Abnormalities of Iron Metabolism and Erythropoiesis in Vitamin E-deficient Rabbits

By Albert C. Chou, Goronwy O. Broun, Jr., and Coy D. Fitch

Rabbits fed a vitamin E-deficient diet developed severe muscular dystrophy in 3–4 wk, but they did not become anemic. Nevertheless, reticulocyte counts increased in deficient rabbits (3.2%) compared to control rabbits (0.9%), and erythroid hyperplasia was evident in the bone marrow. Comparing deficient rabbits to controls, the plasma iron concentration was lower (134.4 versus 206.6 µg/dl); the TIBC was higher (335.9 versus 228.3 µg/dl); the whole blood protoporphyrin concentration was higher (131.6 versus 81.7 µg/dl); and the total iron content was lower in spleen (71 versus 153 µg), higher in skeletal muscle (4956 versus 3054 µg), and unchanged in bone marrow, liver, and heart. Studies of iron absorption and excretion using $^{59}$Fe showed no abnormalities in deficient rabbits. There were abnormalities of ferrokinetics, however. The half-time of disappearance of $^{59}$Fe was shorter (100.6 versus 169.4 min), the plasma iron turnover was greater (1.25 versus 0.95 mg/dl blood/day), and the reappearance of $^{59}$Fe in circulating erythrocytes at day 9 was greater (77.2% versus 57.2%) in deficient rabbits. Anemia induced by phlebotomy accentuated the abnormal iron metabolism of deficient rabbits, and the animals were unable to correct the anemia. These findings show that vitamin E deficiency in rabbits causes abnormal erythropoiesis associated with abnormal iron metabolism and sequestration of iron in skeletal muscle.

Anemia due to vitamin E deficiency has been found in premature human infants and several species of experimental animals, including swine, poultry, fish, and four species of monkey. The primary cause of anemia in premature infants is hemolysis of peripheral erythrocytes, whereas the anemia in monkeys and swine is caused by ineffective erythropoiesis. In rhesus monkeys, vitamin E deficiency causes fatal anemia characterized by erythroid hyperplasia of the bone marrow with multinucleated erythroid precursors, increased ribonucleic acid and deoxyribonucleic acid concentrations in the bone marrow, increased biosynthesis of deoxyribonucleic acid in erythroid cells, trivial reticulocytosis, and a shortened lifespan of circulating erythrocytes. The response of this anemia to vitamin E treatment is prompt and complete.

In contrast to the previously mentioned species, rabbits and most other animals do not develop anemia spontaneously in response to vitamin E deficiency, although they may show many manifestations of the deficiency, including nutritional muscular dystrophy and increased urinary excretion of creatine, allantoin, and free amino acids. In the present work, we chose young rabbits for our experimental animal to take advantage of their unusual susceptibility to vitamin E deficiency. We did not observe spontaneous anemia.
in these rabbits before they succumbed to muscular dystrophy. Nevertheless, we found abnormalities of erythropoiesis within 3-4 wk after the animals were started on the vitamin E-deficient diet. These abnormalities were associated with evidence of iron deficiency, and they were accentuated by phlebotomy. Preliminary accounts of this work have been published.25,26

MATERIALS AND METHODS

Weanling male white New Zealand rabbits, initially weighing approximately 1200 g, were fed the purified vitamin E-deficient diet developed by Young and Dinning22 as modified by Diehl.27 This diet contains all of the essential minerals and vitamins, except vitamin E, and, by weight, 11.3% casein, 29.5% sucrose, 27% corn starch, 25% cellulose, 2.25% cod liver oil, and 2.25% lard stripped of vitamin E. Control rabbits were maintained on the same diet with an additional oral supplement of 8 mg d-a-tocopheryl acetate (1.26 IU/mg; Nutritional Biochemical Corp.) in 0.2 ml corn oil thrice weekly. All of the rabbits received 0.04% sulfaquinoxaline (Pfaltz and Bauer) in their drinking water for 5 days each week as prophylaxis for coccidiosis.28 Food and water were supplied ad libitum.

