

ULTIMATE ERYTHROPOIETIC REPOPULATING  
ABILITIES OF FETAL, YOUNG ADULT, AND OLD  
ADULT CELLS COMPARED USING  
REPEATED IRRADIATION

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The proliferative capacity of somatic cells has been a fundamental issue in biology. About 70 years ago, studies by Carrel (1) and Ebeling (2) convinced most biologists that normal somatic cells could have unlimited proliferative abilities. However, studies of fibroblast cultures over the past 20 years have reversed that opinion (3–6). The modern work has been characterized by two general findings: The cells studied fail to proliferate indefinitely in vitro, and the proliferative capacity declines as the age of the cell donor increases. By far the largest change with age is the reduction in proliferative capacity when cells from fetal tissues are compared with cells from adults (3–6).

Even if normal somatic cells are unable to proliferate indefinitely in vitro, it is critical to determine how they behave in vivo. The earliest precursor cells or stem cells that populate the hemopoietic and lymphoid systems (7–10) must proliferate extensively to perform their normal functions. Their proliferative capacity may be unlimited because their long-term functional abilities may not decline with age (11–19). However, declines may occur with age, especially in lymphoid precursor cells (20–26). An explanation for this disagreement may be that the ratios of precursor cell types change with age (18, 27–29). Reports that fetal stem cells have higher long-term repopulating abilities than stem cells from adults (30–32) are important evidence that their proliferative capacities are limited. However, our initial results suggested that marrow cells from old and young adults have long-term repopulating abilities similar to those of fetal liver cells (18).

In the present report, we measured repopulating abilities of erythropoietic stem cells by competitive repopulation. Equal numbers of cells from fetal, young, or old donors were mixed with a standard dose of pooled marrow with genetically distinguishable hemoglobin. This standard dose is called the competitor marrow. The cell mixtures were injected intravenously into stem cell-deficient hosts. The percentage of hemoglobin produced by each donor indicated its functional ability (17, 18, 33).

Competitive repopulation has proved to be sensitive in detecting small changes

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in long-term repopulating abilities (13, 15, 17, 18, 34–36). In the present study, we also gave the recipients repeated doses of sublethal irradiation. This should greatly increase the sensitivity, because each irradiation greatly reduces the long-term repopulating activity (17, 18). A high level of proliferation is thus required from the surviving stem cells, and this should bring out any subtle defects intrinsic to them. After two or three serial irradiations and 300–400 d, equal numbers of cells from old adult marrows repopulated at least as well as cells from young marrows or even those from 15- or 16-d fetal livers. The least complex explanations for these results are that the earliest erythropoietic precursors (*a*) do not have intrinsic limitations on their proliferative capacities, and (*b*) are present at no greater concentrations in fetal liver than in adult marrow.

### Materials and Methods

*Mice.* C57BL/6J (B6) mice and their F<sub>1</sub> hybrid with WB/ReJ (WBB6F<sub>1</sub>) were bred and maintained at The Jackson Laboratory, which is fully accredited by the American Association for Accreditation of Laboratory Animal Care. All mice used, including aged animals, appeared healthy; they had no signs of leukemia-like cancers on gross autopsy (enlarged spleen, lymph nodes, or thymus) and white cell numbers were normal. The aging colony has been fully described (37). WB-W/+ × B6-W<sup>v</sup>/+ crosses were made to produce W/W<sup>v</sup>, W/+, W<sup>v</sup>/+, and +/+ offspring. The W/W<sup>v</sup> mice are genetically anemic mutants with defective primitive stem cells so that they are repopulated by donor stem cells without further treatment (38). Heterozygous W/+ or W<sup>v</sup>/+ mice are essentially normal hematologically; before being used as recipients, they or +/+ (normal) mice were lethally irradiated with 1,100–1,200 (usually 1,200) rad in a <sup>137</sup>Cs gamma irradiator (Shepherd Mark I; J. L. Shepherd & Associates, Glendale, CA) at a dose rate of 220 rad/min 15–18 h before the marrow was transplanted. Sublethal irradiation (500 rad) was given using the same machine and dose rate. Some of the recipients were also thymectomized at 4–6 wk of age by sucking the thymus from the thoracic cavity into a glass pipette and rapidly closing the incision. In the six experiments described in Tables I–III, all recipients were males and were 3–6 mo old when the radiation and cell transplantation was performed. All recipients were within 1 mo of the same age in the individual experiments. Stem cell donors were also males (B6 +/+ or WBB6F<sub>1</sub> +/+).

