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Hemoglobin Deficit: An Inherited Hypochromic Anemia in the Mouse[1] (42307)

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Abstract. The character and pathogenesis of hemoglobin deficit (gene symbol, *hbd*), an autosomal recessive trait in the mouse, were studied. The main hematological features of hemoglobin deficit are anemia, red cell hypochromia and microcytosis, and reticulocytosis. The absence of raised fecal urobilinogen excretion and frank hyperbilirubinemia and bilirubinuria suggests that excess hemolysis is not the primary cause of the anemia. The raised plasma iron concentration and the failure of the anemia to respond to parenteral iron treatment indicate that the anemia is not due to iron deficiency. The absence of siderocytes and sideroblasts suggests that anemia is probably not due to ferrochelatase deficiency. Thalassemia is excluded by the finding of balanced reticulocyte globin chain synthesis. The markedly elevated levels of free red cell protoporphyrin taken together with the other findings already noted suggest that the anemia of hemoglobin deficit is due to a defect in the erythroid cell iron procurement mechanisms leading in turn to diminished heme and hemoglobin synthesis.

Hemoglobin deficit (gene symbol *hbd*) is the name given to an inherited hypochromic anemia of mice, first observed and investigated by Scheufler (1). It was apparently the result of a spontaneous mutation in an A^Y inbred strain of mice at the Biological Institute of Martin Luther University at Halle-Wittenburg, and was subsequently transferred into the AB/Jena-Halle strain. It is inherited as an autosomal recessive trait, manifested by anemia present in the newborn and persisting through adult life, and not responding to injected iron (1). Blood films were noted to show target cells and red cell polychromasia and there was increased resistance of the red cells to osmotic hemolysis (1).

The present studies were undertaken to confirm and extend Scheufler's original observations and to explore the pathogenetic mechanisms leading to anemia.

Methods. *Animals.* A breeding stock of *hbd/hbd* mice of the AB/Jena-Halle strain was obtained as a gift from Dr. Horst Scheufler of the Martin Luther University, Halle-Wittenberg, and this stock has been maintained by continued inbreeding. These animals were crossed with normal C57BL/6J mice to produce obligate heterozygotes (+/*hbd*) of hybrid strain. The obligate heterozygotes were then intercrossed to produce homozygous affected (*hbd/hbd*), homozygous normal (+/+) and heterozygous (+/*hbd*) animals of hybrid strain. Since it is not possible to distinguish +/+ and +/*hbd* animals, they are referred to as +/? animals. This breeding scheme was then repeated in the process of placing the *hbd* mutation on the C57BL/6J background. Observations have been made on *hbd/hbd* anemic animals of the AB/Jena-Halle and hybrid strain (C57BL/6J–AB/Jena-Halle), obligate heterozygotes of hybrid strain, +/? animals of hybrid strain, and +/+ animals of the C57BL/6J strain. Mice of the MK/RE strain that were either homozygous or heterozygous for the microcytic anemia mutation (*mk*) were obtained from the Jackson Laboratory, Bar Harbor, Maine, and used in tests of allelism of the *mk* and *hbd* mutations.

The mice were housed in plastic cages with stainless-steel lids, fed a standard 4% fat mouse/rat diet (Teklad, Winfield, Iowa) and given tap water *ad libitum.* The principles of animal care promulgated by the U.S. National Institute of Laboratory Animal Sciences were strictly maintained.

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Test for allelism with microcytic anemia. In view of the notable resemblance of hemoglobin deficit to microcytic anemia of the mouse (*mk*), also an autosomal recessive trait, two types of matings were set up to test for allelism between the *hbd* and *mk* mutations. One homozygous male *mk/mk* mouse and one heterozygous male +/*mk* mouse were each mated to homozygous anemic *hbd/hbd* females and all offspring examined for the presence of anemia at 46–90 days of age.

Hematologic methods. Adult and weanling mice were bled by puncture of the orbital sinus and infant mice by incision of blood vessels in the neck. Standard methods, scaled down as necessary, were employed. Hemoglobin was determined as cyanmethemoglobin and microhematocrits were used. Red cells were counted by a Coulter counter after preliminary testing had determined appropriate settings for mouse red blood cells. Reticulocytes were stained with brilliant cresyl blue and 500 cells were counted. Dried blood films were examined with Wright–Giemsa Stain and by Perl's method for iron (2). Bone marrow was fixed and stained by Perls's method for iron. Red cell fragility was studied by a standard method (3).

