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Iron Is Sequestered as Ferritin in Macrophages in Skeletal Muscle of Vitamin E-deficient Rabbits

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Summary

Weanling rabbits were fed a purified diet with or without vitamin E supplementation to evaluate the abnormal sequestration of iron in skeletal muscle associated with vitamin E deficiency. A severe myopathy developed in unsupplemented rabbits within 3 to 4 weeks. At this time, the concentration of soluble nonheme iron in biceps femoris muscles had increased from 2.1 ± 0.4 μg/g wet weight (mean ± SD) for six control rabbits to 4.3 ± 1.4 for 10 vitamin E-deficient rabbits, and total nonheme iron had increased from 5.0 ± 1.2 to 8.4 ± 3.3. Soleus muscles had even greater increases in total and soluble nonheme iron concentrations. Intramuscular injection of iron-dextran caused large increases in total and soluble nonheme iron in noninjected muscle of vitamin E-deficient rabbits, which further exaggerated the difference between the two groups. By radioimmunoassay using an antibody to rabbit liver ferritin, the concentration of ferritin in biceps femoris muscles increased from 0.47 ± 0.18 μg/g wet weight for seven control rabbits to 6.34 ± 1.70 for 14 vitamin E-deficient rabbits. Uptake of intravenously injected transferrin-bound iron into muscle of vitamin E-deficient rabbits was not increased in a short term experiment (6 h), but radioiron did accumulate in muscle in a long term experiment (6 days). There was no trapping of heat-damaged erythrocytes, no phagocytosis of intravenously injected carbon particles, and no erythrophagocytosis in muscle. An immunohistological staining method designed to detect ferritin in tissue sections stained muscle from normal rabbits very scantily but intensely stained macrophages in the muscle of vitamin E-deficient rabbits. We conclude that macrophages in skeletal muscle of vitamin E-deficient rabbits take up iron from transferrin and incorporate it into ferritin, in which form it is relatively unavailable for erythropoiesis because of slow release.

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Young vitamin E-deficient rabbits have high serum iron-binding capacity, low serum iron, and high erythrocyte free protoporphyrin concentrations, and they recover slowly from anemia induced by phlebotomy (2). These abnormalities are secondary to iron sequestration in muscle rather than to a decrease in the total amount of iron in the body (2). Since vitamin E-deficiency is known to cause an extensive necrotizing myopathy accompanied by infiltration of macrophages (1), the present study evaluated the involvement of macrophages in the sequestration of iron.

MATERIALS AND METHODS

Young New Zealand White rabbits initially weighing approximately 650 g were used in all except one experiment. Rabbits

weighing approximately 1200 g were used in that experiment to ensure longer survival. All of the rabbits were fed a purified, vitamin E-deficient diet developed by Young and Dinning (16) and modified by Diehl (6). On a dry weight basis, it contains 11.3% casein, 29.5% sucrose, 27% cornstarch, 25% cellulose, 2.25% cod liver oil, 2.25% lard stripped of vitamin E, and all of the essential minerals and vitamins except vitamin E. Control rabbits received the same diet plus an oral supplement of 8 mg of *d*-α-tocopheryl acetate in 0.2 ml of corn oil 3 times/week. Food and water were supplied *ad libitum*. For 5 days of each week, the water contained 0.04% sulfaquinoxaline (Ralston Purina Company, St. Louis) as prophylaxis against coccidiosis.

In certain of the experiments, control and vitamin E-deficient rabbits received extra iron as iron-dextran (Imferon, Merrell Dow Pharmaceuticals, Inc., Cincinnati) intramuscularly. In still other experiments, some of the rabbits were used to determine the effect of short term vitamin E repletion on iron concentrations in skeletal muscle. For this purpose, four rabbits each received 40 mg of *d*-α-tocopheryl acetate dissolved in 1.0 ml of corn oil by mouth daily when they developed the signs of severe vitamin E deficiency. Immediately prior to and 3 days after initiating vitamin E treatment, blood was obtained and a soleus muscle was removed surgically for iron measurements.

Sodium pentobarbital plus ether or ketamine plus xylazine were used for anesthesia to obtain blood and tissue samples. While anesthetized, the rabbits were killed by exsanguination.

