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The Relative Spatial Distributions of CFU_s and CFU_c in the Normal Mouse Femur

By B. I. Lord, Nydia G. Testa, and J. H. Hendry

Femoral bone marrow was divided longitudinally into two groups of cells of varying size. By assaying CFU_s and CFU_c in the two zones of the marrow, their distributions across the diameter of the femur was determined. It is shown that the concentration of CFU_s increases from the femoral axis (15 $CFU_s/10^5$ bone marrow cells) to the bone surface (44 $CFU_s/10^5$ cells), obeying approximately a square-law relationship. The CFU_c concentration, on the other hand, increases from the femoral axis (32 $CFU_c/10^5$ cells) to a peak value (260 $CFU_c/10^5$ cells) at about 330 μm from the axis and thence falls off again to the bone surface (77 $CFU_c/10^5$ cells). Selective killing of cells in DNA synthesis using the tritiated thymidine suicide technique, in vivo, showed that CFU_s near the bone surface are proliferating at a faster rate than those more distant from the bone, but that CFU_c have a fast proliferation rate irrespective of their position in the distribution. Thus, bone marrow cell populations are shown to conform to a well-defined spatial organization corresponding to the chronologic relationships between marrow cells.

IN A RECENT PUBLICATION[1] it was shown that the hemopoietic spleen colony-forming cells (CFU_s) are not randomly distributed throughout the femoral bone marrow as had formerly been assumed in, for example, estimations of x-ray dose to the hemopoietic stem cell system. Instead there are higher concentrations of CFU_s in regions close to the inside bone surface of the femur than in the center of the marrow cavity. By making a fractionation of the bone marrow cells into axial cells (cells lying closest to the longitudinal axis of the femur) and marginal cells (cells lying closer to the bone surface) and assaying CFU_s concentrations in each region, it was possible to demonstrate that the increasing CFU_s concentrations from the axial to the marginal zones obeyed a linear relationship with the square of the distance from the axis.

Closely related to the CFU_s is a population of cells which can form hemopoietic colonies when grown in agar culture (CFU_c) and which are considered probably to be committed precursor cells of the granulocytic cell series. Analysis of the cell populations within the bone marrow by cell fractionation techniques,[2,3] has shown that the CFU_c is almost certainly derived directly from the CFU_s. In order to define further the geometrical organization of the bone marrow cell populations, these experiments have now been extended to include a study of the distribution of the CFU_c in relation to the CFU_s and in so doing to obtain more information relevant to their relationship.

MATERIALS AND METHODS

Male BDF1 (C57Bl × DBA2) mice, aged 10–12 wk, were used in these studies. For each observation, one femur was removed from each of three donor mice, cleaned, and the bone marrow

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cells fractionated into axial and marginal cells as described in detail previously.[1] The method consists of removing a core of cells (axial cells) from the cavity by inserting a No. 21 gauge needle into the end of the bone and gently pushing a small volume of Fischer's medium through the center. The remaining cells (marginal) are then removed by more rigorous washing with Fischer's medium to form a separate pool of cells. By varying the force of the initial flushing procedure for axial cells, it is possible to vary the relative sizes of the axial and marginal cell populations, and then by assuming a random distribution of the cells with respect to cell size, an approximate position (X) at which the marrow is divided can be calculated. (As far as can be determined from marrow sections, the cells are randomly distributed.) The mean diameter of the femoral shaft measured on transverse sections was found to be approximately 900 μm.[1] The cells were collected and maintained under aseptic conditions. The axial and marginal cells from the three bones were pooled prior to assay.

CFU_s were assayed using the spleen colony assay technique of Till and McCulloch[4] and CFU_c were assayed by culturing in a single-layer, soft-agar system as described by Testa.[5]

In order to check that fractionation of the marrow did not result in changes in the plating efficiency for CFU_c development in agar, a comparison was made between fractionated and unfractionated marrow. Bone marrow from one femur of each of three mice was pooled and assayed for CFU_c. The three contralateral femora were fractionated as above, pooled, and the axial and marginal populations assayed separately for CFU_c. The net CFU_c concentrations in the fractionated and unfractionated marrows were then compared.

