Marrow Transplantation in the Treatment of a Murine Heritable Hemolytic Anemia

By Jane E. Barker and Eleanor C. McFarland-Starr

Mice with hemolytic anemia, sph+/sph−, have extremely fragile RBCs with a lifespan of approximately one day. Neither splenectomy nor simple transplantation of normal marrow after lethal irradiation cures the anemia but instead causes rapid deterioration and death of the mutant unless additional prophylactic procedures are used. In this report, we show that normal marrow transplantation preceded by sublethal irradiation increases but does not normalize RBC count. The mutant RBCs but not all the WBCs are replaced by donor cells. Splenectomy of the improved recipient causes a dramatic decrease in RBC count, indicating that the mutant spleen is a site of donor-origin erythropoiesis as well as of RBC destruction. Injections of iron dextran did not improve RBC counts. Transplantation of primary recipient marrow cells into a secondary host with a heritable stem cell deficiency (W/W') corrects the defect caused by residence of the normal cells in the sph+/sph− host. The original +/+ donor cells replace the RBCs of the secondary host, and the RBC count is normalized. Results indicate that the environment in the sph+/sph− host is detrimental to normal (as well as mutant) erythroid cells but the restriction is not transmitted.

© 1989 by Grune & Stratton, Inc.

MATERIALS AND METHODS

Animals and splenectomy. Recipients were 3-month-old Fl-hybrid males generated by mating W/Bc/Re (WB) females to C57BL/6J (B6) males. Mice with hemolytic anemia (WB6F1-sph+/sph−) or with a hematopoietic stem cell deficiency called dominant white spotting (WB6F1-W/W) and the normal mice (WB6F1+/+) were homozygous for glucose phosphate isomerase, Gpi−/−/Gpi−/− and heterozygous for the β-globin haplotype, Hbb−/Hbb−. The B6 normal male donors were Gpi−/+/Gpi−/− and Hbb−/Hbb−. Splenectomy was performed on primary recipient mice at least 30 weeks after marrow transplantation. The mice were anesthetized with amylene hydrate in 2,2,2-tribromo-ethanol, and a 5-mm incision was made in the left abdominal wall below the ribs. The spleen was located, grasped firmly with blunt-end forceps, and excised. The blood vessels were tied off with surgical thread, and the spleen was removed. The incision was sutured, and the mice were allowed to recover in clean cages under a heat lamp. These mice and six marrow recipients with spleens were injected intraperitoneally (IP) with 0.8 mg Imferon iron dextran (Lakeside Laboratories, Milwaukee) in 0.9% NaCl. Mice were all maintained at the Jackson Laboratory (Bar Harbor, ME), where strict adherence to Federal regulations on animal care results in accreditation by the American Association for the Accreditation of Laboratory Animal Care.

Marrow transplantation. Donors were killed by cervical dislocation, and femurs were removed aseptically. Cells were flushed from the marrow cavity in Dulbecco's phosphate-buffered saline (PBS), dissociated, and washed once. After centrifugation of the cells, the pellet was eluted in PBS, and an aliquot was counted. The cell concentration was adjusted to 2 x 10^7 cells/mL, and 0.1 mL was injected into the tail vein of each recipient. All recipients except the WB6F1-W/W mice were irradiated with γ-rays generated from a ^137Cs source at a dose rate of 220 rad/min. W/W mice require no irradiation for successful transplantation. The primary recipients received either 200 or 500 rad TBI. Blood was removed from the retroorbital sinus of recipients at monthly intervals to determine RBC parameters, hemoglobin phenotype, and GPI phenotype.

RBC parameters. RBCs diluted in saline were counted on a Coulter Counter model ZBI (Hialeah, FL). A single hematocrit tube from each recipient was centrifuged in an Autocrit II, and the...
hematocrit was determined on a microhematocrit reader. Mean cell volume was calculated from the two values.

**Hemoglobin phenotype.** A 1-cm column of packed RBCs from a hematocrit tube was treated with cysteamine, and the hemoglobins were separated by electrophoresis on cellulose acetate. After electrophoresis, the acetate strips were fixed, stained, and cleared. The concentration of the single hemoglobin and of the diffuse major and minor hemoglobins were quantified in a Helena Quick Quant II Spectrophotometer (Helena Laboratories, Beaumont, TX).

