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Quantitative assay for totipotent reconstituting hematopoietic stem cells by a competitive repopulation strategy

STEPHEN J. SZILVASSY*†, R. KEITH HUMPHRIES*‡§¶, PETER M. LANSDORP*‡, ALLEN C. EAVES*†‡¶, AND CONNIE J. EAVES*‡§¶

*Terry Fox Laboratory, British Columbia Cancer Agency, and Departments of †Microbiology, ‡Medicine, §Medical Genetics, and ¶Pathology, University of British Columbia, Vancouver, BC V5Z 1L3, Canada

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ABSTRACT **Although hematopoiesis is known to originate in a population of very primitive cells with both lymphopoietic and myelopoietic potential, a procedure for enumerating such cells has to date not been available. We now describe a quantitative assay for long-term repopulating stem cells with the potential for reconstituting all hematopoietic lineages. This assay has two key features. The first is the use of competitive repopulation conditions that ensure not only the detection of a very primitive class of hematopoietic stem cells but also the survival of lethally irradiated mice transplanted with very low numbers of such cells. The second is the use of a limiting-dilution experimental design to allow stem cell quantitation. The assay involves transplanting limiting numbers of male "test" cells into lethally irradiated syngeneic female recipients together with 1–2 × 10^5 syngeneic female marrow cells whose long-term repopulating ability has been compromised by two previous cycles of marrow transplantation. The proportion of assay recipients whose regenerated hematopoietic tissues are determined to contain ≥5% cells of test cell origin (male) ≥5 weeks later is then used to calculate the frequency of competitive repopulating units (CRU) in the original male test cell suspension (based on Poisson statistics). Investigation of this assay system has shown that all three potential sources of stem cells (test cells, compromised cells, and the host) can under appropriate circumstances contribute to long-term hematopoietic regeneration, thus establishing both the competitive pressure of hematopoietic stem cells in the cotransplanted compromised population and in the host, and the need to use genetic markers to track the specific contribution of the injected test cells. Analysis of the frequency of CRU in test marrow suspensions that varied widely in their CRU content gave similar values when endpoints of either 5 or 10 weeks posttransplantation were used and when either recipient marrow or thymus was used to identify progeny populations. In addition, repopulation of marrow and thymus was found to be associated in most mice injected with limiting numbers of test cells. These findings are consistent with the conclusion that the assay is highly selective for a very primitive, totipotent, reconstituting hematopoietic stem cell and should therefore be particularly useful in future gene therapy-oriented research as well as for more basic studies of hematopoietic stem cell regulation and differentiation.**

The most primitive cell in the hematopoietic system appears to be one that is capable of regenerating and maintaining all lymphoid and myeloid lineages for many months after transplantation (1–5). Nevertheless, despite >2 decades of research to characterize this cell, there is still no quantitative assay that can be used to measure it exclusively. This situation probably reflects our relatively recent appreciation of the fact that the cells present in the hematopoietic tissues of lethally irradiated mice at different times after transplantation of normal marrow cells are derived from different types of precursors (6) and, particularly at later times, cannot necessarily be assumed to be of donor origin (7). For example, it now seems likely that most, if not all, spleen colonies detectable up to 14 days after transplantation are derived from cells [CFU-S; colony-forming unit, spleen (8)] that are not capable of long-term (>4 weeks) hematopoietic reconstitution (9). Two approaches to the development of longer-term assays have therefore been used. The first and technically simplest has been to measure the frequency of cells able to confer protection against a lethal dose of radiation (10). This method has the advantage of being quantitative, but fails to take into consideration the possibility that death may result from the early absence in the recipient of adequate numbers of cells capable of rapid, albeit transient, production of mature blood cells, even in the presence of more primitive cells whose mature progeny may not appear until later times. Another approach has been to measure after 2 weeks the regeneration of CFU-S in the recipient's marrow, thereby extending via a second short-term *in vivo* assay the total time of hematopoietic regeneration to 4 weeks (11). However, the technical complexity of this latter double transplant assay, and potential limitations of its sensitivity, suggest that derivation of stem cell frequencies by limiting-dilution analysis with this approach may be difficult. Moreover, in the absence of adjunct procedures to establish the origin of the cells eventually regenerated in either of these methods, calculated stem cell frequencies may significantly overestimate the actual content of injected long-term repopulating cells.