In one experiment, the donor mice were injected with 1 mCi tritiated thymidine (15 Ci/mmole, specific activity) 30 min before killing and fractionating their femoral marrow cells. The fractions were again assayed for CFU_s and CFU_c. Nonfractionated normal and thymidine-treated mice were also assayed as controls. The loss of CFU_s and/or CFU_c as a result of the S phase cytotoxicity of this dose of tritium was taken as a measure of the proliferative activity of the cells prior to the injection.

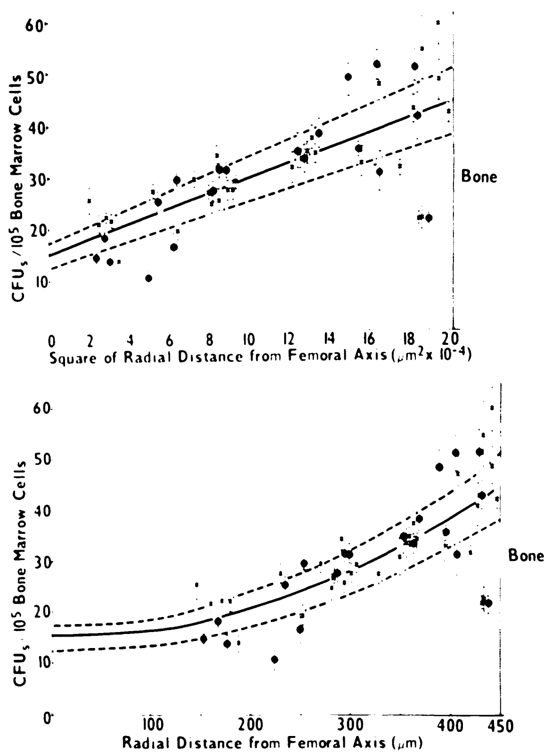

Fig. 1. The relationship between the concentration of CFU_s in femoral marrow and the distance from the central longitudinal axis of the femur. In the upper diagram (*b*), the abscissa is the square of this distance. The points are all plotted at the median values of the square of the distance from the bone's axis for each block of cells considered, i.e., r (axial cells) = $\sqrt{\frac{x^2}{2}}$ and r (marginal cells) = $\sqrt{\frac{R^2 + x^2}{2}}$ where R = radius of cross section of femoral marrow shaft (450 μm). The line shown in the lower curve is that calculated above and transposed to a linear abscissa. The points illustrated ⫱ are data for which parallel CFU_c data were obtained and graphed in Fig. 3. Those illustrated ⫱ include the data published previously.[1]

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RESULTS

The distribution of CFU_s across the diameter of the femoral shaft is shown in Fig. 1A and confirms the basic distribution described in the earlier publication. The data shown on these curves include those previously reported. With the CFU_s concentrations normalized to a mean concentration of 30 per 10^5 bone marrow cells, the regression coefficients were calculated to be *a* (intercept on the CFU_s axis) = 15.2 ± 2.4 CFU_s per 10^5 cells, and *b* (slope) = $(1.44 \pm 0.19) \times 10^{-4}$ CFU_s per 10^5 cells per μm^2. Using these data, the curve shown in Fig. 1B was constructed. Thus the concentration at the center of the cavity is 15.2 ± 2.4 CFU_s per 10^5 cells, and this increases to 44.4 ± 6.3 CFU_s per 10^5 cells at the bone surface.

CFU_c, on the other hand, do not conform to the same square law distribution (Fig. 2). Instead, the distribution is discontinuous; a peak of CFU_c numbers is found about 110 μm from the bone surface. Of the ten points plotted for marginal cells, only one lies significantly higher than the calculated best-fit line for the CFU_c, whereas for the same ten cell suspensions, only one lies significantly lower than the line in the CFU_s distribution (solid circles, Fig. 1 and Fig. 3, which shows the comparable CFU_s distribution determined using the same cell suspensions as those for the CFU_c distribution). It was necessary, however, to establish that fractionation of the marrow did not affect the in vitro plating efficiency of CFU_c. Table 1 shows the results of these tests. It is clear

