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Erythropoiesis in Pyridoxine Deficient Mice[1] (38054)

MANOUTCHEHR KEYHANI, DENNIS GIULIANI, E. REGINA GIULIANI, AND BERNARD S. MORSE
(Introduced by F. P. Chinard)

Division of Hematology of the Department of Medicine, College of Medicine and Dentistry of New Jersey, New Jersey Medical School, Newark, New Jersey

The sideroblastic anemias are a diverse group of disorders characterized by hypochromia, microcytosis, increased serum iron, reticulocytopenia, erythroid hyperplasia and ringed sideroblasts in the marrow (1). The mechanisms leading to ringed sideroblast formation and ineffective erythropoiesis are not clearly understood. A defect in erythroid maturation leading to intramedullary cell death at the intermediate (polychromatophilic) normoblast stage was reported in patients with idiopathic sideroblastic anemia (2). Lead, an agent causally related to the secondary sideroblastic anemias, induces a similar defect in erythropoietin induced erythroid maturation in mice (3). Decreased marrow Δ-ALA synthetase activity was reported in several patients with sideroblastic anemias unrelated to lead toxicity (4). Pyridoxal phosphate is required as a coenzyme for the full activity of Δ-ALA synthetase. Pyridoxine deficiency in animals as well as a decreased ability to convert pyridoxine to pyridoxal phosphate in certain alcoholic patients are both associated with sideroblast formation and impaired utilization of iron for heme synthesis (5, 6).

To further evaluate the relationship between erythroid maturation and impaired heme synthesis, we studied various aspects of erythropoiesis in pyridoxine deficient mice.

Materials and Methods. Ten week old female CF1 mice weighing 25 gm were used for the study. Pyridoxine deficient diet was obtained from Nutritional Biochemicals Company. An identical diet with added pyridoxine (0.0002%) was fed to the control groups. Plethora was induced by the ip injection of washed packed isologous red cells on 2 successive days. Erythropoietin (EP) was given by tail vein injection 6 days after the last injection of red cells. Blood samples, obtained by heart puncture from anesthetized animals were collected in versenate. Plethoric anemals with hematocrits of 57% or less were excluded from the study. Hematological studies were performed by standard technics. Siderocytes were estimated from peripheral blood smears stained with 2% acid ferrocyanide solution. Siderocyte and reticulocyte counts were both performed with the aid of the Miller ocular (7). Tibial marrow cellularity was assessed as previously described (3). Wright's stained bone marrow and spleen smears, prepared by a brush technic, were used for the estimation of nucleated erythroid cells. Blood samples for red cell ^{59}Fe incorporation were collected 18 hr after the tail vein injection of plasma-bound ^{59}Fe. They were washed 3 times with saline, lysed with water, and counted in an auto-gamma scintillation detector. Red cell ^{59}Fe incorporation was based on an assumed blood volume of 6% and 8% of body weight for normal and hypertransfused mice respectively. After the counting was completed, heme was extracted with 2-butanone as previously described (3). Pyridoxine levels were performed by a protozoological assay (8). The values presented in the tables are the mean plus and minus one standard error.

Results. (a) *Pyridoxine levels:* After one

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TABLE I. Red Cell Pyridoxine Levels.

Day[a]	ng/ml
Control	449 ± 27[b]
30	11 ± 2
60	21 ± 4
90	8 ± 1

[a] Refers to number of days on pyridoxine deficient diet.

[b] Mean ± SE, 4–8 mice per point.

month on the deficient diet, red cell pyridoxine levels decreased from control values of 449 ng/ml to less than 21 ng/ml. They remained at this reduced level for the duration of the study (Table I).

