PROLIFERATION AND DIFFERENTIATION OF HIGHLY ENRICHED MOUSE HEMATOPOIETIC STEM CELLS AND PROGENITOR CELLS IN RESPONSE TO DEFINED GROWTH FACTORS

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The differentiation and proliferation of hematopoietic cells are regulated by a complex system of cell–cell interactions and soluble growth factors. Many of the mature cells are short-lived and need to be constantly replenished from earlier progenitors and stem cells. Pluripotent hematopoietic stem cells are defined as cells that give rise to all cells in the myeloid, erythroid, and lymphoid lineages and also have self-renewal capacity. The events that control self-maintenance of stem cells and induction of differentiation into more mature restricted hematopoietic precursors and subsequently into mature cells are currently not well understood. The analysis of these regulatory interactions has been hampered by the complexity of the hematopoietic tissues, the low frequency of the relevant cells, and the heterogeneity of the regulatory factors.

Many of the factors that regulate the differentiation and proliferation of hematopoietic cells have recently become available in recombinant and purified form (for review see reference 1). Several different growth factors can be discriminated by their activities (2). When mouse bone marrow cells are grown in semi-solid medium in the presence of macrophage colony-stimulating factor (M-CSF)[1] the resulting colonies consist predominately of macrophages (3). Similarly, granulocyte-CSF (G-CSF) and erythroprotein (EPO) seem to be lineage specific (4, 5). Most colonies induced by GM-CSF consist of granulocytes and macrophages, but sometimes eosinophils and mixed colonies are found (6–8). IL-5 (Eosinophil-CSF [Eo-CSF]) induces differentiation of bone marrow cells into eosinophils and also acts on B lymphocytes (9–11). IL-3 (multi-CSF) stimulates single lineage colonies of many myeloid and erythroid cells and also induces multilineage colonies containing several different cell types (12–15). IL-3 supports the short-term survival of myeloid/erythroid-restricted progenitors in vitro (14) that give rise to in vivo spleen colonies (CFU-S). On the basis of these findings, a hierarchical model has been proposed that discriminates between

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[1] *Abbreviations used in this paper:* CFU-C, Colony forming unit in culture; CFU-S, spleen colony forming unit; EPO, erythropoietin; 5-FU, 5-fluorouracil; CSF, colony stimulating factor; G-CSF, granulocyte CSF; GM-CSF, granulocyte/macrophage CSF; Eo-CSF, eosinophil CSF; M-CSF, macrophage-CSF.

early- and late-acting factors (1, 2, 16, 17). It suggests that multipotent and perhaps even pluripotent hematopoietic stem cells may be responsive to IL-3. GM-CSF could act on a multipotent progenitor, more restricted than the progenitor responsive to IL-3. G-CSF, M-CSF, and IL-5(Eo-CSF) are believed to stimulate late precursors already committed to the respective lineages.

Several groups have conducted cell separation experiments to characterize the interrelationship of various CSF-dependent progenitor cells. To analyze early progenitors, cells from mice treated with 5-Fluorouracil (5-FU) have frequently been used. 5-FU has been reported to eliminate cycling cells and to affect early progenitors less than late precursors (18, 19). Accordingly, M-CSF–responding cells seem to be more sensitive to 5-FU than IL-3-responding cells (20). Furthermore, 5-FU treated tissues have been reported to be enriched for mulipotential progenitors that may self-renew in vitro (21), as well as high-proliferative progenitor cells (22–24). The populations containing these progenitors are reported to express the Thy-1 (23, 24) and/or Qam-7 antigens (25). Both cell surface antigens are expressed on a variety of bone marrow cells, including murine hematopoietic stem cells (22, 26–30). Precursor cells responding to purified M-CSF express lower levels of the Qam-7 antigen than cells that need M-CSF plus another growth factor (25). The latter cells are primitive myeloid progenitors and seem to be related to pluripotent stem cells (22). Elimination of Thy-1 expressing cells can eliminate the response to IL-3 (31). However, the precursors that are stimulated by M-CSF are believed to express the Thy-1 antigen by some authors (31) but not by others (32). Macrophage colonies are depleted in bone marrow cells expressing Mac-1, and antigen found on mature macrophages (33, 34). Ideally, experiments to identify growth factor responsive progenitors should involve highly purified stem cells, progenitor and precursor cells which should be directly subjected to defined growth factors. With few exceptions defined factors have not been used in these studies. In addition, the enriched progenitor populations often included lymphocytes, which could have produced endogenous growth factors.

We have recently described a population, Thy-$1^{lo}T^-B^-G^-M^-$ cells, which contain most, if not all, pluripotent hematopoietic stem cells found in bone marrow and are depleted of mature lymphocytes and myeloid cells (27, 28). Thy-$1^{lo}T^-B^-G^-M^-$ cells, comprising 0.1 to 0.3% of normal mouse bone marrow, can completely repopulate all the hematopoietic lineages in lethally irradiated mice and are 100 to 200 fold enriched for clonogenic B, T and myeloid-erythroid progenitors (27, 28, 35). In this paper we show that Thy-$1^{lo}T^-B^-G^-M^-$ cells are 370-fold enriched for pluripotent hematopoietic stem cells, emphasizing that the Thy-$1^{lo-}B^-G^-M^-$ population consists of a highly enriched population of stem cells. We had also described another bone marrow-derived subpopulation, Thy-$1^-T^-B^-G^-M^-$ cells, characterized by the absence of Thy-1 and markers expressed on mature myeloid and lymphoid cells (27). This population does not contain stem cells, CFU-S, or T cell precursors, but includes an early B cell precursor (27). These two populations, together with a third population, Thy-$1^+T^+B^+G^+M^+$ cells consisting predominantly of mature T, B, and myeloid cells, represent most of bone marrow. We have analyzed the response of these phenotypically distinct populations to several defined growth factors. Our results indicate that purified progenitor populations can be used to test and extend the hierarchical model of growth factor action directly.

Materials and Methods

Mice. C57BL6-Ly5.2 (originally obtained from Dr. Boyse, Sloan-Kettering Hospital, New York, NY) and BALB/c mice were bred at Lilly Research Laboratories. CBA/J and C57BL/6 mice were purchased from The Jackson Laboratory, Bar Harbor, ME. For some experiments mice were injected intravenously with 5-FU (Sigma Chemical Co., St. Louis, MO) at a dose of 150 mg/kg body weight 2 or 10 d before the experiment.

