Developmental Potential of Hematopoietic Stem Cells Determined Using Retrovirally Marked Allophenic Marrow

By Gary Van Zant, Jau-Jiin Chen, and Kimberly Scott-Micus

Genetic markers of two general types have been used to assess the number of simultaneously productive stem cells in vivo, retrovirus markers and enzyme or hemoglobin variants. Use of the two techniques has led to different conclusions regarding stem-cell population organization, kinetics, and usage. To better understand this discrepancy, we have combined the two methods by retrovirally marking and transplanting stem cell populations of allophenic mice in which all tissues, including the hematopoietic system, are chimeric. Hematopoietic and lymphoid tissues of engrafted recipients were analyzed by Southern blotting to determine the number and extent of participation of individually marked stem cells. Genotypic chimerism of the same tissues was determined by quantitating electrophoretic variants of glucose phosphate isomerase. This procedure permitted the genotypic identification of individual stem-cell clones. The results demonstrate the participation of few pluripotent stem cells in the repopulation and maintenance of engrafted hematopoietic and lymphoid tissues. Furthermore, stem cells used during the period of early engraftment tended to be of one genotype (DBA/2), whereas stem cells used for long-term maintenance tended to be of the other, coexistent genotype (C57BL/6). We propose that this genotypic specificity reflects functional differences in stem-cell subpopulations and their relative prevalence in different mouse strains.

The purpose of these studies was to assess contributions to hematopoiesis of two coexistent stem-cell populations in an experimental paradigm in which allophenic marrow was engrafted in irradiated recipients. Allophenic marrow cells, a mix of hematopoietic and lymphoid cells derived from two mouse strains, are immunologically cotolerant as a result of their temporal coexistence from the 8-cell stage of embryonic development. The genotypically distinct stem-cell populations, when transplanted into irradiated hosts, are put in a setting where together they must replenish the lympho-hematopoietic tissues of the recipient. Intrinsic differences in the abilities of the engrafted stem cells to repopulate and sustain these tissues may be manifested as fluctuations in the genotypic chimerism of lymphoid and blood-forming tissues. We recently used this approach to study allophenic mice compounded from embryos of strains characterized by inherently different stem-cell population kinetics. Allophenic marrow cells derived from a component strain with a relatively high fraction of proliferating stem cells (DBA/2) had a potent competitive advantage in the first crucial weeks of engraftment in comparison with stem cells of the partner strain, C57BL/6. In contrast, the stem-cell population of C57BL/6 origin, with an inherently lower index of proliferation, supplied an increasingly large proportion of blood and lymphoid cells over subsequent periods, in some cases over 1 year. In the present studies we retrovirally marked stem cells of allophenic mice in an effort to determine whether or not chimerism following transplantation, and its pattern of change with time, was because of simultaneous proliferation and differentiation of few or many stem cells.

Factors determining the allocation of stem cells between a proliferatively quiescent reserve pool on one hand and a proliferating, differentiating pool on the other hand, is at present poorly understood. Indeed, the number of stem-cell clones simultaneously contributing to blood cell formation remains unresolved; different experimental approaches have yielded different conclusions. Mintz et al have studied host- and donor-cell contributions to hematopoiesis in congenitally anemic hosts (W locus mutants) injected in utero with a mixture of fetal liver cells from two inbred mouse strains. Erythropoiesis in these chimeras was oligoclonally derived, and in some cases was monoclonal, over the course of about 1 year, with a succession of stem-cell clones contributing to the red blood cell (RBC) pool. These results support a clonal succession model of hematopoiesis proposed by Kay. Studies of radiation chimeras and stem-cell-deficient mice engrafted with retrovirally marked, syngeneic, stem cells have similarly shown that hematopoiesis, under these conditions, was oligoclonal. Some clones temporally waxed and waned and in some mice pluripotent, and stable, clones had productive lifespans of well over 1 year. On the other hand, studies of allophenic mice and radiation chimeras, in which the fluctuation of component genotypes contributing to hematopoiesis was analyzed using binomial statistics, concluded that blood cell formation was polyclonally derived, with most, if not all, stem cells simultaneously active. Our recent studies of hematopoietic chimerism in allophenic mice and in hosts engrafted with allophenic marrow addressed the issue of the genotypic derivation, but not the number, of active stem-cell clones. Given the fact that hematopoiesis in chimeras must have at least two stem-cell sources, we have little further information concerning stem-cell numbers. The present studies were undertaken to address this question using the retroviral-marking technique and show that lymphohematopoiesis, under the conditions of study, is likely derived from few stem cells of both genotypic derivations and that the ratio of contributing clones fluctuates in a predictable temporal fashion, perhaps reflecting genetically
controlled differences in stem-cell proliferation and self-renewal.

