

# Loss of Hematopoietic Stem Cell Self-Renewal After Bone Marrow Transplantation

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The quality of long-term hematopoietic engraftment after bone marrow transplantation (BMT) has not been well characterized. Clinical autologous BMT involves removal of <5% of the total content of the recipient marrow followed by ablation of the remaining marrow and reinfusion. To study long-term consequences of transplanting limited numbers of BM stem cells further, we evaluated the hematopoietic reserve in recipient animals after transplantation of varying quantities of BM. Recipient animals demonstrated a donor BM cell dose-dependent decrease in stem cell content and self-renewal capacity that was not

reflected in peripheral blood (PB) counts or BM cellularity. This decrease was observed after initial BM recovery and did not change with time after transplantation, demonstrating a permanent loss in BM self-renewal capacity. In addition, animals alive at 3 months, a time selected to allow BM recovery, also demonstrated a donor BM cell dose-dependent decrease in survival at 1 year. These results emphasize the importance of optimizing stem cell number in BMT.

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**H**EMATOPOIETIC stem cells have been defined operationally by the characteristics of extended self-renewal and the capacity to mature into one or more differentiated forms. We and other investigators have demonstrated by single or serial transplantation studies<sup>1-9</sup> and by study of bone marrow (BM) exposed to radiation or to certain alkylating agents<sup>6,10-12</sup> that stem cells appear to have a limited proliferative capacity and are not able to divide indefinitely. If stem cell proliferative capacity is limited as has been hypothesized, a small number of stem cells transplanted will require greater proliferative activity to repopulate the host than will a large number of transplanted cells. Therefore, varying the number of donor stem cells transplanted should affect the subsequent proliferative capacity of stem cells in the reconstituted host. We now report a decrease in stem cell content and self-renewal capacity in the recipient that is donor BM cell dose dependent. This decrease does not appear to change with time after transplantation, indicating a permanent loss in BM self-renewal capacity.

## MATERIALS AND METHODS

**Recipient preparation.** Three-month-old male C3H/HeJ recipients (Jackson Laboratory, Bar Harbor, ME) were prepared with 1,250 cGy (700 to 550 cGy, three hours apart) total body irradiation (TBI) from a <sup>137</sup>Cs source (Gamma Cell 40, Atomic Energy of Canada, Ottawa) through opposing portals at 111 cGy/min. This dose was selected for the following reasons. It was well above the ~1,000 cGy required to prevent eight-day spleen endocolony formation and to cause BM death in all animals without BM transplantation (BMT) at 14 to 21 days. Host BM repopulation had not been

observed in C3H recipients prepared with 1,250 cGy and transplanted with 10<sup>7</sup> allogeneic cells.<sup>13</sup> The D<sub>10</sub> for CFUs survival in C3H recipients is 185 cGy.<sup>14</sup> Thus, a dose of 1,250 cGy should allow for a fractionated CFUs survival of only 1.8 × 10<sup>-7</sup>, well below the ~3 × 10<sup>4</sup> CFUs of mice. Fresh donor C3H/HeJ BM was transplanted through the tail vein three hours after host preparation. Mice received 1 × 10<sup>7</sup> (4 × 10<sup>8</sup> cells/kg), 1 × 10<sup>6</sup>, 1 × 10<sup>5</sup>, or 1 × 10<sup>4</sup> donor BM cells, and 28-day survival was 96%, 87%, 31%, and 0%, respectively. The mean number of CFUs transplanted per animal was 536, 53, 5.4, and 0.6, respectively.

**Engraftment studies.** Engraftment studies were performed in recipient C57BL/6 mice (Hbb<sup>b</sup>) (Jackson Laboratory) transplanted with cells from single-locus congenic C57BL/6 mice (Hbb<sup>d</sup>). Erythroid engraftment was measured 3 months after BMT by gel electrophoresis<sup>15</sup> on a scanning densitometer (Hoefer Scientific Instruments, San Francisco). Recipient animals (Hbb<sup>b</sup>) were prepared with 1,250 cGy (700 to 550 cGy, three hours apart) and transplanted with 1 × 10<sup>5</sup> BM cells. Three months after BMT, all ten animals studied were engrafted with 100% donor hemoglobin (Hbb<sup>d</sup>).

