

QUANTITATIVE MEASUREMENT OF HEMATOPOIETIC CELLS OF THE MARROW¹

By DENNIS M. DONOHUE, BEVERLY W. GABRIO, AND CLEMENT A. FINCH
WITH THE TECHNICAL ASSISTANCE OF MARY LU HANSON AND
LORETTA CONROY

(From the Department of Medicine, University of Washington School of Medicine,
Seattle, Wash.)

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In recent years with the availability of more accurate methods, attention has been directed towards the quantitative aspects of both erythropoiesis and leukopoiesis. It has been difficult to measure the precursor cells in the marrow, and their exact number still remains in question. Previous estimates of total marrow cells have utilized three general methods of approach: *a*) direct measurements of volume and cellularity of marrow in animals (1); *b*) calculations based on an assumed maturation time and on the known life span of the erythrocyte (2); and *c*) calculation based on the frequency and the estimated duration of marrow mitosis (3). The first of these methods imposes formidable technical difficulties and cannot be performed in man; the other two involve assumptions not subject to validation.

The present report concerns a direct technique in which the various procedures and assumptions employed are scrutinized. The method employs the erythroid tag Fe⁵⁹ to relate the cells in an aliquot of marrow to the total marrow, as originally proposed by Suit (4).

MATERIALS AND METHODS

1. Experimental subjects. The animals employed in these studies included healthy Sprague-Dawley rats weighing 200 to 300 Gm., New Zealand white rabbits weighing 2 to 3.5 Kg. and 2 to 3 Kg. male Rhesus monkeys. All animals had a blood hemoglobin concentration of over 13 Gm. per 100 ml.

Clinical studies were performed on male patients with controlled tuberculosis who were being subjected to thoracotomy. Prior to operation these patients were afebrile, had red counts of over 4.5 times 10⁶ per cu. mm., and normal leukocyte counts.

2. Preparation and counting of marrow suspension. Rabbits and monkeys were sacrificed by intravenous in-

jections of air or cyanide, rats by exsanguination. Immediately after death of an animal one femur was removed, and the central 60 per cent was carefully cut out, using a bone cutter for femurs of rabbit and monkey and a file and scissors for the rat femur. Marrow was either forced out of the open end of the central shaft by injection of plasma from a syringe into the opposite end, or the shaft was split open and the column of marrow freed. The marrow was then placed in a tube containing isologous heparinized plasma or a 5 per cent albumin solution. Five ml. of plasma was used for monkey marrow, 4 ml. for rabbit and 1.5 ml. for rat marrow. The tube containing the mixture was stoppered and shaken either by hand, as described by Yoffey (5), or on a mechanical shaker at an oscillation of 100 per minute for about three minutes, until cell clumps appeared to be largely dispersed. The suspension was then pressure-filtered through a nylon mesh (MBCO Disposable Filter No. 9331, Macalaster Bicknell Company), as recommended by Thomas (6). Total volume of cell suspension was measured in a graduated centrifuge tube. The suspension was drawn into red cell counting pipettes and diluted with plasma. Counts of approximately 1,000 cells were made in a counting chamber.

An aliquot of the suspension was then centrifuged at 290 G for five minutes and the cell-free supernatant was removed. The concentrated cell suspension, after thorough mixing, was used for the preparation of smears. Differential counts of at least 2,000 marrow cells were made from Wright and Giemsa-stained smears. Unidentifiable cells in these preparations represented 3 to 6 per cent of cells counted. Reticulocyte preparations were also made by placing one drop of the thick cell suspension in a well slide on which one drop of 6 per cent new alcoholic methylene blue had been previously dried. This was mixed and allowed to stand for three minutes, after which smears were made; 2,000 non-nucleated red cells were surveyed for reticulum. All glass surfaces coming into contact with the marrow during preparation and handling of these animal and human suspensions were siliconized.

In human studies, a portion of a rib removed at operation was the source of marrow. This rib fragment was immediately wrapped in parafilm and kept on ice for a period of 30 minutes until the cell suspension could be prepared. Marrow was expressed from the rib by pressure and transferred by a capillary pipette into 2 ml. of

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plasma. Further handling and counting was performed as described for animal studies.

