Amplification of Sca-1 + Lin - WGA + Cells in Serum-Free Cultures Containing Steel Factor, Interleukin-6, and Erythropoietin With Maintenance of Cells With Long-Term In Vivo Reconstituting Potential

By Vivienne I. Rebel, Wiesława Dragowska, Connie J. Eaves, R. Keith Humphries, and Peter M. Lansdorp

Normal murine bone marrow (BM) cells were sorted on the basis of low forward and orthogonal light scatter properties, Sca-1 expression (Sca-1 +), lack of staining with a cocktail of mature hematopoietic lineage markers (Lin -), and binding of wheat germ agglutinin (WGA +). This approach allowed the reproducible isolation of a very small subpopulation (0.037% ± 0.023% of all nucleated BM cells) that was approximately 400-fold enriched in cells capable of reconstituting both lymphoid and myeloid lineages in lethally irradiated recipients. Transplantation of 30 or 10 of these Sca-1 + Lin - WGA + cells resulted in ≥20% donor-derived nucleated peripheral blood cells 3 months posttransplantation in 100% and 22% of the recipients, respectively. When Sca-1 + Lin - WGA + cells were cultured in serum-free medium supplemented with Steel factor, interleukin-6 (IL-6), and erythropoietin (with or without IL-3), a large increase in total cell number, including cells with an Sca-1 + Lin - WGA + phenotype was observed. Single cell cultures showed that 90% to 95% of the input cells underwent at least one division during the first 2 weeks and the remainder died. Interestingly, this proliferative response was not accompanied by a parallel increase in the number of cells with both lymphoid and myeloid repopulating potential in vivo, as quantitation of these by limiting dilution analysis showed they had decreased slightly (1.3-fold) but not significantly below the number initially present. These results demonstrate that Sca-1 + Lin - WGA + cells with long-term repopulating potential can be maintained for 2 weeks in a serum- and stroma cell-free culture, providing a simple in vitro system to study their behavior under well-defined conditions. The observed expansion of Sca-1 + Lin - WGA + cells in vitro without a concomitant increase in reconstituting cells also shows that extensive functional heterogeneity exists within populations of cells with this surface phenotype.

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Within the hematopoietic system there is a continuous generation of lineage-restricted progenitor cells that give rise to the different types of mature blood cells. Hematopoietic stem cells (HSC) are the precursors of lineage-committed progenitor cells and are thus responsible for long-term hematopoiesis. During steady-state hematopoiesis in the adult, most of the HSCs are believed to be in a quiescent state and the mechanisms that control their self-renewal and/or commitment into the different hematopoietic lineages are poorly understood. Clarification of these regulatory processes is not only of fundamental biologic importance, but is also likely to be of practical significance for a variety of clinical applications in bone marrow transplantation (BMT) and gene therapy. One approach of such studies is to study the behavior of purified HSCs under well-defined conditions. The observed extent of this culture system poses problems in the identification of the specific cytokines that provide or may substitute for the support obtained in this system. It has also been reported that the long-term repopulating ability of 5-fluorouracil (5-FU)-pretreated marrow cells cultured in a serum-containing liquid suspension culture in the presence of IL-6 and IL-3 can increase approximately twofold after an initial decrease of similar magnitude. However, such increases have not been found in similar cultures of untreated marrow or highly purified normal marrow cells with long-term in vivo repopulating ability.

We recently showed that primitive human hematopoietic cells with a CD34 + CD45RA + CD71 + phenotype could be...
maintained for a period of up to 50 days in the presence of SF, IL-3, IL-6, and Ep in a defined, serum- and stroma cell-free culture system.25 In view of these observations we were keen to examine the ability of this culture system to sustain primitive murine hematopoietic cells measured in vivo. For these studies we purified long-term in vivo repopulating cells using a combination of previously described approaches26-28 by combining staining with the lectin wheat germ agglutinin (WGA), as described by Visser et al29 and Ploemacher and Brons,30 with staining for Ly6A/E (Sca-1+) and several lineage markers expressed on mature hematopoietic cells, as described by Sprangrude et al.31 The Sca-1+Lin WGA+ cells with low forward and orthogonal light scatter properties were highly enriched for HSCs and showed a dramatic proliferative response when cultured in serum-free medium containing SF, IL-6, and Ep, resulting in large increases in the number of Sca-1+Lin WGA+ cells. Interestingly, transplantation of irradiated recipients with cultured Sca-1+Lin WGA+ cells indicated that the long-term in vivo reconstituting potential of this population was similar to that of the initially purified cells.