MATERIALS AND METHODS

**Allophenic mice.** Allophenic mice were constructed from embryos of C57BL/6 and DBA/2 inbred strains according to techniques described in detail by Mintz and using modifications we have previously described. Briefly, 8-cell embryos were flushed from the uteri of the two strains of mice on the morning of the second day of pregnancy. Pregnancy was determined by the presence of a vaginal plug in spontaneously mated females (day 0). The zona pellucida was removed from the embryos by a brief exposure to 0.5% pronase and they were manipulated together under a dissecting microscope and allowed to aggregate. The chimeric embryos were cultured overnight in the wells of Terasaki plates or glass spot plates containing Brinster’s medium (GIBCO, Grand Island NY). In the afternoon of the next day the embryos, at the early blastocyst stage, were transferred to the uteri of pseudopregnant B6D2F, females mated 2 days previously with vasectomized males. Chimeras were reliably born 17 to 20 days later.

Mice, including B6D2F, hybrids, were obtained through the Animal Resources Division of the National Institutes of Health (NIH) and were housed and maintained according to NIH guidelines. Allophenic mice were housed on encased cage racks supplied with sterilized air; cages, bedding, food, and water were sterilized.

**Glucose phosphate isomerase (GPI) analysis.** RBCs and leukocytes were prepared using discontinuous Percoll (Pharmacia, Piscataway, NJ) gradients exactly as described. Buffy coat cells were collected from the interface of a one-step Percoll (1.095 g/mL) cushion. Cell suspensions were prepared from lymph nodes, thymus, and spleen by squeezing them between frosted ends of microscope slides into culture medium. Wherever possible, connective tissue was excluded from analysis. Resulting cells were lysed by exposure to 0.5% pronase and they were manipulated together under a dissecting microscope and allowed to aggregate. The chimeric embryos were cultured overnight in the wells of Terasaki plates or glass spot plates containing Brinster’s medium (GIBCO, Grand Island NY). In the afternoon of the next day the embryos, at the early blastocyst stage, were transferred to the uteri of pseudopregnant B6D2F, females mated 2 days previously with vasectomized males. Chimeras were reliably born 17 to 20 days later.

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3.5 x 10^6 CPM/μg DNA. Blots were hybridized overnight at 42°C and washed at a final stringency of 0.2 x SSC, 0.25% sodium dodecyl sulfate (SDS) at 65°C. Autoradiograms were exposed for 2 to 6 weeks in cassettes with two intensifying screens (Lightning Plus; Kodak, Rochester, NY).

Copy number quantitation. On each gel we ran at least two lanes containing Sac I cut DNA from a 3T3 fibroblast line carrying exactly one copy per genome of a provirus that contained Neo (a gift of C. Jordan and I. Lemischka). The Neo copy number was obtained for experimental samples by direct comparison with the signal on the film originating from the marked fibroblasts. We carefully quantified the DNA in our samples using Hoechst 33358 staining and quantitative fluorometry based on a DNA standard curve. Five micrograms of DNA of each of the samples, including the Neo standard, was loaded per lane. Comparison of signals from Neo standards on the same gel were routinely within 10% to 15% of one another according to analysis of the film using a computer-assisted image analyzer (BioImage Visage 2000; BioImage, Ann Arbor, MI). Pilot experiments in which we analyzed signals from serial dilutions of DNA from the marked fibroblasts (mixed with appropriate amounts of unmarked DNA so that the same total amount of DNA was loaded per lane) showed that we could accurately quantitate Neo when present at a copy number ranging from 0.1 to 2.0.

