genetically engineered to produce murine IL-3 at a high titer (-70,000 U/ml) was a gift from Dr. T. Sudo (Biomaterial Research Institute, Yokohama, Japan). Purified recombinant human IL-11 was provided by Dr. P. Schendel (Genetics Institute). Purified human Ep was provided by the Genetics Institute Clinical Manufacturing Group. Recombinant murine IL-5 was provided by the Genetics Institute. Pokeweed mitogen spleen conditioned medium (PWM-SCM) was prepared in our laboratory as described (15).

Monoclonal antibodies (mAbs).

See Materials and Methods of Chapter 2.

Purification of progenitor cells.

See Materials and Methods of Chapter 2.

Culture of progenitors in semisolid conditions.

Fifty enriched cells were plated in 1 ml of semisolid methylcellulose culture in a 35 mm suspension culture dish (Becton Dickinson Labware, Lincoln Park, NJ). the materials and methods are described in Chapter 2. The concentrations of cytokines used in culture are as follows: SF, 100 ng/ml; IL-3 CHO-CM, 200 U/ml; IL-5, 12 U/ml; IL-7, 5 ng/ml; IL-11, 100 ng/ml; Ep, 2 U/ml and PWM-SCM, 5%.

Two-step methylcellulose culture assay for lymphohemopoietic progenitors.

See Materials and Methods of Chapter 2.

The single-step assay for the identification of lymphohemopoietic progenitors present in normal mice.

See Materials and Methods of Chapter 2.

Identification of lymphohemopoietic progenitors in primary suspension cultures.

See Materials and Methods of Chapter 3.

RESULTS and DISCUSSION

Analysis of B-lymphoid and myeloid lineage expression by enriched progenitors from normal and 5-FU-treated mice.

The myeloid and B-lymphoid lineage potential of enriched progenitors from 5-FU-treated and normal mice were examined in the two-step and a slightly modified version of the two-step assay in 4 separate experiments, respectively. Briefly, in the two-step assay for lymphohemopoietic progenitors from normal mice, the primary colonies were harvested on day 8, and the cells were plated in myeloid suspension culture containing SF, IL-3, IL-5 and Ep and B-lymphoid suspension culture containing SF and IL-7. The myeloid cultures were examined 4, 6 and 8 days after initiation of culture, and the B-lymphoid cultures were analyzed by FACS analysis for the presence of $B220^+$ pre-B cells after 11 days of secondary culture. Enriched progenitors from 5-FU-treated mice were cultured in IL-11, SF, IL-7 and Ep for 11 days, at which time the primary colonies were harvested and were replated into myeloid suspension culture containing PWM-SCM, IL-3 and Ep and semisolid lymphoid culture containing SF and IL-7. The morphology of the cells present in the suspension culture was examined 2, 4 and 6 days after initiation of the secondary culture, and the lymphoid cultures were examined after approximately 12 days for the presence of pre-B cell colonies.

The cumulative results of the experiments in which progenitors from normal mice were examined are shown in Table 4-1. Individual colonies are grouped together based on their myeloid lineage expression, and they are then subdivided based upon the absence or presence of the expression of the B-lymphoid lineage. From this, the percentage of each colony type with B-lymphoid lineage expression was calculated. In agreement with the previous identification of the percentage of LHP and B-lymphoid progenitors, 18% of the Lin^- $Sca\text{-}1^+$ progenitors gave rise to cells of both the B-lymphoid and the myeloid lineages, and approximately 5% appeared to be restricted to the B-lymphoid lineage (2). The most

common combination of myeloid lineages expressed by individual progenitors was that of the neutrophil (n) and macrophage (m) lineages. Nineteen percent of the colonies expressing n and m were also positive for the expression of the B-lymphoid lineage. The second most common pattern of myeloid lineage expression was m, and similarly, 17% of the macrophage colonies coexpressed the B-lymphoid lineage. Although the progenitors expressing these two combinations, nm and m, account for well over half of the colony forming progenitors, of the remaining progenitors, 55 gave rise to 3 or more myeloid lineages and of these, 22% percent were identified as lymphohemopoietic. Calculation of the mean and standard deviation of these percentages from the 4 separate experiments indicated that there was no significant difference in the percentage of lymphohemopoietic progenitors expressing nm, m, or 3 or more myeloid lineages, although the numbers are low.

The B-lymphoid and the myeloid lineage expression of individual progenitors from mice treated with 5-FU is shown in Table 4-2. The colonies are once again grouped according to their myeloid lineage expression and are subdivided based on the presence or absence of the B-lymphoid lineage expression. Finally, the percentage of each type of colony expressing the B-lymphoid lineage was calculated. Of a total of 61 individual progenitors examined, 43% or 26 were identified as being lymphohemopoietic. The most common myeloid lineage combination expressed by these progenitors was that of the n, m, erythroid (E), mast cell (Mast) and megakaryocyte (M), and 31% of the progenitors giving rise to this combination also expressed the B-lymphoid lineage. Interestingly, the next most common combination of myeloid lineages expressed was n and m, and 64% of these colonies also contained B-lymphoid lineage cells. Only 2 progenitors gave rise to the macrophage lineage alone and neither of these expressed the B-lymphoid lineage.

In Table 4-3 the lymphohemopoietic progenitors identified in Tables 4-1 and 4-2 are grouped based on the number of myeloid lineages they expressed. As can readily be seen, treatment with 5-FU dramatically decreased the percentage of lymphohemopoietic

progenitors expressing only 1 or 2 myeloid lineages. These results support previous observations which suggest that the progenitors giving rise to larger numbers of myeloid lineages are more resistant to the cytotoxic effects of 5-FU, whereas the progenitors that give rise to fewer myeloid lineages are very sensitive to the cytotoxic effects of 5-FU. Knowing that 5-FU inhibits thymidylate synthase creating a thymidylate deficiency that kills more rapidly proliferating cells, these results indicate that the progenitors with greater lineage potentials are proliferating more slowly than are the progenitors with fewer lineage potentials.

