Purification and Characterization of Mouse Hematopoietic Stem Cells

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Mouse bone marrow hematopoietic stem cells were isolated with the use of a variety of phenotypic markers. These cells can proliferate and differentiate with approximately unit efficiency into myelomonocytic cells, B cells, or T cells. Thirty of these cells are sufficient to save 50 percent of lethally irradiated mice, and to reconstitute all blood cell types in the survivors.

THE SEARCH FOR THE HEMATOPOIETIC STEM CELL BEGAN when it was first recognized that animals given lethal doses of irradiation suffered bone marrow failure, and this failure could be reversed by injection of unirradiated bone marrow cells (*1*). It was later shown that these animals were restored in all hematolymphoid cell types by cells of bone marrow donor origin (*2*). The concept of multipotential hematopoietic progenitors derived from the first quantitative experiments on bone marrow restoration of lethally irradiated mice, where limiting numbers of bone marrow cells gave rise to clonal colonies of myeloid-erythroid cells in the spleen (and also in the bone marrow) of the irradiated hosts (*3*). Chromosomal markers were used to show that each spleen colony was unlike any other (*4*), and also that certain distinguishing chromosomal markers in reconstitution experiments could be shared by cells of the lymphoid as well as the myeloid lineages (*5*). However, in most experiments no distinction could be made between repopulation by self-renewing, pluripotent hematopoietic stem cells, and by multipotent, nonrenewing, hematopoietic progenitors. Several cell separation schemes based on density, sensitivity to antimitotic agents, and surface glycoprotein display led to the enrichment of cells responsible for establishing myeloid or erythroid spleen colonies (*6, 7*), but the question of whether lymphoid precursors were also being copurified, or whether an absolute purification of the stem cells had occurred, was not answered.

The isolation of a pure population of self-renewing pluripotent hematopoietic stem cells is a requirement for understanding the developmental biology of the hematolymphoid system, and also would be instrumental for a number of gene, cell, and organ replacement clinical therapies dependent on bone marrow transplantation. Therefore the search for characteristics that unambiguously identify the pluripotent hematopoietic stem cell in any species is desirable.

We have approached the characterization of mouse hematopoietic stem cells by using cell-separation technologies based on monoclonal antibody binding to cell surface "differentiation" antigens—antigens present on some, but not all, cells in a particular population or lineage (*8*). To assay multipotentiality of the isolated cells, we developed limit-dilution assays for clonogenic precursors of B lineage cells in vitro (*9*), and T lineage cells in vivo (*10*), which, along with the Till-McCulloch spleen colony assay for myeloid-erythroid progenitors (*3*), covers most (if not all) hematopoietic lineages. The initial strategy for these selections became clear when it was found that bone marrow B lineage progenitors, assayed by their ability to establish long-term B cell cultures when placed on cloned, pre-B inducing stromal cells (*11*), lack a surface marker, B220, present on most (if not all) pre-B and B cells (*12*). We reasoned that B cell progenitors lacking B220 would also lack expression of cell surface antigens associated with differentiated cells in the other branches of the hematolymphoid tree. Thus, it became possible to negatively select for these progenitors by removing all bone marrow cells expressing selected surface markers characteristic of B cells (B220), granulocytes (Gr-1), myelomonocytic cells (Mac-1), and T cells (CD4, CD8). It was also found that these progenitors expressed low, but significant levels of the cell surface differentiation antigen Thy-1, confirming findings that Thy-1 is a surface marker of reconstituting bone marrow stem cells in mouse (*13*) and rat (*14*). These Thy-$1^{lo}T^-B^-M^-G^-$ (hereafter called Thy-$1^{lo}Lin^-$ to designate the absence of expression of these lineage markers) were remarkably enriched in clonal progenitors for spleen colonies (CFU-S) (~1 of 31 cells injected intravenously), thymic colonies [CFU-T (*10*)] (~1 of 600 cells injected intravenously), and pre-B cultures [~1 of 11 cells placed in Whitlock-Witte cultures of clonal bone marrow stromal cells (*11*)], and represented a 50- to 200-fold enrichment of each of these progenitors (*8*). These cells were also remarkably enriched for hematopoietic stem cells that repopulated all hematolymphoid cells of lethally irradiated mice (*15*). However, estimates of the seeding efficiencies of CFU-S (*16*) and CFU-T (*10*) injected intravenously indicated that the injected populations remained somewhat heterogenous, leaving open the possibility that both stem cells and committed progenitors had been coenriched. We therefore sought markers that could further subdivide the Thy-$1^{lo}Lin^-$ bone marrow cells.