(b) *Pyridoxine deficient diet:* Animals on pyridoxine deficient diet developed a progressive anemia. Hemoglobin values decreased from control values of 13.8 to 8.9 gm% on the 100th day of diet. Red cell indices decreased resulting in hypochromia and microcytosis (Table II). The 18 hr RBC ^{59}Fe incorporation increased to 140% of control by the 44th day of diet but decreased below control values as the study progressed (Table III). The proportion of RBC ^{59}Fe incorporation utilized for heme synthesis was reduced from control values of 83.5% to approximately 60% in deficient animals (Table III). As a result, the incorporation of red cell ^{59}Fe into heme was comparable to control at day 44 but was significantly reduced in the deficient animals thereafter (Table III). The reticulocyte count increased from control values of 2.8% to 6.6% by the day 72 of diet (absolute values of 0.24 ± 0.02 and 0.52 ± 0.08 × 10^6/mm^3, respectively). Siderocytes increased from control values of 0.4% to 8.7% by day 72 (Table II). Sideroblasts were not observed in the bone marrow. There were modest elevations of nucleated erythroid precursors in both the marrow and spleen of deficient mice (Table III). Spleen weight also increased in these deficient animals (Table III).

(c) *Hypertransfusion studies:* Transfusion of 72 day pyridoxine deficient mice was accomplished with washed red cells obtained from pyridoxine deficient donors. The

TABLE II. Hematologic Parameters in Pyridoxine Deficient Mice.

Day[a]	RBC ($\times 10^{-6}$/mm^3)	Hb (g%)	Hct (%)	MCV (μm^3)	MCH ($\mu\mu g$)	MCHC %	Reticulocytes %	Siderocytes %
Control	8.89 ± 0.14[b]	13.8 ± 0.2	41.8 ± 0.6	47.1 ± 1.2	15.6 ± 0.4	33.0 ± 0.3	2.8 ± 0.3	0.4 ± 0.1
44	8.86 ± 0.37	12.0 ± 0.6	37.3 ± 1.6	42.5 ± 1.3	13.7 ± 0.4	32.0 ± 0.5	5.7 ± 0.8	5.4 ± 1.1
72	7.69 ± 0.41	10.1 ± 0.7	32.7 ± 2.1	42.4 ± 1.8	13.1 ± 0.4	30.7 ± 0.8	6.6 ± 2.0	8.7 ± 1.2
100	6.86 ± 0.59	8.9 ± 0.9	29.1 ± 0.9	42.5 ± 1.7	13.0 ± 0.4	30.4 ± 0.6	6.8 ± 1.3	6.0 ± 1.5

[a] Refers to number of days on deficient diet.

[b] Mean ± SE, 8–15 animals per point.

TABLE III. Hematologic Parameters in Pyridoxine Deficient Mice.

Day[a]	RBC ^{59}Fe % of control[b]	RBC heme ^{59}Fe %[c]	RBC ^{59}Fe heme % of control	Serum Fe (μg%)	Spleen weight (mg)	Red cell precursors	
						Tibia ($\times 10^{-6}$)	Spleen (%)
Control	100	83.5 ± 2.2[d]	100	157 ± 26	122 ± 15	1.35 ± 0.12	5.2 ± 1.0
44	140 ± 7.4	65.8 ± 2.2	106.1 ± 3.8	190 ± 13	179 ± 20	1.75 ± 0.21	13.1 ± 1.5
72	95 ± 4.6	56.9 ± 3.4	64.9 ± 3.4	187 ± 11	227 ± 52	1.61 ± 0.14	11.2 ± 1.4
100	80 ± 4.3	60.6 ± 5.9	58.5 ± 6.1	182 ± 22	204 ± 23	1.58 ± 0.20	10.0 ± 2.7

[a] Refers to number of days on deficient diet.
[b] Refers to 18 hr red cell ^{59}Fe incorporation.
[c] 18 hr RBC ^{59}Fe extractable as heme.
[d] Mean ± SE, 8–15 animals per point.

TABLE IV. Marrow Erythroid Response in Hypertransfused Pyridoxine Deficient Mice Treated with Erythropoietin.