If a hierarchy of differentiation exists, then primitive hemopoietic progenitors should have the potential to give rise to many myeloid lineages, and as differentiation proceeds, the resulting more mature progeny should have somewhat restricted lineage potentials. If the conventional view of hemopoietic differentiation holds, and only the most primitive progenitors have lymphohemopoietic potential, then all lymphohemopoietic progenitors must be more primitive than myeloid restricted progenitors. Following this, one would expect a direct relationship between the number of myeloid lineages expressed and the expression of the B-lymphoid lineage to exist. However, examination of the myeloid lineage expression by lymphohemopoietic progenitors present in normal mice indicates that no such relationship exists, and further, treatment with 5-FU removes a great majority of the lymphohemopoietic progenitors with restricted myeloid lineage expression. These results cast doubt on the conventional model of hemopoiesis which proposes a very early divergence of the myeloid and the lymphoid lineages in hemopoiesis.

Previously published observations of the lineage expression of individual blast cells, primitive hemopoietic progenitors, and their daughter cells indicated that the combinations of myeloid lineages expressed by these progenitors were very heterogeneous (16-20), and a total of fifteen different combinations of myeloid lineages expressed by individual progenitors were identified. A stochastic model of progressive random restriction of lineage potential by differentiating progenitors was developed (14). The Lin^-

Sca-1$^+$ progenitors from normal and 5-FU-treated mice were found to give rise to 13 of the 15 combinations previously reported.

The results obtained in this study support a model of hemopoietic differentiation which includes primitive hemopoietic progenitors resistant to the cytotoxic effects of 5-FU, that give rise to actively proliferating lymphohemopoietic progenitors with somewhat restricted myeloid lineage potential that are sensitive to the cytotoxic effects of 5-FU. A modified version of the stochastic model of progressive random restriction of lineage potential (14), in which the entire bone marrow compartment isolated on bone marrow aspiration is represented is presented in Figure 4-1. The shaded cells to the right of the divider represent the largest population of cells present in bone marrow preparations which are removed by density gradient centrifugation, negative selection for lineage committed antigens and Sca-1 sorting. The cells to the left of the divider represent the population of Lin$^-$ Sca-1$^+$ cells present in normal mice. The lightly shaded cells in the population of Lin$^-$ Sca-1$^+$ progenitors represent those cells sensitive to the cytotoxic effects of 5-FU, and the cells with no shading are the cells resistant to 5-FU. As can be seen, both normal and 5-FU treated populations, contain n, m and B-lymphoid progenitors, whereas only very few if any m and B-lymphoid progenitors are present in the population from 5-FU treated mice.

The model presented in Figure 4.1 also incorporates a progressive random restriction of lineage potentials that may result in the formation of a bipotential B-lymphoid/macrophage progenitor that is capable of giving rise to B-lymphoid progenitors and macrophage progenitors. It is important to note that the T-lymphoid lineage is only mentioned in the most primitive pluripotent lymphohemopoietic progenitor. This is because at the present time, conclusions regarding the expression of the T-lymphoid lineage are impossible due to the lack of an *in vitro* culture system permissive for the differentiation T-cell progenitors.

B-lymphoid/macrophage association.

As can be seen in Tables 4-1 and 4-2, virtually all of the lymphohemopoietic progenitors identified from normal and 5-FU-treated mice gave rise to the macrophage lineage. In Table 4-3, the progenitors identified as expressing both the myeloid and B-lymphoid lineages in Tables 4-1 and 4-2 were grouped according to their patterns of myeloid lineage expression. Surprisingly, 22% of the lymphohemopoietic progenitors isolated from normal marrow appear to be bipotential B-lymphoid/macrophage progenitors.

Over the past 15 years, many observations have been published suggesting an association between the B-lymphoid and the macrophage lineages. These observations are somewhat contradictory to the conventional view of hemopoietic differentiation. Some of the first evidence suggesting an association between the B-lymphoid and the macrophage lineage was from observations of leukemic patients, and in cell lines isolated from leukemic patients. Minowada et al. (21) in 1979 isolated a cell line of human origin that had pre-B cell characteristics, but was also positive for the Philadelphia chromosome, a marker for chronic myelogenous leukemia (CML). Since then, a number of investigators have reported cell lines isolated from CML patients exhibiting both B-lymphoid and monocytic characteristics. Several cases of patients with CML who presented with B-lymphoid blast crises have also been reported (22-24). Additional reports of acute leukemia with mixed myeloid and lymphoid phenotypes have also been published (25-28). In 1982 Boyd et al. demonstrated that treatment of the murine pre-B cell line ABLS 8.1 with the DNA demethylating drug, 5-azacytidine, gave rise to clones of macrophage-like cell lines (29). They theorized that alteration of the methylation pattern of the DNA allowed the transcription of a different series of genes, thus prompting the lineage change from pre-B to macrophage. They further speculated that there may be a common progenitor of both the B-lymphoid and the macrophage lineages. In 1986 Holmes et al. established murine cell lines from neonatal bone marrow and fetal liver infected with *ras* containing viruses that coexpressed antigens usually restricted to the B-lymphoid and the macrophage lineage,

B220 and Mac-1, respectively (30). They theorized, that these cell lines were immortalized bipotential B-lymphoid/macrophage progenitors as predicted in a model by Till (2) suggesting that primitive progenitors may express surface antigens of all lineages at very low concentrations and these aid in differentiation (31). Later, this same group examined the $B220^+$ $Mac\text{-}1^+$ cell line isolated from neonatal bone marrow and found that when stimulated with lippopolysaccharide, it was capable of differentiation into pre-B cells or into functional macrophages (32). In the same year, Klinken et al. created Eμ-*myc* transgenic mice. From these mice several pre-B cell lines were isolated. They found that the incorporation of v-*raf* into the Eμ-*myc* pre-B cell lines induced some cells to change lineage production from pre-B cells, to functional macrophages with rearranged immunoglobulin genes (33). Further, Sherr et al. has also demonstrated insertion of *fms*, the CSF-1 receptor, into pre-B cells in long term murine lymphoid cultures produced transformed cell lines capable of producing functional macrophages with rearranged Ig genes when placed in myeloid culture conditions (34). An overriding observation in all of these examples, is that only the macrophage lineage has been found in association with the B-lymphoid lineage. In no case does any other myeloid lineage associate with the B-lymphoid lineage in the same manner. McCulloch interprets these observations as aberrant programming or lineage infidelity (35) whereas, Greaves et al. argue that there is a transient period of lineage promiscuity and the afore mentioned examples are actually progenitors immortalized at specific stages in their differentiation (36).

