Local Clonal Analysis of the Hematopoietic System Shows That Multiple Small Short-Living Clones Maintain Life-Long Hematopoiesis in Reconstituted Mice

By Nina J. Drize, Jonathan R. Keller, and Joseph L. Chertkov

We describe here a technique to study the clonal contribution of primitive stem cells that account for long-term hematopoiesis in the same mouse over a 14-month period. Specifically, irradiated recipient female mice were transplanted with retrovirally marked male hematopoietic progenitors. Bone marrow was then collected repeatedly from local sites from the same mice throughout a 14-month period and injected into secondary irradiated recipients for analysis of donor retrovirally marked day-11 colony-forming unit-spleen (CFU-S-11). We have tracked the temporal in vivo fate of 194 individual CFU-S-derived cell clones in 38 mice reconstituted with such retrovirally marked bone marrow cells. Our data show that long-term hematopoiesis is maintained by a large number of simultaneously functioning small, short-lived (1 to 3 months) clones that usually grow locally with little or no dispersion between different regions of the hematopoietic system. Furthermore, the clones that disappeared were never detected again. The data suggest that normal hematopoiesis is supported by the sequential recruitment of marrow repopulating cells into a differentiation mode.

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Hematopoiesis is a complex process of proliferation and differentiation resulting in the continuous production of large numbers of hematopoietic cells of at least 8 lineages. This process originates in single, pluripotent hematopoietic stem cells (HSCs), which are thought to be capable of self-renewal. A measure of the upper limit of the self-renewal potential of HSCs has never been obtained, although irreversible decline of HSC proliferative potential with passage in vivo has been observed.1,7

The production of large numbers of primitive hematopoietic cells may normally be restricted to early stages of development (eg, fetal life), as suggested by differences in the cycling status of these cells in fetal versus adult life.8 Thus, primitive cells in the normal adult might be anticipated to have high but limited proliferative potential and remain dormant until they are sequentially recruited into active hematopoiesis. This model for hematopoiesis is referred to as clonal succession.9 An alternative model assumes that HSCs are immortal and equal; hence, their activation into cell cycle or their probability of differentiation or self-renewal reflects an average contribution from the entire stem cell pool.10 Therefore, the main controversy concerning the characteristics of HSCs between these models is the potential for unlimited self-maintenance of stem cells. In vivo studies of HSC behavior can be performed by marking HSCs with integrated retroviruses to establish which model is correct.

There are at least 800 to 4,000 retroviral integration sites in the murine genome,11,12 which have allowed investigators to trace the progeny of single primitive stem cells marked with unique integrated provirus when transplanted into irradiated recipients.13-15 These early studies showed that HSCs can give rise to all hematopoietic cell lineages; however, the clonal composition of the hematopoietic system in these reconstituted mice and the ability of HSC to self-renew were not studied. Specifically, studies to determine the fate of individual HSCs were limited due to the need to kill reconstituted animals to obtain bone marrow (BM) cells. However, some retrovirally marked HSC-derived clones were found to persist many months and even for years,16-19 and hematopoiesis was always monoclonal or oligoclonal, with only 1 to 3 HSC clones contributing to constitutive hematopoiesis.14,20

These studies did not evaluate how long the clonogenic cell was in dormancy and what proportion of the clones contributes to mature cells with a long life span, eg, T- and B-memory cells. Nevertheless, even in these studies, the possibility that only a limited number of long-term repopulating HSCs were originally transplanted was not rigorously excluded. Studies to determine the developmental fate of individual HSCs marked with unique radiation-induced chromosomal abnormalities in murine long-term BM cultures also favors the clonal succession of stem cells.21 These limitations were partially overcome by repeated analysis of the peripheral blood of mice reconstituted with marked stem cells in which frequent fluctuations in the contribution of such cells to mature progeny in the circulation were seen during the first 4 to 6 months after engraftment.22,23 However, a stable hematopoietic system later emerged that was dominated by a small number (usually 1 to 2) of clones that was independent of the size of the cell inoculum.

