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Received April 4, 1966. P.S.E.B.M., 1966, v122.

Megakaryocytopoiesis in the Rat with Transfusion-Induced Thrombocytosis.* (31323)

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Tritiated thymidine (^{3}THdR) has been used to study maturation of megakaryocytes in rat bone marrow(1,2). These observations showed that the compartment of recognizable megakaryocytes could be morphologically divided into 3 stages of maturity. It was also clear from evaluation of grain counts that megakaryocytes were not self-maintaining, but were maintained by continuous influx from an unrecognized precursor compartment.

The level of circulating blood platelets is determined by a balance between rate of production from megakaryocytes and rate of destruction. The possibility that this balance is governed by a negative feed-back mechanism, affecting influx from a precursor compartment or the maturation of megakaryocytes was evaluated by observing labeling of megakaryocytes with ^{3}HTdR and differential counts of megakaryocytes in rats with transfusion-induced thrombocytosis.

Materials and methods. Female Sprague-Dawley rats weighing 148-182 g were used as test animals. Larger rats of the same strain served as blood donors for platelet transfusions. Platelet counts were done on cardiac blood, anticoagulated with K_2EDTA, by the method of Brecher and Cronkite(3).

Platelet transfusions were prepared by a modification of the method of Dillard *et al* (4). Cardiac blood was drawn into 0.05 volume of EDTA (1.5% Na_2EDTA and 0.7%

* This investigation was supported by a USPHS fellowship (Dr. Ebbe) (5-F3-AM-8634) from Nat. Inst. of Arthritis & Metab. Dis. and by reasearch grants from Nat. Inst. of Arthritis and Metab. Dis. (5 R01 AM 08263) and Nat. Heart Inst. (HE 07542-03).

NaCl) and pooled in plastic bags (Fenwal Laboratories). It was centrifuged (Model PR-2, International Equipment Co.) at 800 rpm (150 *g*) for 25 minutes, then the plasma was centrifuged at 3000 rpm (2000 *g*) for 25 minutes. The platelet button was resuspended in a small amount of plasma for intravenous infusion. For transfusions calculated to triple the recipient's platelet count, the concentrates had 10-63.4 $\times$ 10^6 platelets/mm^3, and the volumes transfused were 0.3-1.9 ml. The concentrates used to increase the platelet count 6-fold had 44.5-60 $\times$ 10^6 platelets/mm^3, and 1.6-2.0 ml were infused.

Differential counts of megakaryocytes were performed on bone marrow smears stained with Wright's and Giemsa stain. Cells were classified as stages I, II and III as previously described(2). Stage I was larger than myeloid or erythroid cells, had clear basophilic cytoplasm and a high nuclear:cytoplasmic ratio. Stage II had foamy-appearing basophilic cytoplasm with or without localized areas of azurophilic granulation and a lower nuclear:cytoplasmic ratio. Stage III had a low nuclear:cytoplasmic ratio, and the cytoplasm was either basophilic or neutrophilic with azurophilic granules throughout. There was considerable variation in size of cells and in nuclear configuration in each stage. The degree of nuclear segmentation was not a consideration for classification, but, in general, maturation from stage I to III was associated with increasingly prominent segmentation of nuclei. After the large platelet transfusions, 500 megakaryocytes were classified, 250 by each of 2 observers. After the smaller transfusions, 250 cells were counted.

For the studies with ^{3}HTdR, each rat was injected intravenously with 1 microcurie of ^{3}HTdR (Schwarz BioResearch, Inc.), per gram body weight. Autoradiographs of femoral marrow were prepared and background grain counts were estimated as previously described(2). Nuclear grain counts over 250 megakaryocytes were determined for each rat. Platelet transfusions were given before or after the ^{3}HTdR as indicated in the results. Control, non-transfused rats were studied simultaneously with the recipients of platelet transfusions.