**Survival analysis.** Survival of animals starting at 3 months after BMT (a time selected to allow BM recovery) was monitored and compared with that of age-matched controls. Animals were housed four to five to a cage. Sterilized bedding, food, and isolator cage tops were used. Animals were handled with aseptic technique, which included use of gowns, gloves, and masks. Manipulations were performed in a sterile hood. The colony was regularly tested for a panel of viruses by serum titer analysis (Charles River Professional Services, Wilmington, MA). Animals repeatedly tested negative for Sendai virus, pneumonia virus of mice, and mouse hepatitis virus. Statistical significance between survival curves was measured by a two-tail log-rank analysis.<sup>16</sup>

**Recipient hematopoietic reserve.** To measure the quality of long-term hematopoietic engraftment, hematocrit (Hct), BM cellularity, [marrow CFUs content, and CFUs] self-renewal capacity were measured at 3, 6, and 9 months after BMT. No differences in Hct levels were noted (data not shown). BM cellularity was measured by flushing the contents of the hind limb from pooled animals killed by cervical dislocation. A minimum of three animals was pooled for each determination. Lethally irradiated recipient animals (1,250 to 700 to 550 cGy, three hours apart) were then injected with an appropriate number of cells and eight days later were killed for spleen colony determination by the method of Till and McCulloch.<sup>17</sup> The ability of BM stem cells to produce additional stem cells (self-renewal potential) was measured by Rs, a measurement of the proliferation of CFUs over a single 14-day interval.<sup>6</sup> It is calculated by the formula  $R_s = S_n/kS_i$ , in which S<sub>i</sub> is the number of eight-day CFUs injected into a lethally irradiated animal at transplantation and k is a correction factor assumed for seeding the hind

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**Table 1. Cells per Hind Limb × 10<sup>6</sup> After BMT**

Time After BMT (mo)	Age-Matched Nontransplant Controls	Cells Transplanted		
		10 <sup>7</sup>	10 <sup>6</sup>	10 <sup>5</sup>
3	23 ± 0.7	22 ± 5.2	21 ± 2	23 ± 2.9
6	33 ± 10	25 ± 7.8	31 ± 8.2	28 ± 8.5
9	24 ± 1.2	26.7 ± 2.1	23.7 ± 3.4	23.7 ± 1.4
Total	26.6 ± 3.2	24.7 ± 2.7	25.0 ± 2.9	24.9 ± 2.6
<i>P</i> (10 <sup>7</sup> v 10 <sup>6</sup> , 10 <sup>6</sup> v 10 <sup>5</sup> )		NS		NS
<i>P</i> (10 <sup>7</sup> v 10 <sup>5</sup> )		NS		
<i>P</i> (control v 10 <sup>7</sup> , 10 <sup>6</sup> , or 10 <sup>5</sup> )		NS	NS	NS

Values are the mean ± SE of three separate experiments for each time point (n = 3). Each experimental point pooled marrow from three to four mice. Each total represents data from nine experiments (n = 9). The degree of statistical significance is listed for the total means for the 10<sup>5</sup>, 10<sup>6</sup>, or 10<sup>7</sup> groups as compared with the age-matched control group (control v 10<sup>7</sup>, 10<sup>6</sup>, or 10<sup>5</sup>) and within each of the transplanted groups (10<sup>7</sup>, v 10<sup>6</sup>, 10<sup>6</sup> v 10<sup>5</sup>, or 10<sup>7</sup> v 10<sup>5</sup>).

limb (k = 0.1). Animals are killed at 14 days, and the number of eight-day CFUs per hind limb is measured (Sn) for determination of Rs. Past experiments have revealed good correlation between Rs determination and serial transplantability, another measure of BM self-renewal capacity.<sup>6,18</sup>

**RESULTS**

BM cellularity per hind limb at 3, 6, and 9 months after BMT is shown in Table 1. Transplanted animals demonstrated BM cellularity similar to that noted in age-matched nontransplanted control animals. No BM cellularity differences were noted as a function of the number of cells transplanted.

