Differential Regulatory and Compensatory Responses in Hematopoiesis/Erythropoiesis in α- and β-Globin Hemizygous Mice*

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Characterization of hematopoiesis/erythropoiesis in thalassemias from multipotent primitive cells to mature erythrocytes is of fundamental importance and clinical relevance. We investigated this process in α- and β-globin hemizygous mice, lacking the two adult tandemly organized genes from either the α- or β-globin locus. Although both mice backcrossed on a homogeneous background exhibited similar reduced red blood cell (RBC) survival, β-globin hemizygous mice had less severe reticulocyte loss and globin chain imbalance, suggesting an apparently milder thalassemia than for α-globin hemizygous mice. In contrast, however, β-globin hemizygous mice displayed a more marked perturbation of hematologic parameters. Quantification of erythroid precursor subpopulations in marrow and spleen of β-globin hemizygous mice showed more severely impaired maturation from the basophilic to orthochromatophilic erythroblasts and substantial loss of these late precursors probably as a consequence of a greater susceptibility to an excess of free α-chain than β-chain. Hence, only erythroid precursors exhibiting stochastically moderate chain imbalance would escape death and mature to reticulocyte/RBC stage, leading to survival and minimal loss of reticulocytes in the β-globin hemizygous mice. Furthermore, in response to the ineffective erythropoiesis in β-globin hemizygous mice, a dynamic compensatory hematopoiesis was observed at earlier differentiation stage as evidenced by a significant increase of erythroid progenitors (erythroid colony-forming units ~100-fold) as well as of multipotent primitive cells (day 12 spleen colony-forming units ~7-fold). This early compensatory mechanism was less pronounced in α-globin hemizygous mice. The expansion of multipotent primitive and potentially stem cell populations, taken together with ineffective erythropoiesis and increased reticulocyte/RBC destruction could confer major cumulative advantage for gene targeting/bone marrow transplantation. Therefore, this study not only corroborated the strong potential effectiveness of transplantation for thalassemic hematopoietic therapy but also demonstrated the existence of a differential regulatory response for α- and β-thalassemia.

Thalassemia, among the most frequent of inherited diseases, constitutes a heterogeneous disorder based on clinical severity, pathophysiology, and molecular changes. This hemoglobinopathy has been classified into two major groups, α- and β-thalassemia, reflecting impairment or absence of either α or β chain synthesis. The level of globin chain imbalance, resulting from a change in the relative ratio of α- and β-globin chains, appears directly related to the severity of thalassemia in humans. A variety of mutations including deletions, frameshifts, nonsense, and abnormal splicing lead to a thalassemic phenotype (1–5).

Individuals with thalassemia display mild to severe anemia depending on their genotype (6). In symptomatic patients, α- and β-thalassemia display similar abnormal red blood cell (RBC) features including microcytosis, hypochromy, anisocytosis, and poikilocytosis. The excess of one normal globin chain in RBC forms aggregates, leading to premature cell destruction. Thalassemic RBC membranes are rigid, showing instability in the case of β-thalassemia and hyperstability in α-thalassemia (7). Bone marrow from affected individuals usually undergoes erythroid hyperplasia associated with increased production of erythroblasts and moderate to severe splenomegaly. Ineffective erythropoiesis, more prominent in β- than in α-thalassemia, is also observed (8–10). Individuals with severe thalassemia are dependent on regular transfusion. Although chronic transfusion improves survival, it leads progressively to iron accumulation and tissue damage in several organs.

Allogeneic bone marrow transplantation has been successfully used as a therapy for thalassemia. However, the morbidity and mortality associated with this procedure as well as the difficulty in obtaining histocompatible donors remain problematic (11, 12). These problems could potentially be alleviated by the use of autologous bone marrow transplantation following gene therapy correction. In both cases, a detailed characterization of the altered hematopoiesis and erythropoiesis in α- and β-thalassemia is necessary to develop an effective cure for thalassemic patients.

Initial mouse models of α- and β-thalassemia were generated following radiation-induced or genetically induced mutations (13–15). More recently, α- and β-globin hemizygous mice have been produced by targeted deletion of the adult globin genes (16, 17). The α-globin hemizygous mice, with deletion of the two adult α-globin genes, genetically reproduce the human α-thalassemia 1. Similarly, β-globin hemizygous mice correspond genotypically to heterozygous β-thalassemia. A thorough characterization of hematopoiesis/erythropoiesis in these globin hemizygous mice is required to determine the fundamental cellular defects and whether these mice reproduce the human diseases, particularly as these globin hemizygous mice are becoming widely used as models of human thalassemia.

Herein, we report such an investigation in these α- and β-globin hemizygous mice both bred and compared for the first
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time on an identical genetic background. These mice with half their adult α- or β-globin gene content demonstrated thalassemia. On this homogeneous background, the α-globin compared with β-globin hemizygous mice had greater globin chain imbalance in peripheral RBC, a surprising result considering that β-globin hemizygous mice had more severe anemia. However, we demonstrated that the β-globin hemizygous mice had a more severely impaired late erythroid precursor maturation attributed to an increase cell loss upon the onset of α-globin chain expression that occurred earlier than for the β-globin chain. Furthermore, consequent to the ineffective erythropoiesis, the β-globin relative to α-globin hemizygous mice underwent a more pronounced compensatory stimulation of the multipotent primitive cell populations and of early erythropoiesis. Finally, the inverse correlation between the compensatory erythropoietic/hematopoietic stimulation and the severe alterations of hematologic parameters suggested that the level of anemia might provide a reliable index for the potential effectiveness of gene therapy and bone marrow transplantation in thalassemias.

