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Research Article

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## Regulation of Erythropoiesis. XVIII. The Effect of Vincristine and Erythropoietin on Bone Marrow \*

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The *Vinca* alkaloids, vinblastine and vincristine, have been reported to have relatively little effect in some species on the peripheral platelet level as compared with their effect on myeloid and erythroid elements. Boggs and his associates (1) observed marked suppression of erythropoiesis and myelopoiesis in dogs receiving vinblastine, but the level of platelets and megakaryocytes, although not quantified, did not appear to be importantly changed. One explanation of this dichotomy might be that the megakaryocyte is a nondividing cell and as such is not susceptible to the influence of antimitotic agents. If this is the case, then it would appear that the megakaryocytic precursor cells are also little affected. On the basis of morphologic and kinetic observations, the megakaryocytic compartment in the rat has been divided into three stages. The earlier, or stage 1, megakaryocytes have a turnover time of 8 to 14 hours (2). To maintain the stage 1 megakaryocyte in the vincristine-treated animal would require a continual input from a relatively undamaged precursor compartment. If the stem cell is pluripotent, it would follow that the erythroid and myeloid damage is mostly the result of an effect on differentiated cells. Alternatively, there may be a different immediate precursor for the differentiated hematopoietic elements, as we have suggested (3). In an attempt to distinguish between the alternatives, we determined the effect of erythropoietin in the hypertransfused vincristine-treated rat. Hypertransfusion was used to suppress the differentiation of erythroid elements so that the effect of vincristine on precursor cells could be evaluated. The

results to be reported herein appear to favor different immediate precursors and a more primitive pluripotent precursor cell.

### Methods

Adult female Sprague-Dawley rats weighing 150 to 170 g were given vincristine in a single intravenous dose of 0.3 mg per kg. This dose is just below the lethal range in this colony; 0.4 mg per kg resulted in a 90% mortality within 10 days. Rats, when transfused, were given washed, packed homologous red cells by tail vein injection 5 days before the start of the experiment. They received red cells in an amount equivalent to 3.5% of the body weight. One hundred units of human urinary erythropoietin, prepared by alcohol fractionation (4), was given by subcutaneous injection. Blood samples were collected by cardiac puncture from anesthetized animals into Versenate. Peripheral blood values were measured by standard technics. Leukocyte differential counts were based on the evaluation of 200 cells in peripheral smears. Bone marrow smears were made from the split femur by a brush technic. The percentage of erythroid precursors was estimated from differential counts on 500 to 1,000 nucleated cells. The mitotic index was measured in these same preparations. Megakaryocytic differentials were based on evaluation of 100 megakaryocytes. Blood volume was measured from the dilution of intravenously administered  $^{51}\text{Cr}$ -labeled washed homologous red cells. Blood samples for red cell  $^{59}\text{Fe}$  incorporation were collected 20 hours after intravenous administration of plasma-bound  $^{59}\text{Fe}$ . They were lysed with saponin and counted in a well type scintillation counter. The total iron incorporation was based on direct measurement of the blood volume.

### Results

After the administration of vincristine to normal animals, the marrow smears revealed an accumulation of metaphase forms that approximated linearity for the first 4 hours. Extrapolation of this curve to 100% provided an estimate of ~11 hours for the generation time of rat erythroid precursors. By 8 hours, 67% of erythroid precursors potentially capable of dividing and 23% of granulocytic precursors potentially capable of dividing were in metaphase. No postmetaphase forms were

