Temporal Replacement of Donor Erythrocytes and Leukocytes in Nonanemic W^{aJ}/W^{aJ} and Severely Anemic W/W' Mice

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The dominant white spotting, W, locus in the mouse encodes Kit, a receptor molecule with cytosolic tyrosine kinase activity. Mutations in Kit deplete hematopoietic cells by an as yet unknown mechanism, but one that presumably affects the early progenitors of all cell lineages. To examine cell lineage-specific changes caused by different W mutations, we injected genetically marked normal marrow cells into mutant mice and monitored repopulation kinetics. In the present report, we compare repopulation of the various peripheral blood cells in nonanemic W\textsuperscript{aJ}/W\textsuperscript{aJ} and severely anemic W/W' mice administered increasing increments of donor cells. At all doses of cells tested, donor erythrocyte repopulation precedes leukocyte repopulation regardless of the recipient phenotype. There is, in fact, little difference in the rate or extent of nonerythroid repopulation in W\textsuperscript{aJ}/W\textsuperscript{aJ} mice injected with between 6 \times 10^6 and 2 \times 10^7 donor cells. The fact that donor cells rapidly replace erythrocytes, even in the nonanemic W\textsuperscript{aJ}/W\textsuperscript{aJ} host, while other cell lineages become donor type more slowly provides further evidence that mutations at the W locus are especially damaging to erythrocyte progenitors. We suggest that host nonerythroid hematopoietic cells compete with normal cells, probably at the level of early progenitors rather than at the level of the totipotent hematopoietic stem cell. The fact that successively higher doses of donor cells do not markedly alter nonerythroid repopulation kinetics implies that it may be possible to maximize autologous therapeutic marrow transplantation.

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SEVERE MACROCYTIC anemia, deficiency of megalaryocytes, and reduction of mast cell numbers are characteristics of W/W' mice. The hematopoietic defects are ascribed to a deficiency of precursor stem cells. In support of this hypothesis are the following: Firstly, all mice tested with mutations at the W locus, either as homozygotes or as double heterozygotes, accept congenic normal marrow grafts without prior irradiation.\textsuperscript{2} Secondly, the W/W' mice are much more sensitive to total body irradiation than are their normal littermates.\textsuperscript{2} Finally, the W/W' mice are deficient in the hematopoietic lineages descended from the totipotent hematopoietic stem cells (TSC).\textsuperscript{4,4} One would predict that, if the mutant mice do lack TSCs, all host peripheral blood cells would be completely and rapidly replaced after injection of donor +/-+ cells and that the rate of repopulation would be determined primarily by the life span of the host cells.

Previously, we injected 1 \times 10^7 marrow cells that contained just sufficient TSC\textsuperscript{c} to replace the hematopoietic cells in W/W' mice and monitored repopulation.\textsuperscript{10} Complete host erythrocyte replacement preceeded that of leukocytes, platelets, and lymphocytes. Our results suggest that in W/W' mice more severe defects occur in the erythroid lineage than in other lineages derived from the TSCs. The data were unexpected because we predicted, based on the much shorter life span of granulocytes and platelets in the peripheral circulation,\textsuperscript{11} that erythrocyte repopulation would occur more slowly. We concluded that the host could continue to draw from populations of long-lived committed precursors until they were depleted and that no such erythroid population existed in the mutant.

To test the assumption that the erythroid lineage is more severely affected by mutations at the W locus, we have compared peripheral blood cell replacement in mice with different phenotypes, the severely anemic W/W' mice and the nonanemic W\textsuperscript{aJ}/W\textsuperscript{aJ} mice.\textsuperscript{13} W\textsuperscript{aJ}/W\textsuperscript{aJ} mice do not exhibit the classical W phenotype of anemia (for comparisons see Table 1), but do exhibit pigment and fertility defects. The W\textsuperscript{aJ} mutation maps to chromosome 5 and has been shown to involve a rearrangement of the Kit locus.\textsuperscript{13,14} The mast cell growth factor, Mgf, is the ligand for Kit and is deleted in steel, Sl/Sl, mice, which are phenocopies of W/W' mice.\textsuperscript{15}

We have injected sufficient genetically marked +/-+ cells to generate donor peripheral blood cells in all W\textsuperscript{aJ}/W\textsuperscript{aJ} recipients. We show that this dosage varies depending on the severity of the anemia in the W recipient and must be determined by titration. To determine if larger numbers of donor stem cells cause coordinate and complete repopulation of all lineages as they do in a lethally irradiated recipient,\textsuperscript{16} we have injected higher doses of normal marrow cells into both W\textsuperscript{aJ}/W\textsuperscript{aJ} and W/W' mice. We show that, regardless of donor cell dose or recipient phenotype, erythrocyte replacement precedes leukocyte replacement.