RESULTS

Figure 1 shows the contributions to erythropoiesis of three genotypically distinct stem-cell populations—donor (C57BL/6 and DBA/2) and recipient (BDF,)—during 16 months after allophenic marrow engraftment. Genotypic chimerism in the RBC pool was determined by computer-assisted image analysis of electrophoretograms stained to show GPI isozyme patterns. As we previously found, stem cells of all three genotypes may make contributions to the RBC pool at different times after engraftment. Contributions of the donor stem cells followed a predictable and dynamic pattern in which DBA/2 representation was predominant in the first 3 months, far outstripping its representation in the marrow graft (Fig 1, 0 time point). Over the course of the subsequent 13 months, DBA/2 contributions, in this group of 12 BDF, hosts, steadily declined and C57BL/6 representation concomitantly increased. BDF, RBCs were found in some, but not all (5 of 12), hosts during the course of the experiment. This result occurred despite the administration of a supra-lethal irradiation dose (12.0 Gy) to recipients before engraftment.

One of the recipients with BDF, representation is instructive. As seen in Table 1, its RBC picture changed from one entirely of donor origin at 6 months to one almost entirely of host origin at 8 months. During this 2-month period (actually 52 days), a BDF, stem cell, supplying at least erythropoiesis, was apparently activated and in little more than a RBC lifespan (45 days) became the sole source of RBCs. However, this BDF, clone was transiently productive and, at the 12-month bleed, there were no remaining BDF, RBCs.

Of interest to us was whether the dynamic pattern of donor genotypic representation was effected by oligoclonal stem-cell shifts, as suggested by the example depicted in Table 1, or perhaps more commonly, by polyclonal contributions. The decline of DBA/2 blood cells with time, and the concomitant increase in C57BL/6 blood cells, could be because of subtle numerical shifts in a large population of contributing stem cells. Alternatively, the genotypic allocations could be due to dramatic genotypic redistributions in an oligoclonal population and the gradual genotypic changes at the level of mature RBCs may represent the relatively long lifespan of RBCs, in addition to the unknown lifespans and in vivo proliferative potential of erythroid precursors characterized in vitro as CFU-E and BFU-E. Accordingly, we infected allophenic marrow with retrovirus to mark stem cells and engrafted irradiated BDF, hosts. The virus (kindly provided by I. Lemischka) is called CRE gag + 19. Salient features include a deletion in the 3’ long terminal repeat (LTR) to permit the virus to be transcribed only once, and inclusion of part of the viral gag to enhance virus titer. The selectable marker, Neo, was driven by the chicken β-actin promotor.

![Figure 1](image1.png)  
*Fig 1. Genotypic composition of RBCs in engrafted mice as a function of time after allophenic marrow transplant. Chimerism of the RBC populations was determined by electrophoresing RBC lysates, staining for GPI activity, and determining the relative contributions of DBA/2, C57BL/6 (B6), and B6D2F1 (F1) genotypes by computer-assisted image analysis of electrophoretograms stained to show GPI isozyme patterns. As we previously found, stem cells of all three genotypes may make contributions to the RBC pool at different times after engraftment. Contributions of the donor stem cells followed a predictable and dynamic pattern in which DBA/2 representation was predominant in the first 3 months, far outstripping its representation in the marrow graft (Fig 1, 0 time point). Over the course of the subsequent 13 months, DBA/2 contributions, in this group of 12 BDF, hosts, steadily declined and C57BL/6 representation concomitantly increased. BDF, RBCs were found in some, but not all (5 of 12), hosts during the course of the experiment. This result occurred despite the administration of a supra-lethal irradiation dose (12.0 Gy) to recipients before engraftment.*