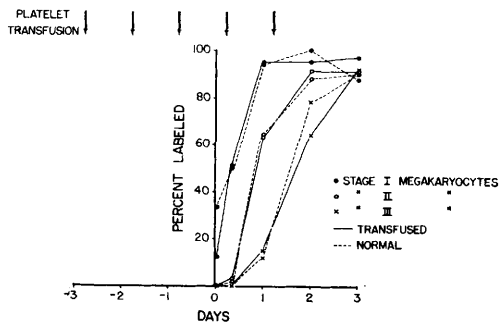

FIG. 1. Labeling of megakaryocytes by ^{3}HTdR in rats with sustained, transfusion-induced thrombocytosis and normal controls. ^{3}HTdR was injected at time zero.

Results. Labeling with tritiated thymidine. Several preliminary observations indicated that acute elevation of the platelet count to about 2.5 times normal did not alter the pattern of labeling of megakaryocytes with ^{3}HTdR. Therefore, studies were done on animals with sustained thrombocytosis and animals in which an acute thrombocytosis of greater than five-fold normal was achieved.

In the first group of rats, platelet transfusions were given daily for 3 days before administration of ^{3}HTdR, and thrombocytosis was maintained with 2 subsequent transfusions (Fig. 1). Each transfusion contained about twice the normal number of circulating platelets, based on a blood volume of 6% of body weight and a platelet count of 1.1 $\times$ 10^6/mm^3. Platelet counts for the first 2 days of the labeling period were at least 3 times normal (Table I). The labeling index of stage I megakaryocytes was initially somewhat lower in transfused rats than the control, but in the range previously observed in normal animals. Thereafter, labeling indices of all megakaryocytes in transfused rats were similar to those of controls (Fig. 1).

In the studies of acute thrombocytosis, ^{3}HTdR was given 30-45 minutes before platelet transfusions to ensure normal labeling of precursor cells and stage I megakaryocytes. The effect of acute thrombocytosis ($\sim$six-fold normal) on the rate of transit of labeled cells through the early megakaryocyte compartments was observed. As can be seen in Table

TABLE I. Megakaryocyte Differentials with Sustained Thrombocytosis.

Days after 1st transfusion	No. of rats	Platelets/mm³ (× 10⁻⁶)	Megakaryocyte differential, % I	II	III
3	2	3.315–3.52	18.8–19.6	27.6–34.0	46.4–53.6
4	1	3.995	9.2	24.0	66.8
5	1	3.87	9.2	32.0	58.8
6	1	2.1	14.4	30.4	55.2
Control	5	1.116 (1.05–1.2)	18.2 (14.4–24.6)	34.5 (24.8–40.4)	47.3 (42.0–60.8)

II, labeling indices in thrombocytotic rats paralleled those of normal controls. Because of the variation seen in groups of normal rats(2), differences between some individual values and their controls cannot be considered significant.

TABLE II. Labeling of Megakaryocytes by ³HTdR in Acute Thrombocytosis.

Treatment	Hours after ³HTdR	Platelets /mm³ (× 10⁻⁶)	Megakaryocyte labeling, % I	II	III
		GROUP 1			
Control	.5	1.05	14.2	0	0
Transfused	8	7.215	48.0	8.3	.7
Control	8	1.13	38.2	1.2	0
Transfused	24	5.75	92.3	48.0	6.0
Control	24	1.11	82.9	57.0	7.7
		GROUP 2			
Control	.75	1.275	19.2	.6	0
Transfused	8	6.63	41.4	9.5	.9
Control	8	1.075	72.5	6.0	0
Transfused	24	5.7	92.3	65.1	9.7
Control	24	1.04	100.0	56.4	10.4

In all of these studies, mean grain counts for megakaryocytes in transfused rats were of the same order of magnitude as those in corresponding controls.

Megakaryocyte differentials. For 3 days after a single transfusion which raised the platelet count to about 2.5 times normal, the differential counts of megakaryocytes of the 3 stages of maturity were the same as in normal rats (Table III).