Assessment of vitamin E deficiency. Measurements of body weight, serum tocopherol concentration (12), susceptibility of erythrocytes to hemolysis by hydrogen peroxide (8), creatinuria (7), and serum iron (11) and iron binding capacity (3) were used to assess the severity of vitamin E deficiency. Shown in Table 1 are data obtained just prior to or at the time tissue samples were obtained for iron and ferritin measurements, which was approximately 3 weeks after beginning the purified diet. The vitamin E-deficient rabbits gained weight slowly, and they had low serum vitamin E concentrations, erythrocytes highly susceptible to hydrogen peroxide hemolysis, creatinuria, low serum iron, and high serum total iron binding capacity. In addition, extreme weakness was apparent when the vitamin E-deficient rabbits attempted to right themselves after being placed on their sides. If not killed at this point in time, these small rabbits invariably died within a week. The same assessment of the larger rabbits yielded similar evidence of vitamin E deficiency 4 to 5 weeks after beginning the purified diet. However, most of the larger rabbits survived for a week or more after they developed severe myopathy.

Iron and ferritin measurements. The serum iron concentration was measured according to the recommendation of the International Committee for Standardization in Hematology (11), and the serum total iron binding capacity was measured by the light magnesium carbonate method (3). Nonheme iron in tissue homogenates was measured by the method of Torrance and Bothwell (14). To measure the soluble fraction of nonheme iron in tissues, the specimens were homogenized in water in ground glass tubes with tightly fitted ground glass pestles and centrifuged at 4°C and 12,000 × *g* for 70 min. The supernatant solutions then were transferred to other tubes and centrifuged again under similar conditions to remove particulate material before measuring nonheme iron (14).

For measurements of ferritin concentrations, tissue homogenates were centrifuged at 4°C and 1500 × *g* for 20 min to obtain a supernatant solution for analysis. Ferritin was measured by standard radioimmunoassay methods (10) using ^{125}I-labeled rabbit liver ferritin, guinea pig antiferritin antibodies to complex the ferritin, and an excess of rabbit anti-guinea pig Immunobeads (Bio-Rad Laboratories; antigen capacity, 3.75 μg/mg beads) to precipitate the ferritin-antiferritin complexes for counting. Rabbit liver ferritin was purified from healthy adult rabbits after repeated injections of iron-dextran as described by Penders *et al.* (13) for use as a standard and as the antigen for production of antiferritin antibodies and for labeling with ^{125}I by the chloramine T method (9).

Antiferritin antibodies were raised in each of four adult guinea pigs by giving an initial subcutaneous injection of 600 μg of the purified ferritin dissolved in 0.5 ml of 0.25 M phosphate buffer (pH 7.2) mixed with 0.5 ml of Freund's complete adjuvant. At weekly intervals thereafter, three booster doses of 600 μg ferritin mixed with 0.5 ml of Freund's incomplete adjuvant were given. The guinea pigs were bled by cardiac puncture under anesthesia 6 weeks after the initial ferritin injection, their sera (approximately 3 ml from each) were pooled, and a partially purified antibody fraction was prepared as follows. The pooled sera were mixed with an equal volume of borate-saline medium (8.37 g boric acid, 6.19 g sodium borate, and 9.38 g NaCl per liter, pH 8.02), after which protein was precipitated by slowly adding solid Na_2SO_4 to a final concentration of 18%. The precipitate was collected by centrifugation and washed once with 18% Na_2SO_4 dissolved in the borate-saline medium. Then, the precipitate was dissolved in a minimum volume of borate-saline medium. Removal of Na_2SO_4 was accomplished by Sephadex G-25 column chromatography and dialysis overnight in borate-saline medium. By Ouchterlony double diffusion immunoassay (10), this antibody preparation gave precipitation lines indicating reactions of identity with purified rabbit liver ferritin and the ferritin in crude soluble preparations of liver, spleen, and skeletal muscle from vitamin E-deficient rabbits. A concentration of antibody approximately equivalent to a 1:100,000 dilution of the original serum pool was used for the radioimmunoassays.