**GPI phenotype.** To determine the relative concentration of donor and host GPI in the peripheral blood cells, we used our modification of the technique of Van Zant et al. Cells collected in a single hematocrit tube were dis渓ased in 10-mL PBS in a 15-mL polystyrene centrifuge tube. The tubes were centrifuged at 760 x g for 10 minutes, and both the supernatant and the cell pellet were saved. The supernatant was centrifuged at 4,500 g to pellet the platelets. The cell pellet was eluted in 1 mL 75% Percoll, underlayered with 2 mL 76.9% Percoll, overlaid with 1 mL PBS, and centrifuged at 1,000 g. Lymphocytes were collected at the PBS-75% Percoll interface; granulocytes were in the Percoll, and RBCs were in the pellet. The cells were washed, and any RBC contaminants in platelet, lymphocyte, or granulocyte fractions were lysed by treatment with 1 mL 1% ammonium oxalate. The cell pellets from each fraction were stored at 4°C overnight and thawed the next day for GPI assays. The GPI phenotype was defined by electrophoresis of the lysates on cellulose acetate according to the technique of Eppig et al. The cellulose acetate plates were fixed in 5% trichloroacetic acid, and the GPI isoforms were quantified by spectrophotometry.

**RESULTS**

The objective of the experiments was to cure the anemia of the sph-sph mice. Mutant and normal littermate control mice were sublethally irradiated rather than lethally irradiated before normal marrow transplantation. Five mutant mice, but none of the +/+ controls irradiated with 500 rad and injected immediately with 6 x 10^6 normal marrow cells were dead within five days. Four of five mutant mice and all five of the normal littermate controls survived 200 rad and injection of 2 x 10^6 normal marrow cells. Two weeks after treatment, the RBC counts of the survivors were at levels expected for untreated mutant and normal mice (Fig 1). Thereafter, the only significant variation was augmentation of RBC counts in the mutant. Despite improvements, the mutant mice never acquired normal levels of RBCs. By 46 weeks, the RBC counts began to decrease. Splenectomy at 49 weeks, as a final resort, depressed the RBC counts even further in the mutant mice but had no effect on the normal mice.

Since incomplete cure of the anemia could result from continued generation of mutant RBCs, various techniques were used to visualize the mutant cells or their products. There was no evidence for the presence of mutant erythrocytes in the peripheral blood. Neither examination of blood smears nor analysis for host hemoglobin showed any trace of the mutant phenotype (data not shown). The hemoglobin in the RBCs of the mutant recipients was 100% donor type (HBS) by 4 weeks, which indicates that normal progenitor cells seeded the host and competed successfully with the host cells. As expected with low-dose irradiation, the normal host cells outcompeted the normal donor cells, and the hemoglobin remained host type (50% HBS).

---

**Table 1. Donor Marker Present in Recipient Peripheral Blood of Sublethally Irradiated sph-sph Mice**

<table>
<thead>
<tr>
<th>Weeks Postinjection</th>
<th>Erythrocytes</th>
<th>Lymphocytes</th>
<th>Granulocytes</th>
<th>Platelets</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>87.5</td>
<td>10</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>92.8</td>
<td>2.5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>12</td>
<td>100</td>
<td>20</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>16</td>
<td>100</td>
<td>10</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>20</td>
<td>100</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>26</td>
<td>100</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>30</td>
<td>100</td>
<td>10</td>
<td>6.7</td>
<td>35.5</td>
</tr>
<tr>
<td>34</td>
<td>100</td>
<td>16</td>
<td>34.5</td>
<td>11</td>
</tr>
<tr>
<td>38</td>
<td>90.5</td>
<td>29.5</td>
<td>40.5</td>
<td>15.5</td>
</tr>
<tr>
<td>49</td>
<td>95</td>
<td>34.5</td>
<td>39.5</td>
<td>-</td>
</tr>
<tr>
<td>55</td>
<td>90.5</td>
<td>21</td>
<td>30.5</td>
<td>18.5</td>
</tr>
</tbody>
</table>

Peripheral blood was fractionated to achieve enrichments of 99.9% erythrocytes, 98% lymphocytes, 80.3% granulocytes, and 100% platelets.
gin was studied after development of adequate separatory techniques 30 weeks into the experiment. Although host erythroid cells disappeared from the peripheral circulation, sph^{w}/sph^{w} lymphocytes, granulocytes, and platelets were maintained for at least 55 weeks. This indicated coexistence of host and donor PHSCs.