Antibodies. Rat mAb 31-11 is specific for the Thy-1 antigen (36), antibody 30H12 detects the Thy-1.2 allele (37). RA3-6B2 detects the B220 antigen, expressed on mouse B lineage cells beginning with the pre-B cell (38). M/70 is specific for the Mac-1 antigen on mouse macrophages (34). A cell line secreting antibody RA3-8C5, specific for the Gr-1 antigen on granulocytes (39) and eosinophils, was kindly provided by Dr. R. Coffman (DNAX Research Institute, Palo Alto, CA). Mature T cells were detected with antibodies GK1.5 and 5.3-6.72, which detect the L3T4 (CD4) antigen (40) and the Lyt-2 (CD8) antigen (37). An antibody specific for the Ly5.2 antigen, expressed on most white blood cells of C57BL6-Ly5.2 mice, was purchased from New England Nuclear (Boston, MA).

Cell Separations. Sorting of bone marrow cells was done as described previously (27). Bone marrow was obtained from the femurs of 3-wk-old mice. Iscove's modified Dulbecco's medium supplemented with 5% FCS, penicillin, and streptomycin was used throughout the procedure. Bone marrow cell suspensions ($\sim 5 \times 10^7$/ml) were incubated with anti-Thy-1 antibodies 31-11 or 30H12. After washing, cells were incubated with FITC-labeled goat anti-rat antibodies (Caltag, South San Francisco, CA), washed again, and incubated with normal rat serum (Cappel Laboratory, Cochranville, PA) to block free binding sites of the goat anti-rat antibodies. A cocktail of RA3-6B2, RA3-8C5, M/70, GK1.5 and 5.3-6.72 antibodies, all biotinylated, was added 1-2 min after addition of the rat serum. Biotinylated antibodies were detected with Texas Red-coupled avidin (Cappel Laboratories). Cells were then resuspended in Dulbecco's PBS supplemented with 3% FCS, 0.02% NaN_3, and 20 μg propidium iodide/ml and were separated with a multi-laser cell sorter at the Flow Cytometry Laboratory at the Salk Institute for Biological Studies (La Jolla, CA). Two argon lasers were used, one was tuned to 488 nm to excite FITC and propidium iodide, the other was used to pump a dye laser tuned to 590 nm for the excitation of Texas Red. A 5 mW HeNe laser is used as a pulse delay monitor to time the charging of the droplets. A volumenometer (Coulter Electronics, Hialeah, FL) in combination with forward and side scatter measurements provide accurate measurement of cell size. Data acquisition and display are via a modified version of the LACEL soft- and hardware, interfaced to a Digital micro-11/73 computer. A detailed description of the instrumentation will be published elsewhere (Trotter, J., manuscript in preparation). Some separations were done with a FACS IV (Becton Dickinson & Co., Mountain View, CA), modified as described (41) and made available through the FACS shared users group at Stanford University. Sorted cells were at least 85% pure as judged by a variety of criteria, including reanalysis by cell sorter, restaining with independent cell surface markers and morphological analysis (28).

CFU-S Assay. Sorted cell populations were routinely tested for the number of day 10 CFU-S to assess enrichment and purity. The assay was done as described previously (27, 42). Frequency of CFU-S in Thy-$1^{lo}T^-B^-G^-M^-$ was 1 in 30-60; Thy-$1^-T^-B^-G^-M^-$ or Thy-$1^+T^+B^+G^+M^+$ cells contained <1 CFU-S in 10^4 cells injected.

Source of Factors. Erythropoietin (step III) was purchased from Terry Fox Laboratories (Vancouver, BC, Canada). Purified G-CSF was a gift from N. Nicola (Walter and Eliza Hall Institute, Melbourne, Australia) and purified M-CSF was a gift of R. Shadduck (University of Pittsburgh, School of Medicine, Pittsburgh, PA). DNAX Research Institute provided purified rIL-3 (43) and provided rGM-CSF and IL-5 in the form of supernatants from Cos 7 cells transfected with the GM-CSF cDNA clone (44) and the IL-5 cDNA, respectively (45). 1 U of G-CSF, M-CSF, IL-3, and GM-CSF is defined as the amount of factor that stimulates half-maximal [^{3}H]thymidine incorporation of an appropriate factor-dependent cell line cultured at 5×10^4 cells/ml. Each factor was then titered in the CFU-C assay and was generally used at twice the concentration required to stimulate optimal colony formation in methylcellulose cultures.

Bone Marrow Colony-forming Assay (CFU-C). Unseparated bone marrow cells or sorted sub-

populations were plated in 35-mm culture dishes (Falcon Labware, Oxnard, CA) containing 1 ml Iscove's Medium (Gibco Laboratories, Grand Isle, NY), 20% FCS, 5×10^{-5} M β-mercaptoethanol, 0.8% (wt/vol) methylcellulose, supplemented with various growth factors, as described previously (46). Colonies consisting of 50 or more cells were scored between days 10 and 21 as indicated.

Radioprotection Assay. 8–10-wk-old C57BL/6 mice were lethally irradiated with 1,140 rad (two times 570 rad at 190 rad/min from a Mark I ^{137}Cs irradiator, with 2–4 h between irradiations). Mice were injected intravenously with graded doses of sorted or unseparated bone marrow cells derived from Ly5 congenic C57BL6-Ly5.2 mice. Percent surviving mice was determined 30 d after injection. All surviving mice were bled 5–8 wk after reconstitution and their blood cells were stained for expression of the donor type Ly5.2 marker. Only mice that showed the majority (≥80%) of white blood cells of donor type were considered in the titration.