MATERIALS AND METHODS

Animals. Mice used in these experiments were bred and maintained at The Jackson Laboratory (Bar Harbor, ME) which is fully accredited by the American Association for Accreditation of Animal Care. Recipients were C57BL/6J(B6)-W/W\textsuperscript{aJ}/W\textsuperscript{aJ} Gpi-P'/Gpi-P', B6-W\textsuperscript{aJ}/W\textsuperscript{aJ} Gpi-P'/Gpi-P', or WBB6F1-W/W\textsuperscript{aJ} Gpi-P'/Gpi-P', males and females. Donors were B6-+++ or Gpi-P'/Gpi-P' or B6-+++/Gpi-P'/Gpi-P' females. Experiments were set up so the difference at the glucose phosphate isomerase (GPI) locus allowed quantitation of enzyme contributed by donor and recipient cells. Donor cells for injection were collected from the femurs in Dulbecco's phosphate-buffered saline solution (PBS; no. 310420AG, Gibco, Grand...
Table 1. Relative Differences Between Normal and Mutant Mice

<table>
<thead>
<tr>
<th>Genotype of Mice</th>
<th>RBC Counts</th>
<th>Hematocrit</th>
<th>Mean Cell Volume</th>
<th>WBC Counts</th>
<th>BFU-E/10⁶ Cells</th>
<th>CFU-S/Femur</th>
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<td>60</td>
<td>84</td>
<td>134</td>
<td>100</td>
<td>27</td>
<td>0</td>
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<tr>
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<td>100</td>
<td>100</td>
<td>100</td>
<td>223</td>
<td>19</td>
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Comparison was made between WBB6F1-x/+ and -W/W⁺ mice and between C57BL/6J-x/+ and -W⁺/W⁻⁺ mice to obtain the percent of normal values.

Abbreviation: CFU-S, spleen colony-forming units.

Island, NY). Cells were dissociated by gentle titration through a 23-gauge syringe needle, washed in 10 mL of PBS, and an aliquot was diluted in 2% acetic acid for nucleated cell counts. In experiment 1, nucleated cells from the same GPI-1B donor pool (eight mice) were adjusted to 1 x 10⁶, 2 x 10⁶, 6 x 10⁶, or 1 x 10⁷ and 0.1 mL was injected into each of five to 10 W⁺/W⁻⁺ recipients. In experiment 2, eight GPI-1A donors provided a mixture of cells whose concentration was adjusted to 6 x 10⁶, 1 x 10⁷, or 2 x 10⁷ for injection into each of 10 W⁺/W⁻⁺ mice. In experiment 3, cells from two GPI-1A donors were adjusted to a concentration of 1 x 10⁷ and 1 x 10⁸ and injected into each of five to 10 W⁺/W⁻⁺ mice. Two 70-μL hematocrit tubes were filled with blood from the orbital sinus of each recipient at various times posttreatment, one for cell separation and one for enzyme analysis.

Cell separation. The technique of Van Zant et al.²⁷ as modified by us²⁸ was used to enrich the various peripheral blood cell populations. Briefly, one hematocrit (150 μL) of blood from the retroorbital sinus was eluted in 10 mL of 1 x PBS and centrifuged for 10 minutes at 670g. Platelets in the supernatant were retrieved by 10 minutes of centrifugation at 4,500g. The pelleted containing lymphocytes, granulocytes, and erythrocytes was eluted in 1 mL of 75% Percoll (no. 17-0891-01; Pharmacia, Piscataway, NJ), underlayered with 76.9% Percoll, and centrifuged at 100g for 10 minutes at 4°C. Any red blood cells (RBCs) remaining in the platelet, granulocyte, or lymphocyte preparations were lysed with 1% (wt/vol) ammonium oxalate. With this method, platelets were obtained free of other cells, erythrocytes were contaminated with 0.001% nucleated cells, lymphocytes were contaminated with 4.3% granulocytes, and granulocytes were contaminated with 19.3% lymphocytes. The enriched populations were stored at −20°C until GPI analysis.

