Separation of Pluripotent Stem Cells and Early B Lymphocyte Precursors with Antibody Fall-3

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Summary

A major goal in the study of hematopoiesis is to obtain populations of primitive stem cells, free of restricted and mature cells. We previously showed that a small population of normal bone marrow, the Thy-1^{lo}Lin$^-$ cells, was highly enriched for pluripotent stem cells that repopulate lethally irradiated mice. These cells also differentiated along the B lymphocyte lineage in response to the stromal elements in Whitlock-Witte cultures. These two hematopoietic activities were entirely contained in and were enriched to similar extents in the Thy-1^{lo}Lin$^-$ population. Here we show for the first time that these two activities can be resolved functionally and phenotypically. The cells that respond to the stroma in lymphoid culture are more sensitive to the cytotoxic drug 5-Fluorouracil than are stem cells. Furthermore, we have derived a new monoclonal antibody, Fall-3, that detects primitive stem cells but does not label the B cell precursor. This indicates that the small Thy-1^{lo}Lin$^-$ population is heterogeneous, containing precursors restricted to the B cell lineage as well as pluripotent stem cells. Antibody Fall-3 defines a novel stem cell antigen, expressed on all primitive stem cells and thus, will be useful in the further characterization and isolation of both stem cells and B cell precursors.

One central issue in hematopoiesis is the characterization of the most primitive pluripotent hematopoietic stem cell. It is well established that a single pluripotent stem cell can give rise to cells of all hematopoietic lineages, the lymphocytes, the myeloid and the erythroid cells (1–3). However, little is known about the process of commitment when stem cells differentiate into restricted precursors. Of particular interest is the differentiation stage at which commitment to the lymphocyte lineages occurs. Identification of this cell would allow to determine whether the stem cell commits directly to T and B cell precursors or via a common lymphocyte progenitor. Also, it is unclear whether commitment to the B cell lineage coincides with Immunoglobulin gene rearrangement. Thus, to define the differentiation events between stem cells and lymphocyte precursors and to analyze what regulates this transition it is critical to separate these cells functionally and phenotypically.

Considerable progress has been made in obtaining populations highly enriched for hematopoietic stem cells from normal mouse bone marrow (4–10). Pluripotent stem cells are defined through their function, namely by their capacity to repopulate all hematopoietic lineages in a compromised host. Besides being able to repopulate lethally irradiated animals, cells in these stem cell enriched populations are active in a variety of in vitro and in vivo assays. For example, stem cell enriched populations have been shown to contain spleen colony-forming units (CFU-S)[1] in vivo (4–9), to give rise to osteoclasts in vitro (11), to generate T lymphocytes upon intra-thymic injection (6), to respond to the stroma in both Dexter and Whitlock-Witte conditions (4, 12), and to give rise to mixed myeloid-erythroid colonies upon stimulation by IL-3 (5). Indeed, the purity of enriched stem cells populations often has been gauged on the basis of clonal responses in these assays as most stem cell assays that are based on repopulation capacity in vivo do not readily allow quantification.

However, it has been difficult to resolve whether pluripotent stem cells themselves respond to these environmental stimuli or whether the stem cell enriched populations also contain lineage restricted precursors whose activity was detected in the various assay systems. In fact, one of these clonal assays that had been previously believed to measure stem cells, the CFU-S assay, detects restricted myeloid-erythroid precursors and not stem cells (13, 14). This indicates that all stem cell enriched populations described to date contained some lineage restricted precursors. Restricted precursors may interfere with or enhance stem cell activity in bone marrow transplantation and in studies designed to identify regulatory signals specific for stem cells. Thus, it is important to define the cellular composition of these stem cell enriched populations.

One of these stem cell enriched populations, the Thy-1^{lo}Lin$^-$ population, is active in most of the assays described above (4–6). It has been reported that the Thy-1^{lo}Lin$^-$ population can be subdivided phenotypically using the cell surface marker Sca-1 (6). Both the Thy-1^{lo}Lin$^-$ population and the stem cell enriched Sca-1–positive subpopulation contain

[1] *Abbreviations used in this paper:* CFU-C, colony-forming unit culture; CFU-S, spleen colony-forming unit; 5-FU, 5-fluorouracil.

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CFU-S. Thus, both populations include in addition to stem cells, restricted myeloid-erythroid precursors. However, the question whether the Thy-1^{lo}Lin$^-$ population contains additional restricted precursors particularly, precursors committed to the lymphoid lineages, was not addressed in these reports.