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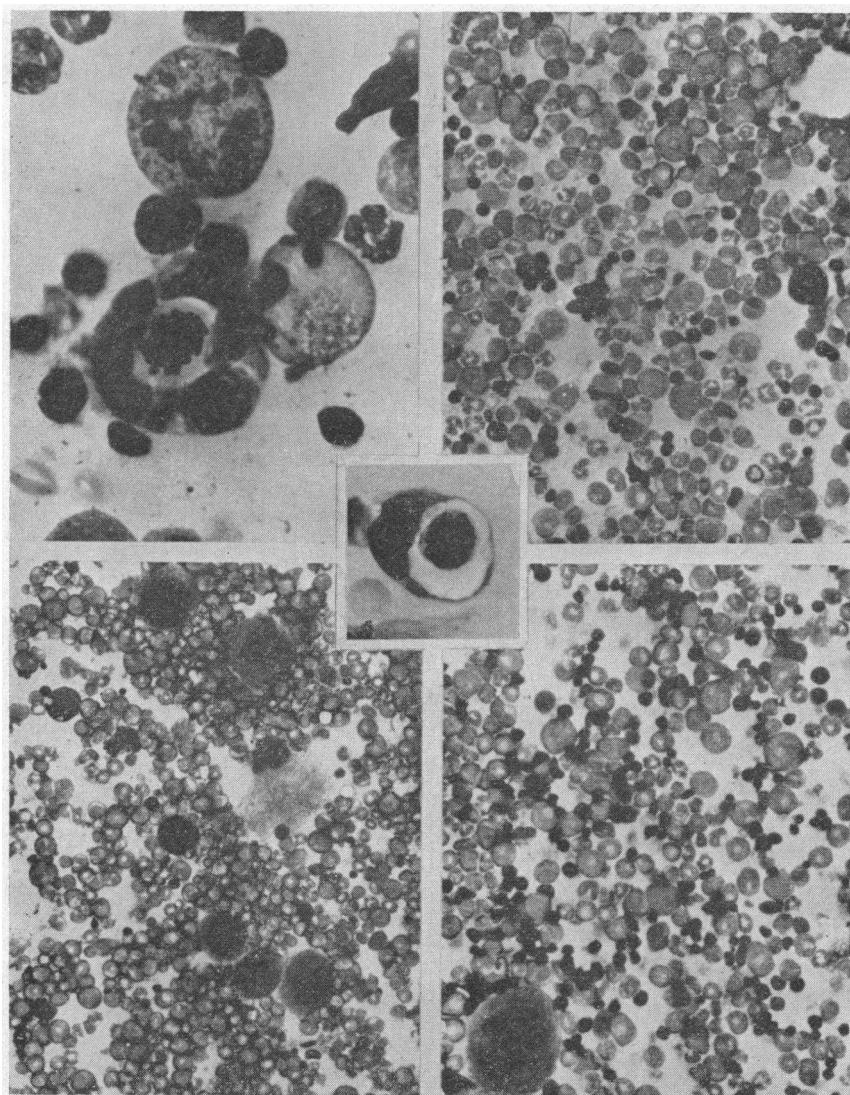


FIG. 1. PHOTOMICROGRAPHS OF BONE MARROW OF VINCRISTINE-TREATED AND CONTROL ANIMALS. Upper left: Marrow from vincristine-treated animals showing abnormal metaphase, karyorrhexis, and phagocytosis. Center: Phagocytosed normoblast in metaphase. The clear zone was characteristic of many such cells. Lower left: Marrow from animal 48 hours after vincristine showing megakaryocytes in all stages and myeloid and lymphoid elements but virtually no erythroid cells. Upper right: Erythroid response in marrow obtained 5 days after administration of erythropoietin to vincristine-treated hypertransfused animals. Lower right: Erythroid response of marrow of hypertransfused control animal 48 hours after receiving erythropoietin.

observed in erythroid cells, whereas such forms were continually noted in myeloid cells. Mitotic figures were not observed among the remaining erythroid precursors at 24 hours. Granulocytic precursors, in contrast, had a mitotic index of 9% at 24 hours. This decreased to 3% after 48 hours.

The morphologic configuration of erythroid and myeloid mitoses was characterized by a disordered

arrangement of chromosomes on the metaphase plate. Chromosomes were frequently blunted, and some were seen loosely within the cell or joined to the main nuclear mass by a narrow bridge. Star, exploded, and imploded metaphases were seen. Karyorrhexis and karyolysis were evident both in arrested and mature cells. Phagocytosis was striking between 2 and 18 hours after vincristine ad-

TABLE I  
*Megakaryocytic differentials in vincristine-treated normal animals*

Treatment	Time*	Animal	Megakaryocyte†		
			Stage I	Stage II	Stage III
	<i>hours</i>		%	%	%
Control		1	16	29	55
		2	20	33	47
		3	22	26	52
Vincristine	17	1	13	36	51
		2	13	21	66
		3	18	27	55

\* Time refers to hours after administration of 0.3 mg per kg of vincristine.

† Stages of maturation of megakaryocytes are as described by Ebbe and Stohlman (2). The values are based on the differential count of 100 megakaryocytes.

ministration. The phagocytes were large mononuclear cells some of which contained as many as 10 ingested cells. Erythroid, myeloid, and lymphocytic elements were undergoing phagocytosis; some of the phagocytosed cells were in metaphase (Figure 1).

There was a striking depopulation of differentiated marrow erythroid elements between the second and fourth days. Infrequent late normoblasts were the only erythroid elements seen. In contrast, all stages of megakaryocytic maturation were present. Absolute numbers of megakaryocytes cannot be accurately quantified, but scanning of the preparation indicated the number of megakaryocytes to be relatively unaffected (Figure 1). Moreover, the effect of vincristine on the individual megakaryocytic compartments appeared to be limited. There was relatively little change in the megakaryocytic differential (Tables I and II). Myelopoiesis was severely depressed but did not

TABLE II  
*Megakaryocytic differentials in vincristine-treated hypertransfused animals*

		Megakaryocyte†		
Treatment	Time*	Stage I	Stage II	Stage III
	days	%	%	%
Control		18.5	25	56.5
Vincristine	1	11	23	66
	2	11	19.5	69.5
	3	17	22.5	60.5
	5	16	29	55
	8	11.5	21	67.5

\* Days refer to the day after 0.3 mg per kg of vincristine.

