Clonal contributions of small numbers of retrovirally marked hematopoietic stem cells engrafted in unirradiated neonatal W/W\(^v\) mice

(blood cell lineages/fetal liver/bone marrow)

BLANCHE CAPEL, ROBERT HAWLEY*, LUIS COVARRUBIAS†, TERESA HAWLEY*, AND BEATRICE MINTZ‡

Institute for Cancer Research, Fox Chase Cancer Center, Philadelphia, PA 19111

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ABSTRACT Mice were repopulated with small numbers of retrovirally marked hematopoietic cells operationally definable as totipotent hematopoietic stem cells, without engraftment of cells at later stages of hematopoiesis, in order to facilitate analysis of stem cell clonal histories. This result depended upon the use of unirradiated W/W\(^v\) newborn recipients. Before transplantation, viral integration markers were introduced during cocultivation of fetal liver or bone marrow cells with helper cell lines exporting defective recombinant murine retroviruses of the HHAM series. Omission of selection in culture [although the vector contained the bacterial neomycin-resistance (neo) gene] also limited the proportion of stem cells that were virally labeled. Under these conditions, engraftment was restricted to a small population of marked and unmarked normal donor stem cells, due to their competitive advantage over the corresponding defective cells of the mutant hosts. A relatively simple and coherent pattern emerged, of one or a few virally marked clones, in contrast to previous studies. In order to establish the totipotent hematopoietic stem cell identity of the engrafted cells, tissues were sampled for viral and inbred-strain markers for periods close to one year after transplantation. The virally labeled clones were characterized as stem cell clones by their extensive self-renewal and by formation of the wide range of myeloid and lymphoid lineages tested. Results clearly documented concurrent contributions of cohorts of stem cells to hematopoiesis. A given stem cell can increase or decrease its proliferative activity, become completely inactive or lost, or become active after a long latent period. The contribution of a single clone present in a particular lineage was usually between 5% and 20%.

Totipotent hematopoietic stem cells (THSC) ancestral to myeloid as well as lymphoid blood lineages have been demonstrated with inbred-strain or chromosomal markers distinguishing donor and host in reconstitution of lethally irradiated or W-mutant mice (1–6). The recent use of retroviral insertions to mark individual cells before transplantation, thus allowing cell clones to be traced, has simplified lineage analysis and confirmed the existence of a stem cell population (7–10). In these retroviral experiments, the hosts were irradiated adults. Irradiation depletes cells at many stages of the hematopoietic hierarchy and makes it possible for various cells with proliferative capacity, including progenitor cells committed to a single specialized lineage, to become engrafted along with self-renewing THSC. The viral marking experiments have revealed a complex pattern of clone relationships within the hematopoietic population but have not distinguished stem cells from progenitors, largely because the time span of all studies (ranging from 8 to 20 weeks) was too short and the lifetimes of most progenitor cells are unknown. In addition, some clones can produce cellular progeny in one lineage at one time and in another lineage at another time (10), thus appearing to be lineage-restricted over short sampling periods.

To focus on the developmental and proliferative potential of the early stem cell under conditions in which cells of later stages do not engraft or persist, we have here used unirradiated neonatal W/W\(^v\) mice as hosts for retrovirally marked hematopoietic cells from normal fetal liver (FL) or bone marrow (BM). The W/W\(^v\) mutant has a THSC defect and subsequently develops a severe anemia, but most other blood lineages appear to be normal (11). The primary defect allows donor cells to become competitively engrafted without irradiation of the host (5). Our experiments with W/W\(^v\) fetal hosts have shown that, irrespective of the transient presence of other donor blood cells, engraftment entails a limited number of THSC, which gradually repopulate all hematopoietic lineages (6). Neonatal hosts offer the convenience over fetuses that the defective W/W\(^v\) segregants from heterozygous matings are recognizable. However, there are some histocompatibility restrictions not found in fetal hosts (12).