Here we present evidence for further heterogeneity in the Thy-1^{lo}Lin$^-$ population. We show that the Thy-1^{lo}Lin$^-$ population contains a precursor restricted to the B cell lineage in addition to pluripotent stem cells. This B cell precursor is characterized by its capacity to establish lymphoid Whitlock-Witte cultures in limiting dilution. B cell precursors and stem cells can be distinguished functionally and phenotypically. A monoclonal antibody, designated Fall-3 that we have derived, allows for the first time the separation of these cells and will be helpful in the further characterization and isolation of both stem cells and B cell precursors.

Materials and Methods

Animals. BALB/c and C57BL6-Ly-5.2 mice were bred either at the Lilly Research facility (La Jolla, CA) or at the Medical Biology Institute (La Jolla, CA). C57BL6-Ly-5.2 mice were obtained originally from Dr. E. Boyse (Sloan-Kettering Cancer Center, New York). 3- to 5-wk-old mice were injected i.v. with a single dose of 150 mg 5-Fluorouracil (5-FU) (Sigma Chemical Co., St. Louis, MO) per kilogram body weight in saline containing 0.1 M $NaHCO_3$ where indicated. C57Bl/6 mice, used as hosts in reconstitution experiments at 3–6 mo-of-age, were either purchased from Jackson Laboratories (Bar Harbor, ME) or bred at the Medical Biology Institute facility.

Derivation of Monoclonal Antibody Fall-3. Lewis Rats were injected i.p. and s.c. with 5×10^5 to 10^6 mouse bone marrow cells depleted of cells that express B220, Gr-1, or Mac-1. Cells were injected three times at 14-d intervals. 3 d after the last immunization spleen cells were fused to the mouse myeloma X63Ag8-653 (15). Supernatants of hybrids were screened on mouse bone marrow cells by immunofluorescence. mAb Fall-3 was detected with goat anti-rat-FITC antibodies (Caltag, South San Francisco, CA). After subcloning of antibody-producing hybrids, mAb were prepared from serum-free culture supernatant by precipitation with 50% saturated ammonium sulfate. The isotype of mAb Fall-3 is IgM as determined in a double diffusion assay using a rat isotyping kit (ICN ImmunoBiologicals, Costa Mesa, CA).

Stem Cell Assay. To measure stem cells we used a combination of the radioprotection and long-term repopulation assays as described previously (5). Briefly, C57BL/6 mice were lethally irradiated with 1,140 rads (2 doses of 570 rads) and reconstituted i.v. with different numbers of bone marrow cells derived from pools of 3-5 Ly-5 congenic C57BL6-Ly-5.2 mice. Age matched cohorts of 6–10 mice were injected for each cell dose. The extent of repopulation in surviving mice was determined 3 to 5 mo later by staining peripheral blood cells. Blood was collected into EDTA solution (3 mg EDTA per ml PBS) and incubated with 2% Dextran for 30 min at 37°C, followed by lysis of residual erythrocytes with Gey's solution. White blood cells were labeled with a biotinylated antibody specific for the donor type Ly-5.2 marker detected by Avidin-FITC. A cell line, A-20, secreting antibodies specific for Ly-5.2 was the generous gift of Dr. S. Kimuro, (Sloan-Kettering Cancer Center, NY). The Ly-5 marker is expressed on most hematopoietic cells with the exception of erythroid cells (16). To determine the restoration of individual lineages, white blood cells were costained with antibodies specific for B220 (mAb Ra3-6B2), Mac-1 (mAb M1/70), Gr-1 (mAb Ra3-8C5), and Thy-1 (mAb 31-11). In all mice tested, donor derived T, B, and myeloid cells were reconstituted in ratios comparable to that seen in unirradiated mice.

This assay does not readily allow the quantification of stem cell frequency. To compare stem cell level we introduce the "stem cell unit" which we define as the number of normal bone marrow cells needed to completely repopulate 50% of lethally irradiated animals. Therefore, 1 stem cell unit = 8×10^4 cells = 100% stem cell activity (see Fig. 1). Further, we calculate: % stem activity in 5-FU bone marrow = 1 stem cell unit/No. of cells for 50% reconstitution (5-FU bm); stem cell activity per femur = (cells per femur/1 stem cell unit) × % stem cell activity; % stem cell activity per femur = stem cell activity per femur 5-FU bone marrow/stem cell activity per femur normal bone marrow.

Cell Separations and Immunofluorescence Analysis. Bone marrow cells were incubated with a saturating concentration of mAb Fall-3 detected with goat anti-rat-FITC antibodies (Caltag). Cells were then separated into Fall-3$^+$ and Fall-3$^-$ subpopulations with a multi-laser cell sorter at the Flow Cytometry Laboratory at the Salk Institute for Biological Studies (La Jolla, CA). The background level for the staining was determined by using either an isotype matched antibody (Fall-84) detected with goat anti-rat-FITC antibodies or just the FITC-labeled second stage antibodies. The background levels determined with these controls did not differ significantly.

