RAPID COMMUNICATION

Expansion In Vitro of Retrovirally Marked Totipotent Hematopoietic Stem Cells

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A large number of biologic, technological, and clinical studies await the development of procedures that will allow totipotent hematopoietic stem cells to be expanded in vitro. Previous work has suggested that hematopoiesis can be reconstituted using transplants of cells from long-term marrow cultures. We have used retrovirus mediated gene transfer to demonstrate that marked totipotent hematopoietic stem cells are both maintained and can be amplified in such cultures, and then subsequently regenerate and sustain lympho-myeloid hematopoiesis in irradiated recipients. Marrow cells from 5-fluorouracil-treated male mice were infected with a recombinant virus carrying the neomycin resistance gene and seeded onto irradiated adherent layers of pre-established, long-term marrow cultures of female origin. At 4 weeks, cells from individual cultures were transplanted into single or multiple female recipients. Southern blot analysis of hematopoietic tissue 45 days posttransplantation showed retrovirally marked clones common to lymphoid and myeloid tissues in 14 of 23 mice examined. Strikingly, for 3 of 4 long-term cultures, multiple recipients of cells from a single flask showed marrow and thymus repopulation with the same unique retrovirally marked clone. These results establish the feasibility of retroviral-marking techniques to demonstrate the maintenance of totipotent lympho-myeloid stem cells for at least 4 weeks in the long-term marrow culture system and provide the first evidence of their proliferation in vitro. Therefore, such cultures may serve as a starting point for identifying factors that stimulate totipotent hematopoietic stem cell expansion.

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MATURE BLOOD CELL production depends on the continual activation of hematopoietic cells with extensive self-renewal, proliferation, and differentiation potential. The most primitive of these hematopoietic cells appear to be individually capable of maintaining normal numbers of a variety of lymphoid and myeloid cell types for many months, as shown by clonal analysis techniques including X-linked isoenzyme measurements,1,2 or genetic marking3-11 in combination with embryonic or adult reconstitution strategies.12 The ability to expand primitive totipotent hematopoietic cells in vitro would provide a significant step toward the further analysis and manipulation of stem-cell behavior and would also have important implications for gene therapy.

In the presence of appropriate combinations of sera, media, and other supplements, bone marrow cultures can be established that maintain hematopoiesis in vitro for many months.13 This is evidenced by the sustained production of mature myeloid elements and various hematopoietic progenitors. In addition, such cultures have been shown to contain cells capable of reconstituting the lymphoid and myeloid elements of supralethally irradiated mice.14-16 The extent to which such repopulation arises from persistent totipotent hematopoietic stem cells as opposed to cells with more restricted developmental potentials is not known. In this study we have used retroviral marking to track the fate of individual repopulating hematopoietic stem cells during and after 4 weeks' maintenance in long-term marrow cultures. Our results provide the first evidence that lympho-myeloid stem cells with long-term repopulating potential can persist in vitro under these conditions. Moreover, we now show that totipotent stem cells are stimulated to undergo self-renewal divisions in these marrow cultures, thereby giving rise to daughter stem cells that can reconstitute both lymphoid and myeloid systems of multiple-recipient animals.

MATERIALS AND METHODS

Retroviral infection and long-term culture. Bone marrow cells from male mice treated 4 days earlier with 5-fluorouracil (5-FU, 150 mg/kg body weight) were cultured in supernatant for 24 hours from a helper-free tkneo 19-2 producer cell line17-18 supplemented with 4 μg/mL of polybrene, 10% pokeweed mitogen-stimulated mouse spleen cell conditioned media,19 and 10% pokeweed-stimulated human leukocyte conditioned medium20 as described in detail previously.21 Mice were 6 to 12 weeks old (C57BL/6J X C3H/HeJ) F1, (B6C3F1) bred and maintained in the animal facility of the British Columbia Cancer Research Centre. Bone marrow cells were then washed and 3 x 10^6 cells plated on previously established 3-week-old irradiated (15 Gy x-ray) female long-term culture adherent layers. These adherent layers were from previously established Dexter long-term marrow cultures22 set up in 25-cm² flasks with an initial inoculum of 3 x 10^7 normal marrow cells. Cultures were given half media23 changes weekly for 4 weeks and then harvested for injection into irradiated (8 Gy) female recipients. Adherent layers were removed with a rubber policeman, washed, and passaged through a 21-g needle. Mice receiving long-term culture cells were killed 45 days later.