MATERIALS AND METHODS

Animals

C57BL/6 (Ly-5.2) and C57B1/6 Ly-5.1:PeP3b (Ly-5.1), were obtained from The Jackson Laboratories (Bar Harbor, ME) and were bred and maintained in the animal facility of the British Columbia Cancer Research Center (Vancouver, BC, Canada). All animals were kept under micro-isolators and provided with sterilized food and acidified water (pH = 3) ad libitum.

Antibodies and Reagents for Immunologic Staining

Hybridomas secreting rat monoclonal antibodies (MoAbs) E13 16-1-7 (anti-Sca-1) and A20-1-7 (anti-Ly-5.1) were kindly provided by Dr. G. Sprangrude (Rocky Mountain Laboratory, Hamilton, MT). The hybridoma M1/70 (anti-Mac-1, myelomonocytic cells) was obtained from American Type Culture Collection ATCC, Rockville, MD). Antibodies were purified from tissue culture supernatants using protein A or protein G affinity chromatography. E13 16-1-7 was directly derivatized with cyanine 5 succinimidyl ester (Cy5) (Biological Detection Systems, Inc, Pittsburgh, PA). A20-1-7 and WGA (Molecular Probes, Inc, Eugene, OR) were labeled with FITC (F-7250; Sigma Chemical Co, St Louis, MO) and M1/70 with biotin-succinimidyl ester (Molecular Probes) by standard procedures. Biotinylated rat antibodies RA3-6B2 (anti-B220, B cells), RB6-8C5 (anti-granulocytes, anti-Gr-1, myeloid cells), and 53.7.3 (anti-Ly-1, T cells) were obtained from Pharmingen (San Diego, CA). Phycoerythrin (PE) conjugated to streptavidin (S-888) was obtained from Southern Biotechnology Associates, Inc (Birmingham, AL). Propidium Iodide (PI) was obtained from Sigma (p-5264) and 7-Amino Actinomycine D (7-AAD) from Molecular Probes.

Preparation of Cell Suspensions

C57BL/6 Pep3b mice were used as the source of donor BM cells. Mice (8 to 12 weeks old) were killed and the marrow cells harvested by flushing the femoral shafts with Hanks' 2% fetal calf serum (HF), using a 3-mL syringe and a 21-gauge needle. The cell suspension was centrifuged on a cushion of fetal calf serum (FCS) and washed once more in HF. Nucleated cell counts were performed using a hemocytometer chamber.

Labeling of Cells

Marrow cells (5 to 10 × 10⁶/mL) suspended in HF containing 5% rat serum (Jackson ImmunoResearch Laboratories, West Grove, PA) were first incubated for 15 minutes at room temperature. The cells were then washed twice at 4°C with biotinylated antibodies against the following lineage (Lin) markers: anti-B220 (1 µg/mL), anti-Ly-1 (2 µg/mL), anti-Gr-1 (0.3 µg/mL), and anti-Mac-1 (0.3 µg/mL), for 30 minutes. After 2 washes with HF the cells were then stained simultaneously at 4°C with Sca-1-Cy5 (3 µg/mL), WGA-FITC (0.1 µg/mL), and SA-RPE (1:500 dilution). Finally the cells were washed twice. PI (2 µg/mL) was added during the second wash step before the resuspension in HF.

Fluorescence Activated Cell Sorter (FACS) Sorting and Cloning of Single Cells

Cells were sorted on a FACStar+ (Becton Dickinson & Co, San Jose, CA) equipped with a 5-W argon and a 30-mW helium neon laser. Specific fluorescence of FITC, RPE, PI, and Cy5 excited at 488 nm (0.4 W) and 633 nm (30 mW) as well as forward and orthogonal light scatter signals were used to establish sort windows. Positivity for each probe was defined as fluorescence that exceeded 99% of matched isotype control antibody conjugates. Cells were collected in sterile eppendorf vials in serum-free medium (see below). For single-cell experiments, purified cells were resorted after collection and single cells were deposited directly into the wells of round-bottom 96-well plate (Nunc, Kamstrup, Denmark) using an automatic cell deposition attachment to the FACStar+ (Becton Dickinson). Medium, supplemented with cytokines (see below), was added to the wells immediately before cloning.

