The Hematopoietic Stem Cells of α-Thalassemic Mice

By Jane E. Barker and Eleanor McFarland

The α-thalassemic mouse has a hereditary microcytic anemia, almost certainly has a shortened RBC life span, and is a potential candidate for cell replacement therapy. In a routine study of bone marrow repopulating capacity using hemoglobin as a cell marker, normal donor marrow cells, but not α-thalassemic donor marrow cells, completely replaced the host cells. Further analysis showed that at least 30 times more α-thalassemic cells were required to outcompete normal donor cells injected simultaneously. The results were more extreme than expected and suggested a defect in a stem cell population as well as in the RBCs. Evidence that the multipotent and erythroid-committed stem cells in α-thalassemic mice are not decreased was shown by CFU-S and CFU-E assays. The combined results indicate that the deletion expresses itself most conspicuously in the RBC population. Tests were also performed to analyze repopulation kinetics in the Hba*+/+ mice. In unirradiated α-thalassemic hosts, the hemoglobin from a normal donor persisted but did not replace the host hemoglobin. Sublethally irradiated α-thalassemic hosts, on the other hand, were easily repopulated with normal cells.

We conclude that the α-thalassemic mouse is a good model for cell replacement therapy.

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Table I. Diagram of Experiments Using Hemoglobin Markers

Repopulation:
1. (Fig. 1)
2. (Table 2)
3. (Fig. 6)

Competitive Repopulation:
1. (Fig. 2)
2. (Fig. 3)

B
Recipient:

Key

Donors:

Treatment:


**Competitive repopulation assays.** Mixtures of cells with different hemoglobin markers were injected into F1 recipients. The recipients were monitored periodically for a change in phenotype. (1) WBB6F1-/+ bone marrow cells carrying diffuse and single hemoglobin were mixed at a concentration of 3 x 10^6 with 3 x 10^6, 6 x 10^6, or 12 x 10^6 bone marrow cells from B6-Hba''/+ mice with single hemoglobin. The mixture was injected into WBB6F1 mice irradiated with 900 rad. Controls were injected with a mixture of 3 x 10^6 WBB6F1 bone marrow cells and 3 x 10^6, 6 x 10^6, or 12 x 10^6 B6-/+ bone marrow cells with single hemoglobin. (2) WBB6F1 bone marrow cells with diffuse and single hemoglobins were mixed at concentrations of 10^6 or 5 x 10^6 with 3 x 10^6 bone marrow cells from B6-Hba''/+ mice with single hemoglobin and injected into lethally irradiated WBB6F1 mice. Lethally irradiated controls were injected with 3 x 10^6 B6-Hba''/+ cells.

**Hemoglobin assays.** Blood samples removed from the intraorbital sinus were centrifuged in a hematocrit tube. For hemoglobin phenotype determinations, a 1-cm column of packed RBCs was treated with cysteamine and electrophoresed on cellulose acetate strips using the technique of Whitney. Following electrophoresis, the cellulose acetate strip was fixed, stained, and cleared. The concentration of the various hemoglobins was quantified in a Helena Quick Quant II (Helena Laboratories, Beaumont, Tex).

**Spleen colony (CFU-S) assay.** Lethally irradiated B6-/+ mice were injected with 4 x 10^6, 6 x 10^6, 8 x 10^6, or 10^7 adult bone marrow cells or 10^6 to 10^7 fetal liver cells from B6-Hba''/+ mice. Bone marrow was from 2- or 10-month-old mice. Liver was from 13- or 14-day fetuses. Lethally irradiated controls were injected with equivalent numbers of B6-/+ cells. The recipients were killed by cervical dislocation ten days later. The spleens were removed and fixed in Telly’s solution. Macroscopic spleen colonies were counted.

**Erythroid colony (CFU-E) assay.** Dissociated marrow cells from B6-/+ or B6-Hba''/+ mice were aliquoted in a mixture that included erythropoietin (Connaught Medical Research Labs, Toronto, Canada) at 0.75 U/mL as described previously. Citrated bovine plasma (GIBCO) was added to promote formation of plasma clots, and the mixture was aliquoted in 0.1 mL microwells and incubated at 36.5 °C in 5% CO2 in air for two days. Clots were removed to microscope slides, fixed, and stained, and the number of colonies was counted microscopically.

**RESULTS**

**Repopulation of sublethally irradiated normal mice.** The initial experiments showed that the stem cells from thalassemic mice were less effective than stem cells from normal mice during repopulation assays. Equivalent numbers of single hemoglobin-containing bone marrow cells from either B6-/+ or B6-Hba''/+ mice were injected into each of five sublethally irradiated WBB6F1-/+ hosts irradiated with 900 rad, a dose we considered from previous experience to be lethal. It appeared that the dose did not destroy all of the WBB6F1 host cells surviving the sublethal irradiation had a selective advantage over the cells donated by the thalassemic but not the normal donor.