† Stages of maturation are as described by Ebbe and Stohlman (2). The values are based on a differential of 100 megakaryocytes. Each value refers to the average of two animals.

TABLE III  
*Peripheral blood values in vincristine-treated hypertransfused animals*

Days after vincristine	Neutrophils $\times 10^{-3}$	Platelets $\times 10^{-6}$	Reticulocytes %
0	16.6 $\pm$ 3.4* (15)†	8.9 $\pm$ 0.35 (20)	0.1 $\pm$ 0.02 (51)
3	7.1 $\pm$ 2.4 (11)	7.1 $\pm$ 0.5 (16)	0.01 $\pm$ 0.01 (11)
4	7.9 $\pm$ 2.2 (12)	6.3 $\pm$ 0.38 (18)	0.05 $\pm$ 0.03 (7)
5	4.8 $\pm$ 0.9 (15)	7.8 $\pm$ 0.4 (24)	0 (18)
6	7.6 $\pm$ 1.2 (18)	7.9 $\pm$ 0.37 (24)	0.02 $\pm$ 0.01 (16)
7	19.0 $\pm$ 1.8 (14)	8.8 $\pm$ 0.5 (24)	0.04 $\pm$ 0.02 (7)
8	27.5 $\pm$ 1.4 (14)	10.7 $\pm$ 0.48 (24)	0.01 $\pm$ 0.01 (9)
9	31.4 $\pm$ 5.2 (10)	12.1 $\pm$ 0.67 (14)	0.04 $\pm$ 0.02 (5)
11	23.4 $\pm$ 4.8 (8)	11.4 $\pm$ 1.0 (11)	

\* Standard error of the mean.

† Numbers in parentheses refer to the number of animals.

reach the same magnitude of depopulation as the erythroid compartment. Minimal myeloid values were seen between the third and fourth days. Bone marrow erythroid recovery began on the fifth day and appeared complete by the seventh day, although even then occasional abnormal mitotic figures were observed.

The changes in peripheral blood parameters after vincristine in hypertransfused animals are given in Table III. In hypertransfused animals only occasional reticulocytes were seen in the peripheral blood before treatment with vincristine. After vincristine, only a rare reticulocyte was seen. In normal animals the number of reticulocytes fell within the first 48 hours, and between the third and fifth day only a rare reticulocyte was seen. Reticulocytes reappeared in the peripheral blood

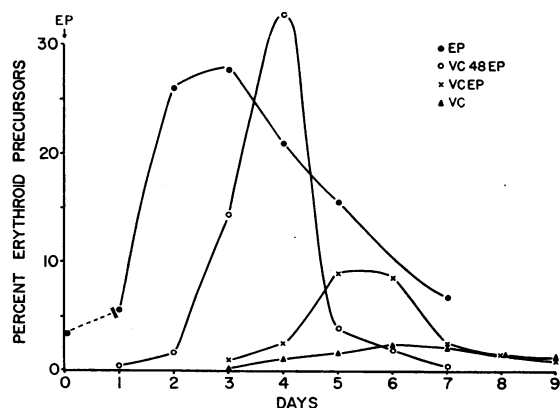


FIG. 2. THE PERCENTAGE OF ERYTHROID PRECURSORS IN THE MARROW OF HYPERTRANSFUSED ANIMALS GIVEN ERYTHROPOIETIN (EP), VINCRISTINE (VC), ERYTHROPOIETIN IMMEDIATELY AFTER VINCRISTINE (VCEP), OR ERYTHROPOIETIN 48 HOURS AFTER VINCRISTINE (VC48EP).

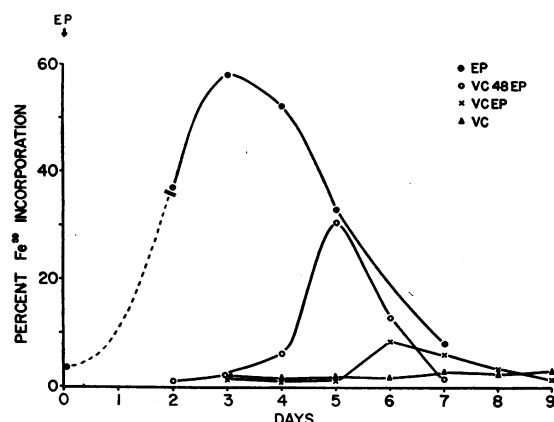


FIG. 3. THE PERCENTAGE OF INTRAVENOUSLY INJECTED IRON INCORPORATED WHEN  $^{59}\text{Fe}$  WAS GIVEN INTRAVENOUSLY 20 HOURS BEFORE THE TIME OF SACRIFICE. All animals were hypertransfused 5 days before injection of vincristine (VC). Erythropoietin (EP) was given either immediately (VCEP) or 48 hours after vincristine (VC48EP).

on the sixth day; on the seventh and eighth days the reticulocyte value increased above the normal range. The nadir of the platelet count was approximately 70% of the control value (Table III). This decrease, although significant, was strikingly out of proportion to the absolute reticulocytopenia and profound granulocytopenia. Granulocytic recovery began on the sixth and progressed to values considerably above normal by the eighth to tenth days.