The number of CFUs per hind limb at 3, 6, and 9 months after BMT is shown in Table 2. Transplanted animals receiving 1 × 10<sup>6</sup> or 1 × 10<sup>5</sup> cells had a significantly lower BM CFUs content per hind limb as compared with age-matched, nontransplanted control animals. Animals receiving 1 × 10<sup>5</sup> cells also had a significantly decreased CFUs content as compared with animals receiving 1 × 10<sup>6</sup> or 1 × 10<sup>7</sup> cells. There was no significant difference between animals receiving 10<sup>7</sup> cells and age-matched, nontransplanted control animals. The decrease in CFUs content was noted at

3, 6, and 9 months after BMT and did not recover with time.

BM CFUs self-renewal at 3, 6, and 9 months after BMT is shown in Table 3. Transplanted animals had a significantly lower marrow CFUs self-renewal capacity (Rs) as compared with nontransplanted control animals. This decrease was most pronounced in the 10<sup>5</sup> BM group but was also noted in animals after BMT with 10<sup>6</sup> or 10<sup>7</sup> cells. Significant differences were noted between all groups. These differences persisted to 9 months after BMT and demonstrated no evidence for recovery with time.

Survival of animals starting at 3 months after BMT was donor BM cell dose dependent; animals transplanted with 10<sup>5</sup> cells had the lowest survival rate (Fig 1). Survival curves were significantly different from each other and from age-matched control curves. Although these results are consistent with the hypothesis of limited BM reserve, we do not know whether the cause of death has anything to do with exhaustion of the proliferative potential of transplanted hematopoietic stem cells (thrombocytopenia, anemia, infection), other transplanted progenitors (stroma), or mechanisms currently obscure. What is clear is that long-term survival is directly related to the number of BM cells injected.

**DISCUSSION**

The BM stem cell compartment appears to have an organizational structure.<sup>6,10-12,18-24</sup> This organization provides control for replication and differentiation of stem cells and allows protection of the earliest stem cells throughout the animal's life.

There are several models for selection of stem cells for commitment to differentiation. The clonal succession model proposes that individual stem cells are selected for clonal expansion, with resulting production of differentiated cells, and that only a few stem cells are clonally active at a specific time.<sup>6,25,26</sup> This model has been supported by both in vitro<sup>27</sup> and in vivo<sup>19,28</sup> studies that used cell markers to follow specific clonal activity. In contrast, another model studying BM after transplantation supports the mechanism of continued activity of all pluripotent stem cells during the recipient's life span.<sup>29</sup>

A model for organization of the stem cell compartment

**Table 2. CFU Content per Hind Limb**

Time After BMT (mo)	Age-Matched Nontransplant Controls	Cells Transplanted		
		10 <sup>7</sup>	10 <sup>6</sup>	10 <sup>5</sup>
3	2,973 ± 360	2,614 ± 100	2,034 ± 243	1,404 ± 295
6	2,838 ± 629	2,292 ± 212	2,947*	1,900 ± 203
9	3,159 ± 80	2,992 ± 256	2,362 ± 497	1,928 ± 294
Total	2,990 ± 204	2,633 ± 143	2,385 ± 258	1,744 ± 160
<i>P</i> (10 <sup>7</sup> v 10 <sup>6</sup> , 10 <sup>6</sup> v 10 <sup>5</sup> )		NS		<.01
<i>P</i> (10 <sup>7</sup> v 10 <sup>5</sup> )		<.001		
<i>P</i> (control v 10 <sup>7</sup> , 10 <sup>6</sup> , or 10 <sup>5</sup> )		NS	<.05	<.001

\*Only two experiments were performed. Values represent the mean ± SE of three separate experiments for each time point (n = 3). Each experimental point pooled marrow from three to four mice. Each total represents data from eight to nine experiments (n = 8 or 9). The degree of statistical significance is listed for the total means for the 10<sup>5</sup>, 10<sup>6</sup>, or 10<sup>7</sup> groups as compared with the age-matched control group (control v 10<sup>7</sup>, 10<sup>6</sup>, or 10<sup>5</sup>) and within each of the transplanted groups (10<sup>7</sup> v 10<sup>6</sup>, 10<sup>6</sup> v 10<sup>5</sup>, or 10<sup>7</sup> v 10<sup>5</sup>).