Prior to the publication of the two-step assay, Cumano et al. published their findings regarding the isolation of a population of $AA4.1^+$, $B220^-$, $Mac\text{-}1^-$ and $Ly6A^+$ cells from day 12 murine fetal liver which contained bipotential B-lymphoid/macrophage progenitors (37). They found no combination of cytokines was capable of replacing the absolute requirement of S17 stromal cells and IL-7 for proliferation of the bipotential B-lymphoid/macrophage progenitors. They established the clonal or single cell nature of

these progenitors by micromanipulation, and they further examined the ability of additional cytokines in combination with S17 cells and IL-7 to support additional myeloid lineage expression by these progenitors. They observed that the only lineage expressed in conjunction with the B-lymphoid lineage was the macrophage lineage. In addition, they determined that these bipotential progenitors had no long term reconstitution potential when transferred into irradiated mice. Therefore, they concluded that the bipotential progenitors were not pluripotent stem cells, but rather, were restricted bipotential B-lymphoid/macrophage progenitors.

Examination of the B-lymphoid and the myeloid lineage potential of the Lin^- $Sca\text{-}1^+$ progenitors of normal mice revealed that only the macrophage lineage is expressed as a single myeloid lineage in conjunction with the B-lymphoid lineage Table 4-1. In addition, examination of the proliferation of lymphohemopoietic progenitors revealed that the last myeloid lineage whose expression was identifiable by progenitors retaining B-lymphoid and myeloid potential prior to the formation of B-lymphoid restricted progenitors was that of the macrophage lineage, supporting the conclusion that a bipotential B-lymphoid/macrophage progenitor is a normal part of B-lymphopoiesis.

Conventional $B220^+$ B cells and $Ly\text{-}1^+$, $B220^+$, $Mac\text{-}1^+$ B cells.

In conducting the flow cytometric analysis of individual colonies in the single-step assay, a population cells expressing both the B220 and Mac-1 surface antigens was identified. This population was most readily seen in colonies grown in the presence of IL-11, SF, IL-7, Ep and IL-3. Interestingly, two major subsets of B cells have been identified as components of the B-lymphoid lineage. The most well understood subset of B cells has been designated "conventional" B cells. They express the pan B cell antigen B220 but not the myeloid lineage antigen Mac-1 (38). These B cells are derived from bone marrow stem cells, and they constitute the predominant population of B cells in normal mice, greater than

99% of all B cells (39). These B cells typically produce the classic primary and secondary antibody responses to T cell-dependent antigens (40, 42).

Hayakawa et al. initially identified a population of B cells that expressed the pan-T surface antigen Ly-1 in 1983. These cells make up the second subset of B cells. (43, 44). Since then, they have been called "Ly-1" B cells, "CD5$^+$" B cells and "B1" B cells. Ly-1 B cells have several very distinctive criteria distinguishing them from "conventional" B-cells. They appear very early in development, approximately day 13 of fetal life, (45, 46) and they maintain extensive self-renewal potential (47). In fact, only fetal and neonatal liver and bone marrow preparations have the potential to reconstitute Ly-1 B cells, while bone marrow preparations from mice older than 8 weeks of age fail to reconstitute even minimal numbers of Ly-1 B cells in recipient mice (39). Hardy et al. has proposed a model of B-lymphopoiesis describing the early divergence of an Ly-1 B cell progenitor which occurs in fetal and neonatal life. This divergence leaves a "restricted" lymphohemopoietic progenitor in adult bone marrow that is incapable of the generation of the Ly-1 subset of B cells. Ly-1 B cells have a very specific tissue localization. Virtually no Ly-1 B cells are identifiable in peripheral lymph nodes and spleen, while almost half of all loosely attached B cells recovered from the peritoneal cavity are Ly-1 B cells (38). These cells appear to home to and remain in the peritoneal, pleural and pericardial cavities at relatively high concentrations (48, 49). Another distinctive characteristic of Ly-1 B cells is their secretion of IgM without T-dependent antigenic stimulation (50, 51). Ly-1 cells are very important in the generation of serum IgM, and a statistically significant correlation exists between the serum IgM level and the number of Ly-1 B cells present in an animal (52). A majority of the antibodies produced by Ly-1 B cells are encoded by germline genes and do not undergo somatic mutation (46-53). Of the IgM antibodies produced on a continuous basis by Ly-1 B cells, many are directed against self antigens and appear to play a role in several autoimmune diseases (37, 46, 54). It has been suggested that these autoantibodies may function in the clearance of senescent cells and removal of denatured proteins as many of their antigenic

determinants are carbohydrates on glycoproteins and glycolipids (45). However, according to Hardy, the most appealing theory as to the specificity, spontaneous production and function of the natural autoantibodies produced by Ly-1 B cells is that these specificity's have been selected into the germ line because such specificity's also react with many antigens present on frequently encountered pathogens. These therefore, may help in defense against pathogens presented to the neonate after the concentration of passive maternal immunoglobulin has begun to decrease and before the active T-dependent immune system is established (45). The final distinctive criterion of Ly-1 B cells is their expression of the pan-B cell antigen B220, the pan-T cell antigen Ly-1, and the myeloid lineage antigen Mac-1.