Six *hbd/hbd* animals of the AB/Jena-Halle strain were each given 0.75 mg Fe as iron–dextran by intraperitoneal injection on Day 0. Four control *hbd/hbd* animals of the same strain were each given an equal volume of normal saline. The response to treatment was assessed by measurement of the hemoglobin concentration, hematocrit, red blood cell count, and weight at 0, 8, and 16 days.

In a second experiment two *hbd/hbd* animals of the AB/Jena-Halle strain were each given 1.0 mg Fe as iron–dextran by intraperitoneal injection on Day 0 and Day 16. Two control *hbd/hbd* animals were each given an equal volume of normal saline. The response to treatment was examined by measurement of the hemoglobin concentration, hematocrit and weight on Days 0, 7, 16, 28, and 59.

Four *hbd/hbd* animals of the AB/Jena-Halle strain were each given 250 μg pyridoxine hydrochloride by intraperitoneal injection on Days 0, 10, and 21. Four *hbd/hbd* control animals of the same strain were each given an equal volume of normal saline. The response to treatment was assessed by measurement of the hemoglobin concentration, hematocrit, red blood cell count, and weight on Days 0, 10, 18, and 33.

For histochemical studies, tissues were fixed in 10% Formalin and embedded and sectioned by standard methods. Sections were stained with hematoxylin and eosin, and by Perl's method for iron. Livers, spleens, and kidneys from anemic (*hbd/hbd*) and control (+/?) animals of hybrid strain were examined.

Biochemical methods. Free erythrocyte protoporphyrin levels (FEP) were determined by a micromethod adapted from Piomelli (4). Urinary porphyrins were determined by scaling down the method of Fernandez *et al.* (5). Littermate mice of the same sex and genotype were confined for 24 hr in a metabolic cage in which the feces could be separated from the urine. Fecal urobilinogen excretion was determined by a method previously used in mouse studies in this laboratory (6) based on methods of Swartz *et al.* (7) and Henry (8). Erythrocyte porphobilinogen deaminase was measured by a previously described micromethod (9). Plasma iron concentration was measured by the method of Carter (10).

Globin chain synthesis. Reticulocyte rich blood from *hbd/hbd* animals and control +/? littermates was collected and incubated as previously described (11), except that incubation was for 2 hr in pooled plasma from the +/? and *hbd/hbd* animals. Red cells were washed and lysed, stroma were removed and globin was prepared as described by Garrick *et al.* (12). α-Globin chains and β-globin chains were separated by a modification of the method of Dintzis (13). A 30-mg sample of globin, dialyzed against 0.2 *M* formic acid–0.02 *M* pyridine, was placed on a column (0.7 × 13.0 cm) of carboxymethylcellulose (Whatman CM-32), equilibrated with the same solution. Globin chains were eluted with the following six chamber gradient (50 ml per chamber): (i) 0.2 *M* formic acid–0.02 *M* pyridine, (ii) 0.6 *M* formic acid–0.06 *M* pyridine, (iii) 0.8 *M* formic acid–0.08 *M* pyridine, (iv) 1.0 *M* formic acid–0.10 *M* pyridine, (v) 1.2 *M* formic acid–0.12 *M* pyridine, and (vi) 1.4 *M* formic acid–0.14 *M* pyridine. Fractions of 4.6 ml were collected and the absorbance at 280 nm for each fraction was measured. A 0.75-ml aliquot of each fraction was mixed with 5 ml of Multisol (Isolab Inc., Akron, Ohio) and counted in a Beckman LS 230 liquid scintillation counter. Counts per minute for the α-

and β-globin peaks were summed and the α/β ratio was calculated. The identities of the α- and β-globin peaks were confirmed by sequencing the first 12 amino acids from the NH_2 terminus using an updated Beckman Model 890B automatic sequencer (14).