Days after EP[a]	Red cell precursors × 10^{-6}/tibia							
	Early		Middle		Late		Total	
	C[b]	D[c]	C	D	C	D	C	D
0[d]	0	0.01 ± 0.01[e]	0.03 ± 0.02	0.05 ± 0.02	0.05 ± 0.03	0.07 ± 0.03	0.08 ± 0.03	0.13 ± 0.04
1	0.06 ± 0.02	0.08 ± 0.02	0.19 ± 0.03	0.17 ± 0.03	0.03 ± 0.02	0.01 ± 0.01	0.28 ± 0.04	0.26 ± 0.04
2	0.02 ± 0.02	0.01 ± 0.01	0.29 ± 0.03	0.44 ± 0.06	1.21 ± 0.18	1.15 ± 0.20	1.52 ± 0.21	1.60 ± 0.31
3	0	0	0.06 ± 0.02	0.08 ± 0.03	0.43 ± 0.07	0.41 ± 0.09	0.49 ± 0.12	0.49 ± 0.11

[a] Refers to the day of sacrifice after administration of 3 units of EP.
[b] Control hypertransfused group.
[c] Pyridoxine deficient hypertransfused group.
[d] Refers to baseline hypertransfused animals that received no treatment.
[e] Mean ± SE, 8–12 animals per point.

TABLE V. Erythroid Response in Hypertransfused Pyridoxine Deficient Mice Treated with Erythropoietin.

Days after EP[a]	Reticulocytes $\times 10^{-6}$/mm^3		18 hr RBC ^{59}Fe %		RBC ^{59}Fe as heme %		18 hr RBC heme ^{59}Fe %	
	C[b]	D[c]	C	D	C	D	C	D
0[d]	0.008 ± 0.002	0.013 ± 0.004	0.03 ± 0.02	0.02 ± 0.01				
1	0.005 ± 0.005	0.002 ± 0.002	0.10 ± 0.07	0.02 ± 0.01				
2	0.018 ± 0.006	0.029 ± 0.018	0.35 ± 0.04	0.48 ± 0.32				
3	0.051 ± 0.015	0.056 ± 0.016	2.64 ± 0.38	3.38 ± 0.35	71.0 ± 3.6	27.8 ± 5.7	0.25 ± 0.03	0.13 ± 0.05
4	0.052 ± 0.011	0.040 ± 0.013	1.02 ± 0.26	0.67 ± 0.12	81.0 ± 2.8	60.0 ± 2.3	2.13 ± 0.32	2.03 ± 0.30
5	0.009 ± 0.004	0.014 ± 0.008	0.05 ± 0.01	0.10 ± 0.04	73.5 ± 2.0	65.7 ± 3.6	0.75 ± 0.21	0.44 ± 0.07

[a] Refers to the day of sacrifice after administration of 3 units of EP.
[b] Control hypertransfused group.
[c] Pyridoxine deficient hypertransfused group.
[d] Refers to baseline hypertransfused animals that received no treatment.

resultant suppression of erythropoiesis in the pyridoxine deficient animals was comparable to that observed in control hypertransfused mice (Tables IV & V).

The iv injection of 3 units of EP resulted in a characteristic wave of erythroid maturation in both control and deficient animals. There was no difference in the wave of marrow erythropoiesis between control and pyridoxine deficient hypertransfused mice. The effective erythroid response, as judged by reticulocytes and red cell ^{59}Fe incorporation, increased to maximum values on day 3 and 4 after the injection of erythropoietin in both groups. This increase of effective response was associated with the rapid decline of marrow erythropoiesis (Tables IV & V). Red cell ^{59}Fe incorporation was higher in the deficient hypertransfused group (3.38 versus 2.64) 3 days after injection of EP. At this time the distribution of red cell ^{59}Fe revealed reduced heme ^{59}Fe and increased nonheme ^{59}Fe in the deficient group. When the RBC ^{59}Fe values were corrected for RBC heme formation, there was no difference between the control and deficient groups (2.03 versus 2.13). The effective response as judged by reticulocytes was also comparable (Table V).

