Comparisons between Bone Marrow Differentials Prepared from Particles and from Random Samples of Aspirate and Determinations of the Dilution of Aspirate with Peripheral Blood Utilizing Radioactive Phosphorus (P³²)

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With the technical assistance of ROSALYN YALOW, PH.D.

The study of bone marrow during life by means of sternal aspiration was first described by Arinkin in 1929. Because of the simplicity and applicability of this procedure it rapidly became an indispensable tool for hematologic evaluation. As is common with any new procedure, different methods of preparing the aspirate were described, with attendant varying interpretations of bone marrow activity. Comparisons of normal bone marrow differentials as reported by several authors and as tabulated by Leitner illustrate that cellular representation varies with the method of preparation.

Young and Osgood advised that differentials be made from smeared random samples of well mixed aspirate fluid. Vogel, Erf and Rosenthal described two methods for preparing differential smears, each made from random samples of the aspirate, and stated that such preparations well represented the bone marrow. They added, however, that when the aspirate exceeded 0.1 to 0.2 cc., there occurred an unpredictable degree of dilution with peripheral blood. Damashek and his co-workers compared random sample smear differentials to trephine biopsy differentials and concluded that aspirate was a part way station located between the peripheral blood and the bone marrow in which was present a variable number of bone marrow cells. Reich, Gordon, Custer, and Limarzi described methods of preparing differential smears from the "buffy coat" of oxalated, centrifuged aspirate fluid. Limarzi stated that such preparations contained marrow elements from which were separated mature erythrocytes. Weller, Davidson, Davis and Innes, Propp, Gorham and Kantor, and others described methods of preparing differential smears from selected particles in the aspirate fluid. Weller stated that such preparations were representative specimens of bone marrow without the distorting influence of peripheral blood. Osgood and Seaman criticized such particle differentials, claiming that adhesive differences among bone marrow cells distorted their proportionality in the differential counts.

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Reviewed in the Veterans Administration and published with the approval of the chief medical director. The statements and conclusions published by the authors are the result of their own study and do not necessarily reflect the opinion or policy of the Veterans Administration.

The authors wish to thank Dr. Bernard Straus and Dr. Bernard Roswit for their cooperation and suggestions and Mr. Sidney Shapiro for his assistance and cooperation in preparing the photomicrographs and illustrations contained herein.
Estimations of bone marrow cellularity have been made from the gross appearance of the aspirate fluid (the size and number of particles), from the height of the "buffy coat" of oxalated, centrifuged aspirates, and from the total nucleated counts of random samples of the aspirate. According to Vogel, Erf and Rosenthal, total nucleated counts reflected the cellularity of the bone marrow with respect to its hypoplastic, normal, or hyperplastic character, but they added that the gross appearance was of little value in determining such cellularity. Other authors stated that wide variations in total nucleated counts occurred among normal individuals and even among several counts performed upon different samples of the same aspirate. Scott and Custer stressed the unreliability of total nucleated counts as estimates of cellularity. Limarzi stated that the height of the "buffy coat" of oxalated, centrifuged aspirates was an accurate estimate of bone marrow cellularity. Schleicher preferred to estimate bone marrow cellularity from the number and size of particles in the aspirate fluid, large particles in the aspirate or aspirates composed largely of particles indicating hyperplastic marrows, and conversely, few or no particles in the aspirate indicating hypoplastic or aplastic marrows. Leitner utilized both total nucleated counts and gross appearances to estimate bone marrow cellularity.

The lack of unanimity of how best to determine bone marrow activity has resulted in conflicting descriptions of normal and abnormal marrows. In an effort to arbitrate these differences we compared random sample smear differentials to particle smear differentials and total nucleated counts to gross appearances. During these studies the importance of aspirate dilution with peripheral blood became manifest, and determinations of such dilutions were made. It is the purpose of this report to describe the results of these studies.

Comparison between Bone Marrow Differentials Prepared from Particles and from Random Samples of Aspirate

To compare the differentials obtained from particle smears and from random sample smears of the same aspirate, 50 consecutive cases with various diseases referred to the Hematology Service for evaluation were studied. Particle smears were prepared from discrete particles in the aspirate; random sample smears were prepared from random samples of the aspirate, particles being disregarded.

