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Genetics

Levels of δ-aminolevulinate dehydratase, uroporphyrinogen-I synthase, and protoporphyrin IX in erythrocytes from anemic mutant mice

(hemolytic disease/reticulocytosis/heme biosynthesis/development)

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ABSTRACT Levels of erythrocyte δ-aminolevulinate dehydratase [ALA-dehydratase; porphobilinogen synthase; 5-aminolevulinate hydro-lyase (adding 5-aminolevulinate and cyclizing), EC 4.2.1.24], uroporphyrinogen-I synthase [Uro-synthase; porphobilinogen ammonia-lyase (polymerizing), EC 4.3.1.8], and protoporphyrin IX (Proto) were measured by sensitive semimicroassays using 2–5 μl of whole blood obtained from normal and anemic mutant mice. The levels of erythrocyte ALA-dehydratase and Uro-synthase showed marked developmental changes and ALA-dehydratase was influenced by the L^v gene.

Mice with overt hemolytic diseases (*ja/ja, sph/sph, nb/nb, ha/ha*) had 10- to 20-fold increases in ALA-dehydratase, Uro-synthase, and Proto compared with their normal controls. Mice with an iron deficiency (*mk/mk*) and mice with hypoplastic anemias (W/W^v, Sl/Sl^d, *an/an*) had mild to moderate increases in these parameters. Elevated enzyme activities and Proto correlated well with the number of reticulocytes. Because all mice with anemias possessed elevated levels of ALA-dehydratase, Uro-synthase, and Proto independent of differences in their genotypes, the increase in these parameters is not likely to be the result of a specific gene defect. The increased enzyme activities and Proto concentration probably reflect increased frequency of young red cells that are still active in heme biosynthesis.

At least 12 anemias induced by single genes have been described among inbred strains of mice. The anemias include the hemolytic diseases *ha/ha, ja/ja, nb/nb, sph/sph,* the iron deficiencies *mk/mk* and *sla/sla,* and the hypoplastic diseases *an/an,* Sl/Sl^d, and W/W^v. These disorders provide very useful material for the study of many current problems in hematology and cell biology and some mouse anemias resemble human conditions sufficiently to be exploited as models of human disorders of erythropoiesis.

Because decreased biosynthesis of heme in erythroid cells is one of the many potential causes of anemia, we have examined the activities of two enzymes and a metabolic intermediate in the heme biosynthetic pathway, namely δ-aminolevulinate dehydratase [ALA-dehydratase; porphobilinogen synthase; 5-aminolevulinate hydro-lyase (adding 5-aminolevulinate and cyclizing), EC 4.2.1.24], uroporphyrinogen-I synthase [Uro-synthase; porphobilinogen ammonia-lyase (polymerizing), EC 4.3.1.8], and protoporphyrin IX (Proto), in the erythrocytes of anemic mutant mice.

In this paper, we report the occurrence of developmental changes in the levels of the two enzyme activities and our findings with regard to the levels of ALA-dehydratase, Uro-synthase, and Proto in the anemic mutants *ha/ha, ja/ja, nb/nb, sph/sph, mk/mk, an/an,* Sl/Sl^d, and W/W^v.

Abbreviations: ALA-dehydratase, δ-aminolevulinate dehydratase; Uro-synthase, uroporphyrinogen-I synthase; Proto, protoporphyrin IX; DTT, dithiothreitol; RBC, erythrocyte.

MATERIALS AND METHODS

Animals. Genetically anemic mutant mice, their littermate controls, and the C57BL/6J and DBA/2J inbred strains were reared in research colonies of the Jackson Laboratory. The origins and hematological, as well as general, characteristics of the various types of anemic mice have been reviewed elsewhere (1–3). The mutants employed in these studies of hemolytic disease included: jaundice (*ja*) (4), spherocytosis (*sph*) (5), hemolytic anemia (*ha*) (1, 6), and normoblastosis (*nb*) (1, 7). Other anemias used were: microcytosis (*mk*) (8), Steel (*Sl*) (9), *W* series (*W*) (10), and Hertwig's anemia (*an*) (11). With the exception of Sl/Sl^d mice, which occur in the F_1 hybrid between inbred strains WC/Re and C57BL/6J, all the homozygous anemic mice were produced in the hybrid WBB6F_1 genetic background. Mice ranged in age from 6 weeks to 1 year. Earlier studies have demonstrated that none of the anemia-producing genes studied are allelic with one another, nor are they allelic with structural genes specifying the β-globin chain of hemoglobin (*Hbb*), and in addition the genetic linkage of *mk* and *ha* to the hemoglobin α-chain locus has been excluded (12). Because the hemoglobins of *sph/sph, nb/nb, ja/ja,* and *ha/ha* mice are electrophoretically normal and have normal solubilities in phosphate buffers, hemoglobinopathies have not been indicated (12). Evidence of immunological disorder has not been forthcoming (2) and no specific defect in glucose metabolism has been described for any of these mutants (12).