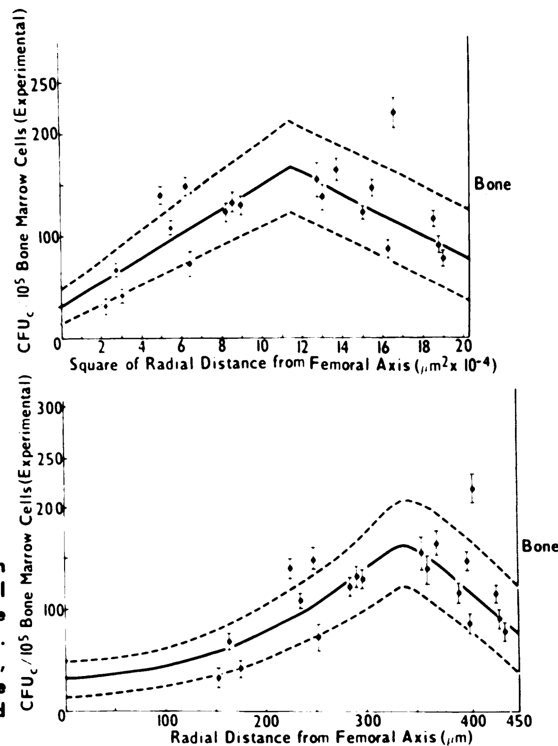

Fig. 2. The relationship between the concentration of CFU_c in femoral marrow and the distance from the central longitudinal axis of the femur. The points are plotted as in Fig. 1, and the line shown in the lower curve is transposed from the line calculated to fit the data in the upper diagram.

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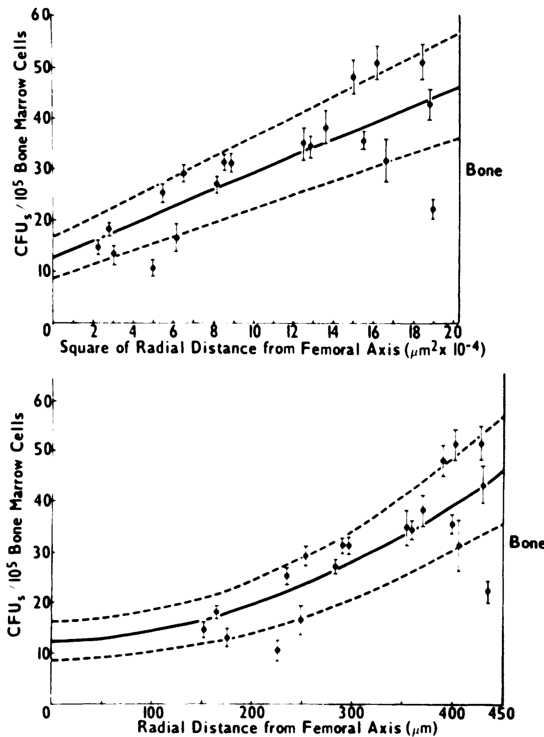

Fig. 3. CFU$_s$ distribution for bones from which parallel CFU$_s$ and CFU$_c$ concentrations were measured (for comparison with Fig. 2).

that the numbers of CFU$_c$ measured are independent of the manner in which they are assayed, and fractionation does not distort their distribution.

Calculation of the regression coefficients for the two segments of the curve based on a mean value of 120 CFU$_c$ per 10^5 bone marrow cells gave values of $a = 32.0 \pm 17.1$ CFU$_c$ per 10^5 cells plated, $b = (1.17 \pm 0.22) \times 10^{-4}$ CFU$_c$ per 10^5 cells per μm^2 for the zone taken from the femoral axis to 340 μm and $a = 281.4 \pm 45.4$ CFU$_c$ per 10^5 cells, $b = -(10.15 \pm 2.84) \times 10^{-4}$ CFU$_c$ per 10^5 cells/μm^2 for the zone extending from 340 μm to the internal bone sur-

Table 1. Effect of Bone Marrow Fractionation on the Plating Efficiency of CFU$_c$

Group	Position of Boundary (X μm)	CFU$_c$ per 10^6 Cells	Ratio CFU$_c$ (A + M) to CFU$_c$ Unfractionated
A + M*	332	1396 ± 45	1.12 ± 0.04
Unfractionated†		1242 ± 65	
A + M*	414	1450 ± 43	1.13 ± 0.05
Unfractionated†		1278 ± 80	
A + M*	421	1168 ± 44	0.95 ± 0.03
Unfractionated†		1228 ± 39	
A + M*	405	1521 ± 95	1.02 ± 0.09
Unfractionated‡		1498 ± 162	
			1.06§ ± 0.05

*A and M assayed separately.

