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Reticulocyte Size and Erythropoietic Stimulation. (26785)

GEORGE BRECHER AND FREDERICK STOHLMAN, JR.

National Institute of Arthritis and Metabolic Diseases, Nat. Inst. of Health, Public Health Service, U. S. Department of H.E.W., Bethesda, Md.

It is generally accepted that reticulocytes are larger than mature red cells(1). The manner in which the oversized reticulocyte matures into a red cell of normal size has not been defined. Recently, Stohlman found that red cells produced during recovery from severe phenylhydrazine anemia have a markedly shortened life span(2). The mean corpuscular volume of the reticulocytes so produced was nearly twice that of normal mature red cells. The results suggested that these macrocytic cells are replaced by cells of more normal size. This thesis was confirmed in the present studies by measuring the distribution of cell sizes; the results indicate that a correlation exists between rapidity of red cell regeneration and size of reticulocytes.

Methods. Groups of female Sprague-Dawley rats weighing 140-160 g were injected s.c. with either 0.1 or 0.3 ml of a 2% solution of phenylhydrazine hydrochloride (PHH) in distilled water on days 0, 1, and 3 of the experiment. A third group served as controls. In one experiment, one group of animals was injected with 0.3 ml PHH on days 0, 1, and 3, then given 650 r whole body irradiation on day 4. Rats were exsanguinated by cardiac puncture at various intervals following last injection.

Mean corpuscular volumes (MCV) were determined from hematocrits measured by a micro method and red cell counts measured with the Coulter Counter(3). Red cell size distribution curves were obtained with a Coulter Counter Model B supplied with an automatic graphout. The Model B is a modification of the basic Coulter instrument which generates impulses proportional to cell volume as individual red cells pass through a 50 micra aperture. An electronic gating device of the Model B* permits discrimination of impulses of different magnitude, corresponding to different cell volumes. The graphout automatically records their frequency distribution. In the resulting graphs, the ordinate represents relative frequencies. The linear scale of the abscissa corresponds to the amplitude of the electronic impulses. The proportionality factor between amplitude of impulses and cell volume can be determined by appropriate calibration and units on the abscissa converted to μ^3. This has been done in Fig. 1. The peaks or modal values of distribution curves are, as a rule,

* Available from Coulter Electronics, Inc., Chicago, Ill.

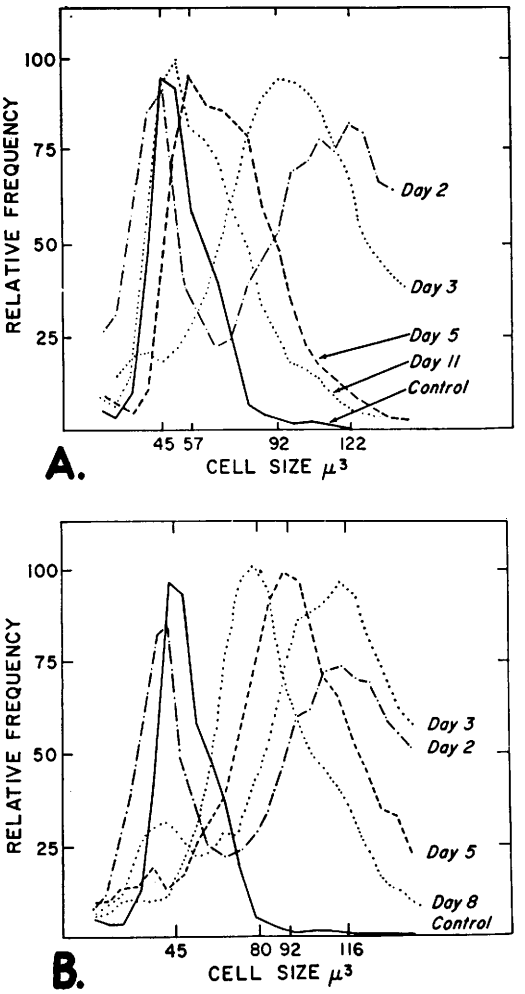

FIG. 1. Size distribution of cells produced in response to phenylhydrazine (PHH) anemia in controls (A) and irradiated rats (B). Days counted from day after last inj. of PHH. All measurements were made on red cell suspensions adjusted to approximately 200,000 cells/ml. Changes in animals' hematocrits are given in text.

