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Labeling of Marrow Cells of Vitamin E-Deficient Monkeys by ^{3}H-Precursors of Nucleic Acids and Protein* (33513)

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When young rhesus monkeys are fed diets deficient in vitamin E, they develop a characteristic syndrome of anemia and muscular weakness after 1–3 years (1, 2). During the anemia the lifespan of the circulating erythrocytes is reduced by about two thirds of normal (3), and the bone marrow is hypercellular (4). There is a granulocytosis but only a slight reticulocytosis (1, 2, 4). Treatment with *alpha*-tocopherol induces reticulocytosis and rapid remission of the anemia and muscular weakness (1–4).

Although erythrocyte survival is reduced, abnormal erythropoiesis is accorded the primary role in producing the anemia of vitamin E deficiency (1, 4, 5). Not only is the bone marrow hypercellular as judged from morphological studies (4) and from measurements of DNA and RNA content (6), there are also abnormalities of the nucleated erythroid cells (4). Porter *et al.* (4), found many of the erythroid cells to be multinucleated, and they describe the nuclei of all nucleated erythroid cells of anemic, vitamin E-deficient monkeys as more deeply staining and homogenous than normal. To further define this abnormality of erythropoises, autoradiographic techniques were used in the present study of thymidine, deoxyuridine, uridine, and leucine incorporation into bone marrow cells.

Materials and Methods. Young rhesus monkeys (*Macaca multatta*) were fed a soybean protein-based, vitamin E-deficient diet [see Table I of Ref. (7)] supplemented with 2% of calcium carbonate and 0.1% of ferrous sulfate heptahydrate at the expense of a similar weight of corn starch. This extra supplement of iron is more than enough to prevent the occurence of iron-deficiency anemia, which otherwise may occur in monkeys fed this diet (7). Control monkeys received on their food 80 mg of *dl-alpha*-tocopheryl acetate dissolved in ethanol three times weekly. Control and vitamin E-deficient monkeys were always studied stimultaneously.

To monitor the course of the anemia, blood was obtained at intervals from an ear vein for determination of hemoglobin concentra-

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TABLE I. Hematologic Data on Peripheral Blood.

Monkey no.	Days on diet	Hemoglobin (g/100 ml)	Reticulocyte count (%)	Hematocrit (%)	Comments
7 controls	288–370	13.6	1.1	45	—
277	370	6.5	2.8	23	In relapse 137 days after 0.1 g of α-tocopherol
274	288	6.6	2.2	19	—
	346	3.9	6.6	14	—
281	288	9.5	3.3	31	—
	346	6.1	3.0	22	Subsequently a remission to 0.1 g of α-tocopherol

tion, hematocrit, and reticulocyte count. When the present studies were performed, the deficient monkeys had the typical muscular weakness and anemia (Table I) of vitamin E deficiency, including the previously described multinucleation and other abnormalities of the nuclei of erythroid cells in the bone marrow (4).

Bone marrow samples of approximately 0.5 ml were obtained by tibial aspiration and routine smears on glass cover slips were made immediately. The remainders of the samples were diluted 1:6 with tissue culture medium (TC 199) and mixed by gentle inversion. For incubation, aliquots of the diluted samples were put into 1-ml test tubes containing

TABLE II. Average Grain Counts per Labeled Cell and Percentage of Labeled Bone Marrow Cells from Vitamin E-Supplemented and Vitamin E-Deficient Monkeys after *in Vitro* Incubation (2 hr) with Tritium-Labeled Thymidine, Deoxyuridine, Uridine and Leucine.