The need for a practical assay that detects donor-derived cells with long-term lymphomyeloid repopulating ability and that allows such cells to be readily quantitated is thus strongly indicated. We have now undertaken experiments to evaluate the potential of a competitive repopulation assay in which lethally irradiated female mice are transplanted with limiting numbers of syngeneic, but male, "test" cells together with a large number (1–2 × 10^5) of "compromised" (twice serially transplanted) marrow cells from syngeneic female mice. This latter population of cells contains a near normal frequency of CFU-S and various *in vitro* clonogenic cells and can alone rescue recipients from the lethal effects of the radiation (12, 13). However, because they have been subjected to two previous cycles of marrow transplantation and regeneration, such cells exhibit a markedly reduced competitive long-term hematopoietic repopulating ability (14). Their coinjection thus serves the dual purpose of ensuring the survival of the recipients and of providing a selective pressure to identify a class of stem cells with a high capacity for competitive long-term repopulation. The frequency of competitive repopulating units (CRU) in the male test cell suspension can then be calculated by Poisson statistics to analyze test cell repop-

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Abbreviations: CFU-S, colony-forming unit, spleen; CRU, competitive repopulating unit(s); 5-FU, 5-fluorouracil.

ulated recipients injected with limiting numbers of test cells (15).

The results of the present study have revealed the importance of genotype analysis to identify the test cell origin of cells in regenerated hematopoietic tissues, even in this completely syngeneic system. In addition, they show that calculated CRU frequencies do not vary when the time of tissue analysis is prolonged from 5 weeks to 10 weeks posttransplant and are the same regardless of whether marrow or thymus repopulation is assessed. These results support the conclusion that both 5- and 10-week endpoints detect the same stem cell population, each member of which has the potential to differentiate along both lymphoid and myeloid lineages.

MATERIALS AND METHODS

Animals. Six- to 12-week-old (C57BL/6J × C3H/HeJ)F_1 (B6C3)F_1 male and female mice bred and maintained in the animal facility of the British Columbia Cancer Research Centre (Vancouver) from parental strain breeders originally obtained from The Jackson Laboratories were used in all experiments.

Preparation of Marrow Cell Suspensions. Bone marrow cells were obtained from the femurs of either normal mice or mice injected i.v. 4 days previously with 150 mg of 5-fluorouracil per kg (day 4 5-FU cells). Cells were suspended in alpha medium containing 2% fetal calf serum with a 21-gauge needle, filtered through a double layer of 20-μm nylon mesh, counted, and then resuspended to a concentration appropriate for injection.

Stem Cell Purification Procedure. Primitive cells were purified from male day 4 5-FU marrow as described (12). Briefly, marrow cells were indirectly labeled with anti-Thy-1.2 and anti-H-2K^b monoclonal antibodies. Cells with high forward and intermediate-to-high orthogonal light-scattering properties, and expressing low levels of Thy-1.2 and very high levels of H-2K^b antigens, were then isolated in a single step with a fluorescence-activated cell sorter (FACS 440; Becton Dickinson).

Competitive Repopulation Assay. In most experiments, lethally irradiated (800–850 cGy of total body x-irradiation; 124 cGy/min) female recipients were injected i.v. with various numbers of male test cells and 1 or 2 × 10^5 compromised marrow cells from female mice. Compromised cells had been subjected to two previous rounds of transplantation and regeneration in female mice (12). In experiments in which male recipients or male compromised marrow cells were used, these were prepared exactly as their female counterparts described above. Recipients of mixed transplants were sacrificed at the times indicated. Marrow and thymus cells were then isolated, pelleted in phosphate-buffered saline, and stored at −20°C before DNA extraction. DNA was purified from marrow and thymus, and the content of male cells was determined by Southern blot analysis with the pY2 plasmid as described (12). This allowed the reproducible detection of a ≥5% male contribution to total DNA. Accordingly, a contribution of ≥5% male cells was set as the requirement for a tissue to be scored as positive in the competitive repopulation assay. CRU frequencies were calculated from the resultant percentage positive recipients by standard limiting-dilution analysis procedures (15).