Reconstitution Experiments

Three- to 6-month-old C57BL/6 mice were used as recipients in all reconstitution assays. Whole-body irradiation was administered in a single dose (900 to 950 cGy, 110 cGy/min) using a Cs137 source. Indicated numbers of cells (counted by FACStar+) were injected in a final volume of 0.2 to 0.3 mL of HF. In some cases, either 2 × 10⁶ "compromised" C57BL/6 marrow cells32 or 4 × 10⁶ normal Sca-1 marrow cells were also injected. Peripheral blood (50 to 100 µL) was obtained by tail-vein puncture 6 to 10 and 15 to 20 weeks after transplantation and the percentage donor-derived peripheral blood cells determined by staining the blood samples directly with an equal volume of HF containing 0.05% azide and 2 µg/mL Ly-5.1-FITC. After 30 minutes of incubation on ice, the red blood cells were lysed by ammonium chloride. The samples were washed twice and 7-AAD (1 µg/mL) was added in the final cell suspension to distinguish dead from viable cells.23 The percentage of donor (Ly-5.1)-derived cells was analyzed on a FACScan (Becton Dickinson). Recipients who showed >20% donor-derived nucleated peripheral blood cells were considered to have been repopulated by the donor cells.

Serum-Free Suspension Cultures

Sorted Sca-1+Lin WGA+ cells were cultured in serum-free medium prepared as described previously.25 Cells were cultured in 1-mL volumes in 24-well plates (Nunc) except for single-cell experiments. The medium was supplemented with human IL-6 at a final concentration of 10 ng/mL, human Ep at 3 U/mL, murine SF at 50 ng/mL, and where specified with murine IL-3 (20 ng/mL). The SF and IL-6 were purified recombinant proteins kindly provided by Dr D.E. Williams (ImmuneX, Seattle, WA). Murine IL-3 was from culture supernatants of COS cells transsected with murine IL-3 and human Ep was obtained from The Media Preparation Ser-
vice of the Terry Fox Laboratory. When the cultures were nearly confluent, viable cells (excluding trypan blue) were counted using a hemocytometer. Phenotypic analysis and resorting of cells with the originally sorted phenotype was performed as described above for fresh marrow cells.

RESULTS

Strategy for Purification of Murine Hematopoietic Cells With Long-Term Reconstituting Ability In Vivo

Staining profiles of murine BM cells. The gating criteria used for the isolation of Sca-1"Lin-WGA" cells of low forward and orthogonal light scatter properties in normal murine BM are shown in Fig. 1. The combination of Lin markers and WGA staining subdivided the cells with low forward and orthogonal light scatter properties that express Sca-1 into distinct subpopulations, the majority (>85%) being Lin"WGA-" (Fig 1D). Although the percentage of Sca-1"Lin-" cells within the selected light scatter gates differed between donors, the ratio between Lin"WGA+" and Lin"WGA-" cells within this subpopulation was rather constant at 2:1. The resulting six-parameter cell purification strategy yielded reproducible staining profiles, in which Sca-1"Lin-WGA" cells with low forward and orthogonal light scatter properties represented 0.037% ± 0.023% (n = 8) of fresh BM (ie, 1 in 2,700 nucleated cells).

In vivo repopulation studies of Sca-1"Lin-WGA" cells. The long-term repopulating ability of the selected population was assessed following injection into irradiated recipients. For these experiments Sca-1"Lin-WGA" cells were injected together with either 2 × 10^5 "compromised" helper cells (marrow cells from secondary transplant recipients) or 4 × 10^5 sorted Sca-1" cells to provide short-term radioprotection. At 6 to 7 and 10 to 15 weeks after transplantation, the percentage of donor-derived nucleated cells in the peripheral blood was determined, by using an allelic difference at the Ly-5 locus between donor (Ly-5.1) and recipient (Ly-5.2). The results of four experiments are presented in Table 1. In line with previous experiments, the sorted Sca-1"Lin-WGA" cells were highly enriched in cells with repopulating ability (365-fold, 95% confidence limits: 85 to 1,600) compared with labeled but unsorted fresh marrow cells. As few as 10 Sca-1"Lin-WGA" of these cells were able to contribute detectably (≥20% Ly-5.1+ nucleated peripheral blood cells) to hematopoietic reconstitution in 44% (four of nine) of the animals analyzed after 6 to 7 weeks, although this percentage decreased to 22% of the animals when these were reanalyzed after 15 weeks (Table 1). Analysis of five of the nine animals that received 10 Sca-1"Lin-WGA" cells at 9 months posttransplantation showed that in one of the five recipients the percentage of donor-derived peripheral blood cells was still greater than 20%, indicating that sorted cells with this phenotype include cells that are...
Table 1. Analysis of Nucleated Peripheral Blood Cells in Recipients 30 to 105 Days After Receiving Different BM Transplants