In 1987 Palacios et al. reported the development of a monoclonal antibody, CC11, (55, 56) capable of identifying two distinct populations of B-lymphoid progenitors present in the marrow of 5-week old mice (57). The two populations were $CC11^+$ and $CC11^-$. The $CC11^+$ cells were capable of proliferating in the presence of IL-3 and they were also characteristic of the subset of B cells known as the Ly-1 B cells with Ly-1 and Mac-1 antigen expression. The $CC11^-$ cells were not capable of proliferating in the presence of IL-1, IL-2, or IL-3, these cells were characteristic of precursors maintained in Whitlock-Witte cultures, "conventional" B cells (57). Unfortunately, no studies have been conducted to determine if $CC11^+$ cells are Ly-1 B cells.

Knowing a distinct subset of B cells exists that express not only the B220 surface antigen, but also the monocyte/macrophage specific antigen Mac-1 and that this population of cells may be capable of proliferation in the presence of IL-3, it is possible that the population of $B220^+$ and $Mac\text{-}1^+$ cells identified in the single step assay are progenitors of Ly-1 B cells. It is important to note that characterization of a Ly-1 B cell is based upon the expression of the Ly-1 antigen and not the Mac-1 antigen. Therefore, further investigation is necessary to determine if this population of $B220^+$ $Mac\text{-}1^+$ cells is related to Ly-1 B cells.

The conclusion that an association between the B-lymphoid and the macrophage lineages is based on the assumption that the two-step and the single-step assays are equally permissive for the differentiation and expression of all lineage potentials of an individual progenitor. In Table 4-4, the incidence of lineage expression by the entire population of enriched Lin^- $Sca\text{-}1^+$ progenitors from normal and from 5-FU-treated mice presented in Tables 4-1 and 4-2 is examined. As can be seen, the macrophage lineage is expressed by greater than 99% of the Lin^- $Sca\text{-}1^+$ progenitors from both normal and from 5-FU treated mice, and similarly, the neutrophil lineage is expressed by 73% and 97% of the same progenitors respectively. Interestingly, the E, B, M and the eosinophil (eo) lineages all have a similar incidence of expression within the populations. The myeloid suspension culture in which the progenitors from 5-FU were assayed, lacked IL-5, a late acting proliferative factor which enhances proliferation of the eosinophils, and this difference probably accounts for the unusually low incidence of identification of the eo lineage in the progenitors from 5-FU treated mice. Finally, the incidence of expression of the Mast lineage in both populations is just above that of the E, B, M and the eo lineages. In previous studies performed in this laboratory, a similar predominance of macrophage lineage expression was noted (16-20). J. Suda et al. concluded that the predominance of expression of the macrophage lineage by progenitors cultured in the presence of PWM-SCM could be due to a higher incidence or rate of production unique to the macrophage lineage, or it could be due to an artifact caused by an increased ability of the macrophage lineage to survive and proliferate in their culture system (14).

In conclusion, culture of enriched hemopoietic progenitors from normal and 5-FU-treated mice in maximally permissive culture systems has resulted in data that casts doubt on the validity of the conventional model of hemopoietic differentiation which postulates an early divergence of common myeloid and common lymphoid progenitors in hemopoiesis. Further, these observations support a stochastic model of the random restriction of lineage potential of differentiating progenitors. However, conclusions concerning the presence of

a bipotential B-lymphoid/macrophage progenitor rely on the assumption that these assays are equally permissive for the expression and identification of all lineages.

Table 4-1. Myeloid and B-lymphoid Lineage Expression of Individual Lin$^-$ Sca-1$^+$ Progenitors from normal mice

Myeloid expression	Number of colonies: B-lymphoid expression -	B-lymphoid expression +	Total	Percentage with B-lymphoid lineage expression
nm	56	13	69	19
m	39	8	47	17
nmEMastMeo	9	1	10	10
nmMast	7	3	10	30
nmEMastM	7	1	8	13
nmMasteo	5	1	6	14
nmeo	5	1	6	17
nmEMasteo	4	2	6	33
nmMastM	3	0	3	0
nmM	1	1	2	50
nmE	0	1	1	100
mEMastM	0	1	1	100
nmEM	1	0	1	0
nmEMast	1	0	1	0
mMast	1	0	1	0
n	1	0	1	0
None		9	9	100

Lin$^-$ Sca-1$^+$ progenitors isolated from normal mice were plated in primary MTC culture containing SF, IL-7, Ep, and IL-11, on day 8 all colonies present were harvested and divided into liquid myeloid culture containing IL-3, SF, IL-5 and Ep and liquid lymphoid culture containing SF and IL-7. Liquid myeloid cultures were examined on days 4, 6 and 8 of secondary culture, while liquid lymphoid cultures were subjected to flow cytometric analysis for the presence of B-220$^+$ cells on day 11 or 12 of secondary culture.

Table 4-2. Myeloid and B-lymphoid Lineage Expression of Individual Lin$^-$ Sca-1$^+$ Progenitors from Mice Treated with 5-FU

Myeloid expression	Number of colonies: B-lymphoid expression -	B-lymphoid expression +	Total	Percentage with B-lymphoid lineage expression
nmEMastM	11	5	16	31
nm	4	7	11	64
nmEMast	3	5	8	62
nmMast	5	1	6	16
nmMastM	2	3	5	60
nmEMastMeo	3	1	4	25
nmMasteo	1	2	3	66
nmEM	2	0	2	0
nmEMasteo	0	1	1	100
nmMastMeo	1	0	1	0
nmE	1	0	1	0
nM	0	1	1	100
m	2	0	2	0

Lin$^-$ Sca-1$^+$ progenitors isolated from 5-FU-treated mice were plated in primary MTC culture containing SF, IL-7, Ep, and IL-11, on day 11 all colonies present were harvested and divided into liquid myeloid culture containing PWM-SCM, IL-3 and Ep and semisolid lymphoid culture containing SF and IL-7. Liquid myeloid cultures were examined on days 2, 4 and 6 of secondary culture, while lymphoid cultures were examined for the presence of pre-B cell colonies on day 11 or 12 of secondary culture.