RESULTS

Origin of Regenerated Cells in the Competitive Long-Term Repopulation Assay. Fig. 1 outlines diagrammatically the competitive long-term repopulation assay procedure. In an initial series of experiments, we found that 10^5 compromised marrow cells alone are sufficient to allow lethally irradiated syngeneic recipients to survive long term (>30 days), although 10^6 such cells are insufficient to outcompete the marrow or thymus repopulating ability of 10^4 day 4 5-FU marrow cells (13). To quantitate more precisely the long-term repopulating potential of the compromised cell population, groups of lethally irradiated female mice were injected with 2 × 10^5 male compromised cells and then sacrificed 10 weeks later. As illustrated in Fig. 2 (group 1), Southern analysis of marrow and thymus with a Y-specific probe revealed the presence of readily detectable (≥5%) donor-derived (i.e., compromised cell) progeny in both myeloid and T-lymphoid lineages of most mice analyzed (10 of 17 = 59%; three experiments). To determine the relative contribution of reactivated surviving host stem cells under these same conditions, the identical experiment was performed with female compromised cells and lethally irradiated male mice. As illustrated in Fig. 2 (group 2), in many mice (10 of 13 = 77%; two experiments) endogenous stem cells were able to compete effectively against 2 × 10^5 compromised marrow cells and contribute significantly to the long-term reconstitution of both the marrow and thymus. Similar results were also obtained in the marrow of 5 of 6 (83%; one experiment) irradiated male mice injected with 10^6 compromised female marrow cells (data not shown).

We next evaluated the repopulating potential of both the compromised cells and the host when these were assessed in the context of the competitive repopulation assay using day 4 5-FU marrow cells as a potential source of cells with a high content of CRU. In these experiments, irradiated recipients were injected with 10^4 day 4 5-FU cells and 2 × 10^5 compromised cells. Male mice were used only for the component being evaluated so that the entire experiment was performed in triplicate a total of three times. Representative results are shown in Fig. 3. They indicate that although 10^4 day 4 5-FU

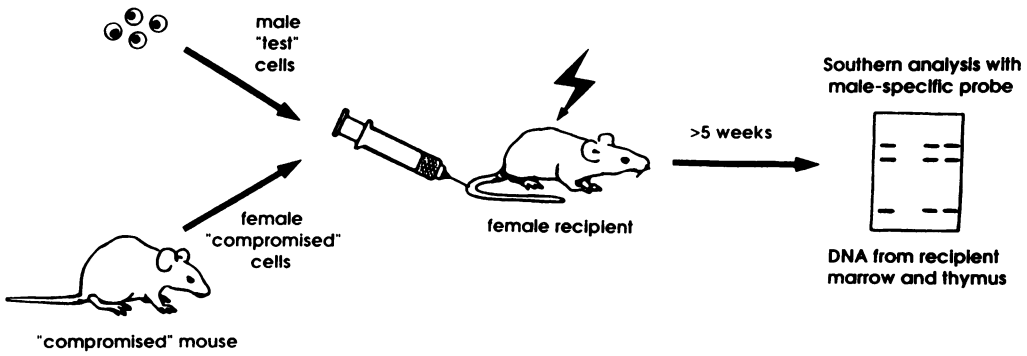

FIG. 1. Schematic representation of the competitive long-term repopulation assay. Lethally irradiated female mice are injected with limiting numbers of male test marrow cells together with 1–2 × 10^5 female compromised marrow cells. The proportion of male cells in the competitively repopulated marrow and thymus 5–10 weeks later is determined by Southern blot analysis with a Y-specific probe.

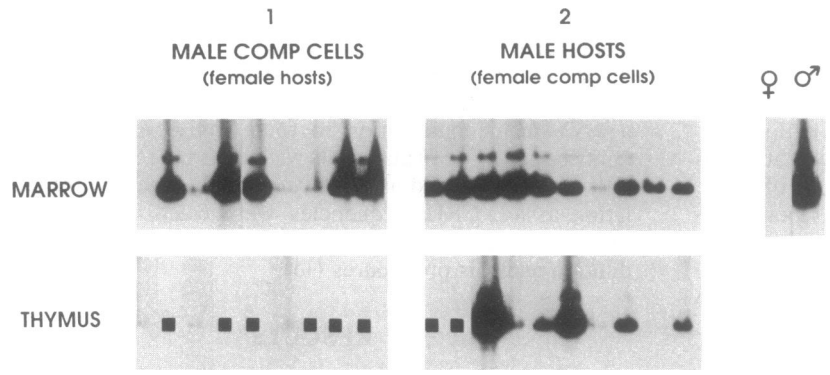

FIG. 2. Representative Southern blot analyses of bone marrow and thymus cells from lethally irradiated mice transplanted 10 weeks previously with 2×10^5 compromised marrow cells. Group 1, male compromised cells, female hosts (9 mice); group 2, female compromised cells, male hosts (10 mice). Solid squares designate lanes that were not loaded with any DNA (because of insufficient numbers of cells in the thymus of these animals).

cells are more competitive in reconstituting the marrow and thymus [i.e., 18 of 18 mice = 100% contained ≥5% of cells derived from the day 4 5-FU cell suspension (group 3)], in some mice (3 of 15 = 20%; three experiments) contributions to long-term hematopoiesis from cells in the compromised population were also detectable (group 4). Similarly, readily detectable contributions from endogenous stem cells in the irradiated host were also found in the majority of mice (group 5; 9 of 14 = 64%; three experiments).