Southern blot analysis. High-molecular-weight DNA was isolated from various tissues and digested with HindIII or EcoRI, which cuts once in the proviral genome and releases a fragment unique to the integration site. Ten micrograms of digested DNA (5 μg for male control lane) was electrophoresed and analyzed by...
Southern blotting using a neomycin fragment from plasmid pMC1 as a probe. To ascertain the origin of repopulating cells, blots were stripped and reprobed with a Y-chromosome-specific fragment from plasmid pY2. To confirm T-cell origin of thymic DNA, all long-term culture recipients were screened by Southern blot analysis of HindIII-digested DNA using a T-cell receptor β1 constant region probe 86T5. In all instances thymic DNAs contained greater than 90% rearranged Cβ alleles (data not shown), similar to results described previously by others.

Colony forming unit-spleen (CFU-S) analysis. DNA for Southern blot analysis was extracted from well-separated individual spleen colonies excised 12 days after injection of 5 × 10^4 to 1 × 10^5 fresh marrow or long-term culture-derived cells into irradiated recipients. For generation of secondary spleen colonies, marrow was isolated from mice 45 days after reconstitution with long-term culture-derived cells.

RESULTS

Maintenance of lympho-myeloid stem cells. Bone marrow cells were isolated from male B6C3F1 mice that had been injected with 5-FU 4 days previously. The cells were then exposed in vitro to supernatant from a cell line producing helper-free recombinant retrovirus carrying the neomycin resistance (neo^R^) gene under conditions previously shown to achieve efficient levels of gene transfer to lympho-myeloid repopulating cells. Cells were washed and then some aliquots were injected directly into irradiated, syngeneic, female recipients to generate spleen colonies. The remaining cells were cultured on irradiated adherent cell layers of pre-established long-term marrow cultures of female origin. After 2 or 4 weeks, cultured cells were harvested and then transplanted into lethally irradiated, syngeneic, female recipients. Eighty-two percent of initial marrow CFU-S in these experiments showed integration of the neo^R^ gene (18 of 22 12-day-old spleen colonies were neo^R^ positive). A high frequency of neo^R^-positive CFU-S (44%, 14 of 32) was also detected in the cultures after 2 to 4 weeks. Analysis of total DNA from the cells in 4-week-old cultures using a restriction enzyme that cuts once in the proviral genome (to allow detection in Southern blots of unique integration fragments) showed a complex pattern of integration events in individual cultures consistent with the presence of multiple active clones in each flask (Fig 1). Southern analysis of DNA from the regenerated marrow, spleen, and thymus of recipients of these same cultured cells (one recipient per culture) assessed 6 to 7 weeks after injection of the cells typically showed more than 50% transplant-derived (male) cells in all three tissues. The presence of a single, uniquely marked clone in all three tissues was also documented (14 of 23 recipients showed 5% and up to 40% of marked-tissue DNA assuming the presence of one copy of the neo^R^ gene, established by comparison to the intensity of the signal obtained when the same blots were rehybridized with a murine granulocyte-macrophage colony-stimulating factor (GM-CSF) probe.)

Figure 1 shows the results of a representative experiment in which three of five mice showed this pattern. Marrow from one of these mice (mouse 3) was further transplanted into secondary irradiated female recipients and day 12 spleen colonies generated. All secondary spleen colonies were found to be both male and marked by the same integration fragment seen in the marrow of the primary recipient (Fig 1), thus confirming that the marked cells had been derived from a single male cell in the original population used to initiate the cultures. The remaining two mice in this experiment did not contain detectable levels of marked cells amongst the male population in either the marrow or thymus, although in one marked spleen cells were found.