Table 4-3. Myeloid Lineage Expression by the Lymphohemopoietic Progenitors from Normal and 5-FU-Treated Mice

Number of myeloid lineages expressed	Percent of lymphohemopoietic progenitors	
	Normal	5-FU-treated
3 or more	36	69
2 (nm)	39	27
1 (m)	24	0

Data shown in Tables 4-1 and 4-2.

Table 4-4. Incidence of Lineage Expression by the Population of $Lin^{-}Sca\text{-}1^{+}$ Progenitors from Normal and 5-FU-Treated Mice

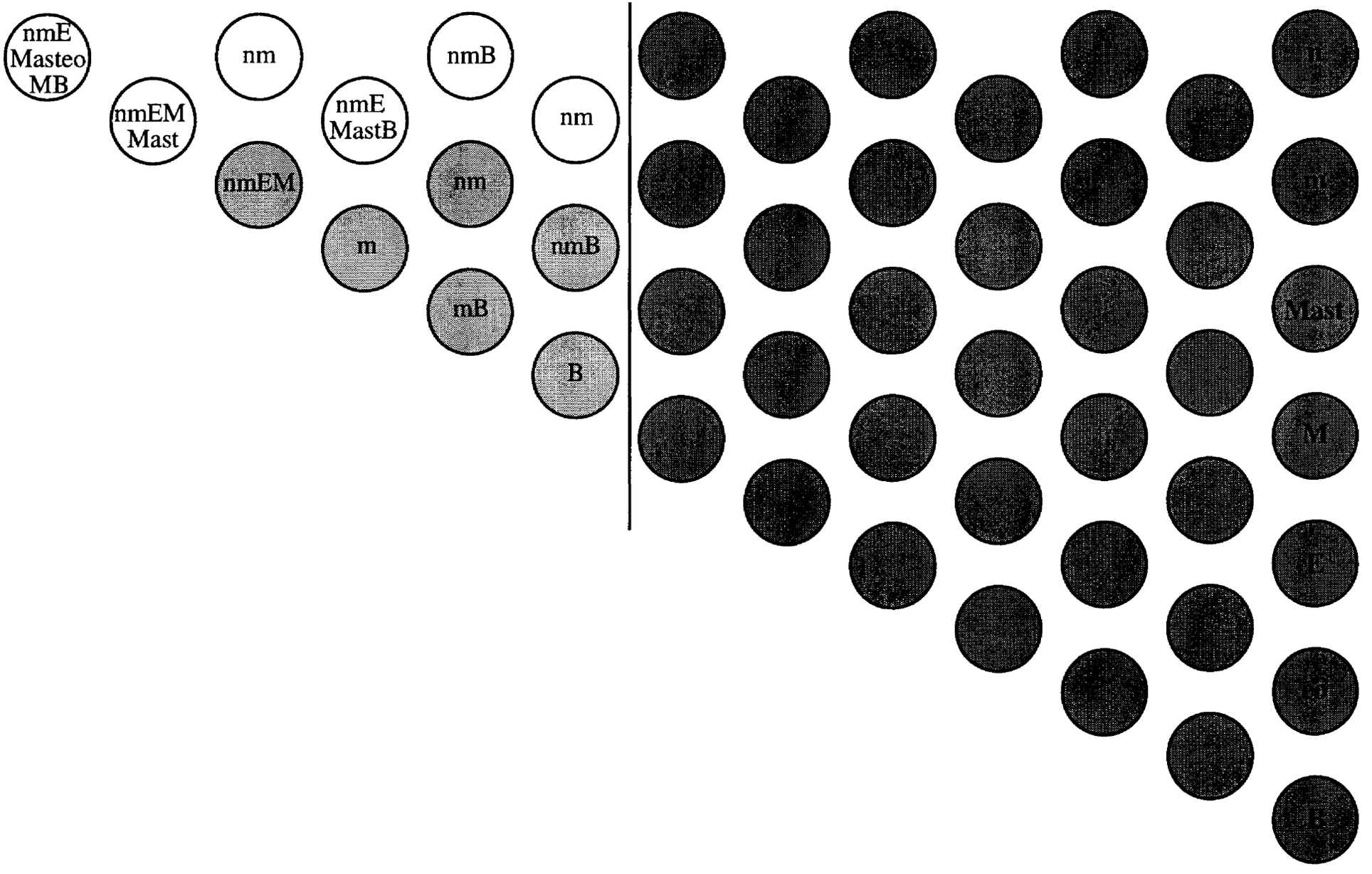
Lineage expression	Percent of progenitors	
	Normal	5-FU-treated
m	100	100
n	73	97
Mast	26	72
E	16	52
M	13	48
B	18	43
eo	15	17

Data shown in Tables 4-1 and 4-2.

Figure 4-1. Modified view of hemopoietic differentiation based on a stochastic model of random lineage restriction, Ogawa et al. In: Leukemia: Recent Advances in Biology and Treatment, pages 391-397, 1985. Alan R Liss Inc., 150 Fifth Avenue, New York, NY 10011. The population represents the cells isolated from marrow on aspiration. The darkly shaded cells are cells those removed by density centrifugation, lineage selection and sorting for the Sca-1 antigen. The remaining population encompasses the populations of lin^- $Sca\text{-}1^+$ progenitors from both normal and 5-FU treated mice. The lightly shaded cells are those Lin^- $Sca\text{-}1^+$ progenitors that are sensitive to the cytotoxic effects of 5-FU. The abbreviations are the same as designated in Figure 4-1.

Lin$^-$ Sca-1$^+$ progenitors, shaded cells are sensitive to the cytotoxic effects of 5-FU.

More mature and mature cells removed by density gradient centrifugation and lineage selection.