To monitor the response to the diet, tocopherol concentrations in plasma were measured by the procedure of Quaife et al.29 as modified by Martinek;30 the susceptibility of erythrocytes to hemolysis by hydrogen peroxide was measured by the method of Gordon et al.;31 and urinary creatine and creatinine concentrations were measured by the Folin method32 with minor modifications. Hematologic determinations including packed cell volumes (PCV), hemoglobin concentrations (Hb), erythrocyte counts (RBC), white blood cell counts (WBC), and reticulocyte counts (Ret) were made according to standard procedures. The plasma iron concentration was measured according to the recommendations of the International Commission for Standardization in Hematology,33 and total iron binding capacity (TIBC) was measured by the light magnesium carbonate method.34 Whole blood protoporphyrin concentrations were measured by the procedure of Heller et al.35

The total iron content of skeletal muscle, heart, spleen, liver, and femoral bone marrow was determined in animals fed the vitamin E-deficient diet for 30 days. A known amount of tissue was digested with mixtures of sulfuric acid, nitric acid, and perchloric acid,36 and the iron concentration of an aliquot of the tissue digest was measured33 after proper pH adjustment using saturated ammonium acetate.

Ferrokinetic studies in control and vitamin E-deficient rabbits were performed using radioiron (59Fe) and plasma obtained from a single rabbit in each group. Blood (8 ml) was drawn from each donor rabbit by cardiac puncture, transferred immediately into a sterilized tube containing 2 ml acid citrate dextrose (ACD) solution, and centrifuged at 1700 g for 15 min at 4°C. The plasma was transferred into another sterilized tube, and 59Fe as ferrous citrate (13-15 mCi/mg) was added to achieve a final radioactivity of 11 μCi/ml. After the 59Fe-plasma mixture was incubated at room temperature for 60 min, 0.1 ml was removed for preparation of a standard. Then 2 ml blood was drawn from each rabbit by cardiac puncture for determination of plasma iron and TIBC, and a measured amount of 59Fe-plasma was injected into a marginal ear vein. Subsequent blood samples (0.5 ml) were withdrawn from the marginal ear vein of the opposite ear at 15, 30, 60, and 90 min, the PCV was measured, and 0.1 ml of each sample was added to a counting vial. Additional samples were drawn on day 3 and daily on days 5-9 to estimate iron utilization. All samples were prepared in duplicate, and all samples from an individual rabbit were counted on the same day. In addition, feces and urine were collected together quantitatively to permit measurement of the loss of 59Fe.

Regression analysis of the radioactivity in the blood samples as a function of time was performed to permit calculation of the zero-time 59Fe activity and the half-time of disappearance of the plasma 59Fe. The plasma iron turnover (PIT) and erythrocyte iron utilization were calculated as described by Bothwell and Finch37 from the plasma iron concentration and whole blood radioactivity at zero time and the whole blood radioactivity at day 9. For these calculations, plasma volume was determined on day 0 using 125I-labeled human serum albumin,38 and red blood cell volume was measured on day 9 using 51Cr-labeled erythrocytes.39 In most cases the erythrocyte utilization of the injected 59Fe at day 6 was the same as at day 9.

To study the effect of vitamin E deficiency on the recovery from an experimental anemia, some
of the rabbits were stressed by blood loss. After 3-4 wk on the diet, when the experimental rabbits showed unequivocal evidence of vitamin E deficiency, rabbits in both groups were made anemic by repeated withdrawal of approximately 10 ml blood by cardiac puncture. PCV, Hb, RBC, and plasma iron concentration were monitored in these samples.

Iron absorption was measured after the rabbits had been subjected to daily bleeding for 8 days. For this purpose, the rabbits were fasted for 5 hr. Then 12 μCi of 59Fe as ferrous citrate (13 mCi/mg), diluted with 0.4 mg carrier FeSO4, was administered directly into the stomach through a small plastic tube. The rabbits were killed 18 hr later, and the gastrointestinal tract and feces were removed. Radioactivity was measured after homogenization of these samples in 250 ml of 0.9% NaCl solution containing 27 μM carrier FeSO4. Iron absorption was defined as the difference between the total radioactivity administered and the total radioactivity recovered from the gastrointestinal tract and feces.

