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Self-renewal of the long-term repopulating stem cell

(hemopoiesis/marrow transplantation/competitive repopulation/colony-forming units, spleen/irradiation)

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ABSTRACT **Self-renewal implies maintenance of all attributes of the original in the offspring and is considered characteristic of the hemopoietic stem cells. Yet, it has been questioned whether one of the most primitive hemopoietic stem cells, the long-term repopulating cell (LTRC), has that capacity. The present experiments demonstrate that single LTRCs can repopulate the lymphohemopoietic system of a lethally irradiated mouse and that the progeny of a single LTRC in a primary recipient again contains LTRCs capable of repopulating lethally irradiated secondary recipients. The transfusion of very small numbers of marrow cells (10,000–20,000 cells containing one or no LTRCs) unexpectedly provided insight into competitive marrow repopulation. At these low levels of stem cells, irradiated host stem cells or their progeny competed successfully with unirradiated donor cells. This parallels the known reemergence and marrow repopulation by host cells when the number of nonirradiated donor stem cells is reduced by serial transplantation.**

Self-renewal is the production of exact duplicates of stem cells with maintenance of all attributes, including the full range of self-renewal of the original. The dual capacity of self-renewal and of differentiation is the current definition of a stem cell. However, the widely accepted model of stem cell kinetics proposed by Kay (1) and extended by Hellman *et al.* (2) postulates a reduction of proliferative capacity at each stem cell division. Their model may exclude exact replication of any stem cell, with the possible exception of the most primitive, pluripotential cell. Self-renewal of even that first-in-line stem cell has been questioned. A recent review (3) of stem cell separation states that there is as yet "no evidence that either symmetrical or asymmetrical division can generate identical daughter cells of these primitive stem cells."

The present paper demonstrates self-renewal of the earliest recognizable stem cell, the long-term repopulating cell (LTRC). Specifically, the progeny of a single LTRC repopulating a lethally irradiated recipient is shown to produce offspring that can again repopulate lethally irradiated recipients. The demonstration does not answer the question whether self-renewal of LTRCs is a unique capacity of the earliest multipotential stem cell or is shared, perhaps to varying degrees, by all stem cells. The resolution of this question appears to require a precise definition of stem cells being investigated, particularly pre-colony-forming units, spleen (CFU-S) that can now be identified as LTRCs, short-term repopulating cells (STRCs), or other pre-CFU-S yet to be characterized. Although LTRCs can apparently self-renew for at least the life-span of the individual, later stem cells may be able to self-renew for only a limited period of time or a limited number of divisions. Such differences must impact on the kinetics of hemopoiesis. These issues are critically reviewed in *Discussion*.

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MATERIALS AND METHODS

The mouse strains and procedures used were those described by Brecher *et al.* (4). Congenic mice that carried distinguishing enzyme markers in all body cells were used as donors of marrow transfusions in order to trace the progeny of stem cells. The enzymes that distinguished donor and recipient cells were phosphoglycerate A and B and glucose phosphate 1A isomerase.

Recipients were irradiated with a uniformly lethal dose of 10.5 Gy or with 10 Gy. The near-lethal 10-Gy dose was used for primary recipients of 10,000–20,000 marrow cells with an estimated average of only one LTRC in the hope of maximizing survival of animals given a single LTRC. The dose killed 30 of 30 mice of the recipient strain in one group and 29 of 30 mice in another. As lethality of 10 Gy thus turned out to be close to 100%, we refer to animals given either 10.5 or 10 Gy as lethally irradiated or simply irradiated.

The marrow of the primary recipients was transfused into secondary, lethally irradiated congenic recipients. Progeny of the donor and host cells in the primary recipients could then be followed and distinguished from the host cells of the secondary recipient carrying a third marker by electrophoresis.