†Unfractionated cells from contralateral femur.

‡Cells from same femur: A and M cells pooled and plated together.

§Mean ratio.

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face. Construction of the distribution related to the linear distance using these coefficients results in the distribution shown in Fig. 3B. At the axis, there are 32 ± 17 CFU_c per 10^5 cells. This rises to a peak of 162 ± 42 at 340 μm and subsequently falls again to 77 ± 43 CFU_c per 10^5 cells at the bone surface.

Each assay gives the average CFU_s and/or CFU_c content for the zone measured. It can be seen, therefore, that where any one zone overlaps parts of both the ascending and descending arms of the distribution, the average value obtained will be lower than the true value at the stated position. This is not applicable for CFU_s because the distribution is continuous, but the distributions shown in Fig. 2 for CFU_c are correct only so long as the axial or marginal cells measured all lie on one side of the peak or the other, respectively. All other points will be underestimates of the true concentrations. Consequently, corrections should be applied to these curves to obtain a more representative CFU_c distribution. Although, by virtue of the methodology available, the distributions can only be approximate, it is important to make these corrections. Thus, the bone marrow cavity was divided into annular zones, each 1 abscissal unit thick, as shown in Fig. 2A. Then, working first from the femoral axis and then from the bone surface, the true average CFU_c concentration for any specific zone was calculated by equating the area under the curve up to and including that zone (based on the experimentally observed average value) with the sum of the areas in that zone and the previous zones. The additional number of CFU_c obtained under the calculated curve for that annular zone was then added to the observed average number for all the annular zones to that point. As a result,

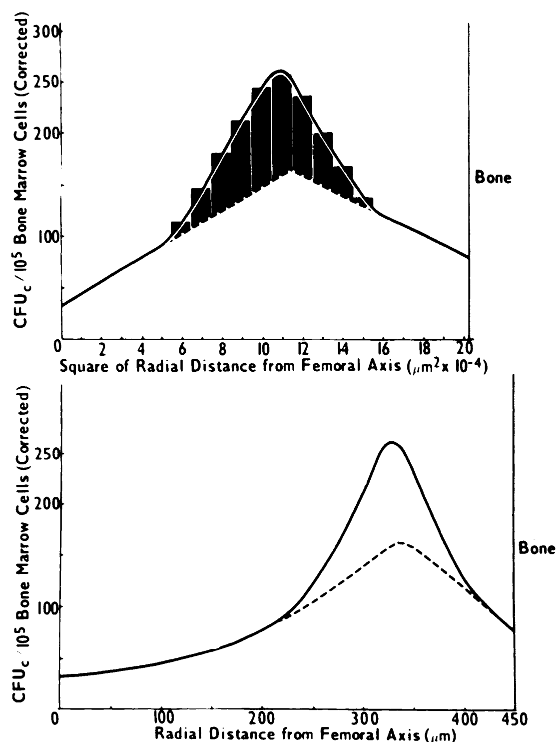

Fig. 4. Distribution of CFU_c in femoral bone marrow corrected for the discontinuity in the experimental distribution.

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Table 2. Tritiated Thymidine Killing (in Vivo) of CFU_s at Different Positions in the Femoral Bone Marrow Cavity

Axial Cells				Marginal Cells		
r μm	CFU_s per 10^5 Cells After ^{3}HTdR Treatment	Percent Killed by ^{3}HTdR	Boundary (X μm)	r μm	CFU_s per 10^5 Cells After ^{3}HTdR Treatment	Percent Killed by ^{3}HTdR
168	19.0 ± 0.9	0	237	360	30.0 ± 3.0	14
264	28.1 ± 2.0	0	373	413	25.2 ± 2.8	39
269	34.3 ± 2.7	0	380	416	9.2 ± 1.1	77
271	33.5 ± 2.7	0	383	418	10.2 ± 1.1	75