	Erythroid cell group[a] ($\bar{X} \pm$ SE[b])		Myeloid cell group ($\bar{X} \pm$ SE)		Undifferentiated cells ($\bar{X} \pm$ SE)	
Precursor	E-supplemented	E-deficient	E-supplemented	E-deficient	E-supplemented	E-deficient
Thymidine						
Grain count	11.8 ± 1.5	29.5 ± 4.3[c]	15.3 ± 1.4	33.2 ± 5.3[c]	31.0 ± 7.7	28.2 ± 9.7
Labeled (%)	28.8 ± 5.9	26.8 ± 2.9	34.7 ± 6.3	32.8 ± 3.3	34.5 ± 5.2	37.4 ± 6.5
Deoxyuridine						
Grain count	23.1 ± 2.3	35.4 ± 3.3[c]	20.3 ± 1.7	35.6 ± 4.8[c]	25.6 ± 3.5	36.8 ± 7.0
Labeled (%)	32.4 ± 8.5	33.5 ± 8.1	36.1 ± 3.1	41.2 ± 3.6	45.2 ± 4.3	44.1 ± 5.4
Uridine						
Grain count	13.5 ± 1.7	23.9 ± 3.2[c]	16.1 ± 2.1	27.4 ± 3.5[c]	23.7 ± 4.9	33.7 ± 6.7
Labeled (%)	35.3 ± 2.9	40.3 ± 3.8	39.3 ± 4.9	46.1 ± 4.4	42.6 ± 4.4	47.0 ± 6.4
Leucine						
Grain count	10.0 ± 1.3	16.3 ± 1.8[c]	11.0 ± 0.8	16.9 ± 2.5[c]	8.3 ± 1.1	22.3 ± 3.9[c]
Labeled (%)	41.7 ± 3.4	36.6 ± 1.6	30.9 ± 4.4	37.1 ± 5.4	42.4 ± 6.1	46.7 ± 7.1

[a] The nucleated erythroid cell group includes blasts and normoblasts and the myeloid cell group includes the blasts, myelocytes, and bands.

[b] The symbols $\bar{X} \pm$ SE are the arithmetic mean ± the standard error of the mean.

[c] Indicates a statistical difference between the E-supplemented and the E-deficient marrow cells with a p value of 0.05 or less. For thymidine-^{3}H and uridine-^{3}H there were 8 samples from 6 vitamin E-supplemented animals taken at the same time 5 bone marrow samples were drawn from anemic vitamin E-deficient animals. The number of samples for deoxyuridine-^{3}H and leucine-^{3}H were: E-supplemented, 5 and 5; E-deficient, 3 and 4, respectively. Three deficient animals were used in this study and where more than 1 sample was taken from the anemic animal, they were taken two months apart.

TABLE III. Average Grain Counts of Labeled Cells and Percentage of Labeled Bone Marrow Cell Types of Vitamin E-Supplemented and Vitamin E-Deficient Monkeys after *in Vitro* Incubation (2 hr) with Tritium-Labeled Thymidine and Uridine.

Cell type:	EB[a] ($\bar{X} \pm$ SE[b])		LBNB ($\bar{X} \pm$ SE)		SBNB ($\bar{X} \pm$ SE)		PNB ($\bar{X} \pm$ SE)		ONB ($\bar{X} \pm$ SE)	
	E-supple.	E-def.	E-supple.	E-def.	E-supple.	E-def.	E-supple.	E-def.	E-supple.	E-def.
Thymidine										
Grain count	24.7 5.4	49.2 6.2[c]	12.7 2.3	24.3 4.2[c]	11.1 1.6	23.8 4.1[c]	5.0 0.7	7.8 1.2[c]	0	0
Labeled (%)	68.9 7.2	87.8 5.2	76.9 7.2	81.4 9.4	64.2 10.0	70.8 9.6	23.7 7.7	10.7 5.4	0	0
Uridine										
Grain count	34.2 2.3	48.9 5.5[c]	14.8 2.2	22.3 3.6[c]	10.5 1.5	21.1 4.0[c]	4.3 0.4	7.9 1.5[c]	2.1 0.4	8.2 1.8[c]
Labeled (%)	93.6 3.9	96.7 2.9	88.5 5.2	78.9 10.1	76.0 7.6	72.5 13.2	41.4 8.1	48.0 12.2	1.9 0.9	2.7 1.1
	MB ($\bar{X} \pm$ SE)		PMC ($\bar{X} \pm$ SE)		MC ($\bar{X} \pm$ SE)		MMC ($\bar{X} \pm$ SE)		BD ($\bar{X} \pm$ SE)	
	E-supple.	E-def.	E-supple.	E-def.	E-supple.	E-def.	E-supple.	E-def.	E-supple.	E-def.
Thymidine										
Grain count	26.8 2.8	61.3 7.6[c]	18.0 1.1	23.8 2.2[c]	11.7 2.5	13.4 3.1	8.4 1.3	13.3 2.2	0	0
Labeled (%)	86.4 6.3	89.2 5.2	71.2 7.5	82.2 9.8	43.2 6.0	31.5 7.5	28.8 8.5	14.7 6.5	0	0
Uridine										
Grain count	40.1 3.0	64.4 8.0[c]	17.9 1.7	24.9 2.3[c]	8.5 1.8	15.2 2.3[c]	7.6 1.3	13.9 2.0[c]	4.5 0.9	7.8 1.5[c]
Labeled (%)	91.9 4.5	96.3 4.0	87.3 4.7	88.1 9.7	60.9 8.3	67.2 9.9	35.2 11.4	55.8 6.7	9.1 4.6	4.5 3.1