For two color immunofluorescence, mAb Fall-3 was detected with PE-labeled goat anti-rat antibodies (Caltag). Cells were washed and then incubated for 1 to 2 min with 25% normal rat serum (Cappel Laboratories, Cochranville, PA), followed by biotinylated lineage specific antibodies (4, 5) detected with Avidin-FITC (Vector Labs, Burlingame, CA). The hybridoma cell line secreting antibody E13 161-1, specific for Sca-1 (6) was obtained from Dr. L. Weissman (Stanford University, CA).

Colony-Forming Assay/Colony-Forming Unit Culture (CFU-C) and Cytokines. Unseparated bone marrow cells or sorted subpopulations derived from 3- to 4-wk-old BALB/c or C57-Ly-5.2 mice were plated in 0.8% (w/v) methylcellulose, supplemented with IL-3 at 400 U/ml, as described previously (5). Recombinant IL-3 (17) in the form of supernatants of appropriately transfected Cos 7 cells was a generous gift of Dr. D. Rennick (DNAX Research Institute, Palo Alto, CA). Colonies consisting of 50 or more cells were scored after 9–11 d of culture. In some experiments colonies were counted again after 3 wk of culture.

Limiting Dilution Analysis for Stroma-Responsive Cells. This assay has been described in detail (4, 18, 19). Briefly, the monoclonal stromal cell line AC-3.5 or its subclone AC-3.GG was seeded into the wells of 96-well plates in RPMI supplemented with 5% FCS (J.R. Scientific, Woodland, CA) and 5×10^{-5} M 2-ME. Cultures were recharged with graded doses of bone marrow cells (one plate for each cell concentration) 2 to 4 d later and then were fed weekly. Wells that contained colonies of at least 10^3 small lymphoid cells were scored by phase microscopy after 10 to 14 d of culture according to morphological criteria. Some experiments were evaluated again 3 wk post reseeding. We showed previously that this assay is linear, and, in fact, detects B lineage cells as all colonies tested contained cells that expressed the B lineage specific marker B220 (4, 19).

Results

Stem Cell Content in Normal and 5-FU Treated Bone Marrow. 5-FU has been used previously to define hematopoietic differen-

tiation stages (20–26). It has been reported that mature cells and late precursors are more sensitive to this drug than primitive precursors or stem cells. We used a single injection of 5-FU to determine whether this treatment would reveal heterogeneity in the Thy-1^{lo}Lin$^-$ populations by differentially affecting two activities completely contained within this population: stem cell activity as assessed in a long-term repopulation assay and the capacity to respond to stromal cells with B cell differentiation. We reasoned that a differential effect of 5-FU on these activities would indicate that the activities are carried by different cells, co-enriched in the same population.

Since all stem cells are found in the Thy-1^{lo}Lin$^-$ population (5), stem cell content measured in unseparated bone marrow reflects stem cell content in the Thy-1^{lo}Lin$^-$ population. The level of stem cells in the bone marrow of normal mice and of mice at day 2, day 4, and day 10 after injection of 5-FU was measured in a long-term repopulation assay in lethally irradiated mice. Host mice were injected with graded doses of donor cells derived from mice that differed in the Ly-5 locus. Peripheral blood cells of surviving mice were stained with a mAb specific for donor type Ly-5 to determine the extent of repopulation. As previously described (27), a number of surviving animals that received low doses of stem cells showed considerable host derived reconstitution. For easier comparison of stem cell activity, the percentage of mice that were completely reconstituted by donor cells ($\geq$ 80%) are depicted in Fig. 1. To allow the survival of 50% of the lethally irradiated animals for at least 3 mo and to obtain $\geq$ 80% reconstitution by donor type cells in these mice, 8 × 10^4 normal bone marrow cells per recipient were needed in these