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Chapter V

General Discussion

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SUMMARY

As discussed by Metcalf, one of the most important issues of which we must always remain cognizant, when interpreting the *in-vitro* and *in-vivo* culture data of hemopoietic progenitors, is the permissiveness of the assay (1). The permissiveness is the ability of a culture assay to support the differentiation of all myeloid and lymphoid lineage cells. The permissiveness is of vital importance, since our inferences concerning the lineage potential and stage of differentiation of a progenitor or a population of progenitors are based solely on our ability to identify the progeny to which the progenitor gives rise. For example, in the first quantitative assay for hemopoietic progenitors, the spleen colony assay (2), the colony forming units designated CFU-S were initially thought to be myeloid restricted progenitors. However, further studies in permissive conditions revealed that many CFU-S are indeed pluripotent (3). As is evident, the major limiting factor of the inferences made about the lineage potential of a progenitor of interest is the permissiveness of the culture system in which it is being studied.

The two-step assay for lymphohemopoietic progenitors of Hirayama, was the first assay capable of the clonal identification of progenitors with B-lymphoid and myeloid lineage potential that was not dependent on stromal cell support (4). The single-step assay for lymphohemopoietic progenitors is a modified version of the two-step assay (5). Both assays are capable of the quantitative identification of lymphohemopoietic progenitors. Alone, each has limitations, but when used in conjunction these limitations can be overcome. For example, identification of the myeloid and B-lymphoid lineage potential of a progenitor of interest in the two-step assay requires the time consuming harvest and division of each primary colony into specific myeloid and B-lymphoid cultures. This process becomes a limitation if the progenitor of interest in the initial pool of colony

forming progenitors cultured is present at a low incidence. The single-step assay, on the other hand, requires that only potentially positive colonies be harvested for flow cytometric analysis at the completion of the culture period. Thus, one advantage of the single-step assay is the accurate identification and study of populations of progenitors with low numbers of lymphohemopoietic and or lymphoid progenitors. Unfortunately, it is impossible to serially examine colonies of interest when using the single-step assay as is done in the two step assay. Since myeloid lineage expression of an individual progenitor is examined at only one time point, identification of the complete myeloid lineage expression of a progenitor although possible, is in some cases not as accurate as by the two-step assay due to the kinetics of the appearance and disappearance of the individual myeloid lineages.

In using the single-step and the two-step assays to address the hypothesis: "Commitment to the individual myeloid and the B-lymphoid lineages occurs randomly as a single event rather than as two events, commitment to myeloid and lymphoid lineage and later commitment of myeloid and lymphoid restricted progenitors to the individual myeloid and lymphoid lineages." I have been able to draw three major conclusions. First, a significant population of lymphohemopoietic progenitors exists in normal mice that appears to be rapidly proliferating (5). This conclusion is supported by two observations: a population of lymphohemopoietic progenitors present in normal mice does not require the presence of an early acting triggering factor to initiate and support lymphomyeloid colony formation; and treatment of mice with 5-FU decreases the number of lymphohemopoietic progenitors present in the bone marrow to approximately one-fourth the number present in normal mouse bone marrow. The second conclusion is that IL-3 positively modulates the proliferation lymphohemopoietic progenitors while inhibiting expression of the B-lymphoid lineage (6). This conclusion is supported by the observation that 48 hour exposure of proliferating lymphohemopoietic progenitors to IL-3 increased the number of clonable B-lymphoid progenitors formed when compared with cultures not containing IL-3. While exposure of proliferating early B-lymphoid progenitors to IL-3 for 48 hours abrogated the

expression of the B-lymphoid lineage in secondary culture. An additional observation of lymphohemopoietic progenitors cultured in IL-3 containing conditions was that the total amount of proliferation of lymphohemopoietic progenitors was decreased, in the presence of an increased rate of proliferation, compared to control cultures without IL-3. The decreased amount of total proliferation can be attributed to the presence of a shorter duration of proliferation of lymphohemopoietic progenitors prior to their commitment, suggesting that IL-3 may provide a stimulus to increase the rate of cellular proliferation and differentiation at the expense of self-renewal. Finally, I was able to conclude that the expression of the B-lymphoid and the myeloid lineages by the population of Lin^{-} $Sca\text{-}1^{+}$ progenitors isolated from normal mice is consistent with the previously established stochastic model of the random loss of lineage potential by individual progenitors (7). This conclusion was supported by the observation that regardless of the number or the type of myeloid lineages expressed by an individual progenitor, approximately 18% of the progenitors gave rise to the B-lymphoid lineage. These observations are very similar to the previous observations of Nakahata et al., T. Suda et al. and J. Suda et al. in which the myeloid lineage expression of individual blast cells and micromanipulated daughter cells was found to be highly heterogeneous which form the foundation of the stochastic model of cell commitment (8-12). In conducting these investigations an apparent association between the B-lymphoid and the macrophage lineage also appeared to be present, as has been postulated by several other investigators. Together, these results support a model of random restriction of lineage potential with a late divergence of the B-lymphoid and the myeloid lineages, possibly resulting in the formation of a bipotential B-lymphoid/macrophage progenitor.

A MODEL OF EARLY B-LYMPHOPOIESIS

As discussed in Chapter I, Cumano et al. proposed a model of B-lymphopoiesis by progenitors isolated from murine fetal liver. Briefly, their model included seven phases of B-lymphopoiesis: phase I was comprised of primitive lymphohemopoietic progenitors; phase II, bipotential B-lymphoid/macrophage expressing AA4 and Ly-6A; phase III, B220 positive B cell progenitors; phase IV, B220 positive B cell progenitors no longer requiring IL-7; and phases V-VII are composed of maturing B-lymphoid cells expressing surface Ig. Although this model incorporates a bipotential B-lymphoid/macrophage progenitor, it emphasizes the stages of differentiation positive for the expression of the B220 surface antigen. In the model of B-lymphopoiesis which I will now propose, emphasis is placed on the elucidation of the stages of differentiation prior to the expression of the B220 surface antigen. In this model, Figure 5-1, B-lymphopoiesis is hypothesized to progress through 4 stages: 1) the lymphohemopoietic progenitor (LHP) proliferative stage; 2) the commitment stage; 3) the early committed B-lymphoid proliferative stage; and 4) the $B220^+$ maturation stage as has been exquisitely characterized by Hardy et al. (13) and is included for completeness.