*Stem Cell Transplantation.* Marrow cell suspensions were prepared using femurs and tibias from each donor. The bones were cleaned, the ends opened, and the marrow washed out by vigorous and repeated flushing of balanced salt solution (Hepes buffered to a pH of 7.4 as described in reference 17) through a 23 gauge needle. Entire livers from fetal mice were minced lightly with ground glass tissue homogenizers. Clumps of cells were dissociated by forcing them through a 1 ml plastic syringe pressed against the bottom of a 4 ml culture tube. Stroma was filtered out with 100-mesh nylon cloth (18). Nucleated cells were counted using a Coulter model ZBI Electronic Cell Counter (Coulter Electronics, Inc., Hialeah, FL), after erythrocytes were lysed using standard lysing agents for white cell counting: Zap Isoton or Zap-oglobin (Coulter Electronics, Inc.) or Ultra-lyse (Clay Adams, Parsippany, NJ). Cells were injected intravenously through 26-gauge needles into the lateral tail veins of warmed mice, usually between 9:00 and 11:30 A.M.

*Competitive Repopulation Assay for Erythropoietic Stem Cells.* This assay was used because the traditional macroscopic spleen colony assay is not proportional to long-term repopulation, the actual function of the earliest precursor cells. Spleen colony numbers are much too high for the repopulation ability of transplanted marrow (16, 17) or circulating white cells (34) compared with fresh marrow, while they are much too low in fetal liver (31). Therefore we measured repopulating ability directly.

The technique used is based on the fact that B6 mice have  $\alpha_2\beta_2^s$  (s, single) hemoglobin, which forms the band migrating furthest on the cellulose acetate gels used, whereas WB mice have  $\alpha_2\beta_2^{\text{dmaj}}$  and  $\alpha_2\beta_2^{\text{dmin}}$  (d, diffuse) hemoglobins. These are the intermediate- and slowest-migrating bands, respectively, with 80 and 20% of the d type hemoglobin. The

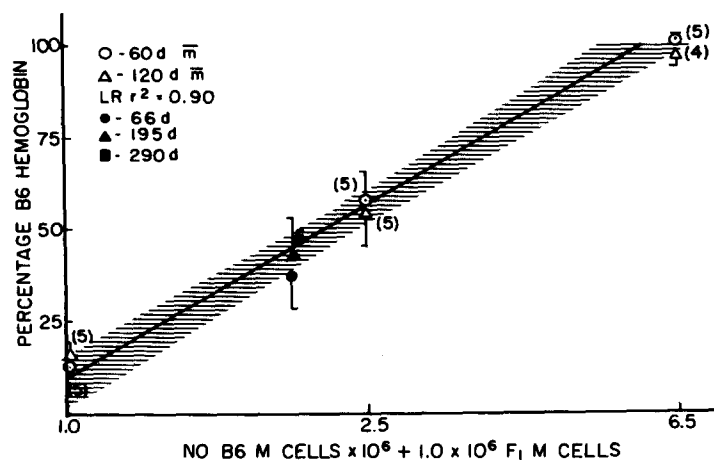


FIGURE 1. 95% confidence limits (shaded area) of the linear regression for the data in a single experiment. The open points are mean values for this experiment at 60 and 120 d, with bars giving the standard error (SE); the number of recipients tested is given beside each point. These data were also used to calculate the correlation coefficient:  $r = 0.951$ , with  $T = 15.96$  and 27 degrees of freedom, so that the correlation is significant ( $P < 0.001$ ). Donors and competitors were 3–5 mo old B6 and WBB6F<sub>1</sub> +/+ females, respectively. The recipients were 4–5-mo-old lethally irradiated WBB6F<sub>1</sub> +/+ females. The shaded points and bars, plotted at a 2:1 ratio of B6 to F<sub>1</sub>, show mean and SE values in a different experiment with three lethally irradiated female WBB6F<sub>1</sub> recipients checked at 66, 195, and 290 d. Each received  $5.4 \times 10^6$  B6 +  $2.7 \times 10^6$  WBB6F<sub>1</sub> +/+ marrow (M) cells.

WBB6F<sub>1</sub> hybrid mice have 50% of each hemoglobin type: three bands with the s comprising 50% and two d bands comprising 40 and 10% of the total hemoglobin. The bands are quantitatively separable by standard electrophoretic methods (39), and the percentages of the B6 and WBB6F<sub>1</sub> types are calculated from the percentage of the s type, as previously illustrated (33). To determine long-term repopulating abilities, B6 and WBB6F<sub>1</sub> cells are mixed and given to WBB6F<sub>1</sub> recipients whose stem cells have been depleted (33). There is little or no hybrid resistance from WBB6F<sub>1</sub> mice against B6 cells (35), and the marrow cell number used,  $1 \times 10^6$  WBB6F<sub>1</sub> +/+ plus  $2.5 \times 10^6$  B6, produce roughly similar amounts of hemoglobin of each type over the lifespan of the recipient (17, 18, 33, 35).

