Mobilized peripheral blood stem cells (PBSC) are used as a source of hematopoietic stem cells for transplantation and gene therapy. It is still unclear, however, whether the PBSC are fully equivalent to normal bone marrow hematopoietic stem cells and whether they are able to provide long-term function of transgene in reconstituted mice. In the present study, mobilized PBSC from male mice were transduced with human adenosine deaminase gene (hADA) and were used for reconstitution of lethally irradiated female mice. At 1\% , 3, 6, 9, and 12 months after reconstitution, the bone marrow cells were repeatedly collected from each mouse under light anesthesia and the number of colony-forming unit-spleen (CFU-S), spleen repopulating ability (SRA), and reconstitution. One to 9 individually labeled clones could be used for reconstitution of lethally irradiated female mice. At 5/2 months posttransplantation, but after an additional 4 months, SRA of mice reconstituted with bone marrow cells was fivefold higher as compared with those engrafted by PBSC. The integration of the human ADA gene was observed during 9 months in about 60% of studied CFU-S. The proportion of marked colonies sharply decreased 1 year following reconstitution. One to 9 individually labeled clones could be shown simultaneously by Southern blot hybridization in the same reconstituted mice during the whole period of observation. The time of clone existence was about 3 months. We conclude that long-term marrow repopulating cells mobilized into circulation by treatment with granulocyte colony-stimulating factor (G-CSF) and stem cell factor (SCF) are capable of maintaining lifelong polyclonal hematopoiesis in reconstituted mice.

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Aspiration of bone marrow cells for DNA analysis. Bone marrow was aspirated repeatedly from the left and right stripping the ADA probe. It was usually possible to obtain 5 to 15 million bone marrow cells from each mouse. Aliquots of bone marrow samples were obtained under light ether anesthesia from individual femurs, in turn, by puncture through the knee joint with a 22-gauge needle. It was usually possible to obtain 5 to 15 × 10⁶ bone marrow cells from the femur of the living mouse. Aliquots of bone marrow from each mouse were injected into six irradiated female recipients for CFU-S analysis and the remaining bone marrow cells were used for DNA isolation.

**Table 1. Donor Cell Characteristics**

<table>
<thead>
<tr>
<th>Donor Cells</th>
<th>Before Gene Transfer Procedure</th>
<th>After Gene Transfer Procedure</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of CFU-S per 10⁶ Cells</td>
<td>No. of CFU-S per 10⁶ Cells</td>
</tr>
<tr>
<td>Peripheral blood</td>
<td>10.3 ± 1.3</td>
<td>2.1 ± 0.2</td>
</tr>
<tr>
<td>Bone marrow</td>
<td>3.0 ± 0.8</td>
<td>1.4 ± 0.2</td>
</tr>
</tbody>
</table>

**Table 2. Survival of Reconstituted Mice**

<table>
<thead>
<tr>
<th>Donor Cells</th>
<th>1.5 mo</th>
<th>9 mo</th>
<th>12 mo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peripheral blood</td>
<td>11/12</td>
<td>7/11</td>
<td>5/11</td>
</tr>
<tr>
<td>Bone marrow</td>
<td>12/12</td>
<td>9/12</td>
<td>6/12</td>
</tr>
</tbody>
</table>

**RESULTS**

One day after the course of cytokine injections, peripheral blood and bone marrow cells were harvested from donor
Table 3. CFU-S Content (per 10^6 cells) in Bone Marrow of Reconstituted Mice

<table>
<thead>
<tr>
<th>Time After Reconstitution (mo)</th>
<th>Donor Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Peripheral blood</td>
</tr>
<tr>
<td>1.5</td>
<td>17.7 ± 5.0</td>
</tr>
<tr>
<td>3</td>
<td>28.6 ± 3.6</td>
</tr>
<tr>
<td>6</td>
<td>8.3 ± 1.2</td>
</tr>
<tr>
<td>9</td>
<td>12.1 ± 2.5</td>
</tr>
<tr>
<td>12</td>
<td>24.3 ± 4.6</td>
</tr>
</tbody>
</table>

Concentration of CFU-S in the bone marrow of normal BDF1 and mice reconstituted with normal bone marrow cells is about 150 to 200/10^6.