Aihara and Klein have produced several monoclonal antibodies to putative pre-T hybridomas (*17*). One of these, E13 161-7, marked several hematolymphoid subsets, including functional bone marrow progenitors of thymic lymphocytes (*18*). The cell surface antigen recognized by this monoclonal antibody has been designated stem cell antigen-1 (Sca-1). We now demonstrate that Sca-1 subdivides the Thy-$1^{lo}Lin^-$ cells into a minor population of Sca-1^+ cells and a major population of Sca-1^- cells. We show that the Thy-1^{lo}-$Lin^-Sca\text{-}1^+$, but not the Thy-$1^{lo}Lin^-Sca\text{-}1^-$ bone marrow subset contains hematopoietic stem cells and CFU-T, although both populations contain CFU-S. We also demonstrate that the degree of enrichment of activities is consistent with the hypothesis that all

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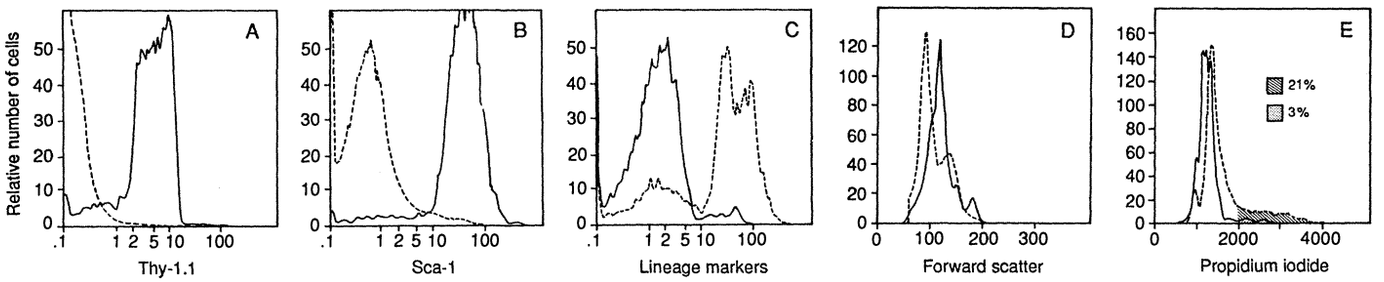

Fig. 1. Purification of pluripotent hematopoietic stem cells. Bone marrow cells were obtained by flushing tibiae and femora of ten C57BL/Ka-Thy-1.1 mice with Hanks balanced salt solution (without phenol red) (Gibco) supplemented with 2 percent fetal calf serum (FCS, Sterile Systems) and 10 m*M* Hepes buffer (Research Organics) (HBSS). Cells were incubated for 20 minutes on ice with directly fluoresceinated, rat antibodies to CD4 and CD8 T cell determinants (antibodies GK-1.5 and 53-6.72, respectively). The cells were washed through a FCS cushion, resuspended in 6 ml of HBSS with 0.6 ml of magnetic beads coupled to sheep antibodies to fluorescein (Advanced Magnetics), and incubated at 4°C for 20 minutes with constant mixing. The labeled T cells were removed by magnetic separation (Bio-Mag Separator, Advanced Magnetics) and discarded. The remaining cells were incubated for 20 minutes on ice with directly fluoresceinated mouse antibody specific for the Thy-1.1 allelic determinant (antibody 19XE5). Magnetic beads were added and incubated as above, and the labeled cells were recovered by magnetic separation. Approximately 2.0 percent of the original cell suspension was recovered. The magnetically separated cells were incubated sequentially with the following reagents, each step being for 20 minutes on ice and being terminated with a washing in HBSS through a FCS cushion: (i) anti-B220, anti-Mac-1, and anti-Gr-1 in one incubation (rat antibodies RA3-6B2, M1/70.15.11.5, and RB6-8C5, respectively; these antibodies define the differentiated hematolymphoid lineages of B cells, macrophages, and granulocytes); phycoerythrin-conjugated goat antiserum to rat immunoglobulin [absorbed with mouse immunoglobulin (Biomeda)]; normal rat immunoglobulin (Pel-Freez Biologicals); biotinylated rat antibody to Sca-1 (antibody E13 161-7); and Texas Red–conjugated avidin (Cooper Biomedical). After the final washing, cells were resuspended in HBSS containing propidium iodide (2 μg/ml). The labeled cells were analyzed and separated with a dual laser FACS (Becton Dickinson), modified as described (*30*) and made available through the FACS shared users group at Stanford University. Cells to be sorted were selected on the basis of an intermediate level of fluorescein staining (Thy-1^{lo}), high right-angle scatter (due to the surface binding of magnetic beads), high levels of Texas Red (Sca-1^+), intermediate forward scatter (to exclude erythrocytes, free beads, and cell aggregates), and low levels of phycoerythrin or propidium iodide (detected together in one FACS channel; this excludes dead cells and lineage marker-positive cells). Sorted populations were more than 90 percent pure with respect to their Thy-1^{lo}Lin$^-$Sca-1^+ phenotype, as assessed by reanalysis on the FACS. (**A** to **C**) The dotted lines indicate expression of the listed antigen by unseparated mouse bone marrow cells, while the solid lines represent a reanalysis of the sorted population. (**D**) Cell size distribution, as assayed by forward scatter, of unseparated bone marrow cells (dotted line) and of the stem cell fraction (solid line). Direct observations of cytospin preparations indicated a homogenous population of cells with a very high nuclear to cytoplasmic ratio and rather dispensed chromatin. Measurements of the nuclei indicated a diameter of 8.3 ± 0.8 μm (mean ± SD) (Fig. 6, top left). (**E**) Cell cycle analysis of unseparated bone marrow cells (dotted line) and sorted hematopoietic stem cells (solid line). Cells were exposed to a lysing agent (American Scientific Products), and the nuclei were analyzed after they were stained with propidium iodide (10 μg/ml).