Fig. 1 illustrates the competitive repopulation assay, giving the relationship between the number of donor cells and the percentage of donor hemoglobin in the recipients after 2 and 4 mo. Each dose of the B6 donor cells was mixed with a constant dose of WBB6F<sub>1</sub> competitor cells and injected intravenously into a recipient. The shaded area shows 95% confidence limits for a linear regression. Values at 60 and 120 d are shown as open symbols; they were pooled in the calculated regression because there is no systematic change with time. This is also illustrated by the solid symbols from a separate experiment in which similar results were found at 66, 195, and 290 d (Fig. 1).

The competitive repopulation technique is extremely sensitive at detecting disparities in long-term stem cell functions between a series of donors whose cells are each mixed with a single competitor pool. Differences between experiments (33) may occur for the same reason: the competition between two cell types to repopulate a recipient magnifies small and even random differences in the repopulating capacities of the cell populations used.

The sensitivity of the assay is illustrated by the effects of transplantation (15, 17, 36). For example, recipients of  $3 \times 10^6$  WBB6F<sub>1</sub> cells and  $7.5 \times 10^6$  B6 cells had  $51 \pm 6\%$  F<sub>1</sub> hemoglobin, while previously transplanted F<sub>1</sub> cells totally failed to compete, producing  $0 \pm 3\%$  donor hemoglobin. This showed at least a 20-fold reduction in repopulating ability

as the result of a single transplantation, although  $10^7$  cells had been transplanted and given 4 mo to recover (17). Such a single transplantation causes little or no reduction in macroscopic spleen colony concentrations (15) and only a twofold reduction in their self-renewal (numbers of colonies per colony) (36). The sensitivity of the assay is also shown by the 20-fold reduction in erythropoietic, competitive repopulating abilities 90 d after mice recovered from 550 rad sublethal irradiation (17).

In the experiments described in Tables I–III, suspensions of stem cells were prepared from individual old, young, or fetal donors. A pool of cells was prepared from young mice of the competitor genotype. B6 mice were used as donors with WBB6F<sub>1</sub> +/+ competitors, and vice versa. Cells from each donor were mixed with a constant number of cells from the same competitor pool. These mixtures were injected intravenously into four to six lethally irradiated or *W/W<sup>v</sup>* recipients of the WBB6F<sub>1</sub> genotype. The percentage of donor hemoglobin in each recipient measured the long-term repopulating ability of the donor's erythropoietic stem cells. The recipient hemoglobins are not completely replaced by donor cells for 50–60 d, so levels of circulating hemoglobins were sampled 75–103 d after the initial transplantation. Then the same recipients were irradiated with 500 rad to require additional rounds of competition repopulation. Blood samples were taken 75–178 d after each sublethal irradiation.

*Hemoglobin Synthesis by Reticulocytes.* Peripheral blood was collected from the retroorbital sinuses, 30  $\mu$ l per mouse in heparinized 10- $\mu$ l and 20- $\mu$ l capillary tubes. The 10- $\mu$ l sample was used to determine hemoglobin concentrations and the 20- $\mu$ l sample was added to 100  $\mu$ l of F-12M medium (12-615A; M. A. Bioproducts, Walkersville, MD) containing 2% fetal calf serum and 50  $\mu$ Ci of <sup>35</sup>S-labeled methionine (S.J.-204; Amersham Corp., Arlington Heights, IL) to label freshly synthesized hemoglobin (40). Cultures were incubated for 3 h at 37°C in 5% carbon dioxide, 95% air. The blood cells were washed twice with cold saline by centrifugation and the pellet was resuspended in 20  $\mu$ l of the cystamine lysing agent (39) used to prepare hemoglobins for electrophoresis. Samples were frozen to lyse the cells and the hemoglobin types were separated by electrophoresis on cellulose acetate as previously described (39). Each of the hemoglobin-containing bands was cut from the cleared cellulose acetate gel, solubilized in 1 ml of Soluene-350 (No. 6003038 diluted 9:1 with distilled water; Packard Instrument Co., Downers Grove, IL), and the <sup>35</sup>S activity was counted by scintillation counting with an Omniflor cocktail (NEF-906B; New England Nuclear, Boston, MA). Background was determined by counting a portion of the gel beyond where the hemoglobin migrated, and background counts were subtracted. Data was expressed as the fraction  $d/d + s$ : (counts in the two diffuse bands)/(total counts in the diffuse + single bands). Two allophenic mice were used in these experiments; they had been prepared by Dr. Peter Hoppe using standard procedures (41) to fuse a B6 embryo with a CBA/HT6 embryo. The latter has diffuse hemoglobin, while B6 mice have single hemoglobin.