Mice and transduced by retroviral vector containing human ADA sequence. After gene transfer, peripheral blood and bone marrow cells were used for reconstitution of lethally irradiated recipients. The relative content of CFU-S after the procedure of gene transfer was only 1.5 to 2 per 10^5, both for peripheral blood mononuclear and bone marrow cells, lower than in the initial inoculum. The number of CFU-S was reduced twice in bone marrow. A more pronounced (fivefold) decrease was observed for peripheral blood mononuclear cells. Moreover, the SRA of freshly harvested donor cells was significantly higher than SRA of cells after gene transfer (Table 1).

The survival of mice reconstituted with peripheral blood cells did not differ significantly from those transplanted with bone marrow cells (Table 2). Donor hematopoiesis was observed in both groups of reconstituted mice. Nine months after reconstitution, the PCR analysis showed that 75.3% ± 6.0% of CFU-S was Y-positive in the group transplanted with peripheral blood mononuclear cells. The same degree of donor CFU-S (62.8% ± 8.5%) was observed in bone marrow-transplanted animals. One year after engraftment the proportion of donor CFU-S decreased to 44.0% ± 9.0% and 42.2% ± 12.4%, respectively.

Bone marrow samples were collected at 1.5, 3, 6, 9, and 12 months after reconstitution from each mouse and the concentration of CFU-S, their self-renewal capacity, and percent of CFU-S marked with hADA gene were determined. It has been shown earlier that 2 to 12 months posttransplantation the concentration of CFU-S in bone marrow of mice reconstituted with 2 to 3 × 10^6 normal (not transduced) bone marrow cells was 150 to 200 CFU-S per 10^6 cells, which is 10-fold higher than that observed in the present experiment following the procedure of gene transfer (Table 3). PCR analysis of individual CFU-S—derived colonies showed that 1.5 to 9 months after reconstitution 30% to 80% of CFU-S were infected with hADA-retrovirus; 12 months after engraftment the proportion of transduced CFU-S decreased to 12% to 25% (Fig 1). The proportion of CFU-S transduced with hADA gradually reached a maximum at 3 to 6 months after reconstitution. At this time 75% to 80% of the CFU-S-11 contained hADA sequences. Thereafter, the percentage of transduced CFU-S decreased, particularly in mice reconstituted with peripheral blood cells, to reach 15% to 20% 1 year after reconstitution.

The influence of hADA sequence integration on the quality of CFU-S was studied by comparison of the proliferative potential of marked and unmarked CFU-S (Table 4). There was no difference in SRA of untransduced CFU-S and those that carried the foreign gene. SRA of bone marrow CFU-S in mice reconstituted with peripheral blood cells did not differ from SRA of initially injected CFU-S 1.5 months after transplantation. However, 4.5 months later a fivefold decrease of their SRA was observed. In contrast, SRA of bone marrow CFU-S from mice reconstituted with bone marrow cells was stable for 6 months.

The DNA from hADA positive colonies was also studied (fivefold) decrease was observed for peripheral blood mononuclear cells. Moreover, the SRA of freshly harvested donor cells was significantly higher than SRA of cells after gene transfer (Table 1).

![Proportion of marked CFU-S in bone marrow of mice reconstituted with peripheral blood mononuclears and bone marrow cells. Each column with error bar represents the mean ± standard error (SE). A total of 10 to 14 CFU-S were analyzed per group. Y-axis, % of marked CFU-S; X-axis, time after reconstitution).](image-url)
different pattern of hADA integration in CFU-S–derived colonies (Fig 2). Integration of 1 to 13 hADA copies per genome can be seen. The unique sites of integration allowed the identification of individual clones. For 12 months, numerous individually marked CFU-S–derived clones were observed in the bone marrow of mice reconstituted both with peripheral blood mononuclear and bone marrow cells. The clonal composition of the CFU-S compartment from different reconstituted mice is shown in Figs 3 and 4. One to nine individual clones were functioning simultaneously. Clonal fluctuation was shown during 12 months of observation. Mono-oligoclonal hematopoiesis was shown mainly in mice reconstituted with peripheral blood mononuclear cells. It was observed in all 5 mice studied 3 months after reconstitution, in 1 mouse (no. 1) 6 months and in 3 mice (no. 4, 33, and 34) 9 months after engraftment (Fig 3). In mice reconstituted with bone marrow cells mono-oligoclonal hematopoiesis was observed only rarely (no. 22 and no. 23 9 months and no. 21 12 months after engraftment) (Fig 4). Most of the animals had polyclonal hematopoiesis during the entire period of observation. The number of clones in an individual mouse changed with time and different clones, as a rule, were observed at different sampling times. The diversity of clones was significantly higher in mice reconstituted with bone marrow cells as compared with the peripheral blood group. Only 4 clones of 115 studied were carrying the same