Thy-1^{lo}Lin$^-$Sca-1^+ cells are hematopoietic stem cells.

The Thy-1^{lo}Lin$^-$Sca-1^+ bone marrow cells are a virtually pure population of primitive myeloerythroid stem cells. Bone marrow stem cells are restricted to a relatively rare subpopulation—the 0.1 to 0.2 percent of cells that bear the Thy-1^{lo}Lin$^-$ phenotype (*8*). This population contains precursors for each hemotolymphoid lineage, including thymocyte precursors (*10*). Another monoclonal antibody, now called Sca-1, also selects most, if not all clonogenic bone marrow precursors of thymocytes and their progeny T cells (*18*). Only 20 to 30 percent of Thy-1^{lo}Lin$^-$ cells are Sca-1^+ (*18*). Using a combination of immunomagnetic bead enrichment of Thy-1^{lo} cells, followed by FACS (fluorescence-activated cell sorter) selection, we obtained a virtually pure population of medium-sized lymphoid-appearing round cells (as shown below). These cells are Thy-1^{lo}Lin$^-$Sca-1^+ (Fig. 1, A to C). By forward scatter analysis they appear as a unimodal peak intermediate in size between bone marrow lymphocytes and large myeloid cells (Fig. 1D). Most, if not all of these cells are in the G_0-G_1 phase of the mitotic cycle; at least more than 97 percent have a 2N amount of DNA (Fig. 1E).

While the splenic colony-forming assay has been long regarded as an accurate reflection of pluripotent hematopoietic stem cell activity, recent evidence indicates that only the late-forming (day 12) CFU-S correlate with true stem cell activity (*19*). The Thy-1^{lo}Lin$^-$Sca-1^+ bone marrow cells contain a 1000-fold enrichment for day 12 CFU-S when compared to whole bone marrow (Fig. 2).

One splenic colony was observed per ten intravenously transferred stem cells. Not all stem cells injected intravenously lodge in the spleen. Several groups of investigators have estimated that the spleen seeding factor (*f*) is 0.1 to 0.2 (*20*). If so, the actual frequency of cells in the Thy-1^{lo}Lin$^-$Sca-1^+ cell population capable of forming macroscopic 12-day splenic colonies is 1 in 1 to 1 in 2 cells.

The temporal evolution of splenic colonies can indicate the level of maturation of their progenitor cells, so that bone marrow cells that generate splenic colonies within 8 days are thought to be already committed to a particular lineage of differentiation, while cells that generate colonies only after 12 days have the characteristics of uncommitted, pluripotent progenitors. We would therefore expect the candidate primitive myeloerythroid precursors to give rise to day 12 spleen colonies, while the more differentiated precursors should give rise predominantly to day 8 spleen colonies. After injection of 200 Thy-1^{lo}Lin$^-$Sca-1^+ cells, an average of 2 day 8 CFU-S was found, compared to 20 day 12 CFU-S. Thus, for this subset the ratio of CFU-S on day 12 to that on day 8 is 10. For the Thy-1^{lo}Lin$^-$Sca-1^- cells the ratio of CFU-S on day 12 to that on day 8 is 0.7, supporting the conclusion that this fraction contains primarily more differentiated progenitors, while the Sca-1^+ cells are more primitive precursors.

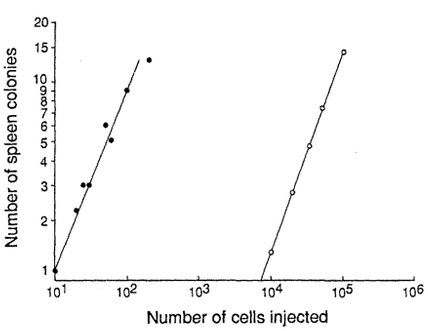

Fig. 2. Spleen colony formation by purified stem cells. Splenic colony-forming unit (CFU-S) activity was assessed 12 days after intravenous transfer of unseparated bone marrow cells or isolated hematopoietic stem cells into lethally irradiated (900 rads) syngeneic mice. Each data point represents the mean from two to four independent experiments with five to ten animals per trial. By linear regression analysis, one splenic colony was formed per ten hematopoietic stem cells transferred [frequency = 0.95 ± 0.08 (SD)], or per 7200 unseparated bone marrow cells [frequency = (1.4 ± 0.3) × 10^{-4}].