Results. *Segregation analysis.* All matings between homozygous anemic (*hbd/hbd*) animals of the AB/Jena-Halle strain yielded 100% anemic offspring. Matings between heterozygous (+/*hbd*) animals of the hybrid C57BL/6J–AB/Jena-Halle strain yielded 34 litters and 288 liveborn offspring. Of the 282 animals surviving to adulthood, 64 (23%) were anemic (*hbd/hbd*) and 218 (77%) were nonanemic (+/+ and +/*hbd*).

Test of allelism. The mating between one *mk/mk* male mouse and two female *hbd/hbd* mice produced one litter from each mating and a total of 10 offspring that were all hematologically normal. The mating between one *mk*/+ male mouse and two female *hbd/hbd* mice produced one litter from each mating and a total of 15 offspring that were all hematologically normal. The absence of anemia in all offspring of the above matings excludes identity or allelism of the *mk* and *hbd* mutations since all offspring of *mk/mk* × *hbd/hbd* matings and 50% of the offspring of *mk/+* × *hbd/hbd* matings would be anemic if identity or allelism was present.

Hematological findings. The results of the hematological findings are shown in Table 1. Homozygous *hbd/hbd* animals were anemic at birth and remained so into adult life. The anemia was most severe during infancy with the lowest hemoglobin concentrations occurring at about 5 days of age. In addition to hypochromia and microcytosis, the examination of peripheral blood smears of adult *hbd/hbd* animals revealed polychromasia, mild to moderate anisocytosis, poikilocytosis, target cells, and reticulocytosis. The mean reticulocyte count (±SD) of 57 adult *hbd/hbd* mice was 7.6 ± 4.6% compared to values of 5.3 ± 3.9% for +/*hbd* animals and 2.5 ± 1.3% in control animals. Heterozygous (+/*hbd*) animals could not be distinguished hematologically from genotypically normal animals. Examination of iron-stained peripheral blood smears and bone marrow smears revealed no siderocytes or excess sideroblasts.

The osmotic fragility of red cells from *hbd/hbd* mice of the AB/Jena-Halle strain was re-

TABLE I. HEMATOLOGICAL DATA[a,b]

	hbd/hbd (AB/Jena-Halle)					+/+ (C57/6J)				
Age	Hb	Hct	RBC	MCV	MCH	Hb	Hct	RBC	MCV	MCH
1	8.23 (7)	30.0 (12)	—	—	—	14.4 (5)	35.0 (5)	4.52 (5)	77.4 (5)	31.9 (5)
5	5.64 (12)	23.3 (12)	2.42 (5)	90.9 (5)	26.4 (5)	10.5 (14)	32.1 (14)	4.29 (14)	74.6 (14)	24.3 (14)
10	5.82 (11)	22.9 (11)	2.69 (4)	74.3 (4)	17.8 (4)	10.0 (6)	31.0 (6)	4.58 (6)	67.7 (6)	21.8 (6)
15	6.21 (18)	26.3 (18)	2.80 (4)	66.1 (4)	17.5 (4)	7.9 (7)	27.0 (7)	4.78 (7)	56.5 (7)	16.5 (7)
21	8.48 (29)	32.3 (29)	5.22 (3)	51.7 (3)	12.5 (3)	10.6 (4)	31.5 (4)	6.70 (4)	47.0 (4)	15.8 (4)
39–58	10.08 (45)	36.7 (45)	9.19 (16)	40.9 (16)	11.7 (16)	13.5 (10)	44.3 (10)	8.03 (10)	55.2 (10)	16.8 (10)
59–80	10.61 (29)	37.9 (29)	9.48 (17)	39.3 (17)	11.6 (17)	14.6 (9)	46.7 (9)	8.73 (9)	53.7 (9)	16.8 (9)
>80	10.70 (8)	37.0 (8)	8.56 (8)	43.3 (8)	12.5 (8)	14.1 (14)	45.7 (14)	8.87 (14)	51.6 (14)	15.9 (14)

[a] All values are means, (*N*) = number of animals.

[b] Age = days, Hb = hemoglobin concentration (g/dl), Hct = hematocrit (%), RBC = red blood cell count ($\times 10^{-6}$)/mm^3, MCV = mean corpuscular volume (uu^3), MCH = mean corpuscular hemoglobin (pg).

duced compared to that of red cells from +/+ mice of the C57BL/6J strain. Fifty percent osmotic lysis of *hbd/hbd* red cells occurred at a saline concentration of 0.41% compared to a figure of 0.50% for control red cells. The osmotic fragility of red cells from +/*hbd* mice of hybrid strain was the same as that of red cells from +/+ mice of the C57BL/6J strain.