We report here that single, or very few, virally marked cells become selectively engrafted in these unirradiated mutant neonates, unlike the situation reported in other hosts. Long-term observations, extending for close to a year in some cases, establish the THSC identity of the clonal initiator cells and enable their histories to be characterized.

MATERIALS AND METHODS

Mice and Genetic Markers. W/W\(^v\)-C57BL/6 mutants were identified at birth by their severe anemia and coat color phenotype (13). FL or BM cells were from hematologically normal congenic (B.C-20 × Hbb\(^b\)/Hbb\(^b\)-C57BL/6)F\(_1\) mice; FL, fetal liver; BM, bone marrow.

Abbreviations: THSC, totipotent hematopoietic stem cells; FL, fetal liver; BM, bone marrow.

*Present address: Department of Experimental Oncology, Ottawa Regional Cancer Centre, Ottawa, ON K1H 8L6, Canada.

†Present address: Centro de Investigación sobre Ingeniería Genética y Biotechnología, Cuernavaca 62270, Mexico.

‡To whom reprint requests should be addressed.

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stably integrated into the target cells and cannot cause viremia in the hosts. There is no evidence for expression in the blood cells of the myc or tk genes present in the vectors.

**Infection and Transplantation of FL and BM Cells.** A suspension of FL cells from 18 to 24 untreated fetuses of 13 days of gestation was prepared in phosphate-buffered saline with 3% (wt/vol) bovine serum albumin as described (6). BM was flushed from hind limbs of female donors (2–3 months old) 48 hr after treatment with morphoracil at 150 mg/kg (15). With both FL and BM suspensions, erythrocytes were lysed, and the remaining cells were washed and cultured as described (14) with one of the virus-producing cell lines. Neither FL nor BM cells were selected in G418 after retroviral infection, although the viruses contained the bacterial neo gene. Nonadherent cells were removed after 24 or 48 hr, concentrated by centrifugation, and resuspended at 10^6 cells per ml in Dulbecco’s phosphate-buffered saline with 3% bovine serum albumin. Approximately 10^5 FL or BM cells were injected into the orbital branch of the anterior facial vein within 24 hr of birth, as described (12). For transfers to secondary hosts, BM was collected from the four limbs of the primary host (without 5-fluorouracil treatment), washed, filtered, and resuspended as usual.

**Blood and Tissue Analyses.** Blood was collected from the incised tail tip or the retroorbital sinus. Erythrocyte lysates were analyzed for strain-specific hemoglobin variants by electrophoresis in cellulose acetate (16). Spleen cells for DNA analysis were obtained from partial splenectomies of mice anesthetized with Nembutal. Lymph nodes, thymus, bone marrow, spleen, and cells isolated from the peritoneal cavity by saline lavage were collected at autopsy. Mast cells, with characteristic morphology in May–Grunwald–Giemsa stain, were obtained by culturing adherent cells from BM for more than 3 weeks in Dulbecco’s modified Eagle’s medium with 10% (vol/vol) fetal bovine serum and 10% conditioned medium from WEHI-3B(D+) cells as a source of interleukin 3 (17). Cells from bone marrow or peritoneal wash were cultured for 2 weeks in Dulbecco’s modified Eagle’s medium with 10% (vol/vol) fetal bovine serum and 10% (vol/vol) conditioned medium from L929 cells as a source of colony-stimulating factor-1 (18). The adherent cells yielded highly enriched populations of macrophages, verified by acid phosphatase staining and by phagocytosis of large numbers of 1-μm latex beads coated with goat IgG anti-mouse IgM. Spleen cell suspensions were fractionated, by panning, into surface-immunoglobulin-positive and -negative populations (19). The surface-immunoglobulin-positive population was recovered and cultured for 3 days in the presence of lipopolysaccharide at 20 μg/ml to stimulate B-cell expansion. The surface-immunoglobulin-negative population was cultured in the presence of concanavalin A at 5 μg/ml for 3 or 4 days to expand the T-cell fraction. At the end of culture, the immunoglobulin-positive population was >95% positive for the early B-cell marker, B-220; the immunoglobulin-negative population was >87% positive for the T-cell marker, Thy-1 (data from fluorescence-activated cell sorter analysis were kindly contributed by R. Randy Hardy, of this institute).