Results

Source of Stem Cells. Hematopoietic cells from normal bone marrow were separated into three subpopulations as described previously (27). Fig. 1 depicts the populations obtained when bone marrow is stained simultaneously with an anti-Thy-1 antibody and a cocktail of antibodies recognizing T, B, and myeloid-specific markers. Population I consists predominantly of macrophages, granulocytes, eosinophils, T cells, pre-B, and B cells. These cells are stained by one or more of the antibodies we used and are summarily called Thy-$1^+T^+B^+G^+M^+$ cells. Population II lacks all markers we assayed (Thy-$1^-T^-B^-G^-M^-$ cells) and contains predominantly erythrocytes and undifferentiated blast cells. Histological analysis of freshly sorted Thy-$1^-T^-B^-G^-M^-$ cells revealed 54% mature red blood cells, 20% nucleated red blood cells, 24% undifferentiated blasts, 0.7% myeloblasts, 0.4% macrophages. Neither the Thy-$1^+T^+B^+G^+M^+$ nor the Thy-$1^-T^-B^-G^-M^-$ population contains detectable levels of stem cells or CFU-S (27). Population III, composing 0.1 to 0.3% of bone marrow, is characterized by expression of low levels of Thy-1 and lack of all other maturational markers. Sorted Thy-$1^{lo}T^-B^-G^-M^-$ cells are >98% undifferentiated blast cells as determined by histological analysis. The Thy-$1^{lo}T^-B^-G^-M^-$ population is ~100–200-fold enriched for B and T lymphocyte progenitor activity, and day 10 CFU-S (27, 28, 35) but is depleted of day 8 CFU-S (Müller-Sieburg, C. E., manuscript in preparation). Furthermore, Thy-$1^{lo}T^-B^-G^-M^-$ cells contain most if not all pluripotent hematopoietic stem cells found in bone marrow. Thy-$1^{lo}T^-B^-G^-M^-$, but not Thy-$1^-T^-B^-G^-M^-$ or Thy-$1^+T^+B^+G^+M^+$ cells, can rescue lethally irradiated mice and repopulate all the hematopoietic cell lineages in these mice (27, 28).

The radioprotection assay is considered the most stringent assay for pluripotent stem cells. To quantitate stem cells in the Thy-$1^{lo}T^-B^-G^-M^-$ population, the dose of unseparated bone marrow cells and Thy-$1^{lo}T^-B^-G^-M^-$ cells needed to protect irradiated mice was compared. We found that 3.7×10^4 bone marrow cells are required to rescue and repopulate 50% of the mice, a dose that is in good agreement with published data (47). In contrast, ~100 Thy-$1^{lo}T^-B^-G^-M^-$ cells, representing a 370-fold enrichment, sufficed (Fig. 2). This 370-fold enrichment parallels the enrichment of Thy$^{lo}T^-B^-G^-M^-$ cells, which compose 0.1 to 0.3% of bone marrow. It also indicates that Thy-$1^{lo}T^-B^-G^-M^-$ cells represent a very highly enriched stem cell population. In agreement with previous studies (27), the Thy-$1^-T^-B^-G^-M^-$ and Thy-$1^+T^+B^+G^+M^+$ cells were unable to rescue lethally irradiated mice (data

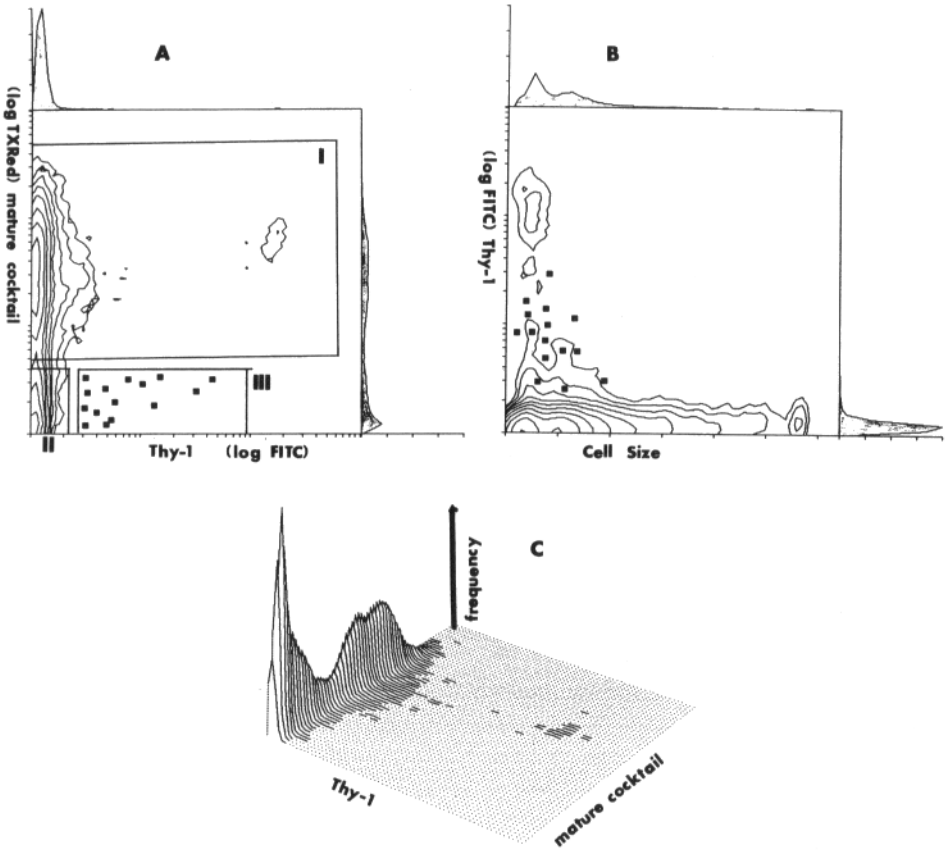

FIGURE 1. FACS profile of mouse bone marrow cells. Cells were reacted simultaneously with biotin-labeled antibodies specific for B220, Mac-1, Gr-1, L3T4, and Lyt-2 (see Materials and Methods for details of antibodies and their specificities) all present in a cocktail and detected with Texas Red–conjugated Avidin. Thy-1-expressing cells were labeled with antibody 31-11 detected with FITC-labeled goat anti-rat antibodies. The windows in *A* are labeled to indicate our designation for each population of cells. Population I: consists predominantly of pre-B and B cells, T cells, macrophages, and granulocytes. These cells express antigens detected by one or more of the antibodies used and are thus designated Thy-1$^+$T$^+$B$^+$G$^+$M$^+$ cells. Population I composes 75–85% of total bone marrow. Population II: Thy-1$^-$T$^-$B$^-$G$^-$M$^-$ cells that lack all markers we used and represent 15–20% of bone marrow. Population III: cells expressing low levels of Thy-1, designated Thy-1^{lo}T$^-$B$^-$G$^-$M$^-$ cells, which make up 0.1–0.3% of bone marrow. *B* shows the cell size of bone marrow, as determined with a Coulter volumenometer, plotted against intensity of FITC staining. For the purpose of easier identification we have marked with: ■. The cells that fall into window III in *A* and represent Thy-1^{lo}T$^-$B$^-$G$^-$M$^-$ cells. Note that these cells are predominantly small. Both *A* and *B* depict on their *x* and *y* axis the corresponding histograms *C* is a three-dimensional depiction of the data in *A*, adding a frequency parameter.

