Separation of Megakaryocytes From Mouse Bone Marrow by Density Gradient Centrifugation

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The density profile of mouse bone marrow megakaryocytes, as determined by discontinuous albumin density gradient centrifugation, was characterized by a single density population (1.088 to 1.119 g/ml) with a peak density of 1.10 g/ml and a maximum enrichment of 15.5. This single population comprised both mature and immature megakaryocytes. The density profile of small, acetylcholinesterase-positive cells, a class of potential megakaryocyte precursors, was almost identical to that of morphologically recognizable megakaryocytes.

Density gradient centrifugation has been used to separate and characterize numerous classes of hematopoietic cells. Separation of megakaryocytes from other hematopoietic cells in bone marrow would be useful both in studying cellular relationships in the stem cell-megakaryocyte-platelet system and in enrichment of megakaryocytes for subsequent biochemical or functional analysis. Nakeff and Maat previously reported the separation and characterization of megakaryocytes from mouse marrow on the basis of cell volume using velocity sedimentation. Populations of megakaryocytes were obtained with cell volumes which were distinct from the pluripotential stem cell and which represented different stages of cytoplasmic maturation. In order to define this cell compartment by another parameter, we have examined the cellular density of megakaryocytes.

In this report, we present data on the enrichment of megakaryocytes from mouse bone marrow using discontinuous albumin density gradient centrifugation, and we describe the density profile of a class of potential megakaryocyte precursors.

MATERIALS AND METHODS

Mice

Male BD$_2$F$_1$ (C57Bl x DBA) mice were obtained from National Laboratories (St. Louis, Mo.) and used at 20–22 wk of age.

Bone Marrow Preparation

Mice were killed by cervical dislocation and their femurs removed. The marrow was collected by flushing each femur with 1 ml of ice-cold phosphate-buffered saline (PBS) with 1% bovine serum albumin (BSA) at mouse serum isotonicity (320 mOsm). Marrow for each gradient was pooled from the femurs of three mice, gently monodispersed through a 25-gauge needle and kept on ice. A 25 µl aliquot of this cell suspension was counted on an electronic particle counter (Celsoscope) in a solution (30 mg/ml) of the cytoplasmolytic agent cetrimide (hexadecyltrimethyl ammonium bromide, Baker, Phillipsburg, N.J.) to obtain the total nucleated cell count.
Gradient Preparation

BSA, fraction V powder (Sigma, St. Louis, Mo., Lot No. 52C-3280) was dissolved in distilled water at 4°C to a concentration of about 40% as determined by refractometry. The pH of this stock solution was 5.2. Its final osmolality was adjusted to 320 mOsm by the addition of sodium chloride (300 mg/ml); it was then membrane-filtered (0.22 μ). A series of BSA solutions from 16% to 38%, in 2% steps, (density 1.050-1.119 g/ml, by pycnometry) were made by diluting the stock solution with sodium chloride (pH 5.2, 320 mOsm). After a final determination of their pH, osmolality, and per cent BSA, fractions were stored at 4°C for no longer than 2-3 wk.

To form the gradient, 0.5-ml aliquots of each BSA fraction, commencing with 38% BSA, were layered in a glass gradient tube (125 x 8 mm I.D.). All procedures were carried out at 4°C to minimize mixing of the fractions by convection; care was taken in layering each fraction to prevent mechanical mixing of the layers.

Gradient Utilization

An appropriate amount of bone marrow (BM) for one gradient run obtained as described above was concentrated by centrifugation at 350 g for 10 min at 4°C and resuspended in 0.6 ml of a 16% BSA solution; 0.5 ml was layered carefully on the formed gradient, which was then centrifuged at 1000 g for 30 min at 4°C. Each fraction was removed with siliconized Pasteur pipettes and transferred quantitatively into 10 ml of PBS.

The 0.1-ml sample of the starting cell suspension not placed on the gradient was washed and concentrated in the same manner as the fractions and was used to determine the total number of nucleated cells and megakaryocytes initially placed on the gradient.