As previously mentioned, the turnover rate of immature megakaryocytes is 8 to 14 hours (2). In view of this and the minimal effect of vincristine on megakaryocytes, it appeared that precursor

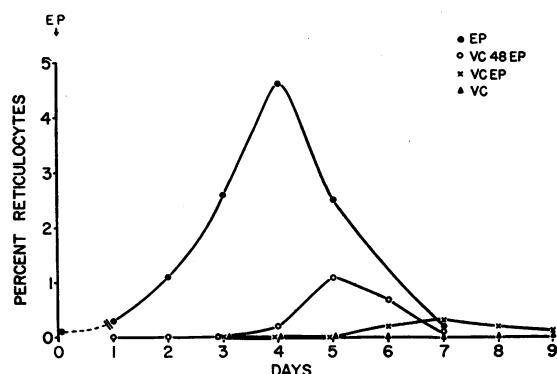


FIG. 4. THE PERCENTAGE OF RETICULOCYTES IN THE PERIPHERAL BLOOD OF HYPERTRANSFUSED ANIMALS GIVEN ERYTHROPOIETIN (EP), VINCRIStINE (VC), ERYTHROPOIETIN IMMEDIATELY AFTER VINCRIStINE (VCEP), OR ERYTHROPOIETIN 48 HOURS AFTER VINCRIStINE (VC48EP).

cells were not substantially affected. To test this hypothesis, we assessed the stem cell response to erythropoietin at intervals after treatment of hypertransfused rats with vincristine. Bone marrow erythroid precursor, 20-hour  $^{59}\text{Fe}$  uptake, and reticulocyte values from these studies are given in Figures 2 to 4 and Tables IV to VI.

Erythropoietin when given to control animals evoked a significant increase in bone marrow erythroid precursors within 24 hours; erythroid hyperplasia reached a maximum on the second or third day. The maximal  $^{59}\text{Fe}$  incorporation of 58% was attained when  $^{59}\text{Fe}$  was given on the second day, and peak reticulocytosis was observed on the fourth day. In contrast, erythropoietin administered immediately after vincristine failed to elicit

TABLE IV  
*Reticulocyte response in vincristine- and erythropoietin-treated hypertransfused animals\**

Days†	VC	EP	VCEP	VC48EP	Control
	%	%	%	%	%
0					$0.1 \pm 0.02^\ddagger$ (51)§
1		$0.3 \pm 0.1$ (4)		0 (3)	
2		$1.1 \pm 0.3$ (6)		0 (6)	
3	$0.01 \pm 0.01$ (11)	$2.6 \pm 0.2$ (12)	0 (5)	$0.02 \pm 0.01$ (10)	
4	$0.05 \pm 0.03$ (7)	$4.6 \pm 0.4$ (6)	0 (9)	$0.24 \pm 0.06$ (7)	
5	0 (18)	$2.6 \pm 0.3$ (3)	0 (15)	$1.1 \pm 0.02$ (7)	
6	$0.02 \pm 0.01$ (16)		$0.24 \pm 0.06$ (15)	0.7 (3)	
7	$0.04 \pm 0.02$ (7)	0.2 (2)	$0.27 \pm 0.05$ (14)	0.05 (3)	
8	$0.01 \pm 0.01$ (9)		$0.19 \pm 0.07$ (9)		
9	$0.04 \pm 0.02$ (5)		0.10 (3)		

\* VC, vincristine; EP, erythropoietin; VCEP, erythropoietin and vincristine given at time 0; VC48EP, erythropoietin given 48 hours after vincristine.

† Days refer to days after treatment; in case of VC48EP, the days after EP.

‡  $\pm 1$  SEM.

§ Numbers in parentheses refer to the number of animals. All animals were hypertransfused, including controls.