Mice were maintained on Jackson Emory-Morse diet 96W. They were fed and watered ad lib. The principles of laboratory animal care as promulgated by the National Society of Medical Research are observed in both laboratories.

Methods. Blood was obtained in a heparinized hematocrit capillary tube by puncture of the retro-orbital sinus. One tubeful of blood (approximately 75 μl) was sufficient to perform all assays and no mice were sacrificed. Packed cell volumes were determined by using approximately 10 μl of blood by using pre-calibrated microhematocrit tubes (no. 1025, Clay Adams). Erythrocyte Proto concentration was determined fluorometrically by using 2 μl of whole blood according to the method of Sassa *et al.* (13). A Hitachi-Perkin-Elmer MPF III fluorescence spectrophotometer equipped with a red-sensitive photomultiplier R446UR was used to determine porphyrin concentrations. Daily calibrations of the instrument was made by using coproporphyrin III (0.1 μM) in 1 M perchloric acid/methanol mixture (1:1, vol/vol) (13).

In some experiments, a Turner III fluorometer also equipped with an R446UR tube was used to determine porphyrin concentrations and the results obtained from the fluorometer were identical to those obtained by the Hitachi fluorescence spectrophotometer. The Fluorescence emission spectrum of porphyrins extracted from erythrocytes obtained from standard

Table 1. Erythrocyte protoporphyrin concentration in normal and anemic mice

Genotype	No. of animals examined	Proto (μg/100 ml RBC), mean ± SEM	P
+/+	21	73 ± 3	
ha/ha	14	923 ± 25	<0.001
ja/ja	8	957 ± 48	<0.001
nb/nb	13	818 ± 27	<0.001
sph/sph	12	1417 ± 25	<0.001
mk/mk	12	363 ± 8	<0.001
an/an	13	130 ± 5	<0.001
W/W^v	9	134 ± 6	<0.001
+/+*	3	70 ± 7	
Sl/Sl^d*	9	167 ± 7	<0.001

* These genotypes were maintained in the hybrid $WCB6F_1$ mice. Others were produced in the hybrid $WBB6F_1$ background.

inbred strains of mice and anemic mutant mice was indistinguishable from that of protoporphyrin IX (13, 14).

ALA-dehydratase activity was determined by using 5 μl of whole blood according to Sassa *et al.* (15). The enzyme assay was performed both in the absence and in the presence of 10 mM dithiothreitol (DTT). The enzyme activity assayed in the absence of dithiothreitol (−DTT) determines the levels of ALA-dehydratase activity that is dependent on the endogenous sulfhydryl groups in the cells, and the activity assayed in the presence of dithiothreitol (+DTT) represents total enzyme activity because DTT activates the inactive form of the enzyme completely (15).

Uroporphyrinogen-I synthase (Uro-synthase) activity was assayed fluorometrically by using 2 μl of whole blood according to Sassa *et al.* (16). Blood samples for ALA-dehydratase and Uro-synthase determinations were frozen and thawed three times in dry ice/acetone prior to the enzyme assay. All determinations were carried out in duplicate and the statistical significance of data was measured by the Student *t* test.

RESULTS

Erythrocyte protoporphyrin (Proto) concentration in normal and anemic mice

Table 1 summarizes the erythrocyte (RBC) Proto levels in normal and anemic mutant mice. The data indicate that erythrocyte Proto levels found in normal $WBB6F_1$ +/+ and $WCB6F_1$ +/+ mice are essentially identical (73 and 70 μg/100 ml of RBC, respectively) and the value for $WBB6F_1$ +/+ is similar to that reported by Kreimer-Birnbaum *et al.* (17). In contrast to normal controls, all congenitally anemic mice possessed erythrocytes with elevated Proto concentrations. In the case of mice with overt hemolytic diseases, Proto concentrations were markedly increased, being 13-, 13-, 11-, and 20-fold greater than the control levels in *ha/ha, ja/ja, nb/nb,* and *sph/sph,* respectively. Microcytic mice (*mk/mk*) with a hypochromic nonhemolytic anemia showed a 5-fold increase in Proto concentration and mice with macrocytic anemias (*an/an,* Sl/Sl^d, and W/W^v) showed increases in erythrocyte Proto levels to a lesser though significant extent. There was no significant correlation between erythrocyte Proto concentrations and erythrocyte counts, packed cell volumes, mean corpuscular volumes, and mean corpuscular hemoglobin concentrations. On the other hand, there appears to be a significant association between erythrocyte Proto levels and the number of reticulocytes, independent of the types of anemia examined (Fig. 1). Thus, this positive correlation between these two variables suggests that the levels of erythrocyte Proto reflect the increased population of young red cells that are still biosynthetically active in Proto formation.