The selective advantage was subsequently monitored by competitive repopulation assays.

**Competition between normal and α-thalassemic stem cells.** Successively larger numbers of either B6-Hba''/+ or B6-/+ bone marrow cells with single hemoglobin were mixed with 3 x 10^6 WBB6F1-/+ bone marrow cells contributing a diffuse hemoglobin marker. The various cell mixtures were injected into WBB6F1-/+ hosts irradiated with 900 rad, a dose we considered from previous experience to be lethal. It appeared that the dose did not destroy all of the host stem cells since two of the recipients injected with 3 x 10^6 normal cells did not switch completely to the donor phenotype (Fig 1). The results (Fig 2) did establish the highly competitive nature of the B6-/+ cells and noncompetitive nature of cells from thalassemic mice. As indicated by the hemoglobin phenotype, the B6-/+ cells repopulated the host with single hemoglobin when the input concentration was only two times that of the co-injected WBB6F1 cells. This means that the B6-/+ cells outcompeted both the recovering host cells and the co-injected cells. The thalassemic cells did not cause a significant elevation of single hemoglobin even at concentrations four times that of the co-injected WBB6F1 cells.
A result of the rapid turnover of Hba'/+ single hemoglobin 36 weeks after injection. The number of WBB6F, cells. The four surviving control mice were present at a concentration six and 30 times and injected with B6-Hba'/+ competitors was much greater than expected if replacement was normal cells, only two of the five recipients had an increase in thalassemic mice. Assays that have been widely used to enumerate multipotent stem cells and erythroid-committed cells (Fig 4). Results showed that there were equivalent numbers of spleen colonies generated from equivalent numbers of normal and thalassemic cells at all of the ages tested. Potential defects during RBC differentiation were monitored by enumerating the colonies formed from the most mature erythroid-committed stem cells using the CFU-E assay (Fig 5). As expected in mice with greater than normal numbers of reticulocytes, there were more colonies generated from Hba'/+ marrow cells than from an equal number of +/+ marrow cells. The findings noted in this section eliminate the possibility of quantitative differences in CFU-S and CFU-E but do not exclude qualitative differences that may affect repopulation kinetics or quantitative differences in the stem cells not measured by these techniques.

Repopulation of unirradiated and sublethally irradiated mice. The 30-fold selective advantage normal cells enjoy over α-thalassemic cells indicated that the anemic mice might accept marrow grafts without undergoing lethal irradiation. It has been shown previously that unirradiated mice homozygous for alleles at the W locus are cured one month after an injection of 10^9 normal bone marrow cells. In the present experiments, B6-Hba'/+ and B6-+/+ mice with single hemoglobin were injected with 2 x 10^6 bone marrow cells from congenic B6-+/+ mice with diffuse hemoglobin. The type of hemoglobin in the recipients was monitored by electrophoresis—a technique sensitive enough to discriminate <1% donor hemoglobin. Results showed that there was donor diffuse hemoglobin present in all of the Hba'/+ mice.

Fig 2. Competition between normal and mutant stem cells during repopulation of sublethally irradiated F1 recipients. WBB6F,+/+ mice with single and diffuse hemoglobin were irradiated with 900 rad and injected with a mixture of bone marrow cells from mice with single hemoglobin (B6) and single-diffuse hemoglobin (WBB6F,). The percentage of S hemoglobin at various intervals after injection is shown for mixtures of 3 x 10^6 WBB6F, cells with 3 x 10^6 B6-Hba'/+ cells (■), 6 x 10^6 B6-Hba'/+ cells (△), 12 x 10^6 B6-Hba'/+ cells (■■) or with 3 x 10^6 B6-+/+ cells (O—O), 6 x 10^6 B6-+/+ cells (△—△), and 12 x 10^6 B6-+/+ cells (■—■). Each point represents the average value from a total of five mice. The SEM varied between 0.3 to 2.2 in mice receiving WBB6F, and B6-Hba'/+ cells and between 0.22 to 5.11 in mice receiving WBB6F, and B6-+/+ cells.

A second experiment was performed to test the extent of the selective advantage of the WBB6F, cells. In this case, WBB6F,+/+ mice were lethally irradiated with 1,000 rad and injected with B6-Hba'/+ and WBB6F,+/+ cells. The B6 cells were present at a concentration six and 30 times that of the WBB6F, cells. The four surviving control mice injected with 3 x 10^6 B6-Hba'/+ cells had primarily single hemoglobin (Fig 3). Even with a 30:1 ratio of thalassemic to normal cells, only two of the five recipients had an increase in single hemoglobin 36 weeks after injection. The number of B6-Hba'/+ cells needed to outcompete the WBB6F, competitors was much greater than expected if replacement was a result of the rapid turnover of Hba'/+ RBCs. Additional lesions were sought in the Hba'/+ stem cells and in the pathway leading to erythroid differentiation.