TABLE V

*Erythroid precursors in bone marrow in vincristine- and erythropoietin-treated hypertransfused animals\**

Days†	VC	EP	VCEP	VC48EP	Control
	%	%	%	%	%
0					3.4 ± 0.3‡ (51)§
1		5.6 ± 0.9 ( 4)		1.2 ± 0.1 ( 3)	
2		25.9 ± 1.1 ( 6)		1.6 ± 0.5 ( 6)	
3	0.1 ± 0.1 (11)	27.6 ± 1.1 (12)	0.8 ± 0.2 ( 5)	14.3 ± 1.5 (10)	
4	1.0 ± 0.2 ( 7)	20.8 ± 2.1 ( 6)	2.5 ± 0.1 ( 9)	32.5 ± 1.4 ( 7)	
5	1.5 ± 0.3 (18)	15.5 ± 1.8 ( 3)	8.9 ± 0.7 (15)	3.9 ± 0.7 ( 7)	
6	2.3 ± 0.5 (16)		8.6 ± 1.0 (15)	2.0 ± 0.7 ( 3)	
7	2.1 ± 0.3 ( 7)	6.9 ( 2)	2.4 ± 0.4 (14)	0.8 ± 0.2 ( 3)	
8	1.5 ± 0.5 ( 9)		1.5 ± 0.2 ( 9)		
9	1.1 ± 0.3 ( 5)		1.0 ± 0.1 ( 3)		

\* VC, vincristine; EP, erythropoietin; VCEP, vincristine given at the same time as erythropoietin; VC48EP, erythropoietin given 48 hours after vincristine.

† Days refer to days after treatment; in case of VC48EP, the days after EP.

‡ ± 1 SEM.

§ Numbers in parentheses refer to the number of animals.

a detectable bone marrow erythroid response until the fifth day, at which time erythroid precursors increased to 9%. Significant elevations of  $^{59}\text{Fe}$  incorporation and reticulocytes were not observed until the sixth and seventh days. The values at this time were 8.4% for iron incorporation and 0.24% for reticulocytes. When erythropoietin was given 48 hours after vincristine, the erythroid response occurred earlier, but was not manifest until 24 to 48 hours after that seen in erythropoietin-treated controls. Vincristine treatment alone resulted in values consistently lower than transfused controls for the 9-day course of the experiment.

Not only was the onset of the erythropoietic response delayed in vincristine-treated animals, but it was also diminished in magnitude. Further,

there appeared to be a dichotomy between the degree of erythroid activity in the bone marrow and the peripheral blood response as judged by reticulocytes or iron incorporation or both. These observations suggested a substantial degree of ineffective erythropoiesis. The presence of bizarre multinucleated erythroblasts, observed in bone marrow smears prepared at the peak of the delayed response, is in concert with such a notion. These cells were not seen in erythropoietin-treated controls.

### Discussion

The data reported herein are consonant with previous reports that a major effect of vincristine is to arrest mitosis in metaphase. The estimate of

TABLE VI

*Iron incorporation\* in hypertransfused and vincristine- and erythropoietin-treated rats†*

Day‡	VC	EP	VCEP	VC48EP	Control
	%	%	%	%	%
0					3.5 ± 0.2§ (51)
1		4.8 ± 0.9 ( 4)			
2		36.9 ± 6.4 ( 6)		0.8 ± 0.1 ( 6)	
3	1.9 ± 0.3 (11)	57.7 ± 1.7 (12)	1.6 ± 0.2 ( 5)	2.7 ± 0.3 (10)	
4	1.4 ± 0.1 ( 7)	51.9 ± 1.9 ( 6)	1.2 ± 0.1 ( 9)	6.1 ± 1.2 ( 7)	
5	1.6 ± 0.1 (18)	32.8 ± 5.1 ( 3)	1.3 ± 0.1 (15)	30.5 ± 4.2 ( 7)	
6	1.6 ± 0.1 (16)		8.4 ± 1.6 (15)	12.8 ± 1.0 ( 3)	
7	2.7 ± 0.2 ( 7)	7.9 ( 2)	6.0 ± 0.5 (14)	1.7 ± 0.3 ( 3)	
8	2.4 ± 0.3 ( 9)		3.3 ± 0.4 ( 9)		
9	2.8 ± 0.9 ( 5)		1.3 ± 0.2 ( 3)		

\* Measured 20 hours after injection of plasma-bound  $^{59}\text{Fe}$ .

† VC, vincristine; EP, erythropoietin; VCEP, vincristine and erythropoietin at time 0; VC48EP, erythropoietin 48 hours after vincristine.

‡ Day refers to day of sacrifice; in the case of VC48EP, the days after EP.

§ ± 1 SEM.

|| Numbers in parentheses refer to the number of animals.

11 hours for the generation time of rat erythroid precursors is in concert with values of 9 to 15 hours derived from either radioiron (5) or tritiated thymidine (6) labeling in the dog. Ana-telophase stages were not observed in the nucleated erythroid cell during the first 24 hours after administration of vincristine. In view of this, and the virtually complete depopulation of the nucleated erythroid compartment, it appears that escape from the mitotic block produced by vincristine is a rare event in the erythroid series.