Radiotracer studies. To evaluate the tissue distribution of radioiron bound to transferrin, 9 ml of blood was obtained from vitamin E-deficient or control donor rabbits and mixed with 1 ml of acid-citrate-dextrose solution (7.3 g citric acid, 22 g sodium citrate, and 24.5 g dextrose per liter). The erythrocytes were removed by centrifugation and 4 ml of plasma was incubated for 60 min at room temperature with 88 μCi of ^{59}Fe (ferrous citrate, 25 μCi/ml; 13–16 mCi/mg iron). The labeled plasma then was injected intravenously (0.6 ml/kg body weight) through an ear vein into control and severely vitamin E-deficient rabbits. Control rabbits received plasma from a control donor and vitamin E-deficient rabbits received plasma from a vitamin E-deficient donor. Radioiron distribution was measured either 6 h or 6 days later by killing the rabbits and removing and mincing selected tissues for counting in a gamma counter. Because of variations in radioactivity in various muscles, all of the muscles of one thigh were pooled for use as a single sample.

Heat damaged ^{51}Cr-labeled erythrocytes were used to explore the possibility of erythrocyte sequestration in skeletal muscle of vitamin E-deficient rabbits. Blood (9 ml) was collected from a control donor rabbit, mixed with 1 ml of acid-citrate-dextrose solution and 1 ml of ^{51}Cr (105 μCi/ml, 50 mCi/mg sodium chromate), and incubated for 30 min at room temperature. The mixture next was incubated in a water bath at 49°C for 10 min to damage the erythrocytes. Then the erythrocytes were collected by centrifugation, washed twice with 2 volumes of 0.9% NaCl, and resuspended in an equal volume of 0.9% NaCl for intravenous injection. Control and vitamin E-deficient rabbits each received 1 ml of this preparation of erythrocytes per kg of body

Table 1. *Comparison of control and vitamin E-deficient rabbits*

	Control	Vitamin E-deficient
Initial body weight (g)	665 ± 142 (10)*	630 ± 153 (13)
Final body weight (g)	901 ± 127 (10)	690 ± 125 (13)†
Hydrogen peroxide hemolysis (%)	1.3 ± 2.6 (10)	96.0 ± 3.2 (11)†
Serum vitamin E (μg/dl)	639 ± 212 (10)	45 ± 15 (11)†
Urinary creatine‡	0.13 ± 0.08 (6)	4.3 ± 2.1 (12)†
Serum iron concentration (μg/dl)	221 ± 24 (10)	174 ± 69 (12)†
Iron binding capacity (μg/dl)	246 ± 14 (10)	359 ± 47 (12)†

* Mean ± SD. The numbers in parentheses are the numbers of rabbits.

† $p < 0.05$ by Student's *t* test.

‡ Expressed as a molar ratio to urinary creatinine.

Table 2. *Nonheme iron in various tissues*

	Control		Vitamin E-deficient	
	Total nonheme iron	Soluble nonheme iron	Total nonheme iron	Soluble nonheme iron
	(μg/g wet weight)			
Muscle				
Biceps femoris	5.0 ± 1.2 (6)*	2.1 ± 0.4 (6)	8.4 ± 3.3 (10)†	4.3 ± 1.4 (10)†
Soleus	7.2 ± 1.3 (5)	1.9 ± 0.6 (5)	14.5 ± 4.2 (7)†	6.7 ± 3.4 (7)†
Soleus‡			14.4 ± 3.3 (4)	5.8 ± 2.6 (4)
Liver	141 ± 127.9 (9)	110 ± 98.5 (9)	64 ± 55 (13)	35 ± 41 (13)†
Spleen	181 ± 53 (9)	150 ± 44 (9)	85 ± 58 (13)†	51 ± 25 (13)†
Heart	22.2 ± 2.4 (10)	6.6 ± 2.3 (10)	21.4 ± 3.9 (12)	8.2 ± 1.3 (12)

* Mean ± SD. The numbers in parentheses are the numbers of rabbits.

† $p < 0.05$ in comparisons between control and vitamin E-deficient rabbits using Student's t test.

‡ After treatment with 40 mg of d-α-tocopheryl acetate daily for 3 days.

weight. The rabbits were killed 21 h later and selected tissues were removed, minced, and counted in a gamma counter.

Histologic studies. Muscle specimens were fixed in 10% buffered formalin, embedded in paraffin, cut into 5-μm specimens, and stained for iron with potassium ferricyanide and counterstained with nuclear fast red. For routine histology and evaluation of the uptake of colloidal carbon by macrophages, similar sections of muscle were stained with hematoxylin and eosin. To study the uptake of colloidal carbon, control and vitamin E-deficient rabbits were injected intravenously with 2.5 ml/kg of body weight of black Pelikan drawing ink (No. 17, Pelikan AG, Hannover, Germany) which had been diluted with 0.9% NaCl to achieve a carbon concentration of 60 mg/ml and centrifuged at 1000 × g for 15 min to remove the larger aggregates. The rabbits were killed 3 h later to obtain muscle specimens.