Selection of Endpoint Time. To examine the effect of time after transplantation on measured CRU frequency, groups of female recipients were cotransplanted with various numbers of male test cells plus 2×10^5 compromised female cells, and tissue repopulation was assessed 5 and 10 weeks later. Three different test cell suspensions were compared in this way: normal marrow, day 4 5-FU marrow, and a highly enriched subpopulation of day 4 5-FU marrow obtained by FACS selection as described in *Materials and Methods*. For each test cell suspension, all available data were pooled and then the frequency of CRU was calculated (Table 1). Fig. 4*A* shows the results that were obtained for day 4 5-FU marrow based on analyses of marrow repopulation in recipients sacrificed 10 weeks after transplantation. The frequency of CRU derived from these data is 1 CRU per 1200 day 4 5-FU cells (95% confidence limits, 1/780 cells to 1/2000 cells). This frequency is similar to the value obtained when recipients were analyzed 5 weeks earlier (Table 1). The frequencies of CRU measured in normal marrow or in the sorted day 4 5-FU marrow subpopulation also appeared relatively insensitive to the time of recipient marrow assessment after 5 weeks, although the frequency of CRU in these test cell suspensions differed by a factor of 30–200.

Comparison of Thymus Versus Marrow Analyses. We have previously used retroviral marking of purified repopulating stem cells to show that the competitive repopulation assay can, and frequently does, detect a stem cell with lymphoid as well as myeloid repopulating potential (16). If this were a consistent selective feature of this assay, we would expect to measure similar CRU frequencies irrespective of whether marrow or thymus repopulation were assessed, given that sufficient time for both to have occurred had elapsed. In each of the experiments described above, both thymus and marrow from the recipients were therefore analyzed for the presence of male test cells and the results of CRU determinations were compared (e.g., Fig. 4 *B* versus *A*). As shown in the complete data set in Table 1, the tissue evaluated, like extension of the time interval prior to tissue evaluation from 5 weeks to 10 weeks, had no consistent effect on the calculated frequency of CRU in the different cell suspensions. Moreover, of the 170 mice in these experiments who were in groups in which some, but not all, animals showed repopulation of at least one tissue [marrow (BM) or thymus (Thy)] with male cells, 141 (83%) showed concordance of tissue repopulation when animals were analyzed on an individual basis (56 BM^+Thy^+; 85 BM^-Thy^-), with only 17% of mice showing discordance (16 BM^+Thy^-; 13 BM^-Thy^+) ($P < 0.005$; G test of independence).

DISCUSSION

A simple *in vivo* assay for a class of primitive hematopoietic stem cells with competitive long-term repopulating ability is described. By limiting-dilution analysis techniques, an estimate of the relative frequency of this stem cell in various test cell suspensions can be obtained. Such estimates are of necessity underestimates since the detection efficiency of the assay procedure is not known, but it is almost certain to be <1. We have therefore suggested the term competitive repopulating unit (CRU) to distinguish between the true number of competitive repopulating cells in a given suspension and the presumably minimum number detectable by the *in vivo* assay described here, as first suggested when the term CFU-S was introduced for the cells measured by the spleen colony assay (8).

As for any assay, it was important to establish the optimal input–output interval and to investigate the sensitivity of the results to variations in this interval. Initially, we chose 5 weeks as a minimum time to detect the progeny of cells more primitive than CFU-S. This was based on previous work by Hodgson *et al.* (11) suggesting that there exists a cell popu-