**Table 4. Self-Renewal Capacity of CFU-S in Bone Marrow of Reconstituted Mice**

<table>
<thead>
<tr>
<th></th>
<th>1.5 mo</th>
<th>6 mo</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Daughter CFU-S/Colony</td>
<td>Daughter CFU-S/Colony</td>
</tr>
<tr>
<td></td>
<td>All Colonies</td>
<td>hADA Positive</td>
</tr>
<tr>
<td></td>
<td>hADA Negative</td>
<td>hADA Negative</td>
</tr>
<tr>
<td>Donor Cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peripheral blood</td>
<td>22.2 ± 3.8</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>25.2 ± 7.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4.2 ± 0.9</td>
<td>7.4 ± 2.2</td>
</tr>
<tr>
<td></td>
<td>4.3 ± 3.5</td>
<td></td>
</tr>
<tr>
<td>Bone marrow</td>
<td>16.2 ± 3.6</td>
<td>19.6 ± 4.1</td>
</tr>
<tr>
<td></td>
<td>12.2 ± 4.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>18.9 ± 8.1</td>
<td>14.2 ± 4.1</td>
</tr>
<tr>
<td></td>
<td>19.8 ± 15.3</td>
<td></td>
</tr>
</tbody>
</table>

Fig 2. Long-term clonal behavior of engrafted hematopoietic stem cells. Analysis of six long-term reconstituted mice. DNA from CFU-S–derived spleen colonies was cleaved with EcoRI and analyzed by Southern blot. Mouse no. 1, 31, and 32 were reconstituted with peripheral blood mononuclears; mice no. 21, 22, and 23 were reconstituted with bone marrow cells. Mouse no. 1, lanes 1 to 3 (clones 10 and 11 on Fig 3) - 6 months; lanes 4 to 11 (clones 28 to 32 on Fig 3) - 9 months; lanes 12 to 20 (clones 38 to 42 on Fig 3) - 12 months after reconstitution. Mouse no. 31, lanes 1 to 3 (clones 1 and 2 on Fig 3) - 3 months; lanes 4 to 11 (clones 2 and 12 to 16 on Fig 3) - 6 months. Mouse no. 32, lanes 1 to 4 (clones 3 and 4 on Fig 3) - 3 months; lanes 5 to 11 (clones 4 and 17 to 19 on Fig 3) - 6 months. Mouse no. 21, lanes 1 to 5 (clones 24 to 28 on Fig 4) - 6 months; lanes 6 to 10 (clones 27 and 34 to 36 on Fig 4) - 9 months; lane 12 (clone 62 on Fig 4) - 12 months. Mouse no. 22, lanes 1 to 7 (clones 29 to 33 on Fig 4) - 6 months; lanes 8 to 10 (clones 57 and 58 on Fig 4) - 9 months; lanes 11 to 12 (clones 63 and 64 on Fig 4) - 12 months. Mouse no. 23, lanes 1 and 2 (clones 34 and 35 on Fig 4) - 6 months; lanes 3 to 6 (clones 59 and 60 on Fig 4) - 9 months; lanes 7 to 13 (clones 65 to 69 on Fig 4) - 12 months after reconstitution.
HEMATOPOIESIS IN MICE RECONSTITUTED WITH MOBILIZED PB CELLS

A 3 months after reconstitution

mouse 31
mouse 32
mouse 33
mouse 34
mouse 35

B 6 months after reconstitution

mouse 1
mouse 31
mouse 32
mouse 33
mouse 34

C 9 months after reconstitution

mouse 1
mouse 4
mouse 33
mouse 34

D 12 months after reconstitution

mouse 1
mouse 34

number of marked colonies

0 1 2 3 4 5 6 7 8 9 10 11 12

Fig 3. Temporal dynamics of clonal fluctuation in mice reconstituted with peripheral blood mononuclear cells. Each horizontal lane represents the number of spleen colonies with a unique integration site. The size of the rectangle indicates the number of spleen colonies with the same unique marker; rectangles with symbols indicate the persistent clones; numbers in rectangles indicate unique clone identification number. (A) Three months after reconstitution; (B) 6 months after reconstitution; (C) 9 months after reconstitution; (D) 12 months after reconstitution. Y-axis, reconstituted animals; X-axis, number of individual colonies.

DISCUSSION

This study represents the detailed longitudinal analysis and comparison of hematopoiesis in mice reconstituted with transduced peripheral blood mononuclear and transduced bone marrow cells. Long-term survival was essentially the same in both groups of reconstituted mice. The proportion of donor hematopoietic precursor cells did not differ during 9 months and decreased sharply 1 year after reconstitution, particularly in the peripheral blood group.

