Competitive Repopulation: A New Assay for Long-Term Stem Cell Functional Capacity

By David E. Harrison

A new assay for the long-term functional capacity of hemopoietic stem cells is reported. Stem cell function in each donor of a particular genotype is assayed by mixing its marrow cells with a constant number of marrow cells from a donor with distinguishable hemoglobin and measuring the relative ability of each donor to populate stem-cell-depleted recipients. For example, cells from many different B6 donors may be assayed by mixing them with a constant number of WBB6F, cells from a single pool and injecting them into irradiated WBB6F recipients. As the ratio of B6 to WBB6F, marrow cells increases from 0:1 to 3:1, the percentage of B6 hemoglobin increases in a linear fashion. This is also found with W8 and WBB6F, or CBA and B6CBAF, mixtures. Correlation coefficients between the percentage of hemoglobin of one donor type and the ratio of cells in the mixture of that type ranged from 0.78 to 0.98 in single experiments, and were 0.68 and 0.75 using data pooled from several experiments.

For stem cell lines to function during a long time in vivo, such as in marrow transplantations, the most important precursor cells are those that are the least differentiated, having maximum proliferative and repopulating ability. The earliest recognized hemopoietic precursor cell detectable using currently available assays is the cell capable of forming a macroscopic spleen colony in a lethally irradiated recipient, the CFU-S. 1 A CFU-S is a single cell that has the potential to proliferate and, in 8–10 days, form a 103–107 cell colony that is producing one or more of the following cell types: erythrocytes, granulocytes, and megakaryocytes. 1, 3 Thus, every cell capable of the brief period of rapid proliferation required to form a macroscopic spleen nodule is counted as a CFU-S. To determine whether the cells in such a colony retain significant amounts of proliferative capacity, they must be dissected and retransplanted into successive irradiated recipients. When this is done, most colonies contain few if any CFU-S, but a small number of colonies contain many such cells. 4 Thus, the proliferative capacity of cells designated as CFU-S varies widely.

Moreover, measurements of CFU-S numbers do not always correlate with long-term functional capacities. This is not surprising, because the CFU-S assay only measures growth for 8–10 days, and it measures only cells growing in the spleen, not the entire hemopoietic system. When life-sparing ability in lethally irradiated recipients is used to measure stem cell function, measurements of CFU-S numbers sometimes fail to predict the outcome, as illustrated by the following three examples. When CFU-S numbers are reduced by hybrid resistance, they grossly underestimate life-sparing ability. 4 W/W genotypically anemic mice have no CFU-S (<0.01% of normal) but have 13% of normal life-sparing ability. 4 Compared to marrow, spleen cells have about 30% the life-sparing ability expected from CFU-S numbers. 7

CFU-S assays also fail to predict relative repopulating abilities of fetal liver cells, normal marrow cells, circulating nucleated cells, and serially transplanted marrow cells. When mixtures of marrow and fetal liver cells, distinguished by chromosome markers, were transplanted into irradiated recipients, most multiplying cells in the recipient’s marrow eventually became the type of the fetal liver donor, although the fetal liver component of the mixture contained 5–10 times fewer CFU-S than the marrow component. 8 When mixtures of marrow cells and circulating nucleated cells from donors with different chromosome markers were transplanted into irradiated recipients, almost all mitoses in recipient marrow became the type of the marrow donor, although the marrow and circulating cells contained similar numbers of CFU-S. 9 A single serial transplantation only reduced CFU-S numbers 0%–20%, but reduced the ability to repopulate irradiated recipients much more severely—50%–90%. 10

These deficiencies in the CFU-S assay demonstrate that there is a need for new types of assays to measure the functional capacity of the earliest precursor cell lines whose progeny populate the hemopoietic system. This article describes such an assay, a procedure that can be used to compare repopulating and proliferative abilities of stem cell lines for as long as several years, and yet is capable of detecting subtle defects in a quantitative manner. Marrow cells from two types of donors with distinguishable hemoglobin are mixed, and the mixtures are transplanted to repopulate lethally irradiated recipients. Thus, both types of stem

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cells in the mixture receive identical stimuli. The transplanted marrow cells compete with each other in repopulating the recipients and in producing erythrocytes. The results of this competition are given by the percentage of erythrocytes in the recipient descended from stem cells of each donor type. These are measured by the percentage of hemoglobin of each donor type.