Details of individual experiments done to determine cell losses in preparation of marrow suspension for counting are found under Results. Radioiron techniques, including the simultaneous use of Fe^{59} and Fe^{55} , have been described previously (7). The method for the determination of hemoglobin was that of Flink and Watson (8). In studies employing paper electrophoresis, material tested was buffered at pH 6.5, 7.0 and 8.6, and the loaded strips were run at 240 volts.

3. *The use of radioiron as a marrow tag.* This method is based on the observation that tracer amounts of radioiron injected intravenously rapidly localize in the erythroid cells of marrow (9). The details of the methods used in animals and man to determine the ratio between the marrow aliquot and the total marrow differed somewhat, although in both instances radioiron was employed to tag the erythroid cells of the marrow.

In animal studies, radioiron was bound to freshly-drawn plasma in a concentration of less than 1 μ g. iron per ml. of plasma and was injected intravenously. Rats received 1 μ c. Fe^{59} , rabbits 5 μ c. and monkeys 10 to 15 μ c. A weighed aliquot of the solution prepared for injection was set aside as a standard. The exact amount of radioactivity injected was also determined. The animals were then killed at the time when radioactivity in circulating blood was minimal, *i.e.*, when most of the radioactivity had been cleared from the plasma and but little activity had yet appeared in circulating erythrocytes. This was found to be at 5 hours in the rat, 6 hours in the rabbit and 12 hours in the monkey. At the time of sacrifice a sample of blood was drawn for radioactivity and cell counting. One femur was removed, and a cell suspension was prepared from the central 60 per cent of the shaft. Residual radioactivity in the filter and bony shells was determined. The supernatant fraction and packed cells of the final marrow suspension were counted separately.

The carcass of the animal was eviscerated and autoclaved for two hours. Bones were then easily separated from soft tissue. The radioactivity of skeleton, liver, spleen and nonskeletal carcass, as well as that of the standard aliquot solution, was determined. A shielded two-inch scintillation crystal detector at a distance of from 6 to 24 inches from the various aliquots was employed for the measurements. The central 60 per cent of the opposite femur was removed and digested in nitric acid. The remaining skeleton, including the ends of the two femurs, was similarly digested. The activity of these two fractions, plus that of a standard, was determined in a vial scintillation counter.

In studies of man, one-half μ c. Fe^{59} per Kg. body weight bound to normal plasma, or in the form of the citrate salt (10), was injected intravenously 14 to 18 hours before surgical removal of a rib. Baseline studies of the patient's blood included red cell count, hemoglobin, hematocrit, reticulocyte count, total and differential leukocyte counts. Blood was drawn for radio-

active determination before injection of Fe^{59} , five minutes following injection, at the time of removal of the rib, and two weeks or more after the operation. A marrow cell suspension was prepared from the rib as described previously. The radioactivity in the centrifuged cells of 1 ml. of marrow suspension was determined.

4. Formulae employed in animals.

$$\text{Cells of suspension} = \frac{\text{ml. of suspension} \times \text{cells}}{\text{per ml.}} \quad \text{I}$$

$$\text{Radioactivity of central femur} = \frac{\text{activity of (suspension cells + suspension supernate + filter + central bone)}}{\text{}} \quad \text{II}$$

$$\text{Cells of central femur} = \frac{(\text{I}) \times (\text{II})}{\text{activity of suspension cells}} \quad \text{III}$$

$$\text{Total marrow cells} = \frac{\text{total skeleton radioactivity} \times (\text{III})}{(\text{I})} \quad \text{IV}$$

Formulae employed in man.

$$\text{Total marrow cells} = \frac{(\text{I}) \times 0.66 \times \text{activity injected}}{\text{activity of suspension cells}} \quad \text{V}$$

The factor 0.66 is employed in man to correct for the amount of radioactivity localized in the marrow at the time of study (see discussion of marrow localization of radioiron).