## Results

*Competitive Repopulation: Fetal Liver, Old Marrow, and Young Marrow.* When we first analyzed the hemoglobins, marrow from old B6 donors competed best, while young B6 marrow or 15-d fetal liver donors gave similar results (Table I, first column). Cells from each had been mixed with equal doses of WBB6F<sub>1</sub> competitor cells. After the first dose of 500 rad, the percentage of donor hemoglobin from the fetal liver cells was reduced twofold and was significantly lower than percentages from old or young marrow cells (Table I, second column). This did not change after the second dose of 500 rad, although the percentage from young adult marrow increased (Table I, third column).

When genotypes were reversed, using fetal, young, and old WBB6F<sub>1</sub> donors and a pool of B6 competitors, fetal cells gave the highest levels of donor hemoglobin initially and remained highest after the first 500 rad of irradiation (Table II, first and second columns); however, these differences were not

TABLE I  
Competitive Repopulating Ability of B6 Donors in Lethally Irradiated WBB6F<sub>1</sub> Recipients

| Donor age, tissue | Percentage donor hemoglobin after: |                       |                       |
|-------------------|------------------------------------|-----------------------|-----------------------|
|                   | 80 d, 101 d                        | 500 rad; 186 d, 207 d | 500 rad; 348 d, 369 d |
| Old marrow        | 84 ± 5 (8)*                        | 63 ± 7 (8)            | 65 ± 12 (7)           |
| Young marrow      | 59 ± 8 (8)                         | 49 ± 10 (7)           | 69 ± 11 (6)           |
| Fetal liver       | 53 ± 3 (7)                         | 21 ± 3 (7)*           | 26 ± 6 (4)*           |

Table I summarizes two experiments with B6 male donors of the following ages: fetal liver, 15 d after vaginal plug observed in mother; young marrow, 3–4 mo; old marrow, 24–30 mo. All data are mean ± SE (*n*). The total cell numbers collected from the livers of each fetal donor averaged  $3.0 \pm 0.4 \times 10^7$  (7); from the femurs and tibias of each young donor,  $7.0 \pm 0.4 \times 10^7$  (8); and from each old donor,  $8.6 \pm 0.4 \times 10^7$  (8). For each donor,  $2.5 \times 10^6$  cells were mixed with  $1.0 \times 10^6$  marrow cells from the same pool of WBB6F<sub>1</sub> +/+ competitors. These mixtures were injected intravenously into lethally irradiated recipients and their hemoglobins were checked after 80 or 101 d. They were given 500 rad 29 or 31 d later and the hemoglobins were checked a second time 75 or 77 d after the irradiation. A second dose of 500 rad was given 271 or 292 d after the beginning of the experiment, and the third hemoglobin analysis was done 76 or 77 d later. The number of recipients per donor at the first two hemoglobin checks averaged 3.4 and 3.3, and was 2.3 by the last one.

\* Values are significantly different from others in the same column ( $P < 0.05$  by the Student-Newman-Keuls multiple range test).

TABLE II  
Competitive Repopulating Ability of WBB6F<sub>1</sub> Donors in Lethally Irradiated WBB6F<sub>1</sub> Recipients

| Donor age, tissue | Percentage donor hemoglobin after: |                       |                       |
|-------------------|------------------------------------|-----------------------|-----------------------|
|                   | 75 d, 103 d                        | 500 rad; 180 d, 208 d | 500 rad; 385 d, 413 d |
| Old marrow        | 33 ± 12 (6)                        | 37 ± 13 (6)           | 33 ± 19 (4)           |
| Young marrow      | 25 ± 7 (6)                         | 37 ± 7 (6)            | 41 ± 17 (5)           |
| Fetal liver       | 50 ± 10 (6)                        | 53 ± 9 (6)            | 35 ± 12 (3)           |

Table II summarizes two experiments with WBB6F<sub>1</sub> +/+ male donors of the following ages: fetal liver, 16 d; young marrow, 7–8 mo; old marrow, 23–28 mo. The total cell numbers [mean ± SE (number of donors)] collected from the liver of each fetal donor averaged  $12.4 \pm 0.8 \times 10^7$  (6); from the femurs and tibias of each young donor,  $11.6 \pm 0.6 \times 10^7$  (6); and from each old donor,  $13.0 \pm 0.6 \times 10^7$  (6). For each donor,  $1 \times 10^6$  cells were mixed with  $2.5 \times 10^6$  marrow cells from the same pool of B6 competitors. The mixtures were injected intravenously in lethally irradiated recipients, whose hemoglobins were checked after 75 or 103 d. They received 500 rad 8 d later, and hemoglobins were checked 97 d after that. The second dose of 500 rad was given in 27 d, and the hemoglobins were last checked 178 d later, 385 or 413 d after the experiment began. Seven recipients were given only the  $2.5 \times 10^6$  B6 competitor cells. They had only B6 hemoglobin throughout the experiment. Other details are the same as in Table I, except that the number of recipients used for each donor averaged 2.2 at the last hemoglobin analysis.

significant by the Student-Newman-Keuls test. After the second irradiation, the percentages of donor hemoglobin from the fetal liver donors dropped to those from the young and old marrow donors (Table II, third column).

