Quantitative studies of blood and bone marrow neutrophils in normal mice


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A number of methods have been described for quantitation of nucleated cells in the bone marrow of various species, most of which involve calculation of marrow cell numbers from indirect data (3, 27-29, 32, 35). Kindred (24) made painstaking counts of cells in sections of an entire bone. Yoffey and Parnell (39) described a method in which the number of nucleated cells was counted in a weighed aliquot of surgically removed bone marrow. A number of investigators (9, 11, 17, 21, 22, 26) have employed this or similar technics in various species as a means of determining the concentration of marrow cells.

More recently, Fruhman (16) described a method by which all nucleated cells could be washed from the tibia of a mouse and counted, thus yielding a figure for the total marrow content within a given bone.

We evaluated the feasibility and accuracy of measurements of the total number of nucleated cells within the marrow cavity of a single bone (humerus) of mice by using a method similar to that of Fruhman (16). We studied 1) the reproducibility of the technic, 2) the completeness of nucleated cell washout from marrow, 3) the possibility that cells may be destroyed and not counted during the procedure, and 4) the proportion of the total marrow hematopoietic tissue that is contained in a humerus.

Leukocyte counts are reported to be extremely variable in mice and to be influenced by a variety of factors (6, 13, 15, 20, 23, 30, 31). Studies were undertaken to determine if consistent leukocyte counts could be obtained in normal mice and to delineate factors which may contribute to the reported inconsistencies in such counts.

MATERIALS AND METHODS

Female mice (C57Bl/6 X DBA/2) F1, bred in our laboratory from parent strains purchased from Jackson Laboratories, ranging in age from 8 to 12 weeks and weighing 18-30 g were used in all experiments. Mice were housed 8-10 per cage and Purina laboratory chow and water were available ad lib.

Bone marrow counts. Mice were killed by cervical dislocation. After the humerus was removed and freed of soft tissue attachments, the epiphyseal cap was detached from the proximal end and the extreme distal tip of each epicondyle (less than 1 mm) was cut off. A 26-gauge needle with an epidermal bevel was inserted into the proximal end of the humerus and to it was attached a syringe containing 5 ml (measured in a volumetric pipette) of a solution of 1% ethylenediaminetetraacetate (EDTA) in 0.9% saline. The solution was forced through the humerus, with the needle positioned in such a manner that fluid flowed freely from both ends of the bone. The cell washout was collected in a vial. The humerus, still mounted on the needle, was immersed in the fluid and approximately 2.5 ml was aspirated back through the humerus and then forced back through the bone into the vial. This was repeated until a total of approximately 15 ml of fluid was passed through the humerus. The cell
suspension was then aspirated three times through a 19-gauge needle into the original syringe in order to obtain a single cell suspension. If any part of the bone was broken or if any fluid was lost during the procedure, the preparation was discarded.

An aliquot of the cell suspension was diluted 1:100 in 1% cetrimide solution in order to lyse nonnucleated erythrocytes (18) and was counted electronically (Coulter Electronics model B, Hialeah, Fla.). Proper settings of the Coulter counter for aperture current, amplification, and threshold were determined empirically with the aid of a particle-size distribution plotter (Coulter Electronics). These settings vary somewhat with different instruments, but with our present model B instrument, settings were as follows: aperture current 3/4; amplification 3/4; lower threshold 30. No change in counts was observed in marrow cell suspensions which were allowed to stand for 2 hr at room temperature either before or after dilution with cetrimide.

The humerus was chosen for study in preference to the tibia or femur since, in a pilot study, better agreement was obtained between the number of cells washed from the right and left humerus of the same mice than between tibias (6 mice) or femurs (4 mice) (an average difference of 8, 23, and 25%, respectively). In addition, the total number of nucleated cells washed from the tibia does not reflect the normal concentration of hematopoietic tissue as will be discussed later.

Smears of bone marrow were made from the femur. After removing the femur and splitting it longitudinally, a portion of the marrow was removed and immediately dispersed in a drop of serum on a cover slip. Differential nucleated cell counts were determined from a 500-cell differential count of smears stained with Wright's stain, and the percent of myeloid cells was also determined from the percent of peroxidase-positive cells which were present (23).

