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Erythrocyte replacement precedes leukocyte replacement during repopulation of W/W^v mice with limiting dilutions of +/+ donor marrow cells

(hemopoietic stem cells/hereditary anemia/cell-specific amplification/bone marrow transplantation)

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ABSTRACT **The severe macrocytic anemia of the stem-cell-deficient W/W^v mouse is alleviated by intravenous injection of normal marrow cells. Donor cells replace the host erythrocytes, but the fate of the more labile blood components, such as granulocytes and platelets, after transplantation into adult mice has not been established. In the present work, the rate of replacement of the various hemopoietic cells in W/W^v transplant recipients was examined by exploiting host–donor differences in cellular markers (hemoglobin and glucose-phosphate isomerase). Limiting dilutions of +/+ parental cells were injected into F_1-hybrid W/W^v anemic mice. A dose of 10^5 donor cells was necessary for the implantation of sufficient multipotent stem cells to alleviate the anemia. Erythrocyte parameters were not significantly different from normal values, and donor cells replaced at least 90% of the host erythrocytes by 20 weeks after injection. At this time, only 10% of the nucleated leukocytes and none of the platelets were donor-derived. The percentage of donor lymphocytes, granulocytes, and platelets increased slowly thereafter but did not completely replace the host elements by 41 weeks. The maintenance of host leukocytes during rapid donor-erythrocyte replacement indicates cell-specific amplification of committed progenitors.**

Marrow transplanted from major histocompatibility complex-matched normal donors is rapidly becoming an accepted treatment for some types of inherited human hemopoietic disorders (1). The efficacy of such a therapeutic measure has been determined by observations of numerous transplant recipients afflicted with a specific heritable insufficiency. A major question posed during these studies is the order of replacement of the various hemopoietic cells in the recipient. In patients where, in order to ablate host lymphoid cells, the pretransplantation treatment has been extremely harsh, the recovery of the immune system is sometimes delayed for many months (2). The donor contribution of granulocytes and platelets has not been extensively monitored in such patients.

Certain animal models for heritable blood disorders permit analyses of replacement kinetics. One such model is the mouse doubly heterozygous for mutations at the dominant white spotting locus, *W*. WBB6F_1-W/W^v mice have a severe macrocytic anemia and less severe deficiencies of granulocyte precursors and megakaryocytes (3, 4). A stem-cell deficit in W/W^v mice allows implantation of donor grafts without prior irradiation. Genetically marked C57BL/6J (B6) parental cells are accepted by the anemic F_1 hybrids. The W/W^v mice do not develop severe F_1-hybrid resistance or graft-vs.-host disease (5, 6). The experiments described here were designed to determine the sequence of hemopoietic cell repopulation in adult W/W^v mice cured with implants of +/+ hemopoietic stem cells. The F_1-hybrid W/W^v mice were injected with successively smaller numbers of genetically marked +/+ parental cells. The object was to implant minimal numbers of pluripotent hemopoietic stem cells (PHSCs). The advantage of such a procedure is that the distribution of the various differentiated descendants of a single (or very few) PHSC is analyzed.