The accumulation of arrested myeloid mitoses was much slower. Approximately 20% of the myeloid cells were in mitosis after 8 hours; a longer generation time for myeloid than erythroid cells has also been estimated from thymidine data (7). Ana-telophase forms could be observed among the myeloid mitoses throughout the first 12 hours. Although the frequency of this mitotic stage was diminished, its continued presence suggests that some of the arrested myeloid cells are capable of escaping the metaphase block. A considerable degree of karyorrhexis and phagocytosis of arrested nucleated precursors was also noted and gives further evidence of their decreased viability. The increased phagocytic activity of the bone marrow is probably secondary to the so-called "odor of death," but a primary vincristine effect on the macrophage has not been eliminated.

Vincristine caused almost complete depopulation of erythroid elements in the marrow but not of megakaryocytic elements. Megakaryocytes, like differentiated erythroid precursors, are apparently fed from a more primitive pool that has not been identified morphologically. Such a notion is supported by kinetic studies of rat bone marrow after flash labeling with tritiated thymidine (2). Thirty minutes after a single dose of intravenous thymidine ~25% of the earliest recognizable (stage 1) megakaryocytes were labeled. Twenty-four hours later, more than 90% of this group were labeled. Grain count data indicated that cell division rarely occurred in this compartment. Thus the major portion of the labeled early megakaryocytes had to be derived from a labeled, unrecognizable precursor pool in DNA synthesis at the time of injection of thymidine. From this study, the turnover rate of the early megakaryocyte was estimated to be of the order of 8 to 14 hours and the total maturation time 60 hours with

a minimum of 43 and a maximum of 75 hours. If differentiation of precursor cells stopped, depopulation of stage 1 megakaryocytes would be anticipated within 16 hours, stage 2 within 36 hours, and virtually complete depopulation of the entire megakaryocytic compartment within 60 hours. The megakaryocytic differential (Tables I and II) showed a slight decrease in stage 1 megakaryocytes relative to control values during the first 24 hours. The values for stage 1 megakaryocytes, however, were within the range of normal values previously reported in this colony (2). Thereafter there were only minor changes in the pattern of megakaryocytic differential. These considerations together with the presence of substantial numbers of megakaryocytes in the bone marrow (Figure 1) lead to the conclusion that differentiation of substantial numbers of megakaryocytes and maturation were not importantly affected by the administration of vincristine. The relatively minor fall in the platelet count observed in the peripheral blood would be in concert with such a notion. The alternative explanation would be that megakaryocytic maturation stopped with a resultant "biological standstill." This is not only unlikely biologically, but appears untenable on other grounds. Had this obtained, the abrupt cessation of platelet production would have resulted in an earlier and more pronounced thrombocytopenia. The platelet life-span in this colony approaches an exponential with a  $t_{1/2}$  of ~1.3 days (8). Accordingly, if platelet production stopped for 4 days, as would be implied by such a biologic standstill, a 90% decrease in the peripheral platelet count would be anticipated. This was not observed (Table III). Finally, it was evident from morphologic evaluation of megakaryocytes that platelet shedding and hence platelet production continued during the period of observation (Figure 1). This in itself would imply continued maturation. From these considerations we conclude that differentiation and maturation of the megakaryocytes continued during the period after administration of vincristine.

The depopulation of the erythroid compartment for a period of ~4 days indicated that differentiation into this compartment was not taking place. Moreover, during this period differentiation into the erythroid compartment could not be induced by massive doses of erythropoietin.

The continued differentiation of precursor cells into the megakaryocytic but not the erythroid compartment could be explained if the immediate precursor cells for both cell lines differed, in which case the megakaryocytic precursors would be presumed to be less sensitive to the effects of vincristine than the erythroid precursors. Alternatively, a selective block of erythroid differentiation in a common precursor cell could explain the observations. Although a final decision between these two alternatives cannot be made with the data at hand, the inability to induce erythroid differentiation with massive doses of erythropoietin together with the morphologic abnormalities seen in erythroid elements, but not in megakaryocytes during the recovery phase, argues in favor of different precursor cells.