In contrast to the conclusions of these studies, the polyclonal origin of hematopoiesis has been established by studies of X-chromosome-linked polymorphism of blood cells.24-27 In addition, the results of competitive repopulation assays argue that at least 10 different cell clones may function simultaneously.28

To best address these conflicting results, we developed a novel technique that allows us (1) to study the fate of individual clones of HSCs at the level of their short-lived progeny to avoid the masking effect of populations of long-lived cells (B and T cells), (2) to study local BM sites rather than peripheral blood, and (3) to detect small clones, preferably at the level of a single progenitor. This method involves...
sequential collection of retrovirally marked cells from the BM of the same mouse for up to 14 months. The clonal composition of these cells was determined by the colony-forming unit-spleen (CFU-S) assay, which is a quantitative, short-term assay for multipotential myeloid stem cells. An additional advantage of CFU-S is their short survival time (about 1 month) both in vivo and in vitro. Thus, this approach permitted us to study the fate of individual BM HSC clones at the level of their short-lived progeny to avoid the masking effect of some populations of long-lived cells. In our experiments, we observed that hematopoiesis was maintained for up to 14 months by the simultaneous functioning of many small short-lived hematopoietic clones that showed little or no dispersion between different regions of the hematopoietic system. In addition, there was no evidence of stable monoclonal or oligoclonal hematopoiesis in these mice.

MATERIALS AND METHODS

Mice and cytokine treatment. Twelve- to 25-week-old male and female BDF1 (C57BL/6 × DBA/2)F1 mice were used as donors and recipients, respectively. Recipient mice were exposed to 1,200 cGy [17]Cs irradiation (dose rate, 18 cGy/min; IPK irradiator; Institute of Radiation Technology, Moscow, Russia). The dose was divided into two equal exposures administered 3 hours apart. Male donors of BM cells were injected with a single intravenous dose of 5-fluorouracil (150 mg/kg body weight, Sigma, St. Louis, MO). Two days later, the femoral BM was flushed out and resuspended in a minimum essential medium (αMEM).

Retroviral vector produces cell line and long-term BM culture. GP + E-86 cells producing a retrovirus containing human ADA cDNA expressed internally from the human phosphoglycerate kinase (PGK) promoter (PGK-hADA) was a generous gift of D.A. Williams (Indiana University School of Medicine). The details of the vector have been previously reported. This cell line produces virus with a titer of 1 × 10^8 CFU/mL. The supernatants from this cell line was shown to be free of helper virus by the 3T3BAG mobilization assay. To rule out the presence of helper recombinant retroviruses in reconstituted mice, serum collected from killed animals was routinely assayed by this method. One day before infection, flasks with confluent producer cells were treated with a single intravenous dose of 5-fluorouracil (150 mg/kg body weight, Sigma, St. Louis, MO). Two days later, the femoral BM was flushed out and resuspended in a minimum essential medium (αMEM).

To generate BM stroma cultures, a modification of the Dexter method of long-term BM cultures (LTBMCs) was used. Briefly, the contents of one femur were flushed into a 25-cm 2 tissue culture flask (Flow, Irvine, CA) containing 10 mL of Fisher’s medium supplemented with 2 mMol/L L-glutamine, 14% horse serum (Vector, Novosibirsk, Russia), 7% fetal calf serum (FCS; Vector), 10^-4 mol/L hydrocortisone sodium succinate (Sigma). The cultures were placed in an incubator at 37°C and 5% CO2. Half of the medium was replaced weekly with fresh medium. Three- to 4-week-old cultures with confluent adherent cell layers (ACL) were irradiated (30 Gy) and used for prestimulation.

Infection of hematopoietic cells with recombinant virus. BM cells (2.5 × 10^7 cells/10 mL) were cultured for 2 days in 0.1% gelatine-treated T-25 flasks at 37°C in αMEM medium supplemented with 20% FCS, recombinant rat stem cell factor (rSCF; 50 ng/mL), and recombinant human interleukin-6 (50 U/mL; Amgen, Thousand Oaks, CA; protocol A). Prestimulated BM cells were transferred onto the monolayer of retrovirus producer cells in the media containing 4 μg/mL polybrene and the same cytokines for 48 hours. For protocol B, BM cells (1.5 × 10^7 cells/10 mL) were incubated for 2 days on irradiated ACL in the same media without cytokines (for prestimulation) and then transferred for 48 hours on the monolayer of op-tovirus producer cells without cytokines. The hematopoietic cells were recovered from retrovirus producer cell line (1.4 × 10^7 cells/flask in protocol A and 0.82 × 10^7 cells/flask in protocol B) and used for reconstitution in irradiated mice (2 to 9 × 10^7 cells injected per mouse).

Analysis of recipient animals. BM samples were obtained under ether anesthesia from individual reconstituted mice 1.5, 3, 6, 9, 12, 13, and 14 months after transplantation. BM was aspirated repeatedly alternately from the left and right femur by puncture through the knee joint with a 22-gauge needle. It was usually possible to obtain 5 to 15 × 10^6 BM cells from the femur of the living mouse. Aliquots of BM from each mouse were injected into 6 irradiated female recipients for CFU-S analysis and the rest of BM cells were used for DNA isolation.