Table 3. CFU Self-Renewal Capacity (Rs)

Time After BMT (mo)	Age-Matched Nontransplant Controls	Cells Transplanted		
		10 <sup>7</sup>	10 <sup>6</sup>	10 <sup>5</sup>
3	37 ± 7.8	15 ± 11	7.4 ± 3.4	2.2 ± 0.8
6	29.8 ± 4.9	13.0 ± 3.9	6.6*	1.6 ± 0.8
9	26.7 ± 8.5	21.2 ± 12.1	7.9 ± 2.9	1.3 ± 0.5
Total	34.6 ± 4.6	16.4 ± 5.7	7.4 ± 1.8	1.7 ± 0.4
<i>P</i> (10 <sup>7</sup> v 10 <sup>6</sup> , 10 <sup>6</sup> v 10 <sup>5</sup> )		<.01	<.001	
<i>P</i> (10 <sup>7</sup> v 10 <sup>5</sup> )			<.001	
<i>P</i> (control v 10 <sup>7</sup> , 10 <sup>6</sup> , or 10 <sup>5</sup> )		<.01	<.001	<.001

\*Only two experiments were performed. Values represent the mean ± SE of three separate experiments for each time point (n = 3). Each experimental point pooled marrow from three to four mice. Each total mean represents data from eight to nine experiments (n = 8 or 9). The degree of statistical significance is listed for the overall means for the 10<sup>5</sup>, 10<sup>6</sup>, and 10<sup>7</sup> groups as compared with the age-matched control group (control v 10<sup>7</sup>, 10<sup>6</sup>, or 10<sup>5</sup>) and within each of the transplanted groups (10<sup>7</sup> v 10<sup>6</sup>, 10<sup>6</sup> v 10<sup>5</sup>, or 10<sup>7</sup> v 10<sup>5</sup>). Statistically significant differences between total means were present for all the above comparisons. No statistical difference was seen at 9 months between the age-matched nontransplant control and the 10<sup>7</sup> transplanted group.

which accounts for in vitro and in vivo findings suggests different mechanisms used to meet different circumstances.<sup>18</sup> In circumstances in which a few stem cells are required to repopulate the compartment, all are subject to proliferative stress and cell division, thus decreasing the average self-renewal capacity. This process is observed in vitro during the first 3 weeks of culture,<sup>18</sup> in utero,<sup>30</sup> and after BMT.<sup>2,6-9</sup> Our current study data suggest that the smaller the number of stem cells transplanted the greater the decrease in average self-renewal observed. In contrast, with an intact BM compartment, end-cell proliferation requires only limited primitive CFUs activity, and clonal succession obtains. Stem cells are recruited as needed without continued proliferative activity of the pool, and the self-renewal capacity remains constant. This is observed in vitro during maintenance of BM cultures,<sup>18</sup> during the life span of the mouse,<sup>6</sup> and after BMT once the stem cell pool reexpands.<sup>28</sup> Thus, after initial activity of all stem cells with loss of self-renewal, BM recovers and self-renewal remains stable although at a lower level. This was demonstrated in the current study: After BMT, animals were able to maintain PB counts and BM cellularity, but a donor BM cell dose-related loss in CFUs content and self-renewal capacity was observed. This loss was present by 3 months after BMT, remained stable for the next 6 months, and did not recover with time.

The murine spleen colony assay provides both the ability to measure pluripotent stem cell content and the self-renewal potential of these cells. BM self-renewal capacity has been measured by either excision of individual spleen colonies and determination of daughter CFUs,<sup>2</sup> by serial transplantability,<sup>6</sup> or by Rs,<sup>6</sup> a measurement of CFUs proliferation in 14 days. Spleen colonies have been commonly measured at eight to 12 days after lethal irradiation and tail-vein injection of a measured number of marrow cells. Day-11 to day-12 colonies appear to be more primitive than day-8 colonies, and the ability of individual colonies to produce daughter colonies (self-renewal) increases with colony size and age.<sup>31-33</sup> None-