EXPERIMENTAL PROCEDURES

Animal Studies

Hemoglobin α-globin mice (Hba1m1Tow/Hbaa) and β-globin mice (Hbbtm1Paz/Hbaa) were a generous gift from Drs. C. Paszty and E. Rubin and from Dr. T. Townes, respectively (16, 17). Both of these lines have been backcrossed for multiple generations (~10) with C57BL/6j inbred mice to avoid the effects of various genetic backgrounds. The α-globin mice (C57BL/6j hemi-α-thal) were identified as previously described (16). The β-globin hemizygous mice (C57BL/6j hemi-β-thal) were also identified following PCR amplification using three primers; two were localized in the wild type β-globin gene, forward (5′-GAG-CAATGTGACAGAAGGAC-3′) and reverse (5′-TGAATGTCGTT-CTGGGTTGTG-3′), producing a 450-bp amplicon and a third neo- 

Fluorescence-activated Cell Sorting Analysis and Quantitation

Flow cytometry analysis was performed on bone marrow and spleen samples from C57BL/6j, C57BL/6j hemi-α-thal, and C57BL/6j hemi-β-thal mice. Bone marrow cells were harvested by flushing one femur with PBS containing 2% fetal calf serum. Spleen cells were suspended in 2% fetal calf serum/PBS by subsequent passage through decreasing size needles (18-, 20-, and 23-gauge). Cells (5 × 10^6 or 7.5 × 10^6) were incubated in 2% fetal calf serum/PBS with antibodies for 30 min on ice according to standard techniques. The labeling with anti-mouse TER119-fluorescein isothiocyanate (0.2 ng) and biotin-conjugated anti-μ-mouse CD71 (transferrin receptor) (0.5 ng) was detected with streptavidin-phycoerythrin (0.2 ng) (BD Biosciences). Apoptosis was defined by phosphatidylserine exposure on erythroid precursors was detected with 1–1.5 μl of Annexin V-fluorescein isothiocyanate-conjugated avidin (BIO/CAN, Toronto, Canada) in 1 ml of PBS. The number of biotinylated cells in circulating blood was determined at t = 0 and monitored at regular intervals by flow cytometry on a FACScan (Becton Dickinson, San Jose, CA).

The abbreviations used are: RBC, red blood cell; hemi-α-thal, α-globin hemizygous; hemi-β-thal, β-globin hemizygous; PBS, phosphate-buffered saline; CFU-E, erythroid colony-forming units; BFU-E, erythroid burst-forming units; CFU-GM, granulocyte/macrophage colony-forming units; CFU-M, macrophage colony-forming units; CFU-GEMM, granulocyte-erythroid-megakaryocyte colony-forming units; CFU-S12, day 12 spleen colony-forming units.

1 The absolute cell loss can be defined as the difference between the expected number of cells and the observed number of cells.

\[
P_{\text{cont}}^{\text{obs}} - P_{\text{cont}}^{\text{cont}} \rightarrow P_{\text{cont}}^{\text{obs}} - P_{\text{f12}}^{\text{f12}}
\]

(1 Eq.)

The expected number of cells based on controls from stage 1 is as follows.

\[
\text{1) subpopulation between two consecutive stages: stage 1} \rightarrow \text{stage 1 + 1; 2) percentage (P) of cells at two consecutive stages for control and hemizygous (P}_{\text{cont}}^{\text{cont}} \rightarrow P_{\text{cont}}^{\text{cont}} \rightarrow P_{\text{f12}}^{\text{f12}}; P_{\text{cont}}^{\text{cont}} \rightarrow P_{\text{f12}}^{\text{f12}}; \text{and 3) total number of cells in a subpopulation (N').}
\]

The expected number of cells based on controls from stage 1 + 1 + 1 as follows.

\[
P_{\text{cont}}^{\text{cont}} \rightarrow P_{\text{cont}}^{\text{cont}} \rightarrow P_{\text{f12}}^{\text{f12}} \rightarrow \frac{P_{\text{f12}}^{\text{cont}}}{P_{\text{cont}}^{\text{cont}}}
\]

(2 Eq.)

Blood was analyzed using flow cytometry-based hematology with the mouse archetype of multispecies software version 2.2.06 (Bayer Advia 120, Tarrytown, NY). A Mie scatter theory was used to determine the volume and hemoglobin concentration for each cell by analysis of low and high angle light scattering as previously described (24). The percentage of hypochromic RBCs (mean cellular hemoglobin concentration, less than 22 g/dl) and the percentage of microcytic cells (volume less than 25 fl) were evaluated by appropriate gating of the cellular hemoglobin concentration mean and the mean cellular volume. Reticulocyte counts were obtained by specific RNA staining with the oxazine 750 dye using the reticulocyte channel of the Bayer Advia 120.

Hematologic Parameters

Finally, the inverse correlation between the compensatory erythropoietic/hematopoietic stimulation and the severe alterations of hematologic parameters suggested that the level of anemia might provide a reliable index for the potential effectiveness of gene therapy and bone marrow transplantation in thalassemias.
Hematopoiesis in α- and β-Thalassemias

Quantification of soluble α- and β-globin chains from reticulocytes was performed following biosynthetic labeling with [3H]leucine (1, 2, and 2.5 h). Soluble globin chain analyses were also performed at shorter time points (10, 20, and 40 min). The globin chain imbalance was more severe for the hemi-αthal than for the hemi-βthal mice. In addition, soluble globin chain analysis was carried out at steady state on peripheral blood. Values are expressed as a percentage of total globin chains ± S.D. n = number of independent mice analyzed.