The splenic colonies produced by both the Sca-1$^+$ and Sca-1$^-$ fractions of Thy-1^{lo}Lin$^-$ bone marrow cells were also evaluated microscopically to determine whether either fraction was relatively enriched or depleted for erythroid or myeloid progenitors. Because the lineage pool of antibodies that led to the Lin$^-$ subset lacked antibodies specific for the erythroid lineage, we might expect the Thy-1^{lo}Lin$^-$Sca-1$^-$ population to be enriched for erythroid-committed progenitors. The results indicated that the Sca-1$^-$ fraction produced a range of colonies similar to that produced by whole bone marrow (Table 1), but were highly enriched in pure erythroid colonies, found at day 9. The Sca-1$^+$ fraction, produced many myeloid and mixed colonies at days 9 and 12 (Table 1).

The Thy-1^{lo}Lin$^-$Sca-1$^+$ bone marrow cells are a virtually pure population of clonogenic thymic precursors. The bone marrow contains a population of clonogenic, thymus-homing precursors (CFU-T) as revealed by their intravenous injection into lethally irradiated hosts (*10*). By limit-dilution analysis these represent ~1 of 35,000 bone marrow cells injected intravenously, and ~1 of 5000 to 8000 cells injected intrathymically (*10*). Colonies of thymocytes derived from the isolated Thy-1^{lo}Lin$^-$Sca-1$^+$ bone marrow cell fraction could be established after the intrathymic injection of as few as four cells (Fig. 3). Intrathymic transfer of ten or more of these cells resulted in thymic colonies in 95 percent or more of the injected thymic lobes. By limiting dilution analysis, the frequency of cells with the ability to respond to the thymic microenvironment is approximately 1 in 5. This is likely to be an underestimate, as only about 30 percent of intrathymically injected bone marrow cells remain in the thymus a few hours after injection (*21*).

Table 1. Histological analysis of early and late spleen colonies derived from Thy-1^{lo}Lin$^-$Sca-1$^-$ and Thy-1^{lo}Lin$^-$Sca-1$^+$ bone marrow cells. Groups of irradiated (900 rads) recipient mice received either Thy-1^{lo}Lin$^-$Sca-1$^-$ cells or Thy-1^{lo}Lin$^-$Sca-1$^+$ cells intravenously. *N* refers to the total number of colonies examined histologically, and is not a measure of the relative number of spleen colonies found on those days.

Day colonies examined	Cell source	Colony morphology (%)			*N*
		Erythroid	Myeloid	Mixed	
8	Thy-1^{lo}Lin$^-$Sca-1$^+$		100		3
	Thy-1^{lo}Lin$^-$Sca-1$^-$	42	42	17	12
9	Thy-1^{lo}Lin$^-$Sca-1$^+$	41	28	31	32
	Thy-1^{lo}Lin$^-$Sca-1$^-$	80	5	15	20
12	Thy-1^{lo}Lin$^-$Sca-1$^+$	27	27	46	15
	Thy-1^{lo}Lin$^-$Sca-1$^-$	27	27	46	11

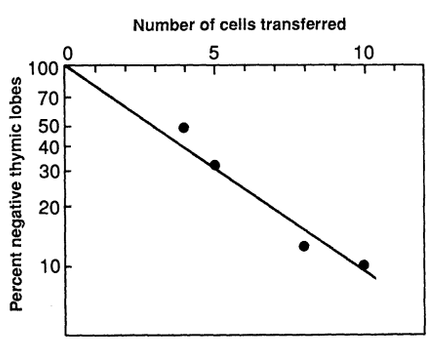

Fig. 3. Thymic colony formation by purified stem cells. Thymic colony-forming unit (CFU-T) activity was assessed 4 weeks after intrathymic transfer of isolated hematopoietic stem cells into sublethally irradiated (700 rads) mice, congenic to the stem cell population at the Thy-1 and Ly-5 loci. Thymic colonies, detected by FACS analysis of cells expressing donor allelic determinants, varied in size from 1×10^5 to 1×10^8 cells. Colony sizes did not vary with the number of cells injected. Each data point represents a single experiment, 10 to 20 thymic lobes per trial. By limiting dilution analysis, one CFU-T was transferred per five hematopoietic stem cells.

The Thy^{lo}Lin$^-$Sca-1$^+$ bone marrow cells are a virtually pure population of multilineage hematopoietic stem cells. The definition of pluripotential hematopoietic stem cells has two components. Each stem cell must be capable of giving rise to progeny in all defined hematolymphoid lineages: limiting numbers of stem cells must be capable of fully reconstituting lethally irradiated mice, leading to their long-term survival. Limiting numbers of Thy-1^{lo}Lin$^-$Sca-1$^+$ bone marrow cells were able to repopulate T cell, B cell, and myeloid lineages when transferred into irradiated mice. Forty Thy-1^{lo}Lin$^-$Sca-1$^+$ stem cells established multiple lineages of the hematolymphoid cells that can be identified by expression of the Ly-5.2 allelic determinant of the T-200 leukocyte common antigen (Fig. 4). This antigen is expressed by all cells of the hematolymphoid lineages with the exception of erythroblasts and erythrocytes (*22*). Approximately 50 percent of the peripheral blood leukocytes are derived from the 40 injected stem cells, with the remaining cells being derived from the 200 syngeneic Thy-1^{lo}Lin$^-$ cells that were transferred along with the 40 congenic stem cells. Thus Thy-1^{lo}Lin$^-$Sca-1$^+$ cells are capable of multilineage reconstitution.