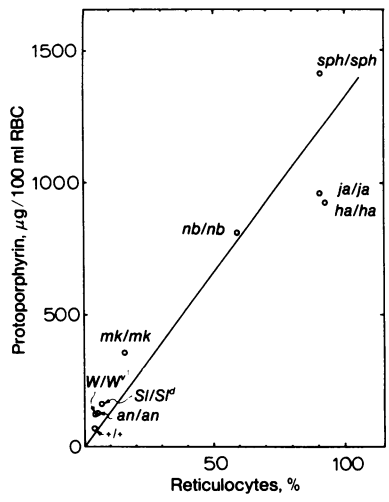

FIG. 1. Relation between erythrocyte protoporphyrin concentration and reticulocyte count. The mean protoporphyrin concentration is plotted as a function of the number of reticulocytes in a total of 100 erythrocytes.

ALA-dehydratase activity in normal and anemic mice

ALA-dehydratase activity in normal and anemic mice is summarized in Table 2. The two wild types (+/+) possessed similar enzymatic activities. When the enzyme was assayed in the presence of dithiothreitol (DTT), it was approximately three times as active as it was in the absence of DTT. The data suggest that approximately 30% of ALA-dehydratase activity in mouse erythrocytes is active and that the rest of the enzyme activity is inactive but can be activated by the addition of DTT under these assay conditions. Mice homozygous for hemolytic disorders had marked elevation of ALA-dehydratase activity both in the absence and in the presence of DTT. For example, the DTT-activated ALA-dehydratase activity was 18-, 14-, 14-, and 18-fold greater in *ha/ha, ja/ja, nb/nb,* and *sph/sph,* respectively, than in normal control mice. W/W^v, Sl/Sl^d, and *mk/mk* mice showed less than 3-fold but significant elevation in ALA-dehydratase activity. The levels of ALA-dehydratase found in *an/an* were not significantly different from those in normal controls.

The ratio of the activated to nonactivated ALA-dehydratase activity has been calculated in order to assess the endogenously active enzyme fraction. The ratio obtained by dividing total enzyme activity (+DTT) by endogenously active levels (−DTT) was significantly lower in anemic mice than in normal controls with the exception of *mk/mk* and *an/an* mice. These data suggest that ALA-dehydratase present in erythrocytes in anemic mice is not only increased, but is also more active without the addition of exogenous sulfhydryl groups.

Uro-synthase activity in normal and anemic mice

Values of Uro-synthase activities in the erythrocytes from normal and anemic animals are summarized in Table 3. *ha/ha, ja/ja, nb/nb,* and *sph/sph* showed approximately 21-, 20-, 16-, and 23-fold increases in Uro-synthase activity, respectively. The mutants *mk/mk,* Sl/Sl^d, and W/W^v showed smaller though statistically significant increases of Uro-synthase activities.

Table 2. Erythrocyte ALA-dehydratase activity in normal and anemic mice

Genotype	ALA-dehydratase* -DTT	ALA-dehydratase* +DTT	Ratio +/-
+/+	158 ± 10 (19)	594 ± 35 (21)	3.78 ± 0.15 (19)
ha/ha	4,570 ± 147 (12)	10,890 ± 294 (14)	2.38 ± 0.13 (12)
ja/ja	4,800 ± 162 (5)	8,650 ± 274 (5)	1.80 ± 0.02 (5)
nb/nb	3,680 ± 88 (6)	8,280 ± 191 (13)	2.30 ± 0.07 (6)
sph/sph	5,010 ± 121 (6)	11,260 ± 211 (12)	2.15 ± 0.04 (6)
mk/mk	524 ± 46 (12)	1,400 ± 49 (12)	2.88 ± 0.23† (12)
an/an	184 ± 10‡ (13)	511 ± 31‡ (13)	2.87 ± 0.23† (13)
W/W^v	519 ± 44 (9)	872 ± 55 (15)	1.83 ± 0.17 (9)
+/+§	215 ± 43 (3)	564 ± 71 (3)	2.67 ± 0.20 (3)
Sl/Sl^d§	664 ± 54 (6)	1,128 ± 54 (15)	1.67 ± 0.05 (6)

Numbers of animals examined are shown in parentheses. For all anemic mice values were significantly greater than normal ($P < 0.001$ except where otherwise noted).
* The enzyme activity is expressed as nmol porphobilinogen formed per ml of RBC per hr, at 37°.
† $P < 0.01$ for these mice.
‡ Not significantly different from values for the wild type.
§ These genotypes were maintained in the hybrid $WCB6F_1$ mice. Others were produced in the hybrid $WBB6F_1$ background.