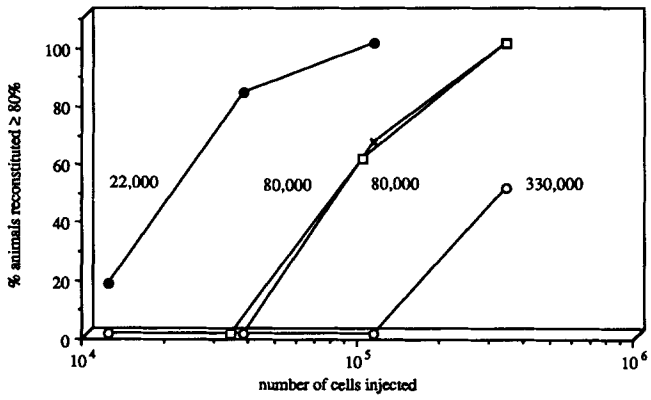

Figure 1. Pluripotent hematopoietic stem cells in normal and 5-FU bone marrow. Stem cells in bone marrow of normal (×), 5-FU day 2 (○), 5-FU day 4 (□), and 5-FU day 10 (●) bone marrow were titrated in the radioprotection assay. Graded doses of cells derived from C57-Ly-5.2 mice were injected into lethally irradiated congenic C57BL/6 mice (Ly-5.1). Full titration using 5 to 6 mice per group was done once. Mid-range individual concentration points were repeated 2 to 3 times. Peripheral white blood cells of surviving mice ($\geq$3 mo) were stained with an antibody specific for the Ly-5.2 antigen to determine the extent of repopulation by donor cells in individual mice. The percentage of mice that contained $\geq$80% of donor (Ly-5.2) cells in their blood is used to compare stem cell levels. The dose of bone marrow cells at which 50% of the animals were fully reconstituted is indicated in the figure.

experiments. The level of pluripotent stem cells was reduced fourfold in 5-FU day 2 bone marrow, while the level of stem cells in 5-FU day 4 bone marrow was similar to that found in normal marrow. Bone marrow from mice treated 10 d previously with 5-FU contained a fourfold higher level of stem cells.

B Cell Precursors in 5-FU Treated Bone Marrow. Thy-1^{lo}Lin$^-$ cells also respond with high frequency to the microenvironment in Whitlock-Witte bone marrow cultures by differentiating along the B cell lineage. These stroma-responsive cells are also entirely contained within the Thy-1^{lo}Lin$^-$ population (4). Thus, the capacity to respond to the stromal cells in Whitlock-Witte cultures of unseparated bone marrow reflects this activity in the Thy-1^{lo}Lin$^-$ population. We wished to determine whether the capacity to give rise to B lineage cells in culture was affected by 5-FU to the same or different extent as the capacity to repopulate irradiated animals.

The frequency of cells that give rise to colonies of at least 10^3 small lymphoid cells in limiting dilution on the monoclonal stromal line AC-3.5 is decreased at least 10-fold in 5-FU day 2 and reduced six-fold in 5-FU day 4 bone marrow compared to normal marrow (Table 1). In contrast, 5-FU day 10 bone marrow contains a four-fold higher frequency of B cell precursors than normal bone marrow. The cultures were counted generally at 2 wk. To determine whether cells from 5-FU treated bone marrow have a delayed response to stromal cells we also evaluated the cultures at 3 wk (data not shown). In agreement with previous observations on normal bone marrow cells (18), we did not observe additional colonies.

Differential Effects of 5-FU. Both radioprotecting and stroma-responsive cells were affected by 5-FU. However, their kinetics of recovery was markedly different. Fig. 2 summarizes and compares the data presented in Table 1 and Fig. 1. Since the radioprotection assay does not allow readily to calculate frequencies we have used stem cell units for comparison (see Materials and Methods for calculations). The fre-

Table 1. *Frequency of B Cell Precursors that Respond to the Stromal Cell Line AC-3-GG in Whitlock-Witte Cultures in Limiting Dilution*

5-FU treatment	Cells per femur	Frequency^{-1}*	Precursors‡ per 10^4	Precursors per femur§
	(× 10^6)			
None	10 ± 2	800 ± 300	12	12 × 10^3
Day 2	9 ± 1	9,167 ± 849	1	10^3
Day 4	4 ± 2	5,600 ± 900	2	10^3
Day 10	1.5 ± 1	170 ± 25	58	9 × 10^3

* Data (± SD) shown are from two representative experiments. Cultures were evaluated 2 wk post seeding.
‡ Data in this column are derived by calculation from the frequency data to facilitate comparison. Values have been rounded to the next integer.
§ Calculation: precursors/femur = (cells/femur) × (precursors/10^4) ÷ 10^4.