not shown). Interestingly, some mice injected with low doses of either unseparated bone marrow or Thy-1^{lo}T$^-$B$^-$G$^-$M$^-$ cells showed predominant repopulation with host-type cells (data not shown). Survival of these mice (which are not included in the titration depicted in Fig. 2) was dependent on injection of a source of stem cells, as mice injected even with high doses of Thy-1$^-$T$^-$B$^-$G$^-$M$^-$ cells (containing my-

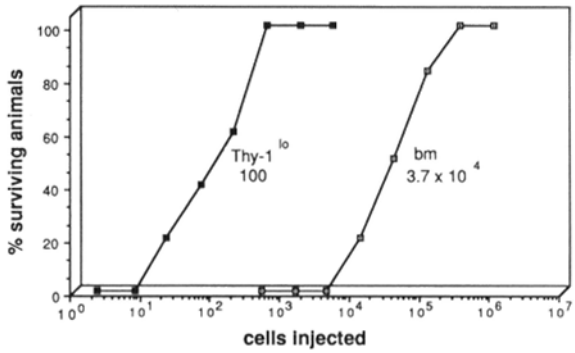

FIGURE 2. Titration of radioprotecting cells. Graded doses of either unseparated bone marrow (⊡) or sorted Thy-1^{lo}T$^-$B$^-$G$^-$M$^-$ cells (■), both derived from C57BL6-Ly5.2 mice, were injected into lethally irradiated congenic C57BL/6 mice (Ly5.1). Groups of six mice were injected for each cell concentration. Surviving mice (>30 d) include only mice that contained a majority of donor cells (Ly5.2) in their blood 5–8 wk after reconstitution. The dose at which 50% of the animals survived is indicated in the figure.

eloid and B lymphocyte progenitors and immature erythrocytes) did not survive more than 2 wk.

The Thy-1^{lo}T$^-$B$^-$G$^-$M$^-$ and Thy-1$^-$T$^-$B$^-$G$^-$M$^-$ Populations Contain Distinct IL-3-responsive Progenitors. IL-3 interacts with a variety of cells. It supports the proliferation of myeloid and mast cell lines and stimulates the generation of single lineage and mixed colonies from bone marrow cells (13, 14, 48). When Thy-1^{lo}T$^-$B$^-$G$^-$M$^-$ cells are stimulated with recombinant purified IL-3 in semi-solid methylcellulose medium, they give rise to colonies with high frequency. One in seven Thy-1^{lo}T$^-$B$^-$G$^-$M$^-$ cells proliferated and differentiated upon stimulation with IL-3, representing a 100-fold enrichment over unseparated bone marrow (Table 1). Analysis of the cellular composition of Thy-1^{lo}T$^-$B$^-$G$^-$M$^-$ derived colonies reveals a

TABLE I

IL-3-dependent Colony Formation by Sorted Bone Marrow Subpopulations

Cells	Percent of bone marrow	Colonies per 10^4 cells: Total	Percent erythroid§	frequency^{-1}‡
Thy-1^{lo}T$^-$B$^-$G$^-$M$^-$	0.1–0.3	1,381 ± 244	35	7
Thy-1$^-$T$^-$B$^-$G$^-$M$^-$	15–20	27 ± 14	3	370
Thy-1$^+$T$^+$B$^+$G$^+$M$^+$	75–85	5 ± 3	0	2,000
Bone marrow	100	15 ± 11	20	666

Sorted cell populations were stimulated with rIL-3 (400 U/ml) in methylcelullose. Erythropoietin (1 U/ml) was added after 4 d of culture to promote erythroid development.

* Colonies counted were standardized to 10^4 cells seeded to allow better comparison and represent the mean of three to nine individual experiments ± SD. Duplicate culture were scored at day 10 of each individual experiment. Cells actually seeded per plate: Thy-1^{lo}T$^-$B$^-$G$^-$M$^-$: 100–5,000; Thy-1$^-$T$^-$B$^-$G$^-$M$^-$: 1–2 × 10^4; Thy-1$^+$T$^+$B$^+$G$^+$M$^+$: 2–10 × 10^4; bone marrow: 10^5.

‡ Frequencies are calculated on the basis of the mean.

§ Refers to both pure and mixed erythroid colonies.

TABLE II
Cellular Composition of IL-3-dependent Colonies

Colony type*	Thy-1^{lo}T$^-$B$^-$G$^-$M$^-$	Thy-1$^-$T$^-$B$^-$G$^-$M$^-$	Thy-1$^+$T$^+$B$^+$G$^+$M$^+$	Bone marrow
G	8	3	2	7
M	18	27	2	14
GM	38	32	7	24
E	1	0	0	1
Eo	5	1	0	4
Mast	1	0	0	2
Mixed‡	42	1	0	15

Sorted cell populations were stimulated with IL-3 and erythropoietin as detailed in Table I. Colonies were counted and sequentially picked for differential staining at day 10.

* Abbreviations used: G, granulocytes; M, macrophages; E, erythrocytes; Eo, eosinophils.

‡ Mixed colonies contained two or more different lineages other than GM; 80% of the mixed colonies contained erythrocytes, 15% megakaryocytes.

large proportion of mixed colonies, as expected from pluripotent or multipotent stem cells (Table II). 40–60% of the colonies contained two or more cell types other than granulocytes and macrophages (Table II). In unseparated bone marrow only 10–25% of the colonies were mixed (Tables I and II).