Irradiated female mice were injected intravenously with 1 to 4 × 10^7 BM cells from reconstituted mice. Individual macroscopic spleen colonies were isolated under dissection microscope 11 days later and used both for DNA analysis and for determination of the CFU-S proliferative quality (number of CFU-S-8 per 11-day-old spleen colony). To determine the proliferative quality of individual CFU-S-11, half of the colony cells were injected into irradiated secondary recipients. The number of daughter colonies were counted 8 days later and the number of colonies generated per one CFU-S-11 was calculated (631 primary CFU-S-11-derived colonies were analyzed). Determination of CFU-S origin in reconstituted mice. Only male mice were used as donors of BM cells; recipients were always female. For identification of CFU-S origin, either polymerase chain reaction (PCR) analysis or Southern blot analysis was used. The primers were chosen in the C-terminal domain of the sex-determining region of mouse Y-chromosome (5'CTTCGTATGGCACAACATTTACG3' [sense] and 5'TGAGTGCTGTAGGTTGTCG3' [antisense]) and a 448-bp fragment of genome was amplified by PCR. Thirty cycles of PCR amplification were performed under the following conditions: denaturation for 45 seconds at 94°C, annealing for 45 seconds at 60°C, and extension for 45 seconds at 72°C. Some membranes were rehybridized with the Y-chromosome probe after stripping the ADA probe.

PCR and Southern blot analysis. Standard procedures were used in the preparation of high molecular weight genomic DNA samples. DNA from total BM and individual spleen colonies was extracted and PGK-hADA provirus was detected by PCR. Primers in ADA coding sequences (5'GACAAGCCCAAGTCTAAGCTC3' [sense] and 5'TGACCCCGAGTCTGCTCC3' [antisense]) amplified a 418-bp fragment of the proviral genome. Thirty cycles of PCR amplification were performed under the following conditions: denaturation for 30 seconds at 94°C, annealing for 30 seconds at 60°C, and extension for 30 seconds at 72°C. DNA from PCR-positive samples was used for standard Southern blotting techniques. Restriction digestion with EcoRI, electrophoresis through a 1% agarose gel, transfer to Hybond N filter, and hybridization with ADA cDNA probe prepared from an HindIII 1.2-kb PGK-hADA fragment or with an PCR-amplified 418-bp fragment of ADA gene were performed as described. Digestion with EcoRI permitted analysis of individual clones of hematopoietic cells, because only one EcoRI restriction site is present within the vector used. The alignment of bands was based on molecular weight standards (λ DNA digested with HindIII).

Statistics. Statistical analysis was performed using the Student’s t-test. The calculation of the number of CFU-S clones was performed using an equation of multinomial distribution (see Appendix).

RESULTS

Experimental strategy. As shown on Fig 1, BM donors were killed 2 days after treatment with 5-fluorouracil (5-
HEMATOPOIETIC CELLS CLONAL SUCCESSION

Donor mice
5 FU day -2
bone marrow
Prestimulation
48 hours

A
rrSCF+IL6

Cocultivation on the
virus producing cell line
48 hours

B
ACL

Recipient mice

reconstituted with

aspiration of bone marrow
from the same mouse
1.5, 3, 6, 9, 12 and 14 months
following reconstitution

secondary
irradiated
recipients

11 days CFU-S

1. PCR and Southern
blot analysis of DNA for
hADA and murine
Y-sequences
2. CFU-S-11 proliferative
quality (CPQ)

Fig 1. Schedule of experiment.

FU). Hematopoietic cells were prestimulated for 48 hours in media with rrSCF and rhIL-6 (protocol A) or on irradiated ACL of LTBMCs (protocol B). To mark the stem cells, after prestimulation, hematopoietic cells were cocultivated on a retrovirus producing cell line for 48 hours with the same cytokines (protocol A) or without cytokines (protocol B). Two to 9 x 10^6 transduced cells (containing 100 to 600 CFU-S-11; Table 1; see below) were collected and injected into irradiated recipients. Over a period of 14 months, BM from individual reconstituted mice was aspirated every 1.5 to 3 months and then injected into secondary irradiated recipients for the determination of CFU-S. Integration of retroviral DNA containing human adenosinedeaminase cDNA (hADA) and the donor origin of individual CFU-S colonies were assessed by PCR. Spectra of individual proviral integration sites were then determined in hADA-positive colonies by Southern analysis.