<table>
<thead>
<tr>
<th>No. of Test Cells Transplanted</th>
<th>% Donor PB Cells*</th>
<th>% Positive Animals†</th>
<th>Total No. of Animals Transplanted</th>
<th>% Donor PB Cells*</th>
<th>% Positive Animals†</th>
</tr>
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<tbody>
<tr>
<td>Total BM</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>800,000</td>
<td>77.8 ± 1.1</td>
<td>100 (5/5)</td>
<td>5</td>
<td>77.8 ± 4.9</td>
<td>100 (5/5)</td>
</tr>
<tr>
<td>400,000</td>
<td>77.6 ± 3.9</td>
<td>100 (5/5)</td>
<td>5</td>
<td>78.4 ± 5.1</td>
<td>100 (5/5)</td>
</tr>
<tr>
<td>200,000</td>
<td>78.2 ± 2.2</td>
<td>100 (5/5)</td>
<td>5</td>
<td>92.0 ± 0.6</td>
<td>100 (5/5)</td>
</tr>
<tr>
<td>100,000</td>
<td>73.7 ± 4.0</td>
<td>100 (3/3)</td>
<td>3</td>
<td>87.3 ± 1.9</td>
<td>100 (5/5)</td>
</tr>
<tr>
<td>60,000‡</td>
<td>54.8 ± 6.5</td>
<td>100 (6/6)§</td>
<td>7</td>
<td>41.0 (5/5)</td>
<td>100 (5/5)</td>
</tr>
<tr>
<td>20,000‡</td>
<td>57.7 ± 16.2</td>
<td>100 (3/3)§</td>
<td>12</td>
<td>75</td>
<td>100 (1/1)</td>
</tr>
<tr>
<td>10,000‡</td>
<td>40.0 ± 18.1</td>
<td>67 (2/3)</td>
<td>6</td>
<td>75</td>
<td>100 (1/1)</td>
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<td>75</td>
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<td>5,000‡</td>
<td>3</td>
<td>0 (0/1)§</td>
<td>7</td>
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Sca-1+Lin-WGA*‡  
250‡   90.7 ± 1.4  100 (3/3)  5  94.0 ± 0  100 (3/3)  
100‡  38.4 ± 13.3  80 (4/5)  11  32.2 ± 15.2  80 (4/5)  
50‡  69.3 ± 12.4  100 (3/3)  5  71.5 ± 16.7  100 (3/3)  
30‡  35.2 ± 10.9  100 (5/5)  5  34.4 ± 12.2  100 (5/5)  
10‡  22.9 ± 7.8  44 (4/9)  27  13.0 ± 3.8  22 (2/9)  
5‡  0  0 (0/1)§  7  75  100 (1/1)  

* Percent donor-derived nucleated peripheral blood (PB) cells is given as the mean ± SEM. Data are pooled from four experiments.  
† An animal was designated as "positive" when ≥20% donor-derived nucleated peripheral blood cells were present at the indicated times after transplantation.  
2 × 10⁶ (‡) "compromised" helper cells or 4 × 10⁶ (‖) Sca-1- cells were coinjected. Both populations were of the same Ly-6 genotype as the recipient.  
§ After first sampling these animals were killed and used in other experiments.