When thrombocytosis was sustained with 5 successive platelet transfusions, the differential counts were normal on the 3rd and 6th days after the initial tranfusion; on the 4th and 5th days, the percentage of stage I was somewhat less than normal (Table I).

In the presence of acute, more severe thrombocytosis, minor alterations in the differential count of megakaryocytes were noted (Table IV). Because of differences among control groups, transfused rats can be compared only with controls drawn from the same population.

In previous studies(2) megakaryocyte differential counts were comparable in 5 different groups of female Sprague-Dawley rats, and similar values were seen in 3 groups of control rats in the present observations (Tables I, III and IV, group 1). In the other 3 groups of control rats (Table IV, groups 2, 3, and 4) there was a shift to the right of megakaryocyte differentials. It appeared that this was due to biological variations, since technical factors could be ruled out, and the 2 observers got similar values. From labeling indices of the group with the most divergent values (group 2, Tables II and IV) it was estimated that transit times of stages I and II were about the same as those with the more usual differential count (Fig. 1, Table II, and ref. 2). This would indicate that total maturation time for mega-

TABLE III. Megakaryocyte Differentials with Acute Mild Thrombocytosis.

Time after transfusion	No. of rats	Platelets/mm³ (× 10⁻⁶) ± S.E.	Megakaryocyte differential, % ± S.E. I	II	III
3 hr	6	2.508 ± .101	16.3 ± 2.4	28.0 ± 1.3	55.7 ± 3.0
1 day	8	2.133 ± .168	14.8 ± 1.8	38.6 ± 2.0	46.6 ± 2.3
2 days	6	2.053 ± .226	21.4 ± 3.7	31.8 ± 1.5	46.8 ± 3.4
3 "	6	1.602 ± .214	16.0 ± 3.6	32.5 ± 1.6	51.4 ± 4.5
Control	31	1.099 ± .021	16.8 ± 1.0	34.8 ± .9	48.4 ± 1.5

TABLE IV. Megakaryocyte Differentials with Acute Marked Thrombocytosis.

Hours after transfusion	No. of rats	Platelets/mm³ ($\times 10^{-6}$)	Megakaryocyte differential, %		
			I	II	III
		GROUP 1			
8	1	7.215	12.0	34.2	53.8
24	1	5.75	19.2	37.6	43.2
Control	4	.98–1.13	17.8–23.0	32.6–38.6	41.4–48.8
		GROUP 2			
8	1	6.63	7.6	22.8	69.6
24	1	5.7	6.4	33.0	60.6
Control	4	1.04–1.275	3.4–9.2	18.8–29.6	62.0–77.8
		GROUP 3			
24	2	6.94–7.22	7.6– 8.2	27.0–31.0	61.4–64.8
Control	2	.97–1.365	10.8–11.6	21.6–26.4	62.0–67.6
		GROUP 4			
48	2	2.49–2.725	8.2–13.2	23.6–25.2	63.2–66.6
Control	2	1.09–1.295	6.4–13.4	20.2–30.6	56.0–73.4

karyocytes was longer in those rats with a smaller proportion of stage I cells.

Discussion. The present findings in normal rats confirmed the previous observations by Feinendegen *et al*(1) and Ebbe and Stohlman (2) of the labeling pattern of megakaryocytes from a single injection of ^{3}HTdR. In the animals with sustained thrombocytosis, this pattern was unchanged indicating that the rate of maturation of recognizable megakaryocytes and the rate of influx from the immediate precursor compartment were normal. Absolute numbers of megakaryocytes were not determined, so it was possible that a new steady state had been established with smaller numbers of megakaryocytes moving through the compartment at a normal rate.