lower than MCV's due to skewing of the curves to the right. MCV's can be computed from the graphs and agree within 4% with MCV's computed as ratio of hematocrit to rbc count. Detailed data on calibration and reproducibility will be published elsewhere. For counting, red cells were suspended in a mixture of 9 parts of the electrolyte and glucose mixture used in Eagle's tissue culture medium and 1 part of distilled water. Size distribution curves were the same in this solution as in plasma.

Partial separation of reticulocytes and mature cells was obtained by centrifugation. Two technics were used. In the first, whole versenated blood was spun, the plasma removed, the cells washed twice in cold Eagle's solution and recentrifuged in a Wintrobe hematocrit tube. The top and bottom layers were then sampled with a Wintrobe pipette. In the second method, whole blood was centrifuged in a 75 mm microhematocrit tube. Top and bottom layers were sampled by a smaller capillary to which plastic tubing was attached for suction, or by breaking the capillary at desired points after marking it with a file. Since only 2 lambda are needed for red cell sizing and similarly small amounts for reticulocyte counts, it was possible to obtain both from a narrow segment of the top and bottom layer respectively. Greater speed and better separation are achieved with the microhematocrit than with the Wintrobe tube. However the latter method was mandatory when additional measurements, such as MCHC, were desired.

Results. In rats given 3 doses of 0.3 ml of phenylhydrazine (PHH), the hematocrit was 26% on the day after last injection, 37% on the 3rd day and returned to a normal value of 42% by the 5th day after last injection. Reticulocytes were 40-55% on the 2nd day and 90-95% on the 3rd day. Two days after the last PHH injection, the erythrocytes were composed of 2 distinct cell populations (Fig. 1, A): cells of normal size with a modal value of 45 μ^3, and cells of more than twice normal size, the modal value being 122 μ^3. The normal sized cells were rapidly eliminated as may be seen by the marked reduction in the cohort of cells with a modal value of 45 μ^3 on the 3rd day after last PHH injection. Thereafter only the larger cells were present, but the distribution curve shifted rapidly toward normal. Modal values were 93 μ^3 on the 3rd day, 57 on the 5th day and 51 on the 11th day after PHH injection. Theoretically, the shift in modal value could be due to shrinkage of cells, selective destruction of larger cells, or production of smaller cells. Shrinkage of the red cells could be eliminated on the basis of measurements of mean corpuscular hemo-

TABLE I. Modal Values of Red Cell Size Distributions after Different Doses of PHH.

Days after PHH	Total dose, 2% PHH, ml	Peaks of size distribution, μ^3		Reticulocytes, %		
		Top	Bottom	Top	Bottom	Whole blood
1	.9	63, 98	45	99	6	45
4	.9	75, 86	86	94	87	93
5	.9	57, 75	75	71	57	64
8	.9	57	69	15	4	9
15	.9	51	75	4	0	1
1	.3	45, 69	45	93	5	15
4	.3	69	45, 69	83	20	53
5	.3	69	63	66	28	40
8	.3	51	69	41	1	9
15	.3	51	69	7	0	2
—	—	45	45	9	1	2

globin concentration (MCHC) and MCV. On the 3rd day after last PHH injection, when 90-95% of cells were reticulocytes, MCV was 100-110 μ^3 (normal ~ 55 μ^3) and MCHC was 27-30% (normal 34-36%). Shrinkage of the oversized reticulocytes to normal sized adult red cells would have resulted in an MCHC of 49-60%. No values over 36% were observed.