Proliferation of totipotent stem cells. To determine whether cells with in vivo lympho-myeloid repopulating potential can also proliferate in long-term marrow cultures,
cells from individual culture flasks were transplanted into several female recipients. Multiple recipients of cells from a single flask showed repopulation of both the marrow and thymus by the same retrovirally marked clone in three of four such experiments (Fig 2).

The results illustrated by flask A are particularly striking. In all five primary recipients, most of the regenerated hematopoietic cells were male and all showed a prominent retrovirally marked clone in both lymphoid and myeloid lineages. In three of these mice (mice 1, 4, and 5) the same unique 6.4-kilobase (kb) *HindIII* proviral fragment was detected. Reanalysis of the same DNA after digestion with a different restriction enzyme (*EcoRI*) that, like *HindIII*, cuts once in the retroviral genome but at a different site, yielded a unique 15.5-kb integration fragment, again common to all nine tissue samples (data not shown). Retroviral proviruses were found to be intact in these cells and in all subsequent recipients. This was determined by Southern blot analysis using restriction enzymes that release the entire provirus (*KpnI*) or the neo<sup>R</sup> insert (*BamHI*) (data not shown). A second fragment of 8.7 kb was seen in both mouse 1 (in marrow) and mouse 4 (in spleen). This second shared fragment must also have been due to the proliferation first in vitro and then in vivo of another marked stem cell as shown by analysis of three secondary spleen colonies generated from the marrow of mouse 1. Two of these spleen colonies (a and c, Fig 2) were found to be retrovirally marked, although the fragments characteristic of each were different. Spleen colony a contained the 8.7-kb fragment, whereas spleen colony c contained the 6.4-kb fragment. All three spleen

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**Fig 2.** Clonal analysis of multiple mice transplanted 45 days previously with either the adherent layer or nonadherent fraction of single long-term cultures A, B, and C. Recipients received either 2 × 10⁶ cells from the adherent fraction or 5 × 10⁶ cells from the nonadherent fraction. Bone marrow (b), spleen (s), and thymus (t) DNA were assessed for proviral integration sites by digestion with *HindIII* (or *EcoRI* where indicated) and hybridization to a neo-specific probe. Blots were reprobed with a Y-specific fragment for assessment of donor origin. Individual 2<sup>nd</sup> spleen colonies (a through c) derived from CFU-S assays of marrow from mice M1, M2, or M3 reconstituted by flask A were similarly analyzed. Mk is a control lane derived from *HindIII*-digested DNA of the viral producer cell line tkneo186<sup>y</sup>-2 showing multiple proviral integration sites.
colonies were male. Similarly, for the other two primary recipients of flask A cells (ie, mice 2 and 3), the clones marked by 5.4- and 5.8-kb fragments, respectively, were able to generate secondary spleen colonies (Fig 2, top right panel). Three of five long-term recipients of marrow cells from mice 1 through 5 (flask A), were kept for 50 days after transplantation before killing for tissue analysis (Fig 3, mice 2*, 3*, and 4*). Two of these tissue analyses (mice 2* and 3*) showed regeneration of the original donor-derived clones in the tissues of secondary recipients. These results show the ability of retrovirally marked lympho-myeloid stem cells to maintain their very extensive proliferative and differentiation potentialities even after maintenance in culture for up to 4 weeks. Furthermore, in the case of flask A, the presence of a common clone in multiple lineages of several recipients indicates that lympho-myeloid stem cells can undergo self-renewal in culture before transplantation.