In previous studies(2), transit times for the morphologic stages of megakaryocytes were estimated as 8-14 hours for stage I, 11-19 hours for stage II, and 24-42 hours for stage III with a total megakaryocyte maturation time of 43-75 hours. If there were a decrease in the influx of cells from the precursor compartment in response to thrombocytosis, there should have been a substantial change in differential counts of megakaryocytes during the subsequent 43-75 hours as the population of pre-existing megakaryocytes matured at a normal rate. A shift toward the mature, stage III cells would have resulted. A similar change would be seen in those rats in which the control values for megakaryocyte differentials were shifted to the right, since stages I and II appeared to mature at about the same rate noted above.

Such changes were not demonstrable in rats with platelet counts of either 2.5 or 6 times normal. The differences noted in a few rats in Table IV could not be reproduced in the other animals. Moreover, there was no pattern of successive depletion of megakaryocytes of increasing maturity as would be expected if influx from the precursor compartment had been diminished in response to thrombocytosis.

In animals in which thrombocytosis was maintained for up to 6 days there was a shift to the right on the 4th and 5th day, but this was not evident on the 6th day at a time when the platelet count was still elevated. The latter might be interpreted as reflecting a new steady state. We are more inclined to feel, however, that these deflections from the control values represent normal variation. Even in the event that this is not the case, the magnitude of decrease in influx must have been small. It was concluded that decreased influx of precursor cells in a chronically thrombocytotic animal does not represent an important homeostatic mechanism.

In studies on the labeling of stage I megakaryocytes during the first 24 hours after increasing the platelet count 6-fold, it was evident that influx from the precursor compartment continued. In the presence of normal maturation of megakaryocytes, the label-

ing index of stage I megakaryocytes increased during the first 24 hours (Table II). This increase could only result from influx of labeled cells.

From grain count data it seemed likely that in transfused rats or in normal rats division of megakaryocytes was at most a rare event.

Late thrombocytopenia after transfusion-induced thrombocytosis was observed in rats by Cronkite(5), and he postulated a negative feed-back mechanism from circulating platelets to megakaryocytes. Subsequent observations(6) that megakaryocyte numbers in spleen and marrow were not changed by transfusion-induced thrombocytosis led to the hypothesis that the feed-back may act by prevention of megakaryocyte degeneration and an orderly flow of cells through the megakaryocyte compartment. The results of the present studies do not support such an hypothesis. In these respects our findings are in agreement with those of Odell *et al*(7) that platelet production, as measured with S^{35}-sulfate, was not apparently changed by transfusion-induced thrombocytosis.

In our studies, emphasis was placed on cytoplasmic maturation of megakaryocytes, as the major distinguishing features of the 3 stages related to basophilia and azurophilic granulation and to a lesser extent the relative amount of cytoplasm per megakaryocyte. Odell *et al*(8) measured the DNA content of rat megakaryocytes microspectrophotometrically and found amounts which could be ordered in a 2^xN type sequence varying from 4N to 64N. In view of the failure of megakaryocytes of morphologic stages II and III to label immediately with ^{3}HTdR(1,2), they proposed that "nuclei of megakaryocytes become polyploid independent of and prior to cytoplasmic maturation." It is conceivable that a negative feed-back may act during the period of DNA replication rather than the phase of cytoplasmic maturation, and that platelet production may be changed by alteration of the number of platelets produced by each megakaryocyte rather than by alteration of maturation rate or total number of megakaryocytes.

Summary. The effect of transfusion-induced thrombocytosis on megakaryocytopoiesis in the rat was evaluated from (1) labeling of megakaryocytes after a single dose of ^{3}HTdR and (2) differential count of megakaryocytes in the bone marrow. Neither parameter was significantly altered by acute or sustained thrombocytosis. It was concluded that thrombocytosis affected neither rate of maturation of recognizable megakaryocytes nor influx of cells into the megakaryocyte compartment from a precursor compartment. These findings do not exclude the possibility that the number of platelets produced by each megakaryocyte could be altered by hypertransfusion.

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Received April 4, 1966. P.S.E.B.M., 1966, v122.