The mouse mutant hemoglobin deficit (gene symbol hbd) is characterized by a severe microcytic anemia that is inherited in an autosomal-recessive manner. To assess the mutation's effect on hematopoiesis, unfractionated bone marrow (BM) from either a mutant C57BL6/J-\textit{hbd/hbd}, Gpi1a\textsuperscript{a}/Gpi1a\textsuperscript{a}, Gpi1a\textsuperscript{a}/Gpi1a\textsuperscript{a}, mouse was injected intravenously into irradiated congeneric C57BL6/J-\textit{hbd/hbd}, Gpi1a\textsuperscript{a}/Gpi1a\textsuperscript{a}, Igh\textsuperscript{a}/Igh\textsuperscript{a}, Thy1\textsuperscript{a}/Thy1\textsuperscript{a}, mice. The congenic recipients of mutant or normal marrow obtained complete red blood cell (RBC) and leukocyte reconstitution, with the exception of one recipient of HBD marrow. After 24 weeks posttransplantation, the normal recipients of HBD marrow obtained a microcytic anemia similar to the donor. These results suggest that the HBD phenotype is caused by a BM defect. We observed that the erythroid lineage derived from donor HBD marrow repopulated more slowly than the normal marrow at 4 weeks posttransplantation. To determine if this difference was a result of an erythropoietic defect, competitive repopulation was performed using either mutant or normal marrow competed against normal congenic marrow. For the erythroid lineage, no significant contribution from HBD marrow was observed. To assess if the RBC block was based on a deficiency of myeloid progenitors, both in vitro and in vivo assays were performed: absolute numbers of bone progenitors were increased, suggesting that the defect results in a late block to erythropoietic differentiation.

This is a US government work. There are no restrictions on its use.

**MATERIALS AND METHODS**

**Animals.** Animals were obtained from the Jackson Laboratory (Bar Harbor, ME) and maintained at the National Institutes of Health
Table 1. Comparison of Blood Parameters for HBD Mice in the NIH Colony and the Original Report

<table>
<thead>
<tr>
<th>Donor</th>
<th>Hct (%)</th>
<th>MCV (fL)</th>
<th>Hgb (g/dL)</th>
<th>MCHC (%)</th>
<th>Protoporphyrin</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL6/J-+/hbd/hbd</td>
<td>39.6 ± 1.7</td>
<td>34.3 ± 1.3</td>
<td>10.3 ± 0.7</td>
<td>26.0 ± 0.7</td>
<td>298.6 ± 25.2 (ZPP)</td>
</tr>
<tr>
<td>C57BL6/J-+/hbd/hbd</td>
<td>46.8 ± 1.7</td>
<td>48.2 ± 1.3</td>
<td>15.4 ± 0.7</td>
<td>32.6 ± 0.9</td>
<td>43.8 ± 5.2 (ZPP)</td>
</tr>
<tr>
<td>AB/Jania-Halle-+/hbd/hbd</td>
<td>37.0</td>
<td>43.3</td>
<td>10.7</td>
<td>28.9</td>
<td>611 (FEP)</td>
</tr>
<tr>
<td>C57BL6/J-+/hbd/hbd</td>
<td>45.7</td>
<td>51.6</td>
<td>14.1</td>
<td>30.9</td>
<td>50 (FEP)</td>
</tr>
</tbody>
</table>

Data in rows 1 (n = 5) and 2 (n = 5) were derived from our colony at the NIH and represent the mean ± SE. Data from rows 3 (n = 8) and 4 (n = 14) are from the original report.2

Abbreviations: Hct, hematocrit; Hgb, hemoglobin concentration. For the protoporphyrin values, (ZPP) = zinc-protoporphyrin in µmol ZPP/mol heme and (FEP) = free-erythrocyte protoporphyrin.