**DNA Analyses.** Genomic DNA was isolated from available tissues from each animal by standard procedures, digested with either EcoRI or BamHI restriction endonuclease, and analyzed by the alkaline Southern transfer method (20). The hybridization probe was a 1-kilobase restriction fragment containing the bacterial neo coding region radiolabeled with 32P by the oligonucleotide priming technique to a specific activity of 1–3 × 10^6 cpm/μg. Blots were hybridized at 65°C in the presence of 7% NaDodSO4, 1% crystalline fraction V bovine serum albumin, and 0.5 M NaPO4 (pH 7.2). The representation of each band was estimated by comparison on each blot with DNA from a cell line carrying a single copy of neo diluted 1:2, 1:10, and 1:20 with C57BL/6 genomic DNA.

**RESULTS**

**Retroviral Infection of FL and BM Cells and Repopulation of W/W*-Mutant Neonatal Mice.** Approximately half of W/ W* neonates die of their anemia within 48 hr of birth even when they are injected with normal uninfected FL or BM cells (data not shown). The remaining half begin to show visible improvement 4–7 days after injection. Coculture of FL or BM cells with virus-producing cells progressively reduced transplantation success. After 24 hr of culture, there was no decrease in the ability to reconstitute and rescue the W/W* mutants: 40% of those receiving FL and 75% of those given BM survived (Table 1). All these survivors, even ones lacking cells with viral sequences, were positive for donor-type hemoglobin (the first tested marker) 7–10 days after injection (ref. 12 and this study). In contrast, coculture of donor cells for 48 hr drastically reduced W/W* host viability: only 6–10% survived to be tested (Table 1). During this time, increasing numbers of dead cells were seen in the cultures, along with an increase in total cell number in the case of FL. Thus, it is likely that the concentration of effective THSC, upon which long-term rescue depends, is continually reduced in culture.

**Repopulation with One or a Few Marked Clones.** Recipients were first tested for donor-type hemoglobin, the earliest indicator of repopulation because the host’s anemia favors rapid expansion of normal cells entering the erythroid lineage (21). Positive animals were partially splenectomized at 11–14 weeks, at which time the spleens were somewhat enlarged, with abundant mitoses, lymphoid and myeloid cell types, and a predominance of cells of erythroid types. DNAs from the spleen samples were tested for the presence and integration pattern of the viral marker. For those based on the HHAM-KK(D) vector, a BamHI restriction digest was used, as the enzyme cuts only once within the virus; for the HHAM-HK(R) vector, an EcoRI digest accomplishes the same result (Fig. 1). The neo-containing radiolabeled probe hybridizes

| Table 1. Transplantation efficiency of retrovirally infected FL or BM cells in unirradiated W/W* neonates |
|-----------------|-----------------|-----------------|-----------------|
| Donor           | Hr in culture*  | No. of hosts    | Survivors at No. % | Hosts with viral sequences No. % |
| FL              | 24              | 25              | 10              | 40              | 7              | 70             |
| BM              | 48              | 17              | 1               | 6               | 0              | 0              |
| BM              | 12              | 8               | 75              | 3               | 33             | 33             |
| BM              | 19              | 2               | 10              | 1               | 50             |                |

*FL or BM cells were cocultivated with a ∆/2 cell line producing a recombinant retrovirus at a titer of 5 × 10^5 colony-forming units per ml.
with a single unique-size junction fragment from each retroviral integration. Approximately twice as many FL as BM hosts were positive for viral sequences, despite the fact that FL donors were not pretreated with 5-fluorouracil. This may reflect the greater numbers of actively cycling stem cells in FL. Since only half as many hosts that received FL survived, the overall yield in both FL and BM experiments was equivalent (±25%) (Table 1).