Completeness of cell removal from the humerus. The effect of forcing 5, 10, 15, and 25 ml of fluid through the humerus on the amount of radioactive iron remaining in the bone and the total number of cells recovered was studied in groups of 5–15 mice. Six hours after the intraperitoneal injection of 0.5 µc 55Fe, mice were killed, humeri removed, and cells washed from the bone. The radioactivity remaining in the bone was determined by counting the bone before and after the washout procedure in a well-type gamma counter. Radioactivity in the washed-out bone plus the cell suspension did not exceed the original activity in the bone, indicating that the bone was counted as efficiently as the cell suspension. Passing 5 ml of fluid through the bone resulted in 32% of the original radioactivity remaining in the bone. This figure was reduced to 22% after 10 ml, 16% after 15 ml, and was not reduced further by flushing with 25 ml. More cells were recovered after passing 10 ml through the humerus than after 5 ml, 6.7 X 10⁶ as compared to 4.7 X 10⁶ but only a slightly larger increment in cell recovery was observed (7.0 X 10⁶) after 25 ml. Since the amount of radioactive iron remaining in bone reached a minimum after 15 ml of fluid was passed through the bone, this volume was selected for use in subsequent experiments.

In later experiments in a group of 25 mice injected with 10 µc of ⁵⁵Fe 6 hr before they were killed, an average of 9% of total radioactivity remained in the bone after 15 ml of fluid was passed through. We therefore concluded that at least 90% of the marrow cells were removed by this procedure.

Studies designed to determine if counts were lost due to cell destruction during the procedure. Thirty percent of the radioactivity flushed from the humerus was in the supernatant portion of the centrifuged cell suspension. This raised the possibility that cells were disrupted during the procedure and not counted. If the radioactive iron in the supernate was derived from disrupted cells, then presumably as manipulation of the cells was increased, more cells should break up and more iron should appear in the supernate. A comparison between the amount of iron in the cell-free supernate of marrow cell washouts after 5, 10, 15, or 25 ml of fluid were passed through the humerus did not reveal any significant difference in amount of radioactivity present (28, 27, 24, and 29%, respectively). A second study was done in which 5 ml of fluid was passed through the humerus with no further mixing of the cell suspension. Ten comparisons of the amount of iron in the supernate of such preparations to the amount of iron in supernates of a washout of the opposite humerus with 15 ml of fluid and subsequent vigorous cell dispersion failed to reveal any differences of significance.

The effect of ultracentrifugation, heat, and perchloric acid treatment on the iron in the supernate was determined. After centrifugation at 12,000 X g for 60 min, a procedure which should remove cytoplasmic particles, 84% of the original activity remained in the supernate fraction. Subsequent heating and centrifugation which should remove the fraction of iron due to hemoglobin and transferrin reduced the activity to 66%. Finally, after the addition of 10% perchloric acid which should remove iron present as ferritin, 29% of the activity remained. Of the residual activity in the supernate, 80–90% was dialyzable at an acid pH but less than 50% at a pH of 7.4. The last observation suggests that a significant proportion of the activity might be due to inorganic iron (10).

As a further attempt to determine if a significant number of cells were ruptured during the procedure, the amount of DNA and number of cells in the washout fluid was determined and DNA per counted cell calculated (8). DNA per milliliter of washout ranged from 30 to 33 µg/ml and the calculated value for DNA per counted nucleated cell was 6 X 10⁻¹² µg. Values of from 5 to 8 X 10⁻¹² µg of DNA per nucleus have been reported by others for cells from liver, kidney (37), and peritoneal exudate (19). If a large number of cells were disrupted and not counted by the washout procedure, an unusually high value for DNA per cell should have resulted.
BLOOD AND MARROW NEUTROPHILS

Estimate of total marrow hematopoietic tissue. The fraction of total hematopoietic tissue which is contained within the humerus was determined from the distribution of $^{59}$Fe in various parts of the skeleton (12, 35). Five hours after the intravenous injection of 0.1 $\mu$g $^{59}$Fe, animals were killed and eviscerated. They were then autoclaved for 2 hr following which bones were freed of soft tissue. The radioactivity in various parts of the skeleton, soft tissue, and blood was determined in a group of five mice.