Discussion. The data presented herein are in accord with some of the reported hematologic effects of pyridoxine deficiency, namely a hypochromic microcytic anemia, hypersideremia, and increased numbers of circulating siderocytes (9, 10). The increased reticulocyte count observed in the present studies is not a feature of pyridoxine deficiency in other species. The extent of this increase indicates a doubling of red cell production. The modest increase of marrow and splenic nucleated erythroid precursors appears consistent with the reticulocytosis. It then follows that ineffective erythropoiesis, if present, plays a minimal role in the development of pyridoxine deficiency anemia in the mouse. The increased reticulocyte count and falling hematocrit further suggests that hemolysis contributes substantially to the severity of the anemia. Moreover, impaired iron utilization, manifest by an increased red cell non-heme iron pool, does not appear to affect the production of

red cells. Alternatively, an earlier release of reticulocytes in anemic mice or even prolonged survival of reticulocytes in pyridoxine deficiency could artificially contribute to the reticulocytosis and thus shift the scale in favor of ineffective erythropoiesis.

Further evaluation of red cell production and erythroid maturation was carried out in pyridoxine deficient mice hypertransfused with blood obtained from pyridoxine deficient donors. The resultant plethora produced a virtually complete suppression of erythropoiesis in both the control and pyridoxine deficient animals. The response to a single injection of EP was comparable in both control and pyridoxine deficient groups. These data indicate a normal stem cell response to EP and a normal marrow maturation sequence of the differentiated nucleated erythroid cell. Iron utilization was impaired in the pyridoxine deficient group at the peak of the effective erythroid response to EP. Red cell ^{59}Fe incorporation was increased in the pyridoxine deficient group and it would appear that this served as a mechanism to compensate for impaired iron utilization; the net effect being comparable utilization of RBC ^{59}Fe for heme synthesis in the control and deficient groups. These observations might be explained by decreased heme feedback on both the formation Δ-ALA and the cellular uptake of iron. Ponka and Neuwirt (11), reported an increased ^{59}Fe incorporation and decreased heme ^{59}Fe formation in rabbit reticulocytes incubated with isoniazid, an agent that interferes with pyridoxine (12). They also attributed the increased uptake to a compensatory mechanism resulting from the inhibition of heme synthesis. In the untreated pyridoxine deficient mice, it appeared that some mechanism initially compensated for the impaired utilization of iron but then by the 72nd to 100th day of diet, red cell ^{59}Fe incorporation fell below control values. In the absence of plasma iron turnover studies, a final conclusion cannot be reached as to whether this represents a quantitative reduction of red cell iron turnover.

It is difficult to reconcile normal marrow erythroid maturation in pyridoxine deficient mice with the defects of erythroid maturation associated with the sideroblastic anemias in humans. Sideroblasts were not observed in the present studies. Siderocytes on the other hand were markedly increased. It has been reported that iron is incorporated at a later stage of erythroid maturation in the rodent as compared to man (13, 14). This appears to offer a reasonable explanation for the dichotomy between sideroblasts and siderocytes in the present studies. Incorporation of iron at earlier stages of erythroid maturation in man may also explain the development of ringed sideroblasts. It also follows that excess nonheme iron accumulation by marrow nucleated erythroid cells might be related to the ineffective erythropoiesis characteristic of these anemias. Sufficient studies are not available to draw firm conclusion about this possibility but inhibition of DNA synthesis (2), abnormal mitochondria (15), and defective erythroid maturation (3) are associated with nucleated erythroid cells containing excess non-heme iron. The lack of such cells in the present study as well as normal erythroid maturation further support this contention.

Summary. Mice maintained on a pyridoxine deficient diet developed a progressive anemia characterized by hypochromia, microcytosis, reticulocytosis, and erythroid hyperplasia of the marrow and spleen. Circulating siderocytes were greatly increased. Hypertransfusion of these animals with red cells obtained from pyridoxine deficient donors resulted in erythroid aplasia. The response to exogenous erythropoietin was comparable to that observed in erythropoietin treated control hypertransfused mice. These data indicate normal erythroid differentiation and maturation in pyridoxine deficient mice.

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