*Table 1. Genotypic RBC Percentages in a BDF, Recipient 6, 8, and 12 Months After Engraftment*

<table>
<thead>
<tr>
<th>Time After Engraftment</th>
<th>6 mo</th>
<th>8 mo</th>
<th>12 mo</th>
</tr>
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<tbody>
<tr>
<td><strong>Donor</strong></td>
<td><strong>Host</strong></td>
<td><strong>Donor</strong></td>
<td><strong>Host</strong></td>
</tr>
<tr>
<td>DBA</td>
<td>B6</td>
<td>F1</td>
<td>DBA</td>
</tr>
<tr>
<td>45</td>
<td>55</td>
<td>0</td>
<td>0</td>
</tr>
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</table>

*Note the steady decline in DBA/2, after early predominance, and the concomitant increase in C57BL/6.*
layer of CRE-gag + 19 virus-producing cells for 2 days, again in the presence of IL-3 and IL-1α, and 2 × 10⁶ cells were injected into lethally irradiated (11.0 Gy) BDF₁ recipients. Figure 2 demonstrates that these manipulations did not alter the dynamics of genotype-restricted stem-cell repopulation of the hosts. As seen first in Fig 1 using uninfected marrow, DBA/2 blood cells, both RBCs and white blood cells (WBCs) in Fig 2, were predominately (about 80%) of DBA/2 origin initially, despite only a 40% representation in the pool of injected marrow cells. Over the ensuing months DBA/2 contributions decreased and C57BL/6 cells became predominant. BDF₁, blood cells were not found in any recipient after the initial 1-month bleeding, a time when residual host RBCs were still present. As we have previously found,² WBCs, in relationship to RBCs, were always skewed toward the C57BL/6 genotype.

We determined that progenitor cells of both genotypes had been retrovirally infected by growing BFU-E and CFU-GM, in the presence and absence of G418, in cultures initiated with the same pool of infected cells that were injected into irradiated recipients (Table 2). Infected cells expressing transduced Neo may survive and grow in the presence of G418, whereas uninfected cells do not survive. The genotype of individual colonies was determined by GPl analysis showed that genotypic representation in precursor populations was almost identical to the overall composition of the marrow (40% DBA/2). The infection rate for BFU-E was 17% to 20%, depending on genotype, and for CFU-GM it was 12% to 15%. Thus, as anticipated, progenitors of both genotypes had roughly equal chances of being retrovirally infected.

Table 3 shows results from a separate experiment in which we assessed the fraction of CFU-S of each genotype infected in allophenic marrow. The marked allophenic marrow was injected into irradiated C57BL/6 and DBA/2 hosts because each permits the growth of only syngeneic colonies. The graft consisted of 43% DBA/2 cells overall and the frequency of CFU-S of the two genotypes was roughly equal (10.7 v 11.4 per 1 × 10⁶ cells injected). The rates of infection for CFU-S of the two genotypes were 36% for C57BL/6 and 52% for DBA/2, based on analysis of 25 to 28 colonies of each genotype. The higher infection rate of CFU-S in this experiment in comparison with the lower values obtained in Table 2 for CFU-GM and BFU-E probably reflects differences between experiments and the fact that the results in Table 2 require expression of Neo whereas only its presence is detected by Southern blotting. Although 12-day CFU-S are arguably not pluripotent stem cells, this experiment demonstrates that cells at least this primitive, of each genotype, are infected in allophenic marrow.