RESULTS

In experiment 1, we transfused each of a group of 15 lethally irradiated mice that carried the enzyme marker glucose phosphate isomerase 1A (G mice) with 10,000 marrow cells that carried the enzyme marker phosphoglycerate kinase A (A cells). Another group of 15 G mice received 20,000 cells from A donors. Three months later, to allow for disappearance of STRCs with a maximum clone time of 12 weeks (5), marrow was harvested from one of the primary recipients transfused with 10,000 cells and from one of the primary recipients of 20,000 cells. The marrow of these two animals used as donors for retransfusion assayed at 47% and 45% G and 53% and 55% A. Eight mice carrying the enzyme marker phosphoglycerate kinase B (B mice) were given a lethal dose of radiation. Four of these, designated 1–4, were then transfused with 5×10^6 marrow cells each from the 20,000 cell recipient. The other four, designated 5–8, were transfused with 5×10^6 marrow cells each from the 10,000 cell primary

Abbreviations: CFU-S, colony-forming units, spleen (equivalent to pluripotential stem cells, counted as colonies in spleen lethally irradiated mice); STRCs, short-term repopulating cells (hemopoietic stem cells that ensure survival of lethally irradiated mice for up to 3 months); LTRCs, long-term repopulating cells (hemopoietic stem cells that ensure survival beyond 3 months or indefinitely); A, B, G mice or cells, carrying genetic markers for phosphoglycerate kinase A or B or for glucose phosphate isomerase 1A, which are separable by electrophoresis.

recipient. (Transfusion of 100,000 cells from the same donors failed to rescue any of a group of eight lethally irradiated recipients.) The blood of the eight secondary recipients was then monitored monthly for 11 months, as shown in Fig. 1. The progeny of irradiated G cells of the primary recipient declined gradually in all eight secondary recipients (Fig. 1*B*), being replaced by A cells at levels of 40% or better by primary donor A cells in five secondary recipients and by the recipients' own B cells at levels of 60% or better in three secondary recipients.

In experiment 2, we transfused 20,000 marrow cells of a 50:50 mixture from A and B donors into each of 30 G recipients. After 3 months, 3 of 15 survivors had only A and G cells with no B cells, 4 had only B and G cells with no A cells, and only 1 had both A and B as well as G cells, while the remaining 7 survivors had reverted to 100% host G cells. That seven of eight or ≈90% of animals had only A or only B cells when the donor marrow suspension had 50% A and 50% B cells confirmed that most animals received a single LTRC so that the repopulating donor cells had only the A or only the B marker. We retransfused marrows from three of the primary recipients. Two had no A cells. One of them had 68% G cells and 32% B cells, and the other had 62% G cells and 38% B cells. From the first of these two primary recipients 5×10^6 marrow cells were transfused into each of five irradiated secondary A recipients. From the second primary recipient, 5×10^6 marrow cells were transfused into each of six secondary A recipients. Five million marrow cells from one of the primary recipients without B cells and containing 68% A and 32% G cells were transfused into six lethally irradiated secondary B recipients. The results are presented in Fig. 2. The blood of all 17 secondary recipients was monitored for 5–6 months.

The 11 secondary recipients of marrow from primary recipients without A cells are graphed in Fig. 2 *A* and *B*. Only 1 of the 11 A recipients of marrow containing only B and G cells showed continued presence of B cells at a high level (40%). These B cells, it should be recalled, had to be derived from the primary donor, which seeded, with 90% probability, a single LTRC in each primary recipient. An additional 2 of the 11 secondary recipients had a small number of B cells, 1 shown in Fig. 2*A* and 1 shown in Fig. 2*B*. In contrast, as shown in Fig. 2*C*, five of six secondary B recipients of marrow containing only A and G cells had stable long-term repopulation by A cells at levels of 30–90% at 6 months. These A cells had to be derived, with at least 95% probability, from a single LTRC seeded in the primary recipient. After 2 of the primary recipients of experiment 1 had been used for retransfusion, the remaining 16 survivors were observed for another 6 months. By that time all but four of them had reverted to 100% host (G) cells. Two of the four had 75% and 78% of G cells, with G cells still increasing. Only two had stable levels of 43% and 55% donor A cells. Similarly, after 3 of the primary recipients in experiment 2 had been used for retransfusion, the remaining 12 mice were observed for another 6 months. By that time the marrow of all but two of these animals reverted to 100% host (G) type.