| Mice                        | Time of biosynthesis | Steady state |
|-----------------------------|----------------------|--------------|
|                             | 1 h                  | 2 h          | 2.5 h        | %      | %      | %      | %      |
| Control: C57BL/6J           |                      |              |              |        |        |        |        |
| Percentage of α chain/totals chains | 47.1 ± 1.0          | 48.7 ± 1.7   | 50.0 ± 0.9   | 50.0 ± 0.9 |
| Percentage of β chain/totals chains | 52.9 ± 1.0          | 51.3 ± 1.7   | 50.0 ± 0.9   | 50.0 ± 0.9 |
| C57BL/6J hemi-αthal         |                      |              |              |        |        |        |        |
| Percentage of α chain/totals chains | 37.1 ± 1.9a         | 37.1 ± 1.5b  | 41.4 ± 2.0c  | 45.9 ± 0.6d |
| Percentage of β chain/totals chains | 62.9 ± 1.9a         | 62.9 ± 1.5b  | 58.6 ± 2.0c  | 54.1 ± 0.6d |
| C57BL/6J hemi-βthal         |                      |              |              |        |        |        |        |
| Percentage of α chain/totals chains | 54.6 ± 0.7c         | 55.6 ± 1.2c  | 55.3 ± 0.9b  | 49.3 ± 0.6 |
| Percentage of β chain/totals chains | 45.4 ± 0.7c         | 44.4 ± 1.2c  | 44.7 ± 0.9b  | 50.8 ± 0.6 |

The equation can be rearranged to give the following,

\[
\frac{P_i^{+}P_{i+1}^{out}}{P_i^{+}P_{i}^{out}P_{i+1}^{+}} \times 100 \quad \text{(Eq. 3)}
\]

The equation can be rearranged to give the following,

\[
\left(1 - \frac{P_i^{+}P_{i}^{out}}{P_i^{+}P_{i+1}^{++}}\right) \times 100 \quad \text{(Eq. 4)}
\]

which equals the percentage of cell loss from the \(P_i\) subpopulation.

**Hematopoietic Progenitor Studies**

*Clonogenic Assays—Clonogenic assays were performed on C57BL/6J hemi-αthal, C57BL/6J hemi-βthal, and control C57BL/6J mice. Progenitor cell analyses were carried out on three hematopoietic tissues: bone marrow, peripheral blood, and spleen. Peripheral blood cells were obtained from the buffy coat, washed twice in Iscove’s modified Dulbecco’s medium plus 5% fetal calf serum and once in PBS; RBC lysis was obtained following incubation in 5 ml of Gey’s solution (8.3 g/liter KHCO₃. pH 7.2) for 2 min at 37 °C, and the cells were resuspended in Iscove’s modified Dulbecco’s medium. Bone marrow cells, peripheral blood mononuclear cells, and spleen single-cell suspensions were plated, respectively, at a density of 10⁵, 10⁶, and 5 × 10⁵ cells/ml in 1% methylcellulose/Iscove’s modified Dulbecco’s medium as previously described (25). All experiments were performed in duplicate for each animal. Erythroid colony-forming units (CFU-E) were counted after 2 days in culture, whereas burst-forming units (BFU-E), granulocyte/macrophage colony-forming units (CFU-GM), and macrophage colony-forming units (CFU-M) were counted at 7 days, and granulocyte-erythroid-macrophage-megakaryocyte colony-forming units (CFUGEMM) was counted on day 11. Results were expressed as the mean ± S.D. from all animals analyzed. For each genotype, the percentage of spleen weight per total body weight was also determined.

**Imbalanced Globin Chains in Both Hemizygous Mice**

The α- and β-globin hemizygous mice (C57BL/6J hemi-αthal and C57BL/6J hemi-βthal) have been generated by targeted deletion of the two adult tandem genes at the α- or β-globin locus, respectively. To characterize the α- and β-globin hemizygous mice on the same genetic background, we backcrossed these mice to the C57BL/6J strain for more than 10 generations. We then investigated whether soluble globin chain levels were imbalanced at three short time periods of de novo synthesis in reticulocytes and at steady state in peripheral blood, which consisted of more than 99.4% reticulocytes and RBC. Short biosynthesis periods in reticulocytes are necessary to detect severe chain imbalances apart from cell destruction that may occur with time. As shown in Table I, in the β-globin hemizygous mice, the biosynthesis of the β-globin chain was significantly decreased at all time points compared with controls (11–14%). For the α-globin hemizygous mice, the α-globin synthesis displayed a stronger decrease compared with controls (17–23%). Thus, both of these globin hemizygous mice have thalassemia, but comparison of relative globin chain levels revealed a more severe imbalance for the α-globin hemizygous mice. When the ratio of soluble globin chains in peripheral blood was evaluated at steady state, it appeared improved for both hemizygous mice (Table I), suggesting loss of reticulocytes. Whereas complete chain balance was attained for the β-globin hemizygous mice, imbalance was still detected in the α-globin hemizygous mice. To assess whether the differential response between the hemi-βthal and hemi-αthal mice is due to an increased tendency of the excess α-globin chains from hemi-βthal to associate with erythroid cell membranes, we have monitored the levels of globin chains in reticulocyte membranes in these mice as a function of total protein (Table II). A significant amount of the globin chain in excess was trapped in reticulocyte membranes for both the hemizygous mice as determined by biosynthetic labeling, whereas no membrane-bound globin chain was detected in controls. However, at all

**Statistical Methods**

Unpaired two-sample Student’s t test was used for statistical analysis; \( p < 0.05 \) was considered significant.