The failure to demonstrate differences does not prove similarity. However, the important point in Tables I and II is that stem cells from fetal donors did not develop increasing advantages over stem cells from adult donors during the rigorous treatments of transplantation, two doses of sublethal irradiation, and the long time periods used. This comparison of stem cells over the range from 15 d fetal life to 30 mo of age showed very minor changes compared with those caused by a single transplantation or sublethal irradiation (17).

The differences in the results given in Tables I and II are not explained by the recovery of WBB6F<sub>1</sub> recipient cells in the experiments summarized by Table II. In each of these experiments, control recipients were given only the B6 competitor cells and all had 98–100% B6 hemoglobin at all hemoglobin determinations. Another explanation for these differences is that the adult B6 marrow donors described in Table I had graft vs. host-responsive cells that damaged the repopulating ability of the WBB6F<sub>1</sub> competitor marrow. The experiments in the following section were performed to test this.

*Use of Immunologically Crippled and Unirradiated Recipients.* Two types of recipients were used in this experiment: immunologically crippled, thymectomized, irradiated recipients (Table III, top 3 lines) and immunologically intact W/W<sup>v</sup> recipients (Table III, bottom 3 lines). The W/W<sup>v</sup> recipients provided an unirradiated environment for the stem cells and prevented damaging graft vs. host reactions from B6 against WBB6F<sub>1</sub> antigens (42). In addition to these results, 12-d fetal liver donors were used in one of the experiments in this set. These failed to produce any donor hemoglobin in three immunologically crippled and two W/W<sup>v</sup> recipients that were followed for 307 d. Liver cells from 15–16-d fetal donors gave intermediate hemoglobin percentages, lower than old marrow donors but higher than young marrow donors when hemoglobins were first analyzed (Table III, first column). This pattern was not altered by the first treatment of 500 rad, but, after the second or third treatments, fetal liver cells produced the lowest amounts of donor hemoglobin (Table III, third and fourth columns). Marrow cells from old donors consistently produced the highest levels of donor hemoglobin in these experiments but they were not significantly higher than the levels produced by fetal cells.

These are similar to the initial results with the same genotypes except that young cells gave higher values in Table I. Again, the important point is that there was no loss in the proliferative capacity of erythropoietic stem cells between the 15th d of fetal development and the end of the adult lifespan. One explanation for this is the clonal selection hypothesis. It predicts that most stem cells do not proliferate and that differentiated cells are produced by a small fraction of the stem cells that cycle and use up their proliferative capacity one, or a few, at a time (43–46). The experiment described in the following section was performed to test this.

*Numbers of Stem Cells Producing Erythrocytes.* Tetraparental mice were made by fusing embryos of two different inbred stains at an early preimplantation stage (41). Such mice have cells of both parent strains in their tissues. We studied

TABLE III  
*Competitive Repopulating Ability of B6 Donors in Immunologically Crippled or Intact W/W<sup>v</sup> WBB6F<sub>1</sub> Recipients*

| Donor age, tissue                                 | Percentage donor hemoglobin after: |                          |                          |                          |
|---|------------------------------------|--------------------------|--------------------------|--------------------------|
|   | 83 d, 98 d                         | 500 rad; 193 d,<br>196 d | 500 rad; 298 d,<br>307 d | 500 rad; 465 d,<br>495 d |
| In thymectomized, lethally irradiated recipients: |                                    |                          |                          |                          |
| Old marrow  | 75 ± 8 (6)                         | 70 ± 10 (5)              | 71 ± 13 (5)              | —                        |
| Young marrow                                      | 30 ± 4 (5)*                        | 30 ± 7 (5)*              | 45 ± 9 (5)               | —                        |
| Fetal liver                                       | 55 ± 6 (2)                         | 43 ± 1 (2)               | 32 ± 13 (2)              | —                        |
| In untreated W/W <sup>v</sup> recipients:         |                                    |                          |                          |                          |
| Old marrow  | 52 ± 3 (6)                         | 57 ± 4 (6)               | 66 ± 8 (6)               | 59 ± 12 (5)              |
| Young marrow                                      | 31 ± 5 (5)*                        | 28 ± 5 (5)*              | 42 ± 11 (5)              | 42 ± 7 (5)               |
| Fetal liver                                       | 43 ± 2 (2)                         | 44 ± 9 (2)               | 41 ± 2 (2)               | 24 ± 13 (2)              |