For histochemical and immunohistological studies, muscle specimens were rapidly frozen in isopentane cooled to −160°C with liquid nitrogen, and 6-μm sections were cut in a cryostat and placed on microslides immediately. These sections were either studied immediately or stored at −70°C until used. Some of the sections were stained for nonspecific esterase according to Yam *et al.* (15) using α-naphthyl acetate as substrate. The remaining sections were used for the localization of ferritin by immunofluorescent staining.

For immunofluorescent staining, tissue sections on microslides were fixed in acetone for 5 min, dried and covered with a dilute solution of the guinea pig antiferritin antibodies described above, and incubated in a moist chamber at room temperature for 30 min. The antiferritin antibodies were prepared in phosphate-buffered saline (0.9% NaCl buffered to pH 7.2 with 10 mM phosphate) at a concentration approximately equivalent to a 1:160 or 1:320 dilution of the original serum pool. Serum from nonimmunized guinea pigs was used as the control. After incubation, the microslides were drained and washed twice with constant gentle agitation in a jar filled with phosphate-buffered saline for 15 min at room temperature. Then the sections were covered with fluorescein-tagged rabbit anti-guinea pig antibody (Miles-Yeda, Ltd., Elkhart, IN) and incubated for another 30 min at room temperature in a moist chamber, after which the excess fluorescein-tagged antibody was removed by washing twice with phosphate-buffered saline as before.

RESULTS

Iron and ferritin measurements. Nonheme iron concentrations in vitamin E-deficient rabbits increased in skeletal muscle, decreased in liver and spleen, and did not change in the heart (Table 2). In skeletal muscle, most of the increase occurred in the soluble fraction, which includes ferritin. To verify that ferritin concentrations in fact increased, a radioimmunoassay was applied to biceps femoris muscles (Table 3). A smaller number of samples of soleus muscles and liver also were available for this assay. In each of these tissues from vitamin E-deficient rabbits, the change in ferritin concentration was in the direction predicted from measurements of nonheme iron. The relative increase in ferritin concentration in muscle was even greater than the increase in soluble nonheme iron.

Table 3. *Ferritin by radioimmunoassay in muscle and liver*

	Control	Vitamin E-deficient
	(μg/g wet weight)	
Muscle		
Biceps femoris	0.47 ± 0.18 (7)*	6.34 ± 1.70 (14)†
Soleus	0.49 ± 0.04 (3)	6.27 ± 2.03 (6)†
Liver	239 ± 69 (3)	90 ± 17.1 (6)†

* Mean ± SD. The numbers in parentheses are the numbers of rabbits.

† $p < 0.01$ by Student's t test.

Since the soleus, a red muscle, contained more iron (Table 2) and was more easily removed surgically than the biceps femoris muscle, it was used to study the effect of vitamin E treatment. No change in iron concentrations was detected after 3 days of treatment with large doses of d-α-tocopheryl acetate (Table 2) despite evidence of other physiological responses. For example, the serum iron concentration increased from 116.7 ± 30 to 176.3 ± 51.9 μg/dl (mean ± SD) in four rabbits, and hydrogen peroxide hemolysis decreased from 96 to 0.3% in the two rabbits tested.

The effect of treatment with extra iron also was studied. In a preliminary experiment, the serum iron concentrations 1 day after intramuscular injection of 25 mg of iron as iron-dextran were 8748 ± 766 μg/dl (mean ± SD) for five control rabbits and 4723 ± 916 μg/dl for seven vitamin E-deficient rabbits. Some of the other effects of treatment with 25 mg of intramuscular iron were evaluated in similar groups of rabbits in a subsequent experiment (Table 4). Six days after injection, more iron still remained in the injected muscle of vitamin E-deficient rabbits (the values ranged from 55 to 296 μg/g wet weight for three vitamin E-deficient rabbits and from 19 to 46 μg/g for six control rabbits). Nevertheless, parenteral iron treatment caused large increases in total and soluble nonheme iron in noninjected muscle of vitamin E-deficient rabbits, which further exaggerated the difference between the two groups. As in the untreated rabbits (Table 2), soleus muscles were more affected than biceps femoris muscles. After parenteral iron, the nonheme iron concentrations of liver and spleen of vitamin E-deficient rabbits approached the values achieved by control rabbits.

