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Effects of Hypertransfusion and Erythropoietin on Labeling of Rat Megakaryocytes by Tritiated Thymidine.* (29424)

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Ninety to one hundred percent of all rat megakaryocytes became labeled during the 48- to 96-hour period after a single intravenous injection of tritiated thymidine (H^3TdR)(1). From this, Feinendegen *et al* (1) concluded that "recognizable megacaryocytic elements originate from unrecognized precursors which continuously synthesize DNA for a period of at least 1 to 3 days prior to maturation into recognizable megacaryocytic precursors." Their conclusion was based on the assumption that availability time of H^3TdR is short.

Initially it was considered that H^3 was available for desoxyribonucleic acid (DNA) labeling for only a few minutes after intravenous injection of H^3TdR because of the rapid plasma clearance of H^3TdR(2,3). Recent studies, however, point to the fact that the H^3 may be available much longer(4-9) either from reutilization or continued availability of H^3, possibly as thymidine itself (9). Thus, an alternative explanation for the megakaryocyte labeling curve might be delayed incorporation of H^3 either directly into megakaryocytes or their precursors.

Reutilization of DNA or its breakdown products has been considered to be minimal because of dilution(3) and rapid degradation by the liver(10). Thus, it has not been thought to influence importantly studies of marrow cell kinetics with H^3TdR. However, extruded nuclei of erythroid cells provide a substantial source of DNA available for local reutilization(11). To evaluate this possibility we studied H^3TdR labeling of rat megakaryocytes after suppression of erythropoiesis by hypertransfusion or stimulation by erythropoietin.

Materials and methods. Female Sprague-Dawley rats, weighing 148-197 g and having free access to Purina rat chow and water, were injected intravenously with H^3TdR,‡ 1 μc/g body weight at time 0. They were serially sacrificed with ether at 30 min, 12 hr, 24 hr and daily thereafter. Bone marrow smears prepared from a split femur were fixed in absolute methyl alcohol. After dipping in Kodak NTB-3 emulsion, the slides were stored for 30 days at —20°C. After developing, they were stained with Giemsa or Wright's and Giemsa stain. For each rat 250 megakaryocytes were classified into 3 groups using the criteria of Feinendegen *et al*(1). These cells were also scored for nuclear grain count, and correction was made for background. Platelets were counted by the method of Brecher and Cronkite(12) from cardiac blood drawn into versene just prior to sacrifice.

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‡ Schwarz Bioresearch, Inc., 1.9 c/mmole, diluted with sterile saline to 500 μc/ml.

TABLE I. Megakaryocyte Differentials and Platelet Counts.

Treatment group	% of megakaryocytes, avg and (range)			Platelet count ($\times 10^{-6}$), avg and (range)
	I	II	III	
Normal	21 (15–33)	26 (19–31)	53 (44–63)	1.178 (.900–1.550)
Hypertransfused	19 (8–35)	28 (16–36)	53 (28–71)	.931 (.600–1.350)
Erythropoietin	18 (6–30)	26 (18–37)	56 (40–74)	1.499 (1.0675–1.7925)

Three experiments were done; in the first, hypertransfused rats were compared with normals; in the others, hypertransfused, erythropoietin-treated and normal rats were compared. Nineteen normal, 15 hypertransfused and 11 erythropoietin-treated rats were used.

Hypertransfusion: 15 rats were transfused intravenously with homologous red cells on the 6th and 7th days prior to injection of H^3TdR. A volume of red cells equivalent to 1.75% of the body weight was given to each. In the first experiment (4 rats) red cells were prepared by a single rapid centrifugation of whole blood, and they were estimated to contain over 1,000,000 platelets/mm^3. In the other 2 experiments (11 rats) the number of platelets was reduced to 125,000-287,000/mm^3 by differential centrifugation.

Erythropoietin: In the first experiment 5 rats were given subcutaneously $\sim$ 35 units (standard A)(13) of erythropoietin on each of 3 days prior to H^3TdR. In the second experiment (6 rats) a similar dosage schedule was followed prior to H^3TdR infusion, but, in addition, erythropoietin administration was continued daily until sacrifice. The erythropoietin was prepared by alcoholic extraction of urine from a patient with congenital hypoplastic anemia.