### Table I

| Time of biosynthesis | Steady state |
|----------------------|--------------|
| 1 h                  | 2 h          | 2.5 h        | %      | %      | %      | %      |
| Control: C57BL/6J    |              |              |          |        |        |        |
| Percentage of α chain/totals chains | 47.1 ± 1.0     | 48.7 ± 1.7   | 50.0 ± 0.9   | 50.0 ± 0.9 |
| Percentage of β chain/totals chains | 52.9 ± 1.0     | 51.3 ± 1.7   | 50.0 ± 0.9   | 50.0 ± 0.9 |
| C57BL/6J hemi-αthal  |              |              |          |        |        |        |
| Percentage of α chain/totals chains | 37.1 ± 1.9a     | 37.1 ± 1.5b  | 41.4 ± 2.0c  | 45.9 ± 0.6d |
| Percentage of β chain/totals chains | 62.9 ± 1.9a     | 62.9 ± 1.5b  | 58.6 ± 2.0c  | 54.1 ± 0.6d |
| C57BL/6J hemi-βthal  |              |              |          |        |        |        |
| Percentage of α chain/totals chains | 54.6 ± 0.7c     | 55.6 ± 1.2c  | 55.3 ± 0.9b  | 49.3 ± 0.6 |
| Percentage of β chain/totals chains | 45.4 ± 0.7c     | 44.4 ± 1.2c  | 44.7 ± 0.9b  | 50.8 ± 0.6 |

\( ^{a} p < 0.0005. \)
\( ^{b} p < 0.001. \)
\( ^{c} p < 0.005. \)
\( ^{d} p < 10^{-5}. \)
\( ^{e} p < 0.001. \)
TABLE II
Analysis of membrane-bound globin chains in α- and β-hemizygous mouse

| Mice                  | Time of biosynthesis | Steady state |
|-----------------------|----------------------|--------------|
|                       | 1 h      | 1.5 h     | 2 h       |          |
| Control: C57BL/6J     | %        | %         | %         | %        |
| Percentage of α chain/total proteins | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | 4.4 ± 3.6 |
| Percentage of β chain/total proteins | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | 3.6 ± 2.9 |
| (n = 3)               | (n = 2)  | (n = 4)   | (n = 11)  |
| C57BL/6J hemi-thal    | %        | %         | %         | %        |
| Percentage of α chain/total proteins | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | 2.2 ± 1.4 |
| Percentage of β chain/total proteins | 25.2 ± 10.3<sup>a</sup> | 36.5 ± 28.4<sup>b</sup> | 35.8 ± 20.7<sup>b</sup> | 19.4 ± 5.9<sup>b</sup> |
| (n = 4)               | (n = 2)  | (n = 4)   | (n = 12)  |
| C57BL/6J hemi-βthal   | %        | %         | %         | %        |
| Percentage of α chain/total proteins | 8.6 ± 5.0<sup>b</sup> | 9.4 ± 3.3<sup>b</sup> | 5.2 ± 1.3<sup>d</sup> | 16.2 ± 2.8<sup>b</sup> |
| Percentage of β chain/total proteins | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | 3.7 ± 1.8 |
| (n = 4)               | (n = 3)  | (n = 4)   | (n = 13)  |

<sup>a</sup> p < 0.01,  <sup>b</sup> p < 0.05,  <sup>c</sup> p < 10<sup>−11</sup>,  <sup>d</sup> p < 0.0005,  <sup>e</sup> p < 10<sup>−4</sup>.  

Analysis of membrane-bound globin chains from erythroid cell ghosts was accomplished following biosynthetic labeling with [3H]leucine (1, 1.5, and 2 h) and at steady state. At steady state, membrane-bound α- and β-chain globins of peripheral blood cells were quantified as the percentage of total protein ± S.D. from urea-Triton-PAGE. Reticulocyte membrane-bound α- and β-globin chain synthesis was determined as the percentage of autoradiographic intensity in function of the total protein evaluated by urea-Triton-PAGE to correct for loading. Of note, values obtained at biosynthesis cannot be compared with steady state. Values are expressed as a percentage of total proteins ± S.D. n = number of independent mice analyzed.

Since these mice suffer from anemia, we determined the survival times of RBCs in the circulation by measuring the turnover of biotin-labeled RBCs using avidin-fluorescein isothiocyanate by flow cytometry. Controls had 50% survival or half-life of biotinylated RBCs at 12.6 ± 2.5 days (Fig. 1). Comparatively, both the hemi-α-thal and hemi-β-thal mice displayed a significantly reduced RBC half-life of 6.9 ± 0.8 (p < 0.02) and 5.7 ± 2.6 (p < 0.05) days, respectively. However, the RBC half-life did not differ significantly between the two types of thalassemic mice even if the hemi-β-thal had a more severe anemia.

Altered Erythropoiesis and Hematopoiesis

The hematopoietic/erythropoietic cell populations from bone marrow, spleen, and peripheral blood of both globin hemizygous mice were analyzed. The differentiation potential of progenitors was evaluated using ex vivo culture clonogenic assays that give rise to differentiated colonies. We quantified the number of primitive multipotent hematopoietic CFU-S<sub>12</sub> cells. Furthermore, the maturation process of erythroid precursors was assessed from bone marrow and spleen.