Thy-1$^-$T$^-$B$^-$G$^-$M$^-$ cells also respond to IL-3 (Table I). The frequency of clonogenic cells in the Thy-1$^-$T$^-$B$^-$G$^-$M$^-$ population is ~50 times lower than in Thy-1^{lo}T$^-$B$^-$G$^-$M$^-$ cells. Furthermore the colonies obtained from the Thy-1$^-$T$^-$B$^-$G$^-$M$^-$ population are almost exclusively restricted to the granulocyte and macrophage lineages (Table II). In particular, Thy-1$^-$T$^-$B$^-$G$^-$M$^-$ cells are depleted of progenitors that can differentiate into erythrocytes or mast cells in our culture conditions. These progenitors are enriched in the Thy-1^{lo}T$^-$B$^-$G$^-$M$^-$ population. This clearly shows that bone marrow contains at least two distinct early progenitors capable of proliferating and differentiating upon stimulation with IL-3, namely cells with the phenotype Thy-1^{lo}T$^-$B$^-$G$^-$M$^-$, which consist of or coenrich with pluripotent stem cells, and a more mature, restricted progenitor lacking Thy-1. Thy-1$^-$T$^-$B$^-$G$^-$M$^-$ cells make up 15–20% of bone marrow (Fig. 1). Thus, despite the low frequency of responder cells, Thy-1$^-$T$^-$B$^-$G$^-$M$^-$ cells contribute two to three times more colonies to the total IL-3 response of bone marrow than Thy-1^{lo}T$^-$B$^-$G$^-$M$^-$ stem cells (Table I).

The large Thy-1$^+$T$^+$B$^+$G$^+$M$^+$ population was depleted of IL-3-responsive colony-forming cells when compared with unseparated bone marrow, Thy-1^{lo}T$^-$B$^-$G$^-$M$^-$, or Thy-1$^-$T$^-$B$^-$G$^-$M$^-$ cells. We have, however, found some colonies derived from this Thy-1$^+$T$^+$B$^+$G$^+$M$^+$ population (Table I). Due to the large size of this population these colonies may represent a significant proportion of the total IL-3 response of bone marrow. Whether these colonies are derived from yet a third, possibly novel progenitor or, as the low frequency and the restricted cellular composition of the colonies suggests (Table II), sorter contamination by Thy-1$^-$T$^-$B$^-$G$^-$M$^-$ cells, is the subject of current studies.

Interestingly, the majority of erythroid cells derived from sorted subpopulations are found in mixed colonies, observable around 10 d of culture (Table III). In several

TABLE III

IL-3-responsive Cells are Affected by 5-FU

Source of bone marrow	Cell population	Colonies per 10^4 cells
Normal	Bone marrow	14 ± 5
	Thy-$1^{lo}T^-B^-G^-M^-$	1,300 ± 260
Day 2 5-FU*	Bone marrow	3 ± 2
	Thy-$1^{lo}T^-B^-G^-M^-$	<5
Day 10 5-FU*	Bone marrow	43 ± 21
	Thy-$1^{lo}T^-B^-G^-M^-$	1,750 ± 290

* Bone marrow cells and Thy-$1^{lo}T^-B^-G^-M^-$ cells were derived from mice that had been injected with 150 mg of 5-fluorouracil per kilogram body weight at 3 wk of age 2 or 10 d before the experiment. Cells were stimulated with IL-3 and erythropoietin as detailed in Table I.

experiments we were unable to identify restricted erythroid precursors, termed CFU-E, in any of the bone marrow subpopulations we tested (data not shown). These precursors, which differentiate upon stimulation with EPO alone, were readily obtained from unseparated bone marrow in 4–6-d-old cultures, even if the cells were incubated with antibodies and run through the cell sorter (data not shown). This indicates that CFU-E are not damaged by the staining or sorting procedure. It is conceivable that CFU-E do need, in addition to EPO, interaction with a yet unidentified cell to proliferate of differentiate. We are currently investigating this possibility by mixing of sorted cell populations.

IL-3-responsive Cells in Bone Marrow of 5-FU-treated Mice. While most Thy-$1^{lo}T^-B^-G^-M^-$ cells are small and resting cells (Fig. 1), ~15% of these cells are in the G_2 or M phase of cell cycle (Tidmarsh, G. A., S. Heimfeld, G. J. Spangrude, I. L. Weisman, and C. E. Müller-Sieburg, manuscript submitted for publication). The drug 5-FU is reported to eliminate cycling cells and is frequently used in studies of early hematopoiesis. It seems to affect late precursors while sparing early progenitors to some extent (18–24). To assess the effect of 5-FU on the IL-3 response of Thy-$1^{lo}T^-B^-G^-M^-$ cells, we used bone marrow of mice injected with 5-FU 2 or 10 d before the experiment. The depletion of late precursors seems to be most apparent 1–2 d after in vivo drug treatment (18, 52). 10 d after 5-FU administration, bone marrow contains a large compartment of Thy-1-expressing cells (other than T cells), many of which are large cycling cells. However, the Thy-$1^{lo}T^-B^-G^-M^-$ population is only slightly increased (Müller-Sieburg, C., unpublished observation). Interestingly, Thy-$1^{lo}T^-B^-G^-M^-$ cells from mice treated with 5-FU 2 d before their isolation, have lost the ability to respond to IL-3 (Table III). This indicates that predominantly the cycling cells in the Thy-$1^{lo}T^-B^-G^-M^-$ population can be stimulated by IL-3. The IL-3 response of Thy-$1^{lo}T^-B^-G^-M^-$ cells is completely restored in the regenerating day 10 post-5-FU bone marrow (Table III). These data are in agreement with previous reports, showing that large Thy-1-expressing cells can give rise to mixed colonies (24).