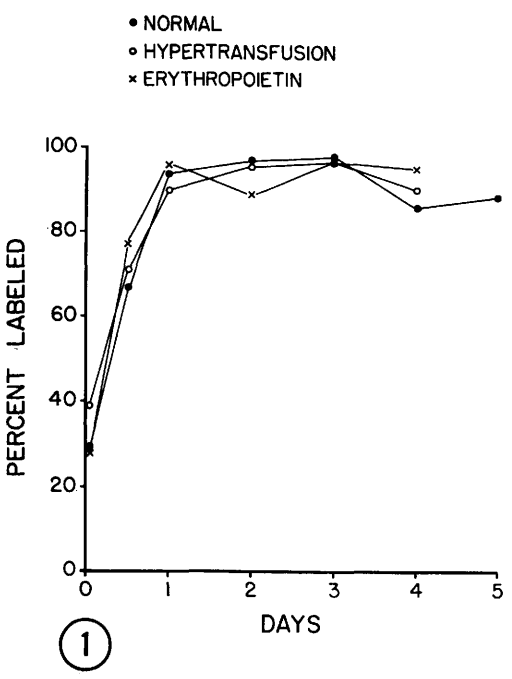
Results. Rat megakaryocytes could be divided into 3 morphologic compartments representing successive stages of maturation. By and large, only the most immature (Group I) megakaryocytes were in active DNA synthesis, 20-30% being labeled within 30 minutes after injection of H^3TdR. In the other groups there was an occasional lightly labeled cell with 2-10 grains above the estimated background in contrast to 100-200 grains in Group I. Rarely, a single heavily labeled lobe was seen initially in more mature megakaryocytes. The labeling index in Group I increased from 20-30% at 30 minutes to nearly 100% at 24-36 hours where it was maintained for 3 days. As would be anticipated, the subsequent labeling indices in Groups II and III megakaryocytes mirrored that in Group I with a minimum transit time from initially labeled to unlabeled compartments of about 8 hours.

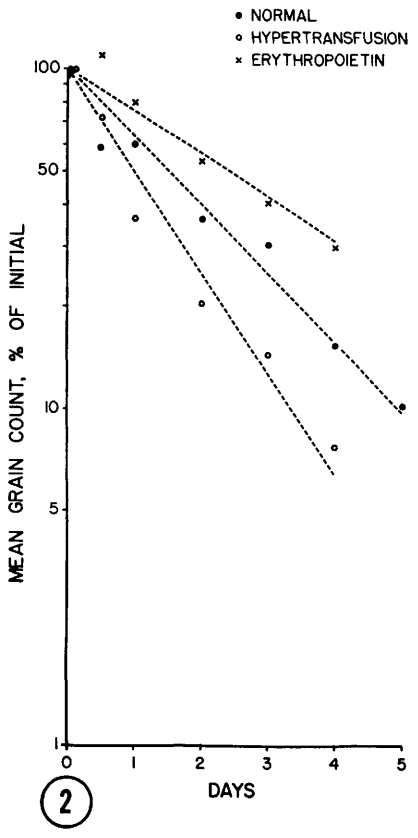
In the hypertransfused rats, hematocrits were 62.8% $\pm$ 0.6, and erythroid activity in the marrow was markedly diminished; this was reflected in reticulocyte values of 0-0.2% in the peripheral blood. In contrast, erythropoietin-treated rats had striking red cell hyperplasia. After 3 injections of erythropoietin, reticulocyte values were 9-10% with hematocrits of 46.5 $\pm$ 1.6; with continued injections reticulocyte values were as high as 17% with hematocrits of 52.3% $\pm$ 1.6. Macrocytosis was demonstrable in erythropoietin-treated rats. Control rats had reticulocyte counts of 2.5-3.8% and hematocrits of 43.4 $\pm$ 0.5.

The megakaryocyte differential was similar in all 3 groups (Table I). The platelet number, however, was significantly increased in the erythropoietin-treated group and decreased in the transfused rats ($p<0.01$) (Table I).

The labeling indices were similar in normal, hypertransfused and erythropoietin-treated rats (Fig. 1). Thirty minutes after injection of H^3TdR, 28-39% of Group I megakaryocytes were labeled; the number of labeled cells increased to 90-98% at 24 hours and remained near this range for 3 days.