We had shown earlier that 50 percent survival of lethally irradiated mice could be achieved with about 4×10^4 bone marrow cells and about 100 Thy-1^{lo}Lin$^-$ bone marrow cells (*23*). In order to quantify the activity of the Thy-1^{lo}Lin$^-$Sca-1$^+$ cells in this study, graded numbers of cells were transferred intravenously into lethally irradiated syngeneic hosts. Only 30 Thy-1^{lo}Lin$^-$Sca-1$^+$ bone marrow cells were necessary to rescue one-half of a group of lethally irradited mice (Fig. 5). In contrast, transfer of as many as 900 Thy-1^{lo}Lin$^-$Sca-1$^+$ cells did not save the mice. Several C57BL/6 mice rescued with <100 Thy-1^{lo}Lin$^-$Sca-1$^+$ cells from C57BL/Thy-1.1 donors were assessed 8 to 12 weeks later for donor reconstitution versus host regeneration. The results indicated that 80 percent of these animals contained donor-derived Thy-1.1 T cells, with most having more than 50 percent of their T cell population derived from the limiting number of Thy-1^{lo}Lin$^-$Sca-1$^+$ stem cells. In subsequent experiments with Ly5 congenic lines, survivors of lethal irradiation given <100 Thy-1^{lo}Lin$^-$Sca-1$^+$ cells were nearly completely recon-

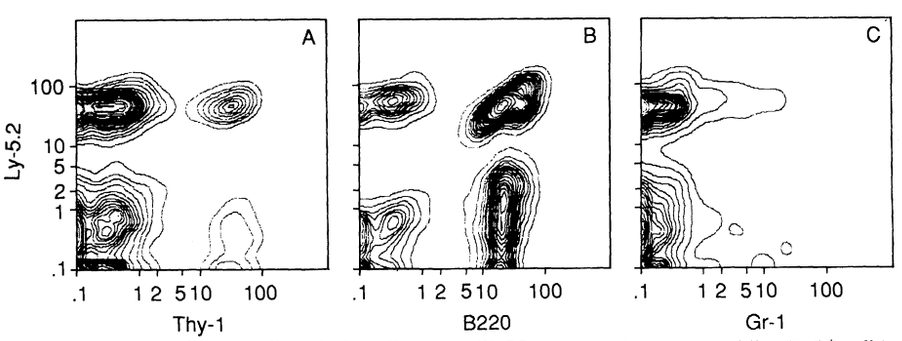

Fig. 4. Multiple hematolymphoid repopulation by purified stem cells. Limiting numbers of hematopoietic stem cells reconstitute multiple hematolymphoid lineages. Forty Thy-1^{lo}Lin$^-$Sca-1$^+$ cells (C57BL/6–Ly-5.2) were transferred intravenously into lethally irradiated (900 rads) Ly-5 congenic mice (C57BL/Ka, Ly-5.1) along with 200 host-derived stem cells. At various times thereafter, donor-derived (Ly-5.2$^+$) cells were detected in the peripheral blood and phenotyped by two-color FACS analysis. Six weeks after reconstitution, 50 percent of the peripheral blood leukocytes in this mouse were derived from the 40 donor hematopoietic stem cells. These included (**A**) 60 percent of the circulating T cells (Thy-1$^+$) cells, (**B**) 50 percent of the B cells (B220$^+$ cells), and (**C**) 50 percent of the neutrophils (Gr-1$^+$ cells).

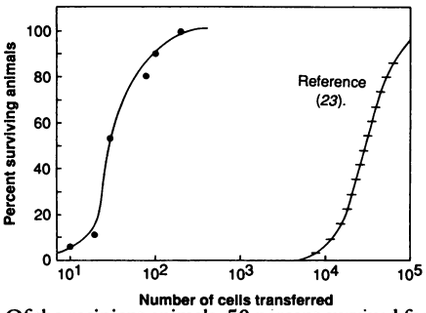

Fig. 5. Protection from lethal irradiation by purified stem cells. Groups of 10 to 20 mice were lethally irradiated (900 rads) and reconstituted with graded numbers of Thy-1^{lo}Lin$^-$Sca-1^+ hematopoietic stem cells intravenously (●). Each point represents the mean from one to two independent experiments, ten animals per trial. Of the recipient animals, 50 percent survived for more than 30 days when 30 cells were transferred. In contrast, we and others have reported that 1.3×10^4 to 3.3×10^4 unseparated bone marrow cells are required to achieve the same level of protection (*6, 23*); the barred curve is derived from (*23*).

stituted in the T, B, granulocyte, and macrophage lineages.