For more than 50 years, a vast literature has accumulated concerning the questions of whether the differentiated hematopoietic cells are partially or completely self-sustaining and whether a unipotent or pluripotent stem cell exists. It became evident from the studies of Alpen, Cranmore, and Johnston (5) on iron labeling of pronormoblasts in bled dogs, together with studies of the effects of hypertransfusion, starvation, and erythropoietin in rodents (9), that the erythroid compartment normally is not self-sustaining but is continually fed from a more primitive precursor compartment. The studies of Ebbe and Stohlman (2) and Feinendegen, Odartchenko, Cottier, and Bond (10) would indicate that the same holds true for the megakaryocytic compartment. Although the data are not so clear-cut in the case of the myeloid elements, it seems likely that this compartment also is fed from a precursor pool (7, 11). The argument concerning whether the precursor cell is unipotent or pluripotent appeared to have been answered by the study of radiation chimeras by Becker, McCulloch, and Till (12) and of the Ph chromosomes in chronic myelogenous leukemia by Whang and her colleagues (13). Both of these studies indicate that at some level a pluripotent precursor cell exists, the marker chromosome being common to all elements. The identity of the common precursor is moot. Yoffey (14) and Cudkowicz, Bennett, and Shearer (15) have suggested a small lymphocyte. Thomas, Flidner, Thomas, and Cronkite (16) suggested a small mononuclear cell, but were uncertain of its

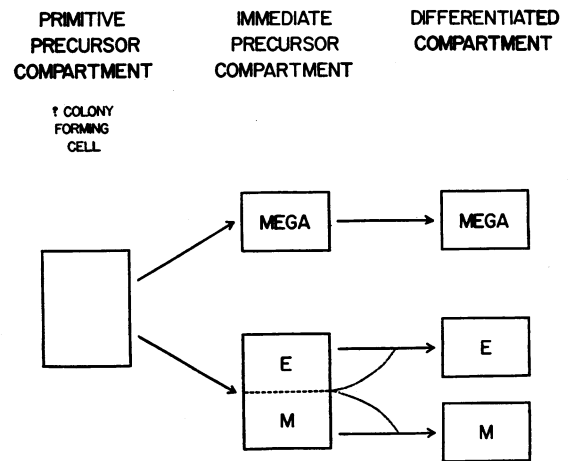


FIG. 5. A TENTATIVE MODEL FOR HEMATOPOIESIS. Mega, megakaryocyte; E, erythroid; M, myeloid. See text for discussion.

identity. It is of interest to note that the small peripheral blood lymphocyte capable of growth in tissue culture does not have the Ph chromosome. There may be functional differences, however, between the small peripheral lymphocyte and that of the bone marrow. In fact, evidence has been adduced by Osmond and Everett (17) and Robinson, Brecher, Lourie, and Haley (18) to indicate that growth potentials may differ between two types of morphologically similar lymphoid cells. Irrespective of the morphologic identity of the colony-forming cell, there is a question that this is the immediate precursor cell for each of the hematopoietic elements. The observations described herein, as well as those of megakaryocytic labeling (2) and the transplantation studies of Blackett, Roylance, and Adams (19) in sublethally irradiated animals, suggest that a more differentiated intermediate precursor may exist. Similarly the observations of Lewis and Trobaugh (20), although not interpreted as such, suggest to us a different immediate precursor cell for each cell line. Accordingly we should like to propose the model for precursor cells shown in Figure 5.

In this model, there is a pluripotent precursor cell similar to the colony-forming cell described by Till and McCulloch (21). This cell in turn gives rise to a more differentiated cell that is the immediate precursor for the individual morphologically identifiable cell lines. The previously discussed evidence indicates that the immediate precursor cell for megakaryocytic elements differs



from that of myeloid and erythroid elements. The data do not permit a final judgment of whether the immediate precursor cell for myeloid and erythroid cells differs, but it seems likely that this is the case. In favor of this are the apparent difference in generation time for the two elements reported previously (7), as well as herein, together with the difference in the rate and degree of depopulation observed under the experimental condition used in the present study.

The relationships of the immediate precursor cells to the primitive pluripotential cell, and the mechanism of regulation, must remain at present to a large extent speculative, although some of the notions to be presented are subject to experimental verification. We should like to suggest that the immediate precursor compartments are to a large extent self-maintaining, but when damaged and perhaps in some instances of severe stimulation, e.g., phenylhydrazine-induced anemia, they are repopulated from the primitive pluripotential compartment. A feedback mechanism, probably negative, to the primitive compartment is thus implicated. An important segment of the immediate precursor compartment presumably is dormant and is triggered into cell cycle either by differentiation, e.g., into erythroid elements, or death of cells within the compartment. Accordingly, increased demand for one or another formed element could be met in large measure by a decreased turnover time of the immediate precursor compartment. Critical to adequate homeostasis would be a control mechanism to prevent the depopulation of the immediate precursor cell. Differentiation of immediate precursor cells does not occur until a critical level of cells is achieved. The signal for differentiation into the erythroid compartment is presumably erythropoietin, and there may be analogs of this for the other formed elements. It would follow, then, that the erythropoietin-sensitive cell is the immediate precursor and not the primitive pluripotential cell.

