# MAINTENANCE OF THE HEMATOPOIETIC STEM CELL POOL DURING SUCCESSIVE TRANSPLANTATIONS USING A METHOD OF QUANTITATIVE ASSAY

**N. L. Samoilina and G. A. Udalov**

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It is generally considered that hematopoietic stem cells (HSC) can not only differentiate into all types of blood cells, but can also maintain themselves, thus ensuring constancy of their pool throughout life [6]. However, an alternative hypothesis has been suggested which does not require the addition of the concept of self-maintenance in order to explain maintenance of the HSC pool throughout life [5]. Experiments with successive transplantations of bone marrow into lethally irradiated animals have not confirmed the ability of HSC to guarantee unlimited self-maintenance [3, 4, 7-9]. The number of possible transfers (from three to eight) with recovery of hematopoiesis by the initial donor cells depended on the number of transplanted cells and the interval between transfers, but the end result was the same – after a definite number of transfers cells of the original donor origin were not restored. Analysis of these data is difficult because of the absence of quantitative information on the number of HSC transplanted and their restoration in the recipients. In the present investigation we used a quantitative method of assessing totipotent HSC (THSC), ensuring long-term recovery of the hematopoietic and lymphoid systems, and, thus, survival of lethally irradiated mice for at least 4 weeks [2]. To describe these cells we used the functional term "hematopoiesis restoring units" (HRU) [1].

In this investigation we studied maintenance of the HSC pool during successive transplantations, using a method of quantitative assay.

## EXPERIMENTAL METHOD

The bone marrow (BM) donors were $(C57BL/6 \times CBA/HT6)F_1$ hybrid mice. The recipients were $(CBA \times C57BL/6)F_1$ females aged 5-10 months. Whole-body irradiation was carried out on an IPK ($^{137}Cs$) apparatus in two sessions separated by an interval of 3 h. The total dose of irradiation was 14.8 Gy and the dose rate 0.19 Gy/min. The mice were kept under ordinary conditions, and after irradiation they were given acidified water and antibiotics. Three limiting dilutions were prepared from all cell suspensions intended for transplantation, and these were injected into three groups of mice in order to determine the concentration of HRU in the test suspension [2]. After different time intervals, cellular repopulation of the three hematopoietic organs (BM, spleen, and thymus) was tested in the surviving recipients. Chromosomal analysis for identification of the donor's T6 marker was carried out by the routine method.

## EXPERIMENTAL RESULTS

**Restoration of Hematopoietic Tissues after Successive BM Transplantations.** One group of recipients, intended for subsequent transfers, was given a large dose of BM ($20 \cdot 10^6$ cells). Another group received minimal doses of BM, as used in the limiting dilutions method. Despite the 400-fold difference in the doses of BM, no differ-

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TABLE 1. Repopulation of Hematopoietic Organs of Lethally Irradiated Mice during Successive Bone Marrow Transplantations

| No. of transfer | Donor of bone marrow | Number of cells injected, $\times 10^6$ | Number of recipients | Time after injection | Repopulation of recipients' hematopoietic organs | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | cell cont. of organs, $\times 10^6$ | | | % of T6 metaphases | | |
| | | | | | BM | spleen | thymus | BM | spleen | thymus |
| 1 | Normal mice | 20±1,6 | 3 | 35 | 12,4±1,7 | 130±12 | 124±26 | 100 | 100 | 100 |
| 1 | The same | 0,05 | 1 | 36 | 15,7 | 112 | 0 | 100 | 100 | — |
| 1 | The same | 0,05—0,075 | 2 | 59 | 12,5±3,3 | 192±45 | 84±36 | 93±9 | 95±7 | 100 |
| 2 | 35-day 1° chimera (20) 100% T6 | 1,475 | 2 | 147 | 21;8±1,2 | 153±41 | 58±25 | 84±23 | 87±18 | 77±33 |
| 2 | The same | 1,475 | 1 | 351 | 18,4 | 145 | 0,75 | 92 | 97 | — |
| 2 | The same | 2,95 | 3 | 199—246 | 18,9±2,2 | 123±62 | 40±11 | 100 | 100 | 100 |
| 3 | 199-day 2° chimera (20; 3) 100% T6 | 2,0 | 1 | 85 | 19,6 | 120 | 11,2 | 100 | 100 | 100 |
| 3 | 246-day 2° chimera (20, 3) 100% T6 | 4,0 | 1 | 125 | 18,3 | 188 | 25,4 | 9 | — | 46 |
| 3 | The same | 4,0 | 3 | 392—501 | 16,8±3,7 | 81±30 | 3,9±4,4 | 96±4 | 93; 100 | — |
| 3 | The same | 1,0 | 2 | 392—470 | 16,5±1,3 | 110±42 | 4,0±3,0 | 100 | 98±3 | — |
| 3 | The same | 0,25 | 2 | 447—474 | 16,6±1,9 | 96±9 | 0 | 59; 0 | 73; 0 | — |
| 4 | 125-day 3° chimera (20; 3; 4) 9% T6 | 2,0 | 6 | 35—63 | 15,8±3,6 | 69±35 | 24±39 | 45±28 | 44±28 | —; 82 |
| 4 | The same | 0,1125±0,0629 | 4 | 29—44 | 13,7±1,2 | 109±62 | 0 | 10,5± 16,7 | 15,3± 23,2 | — |