Table III summarizes two experiments with B6 male donors of the following ages: fetal liver, 15–16 d; young marrow, 4–5 mo; and old marrow, 25–26 mo. Total cell numbers collected from the liver of each fetal donor averaged  $8.6 \pm 4.2 \times 10^7$  (2) while the femurs and tibias from each young donor averaged  $6.8 \pm 0.4 \times 10^7$  (5) and those from each old donor averaged  $7.5 \pm 0.4 \times 10^7$  (6). For each donor,  $2.5 \times 10^6$  cells were mixed with  $1.0 \times 10^6$  cells from a pool of WBB6F<sub>1</sub> marrow. These mixtures were injected intravenously into lethally irradiated recipients that had been thymectomized at 4–6 wk of age or into untreated W/W<sup>v</sup> recipients. The same donors and cell mixtures were used in both types of recipients in each experiment. Hemoglobins were checked initially after 83 or 98 d and the first dose of 500 rad was given 7 or 9 d later. Hemoglobins were checked the second time 91 or 101 d after that and the second dose of 500 rad was given 14 or 33 d later. Hemoglobins were checked for the third time after a total of 298 or 307 d; the third dose of 500 rad was given at 381 or 411 d (by this time only W/W<sup>v</sup> recipients survived), and hemoglobins were checked for the fourth time 84 d later. Other details are the same as Table I except that the numbers of recipients at the first hemoglobin check averaged 4.8 and 4.4; at the second hemoglobin check, 4.2 and 3.8; and at the third hemoglobin check, 2.3 and 3.0 for the recipients in the top and bottom three lines, respectively. At the fourth hemoglobin check, numbers of W/W<sup>v</sup> recipients averaged 2.6 per donor.

\* Values are significantly different from only the higher of the other two values in each half-column ( $P < 0.05$  by the Student-Newman-Keuls multiple range test).

hemoglobin production in vitro from blood samples of two tetraparental mice made from the B6 and CBA/HT6 strains. Stem cells in such mice will produce either s or d hemoglobin. <sup>35</sup>S-labeled methionine was incorporated into 20- $\mu$ l blood samples during 3 h in vitro. Only reticulocytes, the erythrocytes most recently released into the blood stream, synthesize hemoglobin, so labeled hemoglobins were made by a cohort of erythrocytes produced during the past 3 d at most.

Fig. 2 shows the fractions of <sup>35</sup>S counts in d type hemoglobins (d/d+ s) from two tetraparental mice measured at 3-d intervals over 30 d. Measurements made on a normal F<sub>1</sub> hybrid mouse and a B6 mouse are also shown as controls. In mean  $\pm$  SE ( $n$ ), the fractions for T1 were  $0.091 \pm 0.011$  (11); for T2,  $0.117 \pm 0.009$  (10); and for the F<sub>1</sub> control,  $0.499 \pm 0.011$  (7). Using the binomial distribution, the number of active stem cells during a single 3-d period can be calculated as:  $N = [p(1-p)]/s^2$ , where  $p$  is the fraction of the d type, and  $s^2$  is the variance of independent samples (45, 47). With  $s = \text{SE} \times \sqrt{n}$ , for T1,  $s = 0.011 \times \sqrt{11}$  and, with  $p = 0.091$ ,  $N = 62$ ; for T2,  $s = 0.009 \times \sqrt{10}$  and, with  $p = 0.117$ ,  $N = 128$ .

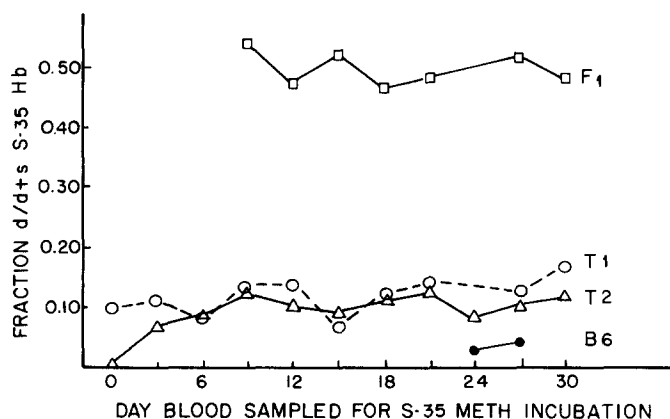


FIGURE 2. The fraction of [ $^{35}\text{S}$ ]methionine-labeled (S-35 meth) diffuse (d), of the total diffuse + single (d + s) hemoglobin bands (Hb) from cellulose acetate gels, from repeated samples at 3-d intervals. The WBB6F<sub>1</sub> control showed a fraction of  $\sim 0.50$  as expected, while the two tetraparental mice (T1 and T2) made by joining early B6 and CBA/H-T6 embryos, averaged  $\sim 0.10$ . Their total hemoglobins measured earlier gave the following d/d + s fractions: (T1) 0.16, 0.14, and 0.07 at 50, 123, and 183 d of age; (T2) 0.21 and 0.10 at 82 and 142 d of age. T1 was 199 d old and T2 156 d old at day 0.