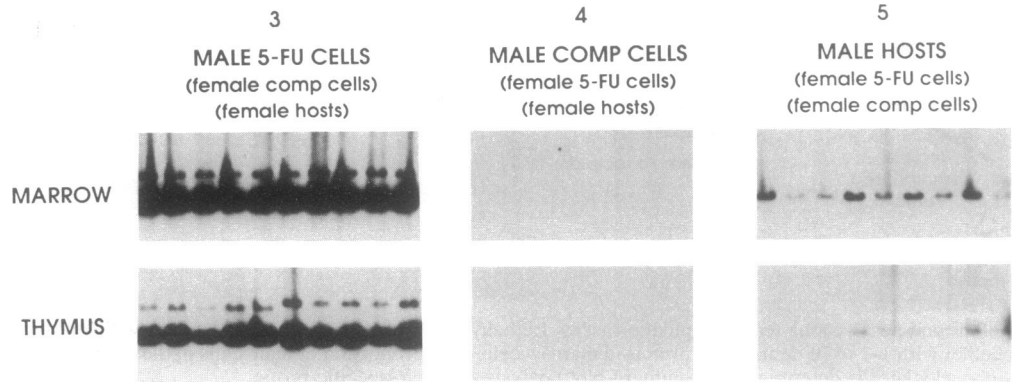

FIG. 3. Representative Southern blot analyses of bone marrow and thymus cells from lethally irradiated mice transplanted 10 weeks previously with 10^4 day 4 5-FU cells and 2×10^5 compromised marrow cells. Group 3, male 5-FU cells, female compromised cells, female hosts (10 mice); group 4, female 5-FU cells, male compromised cells, female hosts (8 mice); group 5, female 5-FU cells, female compromised cells, male hosts (9 mice).

Table 1. Comparison of the frequency of CRU in different marrow cell populations assessed by using different endpoints

Cells	5 weeks posttransplantation		10 weeks posttransplantation	
	Bone marrow	Thymus	Bone marrow	Thymus
Normal bone marrow	10,000 (6200–16,000)	10,000 (6000–16,400)	3600 (1600–8000)	15,700 (7200–34,300)
Day 4 5-FU bone marrow	2,700 (1300–5700)	1,300 (540–3100)	1200 (780–2000)	1,300 (830–2000)
Sorted day 4 5-FU bone marrow*	65 (40–120)	125 (65–250)	125 (65–240)	75 (40–135)

Results were determined by limiting-dilution analysis. Values shown are the reciprocal of the CRU frequency with the corresponding 95% confidence limits defined by ±2 SE (shown in parentheses) based on one to three pooled experiments (20 to 30 mice per experiment).
*CRU frequency in sorted cells is compensated for the 2-fold reduction in seeding efficiency due to antibody coating of the cells as noted (12).

lation in murine marrow that differs from CFU-S and that can be detected by measuring the number of day 13 CFU-S produced in the marrow of primary irradiated recipients 13 days after transplantation. In the present study, we compared the frequency of CRU in normal marrow, in marrow from 5-FU-treated mice, and in a highly purified subpopulation of day 4 5-FU marrow by using either 5- or 10-week endpoints, and we found no evidence that the additional 5 weeks made any difference. This is the result that would be expected if, after 5 weeks under the conditions used (i.e., under conditions of competitive repopulation), the composition of the recipient's marrow already reflected the proliferative activity of the most primitive hematopoietic cells present in the recipient immediately posttransplant.

As a further test of this hypothesis, we compared the CRU frequency derived from assessment of marrow versus thymus repopulation data. The results appear to be independent of the tissue evaluated between 5 and 10 weeks after transplantation. This provides further support for the view that either tissue endpoint detects the proliferative and differentiative activity of the same initial totipotent stem cell population. Additional evidence for this is indicated by the significant association found between test cell contributions to repopulation of the marrow and thymus of individual mice, in spite of considerable variation in the levels attained in either. Some variation in the contribution even of single totipotent cells to different tissues would be expected from previous studies of retrovirally marked clones (4, 5, 16). One possible explanation for this is that different myeloid and lymphoid compartments may have different rates of turnover. Also, some cells with the potential for generating both lymphoid and myeloid progeny may not actually express both because of the mechanisms that determine lineage commitment of totipotent cells *in vivo*. Although the molecular details of these mechanisms are still completely obscure, evidence that stem cell differentiation *in vivo* appears stochastic has been well documented (17, 18). Such a mechanism predicts that a proportion of totipotent cells will not express their full differentiation potential *in vivo* because they happen to become lineage restricted within their first division.