Stage 1: The LHP proliferative stage.

Primitive LHP in this model are those that are present in the population of Lin^- $Sca\text{-}1^+$ progenitors from 5-FU treated mice. The cytokines required for the initiation and support of proliferation by these progenitors have been well studied. They require the synergistic interaction of an early acting triggering factor and an intermediate acting supporting factor, IL-11 and SF for example. Recently, 3 investigators have independently studied the effects of the recently cloned cytokine, flt3/flk2 ligand (FL), on the proliferation and differentiation of primitive hemopoietic progenitors (16, 17) and primitive lymphohemopoietic and committed B-lymphoid progenitors (18). They all observed

somewhat similar result suggesting that FL synergistically supports the proliferation of primitive progenitors while also maintaining them in a somewhat undifferentiated state. As has been defined, the LHP proliferative stage has an approximate duration of 6 days (6). Near the end of this stage, progenitors which I have designated "late stage LHP", are formed. The LHP isolated from normal mice reside at various points within the LHP proliferative stage. A unique characteristic of some late stage LHP is that they do not require an early acting triggering factor to initiate proliferation and support colony formation. However, IL-11 does have a positive synergistic effect and addition increases colony size. As was evident from experiments with LHP isolated from normal mice, the addition of IL-11 to SF resulted in a synergistic increase in colony formation by these progenitors, and thus, perhaps not all late stage LHP are capable of proliferation and colony formation in the absence of IL-11.

Stage 2: The commitment stage.

Following the LHP proliferative stage and the formation of late stage LHP, is the commitment stage. In this stage $B220^-$ B-lymphoid progenitors are generated by LHP as the LHP disappear. As discussed, some controversy as to the lineage potential of the late stage LHP and the divergence of the B-lymphoid and the myeloid lineages exists, be it from pluripotent lymphohemopoietic or bipotential B-lymphoid/macrophage progenitors.

Stage 3: The early committed B-lymphoid proliferative stage.

With the formation of $B220^-$ B-lymphoid progenitors, the early committed B-lymphoid proliferative stage begins. This stage is ultimately characterized by B-lymphoid progenitors that do not express B220. It is this stage of B-lymphopoiesis which is least well understood, and most difficult to study due to the low incidence of progenitors and the lack of known specific markers for cells in this stage. Of major interest, is the duration of this stage and the response of cells in this stage to cytokines and combinations of

cytokines. Hirayama et al. observed that FL was capable of supporting the differentiation of B220$^-$ B-lymphoid progenitors into B220$^+$ progenitors, and that FL and SF synergized enhancing the formation of B220$^+$ cells from B220$^-$ cells. Neither SF or IL-7 alone was capable of supporting the formation of B220$^+$ cells from B220$^-$ cells while in combination they readily supported this differentiation. From this, one could postulate that FL may support an intermediate B220$^-$ progenitor and its differentiation. Another interesting observation is the extremely inhibitory effect of IL-3 on B-lymphoid progenitors in the early committed B-lymphoid proliferative stage. We know that IL-3 is very toxic to the earliest committed B220$^-$ B-lymphoid progenitors. However, we have been unable to definitively determine the effect, if any, of IL-3 on the commitment of lymphohemopoietic progenitors to the B-lymphoid lineage. We are currently unable to identify when the expression of B220 by B220$^-$ B-lymphoid progenitors begin to occur, therefore, we cannot draw a firm conclusion as to the effect of IL-3 on the B220$^+$ pro-B cells as is represented by the dashed lines.

Stage 4: The B220$^+$ maturation stage.

The substages of the B220$^+$ maturation stage have been well defined as has been discussed in Chapter 1. The earliest B220$^+$ progenitors have been shown to express B220 at very low levels (14-15). Interestingly, the earliest B220$^+$ pro-B cells have been shown to be negative for both c-kit and IL-7 receptor expression (19) and non-dependent on these factors (20). However, the combination of SF, IL-7 and Ep in the single-step assay has been shown to support the formation of pre-B cell colonies by lymphohemopoietic and B220$^-$ committed B-lymphoid progenitors from normal mice. It could be argued that further differentiation form B220$^-$ to B220$^+$ requires an additional factor which accessory cells present in the culture may provide in addition to SF and IL-7.

PROPOSED FUTURE INVESTIGATION

The staging of progenitors in B-lymphopoiesis.