Table 2. Blood Parameters for Donors and Their Respective Recipients at Four-Week Intervals Posttransplantation

<table>
<thead>
<tr>
<th>Donor</th>
<th>Baselines</th>
<th>4 wk</th>
<th>8 wk</th>
<th>12 wk</th>
<th>16 wk</th>
<th>20 wk</th>
<th>24 wk</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZPP</td>
<td>+/+</td>
<td>50</td>
<td>27.5 ± 2.3</td>
<td>59 ± 7.1</td>
<td>39.3 ± 3.3</td>
<td>36 ± 2.8</td>
<td>34.7 ± 5.7</td>
</tr>
<tr>
<td></td>
<td>hbd/hbd</td>
<td>325</td>
<td>24.7 ± 2.7</td>
<td>181.7 ± 8.7</td>
<td>249.3 ± 36.5</td>
<td>243.3 ± 27.9</td>
<td>272.5 ± 27.9</td>
</tr>
<tr>
<td>MCV</td>
<td>+/+</td>
<td>47.1</td>
<td>50.4 ± 0.4</td>
<td>47.3 ± 1.0</td>
<td>48.7 ± 1.7</td>
<td>50.3 ± 2.5</td>
<td>43.4 ± 1.3</td>
</tr>
<tr>
<td></td>
<td>hbd/hbd</td>
<td>29.4</td>
<td>51.4 ± 1.0</td>
<td>39.0 ± 0.6</td>
<td>34.7 ± 1.4</td>
<td>34.5 ± 0.9</td>
<td>33.9 ± 1.5</td>
</tr>
<tr>
<td>MCHC</td>
<td>+/+</td>
<td>28.2</td>
<td>29.4 ± 1.3</td>
<td>33.6 ± 0.6</td>
<td>32.6 ± 1.6</td>
<td>31.0 ± 1.2</td>
<td>36.2 ± 1.6</td>
</tr>
<tr>
<td></td>
<td>hbd/hbd</td>
<td>27.4</td>
<td>31.4 ± 0.7</td>
<td>28.5 ± 0.3</td>
<td>27.6 ± 0.9</td>
<td>28.7 ± 1.6</td>
<td>29.2 ± 1.5</td>
</tr>
</tbody>
</table>

Blood parameters for normal and mutant donors are presented in column 1. The congenic recipients of either C57BL6/J-+/hbd/hbd or -hbd/hbd marrow were assayed at 4-week intervals (columns 2-8). Abbreviations and units are the same as in Table 1. For recipients of -hbd/hbd marrow at all time points, n = 6. For recipients of +/hbd/hbd marrow up to 16 weeks, n = 6. For recipients of +/hbd/hbd marrow at 20 and 24 weeks, n = 5. Values are represented as the mean ± SE. For the ZPP and MCV parameters, P < .01. For MCHC, P < .01 at 4, 16, and 20 weeks, P < .05 at 8 weeks, P = .25 at 12 weeks, and P = .1 at 24 weeks. The P values are based on the t-test for equal variance (two-tailed).
mutants from our colony had been backcrossed onto the C57BL/6J background for more than 16 generations at the Jackson Laboratory. To determine if the genetic change had affected the phenotype, blood counts of the HBD mice from our colony were measured and compared with those from the original report (Table 1). RBCs from HBD mice in our colony were smaller than those of previously described mutants. Reduction of the MCHC was more pronounced in our mutants compared with that in the previous report. We also assayed ZPP, an indirect measure of the free erythrocyte protoporphyrin (FEP). The ZPP was higher in the mutant animals. Although the overall phenotype was more pronounced on the C57BL/6J background, we concluded from these results that the anemic phenotype has remained acceptably stable.

To determine if the BM from HBD mice was capable of producing anemia in a normal environment, C57BL/6J-\textit{hbd/hbd}, \textit{Gpi1}^\textit{b}/\textit{Gpi1}^\textit{b} marrow was transplanted into normal congenic recipients of the same genetic background (C57BL/6J-\textit{hbd/hbd}, \textit{Gpi1}^\textit{b}/\textit{Gpi1}^\textit{b}, \textit{Ighc}/\textit{Ighc}, \textit{Thyl}/\textit{Thyl}). A control transplant of normal C57BL/6J-\textit{hbd/hbd}, \textit{Gpi1}^\textit{b}/\textit{Gpi1}^\textit{b} marrow into normal congenic recipients was performed concurrently. By 24 weeks posttransplantation, recipients of HBD marrow had converted to an anemic phenotype (Table 1). These data show that the transplant of BM stem cells from mutant to normal mice was sufficient to produce an anemic phenotype.