Discrete Bone Marrow Stimulation—The bone marrow cellularity was increased in both hemi-α-thal and hemi-β-thal mice compared with controls (Table IV). However, the bone marrow cell populations of the hemi-α-thal mice did not show any significant stimulation of the erythroid compartment (CFU-E and BFU-E) or of other hematopoietic lineages (CFU-GM and CFU-M) (Fig. 2). Accordingly, the numbers of early CFU-S<sub>12</sub> (Table V) and CFU-GEMM (Fig. 2) multipotent cells in hemi-α-thal mice were similar to controls. In contrast, bone marrow from all hemi-β-thal mice displayed a significant 3–4-fold increase in late CFU-E erythroid progenitor cells relative to controls (Fig. 2). This response appears restricted to the erythroid lineage, since all other bone marrow hematopoietic progenitors in hemi-β-thal mice were comparable with controls (Fig. 2). Furthermore, the hemi-β-thal mice showed a moderate increase in CFU-S<sub>12</sub> multipotent cells that could reflect a response to erythroid demand (Table V). This modest stimulation of bone marrow multipotent and progenitor cells is reminiscent of the marrow response observed in the compensatory hematopoietic and erythropoietic mechanism of sickle cell mice (26). This mechanism implicated significant mobilization or relocalization of marrow multipotent cells to the peripheral blood and subsequently their uptake from blood, to colonize the spleen as
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Table III
Altered hematological parameters in α- and β-globin hemizygous mice

| Mice          | n  | Hb  | Hct | MCV | MCH | RDW (V < 25 fl) | Micro (HC < 22 g/dl) | Hypo | Retic |
|---------------|----|-----|-----|-----|-----|---------------|---------------------|------|-------|
| Control: C57BL/6J | 10 | 15.4 ± 1.9 | 51.5 ± 6.2 | 49.4 ± 1.3 | 14.7 ± 1.0 | 13.7 ± 1.1 | 0.2 ± 0.1 | 0.6 ± 0.2 | 3.7 ± 0.7 |
| C57BL/6J hemi-othal | 7  | 12.3 ± 0.9a | 45.7 ± 3.2a | 40.5 ± 0.9a | 10.9 ± 0.2a | 24.4 ± 2.1a | 6.0 ± 1.9a | 4.9 ± 1.2a | 7.1 ± 0.8a |
| C57BL/6J hemi-βthal | 3  | 6.7 ± 1.1a | 25.0 ± 3.0a | 37.3 ± 3.4a | 10.2 ± 0.4a | 36.1 ± 1.3a | 19.9 ± 4.0a | 19.1 ± 8.2a | 22.9 ± 6.0a |

*p < 0.001.

*p < 0.05.

*p < 0.01.

Intravenous injection of biotin-labeled RBCs in mice served to monitor RBC survival at regular intervals (days). The rate of disappearance of biotinylated RBCs was quantified to determine the half-life of the cells. Three mice were assessed for each of the C57BL/6J controls, hemi-othal, and hemi-βthal. Each point represents the mean ± S.D. Values show the decreased half-lives of the hemi-othal and of hemi-βthal RBCs relative to the control C57BL/6J RBCs.

An extramedullary site for rapid differentiation (26). In agreement with the mobilization of marrow multipotent cells to the peripheral blood, early multipotent CFU-S12 were elevated in the hemi-othal (2-fold) and hemi-βthal (4-fold) mice relative to controls (Table V), whereas the differentiated progenitors were not significantly increased (Fig. 2).

Stimulation of Splenic Hematopoiesis/Erythropoiesis—Since peripheral blood multipotent cells could be taken up by the spleen (homing), thus considered an important organ for hematopoietic differentiation, we examined the spleen and splenic hematopoiesis/erythropoiesis in these animals. The number of nucleated cells in the spleen was similar between control C57BL/6J and hemi-othal mice (Table IV). However, a significant 2-fold increase over the normal number of nucleated cells was observed in hemi-βthal. Nonetheless, the hemi-othal and hemi-βthal mice displayed significantly increased spleen/body weight ratios of 1.4- and 7-fold, respectively, relative to C57BL/6J mice (Table IV), which must result from an increase in nonnucleated cells, thus suggesting substantial levels of trapped RBC in the spleen.

Splenec hematopoiesis and erythropoiesis were subsequently evaluated in hemi-othal and hemi-βthal mice. The hemi-othal mice showed a significant 2–3-fold increase in splenic late erythroid CFU-E but not in the erythroid BFU-E, myeloid lineage (CFU-GM and CFU-M) (Fig. 2), and multipotent CFU-GEMM or CFU-S12 cells (Table V). All hemi-βthal mice analyzed showed an increase in both erythroid progenitors, CFU-E (>100-fold) and BFU-E (4-fold). This marked increase in the splenic CFU-E cell population must have derived from earlier progenitors or multipotent cells, because CFU-E cells are not present in peripheral blood. In addition, hemi-βthal mice displayed a significant ~5–10-fold increase in CFU-GM and CFU-M splenic myeloid progenitors, which correlated with the high levels of macrophages undergoing active erythrophagocytosis (data not shown). Quantification of the splenic multipotent CFU-GEMM and primitive CFU-S12 hematopoietic cells in hemi-βthal mice also showed a substantial ~7-fold increase relative to controls (Table V).

Impaired Erythroid Precursor Maturation—Because hemizygous mice were anemic despite a stimulation of their hematopoiesis and erythropoiesis, we determined whether anomalies in later stages of erythroid maturation could occur in bone marrow and spleen. The bone marrow erythroid cell precursors, as characterized by Ter119– and CD71– markers, were increased by ~2-fold in the hemi-βthal mice (49.2 ± 2.6%; n = 3) relative to controls (24.2 ± 2.6%; n = 3, p < 0.0005), correlating with the increase in marrow cellularity. The hemi-othal mice also showed a trend toward an increase in cellularity (29.2 ± 2.2%; n = 2), but that was not statistically significant. In the spleen, erythroid hyperplasia was observed with a 8–10-fold increase in Ter119– and CD71– precursors for the hemi-βthal mice (49.0 ± 3.6%; n = 3, p < 10–5) and 4-fold increase for the hemi-othal mice (22.4 ± 4.0%; n = 2, p < 0.01) relative to controls (5.9 ± 1.4%; n = 3).