Sample Preparation

Samples of the starting cell suspension and each fraction were sedimented quantitatively onto glass slides as follows. A 50-μl sample of PBS with 2% BSA was placed in each chamber. Five minutes later, a 25-μl sample of marrow was placed in the center of the chamber. After complete drying (30 min), the slides were fixed for 10 min in acetone, then stained with May-Grunwald-Giemsa (MGG) as described previously. Slides were then scanned using light microscopy. The morphologically identifiable megakaryocytes in duplicate preparations were counted using the criteria of Odell et al. and then averaged. The total number of megakaryocytes was then expressed per fraction. In those preparations used to identify possible megakaryocyte precursors as defined by Zajicek and Jackson in rodent marrow, slides were stained for 2 hr at room temperature for acetylcholinesterase (AChE) activity using the “direct-coloring” technique of Karnovsky and Roots as modified for mouse megakaryocytes. The nucleated cells in the initial bone marrow sample and in each fraction were counted as described previously.

RESULTS

In order to indicate the extent of enrichment accomplished by this procedure, we define the enrichment ratio (ER) as:

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\text{ER} = \frac{\text{no. megakaryocytes}}{\text{no. nucleated cells}}_{\text{BM fraction}} + \frac{\text{no. megakaryocytes}}{\text{no. nucleated cells}}_{\text{total BM}}
\]

In the starting preparation of normal bone marrow, there were \(57.2 \times 10^3 \pm 3.4\) (± 1 SE) megakaryocytes in \(7.6 \times 10^7 \pm 0.3\) nucleated cells. The peak megakaryocyte fraction (1.1 g/ml) contained 1.16% megakaryocytes, representing an average ER of 15.5 ± 0.17 obtained from six gradients.

Density profiles of morphologically identifiable megakaryocytes were analyzed using preparations stained by either MGG or AChE and classified according to the criteria of Odell et al. Identical profiles were observed characterizing a single “population” of megakaryocytes with an average density of 1.1 g/ml. Figure 1 presents the density profile for megakaryocytes following AChE stain-
Fig. 1. The average density profile of morphologically recognizable mouse bone marrow megakaryocytes obtained from six discontinuous albumin density gradients at pH 5.2 and 320 mOsm. Cell preparations were stained with AChE. (Identical profiles were also obtained following MGG staining.) Megakaryocytes (■) and other nucleated marrow cells (○) were expressed as a per cent of their respective number initially placed on the gradient. Errors shown represent ±1 SE.

Fig. 2. The average density profile of small, AChE-positive BM cells (△) obtained from six gradients. The density profile for morphologically recognizable megakaryocytes (from Fig. 1) is shown as a dashed line. Errors shown represent ±1 SE.
ing which was distinct from that characterizing the majority of the nucleated
cells (average density about 1.08 g/ml). Cell recoveries from six gradients
averaged 96.8 ± 6.8% for nucleated cells and 100 ± 2.7% for megakaryocytes.
Of the total megakaryocytes initially placed on the gradients, 91% comprised
the single density population (1.088–1.119 g/ml) in the presence of only 11% of
the total nucleated bone marrow cells.

No difference between the cell densities of immature (small) and mature
(large) megakaryocytes was observed; both types were present in the single den-
sity population. Following AChE staining, however, the megakaryocytes present
in the gradient in density fraction 1.05–1.088 g/ml (“light” megakaryocytes) stained
less intensely for AChE than megakaryocytes present in density frac-
tions >1.088 g/ml (“heavy” megakaryocytes). In order to determine the density
profile of the small AChE-positive cells which have been described by Jackson7
as being potentially specific cell precursors for megakaryocytes in rodent mar-
row, the AChE stained preparations were rescoped for this particular class of
cells. These cells had not been counted in the determination of the density pro-
file of the morphologically recognizable megakaryocytes (Fig. 1) due to their
relatively small cell size. Analysis of the density profile of this class of precursor
(Fig. 2) revealed a single “population” with an average cell density of 1.1 g/ml
and a distribution that was almost identical to that observed for the recogniz-
able megakaryocytes.