In addition to phenotype analysis, engraftment of RBC and leukocyte lineages was followed at 4-week intervals up to 24 weeks posttransplantation (Fig 1). Recipients of either normal C57BL/6J-\textit{hbd/hbd}, \textit{Gpi1}^\textit{b}/\textit{Gpi1}^\textit{b} or mutant C57BL/6J-\textit{hbd/hbd}, \textit{Gpi1}^\textit{b}/\textit{Gpi1}^\textit{b} marrow showed complete leukocyte reconstitution by 4 weeks posttransplantation (Fig 1A and B). Of the six recipients of HBD marrow, five showed stable reconstitution of the leukocyte lineage for 24 weeks post-transplantation.
Fig 2. Competitive repopulation of HBD and normal marrow against normal congenic marrow. In two independent experiments (A and B), mixtures of either mutant and congenic or normal and congenic marrow were injected into irradiated C57BL6/J-+/hbd/hbd, Gpi1+/Gpi1+, Igfr1+/Igfr1+, Thy1+/Thy1+ recipient mice. The percentage of isotype Gpi1b in the peripheral RBCs and leukocytes indicated the competitive ability of the mutant or normal marrows compared with congenic marrow. Data points indicate the mean for mixtures as indicated under the figure. Error bars represent the SEM. In (A), n = 6 for both the normal:congenic mix and HBD:congenic mixes. The P value for the leukocytes for the HBD:congenic versus the normal:congenic was .0007 at 24 weeks posttransplantation. In (B), n = 6 for normal:congenic and HBD:congenic mixes. At 16 weeks, n = 5 and at 24 weeks n = 4 for normal:congenic mix. The P value for HBD:congenic versus normal:congenic leukocytes was 0.27 at 24 weeks.

weeks. Mouse no. 494 failed to show stable engraftment. All recipients of normal marrow obtained long-term stable reconstitution. The major difference between engraftment of the normal and mutant marrow types was in the kinetics of reconstitution of the RBC lineages. At 8 weeks posttransplantation, the recipients of normal marrow had obtained 100% reconstitution (Fig 1C). In contrast, complete RBC reconstitution of the HBD marrow was obtained at 12 weeks (Fig 1D). As with leukocyte reconstitution, recipient no. 494 did not obtain long-term reconstitution of the donor RBC lineage.

To assess if the delay in erythroid engraftment was caused by a defect in hematopoietic ontogeny, competitive repopulation was performed by mixing equal numbers of nucleated BM cells from donor C57BL6/J-+/hbd/hbd, Gpi1+/Gpi1+ and competitor C57BL6/J-+/hbd/hbd, Gpi1+/Gpi1+, Igfr1+/Igfr1+, Thy1+/Thy1+ mice. In parallel, equal numbers of C57BL6/J-+/hbd/hbd, Gpi1+/Gpi1+ donor BM cells were mixed with C57BL6/J-+/hbd/hbd, Gpi1+/Gpi1+, Igfr1+/Igfr1+, Thy1+/Thy1+ competitor marrow. These mixtures were injected into myeloablated congenic recipients. The percentages of isozymes Gpi1a and Gpi1b were assayed at 4-week intervals up to 24 weeks posttransplantation. In two experiments, no contribution to the RBC population was observed from the C57BL6/J-+/hbd/hbd, Gpi1+/Gpi1+ component for all time points assayed (Fig 2A and B). In addition, the competitive ability of the leukocyte component from HBD marrow was decreased in both experiments. The normal marrow components contributed greater than the expected 50% in both experiments.

To further assess the erythroid defect, myeloid progenitors were assayed from normal and mutant mice (Table 3). An estimation of the absolute number of these progenitors as well as primitive hematopoietic stem cells (PHSC) was also determined (Table 4). The CFU-S contain a mixed population of hematopoietic cells and are thought to represent an
early multipotential progenitor that is distinct and more mature than the pluripotent repopulating stem cells. CFU-S
were generated by injecting normal or HBD marrow into lethally irradiated C57BL6/J-+/hbd recipients. Compared
with normal controls, HBD BM was more concentrated in CFU-S. If corrected for cellularity, we estimate that
there are twice the normal number of CFU-S in HBD mice. Titration effects of fractionated marrow on CFU-S formation
have previously been observed, suggesting that certain cell types inhibit or enhance colony formation. To assess if unfrac-
tionated HBD marrow contains an inhibitor or enhancer of colony formation, CFU-S formation was titrated around the
point reported in Table 3. The slope was not significantly different from 1.0, suggesting that this effect is not present
in unfractionated HBD marrow (data not shown). In vitro colonies derived from BM are thought to represent a precursor
that is more mature than the CFU-S progenitor. BM from the HBD mouse is more concentrated in CFU-GM, CFU-
GEMM, and BFU-E than is the marrow of normal mice (Table 3). Based on cellularity, the absolute number of these
colony-forming progenitors is about three times that of the normal mouse.