The Thy-$1^-T^-B^-G^-M^-$ Population Contains M-CSF, G-CSF, and IL-5 Responders. While Thy-$1^{lo}T^-B^-G^-M^-$ cells from normal bone marrow respond with a very

TABLE IV
Response of Sorted Bone Marrow Subpopulations to IL-3, IL-5, M-CSF, G-CSF, and GM-CSF

Growth factor	Thy-1^{lo}T$^-$B$^-$G$^-$M$^-$	Thy-1$^-$T$^-$B$^-$G$^-$M$^-$	Thy-1$^+$T$^+$B$^+$G$^+$M$^+$	Bone marrow
IL-3	1,360 ± 74	32 ± 4	5 ± 2	10 ± 1
GM-CSF	112 ± 30	15 ± 3	3 ± 1	6 ± 0.4
M-CSF	0.3 ± 0.3	24 ± 1	6 ± 2	8 ± 0.1
G-CSF	0 ± 0	5 ± 2	1 ± 1	2 ± 0.3
None	0 ± 0	0 ± 0	0 ± 0	0.6 ± 0.4
IL-3	1,260 ± 120	26 ± 1	ND	8 ± 0.5
GM-CSF	90 ± 10	21 ± 1		5 ± 0.5
IL-5	0 ± 0	5.5 ± 1		1 ± 0.1
None	0 ± 0	0 ± 0		0.5 ± 0.2

Sorted cell populations were stimulated with indicated growth factors (all used at 400 U/ml) in methylcellulose. Erythropoietin (1 U/ml) was added after 4 d and colonies were counted after 10 d of culture. Data ± SD of two typical experiments are presented. Data are standardized to colonies per 10^4 cells; for details see Table I.

high frequency to IL-3, few or none react to M-CSF, G-CSF or IL-5 (Eo-CSF). The majority of the precursors that give rise to a colony in response to these factors are found in the Thy-1$^-$T$^-$B$^-$G$^-$M$^-$ population (Table IV). Thus, multipotent progenitors and restricted precursors can be separated based on the expression of the Thy-1 antigen. Furthermore, our data confirm that M-CSF, G-CSF, or IL-5 alone do not induce proliferation of stem cells but act on early precursors with restricted differentiation potential.

The enrichment for precursors activated by either G-CSF, M-CSF, or IL-5 within the Thy-1$^-$T$^-$B$^-$G$^-$M$^-$ population is two to threefold when compared with unseparated bone marrow. However, 70–80% of the Thy-1$^-$T$^-$T$^-$B$^-$G$^-$M$^-$ population are mature and nucleated erythrocytes. Thus, the actual enrichment, based on white cell count, is considerably higher and can be calculated to be 10–15-fold. Thus, most of the hematopoietic precursors that are stimulated by M-CSF, G-CSF, or IL-5 are characterized as undifferentiated blast cells that lack Thy-1, B220, Mac-1, Gr-1, L3T4 (CD4), and Lyt-2 (CD8) antigens and can be enriched from normal bone marrow by virtue of this phenotype.

Progenitors and precursors that could form colonies upon stimulation with all the growth factors we have tested were depleted in the Thy-1$^+$T$^+$B$^+$G$^+$M$^+$ population. However, a few colonies were obtained consistently from the Thy-1$^+$T$^+$B$^+$G$^+$M$^+$ population (Table IV). In an attempt to characterize further the cells responding to G-CSF we started to dissect the Thy-1$^+$T$^+$B$^+$G$^+$M$^+$ population. We found that cells responding to G-CSF are depleted both in the Gr-1- or Thy-1-expressing bone marrow subpopulations (data not shown). A more detailed analysis is necessary to resolve the question whether the colonies obtained from the Thy-1$^+$T$^+$B$^+$G$^+$M$^+$ population are due to a low level of sorter contamination by Thy-1$^-$T$^-$B$^-$G$^-$M$^-$ cells or whether they represent a distinct progenitor.

Whether the growth factors anlayzed here act on the same or on different progenitors remains to be clarified. When we stimulated Thy-1$^-$T$^-$B$^-$G$^-$M$^-$ cells with

TABLE V

Additive Effects of Stimulation by Two Growth Factors

Exp.	Growth factor(s)	Thy-1^{lo}T$^-$B$^-$G$^-$M$^-$	Thy-1$^-$T$^-$B$^-$G$^-$M$^-$
1	IL-3	1,390 ± 60	35 ± 5
	GM-CSF	90 ± 20	15 ± 3
	M-CSF	0 ± 0	26 ± 2
	G-CSF	0 ± 0	6 ± 1
2	IL-3 + GM-CSF	1,500 ± 80	49 ± 6
	IL-3 + M-CSF	1,340 ± 50	53 ± 4
	IL-3 + G-CSF	1,290 ± 60	43 ± 5
	M-CSF + GM-CSF	110 ± 20	38 ± 4

Sorted cell populations were stimulated with indicated growth factors (all used at 400 U/ml) in methylcellulose. Erythropoietin (1 U/ml) was added after 4 d and colonies were counted after 10 d of culture. Colonies are standardized to 10^4 cells input (for details see Table I). Concentration of growth factors in mixing experiments was the same as in individual application (400 U/ml each).

a combination of IL-3 and G-CSF, or M-CSF, or GM-CSF, the number of colonies obtained was roughly additive. This indicates that IL-3 acts on different progenitor cells than G-CSF, M-CSF or GM-CSF. A similar additive effect is found when Thy-1$^-$T$^-$B$^-$G$^-$M$^-$ cells are stimulated simultaneously with M-CSF and GM-CSF (Table V). Addition of M-CSF or G-CSF to IL-3 does not increase the number of IL-3-driven colonies in the Thy-1^{lo}T$^-$B$^-$G$^-$M$^-$ population but increases the size of the colonies (data not shown). This again emphasizes that M-CSF and G-CSF do not act on stem cells but on their progeny.