To determine to what extent production of smaller cells can account for the shift in cell size distribution, a number of animals were irradiated on the day after last PHH injection. Erythropoiesis was markedly suppressed. The hematocrit dropped from a normal of 40 to 45% to 17% on the 3rd day after last PHH injection and stayed approximately at this level until the 11th day. Cell size distribution in this group of animals (Fig. 1, B) was at first indistinguishable from that of the non-irradiated rats. The distribution curve again showed 2 populations with normal modal values of 45 and 116 μ^3 on the 2nd day after last PHH injection. The normal sized cells corresponding to the lower modal value virtually disappeared by the 3rd day. Subsequently, however, the modal value of the larger cells changed only slowly in the irradiated rats. It was 92 μ^3 on the 5th day and 80 μ^3 on the 8th day, where it remained until termination of the experiment 11 days after last PHH injection. Control animals irradiated without prior PHH injection had no shift in cell size distribution. Thus, suppression of erythropoiesis by irradiation greatly retarded the rapid shift in the distribution curve observed in rats with a normal erythropoietic response.

To test whether lesser degrees of hemolysis and erythropoietic stimulation would result in cells of more nearly normal size, 2 groups of rats receiving 3 doses of 0.3 ml or 0.1 ml of PHH were compared. In contrast to the double peaks of 45 and 98 μ^3 on the day after last PHH injection in the group with the higher dose, the rats that had received the lower dose had only a broadening of the curve to the right. Three days later the red cells of the high dose group had a single peak with a modal value of 86 μ^3; modal value in the low dose group was 69 μ^3. Production of cells of more nearly normal size in both groups is indicated by the modal values of 63, 51 and 48 μ^3 on days 5, 8, and 15 following the higher dose, and modal values of 63, 63, and 51 μ^3 on the same days in the low dose rats. In both groups the continued presence of larger cells was indicated by the broad tail to the right in all curves. Separate size distribution and reticulocyte counts on the top and bottom layers of centrifuged specimens provided further insight into production of cells of different sizes in the 2 dosage groups (Table I). On the day after last PHH injection, the bottom layer of cells of both groups contained predominantly surviving normal cells with a modal value of 45. The top layer contained 2 populations in both groups. Modal values were 45 and 69 μ^3 in the low dose group, and 63 and 98 μ^3 in the high dose group. In both the high and low dose group there was a gradual reversal in

TABLE II. MCV and MCHC of Red Cells in a Centrifuged Column.

Total dose 2% PHH, ml	Peaks of size distribution, μ^3		MCV, μ^3		MCHC, %	
	Top	Bottom	Top	Bottom	Top	Bottom
.9	75	92	106	109	22	29
.3	63	39, 63	83	73	29	34
—	45	45	57	57	32	34

distribution of smaller and larger cells between top and bottom layers. Reticulocytes continued to be more numerous in the top layer, in keeping with the hypothesis that the very large reticulocytes originally produced are being replaced by successive crops of reticulocytes of more normal size.

The continued predominance of reticulocytes in the top layer appeared to support the thesis that centrifugation results in layering of red cells in accordance with their age. It could be readily shown, however, that this is not always the case. When blood from patients with microcytic hypochromic anemia was examined during the first week of iron therapy, reticulocytes congregated in the middle or bottom layer after centrifugation, strongly suggesting that mean corpuscular hemoglobin concentration (MCHC) rather than age determines the position of red cells in a centrifuged specimen. To test this thesis, MCHC, MCV and size distribution were determined in the top and bottom layers of selected blood specimens of rats that had received 0.9 and 0.3 ml PHH, and of controls. MCHC was lower in the top layer in each instance, while the MCV was larger in one instance, smaller in another, and the same as the bottom layer in the third (Table II).

Discussion. The rapid replacement of the first crop of reticulocytes produced in severe phenylhydrazine anemia has been established by their shortened life span(2). These early reticulocytes, which at one time represent about 90% of all red cells, have an MCV of twice normal but an MCHC only 20% less than normal. This precludes their shrinking to cells of normal size and MCHC. It was to be expected, therefore, that early, greatly oversized reticulocytes will be replaced by cells of more normal size. This could not be established by determination of MCV alone, but was supported by measurement of cell size distribution, which demonstrated the presence of 2 populations of cells with different volumes. When frequency distribution of cell volumes was measured in different cell layers after partial separation of reticulocytes by centrifugation, a general picture of decreasing volumes of successive crops of reticulocytes was observed. It could also be demonstrated that the reticulocytes initially produced after high dosage of phenylhydrazine are almost twice normal size, whereas those produced after a lower dose are only about 1.5 times normal size. The intensity of erythropoietic stimulation thus appears to govern reticulocyte size to some degree. Skipped division may account for the larger size of more rapidly produced cells(4).