The mean grain counts of labeled Group I





megakaryocytes for each treatment group were plotted separately on semilogarithmic paper in each of the 3 experiments. For comparison, each curve was extrapolated to zero time and the intercept (100-200 grains/cell) was called 100%; each value was then expressed as a percentage of this. The mean grain count halving time (T/2) in normal rats was 1.5 days, in hypertransfused rats 1.0 day, and in erythropoietin-treated rats 2.4 days (Fig. 2). The results were similar in animals hypertransfused with platelet-poor or platelet-rich red cells and in rats which received only 3 erythropoietin injections as compared to those in which injections were continued to sacrifice.

Discussion. The development of nearly 100% labeling in the earliest recognizable megakaryocytes within 24 hours after a single intravenous injection of H^3TdR suggests either continuous availability of the H^3 label or, more likely, maturation of precursor cells with a DNA synthesis period of longer duration than the time spent in the Group I megakaryocyte compartment. The latter was estimated as 6-7 hours by Feinendegen *et al* (1) and 8 hours in the present studies. A DNA synthesis period for precursor cells of as long as 3 days(1), however, seems improbable in view of observations with other cell systems. Alteration of this phase of megakaryocyte labeling by transfusion or erythropoietin would not be expected. The estimate of maximum generation time for precursor cells was 1.5 days in normal rats (T/2 of Group I megakaryocytes) indicating an average DNA synthesis period of less than 1.5 days.

The persistence of nearly 100% labeling for 3 days may be due to reutilization of H^3-labeled products of DNA as these become available in the bone marrow from ineffective myelopoiesis, erythropoiesis or megakaryo-

FIG. 1. Labeling indices of Group I megakaryocytes after injection of H^3TdR in normal (●), hypertransfused (○) and erythropoietin-treated (×) rats. Each point is the average of 2 or 3 animals except the day 4, erythropoietin value which represents only one rat.

FIG. 2. Mean grain count, expressed as % of initial value, for labeled group I megakaryocytes after injection of H^3TdR in normal (●), hypertransfused (○) and erythropoietin-treated (×) rats.

cyte nuclei. The T/2's of the Group I megakaryocytes were different in the 3 groups of rats, suggesting that hypertransfusion or erythropoietin changed the amount of H^3 label available for delayed incorporation into DNA. The more rapid disappearance of label in hypertransfused rats and its prolonged presence in erythropoietin-treated animals implicate the red cell nucleus as a probable source of reusable DNA components. Failure to alter the percent label curves in the present studies presumably reflects the inability to influence sources of H^3-DNA other than the erythroid cells.

The decrease in platelet count in hypertransfused rats may have been due to dilution, sequestration, thrombocytolysis or inhibition of platelet production. It is not possible with the data at hand to decide among these alternatives, but dilution and/or sequestration appear most likely. The estimated increase in blood volume of $\sim$ 25% after transfusion paralleled the decrease in platelet count. In addition, the increased viscosity may have led to some sequestration. The higher platelet counts in the erythropoietin-treated animals are not readily explained on mechanical grounds. A true increase in production is suggested. Such increases have not been reported with erythropoietin derived from subjects with normal platelet counts. The erythropoietin used in these experiments, however, was derived from a patient with thrombocytopenia as well as anemia, and it may be that a "thrombopoietin" contaminated the preparation. Changes in production rate due to hypertransfusion or erythropoietin, however, could not explain the observed changes in T/2. If, for example, erythropoietin-treated animals had an increase in production and hence an increased turnover rate of megakaryocytes, then a shortening rather than the observed prolongation of T/2 would be anticipated. Conversely, hypertransfusion and suppression would have lengthened the T/2. The observed alterations in T/2, therefore, are considered to reflect the marked differences in availability of H^3-DNA from red cell nuclei for re-labeling Group I megakaryocytes or their precursors.

Summary. After a single intravenous dose of H^3TdR, there was immediate labeling of about one-third of the immature megakaryocytes in rats. The labeling index increased to 90-98% in 24 hours and remained high for 3 days. This pattern was not altered by hypertransfusion or erythropoietin. The mean grain count halving time of these cells, however, was increased in erythropoietin-treated rats and decreased in hypertransfused rats. These results are interpreted as indicating reutilization of erythroid DNA or its breakdown products by megakaryocytes or their precursors.

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