DISCUSSION

The experiments presented established that single cells seeded in the primary recipients produced additional LTRCs since retransfusion of their marrow leads to high levels of their progeny in 11 of the 25 secondary recipients (Figs. 1 and 2). In experiment 1, all A cells in the secondary recipients must have been derived from the primary A donors, all G cells were from the primary G recipients, and all B cells were from the secondary recipients themselves. The A cells must have been derived in 90% of instances from a single LTRC in the marrow cells transfused into the primary recipient. This can be inferred from the known incidence of LTRCs, which has been found to be between 1 per 30,000 and 1 per 50,000 marrow cells in this strain of mice (4, 6). The low incidence was confirmed by the evidence for seeding of single cells in experiment 2. While the A cells in the primary recipients presumably contained both LTRCs and STRCs initially, the STRCs must have disappeared by the end of 3 months, their maximum survival (5). Thus, the long-term repopulation of the five secondary recipients with >40% A cells (Fig. 1) must have been derived in most, if not all, animals from the progeny of the initial single LTRC of type A transfused into the primary recipient.

In the second experiment, all primary recipients received a mixture of 50% A and 50% B cells. The limitation of transfused marrow cells to 20,000 ensured, as in experiment 1, that almost always only a single LTRC was transfused into each primary recipient, because of the low incidence of LTRCs already noted. That single LTRC must necessarily be either A or B. The absence of A cells from two primary recipients at 3 months identified that single LTRC as B in those two animals. The absence of B cells from the third primary recipient used in retransfusion identified that single LTRC as A. Only 1 of 11 secondary recipients transfused with marrow from the 2 B-only primary recipients had a stable repopulation with B cells of 40% from 2 months on (Fig. 2 *A* and *B*). Another two showed only a temporary presence of B cells of 8% and 16%, most probably technical errors, since the number of B cells is computed as the difference between A and G cells, both determined electrophoretically with measurement errors of at least 5%. Five of six secondary recipients of marrow from a third primary recipient containing no B cells reached stable A cell levels, varying from 30% to 90% at 2–4 months.

The reappearance of donor cells in substantial numbers in 10 of 14 transfusions involving A donor cells, but in only 1 of 11 transfusions involving B donor cells, suggests that A cells

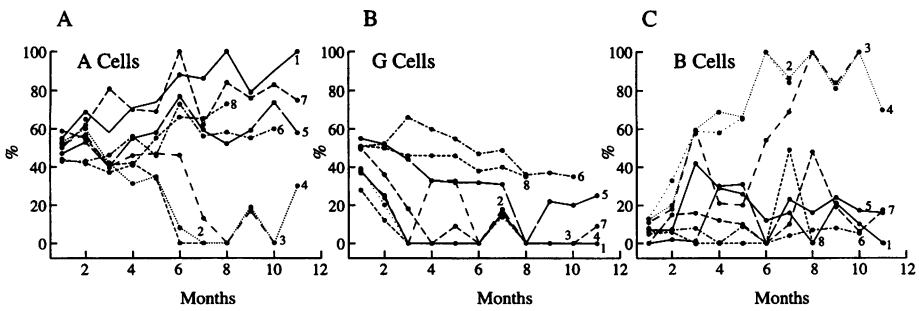

FIG. 1. Analysis of the origin of peripheral blood cells in secondary recipients. Cells of type A (*A*) were progeny of the single A cell transfused into the primary recipient, G cells (*B*) were derived from the primary recipient, and B cells (*C*) were derived from secondary host cells. Note that five of the eight secondary recipients were repopulated predominantly by A cells derived from a single primary donor cell, which self-renewed in the primary recipient.