Radiotracer studies. Six hours after injecting transferrin-bound radioiron, a higher percentage of the injected dose was present in the bone marrow and a lower percentage was present in the liver of vitamin E-deficient than of control rabbits (Table 5).

Table 4. *Effect of iron treatment on nonheme iron in various tissues**

	Control		Vitamin E-deficient	
	Total nonheme iron	Soluble nonheme iron	Total nonheme iron	Soluble nonheme iron
	(μg/g wet weight)			
Muscle†				
Biceps femoris	6.6 ± 0.9 (6)‡	2.8 ± 0.4 (6)	11.5 ± 4.6 (3)§	7.8 ± 4.2 (3)§
Soleus	17.5 ± 3.0 (6)	6.0 ± 2.7 (6)	38.8 ± 15.8 (3)§	23.3 ± 12.0 (3)
Liver	369 ± 64.3 (6)	303 ± 58.7 (6)	340 ± 128 (3)	267 ± 115 (3)
Spleen	344 ± 174 (6)	251 ± 159 (6)	273 ± 139 (3)	214 ± 95 (3)
Heart	42.6 + 20.4 (5)	11.4 ± 2.7 (6)	38.7 (2)	10.8 (2)

* Iron (25 mg) as iron-dextran was injected into the right thigh. The rabbits were killed and the various tissues were obtained 6 days after the iron injection.

† Muscles from the left thigh were analyzed.

‡ Mean ± SD. The numbers in parentheses are the numbers of rabbits.

§ $p < 0.05$ in comparisons between control and vitamin E-deficient rabbits using Student's t test.

Table 5. *Distribution of radioiron 6 h after injection of transferrin-bound radioiron*

	Control	Vitamin E-deficient
	(% of total injected radioiron)	
Thigh muscles*	0.78 ± 0.33 (4)†	0.64 ± 0.26 (6)
Liver	10.4 ± 3.3 (4)	5.9 ± 1.5 (6)‡
Spleen	0.06 ± 0.01 (4)	0.11 ± 0.03 (6)
Bone marrow (one femur)	2.9 ± 0.6 (4)	4.2 ± 0.6 (6)‡

* Total wet weight of thigh muscles: 20.5 ± 2.4 g for control and 17.2 ± 2.9 g for vitamin E-deficient rabbits.

† Mean ± SD. Numbers in parentheses are the numbers of rabbits.

‡ $p < 0.05$ by Student's t test.

Table 6. *Distribution of radioiron 6 days after injection of transferrin-bound radioiron**

	Control	Vitamin E-deficient
	(% of total injected radioiron)	
Thigh muscles†	0.28 ± 0.07 (4)‡	1.06 ± 0.39 (4)§
Liver	15.33 ± 2.79 (4)	6.23 ± 1.39 (4)§
Spleen	0.19 ± 0.04 (4)	0.22 ± 0.16 (4)
Bone marrow (one femur)	0.71 ± 0.11 (4)	0.60 ± 0.10 (4)

* The initial weight of the rabbits was approximately 1200 g.

† Total wet weight of thigh muscles: 52.3 ± 5.4 g for control and 40.1 ± 7.5 g for vitamin E-deficient rabbits ($p < 0.05$).

‡ Mean ± SD. The numbers in parentheses indicate the number of rabbits.

§ $p < 0.01$ by Student's t test.

This greater uptake by bone marrow is in agreement with previous ferrokinetic studies in vitamin E-deficient rabbits (2). At the 6-h interval, thigh muscles of vitamin E-deficient rabbits did not contain an excess of radioiron (Table 5). After 6 days, however, there was a significant excess of radioiron in thigh muscles of vitamin E-deficient rabbits in comparison to control rabbits (Table 6).

In the other radiotracer study, there was no uptake of heat-damaged erythrocytes, labeled with ^{51}Cr, into skeletal muscle of control or vitamin E-deficient rabbits. This study was performed because vitamin E deficiency in certain species causes an exudative diathesis with hemorrhage into tissues (4).

