# CELLS MAINTAINING LONG-TERM HEMATOPOIESIS IN CULTURE CANNOT REGENERATE AFTER IRRADIATION

E. I. Deryugina, N. I. Drize, and I. L. Chertkov

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One of the most important yet most contradictory problems in the discovery of the mechanisms of proliferation and differentiation of hematopoietic stem cells (HSC) is to explain their capacity for self maintenance. Experiments on models of splenic colonies have shown the ability of the cells producing them (CFUs) to form daughter colony-forming cells. Nevertheless, it has been shown repeatedly that the proliferative potential of these cells is lower than that of the original cells, and after the third passage they lose all their ability to produce CFUs [8]. It has accordingly been suggested that HSC are not CFUs, but their earlier precursors, whose function is to maintain hematopoiesis throughout life of the individual [3]. It is these cells, and not any category of precursors, giving rise to colonies in the spleen, that are responsible for the maintenance of long-term hematopoiesis in culture [5]. The fundamental question of whether the HSC maintain themselves or are simply utilized from a population laid down during ontogeny, has not been solved by these investigations.

To study this problem, it was decided to use a model of regeneration of the hematopoietic system after sublethal irradiation of mice. It was shown that the ancestral hematopoietic cells (AHC), by which is meant cells capable of maintaining long-term hematopoiesis in culture, are unable to regenerate after irradiation, and that their number in regenerating (with respect to cell composition and number of CFUs) bone marrow is preserved at the original (immediately after irradiation) level.

## EXPERIMENTAL METHOD

Female (CBA × C57BL/6)$F_1$ (CBF) and (C57BL/6 × DBA/2)$F_1$ (BDF) mice aged 8-12 weeks at the beginning of the experiment, were used. The cultures and mice were irradiated by an IPK $^{137}$Cs source. Mice receiving the hematopoietic cells were irradiated in a dose of 10 Gy. Mice serving as donors of bone marrow were irradiated sublethally in doses of 2 and 4 Gy. Bone marrow for culture was taken from these mice either at one or 2-5 months after irradiation. The number of 8-day CFUs was determined by the splenic colonies method [7]. Bone marrow cells from unirradiated mice, and also from mice irradiated in doses of 2 and 4 Gy 10-19 weeks before the experiment were injected into 10 irradiated syngeneic recipients in a dose of $4 \cdot 10^4$ per mouse. Bone marrow cells from mice irradiated in doses of 2 and 4 Gy 1 h before the experiment were injected into irradiated syngeneic recipients in doses of $2 \cdot 10^5$ and $2 \cdot 10^6$ per mouse respectively. Long-term mouse bone marrow cultures were managed as described previously [2]. Cultures with a confluent layer of adherent cells, formed 3 weeks after explantation of bone marrow from BDF mice, were irradiated in a dose of 12 Gy, and this was followed by a second transplantation of a mixture of bone marrow cells from mice of a different genotype, irradiated sublethally immediately and 2-5 months before the experiment. Cells of neither genotype had a selective advantage when grown on an irradiated supporting layer of adherent cells of either origin (CBF or DBF; data not given), in good agreement with absence of the phenomenon of allogeneic inhibition of CFUs in long-term mouse bone marrow cultures [1, 6]. After culture for 2-6 weeks the number of CFUs of each genotype was determined in the suspension of nonadherent cells, by injecting $2 \cdot 10^5$-$6 \cdot 10^5$ cells

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TABLE 1. Competitive Repopulation in a Culture of Hematopoietic Bone Marrow Cells from Intact and Irradiated Mice of Different Genotypes

| Mice | Dose of irradiation, Gy | Time after irradiation | Number of cells explanted per flask | | Ratio between CFUs (BDF:CBF) | | |
|---|---|---|---|---|---|---|---|
| | | | f.e. | CFUs | initial | after culture | |
| | | | | | | 2 weeks | 4 weeks |
| BDF + CBF | — | — | 0,6 | 3701 + 3970 | 0,9 | — | 2,6 |
| BDF + CBF | — | — | 0,5 | 3638 + 3744 | 1,0 | 1,3 | 2,4 |
| BDF + CBF | — | — | 0,25 | 1378 + 2025 | 0,7 | 0,6 | — |
| BDF + CBF | — | — | 0,6 | 3701 + 444 | 8,3 | — | 4,2 |
| BDF + CBF | 2 | 1 h | 0,6 | 3701 + 268 | 13,8 | — | 25,0 |
| BDF + CBF | — | — | 0,6 | 3701 + 57 | 64,9 | — | 28,6 |
| BDF + CBF | 2 | 5 weeks | 0,6 | 3701 + 79 | 46,8 | — | 25,0 |
| BDF + CBF | — | — | | | | | |
| | 4 | 1 h | | | | | |
| | — | — | | | | | |
| | 4 | 5 weeks | | | | | |

Legend. Here and in Table 2, f.e. denotes femoral equivalent.