The absolute number of PHSC was also estimated (Table 4). This determination was based on the previous estimates of
PHSC concentration as well as the cellularity of the HBD and the assumption that the concentration of PHSC remains
the same. If this assumption is correct, we estimate that there is a 10% decrease in absolute PHSC numbers in the HBD
mouse.

**DISCUSSION**

The results presented here show that HBD BM is capable of producing microcytic RBCs in a normal host environment
and that erythropoiesis is defective in this transplant model. Although these results do not discount a micro-environ-
mental defect for the HBD mouse, we show here that the BM effect is substantial and complex.

Long-term persistence of anemia in recipients of HBD marrow indicates that a defect resides in BM-derived cells.
Only a short-term anemia would have been expected in the recipient if the BM defect was caused by the microenviron-
ment. Control animals that received normal marrow did not obtain a microcytic phenotype. These results indicate that
neither the transplant procedure nor the congenic marker loci on the C57BL6/J background substantially affect the
phenotype.

The recipients of mutant marrow present an anemic phenotype as early as 4 weeks posttransplantation. In fact, histog-
ram analysis of RBC populations from recipients indicates a distinct microcytic population at all intermediate time points
before complete reconstitution of microcytic RBCs (data not shown). These results indicate that the transfer and expansion
of late HBD progenitors is sufficient to produce a defective RBC population in the normal environment. These results
suggest that the established defect is not reversible for late HBD progenitors in a normal environment.

Concomitant with our observations that the HBD marrow-derived cells produce a microcytic phenotype in normal re-
cipients was our observation of the delayed erythropoiesis in the transplant model. To further define this potential de-
fect, the competitive repopulation assay was performed which measures the ability of two different donor popula-
tions to compete in the host environment. We show a striking defect in long-term competitive ability of the HBD ery-
thropoietic lineage. A similar defect has been observed for the α-thalassemic mice, although for a different reason. The
competitive defect is the result of RBC hemolysis, which is not a significant factor in HBD mice. No evidence of excess
hemolysis in the HBD mouse has been observed through analysis of fecal urobilinogen excretion. A severe erythropoietic
defect is also observed in mutants of the W series but is secondary to a PHSC defect. However, we doubt that the HBD
anemia is caused by stem cell lesion. First, we were unable to repopulate an unconditioned HBD mouse with
congenic marrow (data not shown). Second, we show here

**Table 3. Myeloid Progenitor Assays of C57BL6/J-hbd/hbd and C57BL6/J-+/+/hbd Marrow**

<table>
<thead>
<tr>
<th>Type</th>
<th>C57BL6/J-+/+(n = 8)</th>
<th>C57BL6/J-hbd/hbd (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CFU-GM</td>
<td>54.8 ± 2.1</td>
<td>100.5 ± 4.0</td>
</tr>
<tr>
<td>BFU-E</td>
<td>4.6 ± 0.84</td>
<td>7.5 ± 0.96</td>
</tr>
<tr>
<td>CFU-GEMM</td>
<td>1.13 ± 0.64</td>
<td>2.13 ± 0.74</td>
</tr>
<tr>
<td>CFU-S</td>
<td>11.71 ± 0.73</td>
<td>15.42 ± 0.76 (n = 14)</td>
</tr>
</tbody>
</table>

BM cells (4.5 × 10^4) from either a C57BL6/J-+/+/hbd or C57BL6/J-hbd/hbd mouse were grown in methylcellulose media containing recombinant growth factors. Three colony types were scored: CFU-GM, CFU-GEMM, and BFU-E. Each value represents the mean number of colonies per 4.5 × 10^4 BM cells plated from two combined experiments ± SE. P < .05 for all comparisons except CFU-GEMM where P = .32. For CFU-S, either C57BL6/J-hbd/hbd and C57BL6/J-+/hbd/hbd marrow was injected into irradiated C57BL6/J-+/hbd/hbd mice. Ten days postinjection spleen colonies were counted. The means are based on two independent experiments. For the CFU-S comparison, P = .0016.