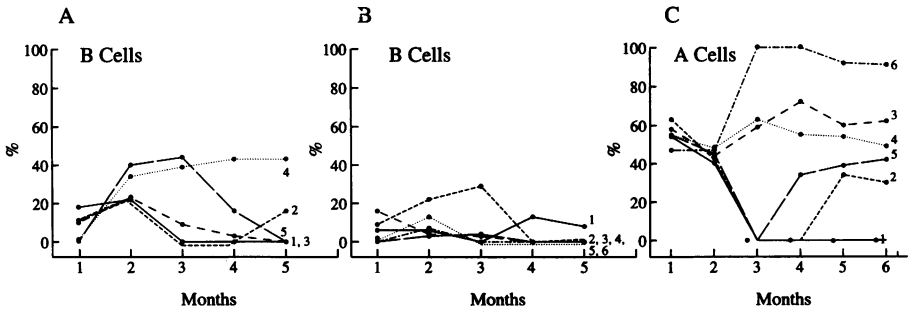

FIG. 2. Origin of peripheral blood cells in secondary recipients of a 50:50 mixture of marrows from A and B donors transfused into primary lethally irradiated G recipients. Note that only one of the secondary recipients of B donor cells had >30% B cells, while five of six secondary recipients of A donor cells had >30% A cells.

more readily replace G cells than do B cells. The evidence of an apparent interaction between B and G cells is somewhat surprising, as transfusions of A cells into B animals or of B cells into A animals gave the expected reciprocal results (unpublished data).

After the presence of multiple LTRCs of donor origin was documented at 3 months in the primary recipients, most of them subsequently reverted to host type as detailed in *Results*. This adds to the evidence that, while a given dose of radiation may be uniformly lethal, it is not necessarily uniformly lethal to all stem cells. This conclusion has long been evident from the reversal to recipient-type marrow of lethally irradiated animals rescued by serially transplanted marrow (7). These observations also indicated that the progeny of irradiated host cells may replace the progeny of nonirradiated transplanted donor cells after recovery from the immediate effects of irradiation. The precondition for such reversal to occur appeared to be the exhaustion of donor LTRCs and STRCs due to serial transplantation or perhaps their reduction to the level of surviving host LTRCs and STRCs. The present observations suggest that transplantation of a single LTRC may keep donor stem cells at a low enough level for surviving host cells to compete in the early posttransplantation period. This interpretation receives substantial support from the observation (5) that it took 3000 separated donor cells, enriched for stem cells, to achieve 100% donor repopulation. Transfusion of lower cell numbers of the same suspension ensured survival of at least 40% of lethally irradiated animals. However, both host and donor cells contributed to repopulation of their marrows. Donor cells, which reached almost 100% initially, were then temporarily and partially replaced by host cells. Eventually, a chimerism with 40–100% donor cells was reached at 9 months. Similar results were obtained by Spangrude *et al.* (8) and Ushida and Weissman (9), with 1000 cells of their suspensions required for 100% donor repopulation, although survival of 50% or better was obtained with 30–100 cells. Recently, M.G.P., D. A. Polikoff, W. Hyun, and G.B. (unpublished data) obtained a sorted population of marrow cells that contained no CFU-granulocyte–macrophage, burst-forming unit, erythroid, or CFU-mix, and 1.6% CFU-S day 13, of which 100 cells rescued 100% of lethally irradiated mice. Engraftment, although maintained for >9 months, never reached 100% and survival with 30 cells was only 50%. Thus, the discrepancy between the number of separated donor stem cells required for 100% survival of lethally irradiated hosts and the excess of separated cells to achieve 100% repopulation by donor cells were common to transfusions of marrow suspensions enriched in LTRCs by three different techniques in two laboratories.

The data reviewed here indicate that a number of stem cells not only survive but survive relatively undamaged when irradiated with doses that are lethal to the untreated controls. A competition between surviving host cells and transfused donor cells thus occurs not only when donor cells are reduced after serial transplantation but also when very few early stem cells are transplanted to begin with. These quantitative relationships thus appear to expand the concept of competitive repopulation.