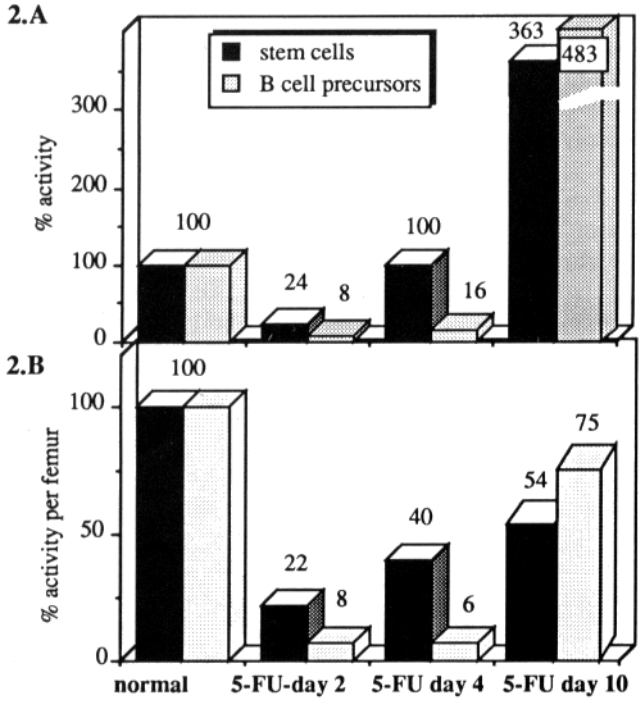

Figure 2. Kinetics of recovery of hematopoietic activities after a single injection of 5-FU. Data are compiled from Fig. 1 and Table 1. Activity in normal bone marrow was set at 100%. For calculation of stem cell units see Materials and Methods. (*A*) Comparison of the level or frequency of activities; (*B*) Comparison of activities based on numbers per femur taking in account the reduced cellularity in 5-FU bone marrow.

quency of B cell precursors and the stem cell level in normal bone marrow was set as 100%. In 5-FU day 2 bone marrow these activities were reduced to 8 and 24%, respectively (Fig. 2 *A*). In 5-FU day 4 bone marrow stem cell levels are the same as in normal bone marrow, while the frequency of B cell precursors is only 16% of normal. Both activities are enriched in 5-FU day 10 bone marrow.

However, injection of 5-FU reduces the cellularity of bone marrow in a time dependent manner. We obtained 9, 4, and 1.5 million cells per femur from mice 2, 4, and 10 d respectively after 5-FU treatment. Therefore, we calculated B cell precursor and stem cell activity per femur in normal and 5-FU treated mice (Fig. 2 *B*). When the stem cell content of normal bone marrow is set as 100%, stem cells in 5-FU day 2, day 4, and day 10 are 22, 40, and 54% of normal, respectively. Thus, the absolute number of stem cells is not fully recovered over the time span examined. This indicates that the increase in stem cell level seen in 5-FU day 10 may be due to elimination of other cells rather than an expansion of stem cells. Similarly, the absolute number of stroma-responsive cells is reduced at all time points analyzed. We found 8, 6, and 75% of the normal response in 5-FU day 2, day 4, and day 10 bone marrow, respectively. However, 5-FU depletes stroma-responsive cells significantly more than radioprotecting cells. This difference is most pronounced in 5-FU day 4 bone marrow (Fig. 2 *B*). Thus, the kinetics of recovery following 5-FU treatment differ for pluripotent stem cells and B cell precursors.

Antibody Fall-3 Separates Stem Cells from B Cell Precursors. The kinetics of recovery after 5-FU treatment indicates that the hematopoietic activities found in the Thy-1^{lo}Lin$^-$ population in normal bone marrow reflect heterogeneity in this population rather than multiple capacities of the same cell (Fig. 2). To define further the cells that carry these activities, we separated normal bone marrow with monoclonal antibody Fall-3. We derived this antibody from a fusion designed to detect subpopulations in bone marrow depleted of mature cells.

Antibody Fall-3 (IgM) stains 15 to 30% of normal bone marrow (Fig. 3). Cells from CBA/J, BALB/c, and C57BL/6 mice are detected to a similar extent. Only minor populations (~5%) of spleen, thymus, and Peyer's patches cells were stained above background. Two-color immunofluorescence analysis indicates that less than 5% of the mature bone marrow cells that express that the lineage markers B220, Gr-1, Mac-1, Thy-1, or CD-8 bear also the Fall-3 determinant (data not shown). This suggests that mAb Fall-3 detects preferentially cells that are not mature granulocytes, macrophages, T or B lymphocytes.

Cells that expressed the cell surface determinant detected by mAb Fall-3 contain most if not all pluripotent stem cells that repopulate all lineages in irradiated mice (Fig. 4 *A*). Only mice that received Fall-3$^+$ cells were stably reconstituted at ⩾ 80% donor type cells in their blood. All mice that were