As with the CFU-S and CFU-T assays (Figs. 2 and 4), these radioprotection results represent a relative enrichment (1000 times) over unseparated bone marrow. The Thy-1^{lo}Lin$^-$Sca-1^+ bone marrow subset represents only about 0.05 percent of all bone marrow cells. Thus the 50 percent reconstitution effected by about 4×10^4 whole bone marrow cells in equivalent to about 20 Thy-1^{lo}Lin$^-$Sca-1^+ cells both numerically and in reconstitution of lethally irradiated mice. It appears unlikely that full long-term hematolymphoid reconstitution and survival of lethally irradiated hosts requires (or utilizes) any cells other than Thy-1^{lo}Lin$^-$Sca-1^+ cells; that is, most, if not all pluripotent mouse hematopoietic bone marrow stem cells are Thy-1^{lo}Lin$^-$Sca-1^+.

The Thy-1^{lo}Lin$^-$Sca-1^+ cells are probably the only pluripotent hematopoietic stem cells. The Thy-1^{lo}Lin$^-$Sca-1^+ bone marrow cells described above certainly constitute the greatest degree of enrichment of hematopoietic stem cells yet reported. However, it is necessary to ascertain whether these cells represent pure hematopoietic stem cells and whether they encompass the entire population of hematopoietic stem cells in the bone marrow.

That they are a population of pure stem cells is likely. (i) These cells have activity potentials approaching 1 for each of the assays for hematolymphoid lineage precursors. The CFU-S assay measures the proliferation and myeloerythroid differentiation potential of stem cells lodging in the spleen (Fig. 6, bottom left), and the recorded experimental number of 1 per 10 cells injected intravenously (Fig. 2) is an underestimate. (i) The reported spleen seeding efficiency of bone marrow stem cells injected intravenously into lethally irradiated animals is between 0.1 and 0.2, increasing the CFU-S activity to 1 in 1 to 1 in 2 cells injected. (ii) The injected cells were ≥90 percent pure and ≥90 percent viable (with no colonies possibly arising from the few contaminants or dead cells). (iii) Only macroscopic colonies were reported, and this leads to an underestimate of the true colony-forming potential. Taken together, the CFU-S potential is much closer to 1 in 1 cell than 1 in 2. The same population of cells scored at approximately 1 in 5 cells injected into the thymus giving rise to a significant clone of thymic lymphocytes (Fig. 6, top right). The retention of intrathymically injected bone marrow precursors of thymocytes is of the order of 30 percent (*21*), and, together with the aforementioned assays of viability and purity, once again the estimated thymic cloning capacity of these putative stem cells is close to one in one. Finally, the enrichment factor for minimal numbers of cells required to restore lethally irradiated animals in all hematolymphoid lineages is exactly that calculated to be the representation of Thy-1^{lo}Lin$^-$Sca-1^+ cells in the bone marrow.

Whether the Thy-1^{lo}Lin$^-$Sca-1^+ cells (Fig. 6, top left and bottom right) are the only true pluripotent stem cells in the bone marrow is more difficult to answer although the evidence appears to lead to a positive answer. As described above, there is no discrepancy between the level of enrichment and the predicted level of radioprotection of lethally irradiated animals. However, the Thy-1^{lo}Lin$^-$Sca-1^- cells do include a significant number of cells that can give rise to CFU-S (Table 1), an activity related to either stem cell activity or primitive myeloid erythroid progenitors. This is not surprising, as our designation of Lin$^-$ included markers of only a subset of the myelomonocytic class (Mac-1 and Gr-1), and did not include markers of the erythroid, megakaryocytic, natural killer, mast cell, or eosinophil lineages—all derived from hematopoietic stem cells. Furthermore, we have shown that Thy-1^{lo}B220$^+$Mac$^-$Gr-1^- cells are potent precursors restricted to the B lineage, while Thy-1^{lo}Mac-1^+B220$^-$ cells are potent myelomonocytic precursors (*25*). Thus, it is likely that a significant percentage of Thy-1^{lo}Lin$^-$Sca-1^- cells are lineage-committed progenitors (for example, erythroid). All these cell types have minimal activity in promoting the survival of lethally