Histologic studies. Sections of muscle from control rabbits bound the antiferritin antibodies minimally, and consequently they stained very scantily after incubation with a fluorescein-tagged antibody to the antiferritin antibodies (Fig. 1*A*). The staining of skeletal muscle from vitamin E-deficient rabbits is illustrated in Figure 1*B*. There was little or no staining of muscle cells, but there was abundant staining of ferritin between the muscle cells, in areas where mononuclear cells were located (Fig. 1, *B* and *D*). There was no staining of muscle from vitamin E-deficient rabbits, however, when unimmunized guinea pig serum was used instead of antiferritin antibodies. The mononuclear cells also took the stain for nonspecific esterase (Fig. 1*C*), as would be expected of macrophages. Similar sections were stained for iron with potassium ferricyanide, but only in the most severely degenerated muscle was a small amount of stainable iron visible in a few mononuclear cells (not shown). The amount of stainable iron in these cells was greatly increased after parenteral treatment with iron (Fig. 1*D*).

Sections stained with hematoxylin and eosin showed extensive hyaline necrosis and infiltration of mononuclear cells in skeletal muscle from vitamin E-deficient rabbits. There was no hemorrhage, no erythrophagocytosis, and no phagocytosis of intravenously injected carbon particles by the mononuclear cells in skeletal muscle.

DISCUSSION

The data presented here are pertinent to four questions. What is the source of the excess iron in skeletal muscle of vitamin E-deficient rabbits? In what storage form is the iron sequestered? What cell types are involved? And, is sequestration due to increased uptake or decreased release? The source almost certainly is transferrin-bound iron since radioiron injected intravenously in this form accumulated in skeletal muscle and since there was no trapping of heat-damaged erythrocytes, no uptake of intravenously injected carbon particles, and no erythrophagocytosis in the muscles of vitamin E-deficient rabbits. It is even more certain that most of the sequestered iron is in the form of ferritin in macrophages since ferritin concentrations were so large and so localized to macrophages. Moreover, extra parenteral iron found its way to macrophages in skeletal muscle of vitamin E-deficient rabbits where it could be detected by staining with potassium ferricyanide.

In regard to the mechanism of iron sequestration, we found no evidence of increased uptake of transferrin-bound radioiron into muscle of vitamin E-deficient rabbits in short term (6-h) experiments. Long term (6-day) experiments, however, revealed an excess of radioiron in muscle of vitamin E-deficient in comparison with control rabbits. We interpret these results to mean decreased turnover of radioiron. Therefore, we conclude that reduced availability of iron for erythropoiesis in vitamin E-deficient rabbits (2) is due to slow release of iron from ferritin stored in macrophages in skeletal muscle.

Of the possible explanations of how vitamin E deficiency could impair iron release, the following two warrant consideration. (a) In the absence of vitamin E, a structurally abnormal ferritin might be produced. This possibility could explain the low ratio of soluble nonheme iron to radioimmunoassayable ferritin which we observed. However, it is more probable that the low ratio