**Legend.** Mean values with standard deviations are given (M ± S); dash indicates no data available. Doses of bone marrow $\times 10^6$ in previous transfers shown in parentheses.

TABLE 2. Recovery of HRU in Recipients' Bone Marrow after Transplantation of Normal Bone Marrow

| Day after transplantation | Number of cells injected, $\times 10^6$ | Number of HRU injected (M ± m) | Number of HRU in BM of recipient | | |
|---|---|---|---|---|---|
| | | | in $10^6$ cells (M ± m) | in all mouse BM* (M ± m) | % of number injected |
| 2 | 20 | 876±228 | 0,4 | 4,67 | 0,53 |
| 13 | 10 | 192±81 | 2,83±0,54 | 825±157 | 430 |
| 35 | 20 | 876±228 | 15,4±3,8 | 3183±785 | 363 |
| 35 | 10 | 192±81 | 6,12±0,74 | 1979±238 | 1030 |

**Legend.** Asterisk indicates that for the calculation it was taken that BM of one femur corresponds to 6% of total BM of a mouse.

ence in recovery of the cell population of BM and the spleen was observed in the two groups of primary (1°) recipients, tested after 5-8 weeks. The only difference was absence of recovery of the thymus in one of the three recipients of a small dose of BM tested. In all 1° recipients the overwhelming majority of dividing cells in the hematopoietic organs belong to the donor strain with the T6 marker. Secondary (2°) recipients, receiving average doses of BM cells from 1° recipients and tested later, did not differ in the cell content of their hematopoietic organs significantly from the 1° recipients (Table 1). The higher cell content of BM was probably linked with the greater age of the 2° recipients at the time of testing compared with the 1° recipients. With the lower of the two doses of cells injected, recovery of the thymus was not observed in one of the three recipients, and a high degree of contamination with recipient's cells was found in two of the three recipients. All 2° recipients of the larger dose of BM were 100% chimeras. Thus after only two transfers of BM a dose-dependent defect of restoration of hematopoiesis could be detected; it was expressed as reduction of competitiveness of the transplanted HSC and absence of maintenance of the cell content of the thymus in some of the recipients. Two long-living secondary chimeras were used for the third BM transplantation (Table 1). In the tertiary (3°) recipients definite differences were observed from the 1° and 2° recipients as regards repopulation of hematopoietic cells. The most stable value still remains the cell content of BM, which did not depend on the dose of BM with a range of 16 times. The cell content of the spleen in the 3° recipients in the late stages of observation was characterized by instability and by a definite lowering of its average values. The most changes were observed in the cell content of the thymus. It was severely depressed not only in old recipients, tested after more than 1 year, but also in those tested after 85-125 days. The frequency of reversions in the 3° recip-

TABLE 3. Ability of HRU to Maintain Their Own Pool during Successive Transfers

| No. of transfer | Transplanted bone marrow | | | | Recipients of bone marrow | | | |
|---|---|---|---|---|---|---|---|---|
| | donor of bone marrow | % of T6 metaphases | number of cells injected, $\times 10^6$ | number of HRU injected (M ± m) | days after transfer | number of HRU (M ± m) | | increase in number of HRU injected |
| | | | | | | in $10^6$ cells | in all BM | |
| 1 | Intact mice | 100 | 20 | 876±228 | 35 | 15,4±3,8 | 3183±785 | 3,63 |
| 2 | 1° chimera (20) | 100 | 2,95 | 45,4±11,2 | 246 | 4,5±1,6 | 1554±551 | 34,2 |
| 2 | The same | 100 | 1,475 | 22,7±5,6 | 351 | 3,6±1,0 | 1103±306 | 48,6 |
| 3 | 2° chimera (10; 3) | 100 | 4,0 | 18±6,4 | 125 | 2,2±0,7 | 683±208 | 37,9 |
| 4 | 3° chimera (20; 3; 4) | 9 | 2,0 | 4,5±1,3 | 35 | 0,5±0,2 | 169±71 | 37,6 |
| 5 | 4° chimera (20; 3; 4; 2) | 48 | 4,0 | 2±0,8 | 28 | — | — | — |