Calculation of total hematopoietic tissue was based on the assumption that the $^{59}$Fe was distributed uniformly throughout the marrow. This assumption was tested in the following manner. Eight mice were injected with $^{59}$Fe and killed 6 hr later. The total number of nucleated cells and total marrow radioactivity was determined in the cell washout from the tibias, femurs, and humeri and radioactivity per nucleated cell calculated. Radioactivity per cell (counts/min per cell) was nearly the same in the humerus and femur (0.018 and 0.020, respectively) but significantly less in the tibia (0.012).

Gross examination as well as microscopic examination of sections of the distal tibia revealed a decreased amount of normal red marrow with predominantly fatty tissue and scattered lymphocytes. Differential nucleated cell counts on smears from the humerus, femur, sternum, and proximal tibia were virtually identical, but smears made from the most distal portion of the tibia contained less erythroid precursors and more lymphocytes.

Tibias were then cut in half and radioactivity per cell was determined for each half. The radioactivity per cell (counts/min per cell) in the proximal tibia was the same as the humerus (0.020 vs. 0.022) with little radioactivity present in the distal tibia (0.003). The exact nature of the cells washed from the distal tibia which accounted for approximately 40% of the total number was not determined. These data suggest that iron distribution in bone does reflect the distribution of marrow erythropoietic tissue. Since the myeloid-to-erythroid ratio was the same in different bones, the calculation of total marrow granulocytes from these data is probably justified. The discrepancy observed between iron per cell in total cell washouts from the tibia as compared to washouts of humerus and femur raises serious questions concerning the use of the tibia (1b) to measure erythroid and granulocyte changes in bone marrow.

Table 1. Distribution of radioactive iron ($^{59}$Fe) in the mouse skeleton

<table>
<thead>
<tr>
<th>Bone</th>
<th>Distribution of Counts in Specific Bones, % of Total Counts in Bone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spine</td>
<td>34.1±2.2</td>
</tr>
<tr>
<td>Rib cage</td>
<td>14.8±1.7</td>
</tr>
<tr>
<td>Skull</td>
<td>13.0±1.0</td>
</tr>
<tr>
<td>Femur (2)</td>
<td>11.8±0.4</td>
</tr>
<tr>
<td>Pelvis</td>
<td>11.7±0.3</td>
</tr>
<tr>
<td>Tibia (2)</td>
<td>6.9±0.3</td>
</tr>
<tr>
<td>Humerus (2)</td>
<td>3.8±0.3</td>
</tr>
<tr>
<td>Radius (2)</td>
<td>2.1±0.4</td>
</tr>
</tbody>
</table>

Values are means ± S.E.; N = 5 mice. * Includes fibula and hindpaws. † Includes ulna and forepaws.

In order to define the mitotic compartment of neutrophil precursors, tritiated thymidine ($^{3}$HTdr) (1 $\mu$g/g body wt) was injected intraperitoneally and 1 hr later mice were killed and autoradiographs prepared from marrow smears. The most mature cell which incorporated $^{3}$HTdr was a cell whose nucleus had a central opening of the diameter of which was slightly less than one-half the diameter of the doughnut nucleus.

The mitotic compartment was divided into the three following types of cells. The myeloblast was defined as a cell with fine nuclear chromatin and without a nuclear opening or cytoplasmic granules. Promyelocytes were defined as cells similar to the myeloblast but with azurophilic granulation and in which a small nuclear opening might or might not be evident. Myelocytes were defined as cells with neutrophilic granulation in which the nuclear hole had a diameter less than one-half the diameter of the doughnut nucleus.

The postmitotic maturation and storage compartment of marrow neutrophils was also divided into three morphological compartments. Segmented neutrophils were defined as cells which had a fine, filamentous strand separating the lobes of the nucleus. Band neutrophils were defined as cells in which beginning segmentation was evident but in which a fine filament had not yet formed. Metamyelocytes were defined as cells in which the diameter of the nuclear opening was equal to or more than one-half the diameter of the nucleus, but in which segmentation was not yet evident.