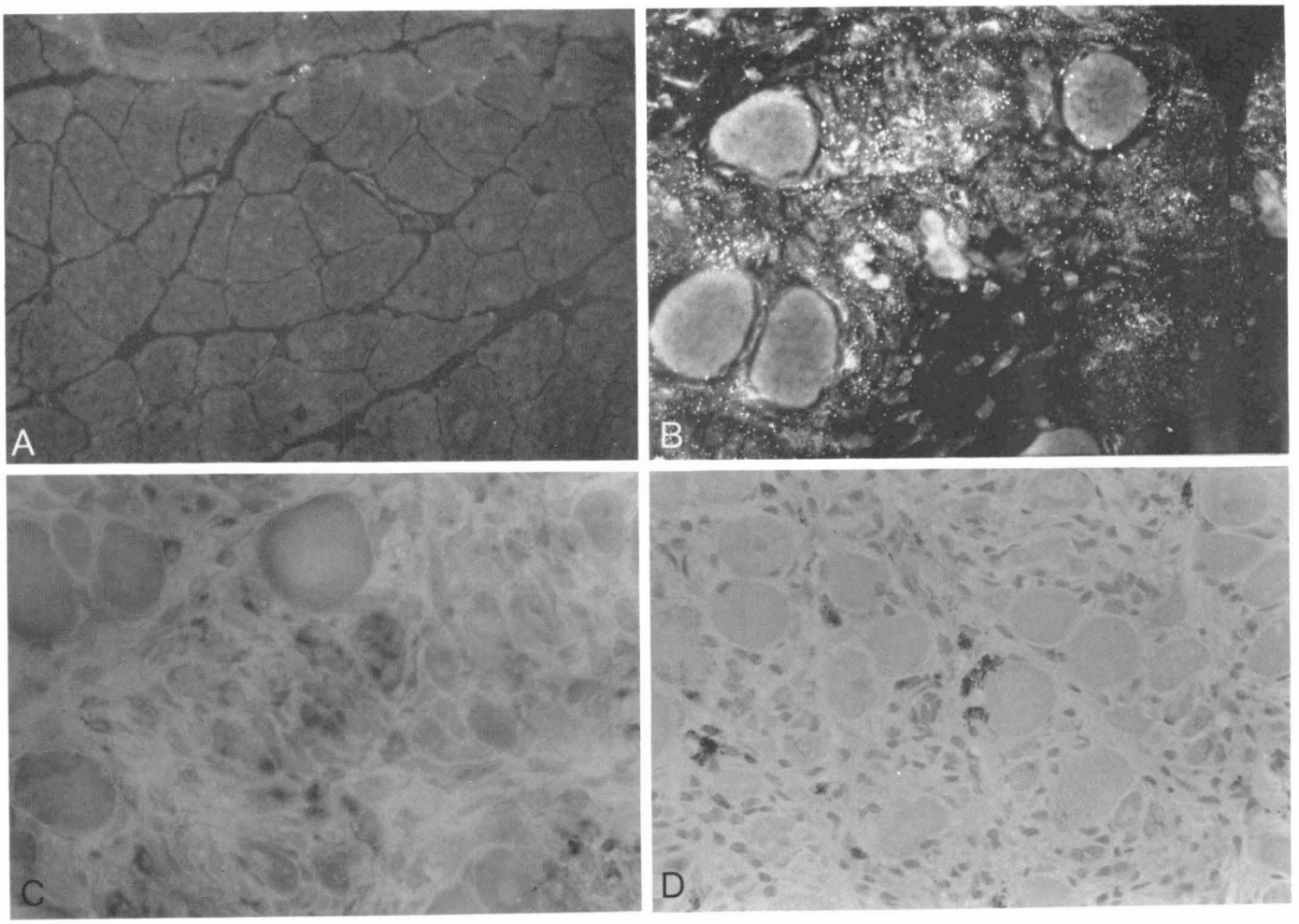

Fig. 1. *A*, immunohistological study of muscle ferritin of soleus muscle of control rabbits. Only scanty ferritin is detectable in normal muscle. *B*, immunohistological study of muscle ferritin of soleus muscle of vitamin E-deficient rabbits. Ferritin is absent in the intact myofibrils but abundant ferritin is present in the tissue between the myofibrils (infiltrating mononuclear cells) and the degenerating myofibrils. *C*, nonspecific esterase activity of soleus muscle of vitamin E-deficient rabbits. Dark staining of cytoplasm indicating enzyme activity is present in many mononuclear cells, especially those infiltrating the degenerating myofibrils. *D*, iron stain of soleus muscles of vitamin E-deficient rabbits receiving iron supplements. Abundant mononuclear cells are located between myofibrils. Stainable iron is present in many of these mononuclear cells.

reflects a difference in the number of macrophages, as our antibody detected ferritin in macrophages but not in normal muscle cells. (b) Vitamin E might be required for the release of iron from macrophages. It might function as a reducing agent directly interacting with ferritin, it might spare other reducing agents which facilitate the release of iron, or it might protect macrophages from oxidant damage. While none of these possible roles for vitamin E in iron release can be excluded, it is noteworthy that the response to vitamin E repletion provided no support for them. Indeed, vitamin E treatment for 3 days had no effect on the iron content of muscle of vitamin E-deficient rabbits.

Finally, since macrophages are known to be heterogeneous in their ability to recycle iron (5), it is possible that the population infiltrating necrotic muscle of vitamin E-deficient rabbits normally may not release iron readily. If such a population of macrophages were present, they would not be expected to respond to vitamin E repletion with a decrease in iron content. Until there is evidence to the contrary, therefore, it is reasonable to suggest that the abnormal iron metabolism of vitamin E-deficient rabbits is due to the presence in necrotic skeletal muscle of a large number of normally functioning macrophages.

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