ients was greatly increased after a small dose of BM. However, even with the maximal dose ($4 \cdot 10^6$) a wide scatter of percentage of donor's metaphases was observed (from 100% to 9%). The 3° chimera selected randomly for the fourth transfer contained the lowest percentage of donor's cells in BM. However, in all 4° recipients (with a dose of $2 \cdot 10^6$ cells) the fraction of donor's cells with the T6 marker was greater than in BM of their immediate donor (Table 1). This fact is evidence that the fraction of tagged BM cells does not directly reflect the fraction of HSC of the corresponding population present. In five of the six quaternary recipients repopulation of the spleen was depressed, and in four repopulation of the thymus was totally absent. These latter appeared exhausted. From 12 to 83% of BM cells were repopulated by donor's HRU. After the fifth transfer, a weak degree of repopulation of BM was observed in the two surviving recipients, but repopulation of the thymus was absent. The dividing cells did not contain the donor's marker.

**Ability of HRU to Restore Their Own Pool after Transplantation.** Changes in the number of HRU during restoration of the recipients' BM was studied 2-35 days after the first transplantation of BM (Table 2). After 2 days the recipients' BM contained an almost undeterminable number of HRU. After 13-35 days, however, their number in the whole BM of the recipients could exceed the number of HRU injected by 4-10 times. The results are evidence of the ability of HRU to proliferate after transplantation. Assuming that all HRU injected survive in the recipients, but for some reason or other they avoid discovery at short intervals after injection, in that case they would have to go through at least 2-4 divisions to maintain the level found 2-5 weeks after transplantation. If, however, the number found after 2 days was close to the real number of HRU which had undergone transplantation, they must have gone through 8-10 divisions during that time.

**Ability of HRU to Maintain Their Own Pool during Successive Transfers.** After four successive transfers the general increase in the number of HRU during the total time of observation (441 days) was about $2 \cdot 10^5$ times (Table 3). Assuming that all injected HRU survive and preserve their properties, this increase would require about 18 divisions. Although direct proof of the ability of HRU to proliferate intensively, it will be evident from the results that this ability is not unlimited. With each successive transfer both the concentration and the whole pool of HRU in BM steadily declined. After injection of small number of HRU, the multiplicity of their increase in the recipients was much greater than after injection of large numbers of HRU (see Tables 2 and 3). Transplantation of fewer HRU evidently requires them to proliferate more rapidly.

The results show that, on the one hand, HRU can proliferate intensively. Each transplantation led to an increase of many times in the pool of injected HRU. On the other hand, proliferation was limited and did not lead to full restoration of the HRU pool. Despite this, after the first and second transfers stable hematopoiesis with no visible defects was established. Thus stable hematopoiesis can be maintained by pools of HRU that differ quantitatively from each other tenfold (Tables 3 and 1). This property of HRU distinguishes this category of HSC from CFU-s, the pool of which was restored virtually completely, not only after one transplantation, but also after another two transfers [8]. Defects of recovery of hematopoiesis begin to be found after the third transfer and later. They are expressed as loss of totipotency of some HRU (a weak degree of recovery of the thymus, or no recovery at all), and also weakening of the competitiveness of HRU, whose repopulating activity was lower than the irradiated recipients' HRU, as shown by the high percentage of metaphases without the donor's marker. Thus after a series of transfers not only the concentration of HRU in BM was reduced, but also their quality, their stem potential, i.e., their totipotency and proliferative potential. The results showed the existence of categories of HRU which, by restoring myelo-

poiesis in BM, are capable of maintaining survival of irradiated mice for 4 weeks, although they are not THSC, because they do not repopulate the thymus. The results of this investigation are in full agreement with the concept of a section of HSC in adult hematopoietic tissue as a hierarchical continuum of cells, heterogeneous with respect to stem potential, and that the position of individual HSC on the hierarchical ladder is determined by the length of their genealogy, i.e, the number of divisions they have gone through [5]. An essential factor of this model is that with stable hematopoiesis most HSC at all level of the hierarchical ladder up to CFU-s are at rest. For that reason, in normal hematopoietic tissue the stem potential of the HSC section is evidently excessive, and to explain the maintenance of hematopoiesis during life there is no need to resort to the concept of "self-maintenance" of HSC. Under conditions of successive transfers of relatively small doses of BM two processes take place: 1) dilution of the original pool of THSC and 2) involvement of THSC in proliferation, leading to depression and, ultimately, exhaustion of their stem potential. The transplantation procedure itself is not traumatic unless it causes proliferation of HSC [9].

Quantitative analysis of the HSC section during intensive proliferation of these cells, evoked by successive transplantation, does not confirm the ability of THSC to maintain themselves in the strict meaning of the term, i.e., to reproduce themselves. In fact, there is convincing evidence that HSC do not have the choice between self-maintenance or differentiation, and during division, differentiation into the next level of development always takes place.

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