**Table 4. Estimation of Absolute CFU-S, CFU-C, and PHSC in Two Femurs and Two Tibias From Either Normal or Mutant Mice**

<table>
<thead>
<tr>
<th>Marrow</th>
<th>Type</th>
<th>CFU-GM</th>
<th>BFU-E</th>
<th>CFU-GEMM</th>
<th>CFU-S</th>
<th>PHSC1</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBD</td>
<td>156,300</td>
<td>11,666</td>
<td>3,313</td>
<td>10,794</td>
<td>399</td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>53,580</td>
<td>4,497</td>
<td>1,104</td>
<td>5,150</td>
<td>440</td>
<td></td>
</tr>
</tbody>
</table>

* Absolute numbers were calculated by multiplying the CFU concentration (Table 3) by the BM cellularity for two tibias and two femurs. The cellularity was calculated based on the mean of BM isolations for the transplant studies. For hbd/hbd mice (n = 3), marrow cellularity was 7.0 × 10^7 cells. For +hbd/hbd mice, marrow cellularity was 4.4 × 10^7.

† For PHSC numbers, the mean engraftment for white blood cells from both competitive repopulation experiments was determined for the HBD and normal marrows. The fraction of HBD to control was multiplied by the estimated frequency of PHSC in the normal mouse. Both the estimate for normal and HBD mice was multiplied by their respective cellularities.

For HBD, estimation of absolute PHSC is as follows:

0.57 PHSC

10^7 BM Cells × 0.73

10^7 cells = 399 PHSC

2.13.
that the HBD marrow can completely engraft into myeloablated hosts by 12 weeks posttransplantation. Excluding a stem cell defect and hemolysis, we examined the myeloid progenitor population to explain the erythroid defect. A defect in this population was not observed. In fact, we estimate an absolute increase in progenitors that form the erythroid colonies. These results indicate that the block to differentiation is at a late step to differentiation.

One explanation for the erythroid expansion defect is that the HBD hematopoietic cells are unable to compete for a circulating component, resulting in a late block to erythroid expansion. This hypothesis is supported by both our phenotype transfer and competitive repopulation results. For the phenotype transfer, HBD engraftment is delayed but finally completed as the normal recipient marrow is ablated from the radiation exposure. For the competitive repopulation, where the normal and HBD donor marrows are equally represented, the HBD erythroid lineage would not be able to compete with normal marrow. In vitro uptake of radiolabeled iron is lower in HBD reticulocytes compared with normal controls whereas transferrin uptake is unaffected. It is possible that iron is the component that is outcompeted by the normal cells.

A defect in erythropoiesis has also been observed for the flexed-tail (fl) mutant. This mutant has a hypochromic anemia in the fetus and newborn that is resolved in adults. A delay in production of cells that incorporate iron into heme is observed in spleen colonies derived from transplanted (fl/fl) adult marrow. Because the anemia is restricted to early development, these results suggest that the defect is only present in an environment of rapid cell proliferation. These CFU-S derived from these adult mice were smaller and deficient in erythrocytes whereas the granulopoiesis was not affected, suggesting a specific defect on the committed erythroid progenitors. In contrast, the CFU-S derived from HBD mice are not smaller in size and do not indicate a gross reduction in erythroid colonies (data not shown). The flexed-tail defect may block erythropoiesis at an earlier step than the HBD mutant.

In conclusion, we have shown a defect in the HBD BM component both in phenotype and erythropoiesis. Given the previous results on a defective iron acquisition in the HBD mouse, the HBD mutant offers an opportunity to study this link between iron metabolism and erythropoiesis. The future determination of the HBD gene product as well as its biochemical function will be crucial.

ACKNOWLEDGMENT

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The Hemoglobin-Deficit Mouse: Analysis of Phenotype and Hematopoiesis in the Transplant Model

Michael L. Bloom and Karen L. Simon-Stoos