Blood leukocyte counts. Blood was collected in heparinized microhematoeit tubes from the orbital sinus, vena cava, aorta, and/or tail. Forty microliters of blood were diluted 1:500 with a 1% cetrimide solution and counts were done electronically in a Coulter counter. Blood leukocyte counts as determined in a Coulter counter were compared to counts of the same blood determined by standard hemocytometer technic. The mean count determined by the two technics was the same but there was less variation with the Coulter counter (Coulter 9,830 ± 150; technologist 1, 9,710 ± 720; technologist 2, 9,800 ± 610; technologist 3, 9,610 ± 340). The leukocyte count tended to increase following dilution if samples were allowed to stand for more than 1 hr.

Blood leukocyte counts were determined. A 200-cell differential count was done on Wright's stained smears made from the same sample of blood from which the leukocyte count was determined.
RESULTS

Total number of nucleated cells in the mouse humerus. In 130 humeri from normal mice, the mean and standard error of the total number of nucleated cells per humerus was 7.15 \times 10^6 \pm 0.8 \times 10^6. Although the number of cells tended to be higher in animals weighing 21–30 g (7.36 \times 10^6) than in animals weighing 16–20 g (7.16 \times 10^6), this difference was not statistically significant (P > 0.2).

Estimate of total marrow hematopoietic tissue. In determining the total marrow hematopoietic tissue from the distribution of \(^{59}\)Fe, 83 \pm 2.2% of injected radioactivity was recovered. Of the recovered radioactivity, 37 \pm 1.7% was present in bone with the major fraction in the spine, followed by decreasing amounts in the rib cage, skull, femur, pelvis, tibia, humerus, and radius (Table 1). Assuming that the \(^{59}\)Fe is distributed uniformly throughout the marrow, these data suggest that a humerus contains 2.8% of the total hematopoietic tissue in the mouse.

Differential nucleated cell counts in mouse bone marrow. Neutrophils and neutrophil precursors constituted 39% of marrow cells as determined on Wright's stained smears and 40% of cells on smears from the same mice which were stained by peroxidase (Table 2). Lymphocytes and erythrocyte precursors were the next most frequent nucleated cells and the myeloid-erythroid ratio was 2:1.

The mitotic compartment constituted 42% and the postmitotic maturation and storage compartment 58% of marrow neutrophils (Table 2). Forty-three percent of marrow neutrophils were bands and segmented cells. These constitute the effective storage compartment of the marrow since they are the only cells which are released into the blood in large numbers under ordinary circumstances (27).

There was no significant difference in differential counts of smears made from the femur, humerus, proximal tibia, and sternum of the same mice.

Blood leukocyte counts. In comparing leukocyte counts from various anatomic sites each mouse was bled only once from any single site. Mice were anesthetized with ether before obtaining blood from the aorta or vena cava. Ether anesthesia resulted in a decrease of approximately 50% in the total leukocyte count in orbital sinus blood with no change in the differential (Table 3). Anesthesia with pentobarbital resulted in a decrease similar to that observed with ether (Table 3). The leukocyte count in vena cava blood in anesthetized mice approximated that in orbital sinus blood and was considerably higher than in the aorta (Table 4). A slight but significantly higher (P < 0.05) percent of neutrophils was present in blood obtained from the aorta.

In nonanesthetized mice there was a slight but significantly (P < 0.05) higher count in orbital sinus blood.
in the afternoon (2:00–3:00 pm, 11,500 ± 200 cells/mm³ in 38 mice) compared to counts in the morning (9:00–10:00 AM, 10,000 ± 200 cells/mm³ in 21 mice). This slight increase in leukocyte concentration in the afternoon was due to an increase in lymphocytes with no increase in the absolute number of neutrophils.

Since clotting was an occasional problem even when blood was collected directly into heparinized tubes, the effect of injecting mice with heparin upon the leukocyte count was studied. One-half hour after heparin injection, the leukocyte count was twice as high as in saline-injected mice (Table 3).