**MATERIALS AND METHODS**

The electrophoretic mobilities of hemoglobins from C57BL/6J (B6) and WB/Re (WB) mice differ as a result of differences at the hemoglobin beta chain locus (Hbb); these strains have Hbb'/Hbb' and Hbb'/Hbb' hemoglobin, respectively.¹¹ Constant numbers of cells from a single pool of WBB6F₁-(Hbb'/Hbb') mouse marrow were used as the source of competitor cells to measure the relative repopulating abilities of different numbers of B6 and WB marrow cells. Lethally irradiated normal WBB6F₁, mice were used as recipients. CBA/CaJ (CBA) mice have the same hemoglobin type as WB; therefore, they and their B6CBAF₁ hybrids were analogous to WB and WBB6F₁, mice.

All donors and recipients were 10–22 wk old when used; they were produced and maintained at the Jackson Laboratory, which is fully accredited by the American Association for the Accreditation of Laboratory Animal Care.

Suspensions of marrow cells were prepared and injected intravenously into all recipients as previously described;¹⁰ measured numbers of cells were mixed and stored on ice before being injected. Whole-body irradiation was delivered to hematologically normal WBB6F₁, +/+ mice at a dose rate of 60–70 R/min 16–18 hr before marrow injections.

To determine percentages of each hemoglobin type, blood was collected from the retroorbital sinus of each recipient and was prepared for electrophoresis using cystamine.¹¹ Hemoglobin concentrations in the electrophoretically separated bands were measured with a Helena Auto Scan densitometer. Figure 1 gives examples of the densitometer readings. The integrated peak areas representing the relative amounts of Hbb' and Hbb' hemoglobin types were totaled, and the results summarized as the percentage of Hbb' type, abbreviated S.

Means, SE ([μ – 1]/√n], linear regressions (L.R) and correlation coefficients (CC) were performed using the programs in the Texas Instruments TI Business Analyst-II calculator. Results are expressed as mean ± SE (numbers of analyses).

**RESULTS**

Mixtures of known amounts of B6 + WBB6F₁ or WB + WBB6F₁, hemoglobin were used to produce the standard curves shown in Fig. 2. The amounts of hemoglobin produced by B6 cells in competition with WBB6F₁, cells were determined from Fig. 2A; the amounts of hemoglobin produced by WB cells in competition with WBB6F₁, or by CBA cells in competition with B6CBAF₁, cells, were determined from Fig. 2B.

Hemoglobin samples from lethally irradiated mice that had received mixtures of marrow cells with various ratios of these cell types were analyzed as shown in Figs. 1 and 2. In each experiment, a constant number of marrow cells from the same pool of F₁ hybrid donors was mixed with marrow cells from pooled donors of a parental genotype. With increasing proportions of parental marrow in the mixtures, the percentages of parental hemoglobin increased (Figs. 3 and 4). When mixtures of B6 + WBB6F₁ marrow cells were injected into lethally irradiated normal WBB6F₁, recipients, percentages of B6 hemoglobin showed a linear increase from 12–25 to 75–90 as the ratio of B6 to WBB6F₁ cells in the mixture increased from 1:1 to 3:1; at 6.25:1, all the hemoglobin was B6 type and this datum was not included in Fig. 3. The percentages of B6 hemoglobin produced by a B6 to WBB6F₁ cell ratio of 2.5:1 varied from 15% ± (5) to 86% ± (4) in different experiments, presumably because of differences in the repopulating abilities of the different marrow donors used. Such differences caused the coefficient for the correlation between percentages of B6 hemoglobin and ratios of B6 to WBB6F₁, marrow cells to be only 0.75 for data pooled from 7 experiments, while correlation coefficients were 0.88 and 0.92 for 2 individual experiments (Fig. 3).

WB cells did not compete with WBB6F₁ as well as B6 cells did. Percentages of WB hemoglobin in mixtures of WB + WBB6F₁, in irradiated recipients showed a linear increase from 4–8 to 25–35 as the

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**Fig. 1.** Densitometer readings from typical mixtures of 0%, 25%, 50%, 75%, and 100% B6 hemoglobin with the balance WBB6F₁, hemoglobin are shown in patterns 1 through 5, respectively. The numbers on the left and right under each pattern are the relative amounts of hemoglobin in the 2 Hbb' (D) bands and in the Hbb' (S) band, respectively, read from the integrator on the Auto Scan recorder.
The correlations between the percentage of WB hemoglobin produced and the WB to WBB6F1 cell ratios were more constant within single experiments, with coefficients of 0.78 and 0.81, than in 4 pooled experiments, with a coefficient of 0.68 (Fig. 3). In a single experiment with CBA and B6CBAF marrow cell mixtures, CBA marrow cells competed very well.