In addition, the proliferative quality of individual CFU-S-11 was determined by injecting individual CFU-S-11-derived colony cells into secondary irradiated recipients to determine CFU-S-8. Therefore, the CFU-S proliferative quality (CPQ) is equal to the number of CFU-S-8 in individual CFU-S-11 spleen colonies.

Finally, the surviving mice were killed 13 to 14 months

Table 1. Donor Cell Characteristics and Survival of Reconstituted Mice

<table>
<thead>
<tr>
<th>Protocol of Transduction</th>
<th>No. of CFU-S per 10^6 Cells</th>
<th>No. of CFU-S Tested for hADA Integration</th>
<th>Percentage of hADA-Positive CFU-S-11</th>
<th>No. of CFU-S (nucleated cells) Injected per Mouse</th>
<th>Survival of Reconstituted Mice (time after reconstitution)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>21.0 ± 3.6</td>
<td>10.0 ± 4.2</td>
<td>26</td>
<td>95</td>
<td>420 ± 64 (2 x 10^6)</td>
</tr>
<tr>
<td>B</td>
<td>7.0 ± 0.5</td>
<td>7.9 ± 2.0</td>
<td>25</td>
<td>71</td>
<td>619 ± 87 (8.8 x 10^6)</td>
</tr>
</tbody>
</table>

* Values are the number of mice that survived/the total number of mice, with percentages in parentheses.
after reconstitution and CFU-S from femurs, tibia, and spleen were tested for hADA in some of the mice. The total DNA from femurs, tibia, spleen, and thymus was analyzed for hADA in all mice.

Characteristics of transduced cells. To determine the number of transduced CFU-S-11 and their proliferative quality, cells from a portion of the each individual spleen colony were injected into secondary recipients for estimation of daughter CFU-S-8 content (proliferative quality) and the remaining cells were used for DNA analysis. The number of CFU-S-11 recovered after gene transfection of BM cells in vitro was threefold higher in cells transduced according protocol A (with exogenous cytokines) as compared with protocol B; however, the proliferative quality of these recovered CFU-S-11 was essentially the same in both groups (Table 1).

The efficiency of hADA integration into CFU-S-11 during 48 hours of transduction was very high (≈90%) for marrow cells transduced by protocol A. A slightly lower fraction (70%) of the CFU-S contained integrated hADA sequences after transduction by protocol B (without exogenous cytokines; Table 1).

Hematopoiesis in reconstituted mice. Injected BM cells that were transduced by different procedures were equally efficient in promoting short-term survival of irradiated recipients (45-day survival rate; Table 1). However, the long-term survival rate declined over time and was about 40% to 50% after 1 year (Table 1). This survival rate is significantly lower than that of fresh BM cells. Specifically, in our laboratory, the average 1-year survival of mice reconstituted with 2 to 3 × 10⁶ fresh BM cells is 80% to 95%. It is possible that the decrease observed here in long-term survival resulted from an exhaustion of donor hematopoietic stem cell activity, eg, due to inability to recover primitive stem cells from monolayer of virus-producing cells. Therefore, we determined the proportion of donor (Y-positive) CFU-S from individual mice over time. In mice reconstituted with BM cells that were prestimulated by exogenous cytokines, the proportion of donor CFU-S was 75% to 85% 9 months after reconstitution and decreased to 45% 12 months after reconstitution. In comparison, after reconstitution with marrow cells prestimulated on ACL, the proportion of donor CFU-S did not decrease from 9 to 12 months and was about 80%. Meanwhile, the long-term survival in both groups of mice was essentially the same. Therefore, decreased long-term survival was not due to the exhaustion of donor hematopoiesis.

The decreased long-term survival may also have been a result of a lower progenitor content in the BM of the reconstituted mice. To test this hypothesis, we studied the CFU-S content in the BM of the transplanted mice. There are approximately 150 to 200 CFU-S per 10⁶ BM cells in mice reconstituted with 2 to 3 × 10⁶ normal BM cells. The number of CFU-S in the BM in the present studies was 10-fold lower than that in normal mice or in mice reconstituted in an equivalent period of time with fresh BM cells. At 1.5 to 12 months after reconstitution, the CFU-S concentration was 12 to 25 CFU-S per 10⁶ BM cells in mice injected with cells transduced by both protocol A and protocol B (data not shown). Therefore, there was a direct correlation between decreased survival rate and decreased CFU-S content in reconstituted mice.