Table 2. Clonogenic Precursors of Both Genotypes in Allophenic Marrow Were Retrovirally Infected

<table>
<thead>
<tr>
<th></th>
<th>Without G418</th>
<th>With G418</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DBA/2</td>
<td>C57BL/6</td>
</tr>
<tr>
<td>BFU-E</td>
<td>13</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>41 (41%)</td>
<td>59</td>
</tr>
<tr>
<td>CFU-GM</td>
<td>12</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>41 (41%)</td>
<td>59</td>
</tr>
</tbody>
</table>

Clonogenic precursors of both genotypes in allophenic marrow were retrovirally infected. Methylocellulose cultures were established with 5 × 10⁶ cells/mL without G418; the cultures with G418 (1 mg/mL) initially contained 2.5 × 10⁶ cells/mL. The percentage of precursors infected was obtained by dividing the number of colonies analyzed from cultures without G418 by the product of the number of colonies in cultures with G418 and the ratio between numbers of cells plated in the two groups.⁵

Abbreviation: B6, C57BL/6.
C57BL/6 and DBA/2 CFU-S in allophenic marrow were retrovirally marked. Allophenic marrow cells (1 x 10⁵) with an overall chimerism of 43% DBA/2 cells were injected into irradiated (1,200 cGy) C57BL/6 and DBA/2 recipients (10 each). Twelve days later individual spleen colonies were dissected from the spleens of host mice and the genotype of each was determined by GPI and the DNA from the same colonies was probed for the presence of provirus.

Reflecting the fact that some cells had multiple proviral integrations and others were not infected (Table 2). The Dra I digest showed a smeared signal, which is consistent with analysis of a cell population consisting of many uniquely marked cells. The ethidium bromide-stained gel showed that the smeared signal was not due to incomplete digestion of the DNA by Dra I (data not shown).

Mice engrafted with this infected marrow were bled 14 to 16 months after transplant (Fig 3, hosts 1 through 3). WBCs from each sample were subjected to three types of analysis: (1) GPI analysis to determine the fraction of each of the donor and host genotypes, (2) Southern blotting of Sac I cut DNA to determine the number of copies of Neo per cell in the population (copy number), and (3) Southern blotting of Dra I cut DNA to determine the number of contributing stem cell clones. Hosts 1 through 3 were selected because they show several major themes from this type of analysis. Hosts 1 and 3 show contributions from a single marked clone and in each case its genotypic identity can be deduced by taking into account the GPI data. Because all WBCs in host 1 were DBA/2 in origin, the contributing marked stem cell was obviously of DBA/2 genotype. If the marked stem cell contributed all of the WBCs, one would have expected a copy number of 1.0, demonstrating 100% labeled cells. However, the copy number was 0.23 implying that 23% of the WBCs were derived from the marked stem cell. Thus, the total number of marked and unmarked clones was a minimum of two: one marked and at least one unmarked.

Analysis of host 3 shows a single marked clone with one integration site contributing 77% of the WBCs. Genotypic analysis showed 81% of the WBCs to be of C57BL/6 origin, a value very similar to the contribution from the marked clone. Thus, we conclude that the marked stem cell was of C57BL/6 origin.

Host 2 demonstrates a case in which we were unable to deduce the genotype of the marked stem cell. The Dra I digest shows three bands that, by densitometry of the film, were of equal strength. Because the likelihood of exactly

<table>
<thead>
<tr>
<th>Genotypes of Spleen Colonies</th>
<th>Spleen Colonies/10⁶ Cells Injected</th>
<th>Colonies Derived From Infected CFU-S</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6 hosts</td>
<td>28/28 C57BL/6</td>
<td>11.4 ± 3.5</td>
</tr>
<tr>
<td>DBA/2 hosts</td>
<td>25/25 DBA/2</td>
<td>10.7 ± 4.0</td>
</tr>
</tbody>
</table>

Fig 3. Genotypic composition and Southern blot analysis of blood cells from hosts 12 months after transplant with a marrow graft whose analysis is similarly presented. Blood cells or cells of the marrow graft were subjected to GPI analysis and analyzed by Southern blot according to procedures outlined. The arrows at the left of each Sac I lane designate that band as 3.2 kb in size. Note that each host's leukocytes were derived from a single marked stem cell (and other unmarked ones as discussed in the text). GPI analysis, in two of the three, permitted the genotypic identity of the marked cells to be determined and, where possible, are labeled with an asterisk. The logic for these designations, based on Neo copy number and GPI chimerism, is discussed in the text.
ENGRAFTMENT BY CHIMERIC BONE MARROW