Response of Sorted Populations to GM-CSF. Progenitors responding to GM-CSF are found in all three populations (Table IV). The progenitors that proliferate in response to GM-CSF are depleted in the Thy-1$^+$T$^+$B$^+$G$^+$M$^+$ population but are enriched in the Thy-1$^-$T$^-$B$^-$G$^-$M$^-$ population to the same extent as G-CSF, M-CSF, or IL-5-reactive cells. In contrast, GM-CSF stimulates a small subset (<10%) of Thy-1^{lo}T$^-$B$^-$G$^-$M$^-$ cells. Slighty more colonies are obtained from Thy-1^{lo}T$^-$B$^-$G$^-$M$^-$ cells cultured in the presence of both IL-3 and GM-CSF than with IL-3 alone, indicating that these factors may act on distinct progenitors (Table V). The analysis of the cellular composition of the colonies derived from Thy-1^{lo}T$^-$B$^-$G$^-$M$^-$ cells after GM-CSF stimulation reveals 10% mixed colonies (Table VI). The GM-CSF response of Thy-1$^-$T$^-$B$^-$G$^-$M$^-$ cells appears to be restricted to the macrophage and granulocyte lineages. Consistent with previous reports (7, 8), our data suggest that GM-CSF can stimulate a subset of multipotent progenitors. Like IL-3, GM-CSF probably can act on at least two distinct progenitors in bone marrow, found in the Thy-1^{lo}T$^-$B$^-$G$^-$M$^-$ and in the Thy-1$^-$T$^-$B$^-$G$^-$M$^-$ populations, respectively.

Discussion

Thy-1^{lo}T$^-$B$^-$G$^-$M$^-$ Cells Are Highly Enriched for Pluripotent Stem Cells and Contain a High Frequency of IL-3-responsive Cells. We have previously shown that a small subpopulation of normal mouse bone marrow, Thy-1^{lo}T$^-$B$^-$G$^-$M$^-$ cells, contains pluripotent hematopoietic stem cells that repopulate and allow the survival of lethally irradiated mice (27, 28). Here we have quantitated stem cell activity in a radio-

TABLE VI
Cellular Composition of GM-CSF-dependent Colonies

Colony type	Thy-1^{lo}T$^-$B$^-$G$^-$M$^-$	Thy-1$^-$T$^-$B$^-$G$^-$M$^-$	Thy-1$^+$T$^+$B$^+$G$^+$M$^+$	Bone Marrow
G	8	5	6	7
M	1	35	6	6
GM	33	37	8	25
E	4	0	0	2
Eo	0	0	0	0
Mast	0	0	0	0
Mixed*	5	0	0	2

Sorted cell populations were stimulated with GM-CSF (400 U/ml) in methyl cellulose. Erythropoietin (1 U/ml) was added after 4 days of culture to promote erythroid development. Colonies were counted and sequentially picked for differential staining at day 10.

* Mixed colonies contained two or more different lineages other than GM.

protection assay and demonstrate that sorted Thy-1^{lo}T$^-$B$^-$G$^-$M$^-$ cells are 370 fold enriched when compared to unseparated bone marrow. Roughly 100 Thy-1^{lo}T$^-$B$^-$G$^-$M$^-$ cells were needed to protect 50% of lethally irradiated mice. Previously reported enrichments for pluripotent stem cells in mice (47) and rats (48) are 180- and 350-fold, respectively. Interestingly, ~3,000 rat stem cells, which had been 350-fold enriched, were needed to protect lethally irradiated rats (48). The shape of the titration curve of stem cells both in mice (Fig. 2; reference 47) and in rats (48) seems to be similar. Thus, the requirement for significantly different doses of rat versus mouse pluripotent stem cells (of similar enrichment) may indicate that the radioprotection assay does not accurately measure frequencies. The absolute frequency of pluripotent hematopoietic stem cells in murine bone marrow remains yet to be determined.

We also show that sorted Thy-1^{lo}T$^-$B$^-$G$^-$M$^-$ cells are 100-fold enriched for progenitors that can proliferate and differentiate upon stimulation with recombinant purified IL-3. Many of the colonies obtained from Thy-1^{lo}T$^-$B$^-$G$^-$M$^-$ cells contain several cell lineages as expected from multipotent or pluripotent stem cells. Thy-1^{lo}T$^-$B$^-$G$^-$M$^-$ cells are >98% undifferentiated blast cells (46), are depleted of mature myeloid, erythroid, and lymphoid cells, lack day 8 CFU-S, and by this criteria are a fairly homogeneous population. Whether the Thy-1^{lo}T$^-$B$^-$G$^-$M$^-$ cells that respond to IL-3 are pluripotent stem cells or multipotent stem cells that coenrich in this population is difficult to say. 5-FU, early after administration, depletes IL-3-responsive cells, indicating that these cells reside in the cycling compartment of the Thy-1^{lo}T$^-$B$^-$G$^-$M$^-$ population (Table III). 5-FU appears to spare some, but not all day 15 CFU-S (18, 19) indicating that IL-3-responsive Thy-1^{lo}T$^-$B$^-$G$^-$M$^-$ cells are distinct from CFU-S. However, little is known about the status of the pluripotent stem cell (as measured by the radioprotection assay) in 5-FU-treated bone marrow. Thus, further analysis is needed to resolve the question whether IL-3 acts directly on pluripotent stem cells. Clearly, Thy-1^{lo}T$^-$B$^-$G$^-$M$^-$ cells represent a population of primitive hematopoietic progenitors, which can differentiate into multiple lineage with a high frequency upon stimulation by IL-3.

It is difficult to deduce the origin of the single lineage colonies derived from Thy-$1^{lo}T^-B^-G^-M^-$ cells upon IL-3 stimulation. It is possible that the Thy-$1^{lo}T^-B^-G^-M^-$ population contains a number of already committed precursor cells. As detailed above, a sorted population is unlikely to be absolutely pure. Alternatively, the single lineage colonies could be attributed to stochastic stem cell differentiation. The stochastic model for stem cell differentiation, formulated by Ogawa et al. (21, 50, 51), suggests that pluripotent stem cells can commit directly to either multipotent or monopotent restricted precursors randomly.

We have previously shown that Thy-$1^{lo}T^-B^-G^-M^-$ cells give rise with a high frequency (20%) to colonies containing mast cells (46). We here confirm this result and demonstrate that mast cells are derived from Thy-$1^-T^-B^-G^-M^-$ cells but not from Thy-$1^+T^+B^+G^+M^+$ or Thy-$1^-T^-B^-G^-M^-$ cells (Table II). Even after prolonged culture (3 wk) in the presence of IL-3, mast cells were not found in 126 colonies derived from Thy-$1^-T^-B^-G^-M^-$ cells (data not shown). This indicates that the precursors for the mast cell lineage and the macrophage-granulocyte lineages may split relatively early in differentiation, possibly at the level of a mulipotent stem cell.