CFU_s numbers normalized to an average value of 27 CFU_s per 10^5 cells to represent an average overall ^{3}HTdR kill of 10%.

r is the position in the distribution at which the points are plotted and represents the midpoint of the axial, or marginal, zones on the square of the radial distance from the femoral axis, i.e., *r* (axial cells) = $\sqrt{x^2/2}$, and *r* (marginal cells) = $\sqrt{(R^2 + X^2)/2}$ where *R* = radius of cross section of femoral marrow shaft (450μm).

the corrected distributions shown in Fig. 4 were obtained. The peak of CFU_c is then found to arise 120 μm from the bone surface. On either side of the peak there is a rapid fall of CFU_c numbers to about 30% of maximum at the bone surface and about 12% along the femoral axis.

Selective removal of S phase CFU_s by tritiated thymidine (^{3}HTdR) resulted in a greater loss of cells in the marginal zones (Table 2). Normally, about 10% of CFU_s in whole marrow preparations are killed by ^{3}HTdR.[6,7] Consequently, the data for Table 2 were normalized to an average of 27 CFU_s per 10^5 bone marrow cells rather than 30 CFU_s per 10^5 bone marrow cells as used for the normal distribution shown in Fig. 2. In the axial region, no loss of CFU_s was observed. It appears, therefore, that CFU_s in the region of the bone surfaces are probably turning over at a considerably higher rate than those more remote from the bone surfaces. CFU_c, on the other hand, have a high degree of killing, 40–50%.[8,9] The data for Table 3, therefore, were normalized to an average of 68 CFU_c per 10^5 cells rather than 120 CFU_c per 10^5 cells as for the normal distribution in Fig. 2. A large variation in the degree of killing was observed, but there was no apparent correlation between the kill and their location in the marrow.

Table 3. Tritiated Thymidine Killing (In Vivo) of CFU_c at Different Positions in the Femoral Bone Marrow Cavity

Axial Cells				Marginal Cells		
r μm	CFU_c per 10^5 Cells After ^{3}HTdR Treatment	Percent Killed by ^{3}HTdR	Boundary X μm	r μm	CFU_c per 10^5 Cells After ^{3}HTdR Treatment	Percent Killed by ^{3}HTdR
168	73 ± 3	50	237	360	104 ± 3	28
264	72 ± 3	35	373	413	71 ± 4	35
269	71 ± 4	57	380	416	32 ± 2	81
271	63 ± 3	36	383	418	19 ± 3	81

CFU_c numbers normalized to an average value of 68 CFU_c per 10^5 cells to represent an average overall ^{3}HTdR kill of 43 per cent.

The values of *r* and *X* are as in Table 2.

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DISCUSSION

Several series of experiments have now established that a considerably higher concentration of CFU_s occurs close to the femoral bone surface than in the center of the bone marrow cavity. This observation is complementary to the findings of Van Dyke[10] and Carsten and Bond[11] who demonstrated that large numbers of CFU_s actually reside in the endosteum and the bone[10] or that it was difficult to remove all the CFU_s close to the bone surfaces.[11] In our earlier experiments,[1] we were unable to obtain more CFU_s by grinding the bone and concluded that the methods used for making cell suspensions were satisfactory for flushing all CFU_s from inside the bone shaft. The present results, however, suggest that CFU_s in the region of the bone surface are in a state of more rapid cell proliferation than those remote from the surface (Table 2). It would appear, therefore, that the bone surface may be an important factor in the maintenance of the CFU_s population.

CFU_c, on the other hand, do not appear to depend on the proximity of a bone surface and show a very clearly defined peak of cell numbers well away from the bone surface. It is possible, therefore, that the endosteum of the femoral marrow cavity forms a matrix upon which CFU_s are largely maintained, and as they differentiate to produce CFU_c, there is a movement towards the center of the cavity, producing this peak of CFU_c inside the marrow. The numbers then fall as the CFU_c themselves differentiate and/or mature.

These data indicate, therefore, that the bone marrow cell populations conform to a well-defined spatial organization which probably corresponds to the relationships between the marrow cells and the development of the various hemopoietic cell lines.

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