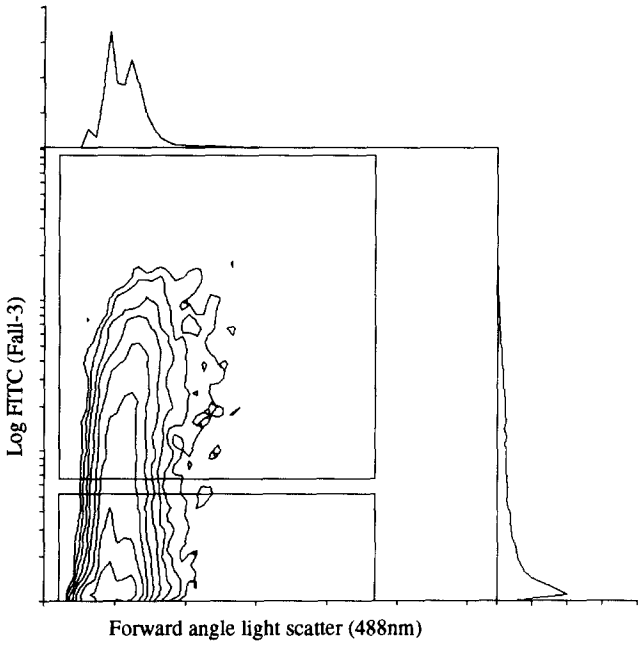

Figure 3. Immunofluorescence profile of normal bone marrow cells stained with mAb Fall-3. A single cell suspension of bone marrow derived from CBA mice was incubated with saturating concentrations of mAb Fall-3, detected with FITC-conjugated goat anti-rat antibodies. Displayed are Forward Scatter versus fluorescence intensity; the corresponding histograms are depicted on the y and x axis. The gate used to separate positive and negative populations is indicated.

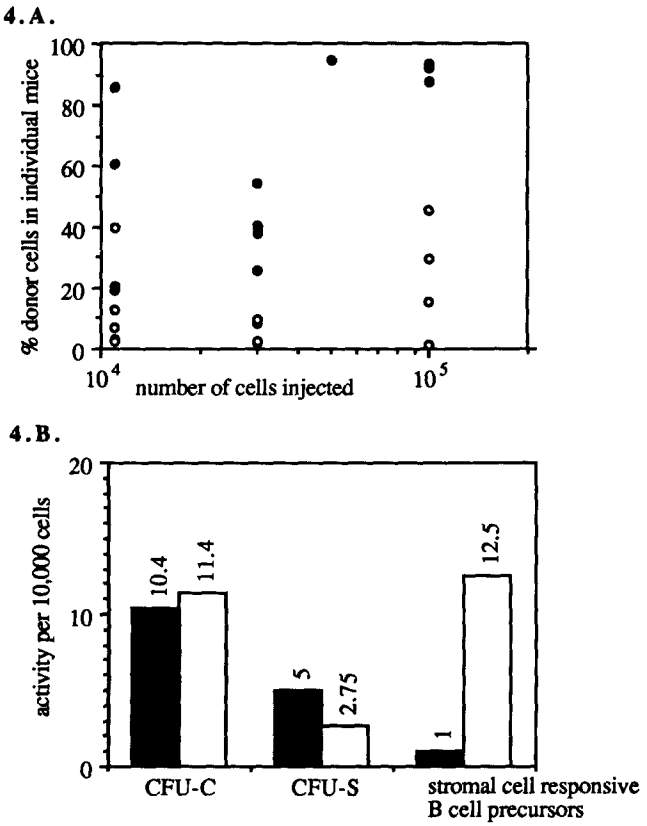

Figure 4. Distribution of hematopoietic activities in sorted Fall-3$^+$ and Fall-3$^-$ bone marrow populations. (*A*) Extent of repopulation by graded doses of sorted Fall-3$^+$ (●) or Fall-3$^-$ (○) cell populations. Groups of 5 to 6 mice were injected per cell dose. Peripheral blood cells were stained for the donor-type Ly-5.2 marker at 5 mo post injection. Donor type T cells, B cells, and myeloid cells were reconstituted in normal proportions in all mice (data not shown). The full titration was done once. An independent experiment using a single dose (4×10^4 cells per mouse) gave comparable results. (*B*) Clonogenic response. IL-3 responsive colonies were evaluated after 10 d of culture, CFU-S at 12 d post injection, and B cell precursors after 2 wk of culture. Data are expressed as activity per 10^4 cells seeded or injected. Fall-3$^+$ (□), Fall-3$^-$ (■).

injected with Fall-3$^-$ cells showed less than 45% donor type cells at 5 mo post reconstitution. Currently, we are analyzing whether this low level of reconstitution by Fall-3$^-$ cells is due to a distinct subset of stem cells or due to a few contaminating Fall-3$^+$ cells in the sorted Fall-3$^-$ population.

In contrast to most radioprotecting stem cells, B cell precursors that respond to the microenvironment in Whitlock-Witte cultures are found predominantly in the Fall-3$^-$ population (Fig. 4 *B*). Thus, pluripotent stem cells and B cell precursors that respond to stromal cells can be separated by their different cell surface phenotype. Interestingly, CFU-C that form a colony upon stimulation with IL-3 and day 12 CFU-S are found both in the Fall-3$^+$ and the Fall-3$^-$ populations (Fig. 4 *B*).