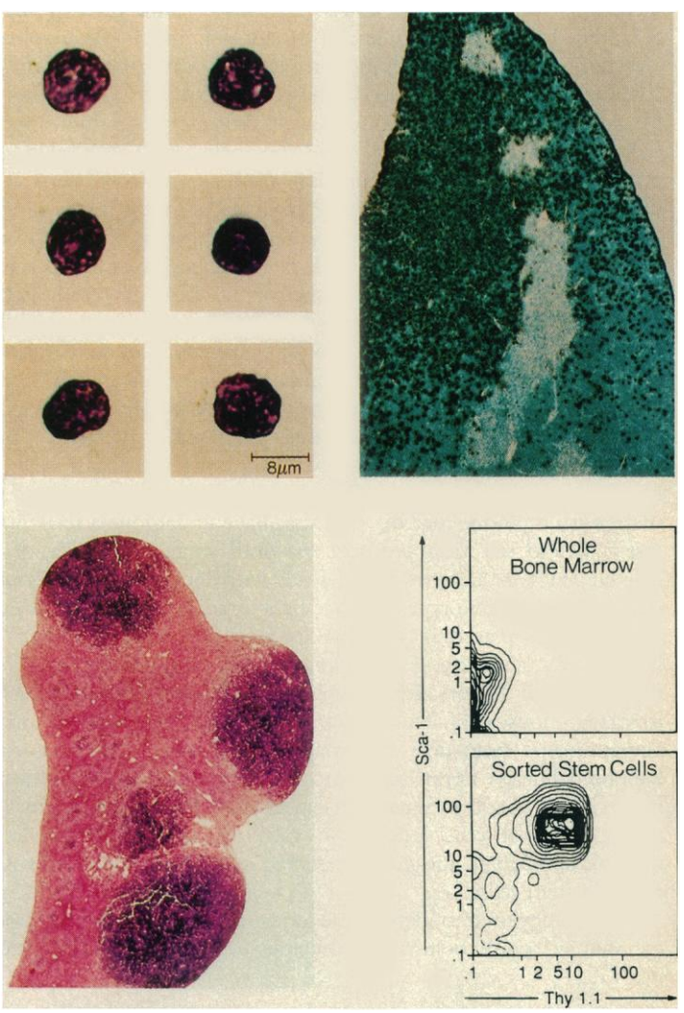

Fig. 6. Characterization of mouse hematopoietic stem cells. (**Top left**) Six Thy-1^{lo}Lin$^-$Sca-1^+ stem cells as stained with hematoxylin after cytocentrifugation. (**Bottom left**) Four macroscopic 12-day spleen colonies after fixation and staining with hematoxylin and eosin. (**Top right**) A high power view of the tip of a Thy-1.2 host thymic lobe containing a single donor colony; the colony, stained with antibody to Thy-1.1, is primarily cortical with scattered medullary cells. Biotinylated antibody to Thy 1.1–coated sections was developed after incubation with avidin peroxidase and diaminobenzidine and then counterstained with methylene blue as described (*18*). (**Bottom right**) FACS analysis of the starting whole bone marrow population (upper) and the isolated stem cell population (lower) stained with antibody to Sca-1 and Thy-1.1.

irradiated animals (*25*). Thus, the major candidates for other bone marrow populations that might contain pluripotent hematopoietic stem cells appear not to have such an activity; and therefore it is unlikely that there exists in the mouse bone marrow a population of pluripotent stem cells that bear markers other than Thy-1^{lo}Lin$^-$Sca-1^+.

How many stem cells actively repopulate the mouse during any defined period following irradiation and reconstitution? We show here that Thy-1^{lo}Lin$^-$Sca-1^+ subset represents approximately 0.05 percent of bone marrow cells, and therefore bone marrow reconstitution with the usual levels between 1×10^5 and 1×10^7 bone marrow cells should yield between 50 and 5000 stem cells. Yet Lemishka *et al.* (*26*), using bone marrow from mice treated with 5-fluorouracil, made retroviral inserts and then cultured the cells for 2 days with interleukin-3 (IL-3) according to a G418 (*neo*^{r}) selection protocol; they found that one or a few predominant retroviral inserts could be found in most, if not all, recognized hematolymphoid lineages. In apparent contrast, we demonstrate here that increasing the numbers of Thy-1^{lo}Lin$^-$Sca-1^+ bone marrow stem cells leads to an increased number of clones found as either spleen colonies or thymic colonies, and one would expect that each of these colonies would continue beyond the 12-day to 4-week interval used for spleen and thymic colony assays. Perhaps the 5-fluorouracil treatment, retroviral infection, IL-3 treatment, and G418 selection procedure results in the reduction of bone marrow stem cell numbers in vitro to one or a few cells. If that were the case, we would have expected very low survivorship of irradiated hosts injected with these cells (Fig. 5). It is also conceivable that certain retroviral inserts became established in a class of poorly self-renewing but highly proliferative multipotential progenitors, cells that can survive only in the presence of coinjected stem cells. A third possibility is that when higher numbers of bone marrow cells are injected we begin to detect regulatory cells that suppress or enhance the emergence of particular hematopoietic stem cell clones. This problem probably cannot be solved until the actual survival of identifiable stem cells—either by phenotype or function—is measured with the bone marrow cells that have undergone a retroviral insertion and selection protocol, or until single stem cells of a defined genotype are introduced, along with another source of stem cells of different genotype, into a lethally irradiated mouse. However, stem cells placed in the thymus give rise only to thymic T lymphocytes, and clonogenic thymic precursors appear to enter the thymus within 3 hours after injection of bone marrow into a lethally irradiated mouse (*27*). This allows us to propose that multipotential hematopoietic stem cells can commit to a single hematopoietic lymphoid lineage upon entry into the appropriate microenvironment (*28*). In that case, we would expect to see lineage restrictions in the progeny of cells lodging in such microenvironments, and multiple clonogenic lineages represented in hosts reconstituted with multiple stem cells.