The decreased long-term survival could also have been a result of decreased proliferative potential of the CFU-S. However, despite the fact that the concentration of CFU-S in the BM of reconstituted mice was significantly reduced, their proliferative quality did not change. Specifically, individual CFU-S-11 from mice transplanted with BM cells produced about 10 to 20 CFU-S-8 (Fig 2). Integration of hADA did not influence the proliferative potential of CFU-S, because the proliferative quality of donor CFU-S was essentially the same in both untransduced and transduced CFU-S-11 colonies (Fig 2A and B). Furthermore, the proliferative quality of individual CFU-S colonies containing 1 to 2 or 3 to 15 copies of hADA sequences per genome was not different (Fig 2C). Taken together, our data suggest that the decreased long-term survival was not due to the proliferative quality of individual CFU-S-11 and that retroviral integration did not effect the proliferative quality of the CFU-S-11.

Dynamics of transduced CFU-S in reconstituted mice. To determine the temporal in vivo contribution of stem cell-derived clones to BM hematopoiesis, we studied 194 individual CFU-S-11 marked colonies in 38 recipients that received retrovirally marked BM cells. Specifically, BM was aspirated from the same mice 1.5 to 14 months after reconstitution and injected into secondary recipients for CFU-S-11 DNA analysis for integration of hADA sequences and for donor-derived Y-specific sequences (Fig 3). For example, mouse no. 13 was reconstituted with BM cells transduced by protocol A, and its DNA contained unique retroviral integration in individual CFU-S-11 6 months after reconstitution (Fig 3B, mouse 13, clones 2 through 7). Interestingly, hADA-containing DNA fragments observed in CFU-S DNA derived from the same mouse after 9 months were not the same as those seen after 6 months (Fig 3B, clones 8 and 9). Similar results are shown for mice no. 12 and 16.

The genotype (unique hADA integration sites) of 113 individual CFU-S clones in mice reconstituted with marrow cells transduced by protocol A after 3 to 13 months is summarized in Fig 3A. For example, in mouse no. 12, 10 donor CFU-S-11--derived colonies were examined after 6 months. Three unique integration patterns were detected with 5, 3, and 2 colonies for each genotype (clones 1 through 3). After 9 months, all four marked colonies had the same genotype (clone 4). After 12 months, we analyzed eight marked colonies and they had five unique patterns, with 2, 1, 3, 1, and 1 colonies for each pattern, respectively (clones 5 through 9). The other mice (mouse no. 13 through 18) are similarly represented. In addition, the hADA-containing DNA fragments observed in individual CFU-S-11 were not detected on Southern blots of total marrow DNA. For example, in mouse no. 16, 9 months after reconstitution, 9 different clones of CFU-Ss were detected (clones 20 through 28), but total marrow DNA showed evidence of only one unique clone that differed from all 8 marked CFU-S--containing clones (Fig 3B, mouse no. 16, bm-9). These findings indicate that the size of the CFU-S--containing clones was usually small in terms of their representation in later compartments of cells.
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A number of daughter CFU-S per 11-day colony

1.5 months 6 months

10 20 30 40 50

all pos neg all pos neg

Fig 2. CPQ from BM of reconstituted mice. BM cells from reconstituted mice were injected into irradiated recipients. Part of the cells from a single 11-day spleen colony were injected to the next irradiated recipients and the number of spleen colonies was estimated 8 days later. DNA from half of the cells from each isolated spleen colony was analyzed by PCR for hADA sequences. Twenty five to 53 CFU-S-11 were analyzed per group. Each column with error bar represents the mean ± SE. all, all analyzed colonies; pos, hADA-positive colonies; neg, hADA-negative colonies. (A) Mice engrafted with BM cells prestimulated with exogenous cytokines (protocol A). (B) Mice engrafted with BM cells prestimulated on irradiated ACL (protocol B). (C) Southern blots of individual clones of CFU-S-11 with the same proliferative quality. The numbers above each lane designate unique clones.

Individual clones were also rarely detected more than once during the whole experiment from individual mice. Among 113 clones examined in 7 mice, we found only 2 persistent clones (Fig 3A, asterisks). Rapid changes in the proportion of donor:recipient hematopoiesis also suggest that the life span of individual clones was typically relatively short (<3 months).