Neither iron–dextran nor pyridoxine hydrochloride produced any significant improvement in the hemoglobin concentration, hematocrit, red blood cell count, or weight of anemic *hbd/hbd* animals of the AB/Jena-Halle strain.

Examination of iron-stained sections of liver, spleen, and kidney from five adult *hbd/hbd* animals and five adult control animals revealed no clear-cut differences between the anemic and control animals with respect to the liver and kidney. A moderate amount of iron deposition was observed in the spleen sections of the control animals, but a lesser amount in the spleen sections of *hbd/hbd* animals.

Anemic *hbd/hbd* animals had mild splenomegaly. The mean weight (±SD) of the spleens from 18 adult *hbd/hbd* animals was 0.145 ± 0.061 g (0.61% body wt) compared to a figure of 0.097 ± 0.024 g (0.38% body wt) for 37 +/? control animals. The differences between the above mean values are statistically significant for both spleen weight and percentage of body weight with $t > 4$ and $P < 0.0005$.

Biochemical studies. The results of biochemical studies are shown in Table II. The FEP level of *hbd/hbd* animals of the AB/Jena-Halle strain was markedly elevated ($t(38) = 4.77$, $P < 0.0005$).

The mean urinary excretion of coproporphyrin by *hbd/hbd* animals of the AB/Jena-Halle strain was not statistically different from that of +/+ or *hbd*/+ animals. Uroporphyrin levels were too low to quantitate accurately.

No significant difference was found among *hbd/hbd* animals of the AB/Jena-Halle strain, +/*hbd* animals of the hybrid strain, and +/+ animals of the C57BL/6J strain in their fecal excretion of urobilinogen.

The level of porphobilinogen deaminase activity was higher in *hbd/hbd* animals of the AB/Jena-Halle strain than in +/+ animals of the C57BL/6J strain and +/*hbd* animals of hybrid strain. However, when the enzyme activity levels were corrected for the differing reticulocyte counts of the three groups of animals, the differences were not significant.

The plasma iron concentration of *hbd/hbd* animals of hybrid strain was significantly higher than that of +/? littermate animals ($t(74) = 2.38$, $P = 0.01$).

TABLE II. BIOCHEMICAL STUDIES[a]

Test	Units	Genotype		
		hbd/hbd	+/*hbd*	+/+
Free erythrocyte protoporphyrin	μg/dl rbc	611 ± 171 (37)	72 ± 19 (21)	50 ± 11 (22)
	μg/10^9 retics	7.5 ± 2.2[b]	2.71 ± 1.37	4.1 ± 2.3
Urinary coproporphyrin	μg/mouse/day	0.210 ± 0.042 (13)	0.300 ± 0.181 (10)	0.284 ± 0.027 (10)
Fecal urobilinogen	μg/mouse/day	74.2 ± 13.0 (10)	76.7 ± 16.4 (10)	83.2 ± 28.2 (58)
Erythrocyte porphobilinogen deaminase	n*M*/ml rbcs/hr	77.5 ± 23.3 (20)	23.3 ± 4.4 (21)	24.4 ± 4.9 (27)
	nM/10^6 retics/hr	10.8 ± 3.5	6.1 ± 2.7	15.9 ± 11.4
Plasma iron	μg/dl	222 ± 44[c] (21)		195 ± 44[d] (55)

[a] All values are means ± 1 SD, (*N*) = number of animals.
[b] $P < 0.0005$ compared to +/+ controls.
[c] $P = 0.01$ compared to +/? controls.
[d] Value for +/? controls rather than +/+ controls.

Globin chain synthesis. The modification of the method of Dintzis (13) used in the current studies produced a good, clear-cut separation of the α- and β-globin chains. The mean α/β ratios (±SD) for 6 +/? animals and 6 *hbd/hbd* animals of hybrid strain were 1.07 ± 0.04 and 1.03 ± 0.05, respectively, and these ratios were not significantly different.