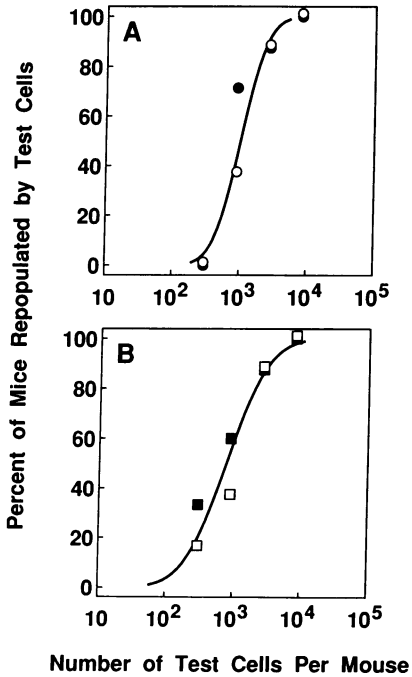

FIG. 4. Percent of recipients (8 animals per group) showing ≥5% repopulation of the marrow (*A*) and thymus (*B*) with male test cells 10 weeks after transplantation of various numbers of male day 4 5-FU marrow cells together with 2×10^5 female compromised marrow cells. The proportion of male DNA was determined by Southern blot analysis of marrow cell DNA from each recipient by using pY2. Circles and squares designate the data for mice from two separate experiments (experiment 1, open symbols; experiment 2, solid symbols).

It is interesting to note that the frequency determined here for CRU in normal B6C3F$_1$ marrow ($\approx 1/10^4$) is in the same range as the frequency of marrow stem cells estimated by other endpoints related to long-term repopulation of the hematopoietic system—i.e., rescuing lethally irradiated mice (10), curing W/W^v mice (19), or analyzing the number of clones contributing to hematopoiesis after 6 weeks (20). Clearly, the precise relationship between the cells detected in each case will depend on comparative measurements with highly purified stem cell populations. The prime advantage of the CRU assay is that it is designed to allow long-term repopulating cells to be specifically quantitated in any strain under such circumstances—i.e., in the absence of other cells that may in the short term influence survival and hence data acquisition. While short-term survival is not a problem when unirradiated W/W^v mice are used for repopulation studies, their own complement of hematopoietic cells may present a much greater competitive pressure than that which can be obtained by using serially transplanted marrow, resulting in a decreased sensitivity of the assay (i.e., decreased efficiency of stem cell detection). Several reports have suggested the feasibility of obtaining highly purified populations of mouse marrow enriched for stem cells with *in vivo* repopulating ability (12, 21–23). These purification strategies have already revealed differences in the extent to which CFU-S may be copurified, suggesting that the populations isolated may be subdivided further (9, 23). Application of the assay described here should help to achieve this goal as an essential step toward the ultimate characterization of lymphomyeloid repopulating cells at the molecular level. Similarly, quantitation of CRU in other tissues (e.g., spleen, blood, yolk sac, and

fetal liver), or in long-term marrow cultures, may provide critical information about stem cell numbers during ontogeny and after genetic, biologic (with growth factors), or pharmacologic manipulation *in vitro*.

Some hematopoietic stem cells able to contribute detectably to the long-term regeneration of both the marrow and thymus were found to be present in the serially transplanted compromised marrow cells as well as in mice given 800 cGy of x-rays. The contribution of such stem cells in the compromised marrow inoculum to the long-term reconstitution of hematopoiesis was largely outcompeted when 20- to 100-fold fewer unseparated day 4 5-FU cells were cotransplanted. However, the contribution of residual host stem cells to the long-term reconstitution of mice rescued with a graft of compromised marrow cells, although minor, remained detectable in many mice even when highly competitive day 4 5-FU cells were cotransplanted at numbers sufficient to dominate the regenerated tissues of most animals. These data are consistent with the concept that the majority of stem cells in the compromised population are qualitatively reduced in their repopulating ability by comparison to normal marrow, whereas the chief effect of the acute irradiation given to assay recipients is to greatly reduce, but not completely eliminate, the number of highly competitive repopulating stem cells. These data also imply that the long-term regeneration of hematopoiesis obtained in the assay we describe is commonly polyclonal with some contributions of progeny from both sources of transplanted cells as well as from the host. This is in apparent contrast to the monoclonal pattern of repopulation reported by several groups after primary transplantation of small numbers of effective marrow cells (3, 4, 24, 25). However, none of these latter studies examined specifically the contribution of residual host stem cells to hematopoietic recovery and many have relied on the use of W/W^v rather than acutely irradiated normal mice as recipients. The present findings thus highlight the likelihood of residual host stem cell activity even in lethally irradiated (800–850 cGy) but otherwise normal syngeneic recipients and encourage the investigation of additional procedures that may allow such host cells to be selectively recruited or suppressed according to experimental requirements.

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