The kinetics of 53 of 82 studied clones in mice reconstituted with marrow cells that were prestimulated on irradiated ACL (protocol B) is shown in Fig 4A (data from mice in which marked CFU-S-derived clones were detected only once in the experiment are not shown). The results observed with protocol B were similar to those obtained with cells from protocol A. In agreement with the above observation,
### A

time after reconstitution (months)

<table>
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<th>Mouse</th>
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<tr>
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### B

![Southern blot analysis](image)

Fig 3. Temporal dynamics of clones in mice reconstituted with BM cells prestimulated with exogenous cytokines (protocol A). (A) Individual clones of HSC-derived CFU-S. Each horizontal lane represents the number of spleen colonies with an unique integration site. The size of rectangle indicate the number of spleen colonies with the same unique marker; rectangles with an asterisk indicate the persistent clones. The numbers in the rectangles represent the unique identification number of clone in each mouse. (B) Analysis of 4 long-term reconstituted mice. DNA from each of the cell populations obtained from reconstituted animals and CFU-S-derived spleen colonies was cleaved with EcoRI and analyzed by Southern blot. The numbers above the lanes are the same as the unique number of clones in the rectangles on (A). bm-9, total DNA from femoral BM 9 months after reconstitution; tib, fem, spl, thy, and pbl, total DNA from tibia, femur, spleen, thymus, and peripheral blood, respectively.
HEMATOPOIETIC CELLS CLONAL SUCCESSION

**A**

**time after reconstitution (months)**

<table>
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<tr>
<th></th>
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</table>

**number of marked spleen colonies**

**B**

Fig 4. Temporal dynamics of clones in mice reconstituted with BM cells prestimulated on ACL without exogenous cytokines (protocol B). (A) Individual clones of HSC-derived CFU-S (see legend to Fig 3A). (B) Analysis of 5 long-term reconstituted mice (see legend to Fig 3A).
Table 2. The Location of Unique Hematopoietic Cell Clones at Different Anatomical Locations in Reconstituted Mice (Killed 13 to 14 Months After Engraftment)

<table>
<thead>
<tr>
<th>Mouse No.</th>
<th>Tibia</th>
<th>Femur</th>
<th>Spleen</th>
<th>Thymus</th>
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<tbody>
<tr>
<td>11</td>
<td>ND</td>
<td>○</td>
<td>□</td>
<td>▽</td>
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<td>12</td>
<td>—</td>
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<td>▽</td>
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<tr>
<td>26</td>
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<td>—</td>
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<td>28</td>
<td>—</td>
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<td>○</td>
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</tbody>
</table>

Symbols define unique clones in an individual mouse; the same symbols are used for different mice.

most of the clones did not survive for more than the period between marrow analyses, ie, 3 months. Only one CFU-S-11 clone persisted at both 3 and 6 months after engraftment (mouse no. 37; Fig 4A, asterisk), but even this clone was no longer detected in the samples of total marrow DNA taken more than 6 months after reconstitution. The unique CFU-S-11-containing clones usually differed from the major clones that were detected in whole BM DNA, again suggesting that their size in these mice also was relatively small. However, the unique markers observed in CFU-S-11-derived colonies were typically absent from the accompanying sample of total marrow DNA, even when multiple clonal CFU-S-11 were detected in the same samples (eg, mouse no. 25 and 26, 9 months after engraftment, and mouse no. 37, 6 months after engraftment).

Interestingly, major clones that repopulated the entire hematopoietic system were also rare. For example, in mouse no. 38, which was killed 14 months after reconstitution, the same unique CFU-S-11 clone was detected in the total DNA sample from the femur and tibia, whereas another clone was detected in the thymus, but no hADA sequences were detectable in the spleen (Table 2 and Fig 4B). Repeated analysis of CFU-S-11 in the same mouse detected the same HSC-derived clone in two spleen colonies derived from femoral BM cells and in the total DNA from the femur and the tibia 1 month later. In mouse no. 26, two unique CFU-S clones were observed 12 months after engraftment. Only 1 month later, 5 different CFU-S clones were detected in tibia of this mouse. One of them was also found in tibia DNA. A unique clone was observed both in the femur and spleen DNA of this mouse; an additional unique clone was detected in its thymus (Table 2 and Fig 4B). Therefore, even major clones often repopulated only part of the hematopoietic system.

The number of hematopoietic cell clones in reconstituted mice. The number of simultaneously functioning clones contributed to hematopoiesis over time was calculated from the data in Figs 3 and 4 from the equation of multinomial distribution (Appendix). This suggested the highest number of clones (∼35) to be functioning in mice reconstituted with BM cells that were transduced with exogenous cytokines and this number remained constant between 3 and 12 months after transplantation (Fig 5). In mice injected with marrow cells transduced without exogenous cytokines, the number of functioning clones was somewhat lower, at about 20 per mouse (Fig 5).