Developmental changes in ALA-dehydratase and Uro-synthase activities

Activities of ALA-dehydratase (Fig. 2) and Uro-synthase (Fig. 3) showed age-dependent changes. Both enzyme activities in erythrocytes from C57BL/6J and DBA/2J mice were significantly higher in young mice (<5 weeks) than in older mice (>6 weeks). The level of the ALA-dehydratase activity found in C57BL/6J +/+ was approximately one-third that observed in DBA/2J +/+ at any given time (Fig. 2). This is in agreement with observations by Russell and Coleman (18), who found that the levels of ALA-dehydratase in the spleen and the livers of mice homozygous for Lv^a locus (e.g., DBA/2J) are approximately three times higher than in those homozygous for the Lv^b locus (e.g., C57BL/6J). Our observations presented in Fig. 2 also indicate that the levels of ALA-dehydratase in erythrocytes are determined by the *Lv* gene. In contrast, Uro-synthase levels were not influenced by the *Lv* locus (Fig. 3).

DISCUSSION

Little is known about the changes in the levels of heme pathway enzymes in weanling animals. The data in this study clearly demonstrate that marked developmental changes in the levels of ALA-dehydratase and Uro-synthase occur in mice from birth to 6 weeks of age. In addition, erythrocyte Proto levels show age-dependent changes (Sassa, unpublished). These enzyme activities and Proto levels appear to be at least 10 times higher in reticulocytes than in mature red cells. Therefore, comparison of the values for these enzyme activities should be made with mice older than 6 weeks or within an age-matched group. Our data also indicate that the level of erythrocyte ALA-dehydratase is influenced by the *Lv* gene and this factor must not be overlooked in the analysis of ALA-dehydratase activity.

Mice homozygous for hemolytic disorders (*ha/ha, ja/ja, nb/nb,* and *sph/sph*) showed marked elevation of erythrocyte Proto concentration and ALA-dehydratase and Uro-synthase activities. These parameters in the heme pathway were also increased in *mk/mk, an/an,* Sl/Sl^d, and W/W^v mice, but to a smaller extent. Since genes coding for these anemias are nonallelic, the increased levels of ALA-dehydratase, Uro-synthase, and Proto are not likely to be the result of a specific gene defect. These changes probably reflect the increased frequency of cells that are still active in heme biosynthesis as an adaptive response to the low oxygen-carrying capacity in anemic mice. This response would be expected to be greatest in mice with the various hemolytic diseases in which the indigenous red cells have half-survival times of less than 2 days (Bernstein, unpub-

Table 3. Erythrocyte uroporphyrinogen-I synthase activity in normal and anemic mice

Genotype	No. of animals examined	Uro-synthase (nmol Uro/ml RBC per hr, 37°) mean ± SEM	*P*
+/+	19	28.0 ± 1.3	
ha/ha	14	581 ± 21	<0.001
ja/ja	8	555 ± 23	<0.001
nb/nb	13	450 ± 9	<0.001
sph/sph	12	635 ± 21	<0.001
mk/mk	12	55.6 ± 1.1	<0.001
an/an	13	38.7 ± 1.4	<0.001
W/W^v	13	46.8 ± 2.0	<0.001
+/+*	3	26.3†	
Sl/Sl^d*	12	64.4 ± 3.0	

* These genotypes were maintained in the hybrid $WCB6F_1$ mice. Others were produced in the hybrid $WBB6F_1$ background.
† Assay was performed on a pooled sample.

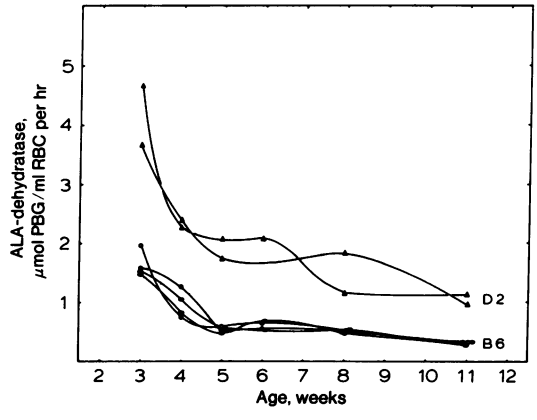

FIG. 2. Developmental changes in erythrocyte ALA-dehydratase activity. PBG is porphobilinogen. D2 = DBA/2J; B6 = C57BL/6J. The various symbols represent individual mice.