The delay in the appearance of A or B cells in the secondary recipients may be due to a lengthy differentiation process of LTRCs as compared with STRCs, which rapidly, although only temporarily, maintain peripheral blood cell levels after marrow transplantation. Alternatively, differentiation may be restricted and self-renewal may predominate initially when only a single or a few LTRCs are seeded. While the mechanism by which this may be accomplished is not apparent, such a development is needed to establish a substantial reservoir of LTRCs. Otherwise, premature differentiation could lead to loss of the limited number of transplanted LTRCs.

In retrospect, the demonstration that LTRCs can self-renew might be considered an unnecessary effort. Such self-renewal may appear inherent in the demonstration that a single LTRC can repopulate the entire marrow and lymphoid system of an irradiated recipient. The question has been raised, however, whether the dual capacity of self-renewal and of differentiation applies to the entire hierarchy of stem cells starting with the LTRCs and ending with the committed stem cells or to the first cell of the hierarchy only. As pointed out by Eaves *et al.* (10) the term hemopoietic stem cell has been used by many authors to apply only to the primordial stem cell capable of sustained self-renewal for the life-span of the individual—i.e., a LTRC. Others reserve the term hemopoietic stem cell for cells that can be separated physically by a particular technique. For instance, some have claimed "functional homogeneity" for the cells they isolated (11). Others have indicated they consider "isolation to homogeneity" a prerequisite for determining the dual capacity to self-replicate and differentiate (3). So far 30–100 cells of such sorted suspensions of marrow cells have achieved only 50% survival of lethally irradiated recipients (11–13). Yet one or two LTRCs have been shown to ensure survival of such animals (4, 7). The need for at least 30 cells of sorted suspension of marrow enriched for stem cells indicates that STRCs or CFU-S keep the transplanted animals alive until LTRCs can differentiate into later multipotential stem cells, which maintain the peripheral blood. The need for cotransfusion of STRCs or later stem cells has been amply demonstrated (13–15). Thus, a homogeneous suspension containing only LTRCs would necessarily fail to ensure survival of lethally irradiated congenic recipients. Conversely, concentrates of bone marrow cells free of LTRCs would lead to death after 1–3 months, the clone time of STRCs. A mixture of those two suspensions would sustain long-term survival. Taken together, these results conclusively establish the homogeneity of a LTRC suspension and the absence of LTRCs of another suspension.

The variations in the definition and use of the term hemopoietic stem cell predate the recognition of heterogeneity of CFU-S and pre-CFU-S. This led to a number of controversies questioning whether hemopoiesis is stochastic or deterministic (16, 17) and whether successive stages of differentiation of stem cells constitute a continuum (1, 2) or separate compartments. We submit that these distinctions are not experimentally verifiable. In contrast, the succession of LTRCs, STRCs, and CFU-S has been verified experimentally by their function, although the different cell types have not been separated physically. They appear to represent steps in differentiation that cannot be skipped, whether viewed as stages of a continuum or as separate compartments.

Other concepts of stem cell kinetics should be similarly questioned. For instance, asymmetric division of an early stem cell has been considered necessary to explain multipotentiality of early stem cells (18). The concept of an asymmetric division implies that the two products of division can be distinguished at the end of a mitosis to exclude that postmitotic influences following a symmetric division are responsible for the different fates of the two offspring. That asymmetric divisions can result in heritable differences between the offspring has now been demonstrated (19). The question remains whether such differences indeed exist at the end of the type of mitosis that resulted in differentiation along different lineages in the two offspring of a blast cell placed into identical environments (20). Without such evidence the alternative cannot be excluded that the division was indeed symmetric and the different fates of the two offspring are due to postmitotic events—e.g., the adherence of different cytokines to the two products of division or minute differences in the identically designed environments in which the offspring were raised. It should also be recalled that Osgood's asymmetric division (18) implied that a stem cell could produce one differentiated offspring and one that would continue to self-replicate as a stem cell, an event that has yet to be demonstrated.