Comparison of kinetics of transduced and nontransduced CFU-S. As was shown earlier, the proliferative quality of transduced CFU-S-11 is not lower than that of CFU-S-11 in fresh BM cell suspension. However, it was important to know the quality of transduced pre-CFU-S, ie, MRC. To determine this, we compared the temporal kinetics of transduced and nontransduced CFU-S-11 in both groups of reconstituted mice. Stable proportions of CFU-S transduced with hADA among Y-positive (donor) CFU-S were observed only in mice reconstituted with the cells prestimulated on ACL (protocol B); there were about 40% to 60% (Fig 6B). Different kinetics of hADA-positive CFU-S were shown in animals reconstituted with BM cells prestimulated with exogenous cytokines (Fig 6A). The proportion of CFU-S transduced with hADA gradually reached a maximum at 6 months after reconstitution. At this time, 80% to 85% of the CFU-S-11 contained hADA sequences. Thereafter, the percentage of transduced CFU-S decreased.

DISCUSSION

Using repeated analysis of the CFU-S-11 progeny of retrovirally marked transplanted MRCs, we showed that many hematopoietic cell clones function simultaneously in reconstituted recipients. The HSC-derived clones had a relatively small size, such that the same clone was not detected in the total tissue DNA sample, even in the cases in which all marked CFU-S originated from the same clone. The vast majority of clones were only detected in individual CFU-S-11 colonies. Thus, analysis of CFU-S-11 origin throughout the life of the same reconstituted recipient mice allowed us to show polyclonal contribution to hematopoiesis for close to the lifetime of reconstituted mice.

Interestingly, HSC progeny did not repopulate the entire hematopoietic system, because unique cell clones detected in specific anatomical locations such that the CFU-S-11 clones in the femur, tibia, spleen, and thymus were often different. Even major clones that were detected in total DNA of a particular tissue and represented at least 10% of hematopoietic cells in this tissue were unequally distributed in different parts of hematopoietic system. The existence of organ restricted bands was often explained by repopulation with cells that were already committed to lymphoid or myeloid lineages.41,42 Our data suggest a different explanation, ie, that there is little or no migration of stem cells between hematopoietic territories.

It was shown here that the vast majority of hematopoietic clones have a short lifetime, usually not longer than 3 months. In fact, we have found only few persistent clones of the 194 studied. In contrast to these observations, only one or few hematopoietic cell clone have been repeatedly observed in BM, spleen, and thymus of reconstituted mice.13,15,41 Differences between our observations and the earlier reports are likely to come from the different sensitivity of detection. Our approach is highly sensitive and allows us to identify small cell clones at the level of a single CFU-
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Fig 5. Calculated probability of the clone number in reconstituted mice 3, 6, and 9 months after engraftment. The number of CFU-S-11 clones was calculated for each individual mouse. Each column with error bar represents the mean ± SE the number of clones from all mice in the appropriate group.

S. All such small clones could not been shown by the usually used methods of Southern blot analysis from total DNA of hematopoietic tissue. Nevertheless, we have also observed lower numbers of major ubiquitous clones, which is in agreement with the earlier findings.42,43

The frequency of cells capable of long-term multilineage reconstitution of irradiated mice has been a subject of active debate for many years, and values ranging from 1 per 10⁴ cells to 1 per 10⁵ cells have been reported.44-46 We report here evidence of CFU-S-11 progeny of more than 100 different MRCs functioning over a 1-year period in two femurs in mice repopulated with 2 to 9 × 10⁶ cells harvested from 4-day cultures of marrow cells from mice treated with 5-FU 2 days previously. This implies a frequency of long-term repopulating cells (MRC) in these culture harvests of 1 per 2 to 9 × 10⁴. We do not know if this is the real content of MRC in BM or if their number was increased during cultivation.

Complex regulation of primitive HSC may be damaged by dissociation of HSC from their microenvironment and by pharmacologic concentrations of cytokines. Transduction according to protocol A recruited MRCs into the cell cycle,
which resulted in a twofold to threefold increase of the number of simultaneously functioning clones. This was followed by selective exhaustion of transduced MRCs, i.e., the progenitors that have been mobilized from dormancy. During transduction, hADA integrate into virtually all CFU-S. The proportion of marked CFU-S decreased up to 25% at 1.5 months and reached a maximal level of up to 80% at 6 months. Hence, even 1.5 months after engraftment, hematopoiesis is maintained by pre-CFU-S cells rather than by CFU-S, which agrees with the findings of Spangrude et al. It is thus possible that transduced MRC are used preferentially over time. 