GPI assay. The cell lysates were applied to cellulose acetate plates and GPI isozymes were separated by electrophoresis as described by Eppig et al.²⁹ After development of the GPI staining reaction, the plates were fixed in 5% acetic acid and the concentration of donor- and host-specific bands was determined by spectrophotometry with the Helena Clinicans 2 (Beaumont, TX). Results are presented as the percentage of donor type GPI in the cell lysate. The assay is sensitive to levels of ≥2% donor GPI.

RESULTS

Repopulation of W⁺/W⁻⁺ mice with donor +/+ cells. Preliminary studies of three groups of five W⁺/W⁻⁺ mice administered 1 x 10⁶, 5 x 10⁶, or 1 x 10⁷ normal marrow cells indicated that at least 1 x 10⁶ donor marrow cells were required before donor differentiated cells could be detected in the peripheral blood. Even with injections of 1 x 10⁷ cells, only one of five mice showed consistent and increasing levels of the donor GPI, as shown in Fig 1’s depiction of the results of experiment 1. At a dose of 2 x 10⁶ +/+ cells, all five recipients had circulating donor cells, indicating sufficient stem cells were provided to replace some of the W⁺/W⁻⁺ cells. Figure 1A shows that, despite the fact the recipients are not anemic by standard tests, erythrocyte replacement does occur. Host cells apparently competed with the donor cells because donor cells did not comprise 100% of the erythrocytes even after 48 weeks.

Replacement of nonerythroid cells (Fig 1B through D) occurred much more slowly at this dose and did not attain the levels of donor erythrocyte cells during the course of the experiment (48 weeks).

The effects of higher doses of 6 x 10⁶ and 1 x 10⁷ cells derived from the same aliquot are also presented in Fig 1A through D. The rate and extent of host cell replacement was increased with both of the higher doses when compared with the lower dose of 2 x 10⁶ cells. Repopulation kinetics, however, were similar regardless of whether mice were injected with 1 x 10⁶ cells or with 6 x 10⁶ cells. At the higher doses, as at the lower dose, host erythroid cells were replaced more rapidly than the nonerythroid cells. After injection of 6 x 10⁶ or 1 x 10⁷ cells, replacement of all lineages was essentially complete (≥90%) by 32 weeks, suggesting that the cells were derived from donor TSC.³⁰

To confirm and extend these findings, each of 10 mutant mice (experiment 2) was injected with either 6 x 10⁶, 1 x 10⁷, or 2 x 10⁷ cells from the same pool. Results are presented in Fig 2A through D and show that the replacement of host nonerythroid cells is delayed regardless of the number of donor cells injected.

The data from these studies provided evidence that the erythroid lineage in the nonanemic W⁺/W⁻⁺ mice was, in fact, affected; that erythroid and nonerythroid cell replacement did not occur coordinately; and that injections of as many as 2 x 10⁷ cells did not overcome the delay in nonerythroid cell repopulation.

Repopulation of W⁺/W⁻⁺ mice with donor +/+ cells. Previous results showed that W⁺/W⁻⁺ mice injected with 1 x 10⁷ cells had delayed replacement of nonerythroid cells.³⁰ In the present experiments, the objective was to compare repopulation kinetics with 10-fold more marrow cells than in the previous experiment. In experiment 3, five severely anemic W⁺/W⁻⁺ mice were injected with 1 x 10⁷ donor cells (Fig 3A). These mice acted as controls and showed similar repopulation kinetics to those previously reported.³⁰ Furthermore, only three of five mice were repopulated with donor erythroid cells after injections of 1 x 10⁷ cells (data not shown), indicating that, as previously reported, at least 1 x 10⁷ cells are required to cure the anemia of all W⁺/W⁻⁺ hosts.