The position of cells in a centrifuged column of blood presumably is governed by the density of the cell. The present data suggest that the MCHC is the principal variant in red cell density. The sequence of events occurring after administration of PHH support this thesis. The MCHC of the very large reticulocytes produced initially (Fig. 1) was lower than that of the normal red cells. Consequently, at this time the larger cells were in the top layer after separation. The continued hemoglobin synthesis in these large reticulocytes eventually resulted in a normal MCHC. We suggest that a new crop of smaller reticulocytes with an MCHC below normal emerged at this time, accounting for the continued accumulation of reticulocytes in the top layer of centrifuged blood.

Our argument implies that eventually reticulocytes of the size of normal adult cells are produced, and that such normal sized reticulocytes replace the normal daily losses from senescence. The top and bottom layers of centrifuged normal blood have, in fact, never shown measurable differences in cell size.

However, minor degrees of excess size might escape detection unless 100% separation of reticulocytes is achieved. The best separation in normal blood with the technique used was 9% reticulocytes in the top layer and 1% in the bottom layer. (Control, Table I). Improved techniques for reticulocyte separation are needed to settle the point.

Summary. The size distribution of peripheral red blood cells of rats recovering from phenylhydrazine anemia is reported. Reticulocytes produced during the first burst of active erythroid regeneration are as much as twice the normal size; degree of macrocytosis is related to the severity of the anemia. The initial crop of oversized cells is replaced by successive crops of reticulocytes of more normal size. It is suggested that separation of reticulocytes by centrifugation depends on their density, which is determined primarily by the MCHC.

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Comparative Effects of Colchicine and Vincaleukoblastine on Bone Marrow Mitotic Activity in Syrian Hamster.* (26786)

GIUSEPPE CARDINALI, GIULIANA CARDINALI, ALFRED H. HANDLER, AND MARIA F. AGRIFOGLIO (Introduced by Sidney Farber)

Children's Cancer Research Foundation and Dept. of Pathology, Harvard Medical School, at Children's Hospital Medical Center, Boston, Mass.

Orsini and Pansky(1) reported the Syrian hamster to be resistant to colchicine. They were unable to observe metaphase arrest in dividing cells in animals treated with doses of colchicine varying from 1 mg to 20 mg/kg of body weight. Since this original report by Orsini and Pansky, the Syrian hamster has been generally accepted as being resistant to colchicine(2).

Recently an alkaloid derived from *Vinca Rosea Linn.,* Vincaleukoblastine, was found to possess, like colchicine, the property of arresting cell mitosis at the metaphase stage (stathmokinetic effect)(3,4,5).

The present study was undertaken to study the comparative effects of Vincaleukoblastine and colchicine in Syrian hamsters.

Methods. Fifty Syrian hamsters† of both sexes, weighing 80-130 g, were used in these experiments. Three groups of 10 animals each were injected intraperitoneally with 1.2 mg/kg, 2.4 mg/kg and 7.2 mg/kg, respectively, of colchicine. They were sacrificed 4 hours after treatment. A fourth group of 10 hamsters was injected with 1 mg/kg of Vincaleukoblastine and also sacrificed after a period of 4 hours. A fifth group of 10 hamsters was used as a control.

Bone marrow from the femurs of all animals was collected and fixed in 70% methylalcohol. Preparations for microscopic study were made according to the Feulgen squash method. Scoring of mitotic figures was done by counting 2000 cells in each preparation. Survival time after intraperitoneal treatment with high doses of colchicine and Vincaleukoblastine was determined in AKR mice, WR rats, and Syrian hamsters.

Results. The results of these experiments, reported in Tables I, II, and III, indicated that, although hamsters seemed to be resistant to the general toxic effects produced by colchicine, as demonstrated by survival time, their bone marrows were only partially resistant to the stathmokinetic effect of this alkaloid. Colchicine, in a dosage of 1.2 mg/kg,

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† Dennen Animal Industries, Gloucester, Mass.