5. *Comparison of Fe^{59} with other marrow tags in determination of total marrow.* In animals, two other methods were employed to establish the ratio between the marrow aliquot from the central femur and marrow from the total skeleton. These involved the quantitative analysis of marrow fractions for porphyrin and hemoglobin. It was necessary to process the carcass at 5° C. in order to prevent postmortem change in these pigments. Bones exclusive of femurs were separated from the carcass and ground in a No. 5 Enterprise food grinder. The ground bones were further pulverized in a Waring Blendor® in an ethyl acetate-acetic mixture (four parts anhydrous ethyl acetate and one part glacial acetic acid). The material was transferred to a sintered glass filter, and repeatedly ground and washed with ethyl acetate-acetic mixture. The final residue was treated with boiling glacial acetic acid. Additional ethyl acetate was added to the filtrate to reconstitute the original 1:4 ratio between glacial acetic acid and ethyl acetate. The extract was then washed three times with 3 per cent sodium acetate, and porphyrins were extracted repeatedly with 10 ml. portions of 3 N HCl until extraction was complete. Porphyrin analysis was performed by using a modified Farrand fluorometer and a Corning glass filter (primary filter No. 5113 and secondary filter No. 2412). The experimental values obtained represented total fluorescence due to protoporphyrin, coproporphyrin and any other fluorescent porphyrin. The femur was similarly prepared and its porphyrin content analyzed. As a check on reproducibility of the method used, as well as on the similarity of the opposite femur, analyses were performed on the femora of three rabbits. The duplicates agreed within a range of 8 per cent.

In using hemoglobin for determination of the femur: total marrow ratio, it was recognized that a considerable portion of hemoglobin was derived from circulating erythrocytes and that this fraction might not bear a constant relationship to marrow. Therefore, radioiron-tagged erythrocytes (Fe^{59}) were injected intravenously into each animal 10 to 20 minutes before sacrifice. The specific activity of circulating hemoglobin was determined, and an appropriate correction for peripheral blood contamination was made in each marrow hemoglobin fraction according to its radioactive content. Hemoglobin extraction from marrow was performed on the ethyl acetate-acetic acid mixture described above, following porphyrin extraction, and after the mixture had been washed with 3 per cent sodium acetate. The extraction was carried out with small volumes of concentrated NH_4OH until the organic phase was colorless. The pigment was then determined by the pyridine-hemochromogen method. In recovery studies on marrow homogenates, an average recovery of 95 (plus or minus 3) per cent of added hemoglobin was achieved in four experiments. The addition of radioactive hemoglobin also indicated that no significant amounts of the radioiron were lost in the various procedures leading to extraction of hemoglobin. Ratios were established between the femur and total skeleton for both porphyrin and hemoglobin. These were compared to results obtained by the use of Fe^{59} labeling of marrow.

RESULTS

1. *Studies relating to preparation of the cell suspension*

There were several problems apparent in the preparation and counting of the cell suspension. It was found that removal of cells from the ends of the femurs was technically difficult due to bone spicules. Therefore, only the central 60 per cent of the femurs was employed. Even in this central portion there was an appreciable amount of marrow and radioactivity which could not be removed without excessive cell trauma. In rabbits this residue amounted to an average of about 10 per cent of the total radioactivity in the marrow. A further loss of about 20 per cent was found on the nylon filter. A significant amount of radioiron in excess of that which could be ascribed to circulating blood was also found in the supernatant fraction of the centrifuged marrow suspension. In rats this averaged 29 per cent, in rabbits 15 per cent, in monkeys 28 per cent and in man 29 per cent. These several losses of radioactivity were collectively large and their manner of production, therefore, of importance in the calculation of re-

sults. In respect to both bone and filter radioactivity, it seemed likely that this activity reflected residual marrow tissue. Activity in the bony shells could be removed by scraping the inner surfaces of the bones. Activity on the filter was, in general, proportionate to the mass of cells retained, and was similarly assumed to reflect activity from trapped cells.

The nature of the radioactivity in the marrow supernatant fraction seemed less certain. After a small correction for that activity attributable to circulating blood content of marrow suspension, the activity of the supernatant fluid was presumed due either to destroyed cells or to iron released from marrow cells. To test the latter possibility, tagged marrow was incubated for two hours at room temperature in saline, in iron-deficient plasma, and in saline solutions containing 0.4 to 4 per cent disodium Versenate®. The supernatant fraction in saline and in the iron-deficient plasma contained 12 per cent of the total activity, similar to the amount in the original marrow supernatant fraction at zero time. Samples in versene contained 14 and 15 per cent. It was concluded that no appreciable amount of iron could reflux from the cell even in the presence of unsaturated transferrin or other chelates.