When doing duplicate counts from two consecutive tubes of blood from the orbital sinus it was noted that the leukocyte count in the second sample was usually lower than in the first. Therefore, the total leukocyte count was determined in each of eight consecutive tubes of blood obtained from the orbital sinus in approximately 1 min (Fig. 1). A progressive decrease occurred until approximately the sixth sample, at which time the leukocyte count became relatively constant. No significant change in the differential count occurred in the consecutive samples and the proportion of neutrophils which were nonsegmented was not changed significantly. The leukocyte count from the seventh orbital sinus sample was 48% of that in the first sample in a group of 25 mice (part 1 of Table 5). This decrease was not merely a local effect in the sampled sinus for after removing six samples from one sinus, the seventh sample taken from the contralateral side yielded a similarly reduced value (part 2 of Table 3). The decrease in leukocyte count was not due to adherence of cells to the sides of the microhematocrit tubes since the same results were obtained if blood was collected directly from the sinus into a measuring pipette or into a microhematocrit tube.

Because of the variation in leukocyte count in consecutive samples from the orbital sinus and different anatomical areas (Table 3), a comparison of the leukocyte count was made in blood from the first and eighth orbital sinus samples and the aorta or vena cava of the same animal. After obtaining the eighth consecutive sample from the orbital sinus, the abdomen was opened and the ninth sample was taken from the aorta or vena cava. Although the counts in the eighth sample of orbital sinus blood approached that in the aorta, they were never equal (part 3 of Table 5).

Leukocyte counts in orbital sinus and tail blood were compared in several groups of mice (Table 6). In one group the orbital sinus was sampled first followed by the tail, and in another group the tail was sampled first followed by the orbital sinus. Although the leukocyte count tended to be higher in the tail if it was sampled first, this difference was not significant statistically ($P > 0.2$). No significant difference was noted in the differential count.

Heating the tail before obtaining blood samples resulted in a decrease in the total leukocyte count with no significant effect upon the differential count (Table 7).

**Discussion**

This method of washing all nucleated cells from the marrow cavity of the humerus, dispensing the cells to obtain a uniform cell suspension, and counting the nucleated cells in the suspension is patterned upon that reported by Fruhman (16) for determining the cellularity of the mouse tibia.

The reproducibility of this technic was reasonably good. An average difference of 8% was observed in the number of cells obtained from the right and left humerus of the same mouse. The standard volume of washout fluid used was that volume beyond which no additional cells or further radioactive iron could be removed from the humerus. If a few firmly adherent marrow cells remained in the washed bone they probably constituted less than 10% of the original marrow cells since an average of 9% of the amount of iron originally present in the entire bone was left in the bone after the washout.
Whether a significant proportion of this residual iron in bone was actually in marrow cells or was within other cells or portions of the bone was not determined.

A second possible problem which would lead to a falsely low count of marrow cells is the possibility that cells were disrupted during the washout procedure and not counted. A significant amount of radioactive iron was present in the cell-free supernate of marrow washout fluid. However, further studies suggested that most supernate iron was not bound to cellular particles and that it was not hemoglobin iron. If a significant number of cells were disrupted and not counted the disruption must have occurred during the passage of the first small volume of fluid through the bone, without further disruption occurring during subsequent washouts and mixing.

The humerus, rather than the tibia or femur, was used since a pilot study suggested that washouts from this bone were more reproducible. Later studies disclosed that the tibia contains a larger proportion of nonerythroid cells than the humerus or femur, suggesting that this bone is the least desirable of these three long bones for study of hematopoietic tissue. Although the femur may be as suitable as the humerus in this type of study the humerus can be removed and washed out more rapidly.

From a total and differential cell count the total number of specific cell types within the marrow cavity can be calculated. The percent of cells which stained with peroxidase corresponded to the percent of neutrophils and neutrophil precursors counted in Wright's stained smears. The percent of peroxidase-stained cells can be determined rapidly and with reasonable accuracy.