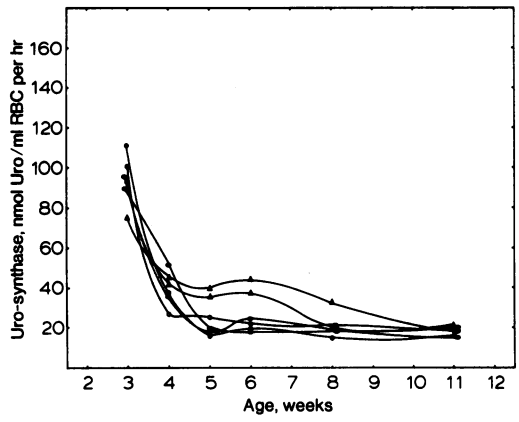

FIG. 3. Developmental changes in erythrocyte Uro-synthase activity. △, ▲ = DBA/2J; ○, ●, ○, ● = C57BL/6J. Each symbol represents an individual mouse.

lished). In fact, increases in Proto, ALA-dehydratase, and Uro-synthase correlate well with the number of reticulocytes and, as has previously been shown, these heme pathway enzymes are strikingly increased when stem cells differentiate along the erythroid pathway (19). Because approximately 90% of red cells are reticulocytes in *ha/ha*, *ja/ja*, and *sph/sph* mice and the nucleated erythroblasts are less than 1.6% in the peripheral circulation (12), the levels of ALA-dehydratase and Uro-synthase or Proto can be considered as the reticulocyte levels of these enzymes or the metabolic intermediate.

Increased levels of erythrocyte proto can be caused by (*i*) inhibition of ferrochelatase, (*ii*) depletion of iron as the substrate for ferrochelatase, (*iii*) decreased breakdown of heme leading to diminished utilization of Proto for heme formation, or (*iv*) increased formation of Proto precursors. In the case of inhibition or decreased activities of ferrochelatase, less heme is formed, thus less heme is catabolized to form bile pigments. Because mice with overt hemolytic diseases have elevated serum bilirubin levels (17) and their red cell survival time is greatly reduced as measured by increased production of ^{14}CO (S. A. Landaw, personal communication), it is unlikely that elevated Proto levels in these mice are the result of decreased ferrochelatase activity or are due to decreased breakdown of heme. The elevation of Proto in these mice is probably due to the increased activities of porphyrin-heme biosynthetic pathway.

In the case of nonhemolytic diseases, a moderate increase in erythrocyte Proto, ALA-dehydratase, and Uro-synthase levels may occur as it does with *mk/mk* mice, which display a moderate increase in the proportion of young red cells. In these mice the serum bilirubin level remains below 1.5 mg/100 ml (17), and no overt hemolysis is observed (12), but there is increased breakdown of red cells as evidenced by an increased ^{14}CO production from their labeled red cells (6). The *mk/mk* anemia thus appears to be a mild but well-compensated disorder. Elevated erythrocyte Proto levels in *mk/mk* mice are not only the result of increased young red cell population, but are also the result of decreased ferrochelatase activity due to iron deficiency that characterizes this disorder. Proto levels are therefore higher in the *mk/mk* mice than would be expected for their reticulocyte counts, as indicated by an upward shift from the regression line in Fig. 1.

The practical importance of this study concerns the effects of red cell aging on the interpretation of certain clinical data based on the erythrocyte enzymes and intermediates. For example, patients with acute intermittent porphyria (AIP) are characterized by an approximately 50% reduction in the level of Uro-synthase activity in the liver, red cells, and cultured skin fibroblasts (20). However, if an AIP patient has the complication of a hemolytic process, the red cell Uro-synthase activity might be falsely elevated. In fact, it has been found that the range of Uro-synthase activities in erythrocytes is considerably greater than that in cultured skin fibroblasts in the same population group (20). The greater variation of erythrocyte enzyme activities may be due to specialized turnover of red cells affecting the levels of enzymes and intermediates.

Using these heme pathway parameters, we have also been able to diagnose mice heterozygous for hemolytic anemias (e.g., *+/ja*, *+/nb*) (S. Sassa and S. E. Bernstein, unpublished). Thus, these assays are not only useful for the characterization of mouse anemias, but also are potentially useful for the diagnosis of mild but well-compensated anemias in humans; e.g., sickle cell traits or thalassemic traits.

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