Fig 4. Genotypic and Southern blot analysis of hematopoietic and lymphoid tissues of engrafted mice. Long-term (hosts 1 through 3) and short-term (hosts 4 through 6) specimens were killed and their BM, spleen, thymus and lymph node were analyzed for GPI and integrated proviral Neo as described in Materials and Methods. The arrow at the left of each panel indicates the location of the 3.2-kb marker on that gel. The genotypic identity of the retrovirally marked stem cells is designated by an asterisk. The logic for these designations, based on Neo copy number and GPI chimerism, is discussed in the text. Note contributions from pluripotent stem cells in the lymphoid tissues of engrafted mice. Long-term (hosts) and contributions from lineage-restricted stem cells are marked. Long-term recipients are consistent with a small number of simultaneously active stem-cell clones contributing to hematopoiesis. The mice in this experiment, and other long-term recipients, were killed 18 months after transplantation. The BM was characterized by a clone not found in spleen, thymus, and lymph node. The lymphoid tissues contained cells derived from a unique, perhaps lymphoid-restricted, progenitor, not found in marrow. Hosts 4 through 6, killed 2, 4, and 5 months, respectively, after engraftment, further demonstrate the presence of marked clones in one or several, but not all, tissues surveyed. Where possible, the genotypic origin of the marked cells is indicated by an asterisk in the GPI summary.

Table 4 presents a summary of 64 recipients analyzed either 1 to 5 months or 7 to 18 months after engraftment. These temporal frames were chosen because of previously reported differences in the stability and numbers of contributing stem cells. The long-term (7 to 18 months) engrafted mice tended to have contributions from marked progenitors that: (1) numbered only one or two, (2) were pluripotent, and (3) were largely of the C57BL/6 genotype. The latter point is in keeping with the previously reported observation that the genotypic makeup of hematopoiesis shifts toward C57BL/6 during aging. In contrast, recipients necropsied at earlier times after engraftment tended to have more marked stem cells represented, many of which were restricted in their differentiative potency. The majority of the earlier clones were of DBA/2 origin, as expected from our previous findings. The marked clone in host 1, for example, was probably C57BL/6 because the copy number in BM and spleen (0.7 to 0.8) most closely fits the C57BL/6 composition of those tissues (67%). The same marked clone was present in both thymus and lymph node, as evidenced by the same-sized marked fragment, albeit at a lower representation. Without the corroborating data from BM and spleen, we would not have been able to deduce the genotype of the marked clone in thymus and lymph node.

A single DBA/2 stem cell was clearly responsible for most, if not all, hematolymphoid cells in host 2. Progeny of two distinctly marked cells were found in host 3, a recipient killed 18 months after transplantation. The BM was characterized by a clone not found in spleen, thymus, and lymph node. The lymphoid tissues contained cells derived from a unique, perhaps lymphoid-restricted, progenitor, not found in marrow. Hosts 4 through 6, killed 2, 4, and 5 months, respectively, after engraftment, further demonstrate the presence of marked clones in one or several, but not all, tissues surveyed. Where possible, the genotypic origin of the marked cells is indicated by an asterisk in the GPI summary.