Five other W⁺/W⁻⁺ mice were administered 1 x 10⁷ cells from the same donor aliquot (Fig 3B). Erythrocyte replacement preceded lymphocyte, granulocyte, and platelet replacement, but the delay was not as long as it was with 1 x 10⁷ cells. By 72 weeks, nonerythroid and erythroid cells were completely donor type in those mice receiving 1 x 10⁷ but not in those mice administered 1 x 10⁷ marrow cells. Results confirmed the severity of W mutations on the erythroid lineage, the delay in nonerythroid replacement, and the inability of high doses of donor +/+ cells to cause
Fig 1. Replacement of \(W^\text{my}/W^\text{mu}\) peripheral blood cells after injection of donor \(+/-\) cells. Lines depict the average donor GPI in five mice injected with \(1 \times 10^6\) (---); \(2 \times 10^6\) (--○--); \(6 \times 10^6\) (++--); or \(1 \times 10^7\) (-----) cells. Graphs shown are for (A) erythrocytes; (B) lymphocytes; (C) granulocytes; and (D) platelets at each time point. Granulocytes and platelets were not present in sufficient numbers until 7 and 5 weeks, respectively, to measure GPI. The standard error of the mean ranged from 0.85% to 5.41% for erythrocytes; 1.07% to 13.38% for lymphocytes; 0.24% to 10.34% for granulocytes; and 1.34% to 7.13% for platelets.

Fig 2. Replacement of \(W^\text{my}/W^\text{mu}\) peripheral blood cells after injection of donor \(+/-\) cells. Lines depict the average donor GPI in 10 mice injected with \(6 \times 10^5\) (■); \(1 \times 10^6\) (▲); or \(2 \times 10^6\) (○) cells at each time point. Cell lineages are the same as in Fig 1. The standard error of the mean ranged from 0.46% to 2.39% for erythrocytes; 1.19% to 4.47% for lymphocytes; 2.87% to 6.35% for granulocytes; and 1.24% to 5.7% for platelets.
CELL REPLACEMENT KINETICS IN W RECIPIENTS

Fig 3. Replacement of W/W peripheral blood cells after injection of donor +/− cells. Anemic recipients were injected with either (A) 1 × 10^5 or (B) 1 × 10^6 donor cells. Bars depict the average donor GPI in erythrocytes (○); lymphocytes (□); granulocytes (■); and platelets (▲) from (A) 10 mice and (B) five mice. The standard error of the mean ranged from 0% to 1.67% for erythrocytes; 4.56% to 8.0% for lymphocytes; 1.89% to 4.0% for granulocytes; and 1.44% to 8.56% for platelets.

equivalent rates of repopulation in both the erythroid and nonerythroid lineages.

DISCUSSION

The major findings of this study are that erythroid precursors are, indeed, affected in the nonanemic W^{a+}/W^{a+} mice as well as in the severely anemic W/W mice; the nonerythroid precursors are also affected but less severely; and repopulation can be optimized by donor cell titration.

We have shown previously by in vitro experiments that the frequency of the erythroid-committed precursors, known as the burst forming units (burst-forming unit erythroid [BFU-E]), is normal or, in fact, increased in W^{a+}/W^{a+} marrow. In the anemic W/W mice, on the other hand, the frequency of BFU-E is dramatically decreased. These data suggest that the erythroid lineage in the W^{a+}/W^{a+} mouse is either not affected or is able to compensate for defects in a less mature progenitor(s). Other studies indicate that erythroid progenitors are affected in W^{a+}/W^{a+} mice because erythrocytes are replaced after injections of 1 × 10^7 normal donor cells^20 and because erythrocyte progenitors compete with normal cells, although with one-tenth the efficiency of congenic +/+ cells, during competitive repopulation assays.19

From results shown in Figs 1A and 2A, it is clear that the erythroid lineage is affected in the W^{a+}/W^{a+} mice, even though the mice are not anemic. Mice injected with just sufficient donor cells to initiate replacement of host cells in all recipients show a progressive increase in the percentage of donor erythrocytes during the experiment. Host erythrocytes are not completely replaced by 48 weeks. In a similar experiment with W/W mice as recipients (Fig 3A), replacement of host erythrocytes is complete by 20 weeks. Results suggest that the W^{a+}/W^{a+} mice, but not the W/W mice, generate sufficient erythrocytes to compete effectively with the host cells. The prolonged ability of W^{a+}/W^{a+} erythrocytes to compete with +/+ cells indicates that the mutation has a milder effect on the progenitors. The findings are in agreement with the proposed affects of the sequence alterations in Kit. Kit/ encodes a receptor for a cytokine linked to an intracellular tyrosine kinase. The theory is that when the receptor is occupied, the tyrosine kinase phosphorylates itself and other cellular proteins essential for mitosis. W^{a+} is a rearrangement of Kit and diminishes the messenger RNA (mRNA)^14 and presumably the level but not the activity of the Kit/ encoded tyrosine kinase. W is a deletion of transmembrane sequences and W is a point mutation in the kinase region, resulting in double heterozygotes with impaired functional activity.21