Discussion

We have analyzed the interrelationship of two early hematopoietic activities contained exclusively in the Thy-1^{lo}Lin$^-$ population. The activities are radioprotecting stem cells and B cell precursors that initiate Whitlock-Witte cultures. Both activities are affected by 5-FU. However, these activities show markedly different kinetics of recovery and are affected to different degrees by 5-FU (summarized in Fig. 2). At 4 d post 5-FU treatment, cells that respond to the stroma in Whitlock-Witte cultures are depleted more strongly than the cells that repopulate irradiated animals. Furthermore, the former cells recover slower than radioprotecting stem cells after a single injection of 5-FU. This indicates that the capacity to respond to stromal cells with B cell differentiation is carried by restricted precursors contained in the Thy-1^{lo}Lin$^-$ population and not by stem cells themselves. Most importantly, B cell precursors and stem cells bear different cell surface antigens and can be separated with mAb Fall-3. Thus, the Thy-1^{lo}Lin$^-$ population is functionally and phenotypically heterogeneous and contains restricted precursors for the B cell lineage in addition to stem cells.

We compared B cell precursors and stem cells in 5-FU day 2, 4, and 10 bone marrow. The most pronounced difference between these two activities was observed at day 4 post 5-FU treatment. The higher incidence of stem cells at this time point could be a reflection of the less severe reduction of this activity earlier in the time course. Alternatively, it is tempting to speculate that the kinetics reflect a precursor progeny relationship. Stem cells may have to replenish themselves to a certain level before being able to generate the B cell precursors. A more detailed analysis involving more time points is necessary to resolve this issue.

Stem Cells in 5-FU Bone Marrow. The frequency of radioprotecting stem cells is enriched in 5-FU day 10 bone marrow when compared to normal marrow. However, we found that the reduction in cell number caused by 5-FU treatment outweighs this enrichment and the absolute number of stem cell units per femur is reduced in bone marrow of 5-FU treated mice over the whole time span tested. Nakano and colleagues reported that the cells that reconstitute erythropoiesis in anemic W/W^v mice were enriched in 5-FU day 2 bone marrow and depleted in 5-FU day 4 marrow when tested in the competitive repopulation assay (26). Using the same assay, Lerner and Harrison (28) did not find any effect of 5-FU on stem cells 2 to 15 d after injection. These results could be reconciled to ours by considering the ages of the 5-FU treated animals. We used 3- to 5-wk-old mice whereas the other studies used adult mice. It is conceivable that stem cells are expanding in young animals resulting in more 5-FU sensitive stem cells in young mice than in older mice.

Besides the difference in the age of the animals, different assay systems for stem cells were used. We define stem cells by their capacity to rescue and fully repopulated lethally irradiated animals. In the competitive repopulation assay the stem cells measured are not necessarily required for survival of the animals as an alternative (competitive) source of stem cells is provided. It is possible that the stringent radioprotection assay reveals damages in the stem cell compartment that could not be revealed with other assays. It has been reported that a single injection of 5-FU resulted in long-term impairment of the lymphocyte lineages; decreases in cellularity and

functional activities were seen for at least 3 wk (25). These results are compatible with a reduction of stem cells by 5-FU as seen in our study.

When we compared the capacity to repopulate mice and the ability to respond to stromal cells, we used two assay systems that required different time spans for completion. The B cell precursor activities were evaluated after 2 wk while the radioprotection assay required 3 mo to ascertain repopulation by stem cells. However, mice that did not receive stem cells died 2 to 3 wk after grafting, indicating that the radioprotection assay requires stem cells to be active at this time. The B cell precursor assay was carried up to 3 wk without revealing additional colonies. Thus, it seems unlikely that the difference in recovery rates of stem cells and B cell precursors was due to a delay in maturation induced by 5-FU but rather is a reflection of different cell types.

Another activity enriched in the Thy-1^{lo}Lin$^-$ population, the capacity to form multilineage colonies in response to IL-3, is also affected by 5-FU. We found 0.2, 10, and 129% of the normal response in Thy-1^{lo}Lin$^-$ cells sorted from 5-FU day 2, day 4, and day 10 bone marrow, respectively (5, data not shown). Consequently, 5-FU appears to affect the CFU-C even stronger than B cell precursors and radioprotecting stem cells. This indicates that these myeloid-erythroid precursors are distinct from B cell precursors and stem cells. Therefore, the Thy-1^{lo}Lin$^-$ population that contains these activities is functionally heterogeneous. Treatment with 5-FU can reduce this heterogeneity. 5-FU day 4 bone marrow contains normal levels of stem cells but at least six-fold lower frequencies of B cell precursors and CFU-C (Fig. 2, data not shown). Thus, the combination of 5-FU treatment and cell sorting, particularly with mAb Fall-3, may result in improved enrichment for pluripotent stem cells.