Table 4. Summary of Stem-Cell Clones Identified in Recipients Necropsied 1 to 5 Months or 7 to 18 Months After Engraftment

<table>
<thead>
<tr>
<th>Recipient Mice Necropsied at</th>
<th>Number of recipients analyzed</th>
<th>More than two marked clones contributing to hematopoiesis</th>
<th>One or two marked clones contributing to hematopoiesis</th>
<th>Same marked clones contributing to both hematopoiesis and lymphopoiesis</th>
<th>Genotype of marked stem cell clones:</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-5 mo</td>
<td>29</td>
<td>17</td>
<td>12</td>
<td>14</td>
<td>DBA/2 88% (15) DB57BL/6 12% (2)</td>
</tr>
<tr>
<td>7-18 mo</td>
<td>35</td>
<td>5</td>
<td>30</td>
<td>28</td>
<td>22% (5) 78% (23)</td>
</tr>
</tbody>
</table>

Summary of stem-cell clones identified in recipients necropsied 1 to 5 months or 7 to 18 months after engraftment. Analyses of marked stem-cell progeny and their genotypic identity are summarized with respect to the time after marrow transplant. Because of constraints discussed in the text, it was not possible to determine the genotypic identity of all marked clones. Results are presented only for those whose identity was unequivocal. The numbers in parentheses are the numbers of clones genotyped.
observations that DBA/2 progenitors were predominant in the first few weeks after marrow transplant (Fig 2).

DISCUSSION

Stem-cell marking and GPI analysis both show oligoclonal hematopoiesis. We have used two methods of genetic analysis to study the number of simultaneously productive cell populations in vivo. These methods, retrovirally marking stem cells and measuring enzyme isotype or hemoglobin chimerism, have previously been used individually to reach different conclusions concerning the organization and usage of the stem-cell population. Studies using marked stem cells have shown that few were simultaneously providing mature blood cells. Moreover, some of the evidence from this line of investigation supports the notion that stem-cell clones are used successively during a mouse's lifetime and that the lifespan of some stem-cell clones may approximate that of the mouse. On the other hand, analysis of the chimerism of hemoglobin variants in either allophenic mice or recipients of transplanted marrow has led others to the conclusion that many, if not all, stem cells were simultaneously productive. Lack of fluctuation in blood cell chimerism in serial blood samplings over many months was consistent with multiple productive stem cells of each genotype damping any alterations of overall chimerism.

The results presented in this report, combining the two methods, demonstrate the participation of few stem cells in hematopoiesis of long-term engrafted mice. We have used a novel donor-recipient combination in which two distinguishable stem-cell populations were present in the allophenic marrow graft. It was possible in turn to discriminate the donor cells from blood cell populations derived from any surviving indigenous stem cells. Thus, the fraction of each component genotype was quantitatively determined in blood cell populations of the host. We have previously used this method to assess blood cell chimerism throughout the lifetime of allophenic mice and in radiation chimeras engrafted with allophenic marrow. Some mice showed dramatic fluctuations in blood cell chimerism consistent with hematopoiesis being oligoclonal. Others had stable chimerism consistent with polyclonal hematopoiesis. Retroviral marking, in the present studies, permitted us to determine, at least in engrafted mice, that hematopoiesis was oligoclonally derived and the accompanying GPI analyses permitted us to determine the genotypic identity of most active stem cells. It is conceivable that hematopoiesis in unmanipulated mice is fundamentally different than in radiation chimeras in that hematopoiesis may be polyclonally derived. The transplantation paradigm involves disruption of stem-cell–stromal-cell associations in the donor and their reestablishment in the recipient, events which have been tied to a decline in the repopulating capacity of the stem-cell population as a whole. Resolution of this question awaits a method to mark stem cells in vivo.

The principal reason for constructing the allophenic mice was to examine genotype-restricted differences in stem-cell function. The current results shed light on the hematopoietic differences between the mouse strains used to construct the embryo aggregates. Immediately after engraftment we found the DBA/2 genotype to be dramatically over-represented in the recipient blood cells in comparison with its representation in the marrow graft. However, with time the RBC and WBC pools of radiation chimeras and allophenic mice became predominately, if not solely, C57BL/6. Here we confirm these earlier findings (Figs 1 and 2) and demonstrate that the effectors of these shifts in chimerism are pluripotent stem cells that shift from predominately DBA/2 at 1 to 5 months to largely C57BL/6 at 7 months or more postengraftment (Table 4). Marked stem cells in the early period were 88% DBA/2, whereas later the differentiating stem cells were largely C57BL/6 (78%).