TABLE 2. Competitive Repopulation in a Culture of Bone Marrow Hematopoietic Cells from Acutely Irradiated Mice and Mice with Hematopoiesis Regenerating after Sublethal Irradiation

| Mice | Dose of irradiation, Gy | Time after irradiation | Number of cells explanted per flask | | Ratio between CFUs (regeneration: acute irradiation) | | |
|---|---|---|---|---|---|---|---|
| | | | f.e. | CFUs | initial | after culture | |
| | | | | | | 2 weeks | 6 weeks |
| BDF + CBF | 2 | 10 weeks 1 h | 0,5 | 1784 + 363 | 4,9 | 0,4 | 1,8 |
| CBF + BDF | 2 | 10 weeks 1 h | 0,5 | 2394 + 262 | 9,1 | 2,5 | 0,5 |
| BDF + CBF | 4 | 10 weeks 1 h | 1,0 | 4297 + 71 | 60,5 | 2,1 | 1,7 |
| CBF + BDF | 4 | 10 weeks 1 h | 1,0 | 4261 + 56 | 76,1 | 0,8 | 0,6 |
| BDF + CBF | 4 | 19 weeks 1 h | 0,5 | 2173 + 88 | 24,7 | 0,5 | — |
| CBF + BDF | 4 | 19 weeks 1 h | 0,5 | 4200 + 60 | 70,0 | 0,2 | — |
| CBF + BDF | 4 | 19 weeks — | 0,5<br>0,006 | 2100 + 33 | 63,6 | 0,7 | — |

per mouse intravenously into immune CBF and BDF recipients (five mice of each genotype). The recipient mice were cross-immunized intraperitoneally by two injections of $2 \cdot 10^7$ splenocytes of the opposite genotype at an interval of 2 weeks. Immune recipients were irradiated not earlier than 2 weeks after the second immunization. If more than 1 month elapsed between the second immunization and irradiation, 3 days before irradiation the mice were given an intravenous injection of $5 \cdot 10^6$ splenocytes of the opposite geneotype. The efficiency of colony formation in the immune mice after injection of syngeneic hematopoietic cells remained unchanged, whereas 91-100% of the CFUs of the genotype used for immunization were inactivated (data not given).

## EXPERIMENTAL RESULTS

It was shown previously that during a second transplantation of a mixture of bone marrow cells from mice of two different karyotypes into culture, the population of dividing nonadherent cells consisted through the period of culture of cells of both karyotypes in a ratio corresponding strictly to that found initially on explantation [5]. On the basis of this

finding it was concluded that the initial ratio is maintained during culture at the AHC level also, for they have no selective advantage. Consequently, by this method it is possible to determine the relative number of HSC in the cell suspensions used on the basis of their progenies.

In this investigation basically the same approach was used to analyze the relative numbers of AHC. The differences were that a mixture of cells from nonsyngeneic partners was explanted into culture, the origin of the AHC was established, not karyologically, i.e., at the myelocyte level, but with respect to much earlier precursors, namely CFUs and, finally, not normal but irradiated hematopoietic cells were studied. The results of testing the adequacy of the system in order to solve the particular problem are shown in Table 1, and they demonstrate that on transplantation of a suspension of normal hematopoietic cells of two different genotypes the relative numbers of CFUs during culture are preserved as a whole within the limits of accuracy of the method used to determine CFUs in the culture (differences with respect to CFUs were less than 2.5 times and were not statistically significant). The same result also was found after explantation of a suspension of bone marrow cells from normal BDF mice and from CBF mice irradiated in doses of 2 and 4 Gy. With the doses used, 12 and 1.5% of CFUs respectively were preserved in the irradiated bone marrow. In the course of 5 weeks, regeneration did not take place in the bone marrow of the mice (7 and 2% of CFUs respectively). In this case also, the original ratio between the explanted CFUs was preserved during culture. Consequently, the results show absence of selective advantage of the cells of the genotypes used, and likewise of selective advantage of the unirradiated cells over irradiated. Absence of regeneration of irradiated CFUs in culture and the suitability of CFUs for estimating the relative number of explanted AHC also were demonstrated.

Table 2 gives the results of competitive repopulation in a culture of hematopoietic cells from the bone marrow of acutely irradiated mice and of mice regenerating after sublethal irradiation. As Table 2 shows, the bone marrow of the mice 10 weeks after sublethal irradiation in a dose of 2 Gy contained 5-10 times more CFUs than immediately after irradiation. Despite this fact, the number of AHC was the same: during 2-6 weeks of culture the ratio of the CFUs in the acutely irradiated bone marrow and in the regenerating marrow did not differ significantly from unity. Even more demonstrative results were obtained by the use of a dose of 4 Gy. In this case, the regenerating bone marrow contained 60-75 times more CFUs than the acutely irradiated marrow. However, the number of AHC in this case also was the same, i.e., no regeneration of AHC took place. Basically the same results also were obtained when bone marrow from mice 19 days after irradiation was used. Direct comparison of the number of AHC in normal bone marrow and 19 weeks after irradiation showed that their number in the regenerating bone marrow was about 0.5% of the number of AHC in the femur of an unirradiated mouse.

On the whole, the results demonstrate that AHC are unable to regenerate. This fact cannot be explained, for example, by their slower regeneration than that of CFUs, for even after 19 weeks, or more than one-quarter of the life span of a mouse, regeneration of the AHC population was not observed. It can thus be concluded that HSC, or at least some of them which are responsible for the maintenance of long-term hematopoiesis in culture, cannot maintain themselves. The results confirm the view that HSC laid down during embryogenesis are utilized consecutively throughout life, one after another, in accordance with the theory of consecutive alternation of clones of hematopoietic cells [4].

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