Similar findings were obtained from analyses of recipients reconstituted with cells from flask B. In this case three of four recipients (mice 2, 3, and 4) showed a common 6.2-kb HindIII fragment in one or more tissues (Fig 2, flask B). Digestion with EcoRI confirmed the presence of a common 6.0-kb band (data not shown). Interestingly, in these recipients substantial variation in the distribution of the neo \(^{+}\) positive clones in different tissues was seen. In mouse 2, bone marrow and spleen were marginally repopulated with this clone, whereas the thymus was strongly marked. In mouse 4, the neo \(^{+}\) signal in the spleen was intense, but in the marrow and thymus it was very weak. In mouse 3, a faint neo \(^{+}\) signal was observed only in the spleen. In contrast, the proportion of male cells in all tissues analyzed from each of these recipients was similar, indicating a significant contribution of other, unmarked clones to at least some lineages in many instances (eg, in the marrow and thymus of mouse 4).

Recipients of cells from a third culture (flask C, Fig 2) showed a more complex pattern of hematopoietic reconstitution by retrovirally marked cells. In two recipients the same two clones appeared to be present. Consistent intensities and cosegregation of restriction fragments suggested that this was due to the presence of two clones marked by multiple integration events rather than several independent clones (Fig 2). One of these clones marked by multiple HindIII fragments and, likewise, multiple EcoRI fragments (flask C, bands indicated by asterisks), appeared to have been derived originally from a totipotential cell because it gave rise to both marrow and thymus cells in mouse 1, although it appeared to have contributed only to the marrow of mouse 2 at the time of killing. The other clone originating from a cell in flask C that was also multiply marked (flask C, bands indicated by dots) was found in both the marrow and the thymus of mouse 1, but appeared restricted to the thymus of mouse 2. These findings illustrate the lineage or tissue restriction of clones that is frequently observed when recipients are analyzed at a single time point. This apparent restriction may simply reflect the detection limit of small subpopulations using Southern analysis, or the different turnover kinetics of mature lymphoid and myeloid cell types in vivo. Alternatively, it may reflect the generation in culture of stem cells.
that have retained extensive repopulating ability but that have become developmentally restricted.28

**DISCUSSION**

We have shown that conventional retroviral-marking techniques can readily detect the persistence of totipotent lympho-myeloid stem cells in 4-week-old long-term marrow cultures. Furthermore, the strategy of transplanting multiple recipients with the contents of a single flask has made it possible to obtain evidence of lympho-myeloid stem cell proliferation in this culture system. In most instances, clonal regeneration of both lymphoid and myeloid tissues was seen in mice killed 6 to 7 weeks after transplantation of cultured cells. In several instances, both short-term (spleen colony formation) and long-term (up to 7 weeks) regeneration of hematopoiesis by retrovirally marked cells in secondary recipients was demonstrable. At least some lympho-myeloid cells harvested from long-term marrow cultures must, therefore, have retained a very extensive potential for self-maintenance, sufficient to allow them to sustain hematopoiesis at a significant level for more than 3 months following transplantation in vivo. The only alternative interpretation, ie, of infection of initially totipotent stem cells with subsequent in vitro expansion of both myeloid and restricted stem cell progeny, seems unlikely given the high frequency with which individual animals contained clonal populations in both lymphoid and myeloid tissues. However, additional experiments, including use of competitive repopulation assays,21 will be required to determine if the totipotent cells indentified here represent the most primitive of lympho-myeloid reconstituting stem cells.

The ability to detect lympho-myeloid stem cell proliferation in culture makes possible the further investigation of the factors to which these primitive cells respond, as well as the nature of this response. Previous analyses of pluripotent hematopoietic cell commitment during colony formation either in vitro or in vivo have provided data consistent with a stochastic model of stem cell renewal and differentiation.29,30 Analogous studies of the progeny of individual pluripotent cells generated under conditions of long-term marrow culture have not been described, although it has been suggested that such conditions may favor the accumulation of lymphoid-restricted stem cells.31 The present studies thus serve as a starting point for delineating the earliest stages of hematopoietic cell development. They also provide impetus for the utilization of long-term marrow cultures for expansion of transplantable human hematopoietic stem cells in vitro, in particular for therapeutic applications requiring the biologic or genetic manipulation of hematopoietic stem cells in vitro.

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**REFERENCES**


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