Another tenet of stem cell kinetics appears based on the assumption that stem cells maintain their number by adjusting their proliferation rate to their numbers. It led to wide acceptance of an inverse relationship between the number of stem cells and the number proliferating as a general law. This relationship has indeed been repeatedly demonstrated for CFU-S during regeneration, although in normal controls CFU-S proliferation varies widely from time to time while the total number of CFU-S remains relatively constant (21).

In conclusion, the data presented demonstrate that LTRCs do self-renew and differentiate into all lineages after seeding of a single donor cell into lethally irradiated mice. This result underscores discrepancies in the present use of the term hemopoietic stem cell and suggests the need for precise identification of the stem cells being discussed. These now include LTRCs, STRCs, CFU-S, and lineage-specific stem cells. Furthermore, functionally, if not physically, separable subdivisions of stem cells may yet be delineated. Such identification would avoid controversies based on concepts that lack experimental support and obscure increasingly detailed knowledge of pre-CFU-S stem cells.

1. Kay, H. E. M. (1965) *Lancet* **1**, 418–419.
2. Hellman, S., Botnick, L., Hannon, E. C. & Vigneulle, R. M. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 490–495.
3. Watt, S. M. & Visser, J. W. (1992) *Cell Proliferation* **25**, 63–97.
4. Brecher, G., Neben, S., Yee, M., Bullis, J. & Cronkite, E. P. (1988) *Exp. Hematol.* **16**, 627–630.
5. Neben, S., Redfearn, W. J., Parra, M., Brecher, G. & Pallavicini, M. G. (1991) *Exp. Hematol.* **19**, 958–967.
6. Micklem, H. S., Lennon, J. E., Ansell, J. D. & Gray, R. A. (1987) *Exp. Hematol.* **15**, 251–257.
7. Wolf, N. A., MacMillan, J. R. & Priestley, G. V. (1983) *Blood Cells* **9**, 415–425.
8. Spangrude, G. J. & Scollay, R. (1990) *Exp. Hematol.* **18**, 920–926.
9. Uchida, N. & Weissman, I. L. (1992) *J. Exp. Med.* **175**, 175–184.
10. Eaves, C. J., Sutherland, H. J., Udomsakdi, C., Lansdorf, P. M., Szilvassy, S. J., Fraser, C. C., Humphries, R. K., Barnett, M. J., Phillips, G. L. & Eaves, A. C. (1992) *Blood Cells* **18**, 301–307.
11. Spangrude, G. J., Smith, L., Uchida, N., Ikuta, K., Heimfeld, S., Friedman, J. & Weissman, I. L. (1991) *Blood* **78**, 1395–1402.
12. Spangrude, G. J., Heimfeld, S. & Weissman, I. L. (1988) *Science* **241**, 58–62.
13. Szilvassy, S. J., Lansdorp, P. M., Humphries, R. K., Eaves, A. C. & Eaves, C. J. (1989) *Blood* **74**, 930–939.
14. Bartelmez, S. H., Andres, R. G. & Bernstein, I. D. (1991) *Exp. Hematol.* **19**, 861–862.
15. Jones, R. J., Wagner, J. E., Celano, P., Zicha, M. S., Celano, P. & Sharkis, S. J. (1990) *Nature (London)* **347**, 188–189.
16. Rosendaal, M., Hodgson, G. S. & Bradley, T. R. (1976) *Nature (London)* **264**, 68–69.
17. Novak, J. P. & Stewart, C. C. (1991) *Br. J. Haematol.* **78**, 149–154.
18. Osgood, E. E. (1961) *Geriatrics* **29**, 208–221.
19. Czerniak, B., Herz, F., Wersto, R. P. & Koss, L. G. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 4860–4863.
20. Suda, T., Suda, J. & Ogawa, M. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 2520–2524.
21. Necas, E., Znohil, V. & Vacha, J. (1988) *Exp. Hematol.* **16**, 231–234.