The number of active stem cells is surely higher than 62 or 128, because it declines as  $s$  increases. The calculated values of  $s$  are too large since they include all sources of variability, not only that due to small numbers of active stem cells. The value of the F<sub>1</sub> hybrid ( $s = 0.011 \times \sqrt{7}$ ) is similar to the values in tetraparental mice. Since the F<sub>1</sub> hybrid has only one type of stem cell, this suggests that little or none of the variance results from limited numbers of active stem cells. In two determinations using B6 blood, the fractions were 0.023 and 0.04 instead of 0. This suggests that one source of variability was the labeled material left behind the fastest-migrating  $s$  band, amounting to 2–4% of its activity. Other sources of variability may be minor levels of contamination in the hemoglobin bands with other labeled proteins, irregularities in the gel, and counting errors.

### Discussion

Our results contradict previous reports (30–32) that equal numbers of fetal liver cells have higher repopulating abilities than adult marrow cells. In two of these cases, CBA mouse strains were used. Different strains may give different results, as shown by Tables I and II in this report. Furthermore, in two cases, erythropoietic repopulation was not specifically studied, because chromosome markers were used without identifying the cell types proliferating (30, 31). In the third case, adult marrow cells failed to compete as well as fetal liver cells in the environment of an 11-d fetus (32). This fits with our finding that cells from 12-d fetal livers failed to compete in repopulating adult donors, if the fetal hemopoietic environment at 12 d and earlier differed so much from the adult in marrow environment that stem cells from one grew less well in the other.

Our competitive repopulation assay should rigorously measure the long-term functional capacity of erythropoietic stem cells, because small declines in proliferative ability are magnified when two populations of genetically distinguishable



stem cells compete in repopulating the recipients (Fig. 1 and references 17, 18, 33). However, requiring the cell mixtures to repeatedly recover from sublethal irradiation should greatly enhance the sensitivity to stem cell defects, because irradiation greatly stresses stem cells (17, 18). A single dose of 560 rad reduced repopulating activity 300–500-fold when the erythropoietic competitive repopulating abilities of WBB6F<sub>1</sub> +/+ recipient type cells were measured 125 d after sublethal irradiation and the injection of  $2.5 \times 10^6$  B6 competitor cells. The recipient's own cells in mice given 560 rad were equivalent in repopulating ability to  $0.73\text{--}1.2 \times 10^6$  (95% confidence limits) fresh WBB6F<sub>1</sub> +/+ marrow cells tested in the same experiment. Since adult mice have  $300\text{--}500 \times 10^6$  marrow cells, this represents a 300–500-fold reduction in effective stem cell numbers.

The surviving descendants of the cells in each mixture must compete to again repopulate recipients after each dose of irradiation. Since these stem cells are in the same recipient and are exposed to exactly the same stresses, only their intrinsic differences in repopulating ability and radioresistance will affect the outcome. After 75–103 d in the recipient, the levels of radioresistance probably are similar for early erythropoietic stem cells descended from fetal liver or adult marrow. Survivors should therefore be determined at random, and even small declines of proliferative ability should be greatly magnified as the different cell types compete to repopulate the recipient.

Stem cells from fetal liver appear to populate adult erythropoietic tissues during development, since spleen colony-forming unit (CFU-S) numbers decline as adult tissues are being populated (48) and fetal liver cells have the capacity to repopulate stem cell-depleted adults (18, 30, 31) and vice versa (32). However, fetal cells must proliferate extensively during the rapid erythropoietic growth from the 15- to 16-d fetal mouse to the mature adult. This would deplete stem cell proliferative capacities, if they were limited. They would also be depleted by the proliferation necessary to produce erythrocytes throughout the adult lifespan. Therefore, fetal liver would show consistently higher long-term repopulating abilities than adult marrow if erythropoietic stem cells had limited proliferative capacities, and cells from young adults would repopulate better than those from old adults.

In fact, the results given in Tables I–III do not support these predictions. Although exact levels of repopulating ability were affected by the strains used, the differences between fetal, young, and old cells were small considering the sensitivity of the competitive repopulation assay. The variations were many times less than those caused by a single transplantation or sublethal irradiation (17).