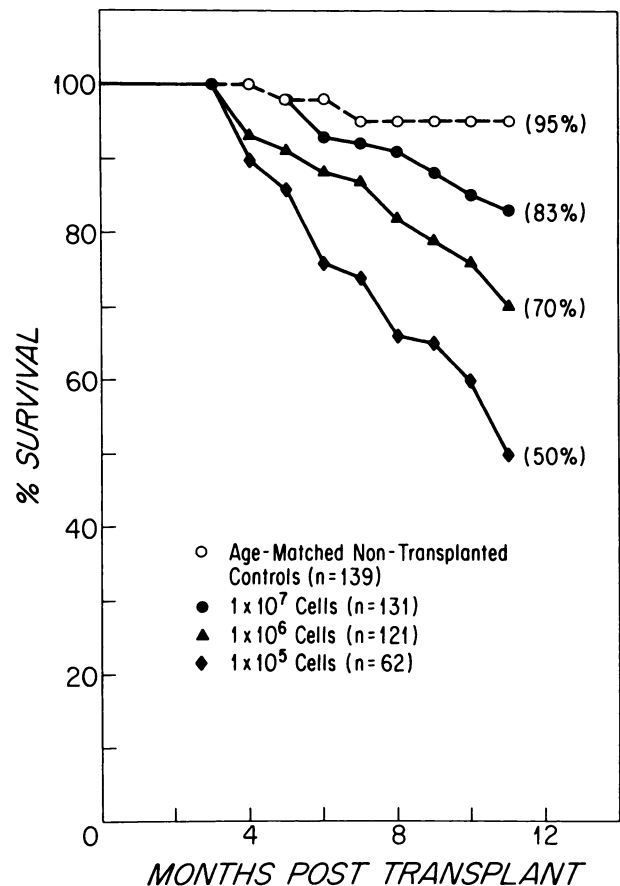


Fig 1. Survival of transplanted animals receiving 10<sup>5</sup>, 10<sup>6</sup>, or 10<sup>7</sup> cells as compared with nontransplanted age-matched control animals. Only animals surviving the transplantation procedure at 3 months after BMT were considered for survival analysis. Statistical significance was as follows: 10<sup>5</sup> v control, *P* < .001; 10<sup>6</sup> v control, *P* < .001; 10<sup>7</sup> v control, *P* < .001; 10<sup>5</sup> v 10<sup>7</sup>, *P* < .001; 10<sup>6</sup> v 10<sup>7</sup>, *P* = .01.

theless, the day-11 to day-12 colony assay does not directly measure self-renewal and, despite the more primitive nature of day-11 to day-12 colonies, the ratio of the number of day-11 to day-12 to day-8 colonies is a poor reflection of BM self-renewal as compared with the excision colony method<sup>34</sup> or with serial transplantability or Rs techniques (P. Mauch, unpublished observations, 1988). In the current experiments, eight-day CFUs were measured. Perhaps an even more profound decrease in stem cell content would have been noted in the 10<sup>5</sup> and 10<sup>6</sup> transplanted groups if day-11 CFUs had been measured. However, the stem cell self-renewal assay (Rs) provided a good correlation between low numbers of stem cells transplanted and decrease in survival and was able to measure directly the decrease (loss) in self-renewal capacity, a measurement not available from 11-day CFUs assay.

Recipient stem cell recovery in animals receiving 1 × 10<sup>5</sup> cells was not observed in this study using a C57BL/6 congenic hemoglobin marker. Previous research by our group has demonstrated that host BM recovery in recipients of congenic BM can be abrogated by increasing the cell dose transplanted.<sup>35</sup> Thus, with the absence of BM recovery with

$1 \times 10^5$  cells transplanted, we would not expect to see such recovery with  $1 \times 10^6$  or  $1 \times 10^7$  cells.

These results have important clinical implications for BMT. Despite seemingly adequate numbers of BM cells and formed blood elements in transplanted recipients, transplantation with smaller numbers of stem cells results in a decrease in BM stem cell content and, perhaps more important, a reduced proliferative capacity of those stem cells. This

reduced capacity may influence host tolerance to cytotoxic agents or other circumstances which require subsequent proliferative demand and may be associated with a decreased long-term survival. Efforts must be made to transplant the largest number of stem cells possible. When feasible, they should be harvested before any large proliferative requirement is made of them.

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