MATERIALS AND METHODS

Animals, Preparation of Donor Cells, and Collection of Cells for Assays. Donors and recipients were chosen for differences in hemoglobin electrophoretic pattern determined by the β-globin locus (*Hbb*) and in glucose-phosphate isomerase (GPI-1, gene symbol *Gpi-1*). Donor mice were 1-month-old C57BL/6J (B6)-+/+ *Gpi-1*^{a}/*Gpi-1*^{a}, *Hbb*^{s}/*Hbb*^{s} females. Recipients were 2-month-old male and female W/W^v F_1 hybrids (W/W^v) generated by mating WB/Re-$W/+$, *Gpi-1*^{b}/*Gpi-1*^{b}, *Hbb*^{d}/*Hbb*^{d} females to B6-+/W^v, *Gpi-1*^{b}/*Gpi-1*^{b}, *Hbb*^{s}/*Hbb*^{s} males. All mice were maintained at The Jackson Laboratory in accordance with the regulations established by the American Association for Accreditation of Animal Care. Donor mice were killed by cervical dislocation and their femurs were removed under aseptic conditions. The femur head and condyles were cut off and the femoral cavity was flushed with 1 ml of phosphate-buffered isotonic saline (PBS). Marrow cells were washed once with 10 ml of PBS and resuspended in 1 ml of PBS. The nucleated cells in an aliquot were counted in a hemocytometer. Within 2 hr of cell collection, 10^5, 10^4, or 10^3 cells were injected intravenously in a volume of 0.1 ml into each of five W/W^v recipients. Two 150-μl hematocrit tubes of blood were obtained from the retroorbital sinus at 6, 8, 10, 16, 20, 24, 28, 32, and 41 weeks after injection. The first tube was used for determination of erythrocyte counts, hematocrits, and hemoglobin phenotype. The second tube was separated at 6, 8, 10, and 16 weeks into erythrocytes and lymphocytes and at 20 weeks and thereafter into erythrocytes, lymphocytes, granulocytes, and platelets for determination of GPI-1 phenotype.

Erythrocyte Counts, Hematocrit, and Hemoglobin Phenotype. Erythrocytes were counted with a Coulter Counter (model ZBI) and hematocrits were determined by centrifuging the microhematocrit tubes in an AutoCrit II (Becton Dickinson) and reading the ratio of packed cells to total blood volume on an Adams microhematocrit reader (Becton Dickinson). Hemoglobin phenotype was determined by electrophoresis on cellulose acetate of an aliquot of the packed cells lysed in cystamine dihydrochloride (7). After electrophoresis, the acetate strips were stained in a 1% (wt/vol) solution of Ponceau S in 5% (wt/vol) trichloroacetic acid, destained in 5% (vol/vol) acetic acid, dehydrated in methanol, cleared in Helena Clear Aid (Helena Laboratories, Beaumont, TX) and

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Abbreviations: PHSC, pluripotent hemopoietic stem cell; GPI, glucose-phosphate isomerase; HBS, single hemoglobin.

air-dried. The concentration of single hemoglobin (HBS) expressed as a percent of the total hemoglobin [HBS plus the diffuse major and minor hemoglobins (HBD)] was measured with a spectrophotometer (Helena Quik-Quant II).

Separation of Peripheral Blood Cells. Successful performance of the experiments required a method of separating the various cell types in the peripheral circulation. Modification of the fixed gradient technique of Van Zant *et al.* (8) proved to be the most useful. The contents of one hematocrit tube of B6 blood diluted in 10 ml of PBS were centrifuged at 670 × *g*. Platelets were retained in the supernatant, and lymphocytes, granulocytes, and erythrocytes were in the pellet. Platelets were subsequently collected by centrifugation of the supernatant at 4500 × *g* and were free of nucleated cells (Table 1). The lymphocytes, granulocytes, and erythrocytes in the initial pellet were enriched by centrifugation in a preformed Percoll gradient. The major changes from Van Zant *et al.* (8) were in the density and amount of the solutions for the fixed Percoll gradients. There was no separation of cells in Percoll at 1.096 g/ml. The best separation was achieved by mixing the cells in 1 ml of Percoll at 1.08 g/ml, underlayering with 2 ml of Percoll at 1.09 g/ml, and overlayering with 1 ml of PBS in a 15-ml centrifuge tube. Centrifugation at 1000 × *g* for 10 min at 4°C enriched the various cell types. Of the nucleated cells at the PBS/Percoll interface, 95.7% were lymphocytes. The granulocytes within the Percoll comprised 80.7% of the nucleated cells. Less than 0.001% of the nucleated cells were found in the erythrocyte pellet at the bottom of the gradient. Erythrocytes were the major contaminants in all fractions and were removed without destroying the other blood elements by lysis in 1% (wt/vol) ammonium oxalate. All cells were washed in PBS, stored at 0°C overnight, and assayed for GPI-1 the next day.