able to give reconstitution of an irradiated animal more than 6 months after transplantation. When 30 Sca-1+Lin-WGA+ cells were transplanted, all animals showed ≥20% donor-derived peripheral blood cells at both 6 to 7 weeks and after 15 weeks. Most mice showing ≥20% reconstitution with donor-derived cells at 6 to 7 weeks after transplantation showed both myeloid and lymphoid repopulation (by light scatter analysis) that was maintained for a further 2 months using either total marrow or Sca-1+Lin-WGA+ cells for transplantation. The only exceptions to this were two animals injected with 10 Sca-1+Lin-WGA+ cells. One of these showed ≥20% donor-derived peripheral blood cells that appeared restricted to the lymphoid lineage and the other showed initially multilineage repopulation of ≥20%, but on second sampling this became restricted to the lymphoid lineage. In several mice showing less than 20% donor-derived peripheral blood cells 6 to 7 weeks after transplantation, the repopulation by donor cells became restricted to the lymphoid system, as determined when the animals were reanalyzed at a later time point. Donor-derived myeloid repopulation alone was not seen in any recipient. A decrease in the percentage of donor-derived peripheral blood cells with time was most noticeable in animals that showed ≤20% donor-derived nucleated peripheral blood cells on first analysis (data not shown). Application of Poisson statistics to an analysis of the number of negative recipients as a function of the number of test cells injected gave a frequency of competitive long-term repopulating units (CRU) of 1 in 13,000 fresh-labeled C57B1/6 Pep3b marrow cells (±SEM = 1/7,400 to 1/23,000), which is similar to the value we previously reported for B6C3F₁ mice using Y-probe analysis of marrow and thymus of reconstituted recipients. Analysis of the data for Sca-1+Lin-WGA+ cells shows a CRU content of 1 in 36 cells of this phenotype (±SEM = 1/23 to 1/57) which represents a fivefold improvement over our previous results.²²

Characterization of Sca-1+Lin-WGA+ Cells Cultured In Vitro

Production of cells and phenotypic analysis. Upon culture of Sca-1+Lin-WGA+ BM cells in serum-free medium supplemented with SF, IL-3, IL-6, and Ep, the total number of cells as well as the number of Sca-1+Lin-WGA+ cells increased significantly (Fig 2). After 7 days of culture, the total number of cells had increased approximately 1,000-fold. At this time the cells were harvested, counted, and reanalyzed by FACS. Cells with the original Sca-1+Lin-WGA+ phenotype were sorted and used to initiate secondary cultures. This was repeated at weekly intervals. After subculturing Sca-1+Lin-WGA+ cells in this way for 4 weeks, the total number of cells had increased greater than 10⁶-fold and the number of cells with an Sca-1+Lin-WGA+ phenotype had increased greater than 1,000-fold. Omission of IL-3 from the culture (Fig 2B) had little effect on the output of either total cells or Sca-1+Lin-WGA+ cells for the first 3 weeks of culture, but after this time the number of Sca-1+Lin-WGA+...
Production of Sca-1+Lin-WGA+ cells in serum-free culture. The number of total cells (■) and the number of Sca-1+Lin-WGA+ cells (▲) present in serum-free cultures containing SF, IL-6, and Ep and with IL-3 (▲, representative of two independent experiments) or without IL-3 (▲, representative of four independent experiments) is shown as a function of time after placing a defined number of highly purified Sca-1+Lin-WGA+ cells into each culture. At weekly intervals Sca-1+Lin-WGA+ cells were resorted from the culture and used to start secondary cultures. The total cell number was calculated from the number of cells produced at each time interval and the dilution factor. To calculate the number of Sca-1+Lin-WGA+ cells present at each time point, the number of total cells was multiplied by the fraction of Sca-1+Lin-WGA+ cells obtained by FACS analysis.

Clonal analysis of the proliferative response of Sca-1+Lin-WGA+ cells. To investigate further the clonal basis of the observed numerical expansion of Sca-1+Lin-WGA+ cells in vitro, freshly isolated single cells with this phenotype were resorted and directly deposited into 96-well plates containing serum-free medium supplemented with SF, IL-6, and Ep. After 13 days, the number of cells in each well was counted. Figure 3 shows the results of two such experiments. Most of the sorted cells (91% and 95%) underwent at least one division and no viable cells were observed in the remaining wells (9% and 5%, respectively). None of the 120 wells examined in each experiment contained only one live cell at day 13, indicating that Sca-1+Lin-WGA+ BM cells either proliferate or die but do not remain quiescent under the culture conditions used. Many individual cells produced up to 100 cells (34% and 52%) and more than 100 cells were observed in 39% and 61% of the wells, respectively. Nineteen percent and 23% of the wells contained more than 10,000 cells. Thus, although greater than 90% of the sorted population showed a proliferative response under the conditions used, most (ie, >80%) of the cell expansion could be attributed to a subpopulation representing approximately one fifth of the original cells.