Prestimulation of donor cells on irradiated ACL without exogenous cytokines (protocol B) resulted in about half of the MRCs marked with an integrated provirus, and this number remained stable throughout the observation period. Because efficient transduction is thought to require proliferating cells, our gene transfer procedure may have led to a recruitment of the majority of MRC into cell cycle. However, we observed hematopoietic clonal succession in reconstituted animals. The clones that disappeared were never detected again, suggesting that MRC can return to a state in which they no longer contribute to the production of more differentiated cells. This ability of primitive HSCs may be preserved in some leukemic stem cells, thus accounting for the existence of leukemia cells without relapse for many years after therapy.

The in vitro manipulations that are required to introduce genetic markers may modify MRC in a way that is largely unpredictable. We have shown here that MRCs manipulated in culture are capable of producing CFU-S with normal proliferative capabilities. However, the number of MRC-derived CFU-S is decreased in the BM of engrafted mice. The radio-protective effect of such cells is also lower than that of normal MRCS. These characteristics of MRCs are hardly caused by integration of the foreign sequences. We compared the proliferative potential of individual CFU-S with and without integrated foreign DNA by measuring their proliferative quality. It was shown that integration of hADA into murine MRC did not change this very important and complex index independently of the number of integrated copies of hADA per genome.

In conclusion, the results of this study challenge prevailing concepts of hematopoiesis in the posttransplant recipient. Under the experimental conditions we used, clones of hematopoietic cells persisting even for a period of 1 year were not observed. Even in mice that appeared to have been reconstituted with a large number of MRC, the proliferative potential they exhibited appeared limited. These MRC appeared to differentiate sequentially and produce small short-living (about 3 months) clones of CFU-S, which were only found in restricted hematopoietic sites. In the BM of one femur of an engrafted mouse, several dozens of such clones could be simultaneously observed. Larger clones that repopulated all hematopoietic territories were rarely detected.

However, our data should be viewed with caution for a number of reasons. A high degree of polyclonality and the small number of marked colonies analyzed in some samples hampered the detection of persisting clones. In the system we used, hematopoiesis was probably reconstituted with a limited number of MRCs (probably not more than 1% of their numbers in a entire mouse). These MRC were separated from the stromal microenvironment, recruited into the cell cycle, and transduced with foreign DNA sequences. It is not clear that normal steady-state hematopoiesis is ever reestablished in this system. To trace HSC lineage in vivo in the absence of gene transfer and cell transplantation, we plan to study the kinetics of individual CFU-S clones marked with unique radiation markers in sublethally irradiated mice.

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APPENDIX

Statistical Procedure for Calculation of the Number of CFU-S Clones

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The assumption of the model is that all clones contribute equally to hematopoiesis. Let \( t = (t_1, t_2, \ldots, t_k) \) is a random sample representing results of test, where: \( t_i - \) the number of colonies belonging to \( i \)-colony \((i = 1, 2, \ldots, k)\), \( \Sigma t_i = N, N - \) total number of tested colonies. Let \( n \) is the true number of colonies, \( n \hat{=} \) estimation of \( n \).

In assumption that the probability of each colony is the same and equals \( 1/n \), the conditional probability of sample \( \hat{\tau} \) in condition of number of colonies equals \( n \) in the following:

\[
P(\hat{\tau}|n) = \frac{n!N!}{(n-k+1)!\prod_{i=1}^{k} t_i \left(\frac{1}{n}\right)^n}
\]

Equation (1) is deduced from multinomial distribution by multiplying it by \( n!/\left(n-k+1\right)! \), in view of the fact that colonies were not "named" previously.

In supposition that the upper limit of possible colony number is \( M \) and a priori probability distribution is uniform a posteriori probability of the true colony number to be \( n \) is

\[
P(n) = \frac{n!}{\Sigma_{j=0}^{M} \frac{j!}{(j-k+1)! \left(\frac{1}{j}\right)^n}}
\]

So the maximum a posteriori probability estimation of true clone number is the following:
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\[ n^3 = \max_{n \geq k} \left( \frac{n!}{(n-k+1)!} \left( \frac{1}{n} \right)^n \right) \]  

(3)

That is, \( n^3 \) estimation is independent of assumption about upper limit of colony number and experimental distribution tested colonies among colonies \((t_1, t_2, \ldots, t_k)\), but only upon the value of \( k \) — the number of different colony in the experiment.

The upper \( 100(1 - \alpha) \) present confidential interval limit for colony number \( n^3 \) is a corresponding percentile of a posteriori distribution function:

\[ F(x) = \sum_{j=k}^{n^3} \frac{j!}{(j-k+1)!} \left( \frac{1}{j} \right)^n \]

(4)

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