GPI-1 Assay. The cell lysates were applied directly to cellulose acetate plates and electrophoresed as described by Eppig *et al.* (9). After electrophoresis, application of the reaction mixture, and development of the GPI-1 banding pattern, the cellulose acetate plates were fixed in 5% acetic acid and the concentration of the donor- and host-specific bands were determined by spectrophotometry with the Helena Quik-Quant II.

RESULTS

W/W^v mice were injected with successive 1:10 dilutions of B6-+/+, *Gpi-1^a/Gpi-1^a*, *Hbb^s/Hbb^s* marrow cells. Erythrocyte counts and mean cell volumes were monitored as indicators of the condition of the host (Fig. 1). Erythrocyte counts increased and mean cell volumes decreased in the mice injected with 10^5 cells, indicating reconstitution, but little change in blood values was noted in the mice injected with lower cell doses. By 16 weeks after injection of 10^5 normal bone marrow cells, the W/W^v mice had nearly normal blood values, which they maintained thereafter.

Table 1. Enrichment of cells isolated from peripheral blood

Fraction	Percentage of formed elements*		
	Platelets	Lymphocytes	Granulocytes
Original supernatant (platelets)	100	0	0
PBS/Percoll interface (lymphocytes)	0	95.7	4.3
Percoll (granulocytes)	0	19.3	80.7
Percoll pellet (erythrocytes)	0	0	0.001

*Erythrocytes contaminating the supernatant, PBS/Percoll interface, and Percoll fractions were destroyed by treatment with 1% ammonium oxalate and were not included in the calculations for those three fractions.

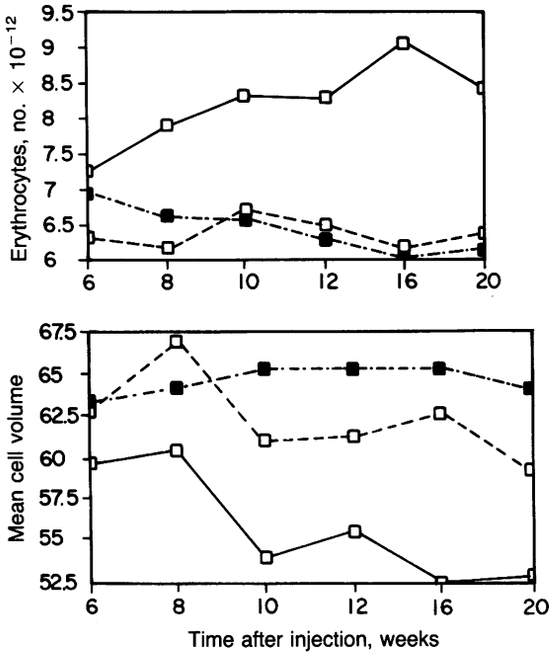

FIG. 1. Erythrocyte parameters during repopulation of W/W^v mice. Erythrocyte counts (*Upper*) and mean cell volumes (*Lower*) are diagnostic in W/W^v mice. Normal values are 10 × 10^{12} cells per liter and 50 fl (50 μm^3), respectively. Mice were injected with 10^5 (□—□), 10^4 (□- -□), 10^3 (■) B6-+/+ cells. Values with a significant difference from W/W^v ($P < 0.01$) include all the erythrocyte counts and mean cell volumes from week 16 of mice injected with 10^5 cells. The standard error of the mean was 0.07–0.54 × 10^{12} per liter for erythrocyte counts and 0.52–2.42 μm^3 for mean cell volume.

Consonant with the alleviation of anemia was the increase in donor marker in the erythrocytes (Fig. 2). By 16 weeks, the donor hemoglobin and GPI-1 almost completely replaced the host markers. The residual 9–12% of host marker is indicative of a small number of host-generated erythrocytes. There is basic agreement between donor GPI-1 levels and donor hemoglobin levels.