Methods and Materials

The skin overlying the sternum was shaved, cleansed, and painted with an antiseptic. A 14 gage Turkel needle with stilet was introduced 1 to 3 millimeters beneath the inner table of the cortex of the sternum at the level of the second or third interspace. The stilet was removed; 0.1 to 0.3 cc. of bone marrow fluid was aspirated into a dry syringe; the stilet was replaced, and the aspirate expelled onto a clean glass slide. A white blood cell counting pipet was immediately loaded with the aspirate fluid and subsequently counted. An estimate of the cellularity of the bone marrow was made by one of us (R.S.F.) dependent upon the number and size of the particles in the aspirate. When marrow particles were absent, additional specimens were aspirated. If sufficient particles were present, the needle was withdrawn and the wound covered with a pressure bandage. Smears were prepared from the aspirate fluid by the following methods:

I. (a) Discrete particles were carefully removed from the aspirate fluid with a capillary
pipet and placed on a clean coverglass. A second coverglass was placed over the first, moderate pressure exerted, and coverglass smears drawn. The coverglass smears were fixed for three minutes with Wright's stain; buffered distilled water was added to the fixed smears, and the smears stained for ten minutes. The smears were dried between sheets of fine filter paper and mounted in balsam on clean glass slides.

(b) When particles were not available in the aspirate fluid random samples of aspirate fluid were used for coverglass smears in a manner identical to (a).

II. A corner of a clean glass slide was dipped into the aspirate fluid at random and the wetted corner placed in the center of a second clean glass slide. Smears were prepared by forward propulsion of the first slide upon the second. The smears were fixed with Wright's stain for two minutes, buffered distilled water was added, the smears were stained for three to five minutes, and dried in air.

Results

A. Gross Appearance Versus Total Nucleated Count

Estimates of bone marrow cellularity as obtained by the gross appearance of the aspirate fluids were compared to the total nucleated counts of the same fluids. These comparisons are tabulated in table 1. It was noted that there was some degree of correlation between the averages of the total nucleated counts and the number of particles in the aspirate fluid. The ranges in the total nucleated counts and the appearance of the aspirates in 2 cases of acute leukemia showed that this correlation did not always exist.

B. Comparisons between Particle Smear and Random Sample Smear Differentials

Differential counts were made on a representative smear prepared by each method from the same specimen of bone marrow aspirate. Both preparations of each specimen were counted by the same individual. In all instances a minimum of 500 nucleated cells was counted in each differential. When possible 1000 to 1500 cells were counted from several different areas. The cases were divided into seven groups for the purpose of comparison on the basis of the following arbitrary criteria:

1. Normally Plastic Bone Marrow. In each of these 10 cases the bone marrow aspirate was normally cellular as determined by both the gross appearance and the total nucleated count. The resulting differentials failed to reveal any abnormality in the cellular elements.
2. Hypoplastic Bone Marrow with Particles. In each of these 5 cases the bone marrow was hypoplastic as determined by gross appearance and total nucleated count, and particles were available in the aspirate.

3. Hypoplastic Bone Marrow without Particles. In each of these 5 cases the bone marrow was hypoplastic as determined by the total nucleated count, but particles were not available in the aspirate fluid.

4. Hyperplastic Bone Marrow in Myelocytic Leukemia and Leukemoid Reactions. In each of these 10 cases the bone marrow aspirate was hyperplastic as determined by the gross appearance, and the peripheral blood revealed a leukocytosis with a "shift to the left" in the granulocytic series.

5. Hyperplastic Bone Marrow in Chronic Anemia (All Kinds). In each of these 10 cases the bone marrow aspirate was hyperplastic as determined by the gross appearance, and the peripheral blood revealed an anemia of some variety other than macrocytic.

6. Hyperplastic Bone Marrow in Macrocytic Anemia. In each of these 5 cases the bone marrow aspirate was hyperplastic as determined by the gross appearance, and the peripheral blood revealed a macrocytic anemia.

7. Bone Marrow in Lymphoma. In each of these six cases a diagnosis of Hodgkin's Disease or lymphosarcoma was established by biopsy either before or after the bone marrow aspiration.