In vivo reconstituting ability of cultured Sca-1+Lin-WGA+ cells. Because the Sca-1+Lin-WGA+ phenotype of freshly isolated normal marrow cells was clearly associated with in vivo repopulating potential (Table 1), it was of interest to determine whether this function was retained by the Sca-1+Lin-WGA+ cells generated in culture. The results of two experiments are presented in Table 2. In the first, the culture was initiated with 1,000 purified cells and by day 14 the total number of cells had increased to a total of 32 × 10^6 cells. Irradiated recipients were then transplanted with 0.1%, 1%, or 10% of the cells in this day 14 culture containing 90, 920, or 9,200 cultured Sca-1+Lin-WGA+ cells or 1, 10, or 100 input cells, respectively. The percentages of regenerated nucleated peripheral blood cells derived from these cultured cells 7 weeks and 15 weeks after transplantation are presented in Table 2. All animals transplanted with 10% of the culture were reconstituted to a level of ≥20% and 20% of these transplanted with only 1% of the culture were similarly reconstituted. Derivation of the long-term CRU content of the cultured cells expressed per initial cell cultured gave a value of 1 in 45 cells (±SEM = 1/16 to 1/122), ie, 1.3-fold lower than the input value. In a second experiment, the results were similar, although fewer transplant recipients were available for reconstitution analyses. These results show that, although the subpopulation of Sca-1+Lin-WGA+ cells had expanded approximately 100-fold during the 2 weeks in culture (Table 2), long-term repopu-
IN VITRO AMPLIFICATION OF Sca-1<sup>+</sup>Lin<sup>-</sup>WGA<sup>+</sup> CELLS

Exp. 1

Fig 3. Analysis of proliferative response by individual Sca-1<sup>+</sup>Lin<sup>-</sup>WGA<sup>+</sup> cells cultured for 13 days in serum-free medium supplemented with SF, IL-6, and Ep. The results presented are for two independent experiments and represent 120 cells each.

Dissociating ability was not increased, although there appeared to have been relatively little loss in the total number of cells with this potential relative to the input population.

DISCUSSION

In this report we have described the in vitro proliferative response of a highly purified subpopulation of normal murine marrow cells and the effect of this culture procedure on the maintenance of long-term repopulating ability for which these cells were selected. The method used to purify cells with this functional property was a combination of two previously published strategies, involving FACS sorting of freshly suspended cells on the basis of low forward and orthogonal light scatter, expression of Sca-1, lack of expression of a combination of markers of various lineages of hematopoietic cells, and stimulation with SF, IL-6, and Ep. The results presented are for two independent experiments and represent 120 cells each.

Table 2. Long-Term Repopulating Ability of Cultured Sca-1<sup>+</sup>Lin<sup>-</sup>WGA<sup>+</sup> Cells