Discussion. The present hematological findings in mice with hemoglobin deficit are generally similar to those originally reported by Scheufler (1), except the present study indicates the anemia to be microcytic rather than macrocytic. Scheufler's demonstration that hemoglobin deficit is inherited as an autosomal recessive trait is also confirmed by the present findings on the segregation of the trait and the lack of detectable hematological abnormalities in heterozygotes.

The red cell morphological abnormalities and decreased red cell osmotic fragility are similar to that found in conditions where there is a deficiency of hemoglobin synthesis. The reticulocytosis and normal to slightly elevated red cell counts in affected adult animals suggest that there is bone marrow erythroid hyperplasia. This presumably represents a response to anemia resulting from diminished hemoglobin synthesis rather than from increased hemolysis because there is no gross evidence of excessive hemoglobin catabolism such as raised fecal urobilinogen excretion, icteric serum, or bilirubinuria.

Scheufler (1) showed that the anemia did not respond to parenteral iron treatment. The present studies confirm this observation and also demonstrate that the anemia does not respond to pyridoxine treatment. The failure of the anemia to respond to parenteral iron treatment taken together with the increased plasma iron levels indicates that the anemia of *hbd/hbd* animals is not due to simple iron deficiency.

A new and striking observation is the finding of a marked elevation of red cell protoporphyrin levels. Even when the raised reticulocyte count of *hbd/hbd* red cells is taken into account the protoporphyrin levels in affected animals still remain about twice as high as those in genotypically normal controls. Similar elevations in red cell protoporphyrin levels are found in congenital erythropoietic protoporphyria, protoporphyria, and iron deficiency (15). In the first two conditions there is a documented deficiency of ferrochelatase (16, 17). However, hypochromic, microcytic anemia does not occur. The elevation of free erythrocyte protoporphyrin in iron deficiency is probably due to underproduction of heme and a relative loss of feedback inhibition on the protoporphyrin synthetic pathway rather than a diminished ferrochelatase activity. The high red cell protoporphyrin levels found in hemoglobin deficit are probably due to intraerythroid iron deficiency rather than an inherited lack of ferrochelatase activity because no evidence of iron accumulation was found in either erythroblasts or erythrocytes. The absence of any significant differences between affected and control animals with respect to either urinary coproporphyrin excretion or red cell porphobilinogen deaminase activity corrected for reticulocyte count argues against a defect in the protoporphyrin synthetic pathway.

The characterization of hemoglobin deficit by Scheufler (1) and the present studies adds another inherited hypochromic, microcytic anemia to the growing list of such disorders in experimental animals. This list now includes flex-tail anemia (18), sex-linked anemia (19, 20), microcytic anemia (21), α-thalassemia (22) and β-thalassemia (23) of the mouse and the anemia of the Belgrade laboratory rat (24).

Apart from some red cell morphological similarities between flex-tail anemia, sex-linked anemia, and hemoglobin deficit, the conditions are otherwise clearly dissimilar. The anemia in flex-tailed mice is transitory, siderocytic, and probably due to a defect in fetal hemoglobin synthesis. Sex-linked anemia is an X-linked trait due to an inherited malabsorption of iron.

Since there are striking similarities between hemoglobin deficit and microcytic anemia including hypochromia, microcytosis, hyperferrinemia, and raised FEP levels, it was imperative to exclude the possibility that both were due to the same mutation or an allele at the same locus. This possibility is excluded by finding no anemic offspring in the progeny of matings between *hbd/hbd* animals and *mk/+* or *mk/mk* animals.

The red cell morphological similarities between hemoglobin deficit and both human and mouse thalassemia raised the possibility that hemoglobin deficit might be due to a deficiency in globin chain production. Thalasse-

mia, however, is excluded by the present findings of balanced globin chain synthesis in *hbd/hbd* reticulocytes and the similarity in α/β globin chain synthesis ratios between affected and control animals.

There are obvious similarities between hemoglobin deficit and the anemia of the Belgrade rat; hypochromia, microcytosis, hyperferrinemia, and raised FEP levels are found in both conditions. It has already been demonstrated that the defect in the anemia of the Belgrade laboratory rat involves a deficiency in the intraerythroid release of iron from transferrin (23).

Based on the current data it appears that the most likely defect in hemoglobin deficit is heme synthesis deficiency due to a failure of the erythroid cells' iron procurement mechanisms.

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