Averages of each cell type in each group were determined for both particle smear (Method I) and random sample smear (Method II) differentials. These averages were compared and are graphically represented in figures 1, 2, and 3. Several distinct differences were evident from these comparisons:

(a) Differentials obtained from particle smears revealed consistently greater percentages of immature cells of the granulocytic and erythrocytic series than did those from random sample smears.

(b) Differentials prepared by Method I were essentially identical to those of Method II when particles were not available in the aspirate.

(c) Differentials obtained from particle smears revealed fewer lymphocytic cells than did those from random sample smears in instances in which there was no primary disease of the lymphatic system.

(d) In certain instances in which there was a primary disease of the lymphatic system differentials obtained from particle smears revealed greater percentages of lymphocytic cells than did those from random sample smears.

(e) Differentials obtained from particle smears revealed certain cellular elements of critical diagnostic significance which were not apparent in those from random sample smears (see fig. 4). This observation was made in the following six cases, the differentials of which are tabulated in table 2.

Cases

Case 5 (Hospital File #118166). A 56 year old white male complained of low back pain and loss of weight. Positive physical findings were limitation of motion due to pain and evidence of weight loss. Initial laboratory data were negative. Roentgenograms revealed a collapsed seventh thoracic vertebra and lytic lesions in the scapula and in the third and seventh ribs posteriorly. Sternal marrow differential by Method I revealed 8.4 per cent plasmacytic cells which were not apparent in that of Method II. Biopsy of a rib lesion was not diagnostic. Bence-Jones protein was never demonstrated in the urine, serum calcium and phosphorus were normal, and serum protein fractionations were normal. No primary malignancy could be demonstrated in any organ system. Because of the possibility of multiple myeloma, the patient was treated with urethane with definite subjective improvement.
COMPARISON OF BONE MARROW DIFFERENTIALS

Fig. 1.—Analyses of bone marrow differentials. (Solid black, Method I; stippled shading, Method II.)
**ROBERT S. FADEM AND IRVING BERLIN**

![Graph](image)

**Fig. 2.** Analysis of bone marrow differentials. (Solid black, Method 1; stippled shading, Method II.)

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**Fig. 3.**—Analyses of bone marrow differentials in six cases of lymphoma.

### Table 2.—Differentials by Method I and Method II in Six Cases

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Fig. 4.—Photomicrographs of bone marrow smears by Method I in six cases. (A is X 90; all others are X 810.)

A. Bone marrow showing selected area for differential counting.
5. Plasmocytes in Case 5.
10. Lymphocytic cells in Case 10.

B. Selected area of A.

Case 9 (Hospital File 113983). A 26 year old male complained of weakness, fatigability, anemia, and undue susceptibility to infections of two years’ duration. Five months prior to hospitalization a diagnosis of idiopathic thrombocytopenic purpura had been made and splenectomy performed without benefit. Physical examination revealed numerous petechiae on the lower extremities and a positive Rumpel-Leeds test. The peripheral blood revealed
an anemia, leukopenia and thrombocytopenia. Clot retraction was poor. Sternal marrow aspirations were performed and the marrow found to be hypoplastic. Differentials by Method I revealed numerous mast cells which were not apparent in those of Method II. A diagnosis of aplastic anemia was made and the patient died after a long hospitalization characterized by episodes of bleeding and repeated infections. Postmortem examination confirmed the clinical diagnosis.

Case 10 (Hospital File #122869). A 36 year old white male complained of massive swelling of the left leg. In 1946, biopsy of an axillary lymph node revealed lymphosarcoma, and the patient received periodic x-ray therapy to recurrently enlarged nodes. Positive physical findings revealed a generalized lymphadenopathy, an enlarged spleen, and a massively swollen left leg. The peripheral blood was normal except for a leukopenia. Sternal marrow aspiration was performed. The differentials of Method I revealed a large proportion of the cellular elements to be mature and immature lymphocytic cells which were not apparent in those of Method II.