| Exp. No. | No. of Cultured Cells Injected<sup>*</sup> | Equivalent of Sca-1<sup>+</sup>Lin<sup>-</sup>WGA<sup>+</sup> Cells at Day 14 | Total Output of Cells at Day 14 | Total No. of Cells Injected<sup>*</sup> at Day 14 | % Donor PB Cells<sup>§</sup> | % Positive Animals<sup>||</sup> | Total No. of Animals Transplanted | % Donor PB Cells<sup>§</sup> | % Positive Animals<sup>||</sup>
<table>
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<tbody>
<tr>
<td>Exp. 1</td>
<td>1,000</td>
<td>32,000,000</td>
<td>3,200,000</td>
<td>9,216 100</td>
<td>60.8 ± 22.4</td>
<td>100 (6/6)</td>
<td>6</td>
<td>56.0 ± 24.0</td>
<td>100 (6/6)</td>
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<td></td>
<td>At Day 13</td>
<td>320,000</td>
<td>922 10</td>
<td>9.4 ± 16.6</td>
<td>20 (1/5)</td>
<td>5</td>
<td>7.6 ± 13.2</td>
<td>20 (1/5)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>At Day 14</td>
<td>32,000</td>
<td>92 1</td>
<td>0.3 ± 0.5</td>
<td>0 (0/4)</td>
<td>4</td>
<td>0.3 ± 0.5</td>
<td>0 (0/4)</td>
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<tr>
<td>Exp. 2</td>
<td>500</td>
<td>4,000,000</td>
<td>250,000</td>
<td>4,924 38</td>
<td>66.0 ± 17.3</td>
<td>100 (3/3)</td>
<td>3</td>
<td>88</td>
<td>100 (1/1)</td>
</tr>
<tr>
<td></td>
<td>At Day 13</td>
<td>50,000</td>
<td>985 7.6</td>
<td>—</td>
<td>—</td>
<td>3</td>
<td>—</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td></td>
<td>At Day 13</td>
<td>10,000</td>
<td>197 1.5</td>
<td>—</td>
<td>—</td>
<td>4</td>
<td>—</td>
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<sup>*</sup> Irradiated recipients were transplanted with parts of a 14-day-old culture (Exp. 1) or parts of a 13-day-old culture (Exp. 2). Presented are the total number of cells injected (*) and the number of Sca-1<sup>+</sup>Lin<sup>-</sup>WGA<sup>+</sup> cells, present in the transplant (†). The corresponding number of original 'day 0' Sca-1<sup>+</sup>Lin<sup>-</sup>WGA<sup>+</sup> cells that had initiated this part of the culture is presented as well (‡).

<sup>§</sup> Percent donor-derived nucleated peripheral blood (PB) cells is given as the mean ± SD.

<sup>||</sup> An animal was designated as 'positive' when >20% donor-derived nucleated peripheral blood cells were present at the indicated time after transplantation.

<sup>†</sup> 2 x 10<sup>6</sup> "compromised" helper cells were cojected.

<sup>#</sup> These animals did not survive.
poietic cells, and WGA binding. In transplantation experiments, 22% of the animals transplanted with only 10 Sca-1+Lin-WGA+ cells and 100% and 80% of the animals transplanted with 30 and 100 Sca-1+Lin-WGA+ cells, respectively, showed donor-derived hematopoietic reconstitution 3 months after transplantation (Table 1). By limiting dilution analysis this gave a CRU content in the sorted population of 1 in 36 cells that may be as high as 5% if it is assumed that 50% of the CRU are lost in vivo because of antibody staining. The corresponding enrichment was calculated to be approximately 400-fold and the recovery 15%. It should be noted that these frequency and recovery values may be underestimated because the repopulation data from one single mouse that did not show any donor-reconstitution after injection with 100 purified cells was not excluded for analysis (Table 1). It is assumed that this was a spurious result, the enrichment factor would increase to 800-fold and the recovery to 30%. Upon culture in serum-free medium supplemented with SF, IL-6, Ep, with or without IL-3 a large increase in total cell number, including cells with a Sca-1+Lin-WGA+ phenotype, was observed. However, this was not accompanied by an increase in cells with long-term repopulating cells, clearly indicating a heterogeneity within the Sca-1+Lin-WGA+ population.

The results obtained with our sorting strategy are similar to those of others in which a low level of Thy-1 expression was used, instead of WGA, in addition to Sca-1 expression and absence of lineage markers to enrich for primitive hematopoietic cells in normal mouse BM. The Sca-1+Lin-Thy-1lo subpopulation has been found to represent approximately 0.05% of the original marrow and an enrichment of 1,000- to 2,000-fold was measured in a variety of assays. However, none of these previous reports included direct comparisons between fresh unsorted marrow cells and sorted Sca-1+Lin-Thy-1lo cells regarding the ability of both cell populations to reconstitute an irradiated animal in both lymphoid and myeloid lineages for prolonged periods of time. The enrichment factor for cells with long-term repopulating ability obtained by selecting for cells with an Sca-1+Lin-Thy-1lo phenotype therefore remains to be established. In our study a clear correlation between the number of purified cells injected and their relative contribution to the regenerated nucleated peripheral blood cells was noticed (Table 1). Furthermore, we observed that in most transplant recipients with less than 20% donor-derived peripheral blood cells such reconstitution was restricted to the lymphoid lineage. Interestingly, such lymphoid cells always included both T and B cells, even if the percentage of donor cell repopulation was very low (results not shown). These results are in agreement with previous findings of Li and Johnson. These investigators suggested to use repopulation by ≥20% test cell-derived nucleated peripheral blood cells as a criterion for detection of at least one transplanted multipotent HSC. We have adopted this criterion in our study.