Approximately 1% of Thy-$1^{lo}T^-B^-G^-M^-$ cells respond to GM-CSF. We can presently not discriminate whether these GM-CSF responders represent a subset of pluripotent or multipotent stem cells or whether they are derived from a few restricted progenitors included in the Thy-$1^{lo}T^-B^-G^-M^-$ population. That the IL-3-responding cells may be distinct from the GM-CSF responder cells is indicated by the observation that the combination of these factors results in roughly additive numbers of colonies. GM-CSF induces a significant number of mixed colonies in the Thy-$1^{lo}T^-B^-G^-M^-$ but not in the Thy-$1^-T^-B^-G^-M^-$ population (Table VI), suggesting that GM-CSF, like IL-3, can stimulate two distinct progenitors distinguished by the presence or absence of the Thy-1 cell surface molecule. Thus, in agreement with previously published data (23, 24, 26–32), our experiments demonstrate that expression of low levels of Thy-1 characterize progenitors with extensive proliferative capacity and multiple differentiation options.

Thy-$1^-T^-B^-G^-M^-$ Cells Contain Restricted Precursors. The Thy-$1^-T^-B^-G^-M^-$ population contains cells able to proliferate in response to all the growth factors tested. In particular, this population contains a high proportion of those cells in normal bone marrow that differentiate and proliferate upon stimulation with either M-CSF, G-CSF, or IL-5 (Eo-CSF). Our results confirm the findings of others, that most M-CSF-responding cells do not express the Mac-1 (33) or Thy-1 (32) antigens. We extend these data to show that neither M-CSF- nor G-CSF -nor IL-5-responding cells express Thy-1, Mac-1, Gran-1, B220, or T cell marker and that these characteristics can be used to enrich the responder cells. Currently we are testing a panel of mAbs raised against Thy-$1^-T^-B^-G^-M^-$ cells and hope to find antibodies that will allow an even better enrichment. Furthermore, these antibodies might allow us to dissect the Thy-$1^-T^-B^-G^-M^-$ compartment in order to establish firmly whether distinct or overlapping progenitor subsets respond to individual growth factors.

The Thy-$1^-T^-B^-G^-M^-$ population also contains progenitors that proliferated in response to IL-3. These progenitors are restricted to the granulocyte and macrophage lineages and contribute the majority of IL-3-responding cells in bone marrow. Thus, the combined response of the Thy-$1^{lo}T^-B^-G^-M^-$ and Thy-$1^-T^-B^-G^-M^-$ populations would account for the large number of granulocyte and macrophage colonies found in unseparated bone marrow upon IL-3 stimulation.

Costimulation with IL-3 plus M-CSF or G-CSF resulted in increased colony formation, indicating that these factors act on distinct precursors contained in the Thy-$1^-T^-B^-G^-M^-$ population. A more detailed analysis is necessary to resolve this issue. At this point it is tempting to conjecture a novel class of progenitors, already committed to the macrophage/granulocyte lineage, that can be stimulated by IL-3 but not by GM-CSF, M-CSF, or G-CSF.

Few if any Thy-$1^{lo}T^-B^-G^-M^-$ cells formed colonies in response to M-CSF, G-CSF, or IL-5. This confirms the previously proposed model (2), that these factors do not act on multipotent hematopoietic stem cells.

Occasionally, progenitors responsive to all the factors we tested were found in the population of predominantly mature hematopoietic cells, the Thy-$1^+T^+B^+G^+M^+$ population. It is possible that further analysis may show that this population contains progenitors distinct from those in the Thy-$1^-T^-B^-G^-M^-$ or Thy-$1^{lo}T^-B^-G^-M^-$ populations. Alternatively, as the low frequency of these progenitors and their restricted differentiation capacity suggests, they may be due to low level sorter contamination of the Thy-$1^+T^+B^+G^+M^+$ population by Thy-$1^-T^-B^-G^-M^-$ cells. Our attempts to dissect the Thy-$1^+T^+B^+G^+M^+$ compartment to further analyze these progenitors cells have failed so far.

Several experiments indicate that the activation of primitive hematopoietic progenitors requires more than one growth factor. IL-1 (hematopoietin-1), M-CSF, and other known cytokines found in conditioned supernatant of a variety of sources may act as necessary co-stimulators with IL-3 (20, 25, 52, 53). Thus, the bone marrow subpopulations Thy-$1^-T^-B^-G^-M^-$ and Thy-$1^{lo}T^-B^-G^-M^-$, both enriched for distinct hematopoietic progenitors, could prove valuable for the characterization of novel growth factors and cofactors.

Summary

Three distinct hematopoietic populations derived from normal bone marrow were analyzed for their response to defined growth factors. The Thy-$1^{lo}T^-B^-G^-M^-$ population, composing 0.2% of bone marrow, is 370-fold enriched for pluripotent hematopoietic stem cells. The two other populations, the Thy-$1^-T^-B^-G^-M^-$ and the predominantly mature Thy-$1^+T^+B^+G^+M^+$ cells, lack stem cells. Thy-$1^{lo}T^-B^-G^-M^-$ cells respond with a frequency of one in seven cells to IL-3 in an in vitro CFU-C assay, and give rise to many mixed colonies as expected from an early mulipotent or pluripotent progenitor. The Thy-$1^-T^-B^-G^-M^-$ population also contains progenitor cells which responded to IL-3. However, colonies derived from Thy-$1^-T^-B^-G^-M^-$ cells are almost exclusively restricted to the macrophage/granulocyte lineages. This indicates that IL-3 can stimulate at least two distinct clonogenic early progenitor cells in normal bone marrow: multipotent Thy-$1^{lo}T^-B^-G^-M^-$ cells and restricted Thy-$1^-T^-B^-G^-M^-$ cells. Thy-$1^{lo}T^-B^-G^-M^-$ cells could not be stimulated by macrophage colony-stimulating factor (M-CSF), granulocyte CSF (G-CSF) or IL-5 (Eosinophil-CSF). The hematopoietic precursors that react to these factors are enriched in the Thy-$1^-T^-G^-B^-M^-$ population. Thus, multipotent and restricted progenitors can be separated on the basis of the expression of the cell surface antigen Thy-1.

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