The data suggest that PBSC are a good alternative to bone marrow cells for transplantation. However, this conclusion comes with reservation. Self-renewal ability of CFU-S from mice reconstituted with bone marrow cells was stable for 6 months after transplantation, while in PBSC reconstituted mice, SRA decreased fivefold by the end of this period. The significance of this effect is not clear, as both types of cells reconstituted hematopoiesis equally well and no selective exhaustion of donor peripheral blood-derived CFU-S occurred during 12 months.

The concentration of CFU-S, as well as their SRA in bone marrow of mice reconstituted with transduced PBSC or bone marrow cells, decreased sharply as compared with mice engrafted with nontransduced bone marrow cells. It is unlikely that this defect of hematopoiesis is induced by intensive treatment of donors with cytokines because similar reduction in CFU-S concentration and their SRA was observed in mice reconstituted with transduced bone marrow cells from nontreated mice (manuscript submitted). The decrease of CFU-S proliferative potential, as measured by SRA assay, cannot be explained by proviral integration. The model of sequential analysis of individual CFU-S allowed the direct experimental study of this problem. There was no difference in the SRA of nontransduced CFU-S and those marker during a 3-month interval (clones 2, 4, and 25 in peripheral blood group, Fig 3, and clone 27 in bone marrow group, Fig 4). Persisting clones were detected in three of six mice reconstituted with peripheral blood cells and in only one mouse engrafted with bone marrow cells. Therefore, the persistence of clones seems to have been longer in the peripheral blood group.
ACKNOWLEDGMENT

The authors gratefully acknowledge the help of Dr Dimitry Ku-prash in choosing primers for PCR analysis of hADA and sex-determining region of Y-chromosome. We express our appreciation to Drs Regina Turetskaya, Sergey Nedospasov, and Sergey Sokol for helpful suggestions in molecular biology techniques. We also thank Dr D. Williams for providing us with the ADA-producing cell line.

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13. Bridell RA, Hartley CA, Smith KA, McNiece IK: Recombinant rat stem cell factor synergizes with recombinant human granulocyte-colony stimulating factor in vivo in mice to mobilize peripheral stem cell clones derived from peripheral blood and bone marrow long-term repopulating cells. The general thesis of colony-forming cells was that the first 4 months postengraftment are characterized by frequent clonal fluctuation. Gradually, however, a stable hematopoietic system emerges, dominated by a small number of clones. Here we report the first continuous analysis of hematopoietic clonal kinetics in the compartment of CFU-S. It was shown that hematopoiesis on the level of CFU-S is polyclonal during at least 12 months after reconstitution. CFU-S both in vivo and in vitro have only short-term repopulating ability. Hence, CFU-S analyzed 3 to 12 months after reconstitution represent progeny of primitive long-term repopulating cells, rather than CFU-S transduced in the initial cell inoculum. One to nine individual clones of simultaneously functioning hematopoietic progenitors have been observed in the bone marrow of mice reconstituted both with PBSC and bone marrow cells at least during 12 months (Figs 3 and 4). Taking into account that only a small number of CFU-S-derived colonies were analyzed for each sampling time and that the majority of them had unique site of integration, the number of simultaneously functioning clones may have reached several dozen. The number of functioning clones in mice reconstituted with bone marrow was higher as compared with PBSC, on the average, about 1.5-fold to 2-fold (Figs 3 and 4). It was also shown early by multinomial analysis of marked clones that after normal bone marrow transplantation, the number of simultaneously functioning clones is higher than was shown here after PBSC engraftment. This suggests that after a 2-week course of cytoxic administration, the content of clonogenic long-term repopulating cells in peripheral blood is somewhat lower than in bone marrow.

The life of a hematopoietic cell clone is about 3 months. Here we observed only four clones (of 115 studied) persisting for 3 months and none for 6 months (Figs 3 and 4). The clones, which disappeared, never returned. These findings, as well as data published elsewhere, support the model of hematopoietic clonal succession, with limited proliferative potential of primitive HSC. However, the reservations are needed since persisting clones may be missed among numerous simultaneously existing clones.
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Long-Term Maintenance of Hematopoiesis in Irradiated Mice by Retrovirally Transduced Peripheral Blood Stem Cells

Nina Drize, Joseph Chertkov, Elena Sadovnikova, Stefan Tiessen and Axel Zander