Fall-3. The antigen defined by mAB Fall-3 is expressed on all pluripotent stem cells that stably repopulate the majority of blood cells for ≥5 mo. Thus, mAb Fall-3 defines a novel stem cell antigen in the same sense as Sca-1 (6) defines a stem cell antigen. However, Sca-1 (Ly 6A/E) and Fall-3 most likely detect different antigens. One notable difference between these antibodies is that mAb Fall-3 stains bone marrow of both Ly-6^a and Ly-6^b mice to a similar extent while Sca-1 is restricted to cells from strains that carry the Ly-6^b allele (29, 30). Sca-1 stains about 7 to 10% of bone marrow cells, most of which are B220 positive (31), while Fall-3 detects about 30% of bone marrow cells, most of which are immature cells. Unlike Sca-1, mAb Fall-3 stains only few cells in thymus, spleen or lymph nodes. Furthermore, two color immunofluorescence analysis on bone marrow cells with Fall-3 and Sca-1 shows that most cells that express one of the antigens lack the other. Only 1 to 2% of bone marrow cells co-express Fall-3 and Sca-1 (Fig. 5). This small population of cells that express both antigens can account easily for all pluripotent stem cells in bone marrow since stem cells comprise a very minor subset of bone marrow. Furthermore, the staining pattern and tissue distribution of Fall-3 suggest that this mAb is not identical to other mAbs that define antigens in the Ly-6 family (32), or to mAbs specific for Qa-m7, another antigen expressed on stem cells (8).

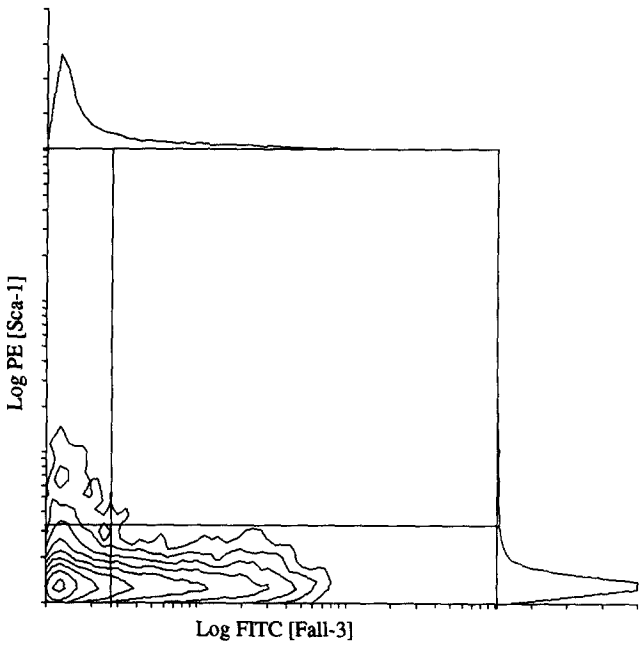

Figure 5. Expression of Fall-3 and Sca-1 antigens on bone marrow cells. Fresh bone marrow cells from C57BL6-Ly-5.2 mice were stained with anti-Sca-1 mAb E13 161-7 detected by PE-labeled goat anti-rat antibodies. Biotinylated mAb Fall-3 was detected with FITC-labeled Avidin. Displayed are PE versus fluorescence intensity; the corresponding histograms are depicted on the y and x axis. The windows used to define positive and negative populations (see Materials and Methods) are indicated.

While most radioprotecting stem cell activity was found in the Fall-3$^+$ population, a number of mice that received Fall-3$^-$ cells survived and contained donor type cells in their blood 5 mo post reconstitution. In these mice, as in the Fall-3$^+$ reconstituted mice, donor derived T, B, and myeloid cells were found. However, in all the mice that received Fall-3$^-$ cells the level of reconstitution was low (Fig. 3 *A*). Currently, we cannot discriminate whether this reflects a stem cell subset with limited proliferation capacity or whether this reconstitution is due to a few contaminating Fall-3$^+$ cells in the Fall-3$^-$ population. The level of the determinant detected by Fall-3 varies considerably on positive cells and staining with mAb Fall-3 does not result in discrete positive and negative populations (Fig. 2). If stem cells would express low levels of the Fall-3 antigen a few stem cells may have fallen within the negative population defined by our sorting gate. We are in the process of testing this hypothesis.

Fall-3 separates most, if not all, primitive stem cells from cells that respond to stromal cells in Whitlock-Witte cultures. Previously, we characterized in detail the Thy-1^{lo}Lin$^-$ cells that can be detected in limiting dilution and initiate long-term (≥6 wk) Whitlock-Witte cultures. The absence of B lineage specific markers, its extensive proliferative capacity, its failure to be transformed by Abelson virus in vitro (32), and its delayed differentiation into mature B cells places this cell very early in the B cell lineage (1). As we are able to separate this B cell precursor from primitive stem cells it will be possible to analyze these Thy-1^{lo}Lin$^-$Fall-3$^-$ cells more thoroughly in vivo and in vitro.

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