Case 13 (Hospital File #118941). A 57 year old white male complained of headache and swelling of the legs. Positive physical findings included a tachycardia and a hepatosplenomegaly. Laboratory studies revealed an anemia, thrombocytopenia, leukocytosis and a marked “shift to the left” in the peripheral blood differential with occasional nucleated red cells. Several bone marrow aspirations failed to reveal particles and differential preparations revealed no abnormality. Subsequently a particle was aspirated and a differential by Method I revealed many immature plasmacytes. Roentgenograms demonstrated lytic lesions in the skull, compression of the eleventh thoracic vertebra, and a diffuse osteoporosis of the vertebral bodies and pelvis. A diagnosis of multiple myeloma with extramedullary hematopoiesis was made. The patient gradually deteriorated and died. Postmortem examination confirmed the clinical diagnosis.

Case 16 (Hospital File #118444). A 53 year old white male with pernicious anemia in relapse was found to have 5.4 per cent rubriblasts in his bone marrow by Method I. He was treated with oral vitamin B12. Two weeks after therapy was instituted another bone marrow aspiration was performed. The differentials by Method I revealed the persistence of rubriblasts which were not apparent in those of Method II. He was subsequently treated with parenteral vitamin B12 and made an uneventful recovery.

Case 14 (Hospital File #118662). A 56 year old white male was referred to the medical service because of a persistent anemia following a hemorrhoidectomy. Sternal aspiration was performed and differential preparations by Method I revealed many immature plasma cells which were not apparent in those of Method II. Roentgenograms revealed circumscribed radiolucent lesions in the skull, femori, humeri, and ribs. Total serum proteins were 10 Gm. per cent with 6.2 Gm. per cent of globulin. Bence-Jones protein was never demonstrated in the urine. A diagnosis of multiple myeloma was made.

2. Quantitative Determinations of the Dilution of Bone Marrow Aspirate with Peripheral Blood Utilizing Radioactive Phosphorus (P32)

To determine quantitatively the dilution of bone marrow aspirates with peripheral blood a method recently described for the estimation of whole blood volume was employed. Such determinations were performed in 10 unselected cases.

Methods and Materials

A. Dilution of Bone Marrow Aspirates With Peripheral Blood

Ten cubic centimeters of heparinized whole blood withdrawn from the antecubital vein was incubated in a centrifuge tube for two hours at 37 C. with 50-150 microcuries of radio-
active phosphorus ($P_{32}$). The activated blood was centrifuged, the supernatent fluid drawn off and discarded, and the activated red cells were washed twice with normal saline. The washed activated cells were reconstituted to their original volume with normal saline, mixed well by inverting several times, and reinjected intravenously into the same subject. Bone marrow puncture was performed, and simultaneous peripheral blood and bone marrow samples were obtained in heparinized syringes ten to fifteen minutes following the injection of the activated cells. The radioactivity of each sample was determined with a Geiger-Mueller counter using identical geometries, and the radioactivity per unit volume of peripheral blood and of bone marrow compared.

In 3 cases the bone marrow was aspirated in two portions, the first measuring 0.1 to 0.2 cc., and the second measuring 1.0 to 3.0 cc. The radioactivities of each of these samples were compared to that of peripheral blood withdrawn simultaneously.

B. Dilution of Aspirate Particles With Peripheral Blood

To determine whether aspirate particles contain peripheral red blood cells the following procedure was employed using the identical technic described above. Five cubic centimeters of bone marrow fluid was aspirated into a heparinized syringe. The radioactivity of 0.5 cc. of this material was determined. The remaining red cells were hemolyzed with 0.1 N hydrochloric acid and the particles separated from the hemolysate. The particles were washed three times with normal saline, and their radioactivity was determined. The radioactivity of an appropriate sample of hemorrhysate was determined. These activities were compared to that of the initial 0.5 cc. aspirate sample.

Results

The dilution of bone marrow aspirate with peripheral blood following the injection of activated red blood cells into the peripheral circulation can be expressed in per cent according to the formula:

$$\frac{\text{Radioactivity of the bone marrow aspirate per cc.} \times 100}{\text{Radioactivity of the peripheral blood per cc.}} = \text{per cent peripheral blood}$$

These studies revealed that there was a dilution of bone marrow aspirate fluid with peripheral blood varying from 61 to 96 per cent (see fig. 5). That the
variations were dependent, in part, upon the total amount of bone marrow aspirated was shown by the second part of Procedure A. In 3 cases, the amount of dilution with peripheral blood observed when small 0.1 to 0.2 cc. samples were aspirated was less than when larger 1.0 to 3.0 cc. samples were aspirated. Such differences were not directly proportional to the amount aspirated (see fig. 5).