It is possible that fetal liver has a higher repopulating ability than adult marrow, but cannot express this in the alien adult marrow hemopoietic environment of the recipients. This is unlikely because these two effects would have to counteract each other by chance so that the fetal liver and adult marrow compete at roughly similar levels. A less complicated hypothesis is that the primitive stem cells responsible for long-term erythropoietic repopulating ability have similar concentrations in 15- and 16-d fetal livers and in young and old adult marrows. This is consistent with findings that stem cell renewal capacity is unchanged from fetal

life through old age (46), although it contradicts the conclusion from long-term marrow cultures that old marrow has a reduced self-renewal capacity (49).

The results described in Tables I–III suggest that erythropoietic stem cells have unlimited proliferative capacities, since they are not reduced by the proliferation required from 15 d of fetal life through 24–30 mo old adulthood. This is consistent with recent reports that repopulating abilities are not affected by repeated treatments with hydroxyurea (36) and that numbers of cells forming macroscopic spleen colonies recover to normal levels after at least 10 monthly treatments with the alkylating agent, triethylenemelamine (TEM) (50). However, an alternative hypothesis explaining these results is that most stem cells reserve their proliferative capacity, while erythrocytes are produced by descendants of a few stem cells at a time (43–46). Recent data (45) supporting this clonal selection hypothesis suggest that 10 stem cells are active over the 40–50-d erythrocyte lifespan, so that each stem cell produces erythrocytes for 4–5 d. This could explain why stem cells do not exhaust limited proliferative capacities during the adult lifespan, but still predicts an advantage of fetal over adult cells, since the earliest stem cells would be diluted during growth and development.

We tested this hypothesis, incubating tetraparental mouse blood with  $^{35}\text{S}$ -labeled methionine to label hemoglobin being synthesized in reticulocyte-circulating erythrocytes produced during the last 3 d. Fig. 2 shows that the fraction of the d hemoglobin type remained nearly constant, although the tetraparental mice have stem cells producing one type or the other. Using measured variances and the binomial distribution, we calculated that 62 and 128 stem cells were active over each 3-d period, contrary to the predictions of the clonal selection hypothesis (43–46). However, even these numbers are much too low, because the variance was similar in the  $F_1$  hybrid control that has only one type of stem cell.

In this report, erythropoietic stem cells were required to recover from two or three repeated doses of sublethal irradiation over more than a year, thus testing ultimate stem cell functions more rigorously than they had been tested by previous studies (18, 30–32). There was no evidence that repopulating abilities were lower in adult mice; in fact, old B6 marrow tended to repopulate better than equal numbers of B6 fetal liver cells. The least complex hypothesis explaining this is that erythropoietic stem cells have unlimited proliferative capacities and are present in similar concentrations in fetal liver and adult marrow.

### Summary

Erythropoietic repopulating abilities of fetal liver cells and young and old adult marrow cells were compared as follows: Equal numbers of cells from a donor of each age were mixed with a constant portion of cells pooled from genetically distinguishable competitors. These mixtures were transplanted into stem cell-depleted recipients, and the proportions of recipient hemoglobin that were donor type measured the relative effectiveness of early erythropoietic precursor cells from the various donors (Fig. 1). At intervals of 3–6 mo, recipients were sublethally irradiated, requiring a new round of competitive repopulation. When B6 mice were used as donors, with WBB6 $F_1$  competitors and recipients, the highest levels of stem cell activity were found using old donors (Tables I, III).

This was true even with unirradiated, immune-competent  $W/W^u$  recipients (Table III). When donors and recipients were WBB6F<sub>1</sub> hybrids, with B6 competitors, fetal cells initially gave higher levels of repopulating ability, and they were similar to the adult and old marrow cells after 400 d and after recovery from two sublethal irradiations (Table II). These effects were mostly insignificant and probably reflect small differences in initial stem cell concentrations that are brought out by the sensitivity of the competitive repopulation assay. Clearly, ultimate erythropoietic stem cell proliferative capacities did not decline as a result of the proliferation required between 15 d of fetal life and old age. Repopulating abilities of 12-d fetal liver cells were not detectable. We also showed that the proportions of newly synthesized hemoglobins made by the two types of stem cells in tetraparental mice remained nearly constant when tested at 3-d intervals over 30 d. Minimum numbers of stem cells producing erythrocytes over a single 3-d period were calculated as 62 and 128, but these are too low, since variances were similar in the tetraparental mice and in the F<sub>1</sub> hybrid control. This contradicts the hypothesis that erythropoietic stem cells reserve limited proliferative capacities by proliferating one or a few at a time. We suggest that erythropoietic stem cells have essentially unlimited proliferative capacities and are found in approximately equal concentrations in the primary erythropoietic organs after 15 or 16 d of fetal life.

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