In two other experiments (A, C) only a single ratio was tested for both WB and B6 mixtures with the same WBB6F1 pool, and in three experiments (B, E, V) only B6 was tested. Zero values were analyzed using hemoglobin from a group of 19 WBB6F1 controls.

It was a long-term effect over the entire hemopoietic system and appeared to be partly radiosensitive.  

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The proportions of hemoglobin types produced were affected by not only the ratios but also the numbers of marrow cells used. Percentages of B6 hemoglobin were 45% ± 15(3), 60% ± 23(3), and 101% ± 1(4) after 100 days in lethally irradiated WBB6F1 recipients of mixtures with a constant 2.5 B6 to 1 WBB6F1 cell ratio, but with numbers of B6 marrow cells 2.5 × 10^6, 2.5 × 10^7, and 2.5 × 10^8, respectively; percentages increased significantly between 100 and 194 days in the recipients of the lowest number of cells, but not in the other groups. This effect suggests that the numbers of marrow cells in the mixtures given to each recipient used in competitive repopulation assays should be 10^6 or more and should not be allowed to vary widely, although the ratios of different cell types are of primary importance. In the data summarized by Figs. 3 and 4, 1.0–3.0 × 10^6 WBB6F1, or B6CBAF, marrow cells were used with appropriate numbers of parental marrow cells to give the ratios listed.

**DISCUSSION**

This study demonstrates that the long-term functional ability of hemopoietic stem cells from a number of different donors of the same genotype could be assayed by competitive repopulation as follows. Stem cells from each donor are mixed with marrow cells from the same pool of stem cells with distinguishable hemoglobin in appropriate amounts, and the mixtures are injected into lethally irradiated F1 recipients. After 60 days or more, the hemoglobin of these recipients is analyzed to determine how much erythrocyte production resulted from cells of each genotype, using procedures analogous to those shown in Figs. 1 and 2. The higher the percentage of a donor’s hemoglobin, the greater is the repopulating and proliferative abilities of the stem cells from the particular donor tested. This was shown by the demonstration with a single pool of parental cells that the higher the ratio of parental cells injected, the higher was the amount of parental type hemoglobin (Figs. 3 and 4). A number of F1 hybrid donors could be compared against a single pool of parental cells using the same procedures.

In earlier studies of stem cell competitive abilities, cells of two types were mixed, injected into irradiated recipients, and their relative repopulating abilities determined by the percentage of mitoses containing the chromosome marker used to distinguish them. However, this technique is limited to measuring the functional abilities of only the two individuals or pools tested relative to each other. To compare the functional abilities of many different stem cell donors, they all must have a distinguishable cell marker, say “A.” Constant numbers of stem cells from each donor with marker A are mixed with a constant number of stem
LONG-TERM ASSAY OF STEM CELL FUNCTION

81

cells from the same pool of a distinguishable donor type, say “B,” and transplanted into a series of identical recipients. The relative repopulating and proliferative abilities from the many different A-type donors are measured by how completely they repopulate recipients in competition with the same B-type cells.10

Markers used for these assays include the T6 chromosome8 and electrophoretically distinguishable hemoglobins. The use of chromosome markers is time-consuming but such markers have the advantage of identifying cells in metaphase. Thus, they can be used to identify differentiated cells that enter mitosis as a result of a specific stimulus, such as a particular mitogen, in vitro.10 Competitive repopulation assays using hemoglobin markers are described here for the first time, although hemoglobin markers have long been used to identify donor marrow.16,17 In this assay, such markers measure the repopulating abilities of erythrocyte precursors quantitatively. The assays are easy, and the cells may be repeatedly tested in the same recipients, since only a small blood sample is required.

Competitive repopulation assays should be useful for comparing long-term stem cell functions in animals of any genotype for which a competitor with distinguishable cells is available. Besides chromosome and electrophoretic hemoglobin markers, many other genetic markers are available.17 It is essential that, whatever combination of donor and recipient genotypes is chosen, dose–response curves, such as those in Figs. 3 and 4, be determined at the onset to define results using the particular combination because F1 hybrid resistance or other interactions may alter the results obtained.

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Competitive repopulation: a new assay for long-term stem cell functional capacity

DE Harrison