A differential count of late erythroid precursors was undertaken to identify whether and at which stage anomalies occur in erythropoiesis (Table VI). Four classes of erythroid precursors can be identified by the staining intensity (low, medium, or high) of specific surface markers upon maturation as shown in Fig. 3 and as previously described (27). From the earlier to the most mature, these precursors are the early proerythroblast (Ter119medCD71high) found in region a, the basophilic erythroblast (Ter119highCD71high) in region b, the late basophilic and polychromatophilic erythroblast (Ter119highCD71med) in region c, and the orthochromatophilic erythroblast (Ter119highCD71low) in region d. In bone marrow from hemi-βthal mice, a preponderance of immature erythroid precursor cells was evidenced by a significant (~2.5–3-fold) increase in basophilic erythroblasts (region b) compared with controls (Fig. 3). In contrast, a significant decrease of ~3-fold in the late erythroid cell population was deduced from the ratio of polychromatophilic (region c) to orthochromatophilic erythroblasts relative to controls (Fig. 3, Table VI). To quantify the percentage of cell loss in a subpopulation observed in Fig. 3 and represented in parenthesis in Table VI, cell loss between two consecutive cell subpopulations was calculated (see “Experimental Procedures”) as (1 – Pfinal/Pinitial) × 100, where Pfinal is defined as percentage of total cells at a specific stage in control versus hemizygous; this value then served to determine the relative cell loss (Table VI). During erythroid maturation, a relative cell loss of ~27% of total bone marrow cells over control was estimated for the hemi-βthal mice by combining the relative cell loss observed between the basophilic to late basophilic/
polychromatophilic erythroblasts (23.4%) and the one occurring in the following maturation stage (3.7%). In the spleen, all of the precursor subpopulations were expanded relative to controls, except for the orthochromatophilic erythroblasts showing a two-thirds decrease (Table VI). Like bone marrow, the spleen of hemi-β-thal mice exhibited ~19.5% destruction of total cells.

**Table IV**

| Mice                             | Bone marrow 10^7 cells/femur | Spleen 10^7 nucleated total cells | Spleen/body weight % |
|----------------------------------|------------------------------|----------------------------------|----------------------|
| Control: C57BL/6J                | 1.2 ± 0.1 (6)                | 10.7 ± 1.4 (5)                   | 0.25 ± 0.06 (29)     |
| C57BL/6J hemi-athal              | 1.9 ± 0.5* (4)               | 11.2 ± 2.7* (7)                  | 0.34 ± 0.1* (30)     |
| C57BL/6J hemi-β-thal              | 1.7 ± 0.4* (5)               | 21.4 ± 7.5* (6)                  | 1.82 ± 0.6* (26)     |

*p < 0.01.

**Table V**

| Mice                             | Number of CFU-S12 | Femur 10^6 blood cells | Spleen |
|----------------------------------|------------------|------------------------|--------|
| Control: C57BL/6J                | 1400 ± 500 (6)   | 1.3 ± 2.0* (4)         | 650 ± 200 (5) |
| C57BL/6J hemi-athal              | 1200 ± 300 (5)   | 2.9 ± 1.8* (5)         | 500 ± 200 (3) |
| C57BL/6J hemi-β-thal              | 2600 ± 900 (4)   | 5.0 ± 2.1* (5)         | 4600 ± 1400* (5) |

*p < 0.05; **, p < 0.02; ***p < 0.01; ****p < 0.001.

**Fig. 2.** Altered hematopoietic progenitors in heterozygous α- and β-globin hemizygous mice. The number of hematopoietic progenitors was quantified by clonogenic assays from bone marrow, peripheral blood, and spleen. The hemi-athal mice showed significant erythroid stimulation in the spleen, whereas the hemi-β-thal mice displayed a generalized stimulation of the erythroid lineage in all hematopoietic tissues. The histograms shown are the mean ± S.D. Results from bone marrow are expressed as the number of progenitors per femur, n = 4 (n, number of mice) for each group. Similarly, data from spleen consist of the number of progenitors per spleen: control mice (n = 5), hemi-athal mice (n = 7), and hemi-β-thal mice (n = 4). Values for the peripheral blood are reported as the number of progenitors/ml of blood: control mice (n = 11), hemi-athal (n = 10), and hemi-β-thal (n = 8). The p values are determined as follows. *, p < 0.05; **, p < 0.02; ***p < 0.01; ****p < 0.001.
Erythroid precursors were quantified in each subpopulation delineated in Fig. 3. Values are expressed as percentage of total cells (P) either in bone marrow or spleen. The percentage of cell loss in a subpopulation is calculated based on the percentage of total cells from two consecutive stages relative to controls as described under "Experimental Procedures." for region b, values are determined from the basophilic to late basophilic/polychromatophilic stage, and for region c, values are determined from late basophilic/polychromatophilic to orthochromatophilic stage. Relative cell loss is determined as the percentage of cell loss in a specific subpopulation by the percentage of total cells (P) of that population. Values shown are mean ± S.D.; n = number of mice analyzed.