DISCUSSION

This study demonstrates the effectiveness of discontinuous albumin density
gradient centrifugation for separating and enriching for megakaryocytes from
mouse bone marrow. A more than 15-fold peak enrichment was obtained
routinely. Megakaryocytes formed mainly a single “population” with a cell
density higher than that characteristic of other nucleated hematopoietic cells.
This finding was true regardless of the state of maturation and, consequently,
the size of the megakaryocytes. This higher density might have reflected either
the degree of polyploidy, the cytoplasmic-to-nuclear (C/N) ratio, or the extent
of cytoplasmic protein synthesis in megakaryocytes, since large amounts of either
DNA or protein, or both, might be expected to increase their overall cell den-
sity. The finding that both immature and mature megakaryocytes were present
in the same density population suggested that the degree of polyploidy (or
DNA content) per se did not play a major role, since both types have similar
ploidy distributions.10 Further, this finding did not reflect solely the C/N ratio
since this also varies markedly during the maturation of megakaryocytes.5
However, it may indicate that there exists a more complex balance between
the density in immature megakaryocytes as a result primarily of a C/N ratio that
is lower than that for mature megakaryocytes and the density of mature mega-

karyocytes as a result of the cytoplasmic content of platelet protein higher than
that present in immature megakaryocytes. Similar investigations with the sepa-
ration and culture of isolated megakaryocytes from guinea pig bone marrow
have been reported in a preliminary fashion by Levine and Fedorko11 and
Levine12 although the recovery of megakaryocytes was much lower and the
peak density less than that reported here for mouse marrow megakaryocytes.
We can only speculate as to the significance of the "light" megakaryocytes. They probably do not reflect a cell loading artifact since separations performed using initial cell numbers 20-fold less than those used in the studies reported here gave essentially the same density profile. We did observe that "light" megakaryocytes stained less intensely for AChE, but the significance of this finding is not clear. Jackson\(^{13}\) has speculated that the degree of AChE activity in individual megakaryocytes may reflect their proliferative state. If this were true, the "light" megakaryocytes would be exclusively immature, since only these particular cells have been shown to be capable of DNA synthesis.\(^{10}\) On the contrary, we found that both mature and immature megakaryocytes were present in the "light" density fractions.

The similarity of the density profile of the small, AChE-positive cells to that of morphologically recognizable megakaryocytes tends to support the formers' designation by Jackson\(^{7}\) as potential megakaryocyte precursors. This similarity may indicate that the small AChE-positive cells also share a similar distribution of DNA content. If so, these cells fit the description of a class of precursor, theoretically proposed in a model of megakaryocytogenesis by Ebbe,\(^{14}\) that may be polyploid but not recognizable as megakaryocytic by conventional morphological criteria. Although the small, AChE-positive cells cannot be separated from recognizable megakaryocytes on the basis of density, they should be separable on the basis of cell volume using velocity sedimentation.\(^{3}\)

The separation and characterization of megakaryocytes from bone marrow by cell size and density raises the possibility of enriching for megakaryocytes on the basis of one parameter using as cellular input megakaryocyte fractions initially enriched on the basis of the other parameter. By combining velocity sedimentation and discontinuous density gradient centrifugation, bone marrow fractions may be obtained with concentrations of megakaryocytes high enough to permit the rapid and simultaneous automatic cell-by-cell analysis of megakaryocyte cell volume, DNA, and cytoplasmic protein content. These studies, now in progress, may enable us to separate and characterize megakaryocyte populations on a multiparameter cellular basis. If we are successful, it should be possible to screen relatively rapidly for various populations of megakaryocytes and their precursors in the marrow of patients suffering from platelet disorders and thus to define the lesion in the megakaryocyte system that accounts for the abnormality.

**REFERENCES**


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