There were strikingly few virally marked clones in the spleens of most of the engrafted animals sampled first at 11–14 weeks after transplantation. Each marked clone had a frequency ranging from 1 in 2 to 1 in 10 total spleen cells. In the single surviving recipient of BM infected for 48 hr in vitro, more numerous integrations were observed, thereby suggesting that the frequency of infected cells increased over the 48-hr period (Fig. 2). However, there were several cases in which cells cultured for 24 hr yielded multiple integrations (Fig. 3, second animal).

**Clonal Shifts over Time and upon Re-Transfer.** In recipients whose spleens were resampled at intervals of 9–35 weeks, clones often showed shifts ranging from changes in intensity to the disappearance or de novo appearance of some clones (Fig. 3). In some cases, there appear to be multiple integrations in a single cell, as in the first animal, in which sequential samplings show the same relative intensity of the bands. In the second animal, however, the two bands (arrows) still present at the second sampling are apparently not marking the same cell clone, since their relative intensity changes.

The contribution of the single clone in the third animal was more than one cell in two at 14 weeks, but by 40 weeks it had entirely disappeared, and the animal died soon after. We have previously observed that an occasional repopulated W-mutant shows a sudden drop in hematocrit followed by death (6, 22, 23). Such animals, as with the third individual in Fig. 3, may have been reconstituted with only one or a few total stem cells, so that excessive proliferative activity may exhaust the clone. This would normally be avoided when more stem cells contribute simultaneously to the hematopoietic population.

Clonal changes observed in these neonatally reconstituted animals over a period as long as 46 weeks demonstrate, as in long-term reconstituted fetuses (23), that there is a reserve population of stem cells that can be activated upon physiological demand. In the fourth animal (Fig. 3), the recipient of BM cultured 48 hr, new marked clones are activated and are making a large contribution at 46 weeks.

**Clonal Segregation in Secondary Hosts.** Bone marrow from an unirradiated adult W/W<sup>r</sup> host, which had received a retrovirally marked BM transplant 36 weeks earlier (14), was transferred to neonatal W/W<sup>r</sup> recipients. Cells from these secondary hosts were sampled by partial splenectomy at 12 weeks. Segregation of long-lived individual clones occurred, as seen in Fig. 4 by emergence of two distinct patterns in the new hosts. Therefore, a number of marked stem cells coexisted in the primary recipient and were capable of extensive proliferation in a secondary host.

**Long-Term Contribution of Single Stem Cells to Clonal Progeny in All Lineages.** One FL recipient and one BM recipient, each with only a single marked cell clone when the spleen was sampled at 14 weeks, were killed at 37 weeks and 32 weeks of age, respectively, and many blood cell types were analyzed for the presence of the clone. In both cases, this single clone, apparently representing an ongoing stem-

![Fig. 2. Unique and simple retroviral integration patterns in DNAs from spleens of individual W/W<sup>r</sup> recipients sampled 13–14 weeks after neonatal transplantation of FL cocultured for 24 hr or BM cocultured for 24 or 48 hr with the virus-producing cells. The 1-kilobase neo probe hybridizes with one unique junction fragment from each integration in an EcoRI digest of cells infected by the HHAM-HK(R) virus. DNA from cells carrying a single copy of the neo gene diluted 1:2 or 1:10 is shown at left.](image)

![Fig. 3. Long-term clonal changes in spleens of four W/W<sup>r</sup> recipients, each engrafted at birth and sampled twice (in a pair of lanes) at the ages noted. The mitotic progeny contributed by a single marked precursor cell can remain the same (first pair of lanes), increase or decrease in separate clones within the same animal (second pair of lanes, arrows), disappear (third pair of lanes), or appear de novo (fourth pair of lanes). The retrovirus, digestion, and probe were as described in Fig. 2. w, Weeks.](image)