Table VI

| Mice                  | Percentage of total cells × percentage of cell loss in subpopulation = percentage of relative cell loss |
|-----------------------|------------------------------------------------------------------------------------------------------|
|                       | Proerythroblasts (region a)                                                                               |
|                       | Basophilic erythroblast (region b)                                                                       |
|                       | Late basophilic/polychromatophilic erythroblasts (region c)                                             |
|                       | Orthochromatophilic erythroblasts (region d)                                                           |
|                       | %          | %          | %          | %          |
| Bone marrow           |                                                       |                                                       |                                                       |                                                       |
| Control: C57BL/6J     | 2.2 ± 1.4  | 13.2 ± 3.9 | 5.5 ± 4.1  | 17.0 ± 5.0 |
| C57BL/6J hemo-thal    | 1.5 ± 0.7  | 17.1 ± 2.7 (0) = 0 | 9.5 ± 3.0 (52) = 5.0 | 13.9 ± 5.2 |
| Spleen                |                                                       |                                                       |                                                       |                                                       |
| Control: C57BL/6J     | 3.2 ± 1.8  | 36.4 ± 4.1 (84) = 23.4 | 5.4 ± 2.1 (68) = 3.7 | 5.3 ± 1.9f |
| C57BL/6J hemo-thal    | 0.4 ± 0.3  | 3.9 ± 2.8   | 2.4 ± 1.0   | 34.3 ± 7.8 |
|                       | 0.3 ± 0.2  | 10.2 ± 3.7f (0) = 0 | 13.3 ± 4.2 (84) = 11.1 | 31.3 ± 4.9 |
|                       | 1.3 ± 0.8f | 27.7 ± 9.1 (38) = 10.6 | 10.5 ± 4.1 (84) = 8.9 | 23.6 ± 9.2f |

\*p < 0.05.
\*p < 10^-6.
\*p < 0.005.
\*p < 10^-8.
\*p < 0.0005.
\*p < 0.02.

DISCUSSION

Characterization of mature and immature erythroid cells in our α- and β-globin hemizygous mice on a homogenous genetic background was undertaken for comparison both between these two mice and to human heterozygous thalassemias. Herein, our studies demonstrate that even with a lower reticulocyte globin chain imbalance, hemi-β-thal mice displayed a more severe thalassemic disorder according to hematologic parameters. Thus, by comparing these mice, a correlation between the severity of the thalassemias and their globin chain imbalance cannot be directly established. Importantly, we also report a difference in the cell loss extent and timing in erythroid precursors from both mice, with more pronounced cell loss in hemi-β-thal mice. In earlier hematopoietic multipotent and progenitor populations, we revealed a stronger hematopoietic response in hemi-β-thal. This was associated with an extramedullary compensatory mechanism, as shown by mobilization of marrow multipotent cells to the spleen for additional erythropoiesis. Together, these results indicate that, for this reciprocal mutation on the same genetic background, both the hemo-thal and hemi-β-thal mice demonstrate thalassemia but with distinct hematologic cellular responses.

More Severe Globin Chain Imbalance in Peripheral Blood from Hemi-α-thal than Hemi-β-thal Mice—The hemo-α-thal and hemi-β-thal mice hemizygous for adult α- and β-globin genes, respectively, are thalassemic based on imbalance of their soluble and membrane-bound globin chains in circulating reticulocytes and RBC. This imbalance was more pronounced in the hemo-α-thal than in hemo-β-thal mice. The decrease in reticulocyte soluble globin chains from adult hemo-α-thal (~17–23%) and hemo-β-thal (~11–14%) correlated with the reduced 1) fetal α-like chains (15%) in primitive erythroid cells of the α-globin hemizygous mice from which our hemo-α-thal with an homogenous background are derived (16 and 2) adult β-globin chains (~8–11%) from a different β-thalassemic mouse model (15, 18, 22) in each case, the percentage of imbalanced globin chain has been recalculated in the function of total α- plus β-globin chains). Furthermore, the soluble globin chain imbalance was more severe in reticulocytes during globin synthesis than in RBC at steady state for both the hemo-α-thal and hemo-β-thal mice. Together, these results provide evidence that erythroid
cells, mainly reticulocytes, with the greatest imbalance of globin chains are eliminated from circulation, affecting a higher proportion of cells in the hemi-thal mice. The greater soluble chain imbalance observed in hemi-thal mice compared with hemi-thal was unexpected based partly on hematologic parameters. Our results indicate, however, that this is not explained by a differential precipitation of excess globin chain in erythroid cell membranes as initially suspected, based on the fact that β-globin chain can form stable soluble tetramers in contrast to α-globin. Altogether, the differential globin chain imbalance responses observed in our mice suggest the existence of a selective process in hemi-thal mice that acts, prior to the reticulocyte stage, against erythroid cells exhibiting the most severe globin chain imbalance.

**Distinct Compensatory Erythropoietic Hematopoietic Response in Hemi-atal and Hemi-βthal Mice**—The affected he-
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matologic parameters in the globin hemizygous mice, particularly a decrease in hemoglobin and hematocrit associated with microcytosis and hypochromia, are typical of human thalassemia. Both hemi-thal and hemi-βthal mice displayed moderate and severe anemia respectively. Notably, these globin hemizygous mice exhibited similar reduced RBC half-life and thus accelerated rates of RBC destruction that correlated with the comparable levels of globin chains in RBC membrane skeletons at steady state. One question that was then raised by our findings in reticulocytes and RBC is whether the anemia in the globin hemizygous mice results from an inadequate and/or distinctive erythroid/erythropoietic response.