An attempt was then made to obtain positive evidence that the radioiron was derived from broken cells. A tagged marrow suspension of high activity was obtained by employing doses of 200 $\mu c.$ to 500 $\mu c.$ of Fe^{59} in donor rabbits. The supernatant fraction from this marrow suspension was concentrated by lyophilization. The centrifuged cells of the suspension were broken by repeated freezing and thawing. The two fractions, containing similar concentrations of radioactivity, were subjected to paper electrophoresis of pH 7.0, 6.5 and 8.6. Both showed a similar distribution of radioactivity among protein fractions (Figure 1). In the supernatant fraction, 80 per cent of the activity moved in the hemoglobin band, while 74 per cent of the activity of the broken cells was in the hemoglobin fraction. It was concluded that the form of radioiron in the supernate was consistent with derivation from broken cells and that most of the radioactivity was present as hemoglobin. Centrifuging the supernatant fraction at 2,000 G for one hour did not result in any sepa-

TABLE I

*Distribution of various nucleated cell types of human marrow **

	Direct smear	In marrow suspension
Granulocytic forms	56.0	56.4
Segmented	12.2	12.4
Band	20.4	20.7
Metamyelocyte	12.9	11.7
Myelocyte	10.5	11.5
Lymphocytic and miscellaneous	4.6	4.1
Eosinophils and basophils	2.4	2.7
Blasts	0.1	0.0
Nucleated red blood cell	35.0	31.7
Smudges	1.9	5.1

* Counts listed are an average of five marrow preparations with counts of 4,000 nucleated red cells on each. Figures are expressed on a percentage basis.

ration of radioactivity, so it was unlikely that the activity was contained in cytoplasmic particles of any appreciable size.

It is our impression that losses due to cell breakdown in the suspension are unavoidable with the present technique. Variations in the filtration process or in the frequency and force of shaking had surprisingly little effect on the amount of radioactivity in the supernatant fraction. Centrifugal forces up to 1,800 G applied to the cell suspension for periods of 15 minutes did not result in an increased radioactivity in the supernatant fluid after mixing and recentrifugation of the marrow cells.

The next consideration was whether or not destruction of marrow cells and loss through filtration altered the relative incidence of the various cell types in the suspension counted. Smears were made of cells remaining on the nylon filter, and differential counts were compared with those of the final cell suspension. While no significant difference was observed, except for disappearance of megakaryocytes from the suspension, the differential counts made from the filter were not considered satisfactory for accurate counting.

Studies were then carried out on five ribs obtained from patients at operation. The nucleated cell differential (4,000 nucleated cells counted) on direct smear was compared with that of the cell suspensions. The values obtained (*cf.* Table I) are interpreted to indicate no recognizable alteration in frequency of nucleated cell types.

Since certain calculations assume that all mature erythrocytes in the marrow suspension represent circulating blood, it was considered desirable,

SOURCE OF SUPERNATANT RADIOACTIVITY

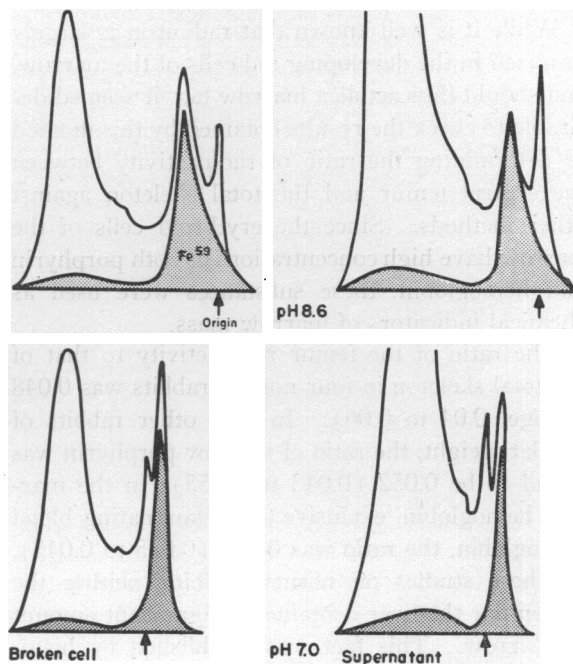


FIG. 1. PROFILES OBTAINED BY PAPER ELECTROPHORESIS OF A CONCENTRATED SOLUTION OF THE SUPERNATE OF A MARROW CELL SUSPENSION (RIGHT) AND THE LYSSED MARROW CELLS (LEFT)