The continued anemia, despite repopulation with normal donor RBCs, was inexplicable. The inability to augment the RBC count by splenectomy was probably due to removal of a major erythropoietic site. Another explanation for anemia in the sph^{w}/sph^{w} recipients of +/+ marrow was exhaustion of iron stores. Two splenectomized mutant mice from the initial experiment and six mutant mice with spleens from a second experiment were injected with two doses of 0.8 mg iron dextran. RBCs were counted before and 2 weeks after the final injection (Table 2). No improvement in blood values was detected.

To determine whether the continued anemia was intrinsic to the donor progenitor cells, marrow from one of the primary mutant recipients and one of the primary normal recipients was transplanted to secondary hosts at 30 weeks. The secondary hosts were W/W" mice with a heritable hematopoietic stem cell deficiency. These animals have a severe macrocytic anemia and accept marrow transplants without prior irradiation therapy. RBC counts increased more slowly in secondary recipients injected with marrow from the sph^{w}/sph^{w} primary recipients (Fig 2). Nevertheless, normal levels were attained by 27 weeks posttransplantation in three of the four surviving recipients of mutant cells. In W/W" recipients of sph^{w}/sph^{w} marrow, the RBCs originated from the +/+ rather than from the sph^{w}/sph^{w} donor. This was evident because the cells had the +/+ hemoglobin (data not shown) and GPI phenotype (Fig 3). Approximately 40% of the lymphocytes, granulocytes, and platelets were also +/+ donor type at 33 weeks. Determination of the source of the remaining 60% of WBC cell progeny was impossible since the two mutants carried the same GPI marker.

**DISCUSSION**

The major finding in the present study was the propensity of marrow transplants to alleviate but not cure anemia in sph^{w}/sph^{w} mice. Complete replacement of anemic WBB6F1 host erythrocytes by normal genetically marked B6 donor erythrocytes normalizes RBC counts in most mice with other types of hereditary anemias. Two exceptions are mice with microcytic anemia (mk/mk) and steel mice (SI/SI'). In the iron-deficient mk/mk mice, iron dextran (Imferon) injection after +/+ marrow transplantation is sufficient to cure the host anemia. Iron injection was not therapeutic in the sph^{w}/sph^{w} recipients of normal marrow in the present study.

The SI/SI' mice have a putative microenvironmental defect and do not support erythropoiesis. In the present study, we showed that the environment in the sph^{w}/sph^{w} mice is also inhospitable to the normal erythron. The normal marrow cells generate erythrocytes that completely replace

---

**Table 2. Effect of Iron Dextran Injections on RBC Counts in sph^{w}/sph^{w} Recipients of Normal Marrow**

<table>
<thead>
<tr>
<th>Mouse No.</th>
<th>Spleen</th>
<th>RBCs x 10^{12}/L</th>
<th>RBCs x 10^{12}/L</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Pretreatment</td>
<td>Posttreatment</td>
</tr>
<tr>
<td>1</td>
<td>−</td>
<td>4.62</td>
<td>4.48</td>
</tr>
<tr>
<td>2</td>
<td>−</td>
<td>5.18</td>
<td>5.38</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>5.98</td>
<td>5.86</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>6.10</td>
<td>5.04</td>
</tr>
<tr>
<td>5</td>
<td>+</td>
<td>3.82</td>
<td>3.74</td>
</tr>
<tr>
<td>6</td>
<td>+</td>
<td>4.92</td>
<td>4.64</td>
</tr>
<tr>
<td>7</td>
<td>+</td>
<td>7.12</td>
<td>7.18</td>
</tr>
<tr>
<td>8</td>
<td>+</td>
<td>4.62</td>
<td>4.50</td>
</tr>
</tbody>
</table>

*Pretreatment counts were performed 4 weeks before ip injection (two doses at weekly intervals) of iron dextran. Posttreatment counts were performed 2 weeks after the final injection.