During donor marrow repopulation, replacement of host lymphocytes as well as erythrocytes was monitored by measuring GPI-1 levels. Donor lymphocytes increased initially in all mice injected with 10^5 cells and in several mice injected with $\leq 10^4$ cells (Fig. 3). Donor lymphocytes ultimately decreased to nondetectable levels in all of the animals injected with the lower doses. The early-appearing donor

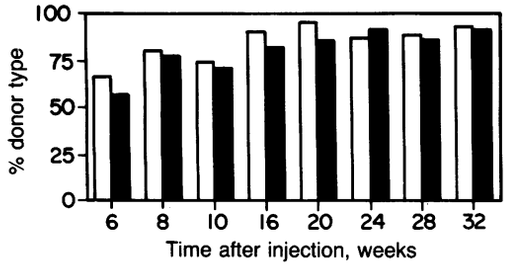

FIG. 2. Donor erythrocyte replacement during repopulation of W/W^v mice. Donor hemoglobin (HBS, □), and GPI-1 (GPI-1A, ■), are indicative of the number of donor-derived erythrocytes. Each of the five recipients was injected with 10^5 cells. Standard errors of the means varied from 1.03% to 3.92% except at 8 and 10 weeks, when they ranged from 4.43% to 11.62%. The variance at 8 and 10 weeks was caused by one mouse that attained the donor phenotype more slowly.

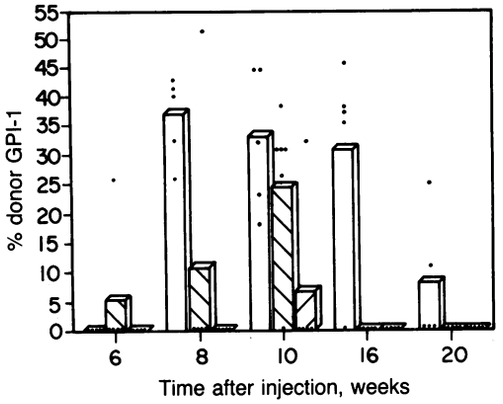

FIG. 3. Timing of donor lymphocyte appearance during repopulation. The percentage of donor GPI-1 in lymphocytes of the mice injected with 10^5 cells (□), with 10^4 cells (□), and with 10^3 (□) has been averaged for presentation at successive time periods after inoculation. The percentage of donor GPI-1 in cells of individual mice is depicted by the dots.

lymphocytes were probably generated from a short-lived precursor.

To determine whether the other peripheral blood components were donor-type, the various cell populations were enriched and examined for donor GPI-1. GPI-1 was monitored at 20 weeks, when the recipients of 10^5 cells were cured and the blood values seemed to have stabilized, and at intervals thereafter. The results of successive assays performed on the mice whose anemia was alleviated by injection of 10^5 donor marrow cells are depicted in Fig. 4. Whereas the erythrocyte population contained an average of 87% donor GPI-1 at 20 weeks after injection, the lymphocytes, granulocytes, and platelets were predominantly host-type. Donor markers increased slowly in the white cells thereafter but did not completely replace the host cells by 41 weeks after injection.

DISCUSSION

The major finding of these studies is that small numbers of normal parental bone marrow cells replace almost all the host erythrocytes but only a small fraction of the lymphocytes, granulocytes, and platelets for up to 32 weeks in W/W^v mice. This has important implications with respect to the primary defect in W/W^v mice and to bone marrow replacement therapy in general.