The strategy used to identify the mouse bone marrow hematopoietic stem cell could be used, in principle, to isolate the human stem cell counterpart. Whether analogous (or similar) markers such as the Thy-1 and Sca-1 are present on human stem cells remains to be determined. A requirement, however, is an assay for human stem cells; there is no direct evidence that in vitro hematopoietic progenitor colony assays can substitute. However, we have an indication that human fetal liver can be used to reconstitute a human lymphoid system in immunodeficient mice (*29*). Perhaps this model will prove sufficient for experiments designed to identify and isolate human hematopoietic stem cells. If so, the use of these cells in bone marrow transplantation, as targets for gene insertion therapy, and for the study of the development biology of the human hematolymphoid system is obvious.

REFERENCES AND NOTES

1. C. E. Ford, J. L. Hamerton, D. W. H. Barnes, J. F. Loutit, *Nature* **177**, 452 (1956).
2. H. S. Micklem and J. F. Loutit, *Tissue Grafting and Radiation* (New York Academy of Sciences Press, New York, 1966).
3. J. E. Till and E. A. McCulloch, *Rad. Res.* **14**, 213 (1961).
4. A. M. Wu, J. E. Till, L. Siminoviteh, E. A. McCulloch, *J. Cell. Physiol.* **69**, 177 (1967).
5. S. Abramson, R. G. Miller, R. A. Phillips, *J. Exp. Med.* **145**, 1567 (1977).
6. J. W. M. Visser, J. G. J. Bauman, A. H. Mulder, J. F. Eliason, A. M. Leeuw, *ibid.*, p. 1576.
7. G. S. Hodgson and T. R. Bradley, *Nature* **281**, 381 (1979).
8. C. E. Muller-Sieburg, C. A. Whitlock, I. L. Weissman, *Cell* **44**, 653 (1986).
9. C. A. Whitlock and O. N. Witte, *Proc. Natl. Acad. Sci. U.S.A.* **79**, 3608 (1982).
10. G. J. Spangrude, C. E. Muller-Sieburg, S. Heimfeld, I. L. Weissman, *J. Exp. Med.*, in press.
11. C. A. Whitlock, G. F. Tidmarsh, C. Muller-Sieburg, I. L. Weissman, *Cell* **48**, 1009 (1987).
12. R. L. Coffman and I. L. Weissman, *Nature* **289**, 681 (1981).
13. J. W. Berman, and R. S. Basch, *Exp. Hematol.* **13**, 1152 (1985).
14. I. Goldschneider, L. K. Gordon, R. J. Morris, *J. Exp. Med.* **148**, 1351 (1978).
15. C. E. Muller-Sieburg, G. F. Tidmarsh, I. L. Weissman, G. J. Spangrude, in *Thy-1: Immunology, Neurology and Therapeutic Applications*, A. E. Reif and M. Schlesinger, Eds. (Dekker, New York, in press).
16. J. E. Till and E. A. McCulloch, *Ser. Haematol.* **2**, 15 (1972).
17. Y. Aihara, H.-J. Buhring, M. Aihara, J. Klein, *Eur. J. Immunol.* **16**, 1391 (1986).
18. G. J. Spangrude, J. Klein, S. Heimfeld, Y. Aihara, I. L. Weissman, in preparation.
19. G. Molineux, R. Schofield, N. G. Testa, *Exp. Hematol.* **14**, 710 (1986).
20. J. N. Hendry, *Cell Tissue Kinet.* **4**, 217 (1971); B. I. Lord, *ibid.*, p. 211; J. E. Till and E. A. McCulloch, *Ser. Haematol.* **2**, 15 (1972).
21. Y. T. Katsura *et al.*, *J. Immunol.* **137**, 2434 (1986).
22. M. Scheid and D. Triglia, *Immunogenetics* **9**, 423 (1979).
23. C. E. Muller-Sieburg, K. Townsend, I. L. Weissman, D. Rennick, *J. Exp. Med.*, in press.
24. L. Siminovitch, E. A. McCulloch, J. E. Till, *J. Cell Comp. Physiol.* **62**, 327 (1963).
25. S. Heimfeld, G. J. Spangrude, I. L. Weissman, unpublished data.
26. I. R. Lemischka, D. H. Raulet, R. C. Mulligan, *Cell* **45**, 917 (1986).
27. G. J. Spangrude and I. L. Weissman, in preparation.
28. M. A. S. Moore and J. T. T. Owen, *Lancet* **ii**, 658 (1967).
29. M. McCune, R. Namikawa, H. Kaneshima, L. D. Schultz, M. Lieberman, I. L. Weissman, in preparation.
30. D. R. Parks and L. A. Herzenberg, *Methods Enzymol.* **108**, 197 (1984); J. W. M. Visser, J. G. T. Baumann, A. M. Mulder, J. F. Eliason, A. M. Leeuw, *J. Exp. Med.* **59**, 1576 (1984).

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