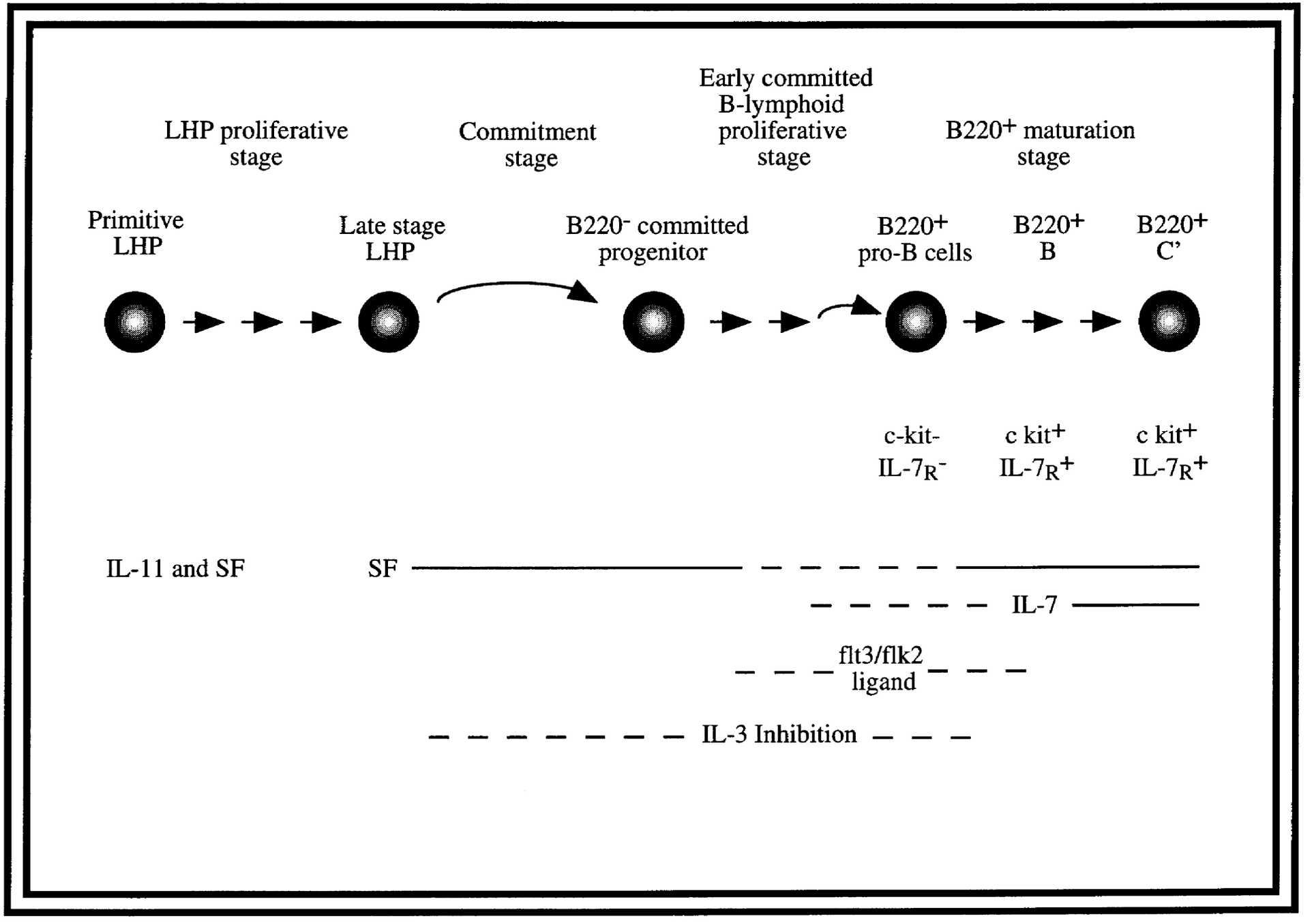
As discussed in the introduction, a great deal of information about B-lymphoid progenitors expressing B220 has been compiled. Far less is known about the stages of B-lymphopoiesis prior to the expression of B220 including late stage lymphohemopoietic progenitors and $B220^-$ B-lymphoid progenitors. I have begun characterization of these less well known stages of B-lymphopoiesis, and I have outlined a model of B-lymphopoiesis by primitive lymphohemopoietic progenitors.

A population of $B220^-$ committed B-lymphoid progenitors is believed by many to give rise to $B220^+$ progenitors. In 1986 Muller-Sieberg et al. in examining early B-lymphopoiesis utilized FACS sorting to isolate a population of $B220^-$ cells from murine bone marrow (21). This population of B-lymphoid progenitors was considered a more primitive population of B-lymphoid progenitors than those present in the $B220^+$ fraction of bone marrow. Later, McNiece et al. isolated a similar population of $B220^-$ progenitors from bone marrow in their investigation of the role of SF in early B-lymphopoiesis (22). Once again, this population was isolated in the belief that the $B220^-$ progenitors present in bone marrow were more primitive B-lymphoid progenitors than those present in the $B220^+$ fraction. Most recently, Hirayama et al. in their investigations of the role of flt3/flk2 ligand on early B-lymphopoiesis by committed B-lymphoid progenitors, isolated a population of $Mac\text{-}1^-$, $TER119^-$, $Ly\text{-}2^-$, $L3T4^-$ and $B220^-$ cells (18). This population was once again considered to be a more primitive population of B-lymphoid progenitors than the $B220^+$ population.

Recent unpublished studies directed at enriching primitive populations of $B220^-$ B-lymphoid progenitors using B220 staining and flow cytometric analysis and sorting, indicate there is no enrichment of early committed B-lymphoid progenitors in the $B220^{low}$

population of committed B-lymphoid progenitors in the early committed B-lymphoid proliferative stage. Future investigations focusing on: 1) the enrichment of lymphohemopoietic progenitors within the various stages, 2) the cytokine requirements of progenitors in the stages of differentiation and 3) the examination and characterization of the progenitors present in fetal, neonatal and adult animals will contribute to a more clear understanding of the processes of B-lymphopoiesis. In pursuing these investigations the following hypothesis should be addressed: "There are several definable and identifiable stages of B-lymphopoiesis prior to the stages characterized by the expression of the B-220 surface antigen." Emphasis should be placed on three specific aims: the demonstration and isolation of late stage lymphohemopoietic and $B220^-$ committed B-lymphoid progenitors; definition of the stages in which they are present; and modeling of these early processes of B-lymphopoiesis.

Figure 5-1. Model of hemopoietic differentiation of lymphohemopoietic progenitors in adult marrow. Primitive lymphohemopoietic progenitors pass through 4 stages in the formation of $B220^+$ pre-B cells in culture. The stage designated the $B220^+$ maturation stage has been well elucidated by Hardy et al. J. Exp. Med. 173; 1213-1225, 1991. The stages prior to the $B220^+$ maturation stage have been characterized using the two-step and the single-step assays for lymphohemopoietic progenitors.


LHP proliferative stage
Commitment stage
Early committed B-lymphoid proliferative stage
B220+ maturation stage
Primitive LHP
Late stage LHP
B220- committed progenitor
B220+ pro-B cells
B220+ B
B220+ C'
c-kit- IL-7R-
c kit+ IL-7R+
c kit+ IL-7R+
IL-11 and SF
SF
IL-7
flt3/flk2 ligand
IL-3 Inhibition

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