[a] Abbreviations: EB = most immature erythroblast; LBNB = large basophilic normoblast; SBNB = small basophilic normoblast; PNB = polychromatic normoblast; ONB = orthochromatic normoblast; MB = most immature myeloblast; PMC = promyelocyte; MC = neutrophilic myelocyte; MMC = neutrophilic metamyelocyte; BD = neutrophilic band and stab.

[b] Arithmetic mean $\pm$ the standard error of the mean.

[c] Indicates a statistical difference between the vitamin E-supplemented and the vitamin E-deficient bone marrow cells with a p value of 0.05 or less. Values for the vitamin E-supplemented marrow cells represent 8 samples taken from 6 monkeys—repeat samples from 2 animals were taken 1 month apart. Values for the vitamin E-deficient marrow cells represent 5 samples taken from 3 monkeys—repeat samples from 2 were taken during the anemic state 2 months apart.

one of the following: thymidine-^{3}H, deoxyuridine-^{3}H, uridine-5-^{3}H, or *l*-leucine-4-5-^{3}H (Nuclear-Chicago, Des Plaines, Ill.): the final concentration was 1 μCi/ml with each compound at a specific activity of 5 Ci/mmole. The incubation temperature was 37° and the gas phase was room air. During the incubation period, samples were occasionally mixed by gentle inversion. After 1 hr and again after 2 hr of incubation, smears were prepared on glass slides. The smears were fixed in absolute methanol for 5 min and in cold trichloroacetic acid solution for 10 min after which they were washed in water before being covered with Kodak NTB3 liquid photographic emulsion (8). The slides covered with emulsion were stored in the cold, 14 days for thymidine-^{3}H and deoxyuridine-^{3}H and 28 days for uridine-^{3}H and leucine-^{3}H. The emulsions were then developed for 2 min at 19° in Kodak D-19, fixed in acidified thiosulfate and stained with Geimsa at pH 5.75 (8). The number of reduced silver grains over each cell (grain count) for at least 1000 marrow cells for each of 4 slides from each sample and for each ^{3}H-labeled compound was recorded. Selected slides were treated with DNase or RNase prior to autoradiographic analysis (9).

Results. With each of the ^{3}H-labeled compounds, the grain count per labeled cell increased between the 1- and 2-hr measurements indicating that either interval was suitable for these studies. For brevity, only the values obtained from the 2-hr interval are given in Tables II and III. In these tables, uptake is expressed both in terms of the percentage of labeled cells and of the grain count per labeled cell. Individual cell types were evaluated separately but with the exceptions to be cited subsequently, erythroid and myeloid cells could conveniently be treated as groups as in Table II. Data for individual cell types are given in Table III.

During the anemia of vitamin E deficiency, there was an increase in the grain count per labeled cell of most but not all cell types (Table III). On the other hand, the percentage of ^{3}H-labeled bone marrow cells was not altered appreciably from control values (Tables II and III).

Not shown in Tables II and III are two exceptions to the general increase in grain counts: (a) leucine-^{3}H labeling of reticulocytes was the same in the vitamin E-deficient as in control bone marrow samples; (b) although approximately 20% of the nucleated erythroid cells were observed to be multinucleated in routine bone marrow smears from the vitamin E-deficient monkeys, most of these cells were absent from the smears prepared for autoradiographic analysis, and none of the remaining multinucleated cells incorporated thymidine-^{3}H.