![Fig. 4. Segregation and long-term duration of clones among secondary (2°) recipients engrafted neonatally. The primary (1°) host was an unirradiated W/W<sup>r</sup> adult given retrovirally infected BM. At 36 weeks of age, he was killed and his BM was transferred to unirradiated W/W<sup>r</sup> neonates subsequently sampled at 12 weeks. The virus was HHAM-KK(D), and the neo probe reveals one unique junction fragment from each integration in BamHI digests of spleen DNAs.](image)
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**DISCUSSION**

Several factors account for the conveniently small number of virally marked THSC clones found in these W/W^v animals. The first is that the hosts were not irradiated; therefore, there has been no imposed depletion of proliferating cells of various stages and types. Although the marrow cellularity of the mutants before transplantation is lower than that in controls of the same strain, there is limited space in the bone marrow as compared to the situation in irradiated animals. Engraftment would thus be expected to involve only the normal early stem cells, due to their competitive advantage over the defective ones of the host. Secondly, the long term of this analysis, in contrast to previous studies, eliminates from consideration any short-lived marked cell clones likely to represent unipotential progenitor cells rather than THSC.

A third reason for the notably few marked donor clones is that selection in the neomycin analog G418 was omitted during culture despite inclusion of the bacterial neo gene in the vector. This decision was based partly on the possibility that the infected stem cells might be eliminated if the neo gene were not expressed in them. The presence of appreciable numbers of engrafted stem cells lacking viral integration was documented by donor-strain phenotypes in the lineages to which they give rise (e.g., by 100% donor-type hemoglobin in erythrocytes). As a result of all of the factors summarized above, a simple pattern of marked clones emerges and is optimal for analyzing the histories of individual THSC.

The THSC identity of these clones is evident from two criteria: they undergo extensive self-renewal, over periods close to a year in some cases, and they produce a wide range of myeloid and lymphoid tissues detected in tests conducted as late as 37 weeks after transplantation.

The hypothesis that a series of stem cells may contribute clones successively to hematopoiesis was first advanced by Kay (24). The model subsequently proposed in this laboratory (23, 25) was based on experiments in which mixtures of FL cells from different inbred mouse strains (without viral marking) were introduced into W-mutant fetuses. Long-term monitoring revealed that cohorts of THSC of at least three genotypes—the maximum number technically detectable in these experiments—could contribute to hematopoiesis at any one time. Representation of individual genotypes waxed or waned, but overall normalcy was maintained. The conclusion was drawn that the hematopoietic stem cell compartment is comprised partly of a small reserve of slowly cycling cells and also of rapidly amplifying clones of progeny cells progressing toward differentiation with declining self-renewal potential. Thus, there may be a substantial disparity in the residual proliferative capacity of THSC, depending on the stage in the progression.

The present data, from retroviral marking of individual stem cell clones, further support this model. It is clear that clonal contributions often change over time, that inactive stem cells may become mitotically active and contribute to all lineages, and that active stem cells may "disappear." When bone marrow was transferred to secondary hosts, the representation of a particular marked clone often changed abruptly, possibly due to sampling of cells from different levels of the total stem cell compartment. From the fact that ≈5–20% of cells were generally attributable to a given marked clone, some five or more proliferating stem cells may be contributing concurrently in these W/W^v mice, although the contributions of unmarked clones cannot be directly evaluated. It is also possible that the number of stem cells recruited into an active cohort may be influenced by the physiological status of the individual at any given time. For example, repopulation of fetuses by FL entails fewer normal stem cells in the mildly defective W/W^v than in the severely defective W/W (23). Yet complete replacement can eventually occur in both.

For the purpose of repairing genetic defects with a retroviral vector introduced into bone marrow stem cells, it will be necessary to achieve a very high efficiency of infection and selection in culture before transfer, to compensate for the shifting contributions of numerous clones.

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