A comparison of the hemi-thal and hemi-βthal mice within the same experiments showed moderate and major compensatory hematopoietic/erythropoietic response, respectively. Splenomegaly in both hemizygous mice indicated that significant numbers of RBC were sequestered, consistent with their removal from the circulation and their reduced half-life. The increased number of splenic nucleated cells in the hemi-βthal mice suggested active hematopoiesis. Confirming this, the early multipotent primitive CFU-S₁₂ hematopoietic population was increased systemically in bone marrow, peripheral blood, and up to 7-fold in spleen. This expansion of the early multipotent primitive population with tissue relocalization and then differentiation supported the existence of a compensatory hematopoietic mechanism in the pathophysiology of thalassemia. This compensatory mechanism involves 1) an increase in bone marrow multipotent primitive cells consequent to severe depletion of erythroid cells, 2) a significant mobilization of bone marrow CFU-S₁₂ to the peripheral blood, 3) homing of such peripheral blood CFU-S₁₂ to the spleen as an extramedullary hematopoietic site, and 4) a high rate of CFU-S₁₂ differentiation at the extramedullary site, which provides an additional and complementary supply to the RBC population. A persistent increase in the differentiation of spleen CFU-S₁₂ was shown by the significant stimulation of hematopoietic/erythropoietic progenitor populations in hemi-βthal mice, which was increased 3–10-fold relative to hemi-thal mice. Furthermore, late erythropoiesis, as evaluated by CFU-E in the spleen, revealed a 40-fold greater stimulation for hemi-βthal relative to hemi-thal mice and supported that RBC homeostasis relies extensively on extramedullary splenic erythropoiesis. In comparison, other mouse models referred to as α- and β-thalassemia displayed a more limited differential stimulation of erythropoiesis, with a 2-fold difference that was restricted to spleen CFU-E, and no differences in their increase of BFU-E, CFU-GM, or CFU-S populations (28, 29). This differential response between the globin hemizygous mice and previous models may be attributed to the particular β-globin haplotype and/or variation in genetic background. The latter would support the existence of genetic modifiers for thalassemia, since the globin hemizygous mice used here were on a homogeneous background. Nevertheless, our data show that erythropoiesis in hemi-βthal mice is considerably stimulated and that the anemia in hemi-thal and hemi-βthal mice most likely results from downstream cellular events rather than from inadequate early erythropoiesis.

Analysis of marrow and splenic erythroblast precursors showed a block in cellular expansion and/or an arrest in maturation after the basophilic erythroblast stage for both globin hemizygous mice. Ineffective erythropoiesis, as defined by low downstream erythroid cell output relative to precursor production, is significant in the hemi-thal and hemi-βthal mice and correlated with the severity of anemia. In the hemi-βthal mice, the more pronounced defect in erythropoiesis from the basophilic erythroblast stage onward probably results from the combined effect of earlier ontogeny of α-globin gene transcription/translation with the relatively greater instability of free α-globin chains compared with β-chains, causing earlier and additional precursor impairment in hemi-βthal relative to hemi-thal mice (30–32). In parallel experiments, ineffective erythropoiesis was also found to be significant in hemi-βthal as evaluated by Annexin V binding to erythroid precursors from bone marrow and spleen. Surprisingly, the levels of apoptosis were much lower than those quantified by the actual calculation of the relative cell loss within erythroid precursor subpopulations. Although the basis for this difference is unclear, Annexin V/phosphatidylserine-exposing erythroid cells in mouse may not be the first or most appropriate recognition signal that triggers removal by macrophages, or apoptotic cells may be eliminated extremely efficiently, becoming marginally detectable. Nonetheless, an impressive number of erythroid progenitors and precursors do not mature to reticulocytes or RBCs. Strikingly, the cell loss is concurrent with globin gene activation. Indeed, the differential timing in cell loss between hemi-thal and hemi-βthal mice correlates with the difference in the onset of α- and β-globin gene activation (30–32). The fact that dramatic cell loss in hemi-βthal occurred subsequent to the basophilic erythroblast stage is seen as one evidence that α-globin expression is significantly increased starting from the basophilic erythroblast stage. Similarly, the precursor stage at which cell loss is first detected in hemi-thal suggests that the activation of the β-globin expression is delayed to the late basophilic/polychromatophilic stage. The massive cell loss in the hemi-βthal mice that begins at the premature erythroid precursors stage and proceeds throughout erythroid differentiation demonstrates that erythroid cell destruction in the hemi-βthal far exceeds that of the hemi-thal mice. Both the extent and timing of cell loss strongly suggests a selective mechanism against precursors with the most severe globin chain imbalance, allowing those with lesser chain imbalances to mature. Interestingly, the ineffective erythropoiesis observed at the polychromatophilic and orthochromatophilic stages in the globin hemizygous mice occurs in the same erythroid subpopulation as in the SAD murine sickle cell model with a mutated β-globin chain (26), indicating that these are key stages for erythroid cell survival in hemoglobinopathies.

Insights into Human Thalassemias from the Hemi-thal and Hemi-βthal Mouse Models—Several features of the hemi-thal and hemi-βthal mice are similar to human α- and β-thalassemias. Consistent with human thalassemia, hematological parameters showed differences in severity between the hemi-thal and hemi-βthal mice. Similarities between human heterozygous thalassemia and globin hemizygous mice also extend to stages of ineffective erythropoiesis in immature and mature erythroid cells and to differences in RBC destruction rate (10, 34). One notable difference between the human and mice diseases is the less affected hematologic parameters in human heterozygous β-thalassemia relative to those in the hemi-βthal mice. A beneficial factor in human heterozygous β-thalassemia could be the reduced proportion of free α-chain pool through formation of additional tetramers such as HbF and HbA₂ and/or through active degradation by a proteolytic pathway in erythroid cells (35–37).

An important finding from this study is the strong general stimulation of multipotent primitive cell populations in hemi-βthal mice. Based on this result, it would be expected that stem cells are undergoing active proliferation and differentiation with a potential cell pool expansion. Three distinct hematopoietic/erythropoietic populations are affected in the hemi-βthal mice: 1) there is an expansion of the multipotent cell pool; 2) there is ineffective erythropoiesis of late precursors as well as
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