RESULTS

After 3-4 wk on the diet, the experimental rabbits developed the typical syndrome of vitamin E deficiency. Their plasma tocopherol concentrations were less than 0.2 mg/dl, hemolysis of their erythrocytes after exposure to hydrogen peroxide was greater than 90%, and their urine showed high creatine to creatinine ratios, with a mean of 1.75. They also showed the clinical signs of severe muscular wasting and weakness. By contrast, the control rabbits remained strong, with plasma tocopherol concentrations above 1.0 mg/dl, erythrocyte peroxide hemolysis less than 5%, and undetectable or only trace amounts of urinary creatine.

The values for RBC, PCV, Hb, WBC, and Ret are shown in Table 1. With the exception of the Ret, these hematologic parameters were not significantly altered by vitamin E deficiency. The vitamin E-deficient rabbits had a significantly higher Ret than the control rabbits and examination of the bone marrow showed erythroid hyperplasia, but there were no megaloblastic changes and no multinucleated erythrocyte precursors. Also shown in Table 1 are the values for plasma iron concentration, TIBC, and whole blood protoporphyrin concentration. These data suggest that the vitamin E-deficient rabbits were iron deficient, yet there was no appreciable difference in the amount of stainable iron in bone marrow preparations from control and deficient rabbits that had not been subjected to bleeding.

The iron content of various tissues from rabbits not subjected to bleeding is

<table>
<thead>
<tr>
<th>Table 1. Hematologic Effect of Vitamin E Deficiency in Rabbits</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
</tr>
<tr>
<td>---</td>
</tr>
<tr>
<td>RBC (×10^12/liter)</td>
</tr>
<tr>
<td>PCV (%)</td>
</tr>
<tr>
<td>Hb (g/dl)</td>
</tr>
<tr>
<td>WBC (×10^6/liter)</td>
</tr>
<tr>
<td>Ret (%)</td>
</tr>
<tr>
<td>Plasma iron (μg/dl)</td>
</tr>
<tr>
<td>Plasma TIBC (μg/dl)</td>
</tr>
<tr>
<td>Blood protoporphyrin (μg/dl)</td>
</tr>
</tbody>
</table>

Values are mean ± SE; numbers in parentheses indicate the number of rabbits.

*p < 0.001.

*†p < 0.05.

*†p < 0.01.
Table 2. Effect of Vitamin E Deficiency in Rabbits on Total Iron Content in Various Tissues

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Total Iron (µg)</th>
<th>Control (6)</th>
<th>Vitamin E-deficient (3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skeletal muscle</td>
<td>3054 ± 184</td>
<td></td>
<td>4956 ± 588*</td>
</tr>
<tr>
<td>Heart</td>
<td>100 ± 8</td>
<td></td>
<td>120 ± 12</td>
</tr>
<tr>
<td>Spleen</td>
<td>153 ± 8</td>
<td></td>
<td>71 ± 6*</td>
</tr>
<tr>
<td>Liver</td>
<td>6183 ± 1192</td>
<td></td>
<td>5684 ± 200</td>
</tr>
<tr>
<td>Bone marrow</td>
<td>2314 ± 226</td>
<td></td>
<td>2314 ± 162</td>
</tr>
</tbody>
</table>

Values are mean ± SE; numbers in parentheses indicate the number of rabbits.

* p < 0.01.
† p < 0.05.
† Values are calculated on the basis that bone marrow of one femur equals 5% of total bone marrow.

shown in Table 2. In vitamin E-deficient rabbits there was a significant decrease in the total iron content of the spleen with no significant change in the iron content of bone marrow or liver. By contrast, the total iron content of skeletal muscle was significantly higher in deficient rabbits than in control rabbits. On a concentration basis, this difference is even more striking because of the decreased muscle mass secondary to the muscular dystrophy in the deficient animals. The iron concentration was 5.87 ± 0.28 µg/g muscle for six control rabbits and 14.70 ± 2.91 µg/g for three vitamin E-deficient rabbits. There was no evidence of hemorrhage into the skeletal muscle of vitamin E-deficient rabbits either by gross inspection or by histologic examination.