The question is which erythroid progenitor(s) is affected? One possibility is that it is a less mature precursor, such as the TSC itself. This suggestion is in accord with the reported blood defects in mice with mutations at the W locus. These mutations include deficiencies of the following hematopoietic lineages descended from the TSC: RBCs, mast cells, megakaryocytes, granulocytes, B lymphocytes, and T lymphocytes. In this report, we have confirmed the fact that cells from the myeloid and lymphoid lineages are affected in the W^{a+}/W^{a+} and W/W mice. All hematopoietic cells tested show varying levels of replacement but they are, indeed, replaced (Figs 1 through 3).

The interesting point is that not all lineages are replaced simultaneously or to the same extent. Nakano et al.22 have also noted a delay in neutrophil replacement in W/W mice even after 200 R total body irradiation and injection of as many as 1 × 10^7 cells. In other studies, they^25 have shown, as have we,^26 incomplete lymphocyte replacement after injection of 1 × 10^7 +/+ or −/− cells into unperturbated W/W mice. Simultaneous replacement by donor RBC and white blood cells (WBCs) occurs after ablation of marrow in normal mice through lethal total body irradiation. All cells generated in this case reputedly arise from the donor lineage-committed hematopoietic cells. By 4 to 6 months posttransplantation, the original supply of lineage-committed cells is gone and there is evidence that the differentiated cells arise from the donor totipotent stem cells. The fact that there is a lengthy delay in nonerythrocyte replacement in both W^{a+}/W^{a+} and W/W mice, regardless of whether the equivalent of one or 10 W-repopulating cells are injected, suggests that the host hematopoietic stem
cells are still functional. After high doses of donor cells, host circulat-269 ing nonerythrocyte cells are present for at least 10 months in the $W^{ae}/W^{ae}$ mice (Fig 2B through D) and for at least 7 months in the $W/W^+$ mice (Fig 3B), long after the cells generated from the donor TSC should have become dominant in a severely TSC-deficient host.

There is other evidence supporting the conclusion that the TSC are not as severely affected in the adult mutant mice. Experiments of Bogs et al. showed that host circulating nonerythrocyte cells are present for at least 7 months in the $W/W^+$ mice, even when stressed with sublethal irradiation, replace their own blood cells but at a slower rate than would a normal mouse treated similarly.

The more likely candidate for the affected cell than the TSC is a more mature progenitor. This cannot be the BFU-E because, as noted earlier, it is present in normal numbers in the $W^{ae}/W^{ae}$ marrow. Instead, it must be either some cell that is capable of contributing to all lineages or several progenitor cells with different responses to the ligand. These could be quantitative or qualitative differences. Identification of the affected cell(s) is complicated by the fact that surviving cells can compensate for the defect by augmenting cell numbers downstream of the affected cell(s) is complicated by the fact that surviving cells can compensate for the defect by augmenting cell numbers downstream of the ligand. This may explain the increased numbers of BFU-E in the $W^{ae}/W^{ae}$ marrow and the normal numbers of granulocytes and of platelets in peripheral blood. The story of the $W$-determined defects in hematopoiesis will remain incomplete until there is further analysis of aberrant hematopoiesis in the mutant mice, coupled with an understanding of the physiologic role of Kit. Certainly, the many $W$-mutations will help provide molecular explanations for the observed phenotypic variations. The surprising finding that up to 10-fold higher doses of donor marrow cells than that necessary to generate host erythrocyte replacement do not speed up nonerythrocyte replacement is difficult to explain. It has been proposed that, in the mouse, there are only a small number of seeding sites available. Once these sites are filled, additional cells are not accepted. This belief has recently been contested. The fact that there is a maximum number of donor cells beyond which replacement of host cells is not improved, injects a cautionary note. Autologous marrow transplantation is a current experimental therapy for a number of human metastatic diseases and a proposed route of gene replacement therapy. If, in fact, there is a dose of marrow with which recovery of cell lineages is maximized, experiments in murine models should be designed to determine what factors contribute to establishing the upper limits.

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Temporal replacement of donor erythrocytes and leukocytes in nonanemic W44J/W44J and severely anemic W/Wv mice

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