Gurney, Lajtha, and Oliver (22) suggested the plethoric rodent as an experimental model to study the extent of "stem cell" damage in radiation injury. The virtue of the "stem cell tolerance test" is that the polycythemic rodent has virtually complete suppression of erythropoiesis and a predictable response to a given dose of erythropoietin. This preparation, then, theoretically should permit

an estimate of the degree of damage to the immediate precursor compartment. In view of the difference between the megakaryocytic and erythroid responses to vincristine, we applied this experimental model to evaluate the effect of vincristine on the immediate precursor of erythroid cells. When erythropoietin was given to the polycythemic animal, within the first several days of the administration of vincristine there was a suboptimal response to erythropoietin. One can infer, then, that vincristine damaged the immediate erythroid precursor compartment. If vincristine's effect was confined mainly to the dividing cell, then a substantial portion of the immediate precursor cells was in cycle even when the demand for differentiation was reduced by hypertransfusion. Extensive damage to the immediate precursor compartment is further supported by the morphologic evaluation of the bone marrow during the initial period of response to erythropoietin. Abnormal multinucleated forms were seen, as were abnormal mitoses. Further, there was a dichotomy between the bone marrow response and peripheral blood, suggesting ineffective erythropoiesis. The degree of ineffective erythropoiesis indicated that there was substantial residual damage to the immediate precursor compartment even after recovery had progressed to the point of permitting differentiation.

Normally a detectable response to erythropoietin is seen in the bone marrow of hypertransfused animals within 24 hours, and by 48 to 72 hours substantial numbers of reticulocytes have been released into the peripheral blood. In contrast, in vincristine-treated animals this response was delayed. When erythropoietin was given at the time of treatment, a small but significant increase in bone marrow erythroid cells was not seen until 5 days after treatment. Since the reticulocytosis was negligible, it is presumed that most of the erythroid precursors died *in situ*. When erythropoietin was given 24 to 48 hours after vincristine, the extent of ineffective erythropoiesis did not appear to be so great, but there was again a delay in response. The length of the delay and the magnitude of the response were related to the interval between administration of vincristine and erythropoietin.

The delay in response to erythropoietin could be explained by continued availability of extra-

cellular erythropoietin. Alternatively, one might suggest that erythropoietin entered the cell during the first 12 hours after injection and interacted with its intracellular target and that the information imparted thereby was stored until regeneration of the immediate precursor compartment reached the point at which differentiation could once again occur. The half-time for plasma clearance of erythropoietin in normal rats has been shown to be of the order of 2.5 to 3 hours (4). In rats with suppressed erythropoiesis, clearance is somewhat slower, the half-time being  $\sim 6$  hours (23). From these observations it would appear unlikely that erythropoietin would be available from extracellular sources for several days, as would be necessary if this were the explanation of the delayed response observed when the hormone was given at time 0. More likely the alternative, intracellular storage of information or perhaps erythropoietin itself, obtains. The difference in the magnitude of response between animals given erythropoietin at time 0, as compared with 48 hours, presumably reflects death from vincristine of erythropoietin-"activated" immediate precursor cells within the compartment. Dilution of information through division may also play a minor role.

A delayed response to erythropoietin is probably a general phenomenon of the regenerating marrow and not peculiar to vincristine-treated animals. There are studies on other regenerating systems that may be interpreted in this light. Preliminary studies in our laboratory indicate that animals given actinomycin D or cytoxan behave like vincristine-treated animals in this respect. Although observations have not been made in hypertransfused irradiated animals, there is evidence in normal irradiated animals that such a delayed response occurs (24). Erythropoietin given immediately after sublethal irradiation produced an effect that was not manifest for several days. Storage of information until regeneration permits differentiation, as described herein, may prove to be a more attractive explanation than the original interpretation that a negative feedback was removed by differentiating immediate precursor cells which then died after one or two abortive divisions. Studies on the effect of erythropoietin of the bone marrow of hypertransfused irradiated animals carried out for several days beyond that in the "stem cell tolerance test" should provide a conclusive an-

swer as to which alternative obtains in the irradiated animal.

### Summary

Studies are presented on the hematopoietic response of rats to vincristine and the effect of erythropoietin on red cell production in these animals. The megakaryocytic compartment appeared to be relatively unaffected during the period of observation, which was interpreted as indicating that there was continued differentiation of precursors into this compartment. In contrast, there was a striking depopulation of the erythroid compartment between the second and fourth days during which differentiation of precursors could not be achieved even with massive doses of erythropoietin. Continued differentiation of cells into the megakaryocytic compartment but not the erythroid compartment suggests different immediate precursor cells; a model for this is presented and discussed. In the hypertransfused vincristine-treated rat there was a delayed response to erythropoietin. It is suggested that the immediate precursor cells for the erythroid compartment are capable of storing information imparted by erythropoietin until such time as regeneration permits differentiation. Morphologic evaluation of the bone marrow suggests that even after recovery has proceeded to a point at which differentiation once again occurs, there is residual damage that leads to ineffective erythropoiesis.

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