A theory to explain genotype-restricted dominance during phases of engraftment. A unified theory to explain genotypic shifts in chimerism during aging in allophenic mice and those observed in short- and long-term transplant recipients of allophenic marrow may involve a hierarchical organization of stem cells in which at least two subpopulations exist. There is ample evidence for multiple stem-cell subpopulations with differing capacities for proliferation and differentiation. In the context of transplantation these populations may differ in two important respects: their capacity for early regeneration, for example, that initially provided by a marrow graft and, secondly, with respect to their capacity for providing long-lived stem cells that have the capacity to be productive over a long time span, one that may approximate the mouse lifespan. The former population, while providing rapid engraftment and recovery of a depopulated hematopoietic and lymphoid system, may be short-lived in comparison with the latter subpopulation. We further hypothesize that DBA/2 and C57BL/6 stem-cell populations differ in the relative proportions of the two subpopulations in their marrows such that DBA/2 is characterized by a preponderance of the former type of stem cells and C57BL/6 is characterized by the latter, long-lived population. In a chimeric setting, when the situation calls for rapid hematopoietic repopulation, DBA/2 stem cells are preferentially selected, by virtue of their wealth of the required subpopulation. However, later during steady-state hematopoiesis, long-lived stem cells are called for and because those are preferentially C57BL/6, the composition of blood and lymphoid tissues slowly becomes predominately C57BL/6. The latter explanation also accounts for the changes observed in aging allophenic mice where C57BL/6 also predominated.

In a comprehensive temporal study of stem-cell function after transplantation, Jordan and Lemischka concluded that engraftment could be divided into two general periods. The first occurred during the first 6 months and was characterized as an unstable period in which blood cell contributions came from several retrovirally marked stem cells whose activity was, in some cases, transient and restricted in developmental potency. The time after 4 to 6 months was a stable period dominated by contributions from one or two pluripotent stem cells with long lifespans. Our results are in agreement with these findings and the theory proposed to explain genotypic predominance during
separate phases of repopulation in our studies may apply to those of Jordan and Lemischka. The donor-recipient combination in their studies was syngeneic, thus, according to the hypothesis, the two temporal phases reflect dominance by the appropriate stem-cell subpopulation, in this case, of the same genotype (C3H/HeJ). It is conceivable that the duration of the two phases would vary from mouse strain to mouse strain depending on the characteristic, and genetically determined, mix of the two stem-cell subpopulations.

Our initial studies on the inbred strains used to construct our allophenic mice uncovered a genetic difference between them based on stem-cell cycle analyses. The fraction of CFU-S killed by hydroxyurea was dramatically higher in DBA/2 mice than in C57BL/6. Interpretation of these results in the light of our current hypothesis suggests that the predominate stem-cell subpopulation in DBA/2, that responsible for immediate replenishment of hematopoiesis, tends to have a larger proportion of cycling cells. Conversely, C57BL/6, because its stem-cell population is dominated by more long-term repopulating cells, tends to have fewer stem cells in cycle. Such cell cycle differences consistently with other theories linking stem-cell proliferation and differentiation. In conclusion, we have found that the gradual decline in DBA/2 representation in the blood of allophenic marrow recipients is not preceded by the demise of the DBA/2 stem-cell population (manuscript in preparation). When long-term recipients no longer harboring differentiating DBA/2 stem cells served as marrow donors, DBA/2 stem cells were reactivated during the early part of the repopulation of secondary recipients. Thus, in keeping with the tenets of the hypothesis, in a competitive setting where two populations coexist each with pre- eminent subpopulations, blood cell genotype reflects the predominant stem-cell subgroup serving the existing hematologic needs of the animal.

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REFERENCES

Developmental potential of hematopoietic stem cells determined using retrovirally marked allophenic marrow

G Van Zant, JJ Chen and K Scott-Micus