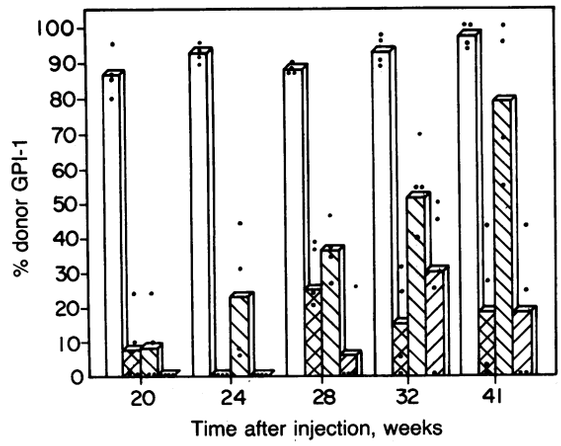

FIG. 4. Donor GPI-1 in the peripheral blood cells at successive times after injection of 10^5 cells. The percent donor GPI-1 present in erythrocytes (□), lymphocytes (□), granulocytes (□), and platelets (□) has been averaged. The dots represent the percentage of donor GPI-1 in cells from each mouse.

The known defects in W/W^v mice indicate the necessity of replacing all myeloid-derived cells. Adult W/W^v mice have a macrocytic anemia, half the normal number of megakaryocytes (10), few mast cells (11), reduced numbers of neutrophils in the marrow (12), and only minor defects in lymphocytes (13, 14). The most severely affected cell detected is an early progenitor cell, the spleen colony-forming unit (CFU-S) (15, 16), whose progeny repopulate the differentiated cells of the myeloid series (17). In most cases where donor contribution to hemopoietic tissue has been monitored, the experience has been that all such tissues in W/W^v mice are repopulated with normal cells (18–21). In experiments where the donor tissue carried a granulocyte marker as well as an erythrocyte marker, there appeared to be parallel replacement of neutrophils and erythrocytes in the circulation (22, 23). Despite the fact that the lymphocytes are not descendants of the apparently defective CFU-S (24), the majority of the mitotic cells in the thymus, and a minority in the lymph nodes, also became donor-type (18–23). Surprisingly, the present report shows stable and selective repopulation of donor erythrocytes, but not other blood elements, during the first 5 months after transplantation.

The reason that our results differ from those obtained previously is not host–donor differences, since others provided B6 parental cells to the anemic F_1-hybrid mice (5, 6, 21, 23). The major alteration in our protocol is in the number of cells injected. Other workers injected $\geq 10^6$ cells, whereas we injected $\leq 10^5$ cells. Limiting dilution injections have been performed by other investigators in order to trace the descendants of a single (or very few) PHSC (25–30). In experiments such as those reported here, where adult W/W^v mice were the recipients, only erythroid repopulation was monitored (25, 26). In experiments where fetal mice homozygous for W mutations were inoculated *in utero*, there were also indications that a small number of PHSCs implanted (27–30). Fleischman *et al.* (28) showed parallel but delayed appearance of neutrophils in two mice with only donor hemoglobin 48 weeks postnatally (28).

We predict the existence of a long-lived, highly proliferative stem cell in the inoculum that exclusively maintains erythropoiesis for prolonged periods of time. Evidence for a long-lived erythroid stem cell was recently presented by Nakano *et al.* (26) from limiting dilution analyses. They measured erythroid but not nucleated cell regeneration in WBB6F_1-W/W^v mice. In this case, the donor mice were females heterozygous for X-linked phosphoglycerate kinase and homozygous for HBS. Hemoglobin analysis indicated when all the WBB6F_1-W/W^v host erythrocytes were replaced. Analysis of phosphoglycerate kinase at intervals thereafter showed that erythrocytes were derived from a single progenitor. Erythrocytes generated for up to 8 weeks expressed only one of the two possible phosphoglycerate kinases. The evidence supports our contention that there is a long-lived, highly proliferative, erythroid-restricted stem cell.

The reason for selective expansion of erythroid cells in mice with a presumptive defect in a multipotent progenitor is unknown. One would predict that cell populations shorter-lived than the erythrocytes, such as the granulocytes and platelets, are likely candidates for more rapid donor-cell replacement. This should be especially true in W/W^v mice. Perhaps with limited numbers of stem cells to draw from, replacement of the most severely deficient population, erythrocytes, is promoted at the expense of the other differentiated cell lines.

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