Ferrokinetic studies showed rapid disappearance of transferrin-bound 59Fe and an increased PIT in the vitamin E-deficient rabbits (Table 3). Reappearance of 59Fe in circulating red cells 9 days after injection also was increased in these rabbits. Nevertheless, red cell iron utilization did not reach 100% in the unbled animals. During the course of the ferrokinetic studies, quantitative collection of feces and urine revealed that 4.0% ± 0.6% (SE) of the injected dose of radioactive iron was excreted by seven vitamin E-deficient rabbits compared to 8.7% ± 2.0% by six control rabbits. Urine obtained from the bladder after the animals were killed showed only trace amounts of radioactivity.

The change of PCV with repeated phlebotomy is shown in Fig. 1. In both groups of rabbits, there was a rapid fall in the PCV as a result of the first 3 days of bleeding. In the control rabbits, the nadir was reached on the fourth day and was followed by a gradual rise in the PCV, despite repeated withdrawal of blood through day 17. The vitamin E-deficient rabbits initially showed less severe anemia than the control rabbits, but with further bleeding their PCV continued to fall and they were unable to correct the anemia.

Table 3. Ferrokinetic Studies in Control and Vitamin E-deficient Rabbits

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Vitamin E-deficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma Iron (µg/dl)</td>
<td>242.2 ± 0.1 (9)</td>
<td>200.1 ± 12.6 (14)*</td>
</tr>
<tr>
<td>t½ (min)</td>
<td>169.4 ± 13.9 (9)</td>
<td>100.6 ± 7.2 (14)*†</td>
</tr>
<tr>
<td>PIT (mg/dl whole blood/day)</td>
<td>0.95 ± 0.09 (9)</td>
<td>1.25 ± 0.09 (14)*†</td>
</tr>
<tr>
<td>RBC utilization (%)</td>
<td>57.2 ± 6.5 (9)</td>
<td>77.2 ± 5.0 (12)*†</td>
</tr>
</tbody>
</table>

Values are mean ± SE; numbers in parentheses indicate the number of rabbits.

* p < 0.05.
† p < 0.001.
The RBC and Ret of control and vitamin E-deficient rabbits subjected to phlebotomy are shown in Fig. 2. Both groups showed the expected decrease in RBC as the blood loss began. After the initial drop, the RBC in both groups remained at a low plateau, but the RBC of the vitamin E-deficient rabbits was higher \( (p < 0.01) \) than that of the control rabbits between the second and sixth days of bleeding. As shown in Fig. 2 the vitamin E-deficient rabbits had a higher Ret \( (p < 0.01) \) before the third day of bleeding. With the continuation of blood loss, this difference in Ret disappeared. After bleeding, both groups of rabbits displayed erythroid hyperplasia in their bone marrows. No multinucleated erythrocyte precursors were seen in the bone marrow of either control or vitamin E-deficient rabbits, and both groups showed absence of or scant traces of stainable iron.

Coincident with the reticulocytosis subsequent to blood loss, the MCV of both groups of rabbits rose. The MCV of vitamin E-deficient rabbits increased only slightly, however, from 62 to 72 fl, whereas that of control rabbits rose from 63 to 100 fl after the eighth day of bleeding. Those vitamin E-deficient rabbits that survived 12 or more days of bleeding produced microcytic, hypochromic erythrocytes. Microscopic examination of blood films revealed no evidence of fragmentation of the red cells.

The MCH of both groups changed little during the first 4 days of bleeding. After day 4 the MCH of control animals increased sharply, from 22 to 28 pg/cell, whereas that of vitamin E-deficient animals remained at its original value until day 6, when it decreased to slightly lower values (20 pg/cell). The mean corpuscular hemoglobin concentration showed equivalent decreases in both groups of rabbits.
Fig. 2. Effect of daily bleeding on RBC and ret of control and vitamin E-deficient rabbits. Data from one experiment shown. Day 0 of the figure corresponds to the 25th day on the diet. Animals were killed after 8th day of bleeding. Mean ± SE for five control and seven vitamin E-deficient rabbits.