Determinations of the radioactivity of the hemolysate and the particles in Procedure B revealed that of the hemolysate to be 100 per cent of that of the initial aspirate sample, while that of the particles was equal to only that of "background." The technic employed, sensitive to 0.5 per cent of the activity of the initial aspirate sample, indicated that there was no detectable P32 in the particles.

DISCUSSION

The results of these studies leave us in firm agreement with Davidson and his associates in their observations that smears made from aspirate particles more accurately represent the bone marrow than do those made from random samples of the aspirate fluid. We noted that with experience, cellular fields could always be found in such particle smears where the cell morphology was excellent, where the normal architectural relationships of the cellular elements were preserved, and where there was little admixture with mature erythrocytes. This method, in our hands, resulted in a high degree of reduplicability of differential counts from different smears of the same aspirate; this was by no means true of random sample smears. Particle smear differentials never failed to reveal cellular elements of diagnostic significance which were revealed in random sample differentials. However, the reverse was true in 6 cases in which cellular elements of primary importance were revealed only in particle differentials.

When particles were not found in the first aspiration, continued aspiration or additional aspirations were usually more fruitful. This proved to be true in several instances in which only after multiple aspirations were particles obtained and diagnoses established. In those rare instances in which particles were not found even after repeated aspirations, differentials from both methods were identical. This demonstrated that the observed differences between particle smear and random sample smear differentials were not inherent in the technics of preparation, but rather, that they were due to specific differences in cellular composition.

In those cases in which there was no primary disease of the lymphatic system, particle differentials revealed significantly fewer lymphocytic cells than did random sample differentials. The higher percentages of lymphocytic cells in random samples were of the mature variety. We felt that such cells were a reflection of the peripheral blood included with the sampling. In 6 cases in which there was a primary disease of the lymphatic system, Hodgkin's disease or lymphosarcoma, we found greater percentages of lymphocytic cells in particle differentials than we did in random sample differentials. These lymphocytic cells were both of the mature and immature variety and were distributed intimately among the normal cellular elements. Because of their appearance and
distribution in the particle smears we felt that they reflected an infiltration of
the bone marrow. In Hodgkin’s disease, most investigators have reported the
bone marrow to be nonspecific,\textsuperscript{24} but Klima\textsuperscript{23} and Varadi\textsuperscript{26} have reported in-
creased numbers of lymphocytic cells in the bone marrow in this disease. In
lymphosarcoma, too, the findings of different workers has been at variance and
inconclusive. Most workers have found some increase of lymphocytic cells in
the bone marrow in this disease.\textsuperscript{27} A study to determine the significance of
lymphocytic cells in the bone marrow in a number of diseases is now in progress.

We cannot state whether the cellularity of the bone marrow is best determined
from the gross appearance of the aspirate, size and number of particles in the
aspirate, or from total nucleated counts performed on the aspirate. Our studies
revealed that the averages of the total nucleated counts correlated closely with
the estimates of cellularity based upon the gross appearance. The ranges of
total nucleated counts were so wide that any single count was unreliable unless
it expressed hypercellularity, i.e., greater than 100,000 cells per cubic milli-
meter. Hypercellular counts expressed the bone marrow cellularity in 2 cases
of acute leukemia when the gross appearance of each was consistent with a
hypoplastic bone marrow, particles being few in number. However, often we
found that the gross appearance agreed more closely with the microscopic ap-
pearance of the marrow than did the total nucleated counts.

Our study whereby we determined the distribution of “tagged” red blood
cells in the peripheral circulation as compared to the bone marrow aspirate
demonstrated conclusively that the aspirate was to a large and unpredictable
degree diluted with peripheral blood. Even in small 0.1 to 0.2 cc. aspirates there
was considerable dilution with peripheral blood. That radioactivity was not
demonstrated nor detected in the aspirate particles indicated that they were
free from the distorting influence of peripheral blood cells.