Enumeration of CFU-S plus CFU-E in normal and α-thalassemic mice. Assays that have been widely used to quantitate multipotent stem cells and erythroid-committed stem cells in mice are the CFU-S^10 and CFU-E^13 assays, respectively. In this report, the CFU-S assay was used to compare the number of colonies found in the spleens of mice injected with various concentrations of B6-+/+ or B6-Hba'/+ 2- or 8-month-old bone marrow cells or fetal liver cells (Fig 4). Results showed that there were equivalent numbers of spleen colonies generated from equivalent numbers of spleen colonies generated from equivalent

Fig 3. Competition between normal and mutant cells during repopulation of lethally irradiated recipients. WBB6F,+/+ mice with single and diffuse hemoglobin were irradiated with 1,000 rad and injected with a mixture of bone marrow cells from WBB6F,-+/+ and B6-Hba'/+ mice. The percentage of S hemoglobin in the positive controls injected with B6-Hba'/+ cells and in the experiments injected with a mixture of B6-Hba'/+ cells and WBB6F,+/+ cells is shown at ten weeks (□) and at 36 weeks (■). Each pair of columns represents data from a single recipient.
These studies have confirmed the fact that α-thalassemic mice have a defective hematopoietic cell population. The most likely candidate is the RBC, although a secondary stem cell lesion cannot be discounted. It has been suspected, but never proved, that Hba+/+ RBCs are short lived. Indirect evidence includes the finding of anisocytosis in RBC smears from one of the Oak Ridge thalassemic mice, reticulocytosis (S.E. Bernstein, personal communication), enlarged and erythropoietic spleens (data not shown), and in the present report, the increased numbers of colonies generated from erythroid-committed stem cells (Fig 5). The results are

**DISCUSSION**

Table 2. Donor Diffuse Hemoglobin in Untreated α-Thalassemic Recipients

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<th>Recipient No.</th>
<th>Percentage of Donor Diffuse Hemoglobin After Injection</th>
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although it is incorporated in hemoglobin, is reused. Radiolabeled glycine-2\(^{14}\)C has been used in vivo to define the RBC life spans of mice with microcytic anemias.\(^{16,17}\)

The competitive repopulation experiments are difficult to interpret if one assumes that the only lesion is the shortened life span of thalassemic RBCs. Thirty times more thalassemic cells are required to outcompete normal WBB6F, cells during repopulation of an irradiated host (Fig 3), whereas only two times more B6+/+ than WBB6F, cells are sufficient (Fig 2). In previous competition experiments between cells from mice with Hertwig's anemia, an/an, and +/+ cells, a fourfold increase in an/an stem cells decreased the contribution of the +/+ hemoglobin in the recipients.\(^5\)

The more extreme results noted with thalassemic mice may be due to a combination of factors. The reduced RBC life span is certainly a major defect but decreased numbers of multipotent stem cells or of erythroid-committed stem cells also had to be considered. Quantitation of colonies generated from CFU-S (Fig 4) and CFU-E (Fig 5) led to the conclusion that the stem cells measured by these assays are not deficient in numbers.

It is possible that a stem cell population has a qualitative defect not detected by these experiments. In fact, results of sublethal irradiation experiments (Fig 6) can be interpreted as indicating the more friable nature of thalassemic when compared to normal stem cells. One must exhibit caution, however, in such an interpretation because equivalent numbers of stem cells may have seeded both hosts, but the need for RBC replacements may have evoked a greater response from the +/+ stem cells in the thalassemic recipient.

The fact that stem cell seeding sites exist in unirradiated thalassemic mice (Table 1) but not in normal mice also suggests some type of stem cell deficit. Regardless of the mechanism by means of which seeding sites are generated in Hba<sup>th</sup>/+ mice, the sites themselves are fortuitous because they allow repopulation of the \(\alpha\)-thalassemic mice following injections with large numbers of donor cells. The classical model for stem cell replacement until now has been severely anemic mice homozygous at the W locus.\(^{11,12}\) The existence of a second model, the thalassemic mouse, with its similarities to human \(\alpha\)-thalassemia-1, should be instructive in developing mild therapeutic measures that are successful and in studying the long-term effects of RBC transfer. Competitive repopulation data indicate further that transfer of a normal allele to Hba<sup>th</sup>/+ stem cells may be an effective means to selectively expand a population of cells expressing the transferred gene product.

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