The clear area is the densitometry reading of the stained protein, the cross-hatched area indicating the distribution of radioactivity in the same strip. It will be observed that a similar relation is found between radioactivity and protein profiles in the two preparations run at two different pH concentrations.

if possible, to verify this assumption. Mature erythrocytes tagged with Fe^{59} were injected into rabbits four to six minutes before sacrifice. To provide extreme conditions, one group of rabbits was made anemic by repeated bleedings and a second group was made polycythemic by transfusion several days before injection of tagged cells. The specific activity of adult red cells in circulating blood and in marrow suspensions for these two groups of animals was determined. The ratio of marrow specific activity to blood activity in six rabbits rendered polycythemic by transfusion was 1.03 plus or minus 0.18 and 1.19 plus or minus 0.12 in four rabbits made anemic by bleeding. These data gave no indication of sequestration of erythrocytes in the marrow, for such cells unmixed with recently injected red cells would result in a specific activity less than unity.

2. Determination of total marrow

While it is well known that radioiron is largely localized in the developing red cells of the marrow, and should thus act as a marrow tag, it seemed desirable to check the results obtained by this method of determining the ratio of radioactivity between the central femur and the total skeleton against other methods. Since the erythroid cells of the marrow have high concentrations of both porphyrin and hemoglobin, these substances were used as chemical indicators of marrow mass.

The ratio of the femur radioactivity to that of the total skeleton in four normal rabbits was 0.048 (range, 0.04 to 0.06). In four other rabbits of similar weight, the ratio of marrow porphyrin was found to be 0.052 (0.043 to 0.055); in the marrow hemoglobin, exclusive of contaminating blood hemoglobin, the ratio was 0.040 (0.035 to 0.045). In these studies on normal rabbits, neither the spleen nor the liver contained a significant amount of marrow. This fact was established by histologic examination, and, in the case of the spleen, by finding less than 0.05 per cent of the injected radioiron in the spleen as compared to 59 per cent in the skeletal marrow.

Skeletal localization of radioiron is an important consideration when the radioiron method is extended to man. In animals studied, the skeletal localization of radioiron at the time of minimal blood activity was determined by direct carcass analysis. Since it was impossible to make similar studies in man, data from the three animal species were used to derive the per cent localization in man. Distribution of radioactivity between skeleton and other body tissues and in circulating blood at the time of sacrifice is shown in Table II. Since there were varying amounts of activity not

yet deposited in body tissues, allowance was made for this on the basis of distribution already present in the tissues. These corrected figures then indicated a marrow localization of 70 per cent in the rat, 69 per cent in the rabbit and 66 per cent in the monkey. It was assumed on this basis that two-thirds of the activity in man would be similarly localized in the erythroid marrow. This refers only to the tissue-marrow distribution at the time of minimal blood activity and only in the normal steady state. It is known that in all of these species, including man, only about 10 per cent of the injected radioiron remains in tissues at two to three weeks after injection. In four patients followed subsequent to the determination of marrow cellularity, 88 per cent of the injected radioactivity was estimated to be present in circulation at three to five weeks.

3. Cellularity of the marrow in animals and man

The data in rats, rabbits, monkeys and man obtained by the radioiron method described are summarized in Table III. The results are expressed in cells per Kg. of body weight to permit comparison between species.

DISCUSSION

The measurement of marrow cellularity requires the preparation and accurate counting of a marrow aliquot and the relation of this marrow aliquot to total marrow. This report is concerned largely with the problems presented by such a method which has previously been briefly described by Suit (4).