---

**Fig 2. RBC counts in secondary W/W" recipients. RBCs were enumerated as described in the legend to Fig 1. Bars are number of cells x 10^{12}/L in the four surviving secondary recipient WBB6F1-W/W" mice. Donors were a WBB6F1-sph^{w}/sph^{w} (C) mouse and a WBB6F1-+/+/ (B) mouse injected with B6-+/+ marrow cells 30 weeks previously. SEM was 0.29 to 0.5.**

---

**Fig 3. Percentage of donor GPI in secondary W/W" recipients. GPI was monitored in peripheral blood cells from the four surviving secondary recipient WBB6F1-W/W". Gpi-1+/Gpi-1" mice injected with marrow cells from primary recipient WBB6F1-sph^{w}/sph^{w} injected 30 weeks previously with B6-+/+ + Gpi-1"/Gpi-1" marrow cells. The peripheral blood was fractionated to enrich RBCs (c), lymphocytes (s), granulocytes (p), and platelets (l). Points show percentage of donor GPI in each of the recipients.**

---

From www.bloodjournal.org by guest on January 30, 2018. For personal use only.
the host RBCs but do not cure the anemia. Transplantation of marrow from the primary recipient to a secondary W/W' host induced normalization of the RBC count in the latter. RBC replacement from the original +/- donor confirmed the suspicion that the environment in sph/sph mice does not fully support the normal erythron.6 Whether there is ineffective erythropoiesis or shortened lifespan of RBCs is not known. The latter is suspected because normal RBCs when transfused disappear more rapidly from sph/sph mice than from +/- mice.3

The cause of lowered RBC counts is not limited to splenic destruction of cells. In the present report, splenectomy of the mutant mice after replacement of host with normal donor RBCs augmented rather than relieved the anemia. Host scavenger cells are an alternate cause of erythroid cell destruction. Unlike the host RBCs, >50% of the host WBCs persist in the sublethally irradiated sph/sph recipient. Host WBCs, because they have had the opportunity to interact with abnormal RBCs, may recognize all erythrocytes as foreign. This would account for the improvement but not cure of hemolytic anemia in sph/sph recipients of normal marrow. In the secondary W/W' host, a requirement for acquisition of normal blood counts would be that most WBCs be W/W' or +/- derived. At the time the W/W' recipients attain normal blood values, <40% of the granulocytes and lymphocytes are from the original +/- donor. The source of the remaining 60% cannot be determined because the WBCs of both the W/W' and the sph/sph mice carry the same marker. However, the WBCs probably originate from the W/W' mice. Recent studies of sequential replacement of blood cells in marrow-transplanted W/W' mice indicate that host WBCs but not host RBCs are maintained long-term.8

The prolonged maintenance of host leukocytes and platelets during complete donor erythrocyte replacement is easier to explain in mice with hemolytic anemia than in W/W' mice. In sph/sph mice, +/- donor erythrocytes are assumed to have a selective advantage because they are longer-lived. There is no need to replace the normal host leukocytes. The W/W' mice, on the other hand, have a putative stem cell deficit that affects all myeloid-derived cells at some level. One explanation for amplification of normal donor erythrocytes but not donor leukocytes is that (as is the case in the sph/sph mice) the host erythrocytes are more severely affected. This interpretation may lead to a reevaluation of the defect in the W/W' mice.

Defining the nature of the unfavorable environment in sph/sph mice is important. The mice share characteristics with severe recessive hereditary spherocytosis patients.17,18 Splenectomy soon after birth appears to relieve the symptoms in humans, although it does not always cure the anemia.18 Both affected humans and mice appear to have inherent difficulties in maintaining RBC counts after treatment with therapeutic measures. Exposition of the environmental defect in mice may lead to use of appropriate prophylactic treatment in humans. Similar therapeutic measures may also permit curative marrow transplantation in the mutant mice. Development of marrow transplantation techniques that result in normalization of mutant RBC counts before use of mutant mice in gene transfer studies is imperative.

ACKNOWLEDGMENT

We thank Drs Selden Bernstein, Elizabeth S. Russell, and Janan Eppig for comments and suggestions during the course of this study and Nancy Hamblen for excellent technical assistance.

REFERENCES

2. Bernstein SE: Personal communication, May 1988
10. Eppig JJ, Kozak LP, Eicher EA, Stevens LC: Ovarian teratomas in mice are derived from oocytes that have completed the first meiotic division. Nature 269:517, 1977
Marrow transplantation in the treatment of a murine heritable hemolytic anemia

JE Barker and EC McFarland-Starr