In complementary studies, pretreatment with DNase removed the ^{3}H-label from the thymidine-^{3}H and deoxyuridine-^{3}H but not from uridine-^{3}H or leucine-^{3}H labeled smears, and RNase treatment removed the ^{3}H-label from the uridine-^{3}H labeled smears but not from the thymidine-^{3}H, deoxyuridine-^{3}H, or leucine-^{3}H labeled smears. Also, washing methanol-fixed, ^{3}H-labeled smears with cold trichloroacetic acid for 1 min resulted in some reduction in grain count, but further treatment with cold trichloroacetic acid produced no greater reduction. These findings are evidence that thymidine-^{3}H and deoxyuridine-^{3}H were incorporated into DNA, that uridine-^{3}H was incorporated into RNA and that leucine-^{3}H was incorporated into protein.

Discussion. The general increase in grain counts of the labeled cells in vitamin E-deficient bone marrow could be due either to increased metabolic activity or to less dilution of the ^{3}H-labeled precursors by small intracellular pools of the compounds. In the case of thymidine-^{3}H, though, the latter explanation may not be applicable, for the intracellular concentration of thymidine normally is small in bone marrow cells (10) and would not be expected to greatly alter the labeling pattern from thymidine-^{3}H. For the other ^{3}H-compounds, our autoradiographic data alone do not permit a choice between the two explanations. However, by considering these new data in the light of earlier studies of bone marrow from vitamin E-deficient monkeys, an argument can be made for increased metabolic activity.

The early studies demonstrated the bone marrow of vitamin E-deficient monkeys to be

hypercellular (4, 6) with an increase in the number of nucleated erythroid cells (4), but it was not possible to decide if the increased number of erythroid cells was due to active proliferation or to prolonged residence in the bone marrow. Finding increased specific activity of bone marrow DNA after an injection of formate-^{14}C (11) failed to resolve the issue for the following reason: There was already evidence of increased granulocytopoiesis in vitamin E-deficient monkeys (1). Thus increased labeling of DNA in myeloid cells would be expected, as was confirmed in the present studies, and possibly could have explained the increased specific activity of bone marrow DNA. This explanation of the data from formate-^{14}C studies may now be discarded since the present work shows that increased labeling of DNA by thymidine-^{3}H and deoxyuridine-^{3}H involves erythroid as well as myeloid cells. Therefore, it is concluded that the increased number of erythroid cells in bone marrow of vitamin E-deficient monkeys is due, at least in part, to active proliferation. In turn it is reasonable to suppose that the proliferating cells have increased metabolic activity and hence increased grain counts from ^{3}H-precursors of nucleic acids and proteins. Furthermore, the increased incorporation of formate-^{14}C, deoxyuridine-^{3}H, and thymidine-^{3}H suggest no alteration of *de novo* DNA synthesis in the labeled bone marrow cells.

If there is active erythropoiesis, why does not the bone marrow compensate for the acceleration of erythroid destruction in vitamin E-deficient monkeys? While an answer to this question at the molecular level is not provided by any study performed so far, it is possible to deduce that many of the extra erythroid cells (presumably including the fragile, metabolically slow multinucleated cells) die in the bone marrow of vitamin E-deficient monkeys. The cause of this premature death merits further study.

Recognition that there is premature death of erythroid cells in the bone marrow, allows an explanation of the shortened half-time of erythrocyte survival in vitamin E-deficient monkeys. With failure of the bone marrow to produce erythrocytes, the average age of circulating erythrocytes would increase. As the erythrocyte population ages, the half time of erythrocyte survival would get progressively shorter and the anemia progressively worse. Thus the intrinsic defect in the erythrocyte of vitamin E-deficient monkeys, which was observed in cross transfusion experiments (12) could be due to aging as well as to production of a defective erythrocyte.

Summary. The incorporation of ^{3}H-labeled thymidine, deoxyuridine, uridine, and *l*-leucine into bone marrow cells of vitamin E-deficient and control monkeys was measured in autoradiographic studies. During the anemic of vitamin E-deficiency there was an increase in the grain count per labeled cell of most cell types in the erythroid and myeloid series, while the percentage of ^{3}H-labeled cells was not altered from control values. Multinucleated erythroid cells in the bone marrow of vitamin E-deficient monkeys were not labeled by thymidine-^{3}H. These findings are evidence that active erythropoiesis associated with premature death of erythroid cells occurs in the hypercellular bone marrow of anemic vitamin E- deficient monkeys.

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