It is assumed that marrow is reasonably homogenous throughout with respect to its composition of hematopoietic cells. While Kindred (1)

TABLE II
Carcass localization of radioiron

Species	Number of subjects	Time after radioiron injection	Skeleton	Tissues	Blood	Calculated total skeletal activity*
		<i>hrs.</i>	<i>%</i>	<i>%</i>	<i>%</i>	<i>%</i>
Rat	4	5	56 (49-67)	24	20 (17-22)	70
Rabbit	4	6	59 (56-65)	26	15 (2-18)	69
Monkey	4	12	64 (53-84)	32	4 (2-8)	66
Man	8	15			2 (1-6)	

* This figure arrived at by assuming a distribution of blood activity between skeleton of other body tissues similar to that of the isotope already localized extramuscularly.

has suggested a 10 per cent difference in the relative number of nucleated erythroid cells in the long bones versus central marrow, a difference of this magnitude is not considered to be critical in the present study. Differential counts of hematopoietic cells performed on human marrow from two or more areas have no important variations. It has been our experience that a marrow aliquot of some size is required for determination of marrow cellularity as outlined here. In these studies on animals and man, in excess of 200×10^6 nucleated cells were contained in the cell suspension. Studies with marrow aspirates containing fewer nucleated cells and more peripheral blood contamination have as yet been unreliable in our laboratory. Since Suit (4) has obtained figures similar to our own employing considerably smaller amounts of radioactivity and obtaining human marrow by needle aspiration, it seems likely that this method may be adaptable to smaller marrow samples.

Surprising numbers of marrow cells, approximately 50 per cent, were left in the bony shell or filter, or were broken down in the cell suspension. The recovery of marrow cells probably could have been improved by more complete filtration of the cells through the nylon filter. Variations in the specific procedure used in preparation of the suspension, *i.e.*, filtration and shaking, seemed to have little effect on the degree of cell breakdown. There was no evidence from differential counts on smears made directly from the marrow, from the cells remaining on the nylon filter, or from the marrow suspension, that there had been any measurable change in the ratio of nucleated hematopoietic elements through the preparation of the suspension. Not only the erythroid-myeloid ratio, but also the myelocytic differential count remained essentially unchanged. Evidence has been presented elsewhere (11), however, that the destruction of nucleated cells is appreciably greater than that of non-nucleated erythrocytes in the suspension.

The contamination of the marrow suspension with leukocytes could be determined from the peripheral leukocyte count and the number of mature erythrocytes in the suspension. By these calculations the circulating granulocyte contribution from the blood was less than 0.1 per cent of the

TABLE III
Marrow cellularity

Species	Number of subjects	Nucleated marrow cells/Kg. $\times 10^6$	
		Average	Range
Rat	4	17	13-22
Rabbit	5	12	9-18
Monkey	5	34	22-44
Man	7	18	11-30

myelocytic cells in the marrow preparation. Likewise, the contribution of radioactivity from the circulating blood in marrow suspension present was less than 1 per cent in all species studied and could therefore be ignored in calculations.

One can accept the radioiron values obtained from marrow aliquot and total skeleton in animals with some assurance, since they represent direct measurements and agree with two other marrow tags (hemoglobin and porphyrin). The use of this procedure in man, however, involves an additional assumption, *i.e.*, that a given amount of iron has localized in the marrow. A figure of two-thirds was arrived at by comparison of data in three animal species. The agreement of the figure obtained in monkeys with the figures obtained in other animals is of particular significance, since activity in circulation at the time of marrow sampling is quite similar to that in man.

The data obtained in these four species are meaningful when they are broken down into figures for erythrocytic and granulocytic cell series. This will be done in detail in a following article (12). It is of interest, however, to compare the over-all figures presented here, varying from 12 to 34×10^6 nucleated marrow cells per Kg. body weight with results obtained by different methods. Kindred (1) found 25×10^6 cells per Kg. in rats, and Patt (3) estimated 49, 17 and 12×10^6 per Kg. for the combined nucleated erythroid and myeloid cells of the marrow of rats, dogs and man, respectively. Osgood (2) estimated 34×10^6 erythroid and myeloid cells per Kg. in man, and Suit (4), employing the technique used here, found 16×10^6 marrow cells per Kg. body weight in man. These various data therefore are in general agreement concerning the size of the total hematopoietic cellular mass.

SUMMARY

A method has been described for determination of the total cellularity of hematopoietic tissue of the marrow, exclusive of megakaryocytes. This has been accomplished by determining the number of cells in an aliquot of marrow and by relating this aliquot to the total marrow. Observations concerning possible technical errors in the method and results found in three species of animals and in man have been described.

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