These observations with “tagged” red cells provided us with a partial explana-
tion of the differences between particle smear and random sample smear differen-
tials and between estimates of bone marrow cellularity from gross appearances
and total nucleated counts. The dilution we demonstrated affected differentials
prepared from random samples of the aspirate by the inclusion of peripheral
blood leukocytes. Such differentials indicated the proportions of cellular ele-
ments in the aspirate fluid rather than those of the bone marrow. We believe
that the greater numbers of mature granulocytes and lymphocytes in random
sample smear differentials expressed this dilution. Total nucleated counts meas-
ured the number of cells in a unit of aspirate fluid and did not necessarily reflect
the number of cells contained in the same unit of bone marrow.

Peripheral blood dilution of aspirate does not explain all of the differences
we have described. Other factors which we believe are important include the
trauma of the aspiration and the adhesive and cohesive qualities of the marrow
cellular elements.

\textbf{Summary}

Estimates of bone marrow cellularity based upon gross appearances and
upon total nucleated counts of the aspirates were compared in 50 cases. These
comparisons revealed some correlation between the gross appearances and the averages of the total nucleated counts. That this correlation did not always exist in individual cases was demonstrated by the wide range in total nucleated counts and by incorrect gross appearance estimates in two cases of acute leukemia.

Differential counts as obtained from particle smears and random sample smears of the same aspirates were compared in 50 cases. Such comparisons revealed certain consistent differences in cellular elements. These differences and their significance were discussed.

The dilution of aspirate fluid and aspirate particles with peripheral blood was determined in 10 cases. Appreciable and unpredictable dilution of aspirate fluid was demonstrated, but none was demonstrated in aspirate particles. This dilution of aspirate fluid with peripheral blood is implicated as the cause of the inaccurate estimations of bone marrow cellularity based upon gross appearances and total nucleated counts. It is also suggested as one of the factors underlying the observed differences in the cellular elements of particle smear and random sample smear differentials.

CONCLUSIONS

1. Neither the gross appearance nor the total nucleated count of the aspirate fluid is a reliable estimate of bone marrow cellularity.
2. Aspirate fluid is composed of a large and unpredictable amount of peripheral blood in which is suspended bone marrow particles.
3. Particle smear differentials determine the patterns of the cellular elements of the bone marrow better than do random sample smear differentials.

ADDENDUM

Recently Berlin, Hennessy, and Gartland have reported their studies on the dilution of aspirate fluid with peripheral blood. They found diluting factors which agree with those reported herein. However, their studies led them to conclude that a total nucleated count of an aspirate fluid was significant "in determining the reliability of a bone marrow differential."

The conclusions drawn by these authors depended upon two considerations which are subject to critical evaluation:

1. As has been shown the adhesive properties of marrow cells differ so that their proportion on glass slide smears (or on coverglass smears) may be different from their proportion in aspirate fluid. This means that the dilution factor, $D$, determined by radioactive measurements of aspirate fluid, does not necessarily express the dilution factor of a cell in a given smear.

2. In interpreting the ratio, $S$, between the observed percentages of $X$ type cells in a marrow and the percentages of the same cells in the peripheral blood, $X_p$, the authors stated that $S$ varied from 0 to 2. This is incorrect because their equation reveals $S$ to vary from $\frac{1}{4}$ to infinity. Notwithstanding, their curves were plotted correctly for values of $S$ greater than $\frac{1}{4}$. It is obvious from their graph that curves, drawn with $S$ equal to values less than $\frac{1}{4}$, deviate considerably from the value $X/Y = 1$ when $T/WBC = 5$ and invalidate their conclusions.

Our disagreement with Berlin and his co-workers has no bearing upon diagnostic results achieved in many diseases of the hematopoietic system such as chronic myelogenous leukemia, chronic lymphatic leukemia, and polycythemia vera. However, for diagnoses in
obscure diseases of the hematopoietic system and for academic and research purposes, when subtle changes in percentages of certain cellular elements are of importance, reliability of bone marrow differentials must not be presumed dependent upon total nucleated counts of aspirate fluid.

REFERENCES


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Comparisons between Bone Marrow Differentials Prepared from Particles and from Random Samples of Aspirate and Determinations of the Dilution of Aspirate with Peripheral Blood Utilizing Radioactive Phosphorus (P32)

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