The amount of injected radioactive iron present in a humerus was 2.0% of the total amount localized in bone. Assuming that this distribution of iron is representative of marrow distribution, the marrow hematopoietic tissue in a mouse is $12 \times 10^6$ nucleated cells per gram of body weight. This figure is similar to figures for marrow mass in other species calculated by Donohue et al. (12) ($18 \times 10^6$ per g in man, $12 \times 10^6$ per g in rabbits, $17 \times 10^6$ per g in rats, and $34 \times 10^6$ per g in monkeys).

Mouse leukocyte counts are reported to be quite variable and to differ considerably from one anatomic site to another (6, 13, 15, 20, 25, 30, 31). Considering the number of factors which induced acute changes in leukocyte counts of venous blood in our studies, the reported variation is not surprising.

Pentobarbital or ether anesthesia resulted in a 50% decrease in venous leukocyte counts. Anticoagulation with heparin resulted in a twofold increase in venous counts. An increase in total count, reflecting a lymphocytosis but not a neutrophilia was noted in afternoon counts as compared to morning counts. Warming the tail before obtaining a venous sample resulted in a decrease in leukocyte count. Repeated venous sampling produced a decrease in count with each succeeding sample until approximately one-third of the blood volume was removed. The decrease with consecutive samples was a general venous phenomenon and not limited to blood from a single area.

Certain of these observations confirm studies by other investigators in mice or in other species with respect to the effect on leukocyte counts of pentobarbital (7, 31) and the time of day (6). However, to our knowledge, the effect of rapid, repetitive venous sampling in mice on leukocyte counts has not been reported. These changes are not limited to this strain of mice for we have obtained identical results in studies of both germ-free and conventional C3H mice (4).

Venous counts, whether obtained from the orbital sinus, vena cava, or tail vein are similar but all venous counts are much higher than counts from the aorta. After a series of samples are obtained from the venous system, venous counts decrease until they approach the count in the aorta.

It seems unlikely that the leukocyte concentration in freely circulating blood is more than twice as high in veins than in arteries in intact undisturbed mice. Rather, this difference is probably induced acutely by the trauma of obtaining a blood sample. The simplest mechanism by which this could occur is by a change in the proportion of intravascular leukocytes which are marginated along vessel walls rather than circulating freely. In man or dog (3) approximately one-half of the leukocytes within blood vessels are marginated. Demargination, with a resultant increase in concentration of circulating leukocytes, is induced by administration of epinephrine or by exercise (2). That the change which occurs in leukocyte count with repetitive venous sampling does not reflect an acute change in the rate at which neutrophils are being released from the bone marrow is suggested by the observation that the ratio of segmented to nonsegmented neutrophils did not change in the blood samples.

If the change in count in venous samples and the difference between arterial and venous samples represents an acute change in the proportion of margined cells, our data suggest that all types of leukocytes marginate with equal facility. Vejlens (36) observed a decrease in leukocyte count between the first and second drops of blood obtained from the finger of human subjects, but no further decrease took place in subsequent drops. He attributed this decrease to marginated neutrophils entering the circulation and being removed by the bleeding.

We would suggest the following explanation for the changes in leukocyte counts in consecutive venous samples and the difference between venous and arterial counts. The trauma of sampling induces a sudden constriction of the peripheral venous circulation with a resultant demargination of large numbers of leukocytes. The first sample from a vein thus contains a number of leukocytes which have been marginated a moment before in addition to those which were freely circulating. As further samples are removed, the number of cells which are demarginating is steadily reduced until the sixth sample, when the leukocyte count remains relatively constant and represents primarily the original level of cir-
culation. Since the aortic count does not increase as the venous count decreases, the demarginated cells must again marginate in the pulmonary circulation and not reach the peripheral arterial blood. Evidence is available to suggest that if an excessive concentration of leukocytes is perfused through the lung, they tend to marginate along pulmonary vessels (1).

Assuming this hypothesis is correct, then counts from the aorta are representative of the size of the circulating leukocyte pool of the mouse and counts from an orbital sinus after removal of six or more samples yield a similar approximation. The first venous samples is representative of the circulating pool plus a portion of the marginal pool.

REFERENCES