The high recovery of cells with long-term repopulating ability reported here (approximately 15%) suggests that a large proportion of the cells in normal mouse marrow with this property have an Sca-1+Lin-WGA+ phenotype. Some losses are likely to have occurred during the staining and sorting of cells and/or as a result of the gating criteria chosen to optimize enrichment. Uchida and Weissman have reported that no long-term repopulating cells were found in the Sca-1+ or Lin+ cell fractions. In our hands, transplantation of 8 × 10^5 Sca-1+ cells into irradiated recipients resulted in less than 10% donor-derived peripheral blood cells at 7 or 15 weeks posttransplantation (data not shown). The content of long-term repopulating cells in the Lin+, WGA-intermediate and WGA- subsets is currently under investigation in view of findings by Ploemacher et al suggesting that some primitive hematopoietic cells may bind only low levels of WGA.

The in vitro proliferative response of Sca-1+Lin-WGA+ cells in serum-free medium supplemented with SF, IL-6, and Ep is in agreement with former published data. After 13 days of culture 39% to 61% of wells that had received a single Sca-1+Lin-WGA+ cell contained more than 100 cells. This result is similar to that found with single Sca-1+Lin WGA+ cell contained more than 100 cells. This result is similar to that found with single Sca-1+Lin-Thy-1lo cells cultured in serum-containing medium supplemented with IL-1, IL-6, G-CSF, GM-CSF, and M-CSF, where 1 in every 1.7 cells (59%) gave rise to a colony of greater than 50 cells. One of the most striking results in our study was the demonstration that in this culture system, in addition to an exponential increase in the total cell number, there was a significant amplification of cells with a primitive Sca-1+Lin-WGA+ phenotype (up to 1,000-fold after 4 weeks). This contrasts with the response of a primitive hematopoietic population of CD34+CD45RA-CD71lo human marrow cells under similar culture conditions, where maintenance but not expansion of cells with the initial phenotype was observed. However, when cultured Sca-1+Lin WGA+ cells were assayed for the long-term repopulating ability the results more closely approximated those for purified human cells in that numbers were maintained at close to input numbers, but not expanded. These results clearly indicate that functional heterogeneity exists within populations of cells with an Sca-1+Lin-WGA+ phenotype.

The in vitro production of Sca-1+Lin-WGA+ cells in culture that lack in vivo long-term repopulating ability underscores the importance of in vivo assays to reliably measure functional properties, particularly after in vitro manipulations, as well as the need for more specific markers for cells with long-term in vivo repopulating potential. It has been reported that Sca-1+Lin-Thy-1lo cells can be subdivided by Rhodamine 123 into a quiescent population that shows long-term repopulation ability (Rh-dull) and a more activated population without this ability (Rh-bright). Interestingly, both Rh-dull and Rh-bright populations show the same proliferative response in culture; however, it is possible that the cytokine combinations used in these in vitro studies are able to rapidly stimulate primitive hematopoietic cells independent of their initial cell cycle status as pre-
viously suggested by Ogawa et al. In addition to a search for markers to obtain more specific purification of long-term repopulating cells, it will be important to evaluate other cytokines for their ability to sustain self-renewal rather than commitment and differentiation events. In this respect it is of interest that expansion of Sca-1+Lin' WGA' cells did not seem to be affected by the presence or absence of IL-3 (Fig 2).

A better understanding of the heterogeneity within the stem cell pool and the mechanisms of self-renewal versus commitment within this population have been long-standing goals in experimental hematology. Curative regimens requiring marrow rescue could be dramatically improved if numerical expansion of hematopoietic stem cells in vitro were possible, or if large numbers of committed cells could be generated without exhausting the most primitive cell types required for long-term reconstitution. The results of this study indicate that the latter type of expansion can already be achieved and underscore the importance of studies using highly purified HSC's subjected to defined culture conditions for the elucidation of HSC biology.

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Amplification of Sca-1+ Lin-WGA+ cells in serum-free cultures containing steel factor, interleukin-6, and erythropoietin with maintenance of cells with long-term in vivo reconstituting potential

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