Fig. 3. Effect of daily bleeding on the plasma iron concentration of control and vitamin E-deficient rabbits. Data are from experiment shown in Fig. 2.
Figure 3 shows the effect of bleeding on the plasma iron concentrations of the two groups of rabbits. Both groups showed significant decreases in plasma iron concentration after 8 days of bleeding compared to the baseline values. The vitamin E-deficient rabbits had a lower baseline prior to bleeding, and the concentration fell to much lower levels after bleeding. These lower plasma iron concentrations occurred even though the amount of blood removed was the same in the two groups.

There was no significant difference in iron absorption between the two groups. Mean gastrointestinal absorption of $^{59}$Fe was 23.2% ± 4.7% and 28.8% ± 3.2% for seven deficient and five control rabbits, respectively.

**DISCUSSION**

In the past, vitamin E has been suggested as an adjunct in the treatment of iron deficiency anemia in man, and the combination of vitamin E and ascorbic acid has been reported to enhance the hematopoietic response to iron in iron-deficient rats. Iron deficiency secondary to vitamin E deficiency has not been reported previously. The observations described in this report show that vitamin E deficiency can induce changes characteristic of iron deficiency in rabbits. These changes include low plasma iron concentration, elevation of plasma TIBC, decrease in percentage saturation of transferrin, increase in erythrocyte protoporphyrin concentration, rapid clearance of transferrin-bound $^{59}$Fe from the plasma, increase in PIT, and an increase in erythrocyte utilization of injected $^{59}$Fe.

Furthermore, phlebotomy accentuated the severity of the abnormal iron metabolism. Repeated bleeding of control rabbits stimulated erythropoiesis and Hb synthesis and resulted in the expected reticulocytosis, macrocytosis, and subsequent rise in Hb. These rabbits maintained a plasma iron concentration only moderately lower than the prephlebotomy level despite increased Hb synthesis. In contrast, although phlebotomy of vitamin E-deficient rabbits stimulated erythropoiesis, the new erythrocytes were normocytic or became microcytic, and there was a dramatic further decrease in plasma iron concentration. This is the same type of response described by Jacobs and Finch in earlier studies of iron deficiency in rabbits. They found a macrocytic, hyperchromic reticulocytosis in rabbits with a sufficient supply of iron. A similar degree of erythroid marrow activity was shown by rabbits with a decreased supply of available iron, but the newly released cells were not as large and had less than the normal content of Hb. An effect of available iron on hemopoiesis after phlebotomy also has been shown in man, dogs, and rats.

Our studies indicate that the iron-deficient erythropoiesis in vitamin E-deficient rabbits is not the result of depletion of total body iron stores. We found neither decreased gastrointestinal absorption nor increased loss of iron in the deficient rabbits. Furthermore, whole-body total iron content, estimated from the data in Table 2, was similar in control and vitamin E-deficient rabbits. The major difference between the two groups of animals was the sequestration of iron in skeletal muscle in vitamin E-deficient rabbits. It appears from these studies that iron is preferentially taken up by the muscle and made unavailable for erythropoiesis, even in the case of markedly stimulated erythropoiesis. The
reasons for sequestration of iron in muscle are not elucidated by the present studies. However, abnormal metabolism of myoglobin in dystrophic skeletal muscle of vitamin E-deficient animals has been reported by Indovina in rabbits and by Bender et al. in guinea pigs.

In addition to deficient synthesis of hemoglobin associated with abnormal iron metabolism, we observed erythroid hyperplasia and mild reticulocytosis in vitamin E-deficient rabbits. The ferrokinetic studies showed that these findings were not due to ineffective erythropoiesis, and we know that they were not simply a compensatory response to occult bleeding. Hemolysis has not been excluded, but it must be before the erythroid hyperplasia can be related unequivocally to the abnormal iron metabolism that we observed. Nevertheless, it is reasonable to suppose at this time that the erythroid hyperplasia and abnormal iron metabolism are related to each other, if not as cause and effect then through a single metabolic defect.

ACKNOWLEDGMENT

The invaluable technical assistance of Patricia Hutcheson and Mary Ann Bockelman are gratefully acknowledged.

REFERENCES

17. Dinning JS, Day PL: Vitamin E deficiency in the monkey. I. Muscular dystrophy,


Abnormalities of iron metabolism and erythropoiesis in vitamin E-deficient rabbits

AC Chou, GO Jr Broun and CD Fitch